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EDITORIAL PREFACE

Jordan Journal of Biological Sciences (JJBS) has had another great year. We have seen a significant increase in articles submission from both regional and international scholars. The editorial board members of JJBS have been very busy throughout the year to maintain excellence in the quality publication of accepted papers. As a result, JJBS has been indexed by CABI's Full-Text Repository, EBSCO and is currently under evaluation to be indexed in National Library of Medicine's MEDLINE\ PubMed system and Elsevier's SciVerse Scopus.

As in the previous two years, this sixth volume of JJBS will include four issues, ten to twelve articles in each issue. In the coming year, it is my vision to have JJBS publishes more outstanding papers and review articles from distinguished scholars in various areas of biological sciences. In addition, I will be working on the inclusion of JJBS in ISI, which will lead to a wider readership and good impact factor. As you read throughout this inaugural volume of JJBS, I would like to remind you that the success of our journal depends directly on the number of quality articles submitted for review. Accordingly, I would like to request your participation by submitting quality manuscripts for review and by encouraging your colleagues to do the same. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscript or not, is the mentoring nature of our review process. JJBS provides authors with high quality, helpful reviews that are shaped to assist authors in improving their manuscripts.

I would like to thank the JJBS International Advisory Board members for their continuous support of JJBS. Furthermore, I would like to thank the JJBS Editorial Board members for their exceptional work and continuous support to JJBS. My thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous financial and administrative support to JJBS.

Moreover, and as always, I would like to highlight and proudly thank the group of authoritative reviewers, both local and international, who have done an outstanding work. We are honored to have you on our review list and many thanks for your valuable mentorship and contributions you provided to authors. Indeed, we count on your excellent reviews to include only high quality articles worthy of publication in JJBS. Together, we strive to make JJBS reach a remarkable rank among other international journals. I very much appreciate your support to make JJBS one of the most authoritative journals in biological sciences.

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
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African Flora as Potential Sources of Medicinal Plants: Towards the Chemotherapy of Major Parasitic and Other Infectious Diseases- A Review

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Abstract

Parasitic diseases, especially those defined as neglected diseases by the WHO, remain a major public health predicament, which affects hundreds of millions of people especially in developing countries. Furthermore, infectious diseases such as HIV, malaria, tuberculosis, diarrhoeal diseases, pneumonia, leishmania and human African trypanosomiasis are responsible for one in two deaths in developing countries, where poverty, limited access to health care, drug resistance and a changing environment make populations particularly vulnerable. As a consequence, herbal medicines have attracted much attention as potential therapeutic agents in the prevention and/or management of parasitic and infectious diseases, as they can yield potential leads to address emerging infections and resistance. Indeed, the use of medicinal plants in the treatment and management of human and animal diseases has long been practiced before the advent of chemotherapy. The present review has endeavoured to provide an overview of the potential of medicinal plants, particularly, those from the African biodiversity to target three common parasitic and infectious diseases, namely malaria, leishmania and human African trypanosomiasis that have plagued humans since time immemorial.

Keywords: Medicinal Plants, Chemotherapy, Leishmania, Human African Trypanosomiasis, Malaria

1. Introduction

It is generally recognized that plants have formed the basis of sophisticated traditional medicine systems that have existed over millennia. Herbal medical products form part of systems of knowledge and practice that has been transmitted over centuries and is evolving fast. There is also no doubt that human beings rely on them directly or indirectly either as alternatives to mainstream medication or as isolated molecules that are derived from medicinal plants. Traditions like Chinese, Ayurvedic, Kampo, Arabic, European Unani, Jamu etc. are all good examples.

In the Western world, the Greeks have contributed substantially to the rational development of the use of herbal medicines. Theophrastus, in his *History of Plants,* dealt with the medicinal qualities of herbs, and noted the ability to change their characteristics through cultivation. Dioscorides had started recording the use of medicinal plants during his travels with the Roman armies. Galen, who practiced and taught pharmacy and medicine in Rome, published several books and he is well known for his complex formulas used in compounding drugs sometimes containing dozens of ingredients ("galenicals") (Heinrich *et al.*, 2004).

During the period 5-12th century, the remains of this western knowledge were preserved in monasteries. However, it was the Arabs who were finally responsible for the preservation of much of the Greco-Roman expertise, and for expanding it to include the use of their own resources, together with Chinese and Indian herbs unknown to the Greco-Roman world (Bashar *et al.*, 2008).

The Arabs were the first to establish privately owned drug stores in the 8th century, and it was Avicenna, the Persian physician and scientist who had contributed much to the sciences of pharmacy and medicine through works such as *Canon Medicinae,* which is regarded as "the final codification of all Greco-Roman medicine" (Bashar *et al.*, 2005)

Nowadays, plant-based systems of medicine continue to play an essential role in health care. The World Health Organization has estimated that approximately 80 % of the world's inhabitants rely mainly on traditional medicines for their primary health care (Anon, 2008).

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Plant products also play an important role in the health care systems of the remaining 20 % of the population residing mainly in the developed countries. It has been estimated that in the United States from 1959 to 1980, about 25 % of prescriptions contained plant extracts or active principles derived from higher plants, and at least 119 chemical substances have been derived from 90 plant species. Of the 119 drugs, 74 % were discovered as a result of chemical studies directed at isolating the active substances from plants used in traditional medicine. In addition, the use of so-called complementary or alternative herbal products has expanded in recent years (Cragg and Newman, 2007).

Whilst it is often acknowledged that traditional medicine works, there still exists gap in the scientific study of the complex products from such traditions. Such is the challenge that faces ethnopharmacology even though it is increasingly being acknowledged that many types of diseases, including such common ones as vector-borne diseases: diarrhea or tuberculosis are still commonly treated and/or managed with herbal medicines.

There is also a growing use of plants to manage or treat HIV/AIDS or other emerging or fast spreading diseases (like viral respiratory diseases). Novel treatment strategies are needed for all diseases and in recent years, herbal medicines from such traditions have received particular attention in the management (prevention or treatment) of chronic diseases like diabetes, chronic disorders and many forms of cancer.

In spite of the availability of antibiotics and vaccines, parasitic and infectious diseases still pose serious challenge in developing countries. Today, we are further away from controlling parasitic diseases than we were 30 years ago (Coker *et al.*, 2008; Nunkoo and Mahomoodally, 2012). Infectious diseases and parasitic protozoa remain a major threat to the health of the population throughout the world. However, while there are few effective medicines for the treatment of many protozoal diseases, therapies for malaria, leishmaniasis and trypanosomiasis related diseases are severe threat to more than 2 billion people who reside in the developing world.

Interest in plant products has been stimulated by the identification of the antiplasmodial activity of the sesquiterpene lactone artemisinin. This experience is not being ignored; on the contrary, it is being reinforced as plants have frequently provided template molecules on which to base further novel structures. This has been the case, at least, for the past 50 years where antimicrobials and anti-parasitic medicines have been identified from the products of fungi and bacteria and this further consolidates the need to look into these resources for novel leads.

It has been observed that parasitic diseases usually cannot be treated with a single compound. It is also a fact that pure natural products while serving as lead structures have restricted use in humans as they have associated toxicity. On the other hand, many natural compounds with a desired profile of activity have low toxicity but cannot progress into preclinical studies because of their low bioavailability and/or poor solubility. Nonetheless, chemists have been able to create safer compounds which are close to the lead compound and have reduced toxicity; greater bioavailability and with reduced toxicity. There are nonetheless, gaps in our knowledge in terms of safety and general interactions with other medicines. Thus, the potential that natural products present in terms of potential development along with the talents of synthetic chemists can contribute to both anti-parasitic drug research and aim at producing new and urgently needed medicines in the future.

Parasitic diseases, especially those defined as neglected diseases by the WHO, remain a major public health problem, which affects hundreds of millions of people especially in developing countries. Among the important life-threatening diseases is malaria and it is the single most important cause of ill health, mortality and poverty in Sub-Saharan Africa. It kills on average 1.5 million people annually and the majority of them being children (WHO, 2010). The re-emergence of malaria in many parts of the world is due to the rapid increase of resistance to most of the available anti-malarial drugs, as well as resistance of vectors to insecticides. Drug-resistant strains of *P. falciparum* are emerging in many endemic areas of the world highlighting the failure of conventional antimalarial drugs. The difficulty over the past decades at creating an efficient vaccine as well as the observed side effects of existing anti-malarial medicines have implied the urgent necessity to research and develop well-tolerated anti-malarials drugs both for the treatment of the prophylaxis and to help eliminate the parasite (Krishan *et al.*, 2006; Willcox and Bodeker, 2004).

2. Malaria

Despite all efforts made to eradicate malaria, the mortality and morbidity still increase each year mainly because of drug resistance of *Plasmodium falciparum*. WHO estimates that with 300-500 million clinical cases and more than 2 million deaths each year, malaria remains one of the three most deadly communicable diseases in the world (WHO, 2010). History has revealed that plants are still important sources of medicine against malaria (Musuyu Muganza *et al.*, 2012).

The classical antiprotozoal drug that has been used against malaria is quinine, extracted from the *Cinchona* bark. Whilst it is still occasionally being used, it has served a very important purpose, especially in the wake of growing resistance to *P. falciparum* – it has been used as a template for the production of newer semi-synthetics such as chloroquine, mefloquine and others, which are under development.

The discovery of Artemisinin, derived from the Asiatic wormwood, *Artemisia annua* has boosted research on plants with potential for treating malaria. The antiprotozoal potential of sesquiterpenes and sesquiterpene endoperoxide in addition to artemisinin has been extensively sought after ever since the potential of the latter has been demonstrated against malaria. The exocyclic methyl lactone found in Parthenin (identified as the allergic principle in some medicinally used plants especially those derived from the Asteraceae family) is showing promise against *P. falciparum*, *in vitro*. Parthenin derivatives with the exocyclic methyl lactone

have been synthesized and retested. Artemisinin has also given the more stable derivative – Artemether. With the observed and confirmed activity of Artemisinin, the antiplasmodial flavonoids (Figure 1) from *Artemisia annua* have attracted renewed interest. Recently, it has been demonstrated that the methoxylated flavonones artemetin and casticin can act synergistically with artemisinin against *P. falciparum in vitro*. Other studies on the *Artemisia* genus have generated interesting results. For instance, *Artemisia vulgaris* from Thailand have led to the isolation of sakuranetin and 7-methoxyaromadendrin which are very active, *in vitro* (Phillipson and Wright, 1991; Pradines *et al.*, 2011; Muthaura *et al.*, 2011).

The Asteraceae family has also contributed other interesting plants - the *Vernonia* species. It was the observations made on wild chimpanzees chewing the young stems of *Vernonia amygdalina* have led to the isolation of vernodalin and vernolides and hydroxyverniladin (Ohigashi *et al.*, 1994). These molecules are nowadays being tested for their antitumor properties (Khalafalla *et al.*, 2009).

Interestingly, while *Artemisia afra* does not contain the exocyclic methyl lactone, it has been used on the continent against malaria (Gathirwa *et al.*, 2007). Recent investigations on this plant are showing interesting applications in controlling mycobacterial applications (Ntutela *et al.*, 2009). Another African Asteraceae showing promise is *Dicoma tomentosa*. This plant is being used in Burkina Faso against malaria. The active compound, with moderate activity, was urospermal A-15-O-acetate. Studies are ongoing to further assess the safety of this plant by the local population (Jansen *et al.*, 2012).

Other molecules isolated from plant sources comprise Lapacho, which contains quinone and also exhibits antiprotozoal properties. It is also used against cancer treatment. *Diospyros* spp. are also used by local people for similar purposes. Prenylated xanthones isolated from plants from the Clusiaceae family, namely *Garcinia cowa*, have demonstrated significant activity against *P. falciparum in vitro*. Cowaxanthone has displayed an antiplasmodial potential comparable to pyrimethamine. While several biological properties (antibacterial, antifungal and cytotoxic) have been attributed to the xanthones, there are very few reports on the antiplasmodial activity of these compounds (Riscoe *et al.*, 2005; Mahabusarakam *et al.*, 2006).

Finally, one species that has shown promise *in vitro* is Cryptolepin, isolated from *Cryptolepis sanguinolenta* (Periplocaceae) was also found to be active, *in vitro*, against *P. falciparum* (Cimanga *et al.*, 1997). Clinical studies have been performed on this plant extract with a view to comparing its efficacy with that of chloroquine using the WHO extended 7 days *in vivo* test to measure patients with parasitaemia response. A number of malaria patients with parasitaemia of 10.000-100.000 *P. falciparum* per 8000 white blood cells were used. The results indicated that the efficacy of an aqueous extract was comparable to that of chloroquine. Interestingly, no recrudescence of parasitaemia occurred after a follow-up period of 21 days. Other clinical symptoms such as fever, headaches etc. cleared faster in the group treated with the herbal preparation (Boye *et al.*, 2002).

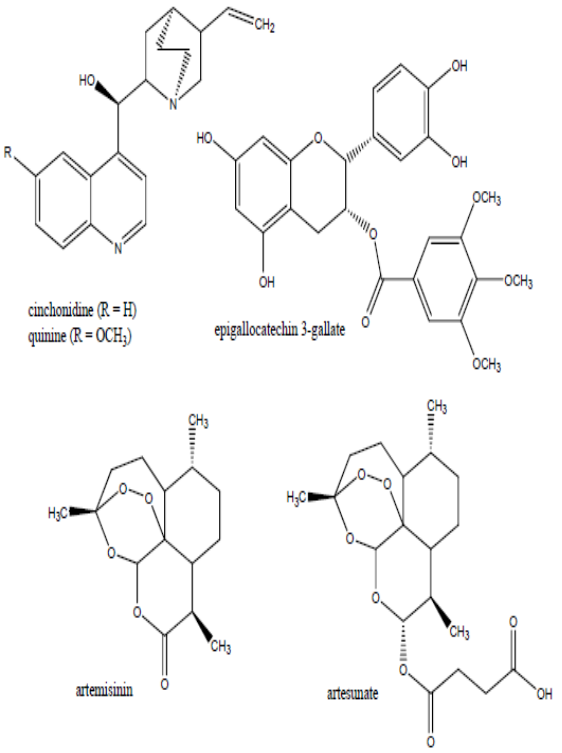

Figure 1. Examples of anti-malarial secondary metabolites. Artesunate is a semisynthetic derivative of artimisinine (Wink, 2012).

3. Leishmania

Leishmaniasis is a severe human disease that is endemic to Africa, America, the Indian subcontinent, subtropical South West Asia as well as many parts of the Mediterranean. In 2009, the disease had accounted for 50, 000 deaths and affected nearly 10 million people worldwide (WHO, 2010). The disease currently threatens about 350 million people in 88 countries around the world, with about 2 million affected annually (Musuyu Muganza *et al.*, 2012). The clinical manifestations vary with the species of the protozoa. They are obligatory intracellular protozoans living in macrophages of mammalian hosts. The extra cellular stage, the promastigote, is introduced into subcutaneous tissues in the human host during the bite of an infected sand-fly vector, *Phlebotomus* (Old World) and *Lutzomyia* (New World). The promastigote is phagocytosed by a mononuclear phagocyte, after which it converts into the obligatory intracellular form, the amastigote. There are two general forms of the disease: visceral leishmaniasis caused by *L. donovani* and *L. infantum* = *L. chagasi* and tegumentary leishmaniasis caused by several dermatropic species of *Leishmania*. This disease is the third largest after malaria and filariasis. Visceral Leishmania causes almost 5000.000 new cases per year globally and almost 90% occurring in just 5 countries (India, Brazil, Bangladesh, Nepal and Sudan). The tegumentary form of the disease, although not as killing as the visceral form,

affects over 1.5 million and causes disfigurement, disability and associated social and psychological stigma.

There is at present, no vaccines against this disease and most of the medicines are mineral based. Unfortunately, such medicines elicit toxic effects, teratogenicity as well as increasing resistance. Antifungal polyene antibiotic is being used in industrialized countries but there have been some relapses in cases of joint infections HIV/*L. infantum*. Often the control of this disease includes the use of pesticides to destroy the vector, improvement of the hygiene and water supplies, as well as targeting the parasite. The latter often takes different forms throughout its lifecycle, making elimination difficult.

The lack of an effective antileishmanial medicine has caused a renewed interest in the study of medicinal plants as source of new chemotherapeutic compounds with better activities and fewer side effects (Khaliq *et al.*, 2009). Natural products in an era of rational drug design still play an important role in the search for new active drugs as they represent valuable lead structures that can de developed further into useful therapeutics. Cragg and Newmann (2007) have shown that many of the drugs approved recently are derived from natural sources. Cordell also reported that 'natural products offer a diversity of structures that simply cannot be matched through even the most active imaginations of the synthetic chemist (Cordell, 2000).

The search from Nature is on and many compounds are still being discovered with good antileishmanial activities. *Garcinia lucida* (Clusiaceae) have yielded chelerythrine derivatives, which manifest significant activities against *L. donovani* (Fotie *et al.*, 2007). Artemisinin from *Artemisia annua* leaves and seeds (Islamuddin *et al.*, 2012, Sen *et al.*, 2007), alkenyl phenol (gibbilimbol B), isolated from *Piper malacophyllum,* has been found, for the first time, to trigger induction of cell-cycle arrest and apoptosis and disrupt the leishmania plasma membrane upon initial incubation, *in vitro*. Derivatives are currently being synthesized for the development of therapeutic agents against both leishmaniasis and Chagas diseases (de Oliviera *et al.*, 2011).

Recent screenings of essential oils from *Coriandrum sativum*, *Lippia sidoides* and the oleoresin from *Copaeifera reticulata* on the promastigotes and amastigotes of *Leishmania chagasi* have shown that the resin of *C. reticulata* could be a viable option for analyzing the *in vivo* therapeutic effects of leishmanicidal plants (Rondon *et al.*, 2012).

A number of natural products have already been screened. Among natural products from marine sources cyclic peptides, various flavonoids, chalcones, lignans, coumarins, iridoids, monoterpenes, saponins, toxoids, curcumin, quinoline alkaloids, and polyketides exhibit interesting anti-leishmanial activities. Active flavanones and flavonoids were reported from *Baccharis retusa*(Asteraceae) and *Kalanchoe pinnata* (Crassulaceae) (Wink, 2012). Warifteine, a Bisbenzylisoquinoline alkaloid, isolated from *Cissampelos sympodialis* is also an attractive candidate as it induces changes in the parasite morphology and notwithstanding the fact that the plant is commonly being used in Brazilian folk medicine against various diseases (da Silva *et al.*, 2012). Allicin (diallyl thiosulphinate), extracted from garlic, has been tested for its anti-leishmanial activity. The low toxicity of mammalian cells of this compound has opened avenues for a combined therapy against leishmania infections (Jesus Corral-Caridad *et al.*, 2012).

A very promising phytochemical, peganine hydrochloride dihydrate was identified as an orally active antileishmanial lead molecule from the seeds of *Peganum harmala*. *P. harmala* Linn, commonly known as 'harmal' belonging to the family Zygophyllaceae, is one of the most important medicinal plants of India. Its different parts are used in traditional systems of medicine for the treatment of variety of human ailments. The potent compound showed a simple structure, easily synthesizable, non-toxic to hosts and induces apoptosis-like cell death in *L. donovani*. The binding interactions between compound 1 from *L. donovani* and DNA topoisomerase I in molecular modeling studies (docking) and experimental studies suggested that the apoptosis like cell death appears to be due to *L. donovani's* topoisomerase I inhibition by 1. Further work is under consideration to study the pharmacokinetic, pharmacodynamic and toxicological studies of compound 1 and also to synthesise the analogues of 1 to develop more potent anti-leishmanial agent than natural lead (Misra *et al.*, 2008).

4. Human African Trypanosomiasis

Human African trypanosomiasis (HAT) is a fatal, if untreated, fly-borne neuroinflammatory disease caused by protozoa of the species *Trypanosoma brucei* (*T.b.*). The increasing trend of HAT cases has been reversed, but according to WHO experts new epidemics of this disease could appear (Seke and Mahomoodally, 2012).

In sub-Saharan Africa, several Trypanosoma species cause important veterinary diseases, but only two cause significant suffering in man: *Trypanosoma b. gambiense* and *Trypanosoma b. rhodesiense*, respectively, causing chronic and acute sleeping sickness. Indeed, HAT is still a considerable burden for quality of life and economy in 36 sub-Saharan Africa countries with 15-20 million persons at risk. HAT may results in a 100% mortality rate if left untreated (Musuyu Muganza *et al.*, 2012). Following joint initiatives of WHO and private partners, the fight against HAT was re-engaged, resulting in considerable breakthrough. Although HAT has had a heavy toll on the health and economy of about 36 African countries, effective and satisfactory chemotherapy has not been found against the causative agents; *Trypanosoma brucei gambiense* which is responsible for chronic form of HAT in West and Central Africa, and *Trypanosoma brucei rhodesiense*, the etiological factor for the acute form of the disease in East Africa.

To this effect, the last few decades have witnessed a plethora of investigations which have been geared to investigate the effect of common traditionally-used medicinal plants in alleviating the cellular changes *in vivo* produced during the *T. b. brucei* infections of rats. A number of medicinal plants and secondary metabolites

isolated from them have been screened for anti-trypanosomal activity. Traditional knowledge was the basis for the selection of plants and one study included *Landolphia uniflora, Momordica balsamina* pulp, *Aloe vera* pulp, *Annona senegalensis, Securidaca longipenduculata* root and root bark. Based on folk medicines they were claimed to possess antiprotozoal activity and alleviate one or many of the clinical symptoms such as intermittent fever, immunosuppression, anemia, jaundice and hepatomegaly commonly associated with trypanosomiasis. Interestingly, it was found that these plants had the potential in the management of HAT due to *T. b. brucei*. *Momordica balsamina* and *S. longipenduculata* were found to possess the highest potential since they are able to control anemia by resisting sudden drop in packed cell volume values (Abubakar *et al.*, 2005).

Recently, Musuyu Muganza *et al.* (2012) reported *in vitro* antiprotozoal and cytotoxic activity of 33 ethonopharmacologically selected medicinal plants from Democratic Republic of Congo. It was found that most of the tested extracts exhibited pronounced ($IC_{50} \leq 5$ µg /ml) or good ($5 < IC_{50} \leq 10$ µg/ml) antiprotozoal activity against one or more of the selected protozoa. A total of 19 plant extracts inhibited *Trypanosoma b. brucei*, especially the extract from Isolona hexaloba stem bark (IC_{50} = 1.95 µg /ml, SI = 16.5); 8 plant extracts were active against *Trypanosoma cruzi*, the extracts from *Enanatia chlorantha* stem bark and *Quassia africana* root bark being the most active with IC_{50} values of 1.87 and 1.88 µg/ml, respectively (SI = 3.0 and 3.3, respectively); 8 plant extracts showed activity against *Leishmania infantum*, with extracts from *Napoleona vogelii* stem bark and *Quassia africana* root bark as the most active with IC_{50} values of 5.66 and 5.04 µg/ml (SI = 11.3 and 1.2). Finally, the authors reported 9 plant extracts inhibited *Plasmodium falciparum* K1 with the extracts from *Quassia africana* (root bark and stem bark) being the most active ones with IC_{50} values of 0.46 and 1.27 µg/ml (SI = 13.7 and 13.6). Extracts from *Enantia chlorantha* stem bark, *Piptadeniastrum africanum* stem bark and *Quassia africana* root bark were cytotoxic for MRC-5 cells ($CC_{50} < 10$ µg /ml).

In another study, extracts of *Hymenocardia acida* stem bark exhibited significant trypanocidal activity whereas *Gardenia erubescens* and *Lophira lanceolata* were effective at Minimum Inhibitory Concentration (MIC) of 20 mg/ml (Abu *et al.*, 2009). Nigerian plants were also evaluated *in vitro* for trypanocidal activity against *T.b. brucei* and *T. congolense* at concentrations of 4 mg/ml, 0.4 mg/ml and 0.04 mg/ml. It was found that extracts of *Khaya senegalensis, Piliostigma reticulatum, Securidaca longepedunculata* and *Terminalia avicennoides* were strongly trypanocidal to both organisms while extracts of *Anchomanes difformis, Cassytha spp, Lannea kerstingii, Parkia clappertioniana, Striga spp, Adansonia digitata* and *Prosopis africana* were trypanocidal to either *T. b. brucei* or *T. congolense*. *Kigelia africana*, from Kenya was also evaluated *in vivo* and was found that the dichloromethane fruits extract of *K. africana* tested at a dose of 2000 mg/kg was effective, curing 60% of the Swiss white mice that had previously been inoculated with *T. b. rhodesiense* KETRI 3798 (Atawodi *et al.*, 2003; Peter *et al.*, 2009).

In another investigation, *Scoparia dulcis* was evaluated on the population of immune cells during a 28 day experimental *T. brucei* infection in rabbits. The result obtained showed that infection resulted in an initial rise in both total WBC and the absolute number of circulating lymphocytes followed by a progressive decrease in total WBC and all WBC subtypes (lymphocytes, monocytes and granulocytes), although the percentage of lymphocytes remained consistently higher than normal throughout the study period. These changes were consistent with the development of trypanosome-induced immunosuppression in their mammalian host and interestingly, treatment with *S. dulcis* at a daily oral dose of 25 mg/Kg body weight was found to significantly reduce the severity of the observed lesions when compared with untreated infected animals. Thus *S. dulcis* was classified as a potential herb that had demonstrated significant potency in protecting against the parasite induced decrease in the population of immunologically active cells (Orhue *et al.*, 2009).

From the above key investigations, it is clear that these findings provide strong evidence of the potential beneficial effects of phytotherapy in the traditional management of trypanosomiasis, which could be subsequently developed into a cost effective alternative medicine to complement treatment of trypanosomiasis.

Nonetheless, several investigations tend to suggest that it is often difficult to probe the exact mode of action by which these plants extracts exhibit their trypanocidal action. Indeed, the possible mechanisms by which these plants extracts and phytochemicals therein carry out this role remain a subject of great speculations and debate in the scientific community. Several possible mechanisms working separately or in concert may account for the observed effect (Orhue *et al.*, 2009).

In one study, it was suggested that different phyto-compounds could be responsible and operate in a synergistic effect for the observed anti-trypanocidal activities. Interestingly, preliminary phytochemical screening of potent plants against trypanosome showed the presence of known bio-active compounds such as saponins, tannins, flavonoids and alkaloids in the crude plant extracts tested. Several authors have also identified or isolated tannins and phenolic compounds, flavonoids and alkaloids in plants that showed significant trypanocidal activities (Freiburghaus *et al.*, 1996). Active natural products include several groups of alkaloids, phenolics, saponins, cardiac glycosides, other terpenoids, and polyacetylenes (common in Apiaceae, Asteraceae and Araliaceae). Although some natural products are active in the sub-micromolar range and show good selectivity, only few have been studied *in vivo* in an animal model. None of these results have been translated into clinical practice (Wink, 2012).

Accumulated evidence also suggest that many natural products exhibit their trypanocidal activity by virtue of their interference with the redox balance of the parasites acting either on the respiratory chain or on the cellular defenses against oxidative stress. For instance, the observed trypanocidal activity of *K. africana* extract was

justified due to the increase of oxygen consumption and stimulation of hydrogen peroxide production in the protozoan cell. Trypanosoma do not have the same biochemical mechanism as mammalian cells for dealing with excess peroxide and consequent oxygen free radicals (Peter *et al.*, 2009). Furthermore, it is proposed that natural products possess structures capable of generating radicals that may cause peroxidative damage to trypanothione reductase that is very sensitive to alterations in redox balance. It is also known that some agents act by binding with the kinetoplast DNA of the parasite.

On the other hand, investigations undertaken by Freiburghaus *et al.* (1996) and Atawodi *et al.* (2003) have clearly indicated that different solvent extracts of the same plant may exhibit different trypanocidal activity just as extracts of different parts of the same plants. Therefore, the statement that a plant is trypanocidal or not should be taken within the context of the solvent used and the parts investigated. On the other hand, out of the 40 plant extracts tested by Nibret and Wink *et al.* (2010) and Nibret *et al.* (2010), the dichloromethane extract from stem bark of *Warburgia salutaris* (claimed to be used against many pathologies in many parts of Africa) was found to exhibit the most potent trypanocidal activity. The trypanocidal activity was suggested to be due to the drimane sesquiterpenoids (warburganal, polygodial). Concerning the mechanism, it was proposed that the two sesquiterpene aldehydes, warburganal and polygodial, formed covalent bonds with amino groups of proteins and affect a vast number of cellular activities. In another groundbreaking *in vitro* study, the authors were able to isolate the pregnane glycosides from genus *Caralluma* (*C. Penicillata, C. tuberculata* and *C. russelliana*) and evaluated for the trypanocidal activity. It was found that the penicilloside E possess the highest anti-trypanosoma activity followed by caratuberside C, which exhibited the highest selectivity index (Abdel-Sattar *et al.*, 2009).

Studies have shown that it is probable that the etiology of trypanosome-induced leucopenia in rabbit may be similar to the case with trypanosome-induced anemia. There has been striking indications that the onset of anemia in HAT may be strongly related to disruption of erythrocyte membrane caused directly by parasite attack on red cells. It has also been suggested that products secreted by the parasite may play a significant role in the disruption of red cell membrane.

Reduction in red cell membrane sialoglycoprotein secondary to elevated activity of plasma sialidases promotes the rapid destruction of erythrocytes. A role for parasite and macrophage-derived free radicals and proteases in the pathogenesis of trypanosome-induced anemia has also been postulated (Orhue *et al.*, 2009). The possibility that *S. dulcis* or certain components of the herb may help stabilize the membrane of blood cells cannot be out rightly dismissed. Specifically, the anti-oxidant or free radical scavenging properties of *S. dulcis*, may play vital roles in this regard especially against the backdrop of the role of free radicals in the pathogenesis of *T. brucei* infection. Furthermore, increased production of blood cells may help in replenishing of these cells. In the absence of any evidence of possible trypanocidal activity for the herb, it does not seem an attractive option to speculate that the higher level of immunological cells in treated animals could be due to the destruction of the parasite by agents native to the plant (Orhue *et al.*, 2009).

Trypanosomes lack catalase and glutathione peroxidases but have evolved a unique system with trypanothione to detoxify hydroperoxide. Trypanothione is built from two molecules of glutathione and one molecule of spermidine (Figure 2). Inactivation of the enzymes of trypanothione, spermidine or glutathione biosynthesis or of trypanothione directly will lead to death of the parasite. The synthetic drug eflornithine (see above) inhibits ornithine decarboxylase which is important for spermidine biosynthesis. It is likely that secondary metabolites which can bind to the SH-group of trypanothione exhibit anti-trypanosomal activity. This has been shown for polyacetylenes which carry a reactive triple bond that can easily alkylate SH groups; e.g., the polyacetylene Carlina oxide from *Carlina acaulis* (Asteraceae) (Figure 2) and polyacetylenes from ginseng (*Panax ginseng*) have significant cytotoxic activity against *T. b. brucei* but are hardly toxic to human cells.

On the other hand, (Adeyemi *et al.*, 2009) have showed that *Psidium guajava* leaf extract has trypanocidal properties and has attributed these effects in parts to the broad antimicrobial and iron chelating activity of flavonoids and tannins respectively. They have also proposed that iron chelation is an effective way of killing trypanosomes and the prime target is the enzyme, ribonucleotide reductase whose activity is central to DNA synthesis prior to cell division as depicted in trypanosomiasis infection.

Another target is DNA topoisomerase I; camptothecin, a known inhibitor of DNA Topo I also inhibits *T. brucei* with an IC_{50} of 1.5 μM. The aporphine alkaloid dicentrine which is present in Papaveraceae and Lauraceae inhibits DNA Topo II and is active against trypanosomes. Inhibitors of farnesyl transferase and tubulin polymerisation (e.g., vinblastine, sanguinarine) have substantial anti-trypanosomal activities (Wink, 2012).

Moreover, a plant with high *in vitro* trypanocidal activity may have no *in vivo* activity and vice versa, because of peculiarities in the metabolic disposition of the plant's chemical constituents. Therefore, plants found to be active in the above-mentioned investigations must be tested *in vivo* and tested clinically before a definite statement can be made on their trypanocidal potentials (Atawodi *et al.*, 2003).

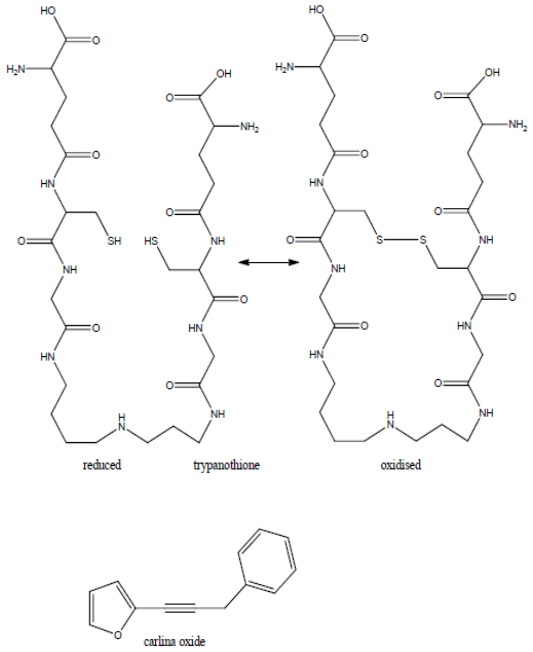

Figure 2. Reduced and oxidised form of trypanothione and carlina oxide which can block the SH-groups of trypanothione (Wink, 2012).

5. Conclusion And Future Perspectives

Evidently, many investigators indicate that traditional medicines might offer potential template molecules in the drug discovery process. Indeed, the discovery of compounds with anti-parasitic and antimicrobial activities from traditional medicinal plant remedies remains a medically and potentially challenging task. For malaria and trypanosomiasis quite a number of medicinal plants and isolated natural products such as alkaloids, phenolics, saponins, cardiac glycosides, and terpenoids, amongst others have already been tested, but for most of the other parasitic diseases such information is largely missing.

On the other hand, to adventure in such an endeavor, highly reproducible and robust innovative bioassays are needed in view of our limited understanding of the multi-factorial pathogenicity of such infectious diseases. To this effect, it is of utmost importance for investigators to embark and devise new automated bioassays with special emphasis on high through-put procedures that can screen and process data from a panoply of phytochemicals within shorter time lapse. Additionally, these procedures should also attempt to rule out false positive hits and de-replication methods to remove nuisance compounds. The ultimate goal will be to establish structure-activity phytochemical libraries to boost new antimicrobials drug discovery.

Nonetheless, despite continuous comprehensive and mechanism-orientated evaluation of medicinal plants worldwide, there is still a dearth of literature coming from the last decade's investigations addressing procedures to be adopted for quality assurance, authentication and standardization of crude plant products. Finally, above and beyond simple *in vitro* and *in vivo* assays, randomized controlled trials using standardized products or products containing pure plant extracts must be carried out and reported for each claim.

References

Abubakar A, Iliyasu B, Yusuf AB, Igweh AC, Onyekwelu NA, Shamaki BU, Afolayan DO and Ogbadoyi EO. 2005. Antitrypanosomal and haematological effects of selected Nigerian medicinal plants in Wistar rats. *Biokemistri*., **17**: 95-99.

Abu AH, Uchendu CN and Ofukwu R.A. 2009. *In vitro* anti-trypanosomal activity of crude extracts of some Nigerian medicinal plants. *J. Appl. Biosc*., **21**: 1277-1282.

Abdel-Sattar E, Shehab NG, Ichino C, Kiyohara H, Ishiyama A, Otoguro K, Omura S and Yamada H. 2009. Antitrypanosomal activity of some pregnane glycosides isolated from Caralluma species. *Phytomedicine*., **16**: 659-664.

Adeyemi OS, Akanji MA and Oguntoye SA. 2009. Ethanolic leaf extract of *Psidium guajava*: Phytochemical and trypanocidal activity in rats infected with *Trypanosoma brucee brucei. J. Med. Plants Res.,* **3**: 420-423.

Anon. 2008. World Health Organisation. www.who.int/mediacentre/factsheet/fs134/en

Atawodi SE, Bulus T, Ibrahim S, Ameh DA, Nok AJ, Mamman M and Galadima M. 2003. *In vitro* trypanocidal effect of methanolic extract of some Nigerian savannah plants. *Afri. J. Biotechnol.,* **2**: 317-321.

Bashar S, Hassan A and Omar S. 2005. Tradition and Perspective of Arab Herbal Medicine: A Review. *eCAM*, 1-5.

Bashar S, Hassan A and Omar S. 2008. Arab Herbal Medicine. In: Ronald R. Watson and Victor R Preedy (Eds), Botanical Medicine in Clinical Practice, CABI, UK.

Boye GL, Bugyei K, Addy ME, and Ofori-Adjei D. 2002. Clinical efficacy of Phyto-Laria, a teabag formulation of *C. sanguinolenta* root, The Food and Drugs Board, Medical School, University of Ghana.

Cimanga K, De Bruyne T, Pieters L, and Vlietinck AJ.1997. *In vitro* and *in vivo* antiplasmodial activity of cryptolepin and related alkaloids from *Cryptolepis sanguinolenta*. *J. Nat. Prod.*, **60**: 688-669.

Coker R, Atun R, and McKee M. 2008. Health Systems and the Challenge of Communicable Diseases Experiences from Europe and Latin America. McGraw-Hill Education, Open University Press. England.

Cordell G. 2000. Biodiversity and drug discovery - a symbiotic relationship. *Phytochemistry.,* **55**: 463-480.

Cragg G and Newmann GM. 2007. Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod*., **70**: 461-477.

da Silva E, Dias Rayol C, Lys Medeiros P, Figueiredo R, Piuvezan M, Brabaso-Filho J, Fernandes Marinho A, Silva T, Militao G, Pimentel Cassilhas A, and Paes de Andrade P. 2012. Study of the activity of 3-benzyl-5-(4-chloro-arylazo)-4-thioxo-imidazolidin-2-one against *Schistosomiasis mansoni* in Mice. *Scientific World J.,* **2012: 520524.**

de Oliviera A, Mesquita JT, Tempone AG, Lago JH, Guimaraes EF and Kato MJ. 2012. Leishmanicidal activity of an alkenylphenol from piper malacophyllum is related to plasma disruption. *Exp. Parasitol.,* **132**: 383-387.

Fotie J, Bohle DS, Olivier M, Gomez MA, and Nzimiro S. 2007. Trypanocidal and antileishmanial dihydrochelerythine derivatives from *Garcinia lucida*. *J. Nat. Prod.,* **70**: 1650-1653.

Freiburghaus F, Kaminsky R, Nkunya MH, and Brun R. 1996. Evaluation of African medicinal plants for their *in vitro* trypanocidal activity. *J. Ethnopharmacol*., **55**: 1-11.

Gathirwa JW, Rukunga GM, Njagi ENM, Omar SA, Guantai AN, Mathaura CN, Mwitari PG, and Ndiege IO. 2007. *In vitro* antiplasmodial and *in vivo* antimalarial activity of some plants

traditionally used for the treatment of malaria by the Meru community in Kenya. *J. Nat. Med*., **61**: 261-268.

Heinrich M, Barnes J, Gibbons S and Williamson EM. 2004. Fundamentals of Pharmacognosy and Phytotherapy. Churchill Livingstone, Elsevier Science Ltd., UK

Islamuddin M, Farooque A, Dwarakanath B, Sahal D and Afrin F. 2012. Artemisia annua leaves and seeds mediate programmed cell death in *Leishmania donovani*. *J. Med. Microbiol*., **61**: 1709-1718.

Jansen O, Tits M, Angenot L. Nicolas J, De Mol P, ikiema J and Frédérich M. 2012. Anti-plasmodial activity of *Dicoma tomentosa* (Asteraceae) and identification of urospermal A-15-O-acetate as the main active compound. *Malaria J.* **11**: 289 doi:10.1186/1475-2875-11-289.

Jesus Corral-Caridad M, Moreno I, Torano A, Dominquez M and Alunda JM .2012. Effect of allicin on promastigotes and intracellular amastigotes of *Leishmania donovani* and *L. infantum*. *Exp. Parasitol*., **132**: 475-482.

Khaliq T, Misra P, Gupta S , Reddy KP, Kant R, Maulik PR, Dube A and Narender T. 2009. Peganine hydrochloride dihydrate an orally active antileishmanial agent. *Bioorg. Med. Chem. Lett.,* **19**: 2585-2586.

Khalafalla M, Abdellatef E, Daffalla HM, Amr A. Nassrallah AA, Aboul-Enein KM, David A. Lightfoot DA, Cocchetto A and El-Shemy HA. 2009. Antileukaemic activity from root cultures of *Vernonia amydalina*. *J. Med. Plants Res.*, **3**: 556-562.

Krishna S, Woodrow CJ, Staines HM, Haynes RK and Mercereau-Puijalon O. 2006. Re-evaluation of how artemisinins work in light of emerging evidence of *in vitro* resistance. *Trends Mol. Med*., **12**: 200-205.

Mahabusarakam W, Kuaha K, Wilairat P and Taylor WC. 2006. Prenylated xanthones as potential antiplasmodial substances. *Planta Med.,* **72**: 912-916.

Misra P, Khaliq T, Dixit A, SenGupta S, Samant M, Kumari S, Kumar S, Kushawaha P, Majumder H, Saxena A, Narender T, and Dube A. 2008. Antileishmanial activity mediated by apoptosis and structure-based target study of peganine hydrochloride dihydrate: an approach for rational drug design. *J. Antimicrob. Chemther*., **62**: 998-1002.

Musuyu Muganzaa D, Fruthb BI, Nzunzu Lamia J, Mesiaa GK, and Kambua, OK. 2012. *In vitro* antiprotozoal and cytotoxic activity of 33 ethnopharmacologically selected medicinal plants from Democratic Republic of Congo. *J. Ethnopharmacol*., **7**: 301-308.

Muthaura CN, Keriko JM, Derese S, Yenesew A, Rukunga GM. 2011. Investigation of some medicinal plants traditionally used for treatment of malaria in Kenya as potential sources of antimalarial drugs. *Exp. Parasitol*., **3:** 609-626.

Nibret E. and Wink M. 2010. Volatile components of four Ethiopian *Artemisia* species extracts and their *in vitro* antitrypanosomal and cytotoxic activities. *Phytomedicine*., **17**: 369-374.

Nibret E, Ashour ML, Rubanza CD, and Wink M. 2010. Screening of some Tanzanian medicinal plants for their trypanocidal and cytotoxic activities. *Phytother. Res*., **24**: 945-947.

Ntutela S, Smith P, Matika L, Mukinda J, Arendse H, Allie N, Estes DM, Mabusela W, Folb P, Steyn L, Johnson Q, Folk WR, Syce J and Jacobs M. 2009. Efficacy of Artemisia afra Phytotherapy in experimental tuberculosis. *Tuberculosis*., **89**: S33-S40.

Nunkoo D and Mahomoodally F. 2012. Ethnopharmacological survey of native remedies commonly used against infectious diseases in the tropical island of Mauritius. *J. Ethnopharmacol*., **28**: 548-564.

Nhigashi H, Huffman M and Izutsu D.1994. Towards the chemical ecology of medicinel plant use in chimpanzees: the case of Vernonia amygdalina, a plant used by wild chimpanzees possibly for parasite-related diseases. *J. Chem. Ecol*., **20**: 541-553.

Orhue N, Nwanze E and Okafor A. 2009. *Scoparia dulcis* protects against *Trypanosoma brucei* induced immunosuppression in experimentally infected rabbits. *Afri. J. Food Sci*., **3**: 172-175.

Peter O, Magiri, E., Auma J, Magoma G, Imbuga M and Murilla G. 2009Evaluation of in vivo antitrypanosomal activity of selected medicinal plant extracts. *J. Med. Plants Res*., **3**: 849-854.

Phillipson D and Wright C. 1991. Can ethnopharmacology contribute to the development of antimalarial agents? *J. Ethnopharmacol.,* **32**: 155-165.

Riscoe M, Kelly JX and Winter R. 2005. Xanthones as antimalarial agents: discovery, mode of action, and optimization. *Curr. Med. Chem*., **12**: 2539-2549.

Rondon FC, Bevilaqua CM, Accioly MP, Andrade-Junior HF, Lima JC and Magalhaes HC. 2012. *In vitro* efficacy of *Coriandrum sativum, Lippia sidoides* and *Copaifera reticulata* against *Leishmania chagasi. Rev. Bras. Parasitol. Vet.,* **21**: 185-191.

Seke P and Mahomoodally F. 2012. New insights in staging and chemotherapy of African trypanosomiasis and possible contribution of medicinal plants. *Scientific World J*., **2012**: 436039.

Sen R, Bandyopadhyay S, Dutta A, Mandal G, Ganguly S, Saha P and Chatterjee M. 2007. Artemisinin triggers induction of cell-cycle arrest and apoptosis in Leishmania promastigotes. *J. Med. Microbiol.,* **56:** 1213-1218.

WHO 2005. Leishmaniasis background information. http://www.who.int/leishmanisis/en/

WHO 2010. World Malaria Report 2010, Available at http://who.int/malaria/worldmalariareport2010/worldmlariareport .pdf.

Willcox ML and Bodeker G. 2004. Traditional herbal medicines for malaria. *BMJ*., **329**:1156-1159.

Wink M. 2012. Medicinal Plants: A source of anti-parasitic secondary metabolites. *Molecule.,* **17**:12771-12791.

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Sodium Azide Induced Complementary Effect of Chromosomal Stickiness in *Brassica campestris* L.

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Abstract

Present investigation reports the mutagenic efficacy of sodium azide with a view to study its effects on meiotic behavior and phenotypic quantitative traits of *Brassica campestris* L. Seeds were treated with three concentration of sodium azide i.e. 0.3%, 0.5% and 0.7%, along with control. Morphological/phenotypic parameters studied were plant height, number of primary branches, number of secondary branches, seeds/siliqua, husk yield, seed yield and 1000-seed weight. All these traits showed the significant positive shifts in mean at 0.3% and 0.7% doses of azide as compared to control and 0.5% dose. However, cytogenetic assessment of the treated plants clearly revealed the preponderance of stickiness distributed in all the phases of meiotic division that persisted up to the tetrad stage. The phenomenon displayed mild to severe case of stickiness on the basis of number of genome involved during meiosis. Manifestation of stickiness has also been resulted in several other meiotic abnormalities such as bridge (1.88%), unorientation (0.64%), micronuclei (1.38%), asynchronous division (0.77%), and precocious chromosomes (3.89%) along with some other anomalies (laggards, univalents etc.). Frequency of these anomalies along with severe stickiness was higher at 0.5% dose of azide that must have compromised with pollen fertility resulting declined fertility rate. Though, pollen fertility at 0.3% and 0.7% doses had not been much affected and had fallen near control. Thus, from present investigation inference can be drawn that the sodium azide induced stickiness might have resulted in some meiotic mutant that has imposed some novel genetic combinations thereby positively affecting morphological traits of the crop.

Key Words: *Brassica campestris* L.; Mutation Breeding; Pollen Fertility; Sodium Azide; Stickiness

1. Introduction

Economies all over the world understand that rapid urbanization and industrialization are the surest ways of achieving accelerated economic growth (Oliveria, 2011). However, these cause many other problems such as population explosion, lack of agriculture field and environmental pollution that ultimately results in an increasing food scarcity. There is a growing food shortage problem in many areas around the world and is becoming a matter of worldwide concern. Thus, to overcome the drastic situation of food shortage and increasing world food security, mutation breeding is an effective, inexpensive and dependable approach. Since food crop varieties embedded with various induced mutations have contributed to the significant increase of crop production (Kharkwal and Shu, 2009). It paves a path to induce genetic variability in some economic important self pollinated crops where crossing/hybridization is quite difficult viz. wheat **(**Srivastava *et al.*, 2011**)** and fenugreek **(**Chaudhary and Singh, 2001; Basu *et al.*, 2008). Hence, utilization of mutagenesis, undoubtedly, capable of increasing genetic variability in number of crops as reported by (Mahandjiev *et al.*, 2001; Tai *et al.*, 2007; Khan and Goyal, 2009; Kozgar *et al.*, 2011; Srivastava *et al.*, 2011). Mutations are the tools that being used to study the nature and function of genes which are the building blocks and basis of plant growth and development thereby, producing new raw materials for genetic improvement of economic important crops (Adamu and Aliyu, 2007). Till to date, several mutagens have been known to us that are used in mutation breeding and proved to be valuable in the achievement of crops with beneficial and desired traits such as high yield and resistant mutant (Mahmoud *et al.*, 2006; Tomer *et al.*, 2007; Basu *et al.*, 2008; Srivastava *et al.*, 2011).

Chemical mutagenesis is considered as an effective means in improving the yield and quality traits of crop plants (Srivastava *et al.*, 2011). Among numerous alkylating chemical mutagens such as ethyl methane sulphonate (EMS), sodium azide, hydrazine hydrate etc., sodium azide had been and is still used as potent mutagens in variety of crop improvement programs. Its mutagenic potential has been reported in several

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screening assays (Raicu and Mixich, 1992; Grant and Salamone, 1994; Srivastava and Kapoor, 2008; Srivastava *et al.*, 2011). Notwithstanding the potency, sodium azide is marginally mutagenic in humans and animals (Sadiq and Owais, 2000) thus safe to use.

Brassica is the important oilseed crops throughout the world which rank third among the oilseed crops after soybean and oil palm in production of vegetable oils (Kauser *et al.*, 2006). It is the most economically important genus in the Brassicaceae family (syn. Cruciferae) which contains little fat, and is sources of vitamins, minerals, and fiber (Cardoza and Stewart, 2004). The plant is also used to produce a high quality protein and after extraction of this oil, the residual high protein meal can be soaked in water and fed to cattle (Downey, 2003).

Thus, considering the above points in mind, the present study was performed to appraise the mutagenic effectiveness and efficacy of sodium azide on the chromosomal behavior and morphological characters of *Brassica campestris* L. Also, it was aimed to obtain some desirable mutant lines.

2. Materials and Methods

2.1. Seeds Procurement and Treatment

Inbred seeds of cultivar *Brassica campestris* L. accession number-IC363713 were obtained from National Bureau of Plant Genetic Resources (NBPGR), New Delhi. Seeds were pre-soaked for 6 h in distilled water and later blotted, dried. Thereafter dried seeds were treated with freshly prepared sodium azide (NaN_3) solution for three doses (0.3% 0.5% and 0.7%, w/v) for 5 h at room temperature (26±2ºC) with intermitted stirring (Srivastava *et al.,* 2011). Meanwhile, some seeds were kept in distilled water to maintain control sets. Seeds were washed thoroughly with running water and blotted with filter paper in separate petri plates each and sown in nursery pots in triplicates.

2.2. Meiotic Preparation

Unopened flower buds of *Brassica campestris* L. were collected in vials from adult plants at an early winter's morning. Buds were fixed in glacial acetic acid – alcohol (1:3, v/v) for 24 h at room temperature and transferred to 70% alcohol, refrigerated at 4ºC until use. The anthers were squashed in 2% standard aceto-carmine stain (Fürste, 1962) and the slides were covered with cover slips following gentle tapping. After preparation slides were observed under optical microscope (Olympus CH20i) and photomicrographs of pollen mother cells (PMCs) were made using pinnacle PCTV capture device. Habib *et al.* (2004) reported that chromosomes of *Brassica* species are very small and their identification through the ordinary cytogenetic techniques is extremely difficult. Thus, meiotic analysis of *Brassica campestris* L. has become really complicated yet it was done by the preparation of large number of slides and their thorough selection for each stage of meiosis. Ten slides per plant were prepared and 10fields/slide was cytologically tested. Morphological observations were made in three replicates per dose whereas five plants per replicate were randomly sampled along with control. Frequency of the meiotic abnormalities was documented and pollen fertility was estimated using a glyceroacetocarmine (1:1) mixture as well.

2.3. Statistical Analysis

Variations of the different studied morphological parameters were subjected to one-way variance analysis (ANOVA) and Duncan's test *($p<0.05$)* using Statiatica-8 software (Stat Soft).

3. Results

3.1. Chromosomal Study

Cytological study revealed the normal course of meiotic chromosomes comprised 10 bivalents at diakinesis (2n=20) and its segregation into 10:10 at anaphase (Figures A, B). While treated sets shared an array of abnormalities distributed in all the phases of reduction division namely stickiness (Figures C-L), Unorientation-0.64%, (Figures F, G, I, L), micronuclei-1.38%, precocious chromosomes-3.89% (Figure J), incomplete bridge-1.88% (Figure L) and asynchronous division-0.77%. Some other anomalies such as laggards (Figure G), univalents and picnotic nuclei have also been registered in low frequency. However, among the all anomaly observed, the most prominent anomaly documented was stickiness, found to be distributed in various phases of meiosis i.e. metaphase-I/II to anaphase-I/II. It ranged from mild to severe on basis of number of genome involved. Mild stickiness has been documented at 0.3% and 0.7% doses in which few chromosomes were involved that might be resulted in to aneuploids bearing desirable traits. While intense or severe case of stickiness was found at the medium dose of azide i.e., 0.5% (Table 1). The latter has resulted in clumping or completely intermingling of genome due to which chromosome has lost its distinctiveness completely and resulting genome damage. Whereas occurrence of 0.03% stickiness at control might be due to environmental factor (Table 1).

Despite stickiness, many other abnormalities have been recorded which ranged from 0.03% to 12.88%, where the highest dose has lesser values of aberrations as compared to 0.5%. Further, pollen fertility recorded in 0.3% and 0.7% doses was found to be 96.68% and 94.63%, respectively as compared to control which possessed 99.81% pollen fertility rate. However, the intermediate dose registered a sharp fall in its mean i.e. 73.58% (Table 1).

3.2. Morphological Variability

In present investigation various doses of sodim azide have considerably affected some phenotypic traits of the present crop. Phenotypic characters used to assess the effects of mutagen were plant height, number of primary branches, number of secondary branches, number of seeds/siliqua, husk yield, seed yield and 1000-seed weight. Table 2 clearly depicted that the 0.3% and 0.7% doses of sodium azide were significant and found to be in positive correlation with higher value of number of branches, seeds/siliqua, husk yield, seed yield and 1000-

seed weight as against controls and 0.5% dose of azide. More surprisingly, it was recorded that the lowest as well as the highest doses were promising in inducing desirable traits in the present crop. On the other hand, 0.5% dose was negatively correlated with yield related traits with the greatest share of meiotic aberration (Table 1). As far as plant height is concerned, treated plants were significantly shorter as compared to control ($p<0.05$), which is in fact a desirable trait. Since it prevents plants from lodging as lodging damage might result in reducing the yield capacity of crop.

4. Discussion

The prime strategy in mutation breeding has been to upgrade the well-adapted plant varieties by altering one or two major traits which limit their productivity or enhance their quality (Srivastava *et al.*, 2011). Thus, in view of the above statement, it has found that sodium azide has well suited for mutation breeding since the mutagenic effects of azide appear soon after sowing the seeds and can be observed by naked eyes (Srivastava *et al.*, 2011). Moreover, due to its ease in availability and in being reasonably priced, could be effortlessly utilized in mutation breeding program. Many researchers have been exploited the mutagenic effectiveness of sodium azide in different agronomic crops such as *Triticum aestivum*, *Trigonella foenum-graecum*, *Nigella sativa* and *Plantago ovate* (Srivastava and Kapoor, 2008; Prabha *et al.*, 2010 a, b; Srivastava *et al.*, 2011).

Cytological analysis provides a genetic basis for chromosomal behavior at different stages of cell cycle and hence provides an authentic mean to determine the efficiency of mutagens. Mutagens may cause error in the normal behavior of chromosome. Hence, any disturbance in normal cytological behavior of chromosomes (either positive or negative) reflects in phenotypic traits of plants. As in the present case, positive effects of mutagens have been displayed by the obtainment of meiotic sticky mutant having enviable trait(s) at 0.3% and 0.7% doses of chemical mutagen. Meiotic observations presented in Table 1 clearly showed the dominance of chromosomal stickiness at all the doses of azide. However it had also taken together with meiotic manifestation mainly at 0.5% dose resulting marked reduction in pollen fertility.

Sticky chromosomes were first reported in maize by Beadle (1932), and he attributed such irregularity to a mutation caused by a recessive gene called sticky (*st*). Bione *et al.* (2000) reported that the phenotypic manifestation of stickiness may vary from mild, when only a few chromosomes of the genome are involved, to intense that may involve the entire genome. In the present case, prevalence of stickiness at medium dose (0.5%) could be arisen from improper folding of the entire chromosomes into single chromatids and chromosomes, as a result of which chromatin fibers intermingled (Klasterskii *et al.*, 1976) hence causing genome damage. Such genome damage could be the reason for decreased pollen fertility at this particular dose. However, mild stickiness registered at two optimal doses (0.3% and 0.7%) might be resulted in aneuploids bearing peculiar and enviable genomic constitution resulted in higher mean value of some quantitative parameters over control.

Occurrence of mild stickiness at utmost dose (0.7%) could be due to the effect of azide that might have induced meiotic mutant having beneficial traits over 0.5% dose. Plenty of reports, however, are available which showed the stimulatory effects of lower dose of treatment (Luckey, 1980; Kim *et al.*, 2004) and similar finding has been reported in present case as well.

Table 1. Effect of sodium azide on meiotic courses of *Brassica campestris* L.

Conc.%	TCO	% stickiness at metaphase (M)		% stickiness at anaphase (A)		Oth.Ab.%	Pollen fertility %
		I	II	I	II		
Control	1021	-	-	-	-	0.03	99.81 ± 0.07*
0.3	1005	1.19±0.11*	1.21 ± 0.10	1.04 ± 0.09*	0.85 ± 0.18	3.57	96.68 ± 0.97
0.5	918	10.45±0.29	10.20 ± 0.35	9.04 ± 0.19	7.31 ± 0.20	12.88	73.58 ± 2.45
0.7	922	4.17 ± 0.28	5.04 ± 0.33	4.17 ± 0.10	3.53 ± 0.32	7.28	94.63 ± 1.20

TCO- total cell observed, Oth. Ab.- other meiotic anomaly (laggards, precocious chromosomes, bridge etc.)
*Mean±SE

Table 2. Effect of sodium azide on some yield attributing traits of *Brassica campestris* L.

Conc. %	Plant height (m)*	No. of primary branches*	No. of secondary branches*	Seeds/siliqua*	Husk yield(g)*	Seed yield(g)*	1000-seed weight(g)*
Control	1.57±0.38^{b}	4.0±0.77^{a}	7.4±1.43^{a}	19.7±1.52^{b}	14.3±0.63^{b}	15.57±0.46^{c}	2.50±0.04^{b}
0.3	1.30±0.67^{a}	5.8±0.66^{b}	13.0±2.48^{c}	21.8±1.29^{c}	17.7±2.72^{c}	16.43±2.24^{c}	3.28±0.21^{d}
0.5	1.22±0.38^{a}	3.75±0.37^{a}	8.0±0.63^{ab}	19.5±1.71^{a}	11.9±1.70^{a}	11.44±2.87^{a}	2.24±0.10^{a}
0.7	1.32±0.11^{a}	5.75±0.74^{b}	8.75±1.93^{b}	20.0±0.64^{a}	12.3±2.52^{a}	13.82±1.87^{b}	2.83±0.05^{c}

*Mean±SE, For a given means within each column of each section followed by the different lowercase letter are statistically significant at $p < 0.05$.

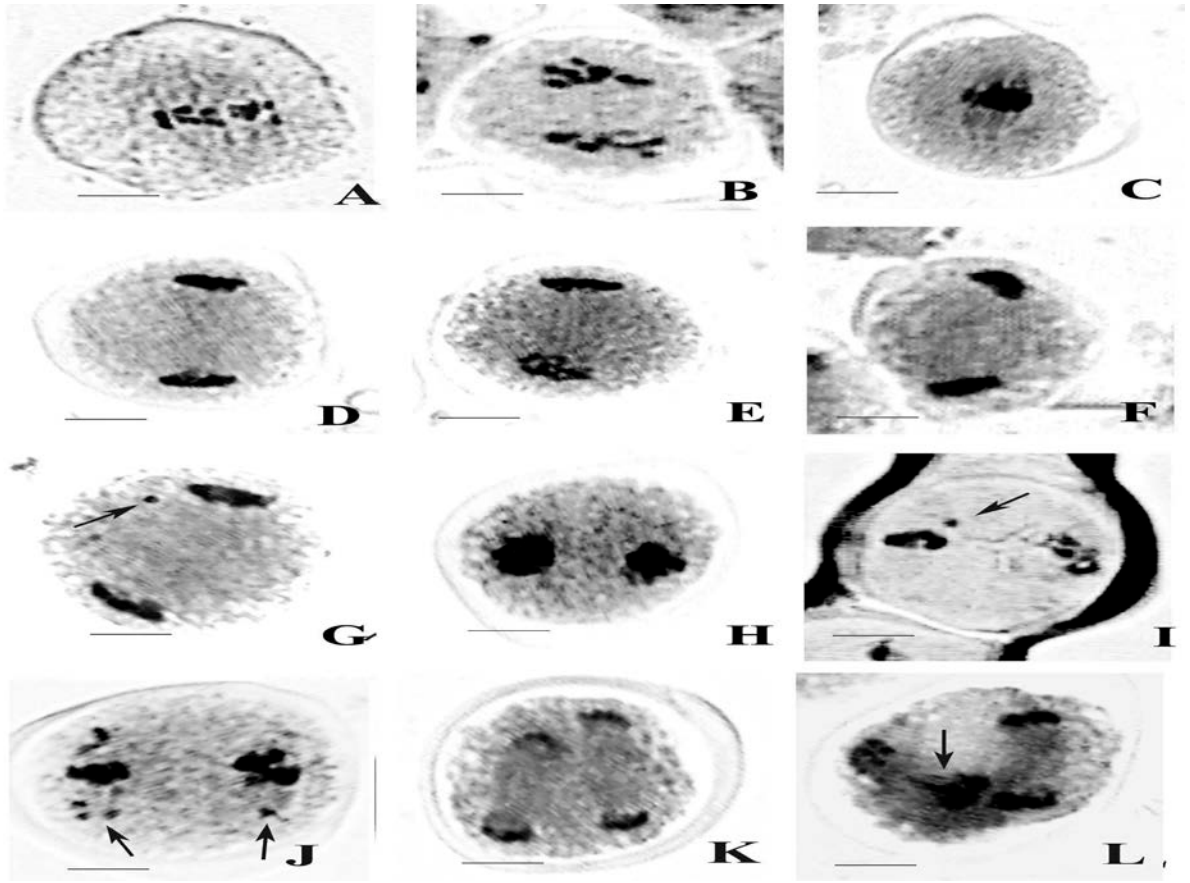

Figure 1. A. Normal metaphase (n=10); **B**. normal anaphase (10:10); **C-** stickiness at metaphase-I; **D-** stickiness at anaphase-I; **E**. stickiness at only one pole of anaphase-I; **F**. intense unoriented sticky mass of chromosomes observed at both pole of anaphase-I; **G**. unoriented sticky mass of chromosomes with laggard; **H**. clumping at metaphase-II; **I**. genome elimination with sticky metaphase-II; **J.** multi precocious condition at metaphase-II; **K.** stickiness at anaphase-II; **L.** unoriented sticky anaphase-II with incomplete bridge.(40×, scale bar=4.2µm).

Several agents have been reported to cause chromosome stickiness, including physical mutagens (Rao and Rao, 1977; Al Achkar *et al.*, 1989), temperature (Eriksson, 1968), and chemicals (Kumar and Singh, 2002; Srivastava and Kapoor, 2008; Kumar *et al.*, 2010). Proper interpretation for the occurrence of stickiness is still yet to be known, however, according to Pagliarini (2000), it may be of either hereditary, caused by mutation in the structural genes coding for them or induced by the direct action of mutagens. Recent reports suggest that chromosome stickiness may be under genetic control, or rather, it may be controlled by a single pair of genes, two pairs of genes or by the interaction of several genes which may be recessive or dominant (Kiihl *et al.*, 2011) resulting into meiotic mutants. Present study also contradict the finding of Kumar and Singh (2002), who noticed the detrimental impact of stickiness on meiotic course and pollen fertility provoked by another chemical mutagen EMS.

During morphological studies, positive impact of some specific doses of chemical mutagens has been verified by assaying some phenotypic traits. Result shows the considerable decrease in plant height in treated sets over control that is in fact desirable as it prevents crop from lodging damage. Since due to lodging, falling of the crops occurs at the time of harvesting, which results in the significant reduction in the yield due to stem breakage at the ground level (Islam and Evans, 1994).

In most of the cases, relationship of different quantitative traits is not positive therefore, difficult to manipulate through mutation breeding yet a trait, number of seeds per siliqua is inherited monogenically and therefore easy to manipulate (Sinhamahapatra *et al.*, 2010). However, in present case this particular trait has been efficiently manipulated by mutation breeding experiment along with some other important parameters as presented in Table 1.

Conclusively, on the basis of our observations, it is worth suggesting that the azide has induced some sticky mutant that had definitely provoked some advantageous variations at gene level which affected the yield and yield attributed traits through mutagenesis. Thus, these meiotic mutants induced via sodium azide, signifying a complementary and regulatory effect of stickiness during microsporogenesis of *Brassica campestris* L. beyond the some instances of impaired male meiosis.

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References

Adamu A K and Aliyu H. 2007. Morphological effects of sodium azide on tomato (*Lycopersicon esculentum* Mill). *Sci World J.,* **2(4):** 9-12.

Al Achkar W, Sabatier L and Dutrillaux B. 1989. How are sticky chromosomes formed? *Annu. Genet*., **32**: 10-15.

Basu SK, Acharya SN and Thomas JE. 2008. Genetic improvement of fenugreek (*Trigonella foenum-graecum* L.) through EMS induced mutation breeding for higher seed yield nuder wesrern Canada prairie conditions. *Euphytica*, **160:** 249-258.

Beadle GW. 1932. A gene for sticky chromosomes in *Zea mays*. *Indukt. Abstamm. Vererbungle*, **63(1):**195-217.

Bione NCP, Pagliarini MS, DE and Toledo JFF. 2000. Meiotic behavior of several Brazilian soybean varieties. *Genetics and Mol Biol.*, **23(3):** 623-631.

Cardoza, V and Stewart Jr, CN. 2004. *Brassica* biotechnology: progress in cellular and molecular biology. *In vitro* cell. *Dev. Biol. Plant*., **40**: 542–551.

Chaudhary AK and Singh VV. 2001. Inheritance pattern and growth habit in fenugreek (*Trigonella foenum-graecum* L.). *Indian J Genet Plant Breed*, **61(4):**369-371.

Downey RK. 2003. Mustard. In: Katz SH and Weaver WW. **Encyclopedia of Food and Culture**. Gale virtual reference library. New York: Scribner. ISBN 0684314169.

Eriksson G. 1968. Temperature response of pollen mother cells in Larix and its importance for pollen formation. *Stud. For. Suec*, **63**: 121-132.

Fürste K. 1962. Welsches Weidelgras (*Lolium multiforum*)-Tetraploid, polyploidisierung, züchterische Bearbeitung und vergleichende Untersuchungen mit Diploiden. *Zeitsch. Pflanzenzucht*, **47**:369-387.

Grant WF and Salamone M. 1994. Comparative mutagenicity of chemicals selected for test in the international program on chemical safety collaborative study on plant systems for the detection of environmental mutagens. *Mutation Res*., **310**: 187-209.

Habib A, Shahida H and Khan A. (Jul - Dec, 2003) & 3-4 (Jan - Jun, 2004). Genome biology of the cultivated *Brassica*. *Quarterly Science Vision*, **9**: 1-2.

Islam N and Evans EJ. 1994. Influence of lodging and nitrogen rate on the yield attributes of oilseed rape *(Brassica napus* L.). *Tag Theoretical and Applied Genetics*, **88(5)**: 530-534.

Kauser R, Athar HR and Ashraf M. 2006. Chlorophyll florescence: a potential indicator for rapid assessment of water stress tolerance in canola (*Brassica napus* L.) *Pak. J. Bot*., **38(5):** 1501-1510.

Khan S and Goyal S. 2009. Improvement of mungbean varieties through induced mutations. *Afr J Plant Sci*, **3**: 174-180.

Kharkwal MC and Shu QY. 2009. The role of induced mutations in world food security. In: Shu QY (Ed.) **Induced Plant Mutations in the Genomics Era**, Food and Agriculture Organization of the United Nations, Rome, Italy, 33-38.

Kiihl PRP, Pereir ARA, DE Godoy SM, Colauto Stenzel NM and Risso-Pascotto C. 2011. Chromosome stickiness during meiotic behavior analysis of *Passiflora serrato-digitata* L.(Passifloraceae). Ciência Rural, *Santa Maria*, **41(6)**: 1018-1023.

Kim JS, Baek MH, Lee YK, Lee HY and Park YI. 2004. Stimulating effect of low dose gamma-ray radiation on the growth and physiological activities of Chinese cabbage cultivars. Proceedings of the 12th international congress on photosynthesis, Brisbane 18–24 August 2001, CSIRO Publishing, doi:10.1071/SA0403244

Klasterskii I, Natarajan AT and Ramel C. 1976. An interpretation of the origin of subchromatid aberrations and chromosome stickiness as a category of chromatid aberrations. *Hereditas*, **83**: 153.

Kozgar MI, Goyal S and Khan S. 2011. EMS induced mutational variability in *Vigna radiata* and *Vigna mango*. *Res J Bot*, **6**: 31-37.

Kumar G and Singh V. 2002. EMS induced chromosomal stickiness in *Hordeum valgare*. *The Nucleus*, **45(3):** 139-142.

Kumar S, Arya SK, Roy BK and Singh AK. 2010. The effects of 2,4-dichlorophenoxy acetic acid and isoproturon herbicides on the mitotic activity of wheat (*Triticum aestivum* L.) root tips. *Turkish J Biol*., **34**: 55-66.

Luckey TD. 1980. **Hormesis with Ionizing Radiation**. CRC press, Inc.Boca Raton.

Mahandjiev A, Kosturkova G and Mihov M. 2001. Enrichment of *Pisum sativum* gene resources through combined use of physical and chemical mutagens. *Israel J of Plant Sci*., **49 (4):** 279-284.

Mahmoud AA and AL-Twaty N. 2006. Effect of gamma irradiation and sodium azide on some economic traits in tomato. *Saudi J Biological Sci*., **13(1):** 44-49.

Oliveria. 2011. Rapid urbanization and its effects on the environment, http://socyberty.com/issues/rapid-industrialization-and-its-effects-on-the-environment.

Pagliarini M S. 2000. Meiotic behavior of economically important plant species: the relationship between fertility and male sterility. *Genetics and Mol Biol*., **23(4):** 997-1002.

Prabha R, Dixit V and Chaudhary BR. 2010a. Comparative spectrum of sodium azide responsiveness in plants. *American-Eurasian J Agric & Environ Sci,* **8(6):** 779-783.

Prabha R, Dixit V and Chaudhary BR. 2010b. Sodium azide-induced mutagenesis in fenugreek (*Trigonella foenum graecum* linn). *Legume Res*, **33(4):** 235 – 241.

Raicu P and Mixich F. 1992. Cytogenetic effects of sodium azide encapsulated in liposomes on heteroploides cell cultures. *Mutation Res*., **283**: 215-219.

Rao PN and Rao RN. 1977. Gamma ray induced meiotic chromosome stickness in tomato. *Theor Appl Genet*, **50**: 247-252.

Sadiq MF and Owais WM. 2000. Mutagenicity of sodium azide and its metabolite azidoalanine in *Drosophila melanogaster*. *Mutation Res*., **469**: 253-257.

Sinhamahapatra S P, Raman RB, Roy S, Roy US, Raut NM and Ashok K.V, 2010. Breeding for an ideal plant type in yellow sarson *(Brassica rapa* L. yellow sarson). *Electronic J of Plant Breeding,* **1(4):**689-694.

Srivastava A and Kapoor K. 2008. Seed yield is not impaired by chromosome stickiness in sodium azide treated *Trigonella foenum-graecum*. *Cytologia*, **73(2)**: 115-121.

Srivastava P, Marker S, Pandey P and Tiwari ZDK, 2011. Mutagenic effects of sodium azide on the growth and yield characteristics in wheat (*Triticum aestivum* L. em. Thell). *Asian J Plant Sci.*, **10(3):** 190-201.

Tah PR. 2006. Induced macromutation in mungbean (*Vigna radiata* (L.) Wilczek]. *Int J Bot*, **2**: 219-228.

Tai YS, Bragg J and Meinhardt SW. 2007. Functional characterization of toxa and molecular identification of its intracellular targeting protein in wheat. *Am J Plant Physiol*, **2**: 76-89.

Tomer AK, Tyagi K and Lal JP. 2007. Selection of promising drought tolerant mutant lines in lentil (*Lens culinaris* Medik.). *Indian J Genet*, **67(2):** 140-144.

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Effects of an Ecdysteroid Analog (RH-0345) on the Ovarian and Testicular Components of *Eupolybothrus nudicornis* (Myriapoda: Chilopoda)

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Abstract

We studied the effects of one ecdysteroid analog (RH-0345) on the ovarian and testicular components of the centipede *Eupolybothrus nudicornis* used as a model of predators of soil fauna. The injection of 10 µg of RH-0345 significantly reduced protein, lipid and carbohydrate concentrations in both the ovarian tissue of females and testicular tissue of males collected in spring or autumn. In consequence, this study shows that ecdysteroid analog pesticides can affect the reproduction of other arthropods than insects by modifying the level of metabolites in male and female gonads.

Keywords: Myriapoda, *Eupolybothrus nudicornis*, Ecdysteroid Analogs, Reproduction, Gonad Components, Carbohydrates, Proteins, Lipids

1. Introduction

Today, the use of pesticides in order to control pests causes much concern in the general population. Since it is virtually impossible to control all pest species without resorting to some use of pesticides, these are required to be specific and environmentally safe. In this context, natural and synthetic compounds capable of interfering with the processes of growth, development, reproduction and metamorphosis of the target pests have been developed in the search for safer insecticide technologies. These chemicals have been called insect growth regulators (IGRs) (Hoffmann and Lorenz, 1998). They work at extremely low levels, they are very specific and are not toxic to mammals; several compounds are already used. Among these compounds, novel insecticides that mimic the action of the molting hormone, the steroidal 20-hydroxyecdysone, are currently developed by the industry.

Bisacylhydrazines are non-steroidal ecdysteroid agonists of 20-hydroxyecdysone and exhibit their insecticidal activity via interaction with ecdysteroid receptor proteins (Dhadialla *et al.,* 1998). Although the effects of these compounds are well known in insects (Dhadialla *et al*., 1998), we know almost nothing about the effect of these compounds on other terrestrial arthropods. In previous studies, Daas *et al*. (2005 and 2007) used the centipede *Eupolybothrus nudicornis* (Gervais, 1837) (= *Eupolybothrus elongatus*, = *Bothropolys elongatus*) as a biological model to test the influence of ecdysteroid analogs on one of the predators of the soil fauna, this species could be directly affected by exposure to these compounds or affected following ingestion of preys containing these molecules. These authors showed that the injection of sublethal doses of two ecdysteroid analogs (RH-0345 or RH-5992), induced a significant decrease in hemolymph protein concentrations in males and females. A significant decrease in hemolymph lipid concentrations in males and females was also observed, except for females collected in spring and intoxicated with RH-0345 (Daas *et al*., 2005). Moreover, the injection of sublethal doses of RH-0345 and RH-2485 induced a significant reduction of total body and ovarian weights of female individuals (Daas *et al.,* 2007). Nevertheless, the decrease in ovarian weight was proportional to the decrease in total body weight because the gonadal somatic index remained constant and was the same among controls and intoxicated animals. These two compounds also reduced significantly the total protein concentrations in both the hemolymph fluid and ovarian tissue of females.

In order to better characterize the possible effect of ecdysteroid analogs on the reproduction of the centipede *E. nudicornis*, we tested the influence of RH-0345 on the ovarian and testicular components of individuals collected in spring or autumn.

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2. Materials and Methods

2.1. Animals and Housing

Experiments were conducted on mature females and males *Eupolybothrus nudicornis* collected during the spring or the autumn of 2008 from the area of Nechmaya (northeast Algeria) (Figure 1). This area is considered to be free of pollution. Animals were maintained individually in plastic boxes of 500 ml containing humid earth and covered with a moistened filter paper. They were fed three times a week with insects (cockroach larvae, flies and mosquitoes) and spiders.

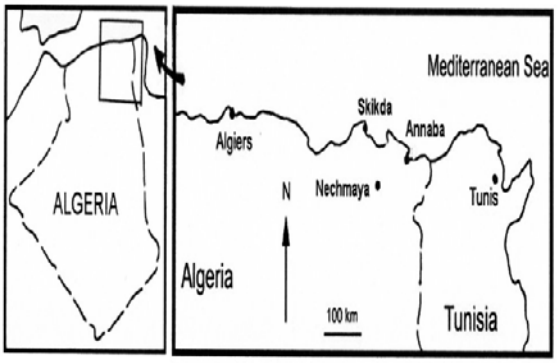

Figure 1. Map of Algeria showing the location of the site where individuals were collected

2.2. Chemicals And Toxicity Tests

RH-0345 (halofenozide) was developed by Rohm & Haas Company (Pennsylvania, USA). It was a gift by Prof. G. Smagghe (Laboratory of Agrozoology, University of Gent, Belgium). RH-0345 was dissolved in acetone to prepare a concentration of 3.33 µg/µl for experimental use. 3 µl were injected by means of a microsyringe between the third and fourth dorsal segment. Toxicity tests were performed during a 15-day period. Dose levels injected were based on previous finder range test (Daas *et al.*, 2005; 2007) and were chosen in order not to be lethal during the experiment. The amount of carbohydrates, lipids and proteins in ovaries and testicules were measured 5, 10, and 15 days after injection.

Control animals were maintained in the same conditions and injected with 3 µl of acetone.

2.3. Analytical Methods

Proteins, carbohydrates and lipids were extracted from the same gonad sample following the procedure of Shibko *et al.,* (1967). In brief, after dissection, each sample of gonad was individually homogenized in 1 ml of trichloroacetic acid (20%) and then centrifuged (5,000 g for 10 min at 4°C). The supernatant was used for the determination of carbohydrates as described by Duchateau and Florkin (1959) using anthrone as reagent and glucose as standard, while the pellet added with a mixture of ether and chloroform (1V/1V) was subjected to a second centrifugation (5.000 g for 10 min at 4°C). The resulted supernatant was used to quantify the lipids based on the vanillin method of Goldsworthy *et al.* (1972), Finally, protein concentration was determined in resulting pellet using the Bradford (1976) assay with bleu brilliant of coomassie (G 250, Merck) as reagent and bovine serum albumin (Sigma) as standard.

Data were expressed as µg of proteins, lipids or carbohydrates per mg of ovaries or testicules.

2.4. Data Analysis

The results were expressed as means ± standard deviation (S.D.). One-way ANOVA was used to compare the amounts of metabolites in ovaries and testicules from acetone injected animals (controls) and animals exposed to RH-0345 as a function of time. This analysis was followed by application of the Student-Newmans-Keuls multiple comparison method. The level of significance was set at $p < 0.05$.

3. Results

3.1. Ovarian Protein Concentrations

We obtained the same results with females collected in spring or autumn (Figures 2A and B). The ovarian protein concentrations of control females collected in spring or autumn remained stable the first 5 days and then slightly increased from 10 to 15 days but differences were no significant (P>0.05). The ovarian protein concentrations of females collected in spring were significantly (P<0.05) reduced compared to those of controls from 5 to 15 days after exposure (Figure 2A) whereas those of females collected in autumn were significantly (P<0.05) reduced from 10 to 15 days (Figure 2B). The ovarian protein concentrations were the same for females collected in spring or autumn.

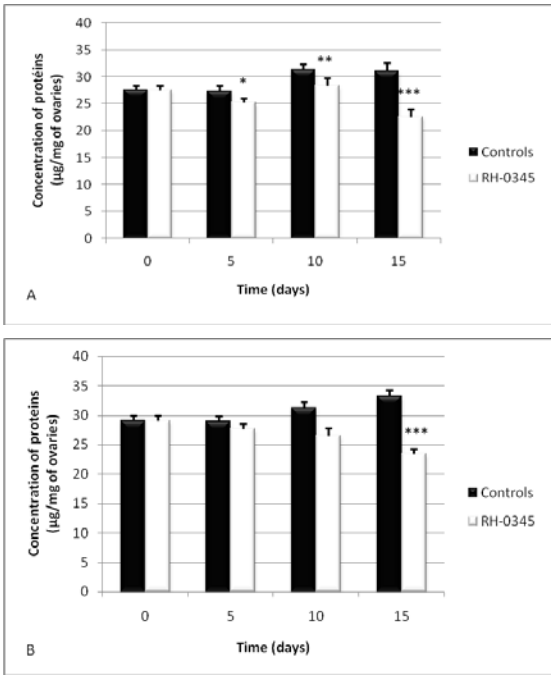

Figure 2. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* ovarian protein concentrations. A, females collected in spring; B, females collected in autumn. Means ± SD of 5 replicates. *$p < 0.05$; **$p<0.01$; ***$p<0.001$.

3.2. Testicular Protein Concentrations

The same results were obtained with males collected in spring or autumn (Figures 3A and B). The testicular protein concentrations of control males collected in spring or autumn decreased significantly (P<0.05) 5 days after beginning of the toxicity tests. Then we noticed an increase of the testicular protein concentrations 15 days and from 10 to 15 days after the start of the experiment for males collected in autumn and spring respectively. The testicular protein concentrations of males collected in spring or autumn were significantly reduced compared to those of controls from 5 to 15 days after exposure. No significant differences (P>0.05) were observed between testicular protein concentrations for males collected in spring or autumn.

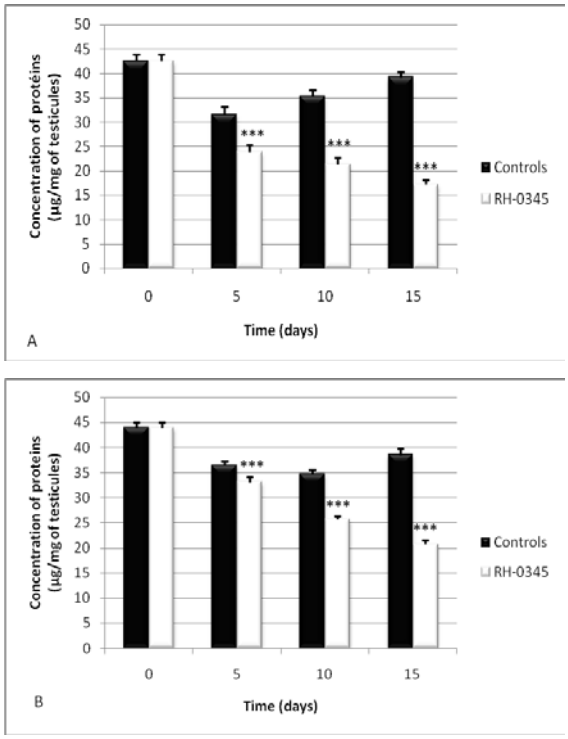

Figure 3. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* testicular protein concentrations. A, males collected in spring; B, males collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

3.3. Ovarian Lipid Concentrations

We obtained the same results with females collected in spring or autumn (Figures 4A and B). The ovarian lipid concentrations of control females slightly increased during the toxicity tests but differences were no significant (P>0.05). The ovarian lipid concentrations of females collected in spring or autumn were significantly (P<0.05) reduced compared to those of controls from 5 to 15 days after exposure. The ovarian lipid concentrations were the same for females collected in spring or autumn.

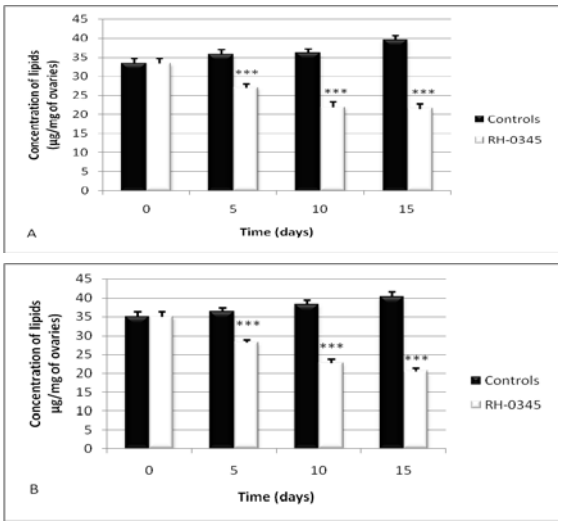

Figure 4. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* ovarian lipid concentrations. A, females collected in spring; B, females collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

3.4. Testicular Lipid Concentrations

The same results were obtained with males collected in spring or autumn (Figures 5A and B). The testicular lipid concentrations of control males decrease significantly (P<0.05) 5 and 10 days after the beginning of the experiment and then increased or remained stable for males collected in spring or autumn respectively. The testicular lipid concentrations of males collected in autumn (Figure 5B) were significantly reduced (P<0.05) compared to those of controls from 5 to 15 days after exposure whereas those of males collected in spring (Figure 5A) were significantly reduced from 10 to 15 days. No significant differences (P>0.05) were observed between testicular lipid concentrations for males collected in spring or autumn.

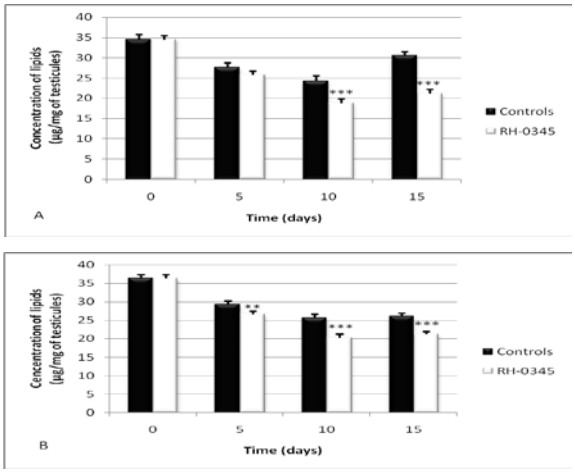

Figure 5. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* testicular lipid concentrations. A, males collected in spring; B, males collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

3.5. Ovarian Carbohydrate Concentrations

Globally we obtained the same results with females collected in spring or autumn (Figures 6A and B). The ovarian carbohydrate concentrations of control females

remained stable. The ovarian carbohydrate concentrations of females collected in spring or autumn were significantly reduced (P<0.05) compared to those of controls from 5 to 15 days after exposure. No significant differences (P>0.05) were observed between ovarian carbohydrate concentrations for females collected in spring or autumn.

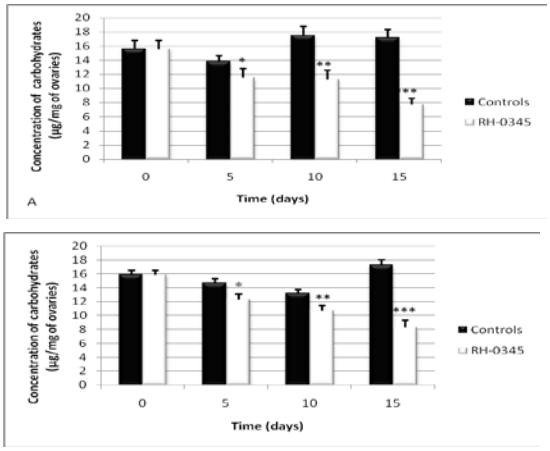

Figure 6. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* ovarian carbohydrate concentrations. A, females collected in spring; B, females collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

3.6. Testicular Carbohydrate Concentrations

Globally we obtained the same results with males collected in spring or autumn (Figures 7A and B). The testicular carbohydrayte concentrations of control males remained stable. The testicular carbohydrate concentrations of males collected in autumn (Figure 7B) were significantly reduced (P<0.05) compared to those of controls from 5 to 15 days after exposure whereas those of males collected in spring (Figure 7A) were significantly (P<0.05) reduced from 10 to 15 days. No significant differences (P>0.05) were observed between ovarian carbohydrate concentrations for females collected in spring or autumn.

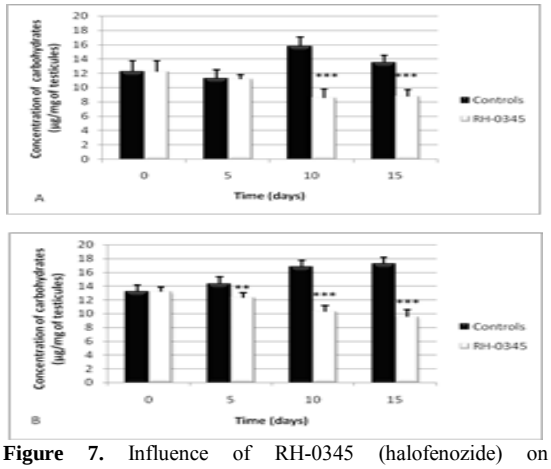

Figure 7. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* testicular carbohydrate concentrations. A, males collected in spring; B, males collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

4. Discussion

The developmental physiology of myriapods as that of insects depends on different hormones and neurohormones but the molting and the juvenile hormones are the principal ones which control different processes such as growth, molt, and metamorphosis (Rees, 1995). Ecdysteroids which constitute the molting hormone have been considered as the main key in the control of the molt in immature stages of insects (Gäde *et al.*, 1997). Today, it is well accepted that ecdysteroids also play an important role in the processes which regulate the reproduction of insects such as meiotic reinitiation in oocytes, vitellogenesis, ovogenesis and growth of the spermatocytes (Wigglesworth, 1984; Hagedorn, 1985; Jacob, 1992; Lanot *et al.*, 1987; Yamashita and Susuki, 1991).

Hence, Robbins *et al.* (1968, 1970) reported that high concentrations of natural ecdysteroids had chemosterilizing properties in several insects. The goal of our study was to test the effects of one ecdysteroid analog (RH-0345) on the ovarian and testicular components of other arthropods than insects on which we have only limited evidence.

Bisacylhydrazines are non-steroidal agonists of 20-hydroxyecdysone (20E) and exhibit their insecticidal activity via interaction with the ecdysteroid receptor proteins. In cases where these compounds have produced lethal effects, the symptoms have been similar to those expected from a state of ecdysteroid excess, called hyperecdysonisms (Williams, 1967).

Proteins play a fundamental role in the functioning of the organism (Mahler and Cordes, 1969). They can assure the biochemical catalysis, the hormonal regulation and can be integrated in the cell as structural components just as lipids and sugars (Jacob and Monod, 1961). We showed that the ovarian and testicular protein concentrations of individuals (males or females) are significantly reduced after injection of a sub-lethal dose of RH-0345. These results are in accordance with previous studies of Taibi *et al.* (2003) who showed that ovarian protein concentrations of female adult beetles of mealworm *Tenebrio molitor* significantly decreased 2 and 4 days after topical treatment with 10 µg/insect of RH-0345. On the other hand, another ecdysteroid agonist, RH-5849 stimulates *in vitro* the protein synthesis by the fat 2body of the rice moth *Corcycra cephalonica* (Ashok and Dutta-Gupta, 1991). Maiza *et al.* (2004) showed that treatment of the German cockroach *Blattella germanica* with RH-0345 applied topically (10 and 20 µg/insect) and a carbamate insecticid benfuracarb orally administrated (at 2%) reduced ovarian amount of proteins while topical application of the juvenile hormone analogue methoprene (1 and 10 µg/insect) increased it during the sexual maturation.

Previous studies using certain steroid ecdysone analogs have shown that these compounds affected the ovarian growth in *Spodoptera exempta, S. exigua, S. littoralis* and *Leptinotarsa decemlineata* (Smagghe and Degheele 1992; 1994) and inhibited the ovarian development of *Musca domestica* and *Tribolium confusum* (Robbins *et al.*, 1968; 1970). Moreover, it has been shown that treatment of *Spodoptera exigua* and *Leptinotarsa decemlineata* with RH-0345 and RH-5992 induced a decrease of *hemolymphatic* protein concentrations before the death of individuals (Smagghe *et al.*, 1996). Nevertheless, treatment of the mealworm *T. molitor* with an analog of the juvenile hormone (pyriproxyfen) induced an increase of *hemolymphatic* protein concentrations (Aribi *et al.*, 2001; 2006). Besides, another growth regulator, RH-5992 (tebufonozide) reduced oocyte growth in *Plodia interpunctella* (Salem *et al.*, 1997).

Lipids are essential as a source of energy in arthropods (Beenakers *et al.*, 1985). They are synthetized and stored in the fat body (Keeley, 1985; Van Hensden and Law, 1989) and then transported *via* the hemolymph to organs such as ovaries (Kilby, 1963; Wigglesworth, 1984; Chino *et al.*, 1981) where they are used for vitellogenesis (Downer, 1985; Keeley, 1985). We showed that the ovarian and testicular lipid concentrations of individuals (males or females) were significantly reduced after injection of a sub-lethal dose of RH-0345.

During this study, we noticed that ovarian lipid concentrations of control females collected in spring or autumn increased during all the exposure time. On the contrary, we observed fluctuations of testicular lipid concentrations of control males collected in spring or autumn. These contrasting results could be explain by the change of the environmental conditions and the external factors whom probably induced physiological disturbances of reproduction and development as was demonstrated by Scheffel (1987) and Descamps (1988; 1992) during the study of the effect of weather conditions on the life cycle of another Chilopoda *Lithobius forficatus.*

The decrease of the lipid concentration in the ovaries of females after treatment with RH-0345 could be due to a slowing down of the passage of these metabolites towards ovaries via the hemolymph. Topical application of chitin inhibitors such as diflubenzuron at 0.5 µg/insect also disturbed growth and development of oocytes of the coding moth *Cydia pomonella* (Soltani and Soltani-Mazouni, 1992) and reduced lipid concentrations in the fat body of the mealworm *Tenebrio molitor* (Khebbeb *et al.*, 1997). Our results are in accordance with previous studies of Padjama and Rao (1994) who showed a decrease of lipid concentrations in viscera, mantle and foot of the freshwater snail *Bellamya dissimilis* after treatment with sublethal concentrations of an organochloric (Endosulfan) and three organophosphate pesticids (Methyl parathion, Quinalfos and Nuvan). Daas-Maamcha (2005) also showed that treatment of *E. nudicornis* females collected in spring or autumn with different ecdysteroid analogs (RH-2485, RH-5992 and RH-0345) induced a decrease of the protein and lipid concentrations in the hemolymph. A similar effect has been observed by Daas *et al.* (2003) after injection of 20-hydroxyecdyson to the females of the same species.

Carbohydrates which represent an indispensable source of energy for living organisms are used in a immediate way as glucose or in the form of reserve as glycogen (Wigglesworth, 1984). Tissular carbohydrate rates are strictly connected to the physiological events such as molt and reproduction (Wiens and Gilbert, 1968). We showed that the ovarian and testicular carbohydrate concentrations of individuals (males or females) were significantly reduced after injection of a sub-lethal dose of RH-0345. Soltani (1990) noticed a significant decrease of carbohydrates (trehalose) concentration in the hemolymph of pupal stages of mealworms *T. molitor* 3, 4, 5, 6 and 7 days after injection of 10 µg of 20-hydroxyecdysone

It is well recognised that ecdysteroid analogs are toxic to insects. The synthetic nonsteroidal ecdysone agonist RH-0345 is an excellent insect control agent because it induces feeding inhibition and precocious incomplete molting, thus causing high larval mortality (Dhadialla *et al.*, 1998). RH-0345 has an overall insect control spectrum with accentuated soil-systemic efficacy against scarabid beetle larvae, cutworms, and webworms. Based on its reported narrow pest control spectrum and its structural and mechanistic similarity to the other bisacylhydrazines, it is expected to have low toxicity to non-target arthropods (Dhadialla *et al.*, 1998). Nevertheless, our study showed that this pesticide may affect the reproduction of other arthropods than insects such as centipedes.

The decrease of protein, lipid and carbohydrate concentrations in both the ovarian tissue of females and testicular tissue of males after treatment with RH-0345 may be explain by the interference between this compound and natural ecdysteroids thus disturbing the endocrine regulation of oogenesis and spermatogenesis as in *Tenebrio molitor* (Amrani, 2007; Boukachabia *et al.*, 2003; Taibi *et al.*, 2003).

References

Amrani L. 2007. Bioactivité de deux régulateurs de croissance, le dérivé de l'imidazole (KK-42) et le mimétique des ecdystéroïdes (RH-0345) sur la reproduction de *Tenebrio molitor:* morphométrie de l'ovaire, potentiel reproducteur et production hormonale. PhD thesis, Badji Mokhtar University, Annaba, Algeria.

Aribi N, Lakbar S, Smagghe G and Soltani N. 2001. Comparative action of RH-0345 and pyriproxyfen on molting hormone production and protein analysis in mealworm pupe. *Med Fac Landbouw Univ Gent*, **66**:445-454.

Aribi N, Smagghe G, Lakbar S, Soltani-Mazouni N and Soltani N. 2006. Effects of pyriproxyfen, a juvenile hormone analog on development of mealworm, *Tenebrio molitor. Pest Biochem Physiol*, **84**:55-62.

Ashok M and Dutta-Gupta A. 1991. *In vitro* effect of nonsteroidal ecdysone agonist RH- 5849 on fat body acid phosphate activity in rice moth, *Corcycra cephalonica* (Insecta). *Biochem Int*, **24**:69-75.

Beenakers AM, Van Der Host DJ and Van Marrewijk WJ. 1985. Insect lipids and lipoproteins and their role in physiological processes. *Prog Lipid Res*, **34**:19-67.

Boukachabia A, Soltani-Mazouni N, Smagghe G and Soltani N. 2003. Comparative effects of some ecdysteroid agonists in mealworms : Ecdysteroid amounts and protein analysis in developing ovaries under *in vivo* conditions. *Comm Appl Biol Sci Ghent University*, **68(4A):**307-312.

Bradford MM. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal Biochem***, 72**:248-254.

Chino H, Katas H, Dawner, RC and Hard Takahashi K. 1981. Diacylglycerol, carrying lipoprotein of haemolymph of the American cockroach: purification, characterization and function. *J Lipid Res*, **22**:7-15.

Daas O, Daas T and Descamps M. 2003. Étude de paramètres biologiques chez *Eupolybothrus elongatus* en vue de leur utilisation pour l'évaluation de la toxicité de composés chimiques utilisés en agriculture. *Bull Soc Zool Fr*, **128**:239-246.

Daas O, Daas T, Scaps P and Descamps M. 2005. Comparative toxicity of two ecdysteroid analogs (RH-0345 and RH-5992) and of a JH mimic (pyiproxyfen) on *Eupolybothrus elongatus* (Myriapoda, Chilopoda) hemolymph components. *Bull Soc Zool Fr*, **130**:303-313.

Daas O, Scaps P, Bouzerna N and Descamps M. 2007. Effects of two ecdysteroid analogs (RH-0345 and RH-3485) on the total growth of *Eupolybothrus elongatus* (Myriapoda, Chilopoda). *Life and Environment*, **57**:39-43.

Daas-Maamcha O (2005) Etude biologique de deux espèces de Myriapodes Chilopodes: *Eupolybothrus nudicornis* et *Lithobius forficatus*. Effets de traitements hormonaux et insecticides sur l'ovogenèse. PhD thesis, Badji Mokhtar University, Annaba, Algeria.

Descamps M. 1988. Ecdysteroid changes during the life cycle of the centipede *Lithobius forficatus* (Myriapoda, Chilopoda). *Adv Comp Endocrinol*, **1**:193-196.

Descamps M. 1992. Influence de la 20-hydroxyecdsone sur les synthèses d'ARN dans les ovocytes de *Lithobius forficatus* (Myriapoda, Chilopoda). *Bull Soc Zool Fr*, **117**:139-147.

Dhadialla TS and Carlson GR. 1998. New insecticides with ecdysteroidal and juvenile hormone activity. *Ann Rev Entomol*, **43**:545-569.

Downer RGH. 1985. Lipid metabolism. In: Kerkut GA, Gilbert L (Eds.), Comprehensive insect biochemistry, physiology and pharmacology. Pergamon Press, Oxford, **10**:77-113.

Duchateau G and Florkin M. 1959. Sur la tréhalosémie des insectes et sa signification. *Arch Int Physiol Biochem*, **67**:306-314.

Gäde G, Hoffman KH and Spring JH. 1997. Hormonal regulation in insects: facts, graps and futur direction. *Physiol Rev*, **77**:963-1032.

Goldsworthy GJ, Mordue W and Guthkelch J. 1972. Studies on insect adipokinetic hormones. *Gen Comp Endocrinol*, **18**:545-551.

Hagedorn HH 1985. The role of ecdysteroids in reproduction. In: Kerkut GA, Gilbert L (Eds.), **Comprehensive Insect Biochemistry, Physiology and Pharmacology**. Pergamon Press, Oxford, 8:205-262.

Hoffmann KH and Lorenz MW. 1998. Recent advances in hormones in insect pest control. *Phytoparasitica*, **26**:323-330.

Jacob M. 1992. *In vitro* spermatogenesis in *Oryctes rhinoceros* (Coleoptera, Scarabaeidae): the role of ecdysone and juvenile hormone. *Zool Sci*, **9**:457-461.

Jacob F and Monod J. 1961. Genetic mechanism in the synthesis of proteins. *J Biol Med*, **3**:318-321.

Keeley LL. 1985. Physiology and biochimisty of fat body. In: Kerkut GA, Gilbert L (Eds.), **Comprehensive Insect Biochemistry, Physiology and Pharmacology**. Pergamon Press, Oxford, 3:211-248.

Khebbeb MEH, Delachambre J and Soltani N. 1997. Ingested diflubenzuron disturbed lipid metabolism during the sexual maturation of mealworm *Tenebrio molitor*. *Med Fac Landbouw Univ Gent*, **62**:557-564.

Kilby BA. 1963. The biochemistry of the insect. *Fact Adv Insect Physiol*, **1**:112-174.

Lanot R, Thiebold J, Lagueux M, Goltzene F and Hoffmann JA. 1987. Involvement of ecdysone in the control of meiotic reinitiation in oocytes of *Locusta migratoria* (Insecta, Orthoptera). *Dev Biol*, **12**:174-181.

Mahler HR and Cordes EH. 1969**. Biological Chemistry**. Harper and Row, New York.

Maiza A, Kilani-Morakchi S, Farine JP, Smagghe G, Aribi N and Soltani N. 2004. Reproductive effects in German cockroaches by ecdysteroid agonist RH-0345, juvenile hormone methoprene and carbamate benfuracarb. *Commun Agric Appl Biol Sci*, **69(3)**:257-266.

Padjama RJ and Rao MB. 1994. Effect of an organochloric and three organophosphate pesticids on glucose, glycogen, lipid and protein contents in tissues of freshwater snail, *Bellamya dissimilis* (Müller). *Bull Environ Contam Toxicol*, **53**:142-148.

Rees HH. 1995. Ecdysteroid biosynthesis and inactivation in relation to function. *Eur J Entomol*, **92**:9-39.

Robbins WE, Kaplanis JN, Thompson JN, Shortino TJ, Cohen CF and Joyner SC. 1968. Ecdysones and analogs: effects on development and reproduction of insects. *Sciences*, **1611**:1158-1160.

Robbins WE, Kaplanis JN, Thompson JN, Shortino TJ and Joyner SC. 1970. Ecdysones and synthetic analogs: molting hormone activity and inhibitive effects on insect growth, metamorphosis and reproduction. *Steroids*, **16**:105-125.

Salem H; Smagghe G and Degheele D. 1997. Effects of tebufenozide on oocyte growth in *Plodia interpunctella*. *Med Fac Landbouw Univ Gent*, **62**:9-14.

Scheffel H. 1987. Häuntungsphysiologie der Chilopoden: Ergebnisse von Untersuchungen an *Lithobius forficatus* (*L.*). *Zool Jb Physiol*, **91**:257-282.

Shibko S, Koivistoinen P, Tratnyeck CA, Newhall AR, Friedman L. 1967. A method for sequential quantitative separation and determination of protein, RNA, DNA, lipid, and glycogen from a single rat liver homogenate or from a subcellular fraction. *Analyt Biochem***, 19**:514-528.

Smagghe G and Degheele D. 1992. Effects of RH 5849, the first nonsteroidal ecdysteroid agonist, on larvae *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). *Arch Insect Biochem Physiol*, **21**:119-128.

Smagghe G and Degheele D. 1994. Action of the nonsteroidal ecdysteroid mimic RH 5849 on larval development and adult reproduction of insects of different orders. *Invert Reprod Dev*, **25**:227-236.

Smagghe G, Ealen H, Verschelde E, Richter K and Degheele D. 1996. Differential effects of nonsteroidal ecdysteroid agonists in Coleoptera and Lepidoptera : Analysis of

evagination and receptor binding in imaginal discs. *Insect Biochem Mol Biol*, **26**:687-695.

Soltani N. 1990. Action du diflubenzuron et de la 20-hydroxyecdysone sur les glucides et les protéines hémolymphatiques chez la nymphe de *Tenebrio molitor*. *Ann Soc Ent Fr*, **26(4):**575-584.

Soltani N and Soltani-Mazouni N. 1992. Diflubenzuron and oogenesis in the coding moth, *Cydia pomonella* (L.). *Pesticide Sci*, **34**:257-261.

Taibi F, Smagghe G, Amrani L and Soltani-Mazouni N. 2003. Effect of ecdysone agonist RH-0345 on reproduction of mealworm, *Tenebrio molitor*. *Comp Biochem Physiol*, **135**:257-267.

Van Hensden HC, Law JH. 1989. An insect transport particule promotes lipid loading from fat body to lipoprotein. *J Biochem*, **246**:17287-17292.

Wiens AW and Gilbert T. 1968. Regulation of carbohydrate mobilization and utilization in Leucophaea maderae. *J Insect Physiol*, **13**:779-794.

Wigglesworth VB. 1984. **Insect Physiology**. Chapman and Hall, London

Williams CM. 1967. Third-generation pesticides. *Sci Am*, **17**:13- 17.

Yamashita O and Suzuki K. 1991. Roles of morphogenetic hormones in embryogenic diapause. In: Gupta PA (Ed.), **Morphogenic of Arthropodes. Roles in Histogenesis, Organogenesis and Morphogenesis**. Rutgers University Press, New Brunswick, 3:81-128.

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Evaluation of the Performance of Different Maize Varieties against *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae) Infestation in the Niger Delta Region of Nigeria

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Abstract

The performance of different maize varieties aginst *Sitophilus zeamais* infestation was evaluated in a laboratory. Seventeen maize varieties comprising fourteen hybrids (ACR.97 TZL COMP.1-W, ACR.8328 BNC7, TZL COMP.4C2, OBA SUPER 1 and 2, SINE 9449-SR, IWD SYN C3F2, TZL COMP.1SYN STR-Y, TZSR White and Yellow; 95TZEE-W, MASYN VAR-3 F2, ADV.NCRE-STR and BG 97 TZE COMP.3XL) and three local cultivars (Akparike, Bende and Ogbia muno) were screened to ascertain their level of susceptibility to *S. zeamais* in the study area. Number of adults that emerged from the 17 varieties differed significantly ($P \leq 0.05$) and ranged from 2.00 for the improved variety ADV.NCRE-STR to 62.0 in the local cultivar Bende. Significantly higher weight losses were recorded on local cultivars. Heavier males emerged from the local cultivars Akparike and Bende and lighter ($P \leq 0.05$) weights were recorded on Oba super 1, TZL Comp.4C2, TZSR White, ADV.NCRE-STR and MASYN VAR-3F2. Grain hardness test showed that the hybrid variety MASYN VAR-3F2 (275.12N) was the hardest followed by TZSR-Y (259.42N) and the softest were local varieties Akparike (116.62N) and Bende (91.65N). Seed coat thickness result indicated that the local variety Akparike (0.38mm) had the highest value of seed coat thickness followed by MASYN VAR-3F2 (0.22mm) and the least seed coat thickness was BG 97 TZE COMP.3XL (0.03mm). Results indicated that the local cultivars commonly cultivated in the Niger Delta (Bende, Akparike and Ogbia muno) supported higher *S. zeamais* adult progeny than the improved varieties which had harder seeds and thicker coats. The fact that Akparike which has thick testa was susceptible shows that physical factors alone are not responsible for the observed resistance.

Key Words: *Sitophilus zeamais,* Maize, Variety, Susceptibility and Grain Hardness

1. Introduction

Maize is a major food crop and source of animal feed in Africa, the Americas and Asia as well as a feed for livestock in these regions (Bergvinson, 2000). It is the third most important cereal after wheat and rice globally and the most widely distributed (Purseglove, 1981; Siwale *et al.*, 2009). It is popular for being more resistant to pests and diseases and easier to store and process than traditional food cereals including sorghum and millets in Kenya (Karaya *et al*., 2009).

The major constraint to utilization of maize in the tropics and subtropics is the attack by maize weevil (*Sitophilus zeamais*) (Akob and Ewete, 2007). It is the principal post-harvest pest and infestation commences in the field as soon as maize cobs begin to turn yellow (Haines, 1991). Adult weevils and larvae feed on undamaged grains and frequently cause severe powdering, rendering the product unfit for human consumption (Ofuya *et al.*, 2008). Partially damaged maize grains manifest loss in weight, poor marketability, quality deterioration and low viability (Enobakhare and Law-Ogbomo, 2002). Enobakhare and Law-Ogbomo (2002) and Lale and Kartay (2006) have shown from their studies that some cultivars of maize are relatively resistant to *S. zeamais* attack.

2. Materials and Methods

2.1. Laboratory Screening Of Maize Varieties Against S. zeamais Infestation

A laboratory screening for the most resistant and susceptible maize varieties against *S. zeamais* in the Niger Delta area of Nigeria was carried out under laboratory conditions [temperature (30 ± 2^0C), relative humidity ($65\pm5\%$)] at the General Laboratory of the Faculty of Agriculture, University of Port Harcourt. Seventeen maize varieties comprising fourteen hybrids [ACR.97 TZL COMP.1-W, ACR.8328 BNC7, TZL COMP.4C2, OBA SUPER 1 and 2; SINE 9449-SR, IWD

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SYN C3F2, TZL COMP.1SYN STR-Y, TZSR White and Yellow; 95TZEE-W, MASYN VAR-3 F2, ADV.NCRE-STR and BG 97 TZE COMP.3XL] and three local cultivars (Akparike, Bende and Ogbia muno) that are normally grown by local farmers in the study area were screened. The fourteen hybrid varieties were obtained from the International Institute of Tropical Agriculture (IITA), Ibadan germplasm, while the three local cultivars were purchased from open markets in Ali-Brada and Mile 3 both in Rivers State, Nigeria. The experimental jars and maize varieties were all sterilized thermally in a hot-air Gallenkamp oven at 60ºC for 2 hours to kill any pest and pathogen that might be present and allowed to acclimatize for 24 hours to laboratory temperature (30ºC) in the laboratory (Atijegbe, 2004; Zakka, 2005). *S. zeamais* culture was raised in the laboratory from infested maize collected in Choba market in Rivers State and reared on a susceptible local maize variety (Coma) in 1-Litre Kilner jars which were left on an open air shelf in the laboratory. To standardize the age of the progeny, the adults were removed after seven days by sieving and the eggs laid were allowed to form the sub-culture. The emerging F_1 were later used for the experiment (Zakka, 2005).

2.2. Sitophilus zeamais Performance On Different Maize Varieties

Six pairs of 2-3 day-old adult *S. zeamais* were introduced into separate jars containing 20g of each maize variety weighed on a sensitive Mettler balance (A & D Electronic balance FX-6000) and left for seven days to oviposit. On the seventh day, the adults were removed by emptying the content of each jar on a piece of white cloth and removing the adults with a soft entomological brush. Subsequently, the content of each jar was carefully returned into the jar and kept in its original position on the shelf and left undisturbed for 37 days to enable the insects to complete their development. The jars were examined daily to observe the emergence of teneral adults so as to determine their daily emergence pattern and developmental rate. Adult count started 15 days after ovipositing females were removed and counted until no teneral adult(s) emerged in each jar for five consecutive days (modified after Throne *et al*., 2002). Each treatment was replicated three times in a completely randomized design (CRD) and kept on open air shelves.

2.3. Sitophilus zeamais Teneral Adult Weight by Sexes

The weight of adult insects by sex was determined by collecting ten males and ten females that emerged from each maize variety by random selection and weighed and the weights recorded. The sexing was done using the snout characteristics such as color and shape, body length and size (Halstead, 1963; Tolpo and Morrison, 1965). The weight of the various maize varieties was taken in batches using an electronic sensitive balance J2003 model and then dried in an oven at 130°C for one hour and cooled for 2 hours before re-weighing. The procedure was repeated until a constant value was obtained. The percent moisture content was calculated as weight of moisture/weight of wet sample x 100 (Obeng-Ofori and Boateng, 2008)

Thus: Mw = $\frac{Wm}{Wo} \times 100\%$

Where Wm=water weight in grain at Mw

Wo= total grain weight at Mw

Since Wo=Wm+Wd

Where: Wd=dry matter weight at Mw moisture content

Therefore Mw = $\frac{Wm}{(Wm+Wd)} \times 100\%$

2.4. Grain Physical Parameters

Seed coat thickness was measured using a Digimatic Vernier Caliper (model Mitutoyo Electronic Digital Caliper Japan) and seed weight loss was determined as described by Lale (2002):

%weight loss = $\frac{[UaN - (U + D)]}{UaN} \times 100$

Where U= weight of undamaged fraction in a sample

N=total number of grains in a sample

Ua=average weight of one undamaged kernel

D= weight of damaged fraction in a sample

This was confirmed by the modified gravimetric method of Compton *et al*. (1998) by counting damaged grains and weighing of the final samples using the formula

%weight loss = $\frac{Pnd - Pfa}{Pnd}$

Where Pnd= weight of non-damaged kernels

Pfa= final weight of sample

Grain hardness was determined using CBR Compression machine in the Department of Applied Geology in the Federal University of Technology, Akure, Nigeria. Ten grains were randomly selected from each variety/cultivar and tested for hardness. Each grain was carefully placed in a vertical position on the stage meter and crushed. The hardness of the grain was obtained by multiplying the value obtained from the machine by a factor (23.8N/div). Susceptibility index (Dobie, 1974), was determined in order to assess the resistant and susceptible maize varieties.

$$SI = \frac{LogY}{T} \times 100$$

Where SI = susceptibility index

Log Y= log number of F_1 emerged adults

T= mean developmental periods (days)

3. Results

Table 1 shows grain physical properties. Data on seed coat thickness indicated that Akparike, a local cultivar, had the thickest seed coat which was followed by an improved cultivar MASYN VAR-3F2 though it did not differ significantly from most of the other improved varieties. The thinnest seed coat was recorded in BG97 TZE Comp.3XL and local varieties Bende and Ogbia muno. Grain hardness indicated that varieties MASYN VAR-3F2 and TZSR-Y were the hardest though the latter did not differ significantly from TZSR-W and Oba super 2. The four varieties with the softest kernels were ranked in a decreasing order as follows: Ogbia muno > Akparike > ADV.NCRE-STR > Bende.

Table 1. Grain hardness and seed coat thickness as physical parameters.

Maize variety	Grain physical parameter	
	Grain hardness(N)	Seed coat thickness(mm)
ACR 97 TZL Comp. 1-W	227.75^{cd}	0.0750^{c-f}
IWD SYN C3F2	194.65^{ef}	0.0766^{c-f}
OBA SUPER 1	226.10^{cd}	0.0618^{d-f}
ACR.8328 BNC7	216.82^{c-e}	0.1580^{b-e}
TZL Comp.4C2	171.36^{f}	0.1982^{bc}
SINE 9449-SR	211.8^{de}	0.1776^{b-d}
ADV.NCRE-STR	106.13^{gh}	0.1120^{b-f}
BG 97 TZEComp.3XL	134.46^{g}	0.0253^{f}
AKPARIKE	116.62^{gh}	0.3820^{a}
TZL Comp. 1SYN STR-Y	206.09^{de}	0.1872^{b-d}
BENDE	91.65^{h}	0.0448^{ef}
95 TZEE-W	203.25^{de}	0.1710^{b-d}
MASYN VAR-3 F2	275.12^{a}	0.2240^{b}
OBA SUPER 2	242.66^{bc}	0.1790^{b-d}
TZSR White	240.37^{bc}	0.0988^{b-f}
TZSR Yellow	259.42^{ab}	0.0960^{c-f}
OGBIA MUNO	131.65^{g}	0.0643^{d-f}

Means with the same letters in the same column are not significantly ($P \leq 0.05$) different by Student-Newman-Keuls test

Table 2 shows that the mean number of adults that emerged from the 17 varieties were significantly different ($P \geq 0.05$) and ranged from 2.00 for the improved variety ADV.NCRE-STR to 62.0 for the local variety Bende. The number of adults that emerged on the improved varieties ranged in the increasing order: ADV.NCRE-STR < TZL Comp.4C2 < IWD SYN C3F2 < TZSR White < BG 97 TZE Comp.3XL < SINE9449-SR < OBA SUPER 1. The effect of pest activities on weight losses is also shown in Table 2.

Table 2. Mean number of adult progeny and grain weight (g) loss on 17 different maize varieties.

Maize variety	Number of emerged adults	Grain weight loss (g)
ACR 97 TZL Comp. 1-W	23.00^{cd}	2.20^{b-d}
IWD SYN C3F2	04.33^{f}	0.40^{de}
OBA SUPER 1	16.00^{de}	1.56^{b-e}
ACR.8328 BNC7	17.00^{de}	1.76^{b-e}
TZL Comp.4C2	02.67^{f}	0.27^{e}
SINE 9449-SR	10.67^{ef}	1.00^{c-e}
ADV.NCRE-STR	02.00^{f}	0.20^{e}
BG 97 TZE Comp.3XL	05.00^{f}	0.56^{c-e}
AKPARIKE	46.00^{b}	5.33^{a}
TZL Comp. 1SYN STR-Y	19.67^{c-e}	1.90^{b-d}
BENDE	62.00^{a}	7.67^{a}
95 TZEE-W	19.67^{c-e}	1.96^{b-d}
MASYN VAR-3 F2	19.67^{c-e}	1.96^{b-d}
OBA SUPER 2	24.00^{cd}	2.46^{bc}
TZSR White	04.67^{f}	0.53^{c-e}
TZSR Yellow	17.00^{de}	1.73^{b-e}
OGBIA MUNO	27.67^{c}	3.26^{b}

Means with the same letters in the same column are not significantly ($P \leq 0.05$) different by Student-Newman-Keuls test

A significantly higher weight loss was recorded in the local varieties Bende and Akparike and closely followed by Ogbia muno and in the improved varieties Oba super 2 and ACR97 TZL Comp.1-W, although they did not differ significantly from those of BG97 TZE Comp.3XL, TZSR White and TZSR Yellow. The least weight losses were recorded on the improved varieties ADV.NCRE-STR, TZL Comp.4C2 and IWD SYN C3F2.

Table 3 shows the mean weights of male and female adults that emerged from each variety , and it indicates that heavier males emerged from the local cultivars Akparike and Bende than those that emerged from the improved varieties 95TZEE-W, SINE 9449-SR, TZL Comp.1SYN STR-Y, IWD SYN C3F2. However, lighter ($P < 0.005$) weights were recorded on Oba super 1, TZL Comp.4C2, TZSR White, ADV.NCRE-STR and MASYN VAR-3F2, Oba super 2, TZSR Yellow, BG97TZE Comp.3XL, ACR.8328BNC7 and ACR97TZL Comp.1-W varieties. Heavier females were recorded in the local cultivar Akparike which was closely followed by those from ACR97TZL Comp.1-W, TZL Comp. 1SYN STR-Y, 95TZEE-W, TZSR Yellow, SINE 9449-SR, ACR.8328 BNC7, Bende and Obgia muno. Lighter adult females emerged from IWD SYN C3F2, TZSR White and ADV.NCRE-STR, BG97 TZE Comp.3XL, MASYN VAR-3F2, Oba Super 2 and Oba Super 1.

Peak of emergence was recorded within the first 7 days on all the varieties (Figures 1a, 1b, 1c and 1d) and two peaks were recorded on Akparike and Ogbia muno varieties (Figures1a and 1c); however, highest peaks were recorded on the local cultivars (Figures 1a, 1b and 1c) with Bende (Figure 1b) recording the highest peak and an improved variety ACR.8328BNC7 had irregular peaks (Figure 1d).

Table 3. Mean weight (g) of males and females of *S. zeamais* that emerged from 17 different maize varieties.

Maize variety	Weight (mg)	
	Male	Female
ACR 97 TZL Comp. 1-W	2.9^{b-e}	5.7^{b}
IWD SYN C3F2	3.3^{a-d}	2.7^{e}
OBA SUPER 1	2.4^{e}	3.9^{c-e}
ACR.8328 BNC7	2.8^{c-e}	5.2^{bc}
TZL Comp.4C2	2.4^{e}	3.0^{e}
SINE 9449-SR	3.4^{a-c}	4.6^{b-d}
ADV.NCRE-STR	2.5^{e}	3.0^{e}
BG 97 TZE Comp.3XL	2.8^{b-e}	3.1^{de}
AKPARIKE	3.9^{a}	7.5^{a}
TZL Comp. 1SYN STR-Y	3.3^{a-d}	5.9^{b}
BENDE	3.6^{a}	5.2^{bc}
95 TZEE-W	3.4^{ab}	5.9^{b}
MASYN VAR-3 F2	2.7^{e}	3.8^{c-e}
OBA SUPER 2	2.8^{de}	3.9^{c-e}
TZSR White	2.6^{e}	2.7^{e}
TZSR Yellow	2.8^{de}	6.1^{b}
OGBIA MUNO	2.9^{b-e}	5.4^{bc}

Means with the same letters in the same column are not significantly ($P \leq 0.05$) different by Student-Newman-Keuls test

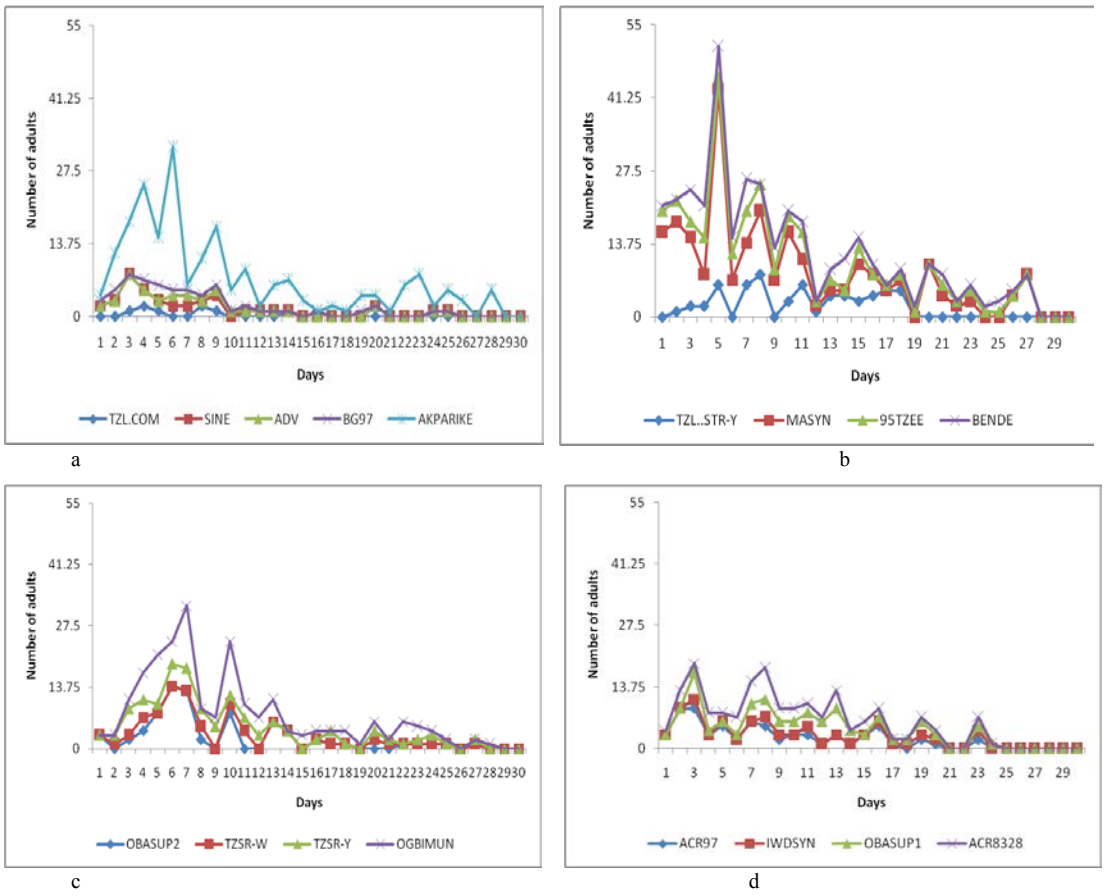

Figure 1 a-d. Emergence pattern of *S. zeamais* on different maize cultivars

4. Discussion

The result of this study indicates that the grains of the local varieties had thinner seed coats and softer kernel compared with the improved varieties which had thicker seed coats and harder kernels. These local varieties supported higher *S. zeamais* adult progeny than the improved varieties. The physical characteristics of the seed coat affect oviposition and/or egg-hatch in beetles (Lale and Makoshi, 2000; Lale and Kartay, 2006). These physical factors, among others, are most likely to be the contributory factors that affected the differences in the development of the adult *S. zeamais* that eventually emerged from the different varieties. The resistance exhibited by the improved varieties has been attributed to mechanical barriers provided by thick testae and hard grains (Lale and Yusuf, 2001; Ashamo, 2001; Lale and Kartay, 2006). The fact that Akparike which has a thick testa was susceptible shows that physical factors alone are not responsible for the observed resistance. In earlier studies hydroxycinnamic acids (phenolics), E-ferulic acid and protein content and other chemical factors have been implicated (Dobie, 1977; Serratos *et al*., 1987; Arnason *et al*., 1992).

Although the precise mechanism by which the factors impede *S. zeamais* development is yet to be well understood, the result from this work suggests that the soft texture of the local varieties may have contributed at least in part to their susceptibility. The seed coat thickness and grain hardness served as a barrier to the penetration of the endosperm by *S. zeamais* amongst the improved varieties which proved to be less susceptible to infestation. It is therefore, possible that the harder endosperm of the improved maize varieties poses some degree of difficulties for the development of the beetles thereby interfering with their ability to obtain the desired nutrient for oviposition and growth thus resulting in poor development (Lale and Yusuf, 2001). The higher weight losses observed in the local cultivars after a period of 35 days of the experiment can be attributed to the higher numbers of adult progeny that developed in these cultivars thus indicating a greater preference of such cultivars by *S. zeamais* as suitable substrates for development. The implication of this is that if theses maize varieties are left unprotected it could lead to economic weight losses in those maize cultivars as compared to the improved varieties.

Differences in weights of male and female beetles that developed in different substrates were observed in earlier studies (Danho *et al*., 2002; Zakka, 2005). The improved variety ADV NCRE-STR that proved relatively resistant to the pest had heavier adults emerging from it; this could be due to its ability to supply nutrients that can encourage robust physical development of the pest but possibly possesses an inhibitory factor that perhaps affects oviposition, larval or pupal development. In contrast, those improved varieties that did not support much development had equally lighter adults emerging from them, and this might be attributed to lack of required nutrients for optimal development.

Adedire (2001) gave the life cycle of *S. zeamais* to be about 35 days. Adult emergence from ACR97TZL Comp1-W, ACR.8328 BNC7, SINE 9449-STR, BG97TZE Comp.3XL, Akparike, Bende, MASYN VAR-3F2, 95TZEE-W and Oba Super 2 commenced on the 37th day of introduction and late emergence on improved varieties IWD SYNC3F2 and ADV NCRE-STR commenced on the 39th and 42nd day, respectively. This delayed development was reported by Lale and Maina (2002) on groundnut cultivars 'Jato', 'Yar Damboa' and 'Kampala' which restricted development by prolongation of the bruchid's life cycle. Adult emergence stopped early (10 days after commencement) on varieties ADV NCRE-STR and TZL Comp.4C2 while it lasted for up to 30 days on the susceptible local cultivars Ogbia muno and Akparike. The relative resistance shown by the improved varieties especially variety ADV NCRE-STR to *S. zeamais* infestation through prolongation of development and reduction of adult progeny may be attributed principally to antibiotic factors in the endosperm and difficulty in obtaining optimum quantity of nutrients needed for growth from the harder endosperm (Jansen and Nylin, 1997; Barros and Zucoloto, 1999). Other known factors that determine suitability as a breeding medium are morphology, environmental conditions, age and size of individuals (Stejskal and Kucerova, 1996; Johnson and Kistler, 1987) and competition (Siemens *et al*., 1991).

The highest number (37/day) of adult progeny recorded on Bende gives a clear indication of the pest load it can accommodate thus making it highly susceptible to infestation. If such an emergence level is continued over the emergence period of 30 days recorded, then within a short time the whole crop stands the risk of having 100% devaluation thereby leading to high grain loss. From the rate of emergence of the adult progeny on the local cultivars (Figs 1a, 1b and 1c), it means that if they are left unprotected, infestation could build up to an economic damaging level within the first few days of storage, thereby making the farmer to incur high quantitative and qualitative losses. The discovery of ADV NCRE-STR, TZL Comp.4C2, IWD SYN C3F2 and other improved varieties which are resistant to *S. zeamais* infestation in comparison with the local cultivars grown in the Niger Delta underlines the need for a change from the cultivation of the local cultivars in preference for the improved varieties for maize. It may also lead to the extension of the shelf life of maize in store, though it will require extensive extension work to sell the idea to the local farmers. It is also important to note that as Cavanaugh *et al*. (1995) reported, increased hardness of endosperm of grains in maize is desirable for dry milling, storage and export purposes; breeding intentionally for grain hardness would be an effective strategy for reducing the menace of *S. zeamais* on maize grains in storage.

5. Conclusion

The results of the study suggest that in order to improve maize storage, farmers need to adopt the cultivation of the improved cultivars. They also give indication of the need for breeders to develop cultivars with thick testae and hard endosperm (Cavanaugh *et al*., 1995; Lale and Yusuf, 2001; Lale and Modu, 2003; Lale and Kartay, 2006).

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References

Adedire CO 200.1 Biology, Ecology and Control of Insect Pests of Stored Cereal Grains. In: Ofuya T.I and Lale N.E.S. (Ed) **Pests of Stored Cereals and Pulses in Nigeria: Biology, Ecology and Control**. Dave Collins Publishers 174 pp.

Arnason JT, Conilh de Beyssac B, Philogene BJR, Bergvinson, D, Serratos JA and Mihm, JA 1997. Mechanisms of resistance in maize grains to the maize weevil and the larger grain borer. *Crop Science.* **43**:2043-2049.

Ashamo MO. 2001. Varietal resistance of maize to the maize weevil, *Sitophilus zeamais* Motsch.(Coleoptera: Curculionidae). *J Plant Disease and Protection*, **108**: 314-319.

Akob CA and Ewete FK. 2007. The efficacy of ashes of four locally used plant materials against *Sitophilus zeamais* (Coleoptera: Curculionidae) in Cameroon. *Inter J Tropical Insect Sci.*, **27**:21-26.

Atijegbe SR. 2004 Infestation of smoked fish in Ghana. M.Phil. Thesis in Entomology University of Ghana, Legon, Ghana. 103pp.

Barros HCH and Zucoloto FS. 1999. Performance and host preference of *Ascia monuste* (Lepidoptera: Pieridae). *J Insect Physiol.,* **45**:7-14.

Bergvinson D J. 2000. Storage Pest resistance in maize. Maize Research Highlights 1999-2000.

Cavanaugh KJ, Zehr BF, Nyquist WE, Hamaker BR and Crane PL. 1995. Response to selection for endosperm hardness and associated changes in cycles in agronomic traits after four cycles of recurrent selection in maize. *Crop Sci. Society of Amer*., **35**:745-748.

Compton JAF, Flovo A, Ofosu A, and Agbo B. 1998. The modified count and weight method and improved procedure for assessing weight loss in stored maize cobs. *J. Stored Product Res*., **34(4):**277-285.

Danho M, Gasper C and Haubruge E. 2002. The impact of grain quantity on the biology of *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) oviposition, distribution of eggs, adult emergence, body weight and sex ratio. *J Stored Product Res.,* **38(3)** :259-266.

Dobie P. 1974. The laboratory assessment of the inherent susceptibility of maize varieties to post-harvest infestation by *Sitophilus zeamais* Mot. (Coleoptera: Curculionidae). *J Stored Products Res.*, **10**:183-192.

Dobie P. 1977. The contribution of the stored product centre to the study of insect resistance in stored maize. *Tropical Stored Product infestation*. **34**:7-22.

Enobakhare DA and Law-Ogbomo KE. 2002. Reduction of post harvest loss caused by *Sitophilus zeamais* (Motsch) in three

varieties of maize treated with plant products. *Post Harvest Sci.,* **1**:1-6.

Haines CP. 1991. **Insects and Arachnids of Tropical Stored Products: their Biology and Identification**, NRI, Chatham, Kent (UK).

Halstead DGH 1963. The separation of *Sitophilus oryzea* (L.) and *S. zeamais* Motschulsky (Coleoptera: Curculionidae) with a summary of their distribution. *Entomology Monthly Magazine* **94:** 72-74.

Jansen DH and Nylin S. 1997. Seed-eaters versus seed size, number, toxicity and dispersal. *Evolution* **23**: 1-27.

Johnson C and Kistler R A. 1987. Nutritional ecology of bruchid beetles. In: Slansky, F, Jr. and Rodriguez, J.G. (eds**.), Nutritional Ecology of Insects, Mites, Spiders and Related Invertebrates**, John Wiley, New York. Pp. 259-276.

Karaya H, Njoroge K, Mugo S and Nderitu H. 2009. Combining ability among twenty insectresistant maize inbred lines resistant to *Chilo partellus* and *Buseola fusca* Stem borers*. Inter. J. Plant Production.* **3(1)**: 115-126.

Lale NES and Maina YT. 2002. Evaluation of host resistance, solar heat and insecticidal essential oils for the management of *Coryedon serratus* (Olivier) (Coleoptera: Bruchidae) infesting groundnut seeds and tamarind pods in storage. *J. Plant Disease and Protection,* **109 (4):** 410-420.

Lale NES and Yusuf BA 2001. Potential of varietal resistance and *Piper guineense* seed oil to control infestation of stored millet seeds and processed products by *Tribolium castaneum* (Herbst). *J. Stored Product Res*., **37**: 63-75.

Lale NES and Makoshi SM. 2000. Role of chemical characteristics of the seed coat in the resistance of selected cowpea varieties to *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) in Nigeria. *Inter. J. Pest Management*, **46(2):** 97-102.

Lale NES. 2002. **Stored Product Entomology and Acarology in Tropical Africa** (First Edition) Mole Publications (Nigeria) Ltd, Maiduguri 204pp.

Lale NES and Modu B. 2003. Susceptibility of seeds and flour from local wheat cultivars to *Tribolium castaneum* infestation in storage. *Tropical Sci*., **43**:174-177.

Lale NES and Kartay MO. 2006. Role of physical characteristics of the seeds in the resistance of local cultivars of maize to *Sitophilus zeamais* infestation in storage. *Tropical Sci*., **45**:112-114.

Obeng-Ofori D and Boateng BA. 2008. Global population growth, crop losses and post harvest technology. In: Cornelius, E.W and Obeng-Ofori, D. (Editors). **Post Harvest Science and Technology**. Teaching and Learning Innovation Fund (TALIF) Smartline Publishers Accra, Ghana, 504pp.

Ofuya TI, Idoko JE and Akintewe LA. 2008. Ability of *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) from four locations in Nigeria to infest and damage three varieties of maize, *Zea mays* L. *Nigerian J. Entomol*., **25**:34-39.

Purseglove JW. 1981. **Tropical Crops, Monocotyledons**. Longman.

Pixton SW. 1967. Moisture- Its significance and measurements in stored products. *J. Stored Product Res*., **3**:35-47.

Serratos JA, Arnason JT, Nozzolillo C, Lambert JDH, Philogene BJR, Fulcher G, Davidson K, Peacock L, Atkinson J and Morand P. 1987. Factors contributing to resistance of exotic maize populations to maize weevil, *Sitophilus zeamais*. *J. Chemical Ecology,* **13**:751-762.

Siemens DH, Johnson, CD and Woodman RL. 1991. Determinants of host range in bruchid beetles. *Ecology* **72**: 1560 - 1566.

Siwale J, Mbata K, Microbert J and Lungu D. 2009. Comparative resistance of improved maize genotypes and landraces to maize weevil. *African Crop Sci. J*., **17(1)**:1-16.

Stejskal V and Kucerova Z. 1996. The effect of grain size on the biology of *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). I. Oviposition, distribution of eggs and adult emergence. *J. Applied Entomol*., **120:** 143-146.

Throne JE, Baker JE, Messina FJ, Kramar KJ, Howard JA. 2002. Varietal resistance. In: Subramanyam BH, Hagstrum DW (Eds.) **Alternatives to Pesticides in Stored-Product** IPM. Kluwer Academic Publishers, Norwell, MA 165-192pp.

Tolpo NC and Morrison EO. 1965. Sex determination by snout characteristics of *Sitophilus zeamais* Motschulsky. *Texas J. Sci*., **17**:122-124.

Zakka U 2005. Development of *Dermestes maculatus* (De Geer, 1776) Coleoptera: Dermestidae on different fish substrates. MPhil. Thesis in Entomology University of Ghana. Pp128.

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Total Phenol, Antioxidant and Cytotoxic Properties of Wild Macrofungi Collected from Akure Southwest Nigeria

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Abstract

Three wild macrofungi, *Lenzites betulina* (Fries), *Trametes vesicolor* (Lloyd) and *Coriolopsis polyzona* (Pers) collected in Akure southwest of Nigeria were assessed for their total phenol, antioxidant and cytotoxic activities. The antioxidant and cytotoxic properties of the hexane, ethyl acetate and ethanol extracts from the fruit bodies of these macrofungi were assessed using DPPH scavenging capacity, inhibition of the formation of thiobarbituric acid reactive species (TBARS), and the protein-binding dye sulforhodamine B (SRB) microculture assay to measure cell growth using six human cancer cell lines. Total phenol content ranged from 8.22µgGAE/mg to 60.54 µgGAE/mg. The DPPH and inhibition of the formation of TBARS ranged between 7.97% to 91.18% at 1000µg/mL. Ethanolic extracts (LET, TET and CET) with higher phenol content exhibited better antioxidant property. The inhibition of human cancer cell lines varies from one extract to the other. However, ethanolic extract of *Trametes vesicolor* (TET) demonstrated the best cytotoxic activity with 100% inhibition of HCT-5, MCF-7 and SKLU-1 human cancer cell lines. The study suggests that these three wild macrofungi could be source of effective antioxidant and anticancer agents.

Keywords: Wild Macrofungi; Total Phenol; Antioxidant; Anticancer.

1. Introduction

Macrofungi have long been used as valuable food source and as traditional medicines around the world, especially in the orient (Wasser, 2002). Macrofungi are known to produce large and diverse variety of secondary metabolites (Liu, 2007). These secondary metabolites have health promoting properties such as antioxidant, antimicrobial, anticancer, cholesterol lowering and immunostimulatory effects (Anderson, 1992; Mizuno, 1999; Mau *et al*., 2004). Some common bioactive compounds isolated from these macrofungi include glycolipids, compounds derived from shikimic acid, aromatic phenols, fatty acid derivatives, polyacetylamine, polyketides, nucleosides, sesterterpenes, and many other substances of different origins (Lorenzen and Anke, 1998; Wasser and Weis, 1999; Mizuno, 1999; Liu, 2007).

The number of these macrofungi on earth has been estimated to be 140,000 out of which only 14,000 (10%) are identified (Hawksworth, 2001). The pharmacological potential of about 90% of these macrofungi is yet to be explored. A large number of the unknown species of mushrooms which may possess health promoting properties are not well studied especially in Africa (Oyetayo, 2011). There are no data on these macrofungi and their medicinal potentials.

The search for safe and effective pharmacological substances had increased of recent. Bioactive compounds obtained from macrofungi maybe the answer to these novel pharmacological agents. The antioxidative and free radical scavenging properties of mushroom have been reported (Mau *et al*., 2002; 2004; Ferreira *et al*., 2007). Chinese Shiitake mushroom (*Lentinus edodes*) has also been reported to possess both anti-tumour and antimicrobial properties (Jong and Birmingham, 1993). Natural products have been the source of most of the active ingredients of medicines (Harvey, 2008). Natural substances provide a large reservoir for screening of anti-HIV-1 agents with novel structure (Liu, 2007). The present study seeks to assess the total phenol, antioxidant and cytotoxic effects of hexane, ethyl acetate and ethanol extracts obtained from three wild non-edible macrofungi, *Lenzites betulina* (Fries), *Trametes vesicolor* (Lloyd) and *Coriolopsis polyzona* (Pers), collected from Akure, South West region of Nigeria.

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2. Materials and Methods

2.1. Collection of Macrofungi

Lenzites betulina (Fries), *Trametes vesicolor* (Lloyd) and *Coriolopsis polyzona* (Pers) were collected in the wild between June and September, 2011 in forest around the campus of Federal University of Technology, Akure, Nigeria (Lat. 07º 14^{I}N Long. 05º 11^{I}E). The morphological and ecological characteristics of these macrofungi were recorded in their natural habitats. Dried samples of the macrofungi were numbered and kept in polythene bags. The collected macrofungi were identified based on their macroscopic and microscopic characteristics and the related literature (Watling, 1973; Moser, 1983). Voucher specimens of dried macrofungi were deposited in the herbarium of Department of Microbiology, Federal University of Technology, Akure, Nigeria.

2.2. Preparation of Macrofungi Extracts

The method described by Yu *et al*. (2006) was adopted with slight modification. Briefly, dried samples of *Lenzites betulina*, *Trametes vesicolor* and *Coriolopsis polyzona* were ground into fine powder with an electric mill. The bioactive components were sequentially extracted from non-polar to polar solvents using hexane, ethylacetate and ethanol. The extraction by the solvent was performed in Erlenmeyer flask at room temperature for 48 h. The extracts obtained were dried to constant weight in a laboratory hood overnight (12 hours). The extracts were designated LHE (hexane extract of *L. betulina*), LEA (ethyl acetate extract of *L. betulina*), LET (ethanol extract of *L. betulina*), THE (hexane extract of *T. vesicolor*), TEA (ethyl acetate extract of *T. vesicolor*), TET (ethanol extract of *T. vesicolor*), CHE (hexane extract of *C. polyzona*), CEA (ethylacetate extract of *C. polyzona*) and CET (ethanol extract of *C. polyzona*).

2.3. Total Phenol Determination

The method of estimating total phenols as described by Singleton *et al*. (1999) was used. Fifty microlitre (50µL) of extract was added 250µL of undiluted Folin-Ciocalteau-reagent. After 1 min, 750µL of 20% (w/v) aqueous Na_2CO_3 were added and the volume was made up to 5.0mL by adding 3.95mL of water. The control contained all the reaction reagents except the extract. The preparation above was incubated for 2 h at 25^{o}C and the absorbance was measured at 760nm and compared to a gallic acid calibration curve. Total phenols were determined as gallic acid equivalents (mg gallic acid/g extract) in triplicate. Each experiment was repeated three times and the results were expressed as average values.

2.4. Scavenging Effect of Extracts on DPPH Radicals

The method of Blois (1958) was used in determine the effect of extracts of *Lenzites betulina*, *Trametes vesicolor* and *Coriolopsis polyzona* on DPPH• radicals with some modifications. A solution of DPPH (0.5mmol/L) in ethanol and in 0.05 mol/L acetate buffer (pH 5.5) was prepared. Extract in solution (0.1mL of 2mg/mL) was mixed with 2mL of acetate buffer, 1.9mL of absolute ethanol and 1mL DPPH solution. The mixture was shaken immediately after adding DPPH and allowed to stand at room temperature in dark for 30 min. The decrease in absorbance at 517nm was measured using Ultra Microplate Reader (Elx 808, BIO TEK Instruments Inc). BHT was used as positive control and the sample solution without DPPH was used as blank. The radical scavenging activity was measured as a decrease in absorbance of DPPH and calculated as:

$$\text{Scavenging activity (\%)} = \frac{Ab - (As - Asb)}{Ab} \times 100$$

Where Ab, As and Asb are absorbances at 517nm of DPPH of the blank, extract or control and sample blank respectively.

2.5. Estimation of Lipid Peroxidation.

Thiobarbituric acid reactive species (TBARS) levels were measured using rat brain homogenates according to the method described by Ng *et al*. (2000) with some modifications. Adult male Wistar rats (200-250 g) were provided by the Instituto de Fisiologı´a Celular, UNAM, and their use was approved by the Animal Care and Use Committee. Rats were maintained at 25 °C on a 12/12 h light/dark cycle with free access to food and water and killed under mild ether anesthesia. Cerebral tissue was rapidly dissected from the whole brain and homogenized in phosphate-buffered saline (PBS; 0.2 g of KCl, 0.2 g of KH_2PO_4, 8 g of NaCl, and 2.16 g of $NaHPO_4 \cdot 7\ H_2O$/L, pH 7.4) to produce a 1 in 10 homogenate (w/v) (Rosato *et al*., 2002). The homogenate was centrifuged for 10 min at 3400 rpm, and the resulting pellet was discarded. The protein content of the supernatant was measured according to the method of Lowry *et al*. (1951), and samples were adjusted to 2.5 mg of protein/mL with PBS. The supernatant (400 μL, 1 mg of protein) was preincubated with sample (50 μL) at 37 °C for 30 min, then peroxidation was initiated by the addition of 50 μL of freshly prepared FeSO4 solution (final concentration) 10 μM), and the sample was incubated at 37 °C for an additional 1 h (Ng *et al*., 2000). The TBARS assay was determined as described by Ohkawa *et al*. (1979) with the modification, 0.5 mL of TBA reagent (1% thiobarbituric acid in 0.05 N NaOH and 30% trichloroacetic acid, 1:1) was used and the final solution was cooled in ice water bath for 10 min, centrifugated at 10000 rpm for 5 min, and then heated at 95 °C in a boiling water bath for 30 min. BHT and α-tocopherol were used as positive controls. Then, the absorbance was measured at 532 nm in the Ultra Microplated Reader (Elx 808, BIO TEK Instruments Inc). Results are expressed as nanomoles of TBARS per milligram of protein, with percent inhibition after 30 min calculated as the inhibition ratio (IR), where C = absorbance of the control and E = absorbance of the test sample.

$$\text{IR (\%)} = [(C - E)/C] \text{ X } 100$$

These values were plotted against the log of the concentrations of individual extracts. Each experiment was replicated three times and the results were expressed as average values.

2.6. Cytotoxicity Assay

The extracts were screened *in vitro* against human cancer cell lines: U251 (human glioblastoma), PC-3 (human prostatic adenocarcinoma), K562 (human chronic

myelogenous leukemia), HCT-15 (human colorectal adenocarcinoma), MCF-7 (human breast cancer cell line) and SKLU-1 (human lung adenocarcinoma). Human cancer cell lines were supplied by National Cancer Institute, USA. The protocol described by Monks *et al*. (1991) for assessing human tumor cytotoxicity was adopted. The cell lines were cultured in RPMI-1640 medium which was supplemented with 10% fetal bovine serum, 2mM L-glutamine, 10,000 units/ml penicillin G, Sodium, 10µg/mL streptomycin sulfate and 25µ/mL amphotericin B (Gibco) and 1% non-essential amino acids (Gibco). The cultures were maintained at 37^{o}C in a 5% CO_2 humidified atmosphere. The viability of the cell exceeded 95% as verified by trypan blue method.

The cells were removed from the tissue culture flasks by treatment with trypsin, and diluted with fresh media. One-hundred-microliters cell suspension aliquots, containing 5000 – 10,000 cell per well, were transferred into 96 well microtiter plates and incubated at 37^{o}C for 24h in a 5% CO_2 atmosphere. Stock solution of extracts initially dissolved in DMSO (20mM) were prepared and further diluted to a final concentration of 50µg/mL. One hundred microliter aliquots of diluted solution of extracts were added to each well. The cultures were exposed for 48h to the extracts at concentration of 50µg/mL. After the incubation period, cells were fixed to the plastic substratum by the addition of 50µL of cold 50% aqueous trichloroacetic acid. The plates were incubated at 4^{o}C for 1h, washed with tap water and air-dried. The trichloroacetic-acid-fixed cells were stained by the addition of 0.4% sulforhodamine B (SRB). Free SRB solution was then removed by washing with 1% aqueous acetic acid. The plates were then air-dried, and the bound dye was solubilized by the addition of 100µL of 10mM-unbuffered Tris base. The plates were placed on a shaker for 5 min, prior to analysis. Optical density was determined in the Ultra Microplated Reader (Elx 808, BIO TEK Instruments Inc) while the absorbance was measured at 515nm. Each experiment was repeated three times and the results were expressed as average values.

3. Results and Discussion

Higher fungi are a major source of biological active natural substances among many diverse organisms which provide a rich variety of active metabolites (Liu, 2007). There are potentially many bioactivities and novel compounds still to be discovered in higher fungi since until now only a few numbers of higher fungi have been biologically and chemically investigated (Liu, 2007; Oyetayo, 2011). Nigeria is extraordinarily rich in higher fungi. However, there are few data on the medicinal uses of these fungi. The current study reports the total phenol, antioxidant and cytotoxic effects of extracts of three wild mushrooms, *Lenzites betulina* , *Trametes vesicolor* and *Coriolopsis polyzona* collected from Nigeria.

The total phenol content of the extracts ranged from 8.22µgGAE/mL to 60.54 µgGAE/mL (Figure 1). The highest phenolic content was recorded for CET (60. 54 µgGAE/mL). Generally, the phenolic content of ethanolic extracts was higher than extracts obtained with hexane and ethyl acetate. It has been reported that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g is ingested daily from a diet rich in fruits and vegetables (Tanaka *et al*., 1998).

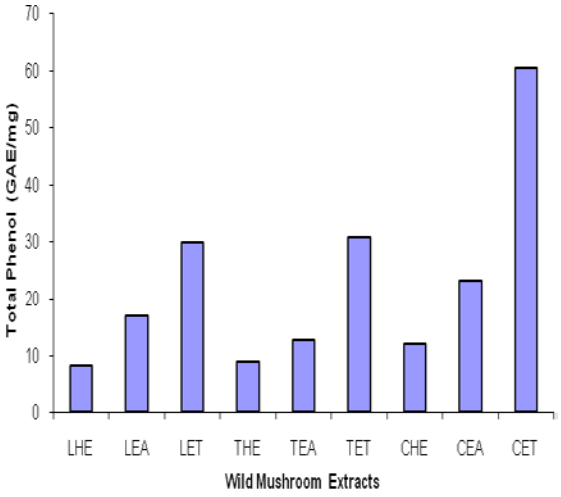

Figure 1. Total Phenol Content (GAE/mg) of Extracts of Wild Mushrooms.
Values are means of replicates (n=3). LHE: hexane extract of *L. betulina*, LEA: ethyl acetate extract of *L. betulina*, LET: ethanol extract of *L. betulina*, THE: hexane extract of *T. vesicolor*, TEA: ethyl acetate extract of *T. vesicolor*, TET: ethanol extract of *T. vesicolor*, CHE: hexane extract of *C. polyzona*, CEA: ethylacetate extract of *C. polyzona* and CET: ethanol extract of *C. polyzona*.

The ability of extracts to scavenge for DPPH radicals is presented in Table 1. The extracts displayed concentration dependent DPPH scavenging activity. The scavenging activity of extracts at 1000µg/mL ranged from 14.65% to 77.51%. Ethanolic extracts displayed better DPPH scavenging capacity when compared with the other extracts. This may be as a result of higher phenolic contents of ethanolic extracts (29.88 µgGAE/mL to 60.54µgGAE/mL). It had been reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic compounds (Velioglu *et al*., 1998). In this study, the correlation coefficient of DPPH activity of extract with the phenolic content was found to be 0.996.

The inhibition of the formation of thiobarbituric acid reactive species (TBARS), a measure of lipid peroxidation was concentration dependent and it ranges from 7.97% to 91.18% at 1000µg/mL. Generally, the ethanolic extracts exhibited higher TBARS inhibition (85.46% to 91.18%) when compared to the other extracts (Table 1). The higher TBARS inhibition exhibited by ethanolic extracts may be as a result of higher phenolic content present in it. In general, a correlation between higher antioxidant activity and larger amount of total phenolics was found in the mushroom extracts. The inhibition of the formation of TBARS by ethanolic extracts was higher than the second positive control, tocopherol (63.78%).

Table 1. Scavenging of DPPH radicals (%)* and inhibition of the production of TBARS (%)*in rat brain homogenate by Wild Macrofungi Extracts

	DPPH Scavenging			Inhibition of TBARS production		
Extracts	**10µg /ml**	**100 µg /ml**	**1000 µg /ml**	**10µg /ml**	**100 µg /ml**	**1000 µg /ml**
LHE	0.68	2.95	34.19	0.00	0.00	7.97
LEA	3.14	3.55	39.89	0.00	0.00	25.91
LET	2.37	6.54	70.99	0.00	2.78	90.90
THE	1.45	1.84	14.65	0.00	0.00	18.68
TEA	1.31	3.68	25.44	0.00	4.77	58.07
TET	2.98	10.12	43.77	0.00	1.91	91.18
CHE	0.87	2.85	21.28	0.00	0.00	11.13
CEA	3.38	11.61	44.20	8.37	11.97	75.81
CET	4.55	17.16	77.51	0.00	16.08	85.46
BHT	43.42	87.87	88.22	96.26	97.00	97.90
Tocopherol	35.89	90.02	90.71	63.78	98.54	98.80

Growth inhibition of human cancer cell lines by extracts is presented in Table 2. The inhibition of cell lines varies from one extract to the other. However, the following extracts, THE, CHE, TEA, CEA and TET exhibited significant inhibition of the various cell lines. Overall, ethanol extract of *Trametes vesicolor* (TET) showed the highest cytotoxic activity with 100% inhibition of HCT-15, MCF-7 and SKLU-1 cancer cell lines. A *β*-glucans, krestin from cultured mycelia biomass of *Trametes versicolor* (Turkey Tail) had earlier been reported to possess antitumour activity (Ikekawa 2001; Wasser, 2002). The least cytotoxic effect was exhibited by ethyl acetate extract of *Lenzites betulina* (LEA). Growth inhibition of K562 was the lowest when compared to the inhibition of other cell lines by the extracts.

Table 2. Cytotoxic Effects (%)* of Wild Macrofungi Extracts on Human Cancer Cell lines.

Extracts	U251	PC-3	K562	HCT-15	MCF-7	SKLU-1
LHE	NA	NA	41.85	17.65	37.93	25.15
LEA	NA	23.55	NA	NA	NA	NA
LET	NA	11.65	3.50	22.00	22.31	14.28
THE	13.38	51.95	22.28	41.45	63.78	25.15
TEA	45.46	78.80	NA	76.77	92.26	98.12
TET	46.15	84.97	12.77	100	100	100
CHE	12.90	37.77	16.58	61.12	52.23	56.47
CEA	76.68	59.99	NA	95.13	74.80	92.74
CET	NA	18.77	28.50	15.70	21.00	12.90

*Values are mean of replicates (n=3). NA: No Activity. U251:human glioblastoma, PC-3:human prostatic adenocarcinoma, K562: human chronic myelogenous leukemia, HCT-15: human colorectal adenocarcinoma, MCF-7: human mammary adenocarcinoma and SKLU-1: human lung adenocarcinoma.

In conclusion, significant DPPH scavenging effect, inhibition of the formation of TBARS and growth inhibition of cancer cell lines demonstrated by extracts indicates that these wild macrofungi contain bioactive compounds that may be used in ameliorating the problem of free radicals and cancerous cells. Further works of isolation, purification, identification and bioassay of specific bioactives from these macrofungi will be the next focus of this research.

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References

Anderson JB and Stasovski E. 1992. Molecular phylogeny of Northern Hemisphere species of *Armillaria. Mycologia,* **84**: 505-516.

Blois MS. 1958. Antioxidants determination by the use of a stable free radical. *Nature*, **181**: 1199-1120.

Ferreira ICFR, Baptista P, Vilas-Boas M and Barros L. 2007. Free-radical scavenging capacity andreducing power of wild edible mushrooms from northeast Portugal: individual cap and stipe activity. *Food Chem.,* **100**: 1511-1516.

Harvey AL. 2008. Natural products in drug discovery. *Drug Discov. Today*. **13 (19/20):** 894 901.

Hawksworth DL. 2001. The magnitude of fungal diversity: The 1.5 million species estimaterevisited. *Mycolog. Res*. **105**: 1422–1432.

Ikekawa T. 2001. Beneficial effects of edible and medicinal mushrooms in health care. *Int. J. Med. Mushrooms* **3**:291–298.

Jong SC and Birmingham JM. 1993. Medicinal and therapeutic value of the Shiitake mushroom. *Advan. Appl. Microbiol***.,** **39**: 153-184.

Liu J-K. 2007. Secondary metabolites from higher fungi in China and their biological activity. *Drug Discov. Ther*. **1(2):** 94 – 103.

Lorenzen K and Anke T. 1998. Basidiomycetes as a source for new bioactive natural products. *Cur.Organ. Chem*. **2**:329-64.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.,* **193**: 265-275.

Mau JL, Chang CN and Chen CC. 2002. Antioxidant properties of several medicinal mushrooms. *J. Agric Food Chem***.,** **50**: 6072-6077.

Mau JL, Chang CN, Huang SJ and Chen CC. 2004. Antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* mycelia. *Food Chem.*, **87**: 111-118.

Mizuno T. 1999. The extraction and development of antitumor active polysaccharides from medicinal mushrooms in Japan-Review. *Inter. J. Medicinal Mushrooms*, **1:** 9-30.

Monks A, Scudiero D, Skehan P, Shoemaker R, Paul K, Vistica D, Hose C, Langley J, Cronis P, Vaigro-Woff A, Gray-Goodrich M, Campbell H, Mayo J and Boyd M. 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J. Nat. Can. Inst*. **38(11)**: 757 – 766.

Moser M (1983) Die Röhrlinge und Blätterpilze (Polyporales, Boletales, Agaricales, Russulales). In: Gams H (Hrg.) Kleine Kryptogamenflora, Band II b/2. Basidiomyceten, 2. Teil, 5. Aufl. Gustav Fischer Verlag, Stuttgart, pp 1–532.

Ng TB, Liu F and Wang ZT. 2000. Antioxidative activity of natural products from plants. *Life Sci.*, **66**, 709-723.

Ohkawa H, Ohishi N and Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, **95**: 351-358.

Oyetayo VO. 2011. Medicinal uses of mushrooms in Nigeria: Towards full and sustainable exploitation. *Afr. J. T radit. Complement. Altern. Med*. **8 (3):** 267-274.

Rossato JI, Ketzer LA, Centuriao FB, Silva SJ, Lu¨dtke DS, Zeni G, Braga AL, Rubin MA and Rocha BT. 2002. Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain. *Neurochem. Res.,* **27**: 297-303.

Singleton V, Orthofer R, Lamuela-Raventos R. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In L. Packer (Ed.). **Oxidants and Antioxidants, , part A, Methods in Enzymology** (Vol.299). New York: Academic Press, pp. 152 – 178

Tanaka M, Kuei CW, Nagashima Y and Taguchi T. 1998. Application of antioxidative maillrad reaction products from histidine and glucose to sardine products. *Nippon Suisan Gakkaishil***, 54**: 1409–1414.

Veglioglu YS, Mazza G, Gao L and Oomah BD. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Chem***., 46**: 4113-4117.

Wasser SP. 2002. Medicinal mushrooms as a source of antitumour and immunostimulating polysaccharides. *Appl. Microbiol. Biotech*. **60**: 258 - 274.

Wasser SP and Weis AL. 1999. Therapeutic effects of substances occurring in higher basidiomycetes mushrooms a modern perspective. *Crit. Rev.Immunol*., **19 (1):** 65-96.

Wu Y, Sun H, Qin F, Pan Y, Sun C. 2006. Effects of various extracts and polysaccharidefrom edible mycelia of *Codyceps sinensis* on cellular and humoral immune responseagainst ovalbumin in mice. *Phytother. Res*. 20: 646 –652.

Watling, R. 1973. **Identification of the Larger Fungi**. Hulton, Amersham.

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Isolation, Characterization and Determination of Antimicrobial Properties of Lactic Acid Bacteria from Human Milk

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Abstract

Breast milk has a distinctive combination of proteins, carbohydrates, minerals, lipids and various vitamins that promote the proper growth, development and immunity of the infants. That's why it is considered to be a complete food for new born babies. Moreover, it is also rich in various bioactive compounds which promote the maturation of immune system as well as develop body's defense against infections. Among these bioactive agents, probiotic bacteria were isolated from human milk in this research work using selective MRS media. Two *Lactobacillus spp.* were isolated from each of the two breast milk samples, were observed as potential probiotics, and identified using morphological and biochemical tests. These bacteria were facultative anaerobic, gram positive, catalase negative and non-endospore forming. They showed tolerance against 0.3% bile concentration and 1-10% NaCl. Sugar fermentation patterns of both isolated bacteria also greatly varied. Isolate-1 from both sample 1 and 2 showed antimicrobial activity against *Shigella flexneri, Shigella dysenteriae, Vibrio cholerae and Salmonella typhi.* Isolate-2 from sample 1 and 2 showed antimicrobial activity against *Shigella flexneri, Shigella dysenteriae, Vibrio cholerae, Salmonella typhi, Staphylococcus epidermidis* and *Pseudomonas* spp. The addition of breast milk probiotics to infant formulas could be a new alternative to mimic some of the functional effects of human milk in children who are not breastfed

Keywords: Human milk, *Lactobacillus* spp., Sugar fermentation pattern, Quantification of organic acid, Bile tolerance, Antimicrobial activity.

1. Introduction

Probiotics are live microorganisms which are defined by the World Health Organization/ Food and Agricultural Organization (2001) as: "Live microorganisms whose administration in adequate amount to the body is able to confer a health beneficial effect on the host". The most common types of microbes which are used as probiotics are lactic acid bacteria (LAB) and Bifidobacteria.

A number of genera within Firmicutes phylum like *Lactobacillus, Lactococcus, Lactosphaera*, *Carnobacterium, Enterococcus*, *Streptococcus, Tetragenococcus, Oenococcus*, *Pediococcus, Weissella, Melissococcus, Vagococcus* constitute lactic acid bacteria.(Ercolini *et al.*, 2001; Jay, 2000; Holzapfel *et al.*, 2001). LAB are Gram-positive bacteria (Fooks *et al.,*1999) able to ferment carbohydrates into lactic acid and energy (Jay, 2000). Some LAB differ in their metabolic pathway for example homofermentative bacteria like *Lactococcus* and *Streptococcus* produce two lactate molecules from one glucose molecule while heterofermentative bacteria like *Leuconostoc* is able to convert one molecule of glucose into ethanol, lactate and carbon dioxide (Caplice and Fitzgerald, 1999; Jay, 2000; Kuipers *et al.*, 2000).

Furthermore, lactic acid bacteria yield some organic compounds that contribute to the aroma as well as flavor of the fermented products. (Caplice and Fitzgerald,1999).

Human milk is a complex biological fluid that is species-specific and completely fulfills both nutritional and microbiological requirements of the new born. Breast milk boosts up immune system and builds body defense against various infectious diseases which makes it superior to other food supplements for infants. Various bioactive compounds like immunoglobulins, lysozyme, antimicrobial acids, oligosaccharides, glycoproteins for example lactoferrin, polyamines, immune cells and bioactive peptides present in breast milk that are responsible for it's anti-infective effect (Saavedra JM, 2002; Isaacs CE, 2005). These bioactive compounds of human milk play a major role in the regulation of the anti-inflammatory system. Due to immunomodulatory action of human milk, the incidence as well as severity of various infectious diseases like tetanus, poliomyelitis and diphtheria is lesser in breast-fed infants than those fed with other food formulae (Hahn-Zoric M. *et al.*, 1990). The addition of breast milk probiotics to infant formulas could be a new alternative to mimic some of the functional effects of human milk in children who are not breastfed. That is why breast milk was selected as source of probiotic bacteria in this study. In human milk, most

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frequently occurring LAB are *Lactobacillus*, *Lactococcus* and *Enterococcus* (Federico Lara-Villoslada1 *et al.*, 2007).

Therefore the present research work was undertaken to isolate and characterize lactic acid bacteria from human milk and to study the antimicrobial properties of isolated probiotic bacteria.

2. Materials and Methods

2.1. Collection of samples

Human breast milk used in this study was obtained from two healthy mother volunteers in Khulna Medical College Hospital. The samples were collected in sterile carriers and stored on ice until delivery to the laboratory.

2.2. Media preparation

The lactic acid bacteria Lactobacillus spp. were isolated from breast milk samples using selective MRS broth and MRS agar media (De Man, Rogosa and Sharpe, 1960). Additionally, 0.5% salicin and 0.05% cysteine were added to MRS to improve the specificity of this medium for isolation of Lactobacillus acidophilus and Lactobacillus delb. ssp. bulgaricus, respectively (Hartemink *et al.,* 1997; Lankaputhra *et al.,* 1995; Shah, 2000). The pH of the media was adjusted to 6.5 and 5.2, respectively, using a digital electrode pH meter.

2.3. Isolation of bacteria

Lactobacillus isolate-1 from sample 1 and 2 was obtained using MRS-salicin medium at pH 6.5. One milliliter of each sample was 4-fold serially diluted (l0-1 to l0-4) in 0.15% sterile peptone water. For this agar, the spread-plate technique was used. After solidifying, the plates were incubated anaerobically at 37°C for 24 h. For the isolation of Lactobacillus isolate-2 from the both samples, selective MRS-cystine agar medium at pH 5.2 was used. Then one milliliter of each sample was 4-fold serially diluted (l0-1 to l0-4) in 0.15% sterile peptone water. The plates were incubated anaerobically at 45°C for 72 hr. The cultures were subjected to five sub-cultures to obtain the bacteria in pure culture. The cultures were maintained in MRS broth at pH 6.5.

2.4. Identification

The isolated bacteria were identified as Lactobacillus spp. by observing their morphological characteristics and by means of several biochemical tests.

2.5. Sugar fermentation test

MRS broth at pH 6.5 was put into a screw capped test tube and phenol red (0.01 g per L) was added into the tube as pH indicator. The medium was autoclaved at 121°C for 15 min. After autoclaving, 1 ml of different types of sugar solutions (10%) (filtered and sterilized) were inoculated into the different tube. Then 200 µl of an overnight bacterial culture was inoculated into the broth medium and incubated anaerobically at 37°C for 24 h. As a pH indicator, phenol red was included in the medium; acid production changed the medium from its original color to yellow. After adding the proper amount of broth, Durham tubes were inserted into each culture tube in order to observe gas production.

2.6. NaCl tolerance test

For the determination of NaCl tolerance of isolated lactobacilli, 10 test tube containing MRS broth were adjusted with different concentration (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% (v/v) fresh over night culture of Lactobacillus and incubated at 37°C for 24 h. After 24 h of incubation their growth was determined by observing culture medium turbidity.

2.7. Quantification of organic acid and determination of pH value

One percent (v/v) 24 h active culture of lactobacillus was used to inoculate 10% sterilized skim milk obtained from whole milk (Vita Co-operative Bangladesh Ltd). The initial pH (6.62) was determined by a digital electrode pH meter. The inoculated skim milk was incubated at 37°C for 72 h and samples were collected at 24 h, 48 h and 72 h. Samples having coagulated milk were separated by filtration. The pH of the separated liquid was recorded using a digital electrode pH meter and quantification of organic acid was performed through titration with 0.1 N NaOH.

2.8. Bile tolerance activity

Bile tolerance of lactobacilli isolated from human milk was determined using the protocol described by Graciela and Maria (2001).

2.9. Antimicrobial activity of metabolites produced by Lactobacillus isolates

Modified agar well diffusion method of Schillinger and Lucke (1989) and Toba *et al.* (1991) was used to detect antimicrobial activities of cell free supernatant (CFS) produced from the Lactobacillus isolates. These assays were performed in duplicate. Twenty milliliters of nutrient agar medium (MHA, Difco) were poured into each plate. The pathogenic strains (listed in table-8) obtained from bacterial stocks preserved in animal cell culture laboratory of Biotechnology and Genetic Engineering discipline, Khulna University, Bangladesh, were adjusted to109 cfu/mL by adding sterile water, and spread on the surface of nutrient agar plate. Four wells of 4 mm diameter were cut into these agar plates using a sterile tip and 15 µL, 20 µL, and 25 µL of the cell free supernatant (CFS) collected from 72 hour old bacterial culture were poured into three wells and MRS broth was placed in one well as negative control. The pH of the cell free supernatant was 6.1 and the MRS broth was adjusted to the same pH. The plates were incubated aerobically overnight at 37°C. The plates were examined for zones of inhibition.

3. Results

3.1. Identification

Bacteria isolated from human breast milk were identified as *Lactobacillus* spp. by observing their colony morphology, physiological as well as biochemical

characteristics. The isolates which grew on MRS agar *media* with 0.5% salicin and 0.05% cysteine had small, circular, white-creamy color, convex and nontransparent colonies. Microscopically they were Gram-positive, rod shaped, non-motile, catalase negative, and lacked endospores.

3.2. Sugar fermentation pattern

Isolated bacteria were identified up to species level on the basis of their growth at different temperatures and sugar fermentation tests as recommended by Harrigan and McCance (1976). Four isolates were selected from two samples and subjected to sugar fermentation test. In the sugar fermentation patterns, mainly acid and no gas production was observed. The acids and gas production results are presented in Table-1.

Table1.Sugar fermentation patterns of the four isolates from human milk samples

Sample no.	Name of the bacteria isolate	Rib	Sor	Manni	Sucr	Fruct	Cello	Sal	Lact	Gas Production
1	Isolate-1	+	-	+	+	+	+	+	+	no
	Isolate-2	+	-	-	-	+	-	-	+	no
2	Isolate-1	+	-	+	+	+	+	+	+	no
	Iolate-2	+	-	-	-	+	-	-	+	no

Legend. Rib=Ribose, Sor=Sorbitol, Manni=Mannitol, Sucr=Sucrose, Fruct=Fructose, Cello=Cellobiose, Sal=Salicin, Lact=Lactose, (+) means good fermentation and acid production, (-) means no fermentation and no acid production.

3.3. Tolerance to NaCl

The identified lactobacilli (isolate -1 and isolate-2 from sample 1 and 2) from human milk were able to tolerate 1-10% NaCl. The results are presented in Table 2.

Table 2. Tolerance of *Lactobacillus* isolate-1 and isolate-2 to NaCl

Concentration of NaCl (%)	Isolate-1		Isolate-2	
	Sample-1	Sample-2	Sample-1	Sample-2
1	+++	+++	+++	+++
2	+++	+++	++	++
3	+++	+++	++	++
4	+++	+++	++	++
5	++	++	++	++
6	++	++	+	+
7	++	+	+	+
8	++	+	-	-
9	+	+	-	-
10	-	-	-	-

Legend: (+++) maximal growth, (++) good growth, (+)minimal growth, (-) no growth.

3.4. Quantification of organic acid and determination of pH value

The identified lactobacilli from human milk (isolate-1 and isolate-2 from both samples) coagulated the skim milk and produced organic acids in the sterilized skim milks which were detected by titrimetric methods. The results are presented in Table 3.

Table 3. Organic acids (%) and pH in skim milk produced by *Lactobacillus* isolates from sample-1 and sample-2 of human breast milk

Sample no.	Isolates	Incubation time (Hour)	Incubation temp. (C)	Organic acid (%)	Initial pH of skim milk	pH at end of incubation
1	Isolate-1	24	37°	2.98	6.61	6.20
		48	37°	5.225		5.21
		72	37°	9.178		4.13
	Isolate-2	24	37°	2.112		6.01
		48	37°	3.423		6.12
		72	37°	4.714		5.89
2	Isolate-1	24	37°	2.90	6.61	6.16
		48	37°	4.210		5.87
		72	37°	6.217		3.93
	Isolate-2	24	37°	2.523		6.01
		48	37°	3.735		5.96
		72	37°	5.172		5.96

3.5. Bile tolerance test

Isolated lactobacilli were screened for their ability to tolerate bile salts by spectrophotometery. Results comparing the tolerance of the different isolates to bile salts are presented in Tables 4, 5, 6 and 7.

3.6. Antimicrobial test

Lactobacillus isolate-1 from sample 1 and 2 showed antimicrobial activity against *Shigella flexneri, Shigella dysenteriae, Vibrio cholerae* and *Salmonella typhi. Lactobacillus* isolate-2 from both samples showed antimicrobial activity against *Staphylococcus epidermidis, S. flexneri, S. dysenteriae, V. cholerae, S. typhi,* and *Pseudomonas spp*. Results are presented in Tables 8 and 9 and shown in figures 1 and 2.

Table 4.: Bile salt tolerance of *Lactobacillus* isolate-1 from sample-1 in MRS broth

Concentration of bile salt (%)															
0.05			0.1			0.15			0.2			0.3			
Incubation time (hour)															
4	8	24	4	8	24	4	8	24	4	8	24	4	8	24	
Spectrophotometer reading															
1.02	1.927	2.196	1.055	1.940	2.156	1.044	1.974	2.214	0.987	1.901	2.214	0.894	1.887	2.135	

Table 5. Bile salt tolerance of *Lactobacillus* isolate-1 from sample-2 in MRS broth

Bile salt concentration (%)															
0.05			0.1			0.15			0.2			0.3			
Incubation time (hour)															
4	8	24	4	8	24	4	8	24	4	8	24	4	8	24	
Spectrophotometer reading															
0.916	1.886	2.358	0.948	1.961	2.545	1.045	1.935	2.644	1.168	1.948	2.448	1.172	1.960	2.685	

Table 6. Bile salt tolerance of *Lactobacillus* isolate-2 from sample-1 in MRS broth

Concentration of bile salt (%)															
0.05			0.1			0.15			0.2			0.3			
Incubation time (hour)															
4	8	24	4	8	24	4	8	24	4	8	24	4	8	24	
Spectrophotometer reading															
1.022	1.007	2.101	1.026	1.005	2.131	1.043	1.032	2.147	1.018	1.030	2.116	1.031	1.006	1.851	

Table 7. Bile salt tolerance of *Lactobacillus* isolate-2 from sample-2 in MRS broth

Bile salt concentration (%)															
0.05			0.1			0.15			0.2			0.3			
Incubation time (hour)															
4	8	24	4	8	24	4	8	24	4	8	24	4	8	24	
Spectrophotometer reading															
1.104	1.950	2.711	1.276	1.974	2.521	1.144	1.948	2.440	1.220	1.933	2.180	1.238	1.916	2.246	

Table 8 . List of the pathogenic bacteria used in antimicrobial assay

Bacteria no.	Name of the bacteria
1	*Staphylococcus aureus*
2	*Staphylococcus epidermidis*
3	*E. coli*
4	*Shigella flexneri*
5	*Shigella dysenteriae*
6	*Vibrio cholera*
7	*Enterococcus faecalis*
8	*Salmonella typhi*
9	*Pseudomonas spp.*

Source: Animal cell culture laboratory of Biotechnology and Genetic Engineering discipline, Khulna University, Bangladesh.

Table 9. *In vitro* antibacterial activity of *Lactobacillus* isolates

Bacterial strains	Diameter of zone ofinhibition in mm					
	Isolate-1 from sample 1 and 2			Isolate-2 from sample 1 and 2		
	Vol. 15µl / well	20µl / well	25µl / well	15µl / well	20µl / well	25µl / well
1.*Staphylococcus aureus*	Nil	Nil	Nil	Nil	Nil	Nil
2.*Staphylococcus epidermidis*	Nil	Nil	Nil	9	16	17
3. *E. coli*	Nil	Nil	Nil	Nil	Nil	Nil
4. *Shigella flexneri*	13	15	18	15	17	19
5. *Shigella dysenteriae*	10	16	17	14	17	22
6. *Vibrio cholera*	13	18	9	13	17	22
7. *Enterococcus faecalis*	Nil	Nil	Nil	Nil	Nil	Nil
8. *Salmonella typhi*	13	17	20	7	13	15
9. *Pseudomonas spp.*	Nil	Nil	Nil	15	16	19

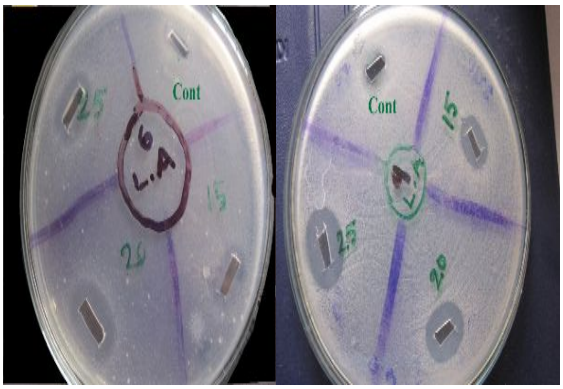

Figure 1. Antimicrobial activity of *Lactobacillus* isolate-1 against *Vibrio cholera* and *Shigella flexneri* (fromleft to right). The wells depicted as Cont in each plate contained blank MRS media as negative control.

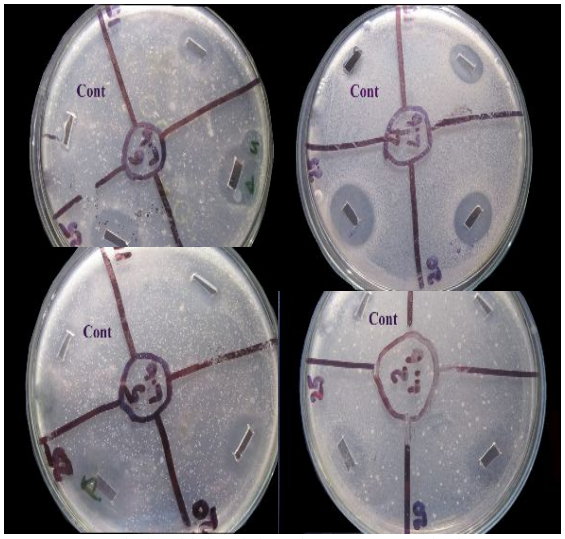

Figure 2. Antimicrobial activity of *Lactobacillus* isolate-2 against *Shigella flexneri, Vibrio cholera, Shigella dysenteriae* and *S. epidermidis* (clockwise from upper left side). The wells depicted as Cont in each plate contained blank MRS media as negative control.

4. Discussion

On the basis of colonal, morphological and biochemical characteristics (gram positive, catalase negative, endospore absence, non-motile, tolerance to inhibitory substances e.g., 1-10% NaCl, milk coagulation activities, sugar fermentation pattern, bile tolerance activity and antimicrobial activity), the isolates were identified as *Lactobacillus* spp.. The colonies of *Lactobacillus* isolate-1 from sample 1 and 2 are presumed to be *Lactobacillus acidophilus* and appeared rough, dull white, 0.1-0.5 mm in diameter, and demonstrated medium to short rods. The colonies of *Lactobacillus* isolate-2 from both samples are presumed to be *Lactobacillus delbrueckii* ssp. Bulgaricus and appeared cottony, rough, irregular, white,1.0 mm in diameter, and demonstrated long rods in chains. Earlier studies by Vamanu *et al.* (2005), Emanuel *et al.* (2005), Lilia et al. (2002), Oyetayo (2004), and Eduardo et al. (2003) have found similar aforementioned characteristics in isolated lactobacilli.

Isolate-1 from both samples tentatively identified as *Lactobacillus acidophilus* and isolate-2 (tentatively identified as *Lactobacillus delbrueckii* ssp. Bulgaricus) did not produce gas from glucose and other sugars and also showed variation in sugar fermentation patterns. Lactobacillus isolate-1 fermented all the sugars used except sorbitol. On the other hand, *Lactobacillus* isolate-2 from sample 1 and 2 fermented only three sugars (ribose, fructose, lactose) among eight sugars. This observation is consistent with the studies of Shah *et al.* (2000), and Azizpour *et al.* (2009), who found similar fermentation patterns in their isolated lactobacilli.

NaCl is an inhibitory substance which antagonizes the growth of certain types of bacteria. All of the isolates were able to grow at 1-7% NaCl concentration. Isolate-2 did not grow at 8 %, 9% and 10% NaCl concentration, however, isolate-1 grew at these concentrations. Elezete and Carlos (2005) isolated lactobacilli from gastrointestinal tract of swine that were tolerable to 4-8% NaCl. Schillinger and Lucke (1987) were able to grow lactobacilli isolated from meat and meat products in the presence of 7.5% NaCl and these results are similar to the findings of this present study.

Results have shown that organic acid production increased with the incubation time while pH of the media decreased with the increasing acid production. After 72 h incubation at 37°C, highest acidity (9.178%) and lowest pH (4.13) were observed for *Lactobacillus* isolate-1 from sample 1 and highest acidity (4.714%) and lowest pH (5.89) were observed for *Lactobacillus* isolate-1 from sample 2. On the other hand, after 72 h incubation at 37°C, highest acidity (6.217%) and lowest pH (3.93) were observed for *Lactobacillus* isolate-2 from sample 1 and highest acidity (5.172%) and lowest pH (4.89) were observed for *Lactobacillus* isolate-2 from sample 2. These findings are consistent with previous research findings of Haddadin *et al.* (2004), and Rashid *et al.* (2007).

Although the bile concentration of the human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 h (Prasad, *et al.,* 1998). According to our findings, all of the study isolates are able to grow up in 0.05-0.3% bile salts.

The capacity of substances to inhibit microbial growth is referred to as antimicrobial activity. Isolate-1 showed antimicrobial activity against *S. flexneri, S. dysenteriae, V. cholerae* and *S. typhi*. Diameter of zones of inhibition ranged from 10 mm to 20 mm. Isolate-2 showed antimicrobial activity against *S. epidermidis, S. flexeneri, S. dysenteriae, V. cholerae, S. typhi* and *Pseudomonas* spp. Diameter of zones of inhibition ranged from 7 mm to 22 mm. As the isolated lactic acid bacteria inhibited these pathogenic strains successfully, it may be expected that addition of these human milk probiotics to commercial food products for infants would confer effective protection against infections caused by these pathogens.

5. Conclusion

Lactic acid bacteria were isolated from human milk in pure culture and various properties of isolated bacteria were determined. All of isolates showed tolerance to bile salt, organic acid production and antimicrobial activity against some indicator microorganisms. Phenotypic identification effectively differentiated the isolates especially sugar fermentation patterns. Two different isolate strains were identified and these could be used as potential probiotic strains.

6. Recommendations

Future research work regarding adhesion to mucosal surface, clinical studies for human health, strain stability, bacteriophage resistance, viability in products, antibiotic resistance should be carried out.

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References

Azizpour K, TkmechiA and Agh N. 2009. Characterization of lactic acid bacteria isolated from the intestines of common carp of West Azarbaijin, Iran. *J Ani Vete Adv*., **8(6):** 1162-1164.

Caplice, E and Fitzgerald GF. 1999. Food fermentation: role of microorganisms in food production and preservation. *Int. J. Food Microbiol*. **50**, 131-149.

De Man J C, Rogosa M and Sharpe ME. 1960. A medium for cultivation of lactobacilli, *J. Appl. Bacteriol*., **23**: 130–135.

Eduardo LC JR., Carmina VP, Rivera MARV and Cabrera EC. 2003. Antimicrobial activity of probiotics from milk products. *Phil. J. Microbiol. Inf. Dis*. ,**32**: 71-74.

Eecolini D, Moschetti G, Blaiotta G and Coppola S. 2001. Behavior of variable V3 region from 16S rDNA of lactic acid bacteria in denaturing gradient gel electrophoresis. *Curr. Microbiol.* **42**: 199-202

Elizete DFRP and Carlos SR. 2005. Biochemical characterization and identification of probiotic *lactobacillus* for swine. *B.CEPPA. Curitiba*. **23:** 299-310.

Emanuel V, Adrian V, Ovidiu P and Gheorghe, C. 2005. Isolation of *a Lactobacillus plantarum* strain used for obtaining a product for the preservation of fodders. *Afr. J. of Biot.* , **4**: 403-408.

FAO/WHO. 2001. Food and Agriculture Organization of the United Nations/ World Health Organization.

Federico Lara-Villosladal, Olivaresl M, Sierral S, Rodriguez J M and Xaus J. 2007. Beneficial effects of probiotic bacteria isolated from breast milk. *British J Nutrition*, **98**, Suppl. 1, S96–S100.

Fooks LJ, Fuller R and Gibson GR. 1999. Prebiotics, probiotics and human gut microbiology. *Int. Dairy J*. **9**:53-61.

Graciela, F DE V and Maria, P T. 2001., Probiotic Properties of Lactobacilli. In: Spencer J F T and Spencer A L R D(Eds). **Food Microbiology Protocols,** Totowa, New Jersey: Humana Press Inc., p. 173.

Haddadin MSY, Awaishe SS and Robinson RK. 2004. The Production of Yoghurt with Probiotic Bacteria Isolated from Infants in Jordan. *Pak. J. of Nutr*, **3 (5),** 290-293.

Hahn Zoric M, Fulconis F, Minoli I, Moro G, Carlsson B, Bottiger M, Räihä N and Hanson L. 1990. Antibody response to parenteral and oral vaccines are impaired by conventional and low protein formulas as compared to breast-feeding. *Acta Paediatr Scand* **79**: 1137–1142.

Harrigan WF and Mccance ME. 1976. **Laboratory Methods in Food and Dairy Microbiology**, p. 19-20. Academic Press, London, UK.

Hartemink R, Domenech VR and Rombouts FM. 1997. Lamvab – A new selective medium for the isolation of *lactobacilli* from faeces, *J. Microbiol. Meth*.. **29:** 77–84.

Holzapfel WH, Haberar P, Snell J, Schillinger U and Huicin't Veld J. 1998. Overview of gut flora and probiotics. *Int. J. of Food Microbiol.* **41**:85-101.

Isaacs Ce. 2005 Human milk inactivates pathogen individually,additively and sinergistically. *J Nutr* **51:** 1286–1288.

Jay J.M. 2000. Fermentation and fermented dairy products, pp. 113-130. In : **Modern Food Microbiology**, 6th edition. An Aspen Publication, Aspen Publishers, Inc. Gaithersburg, USA.

Kuipers OP, Buist G and Kok J. 2000. Current strategies for improving food bacteria. *Res. Microbiol*. **151**: 815-822.

Lankaputhra WEV and Shah NP. 1995. Survival of *Lactobacillus* and *Bifidobacterium* spp. in the presence of acid and bile salts. *Cult. Dairy Prod. J*. **30**: 2–7.

Lilia T, Stefanova S and Yordanova T. 2002. Identification of lactic acid bacteria isolated from Katyk, goat's milk and cheese. *J. of Cult. Collections*. **3**: 48-52.

Oyetayo VO. 2004. Phenotypic characterization and assessment of the inhibitory potential of *Lactobacillus* isolates from different sources. *Afri. J. of Biot*. **3**: 355-357.

Prasad J, Gill H, Smart J and Gopal PK. 1998. Selection and characterization of *Lactobacillus* and *Bifidobacterium* strains for use as probiotic. *Int. Dairy J*. **8**:993-1002.

Rashid MHU, Togo K, Ueda M and Miyamoto T. 2007. Probiotic characteristics of lactic acid bacteria isolated from traditional fermented milk 'Dahi' in Bangladesh. *Pak. J. of Nutr.* **6 (6):** 647-652.

Saavendra JM. 2002. Probiotic agents: clinical applications in infants and children. In: NCR Ra¨iha¨ and FF Rubaltelli,(Eds) **Infant Formula: Closer to Reference**, pp. 15–27. Philadelphia: Lippincott Williams & Wilkins.

Schillinger U and Lucke FK. 1987. Identification of lactobacilli from meat and meat products. *Food Microbiology*. **4**: 199-208.

Shah NP. 2000. Probiotic bacteria: selective enumeration and survival in dairy foods. *J. Dairy Sci*., **83:** 894–907.

Vamanu A, Vamanu E, Drugulescu M, Popa O and Campeanu, G. 2005. Identification of a lactic bacterium strain used for obtaining a pollen-based probiotic product. *Turk J Biol*. **30**: 75-80.

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Challenges Towards *Hypericum sinaicum* Conservation in South Sinai, Egypt

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Abstract

Hypericum sinaicum is one of the near endemic plant species in Saint Katherine Protectorate. Found only in Sinai and Northwest Saudi Arabia, there are many challenges to the conservation of this species. The aim of this study is to: (1) identify and rank the different threats, and to identify their underlying root causes, as well as the barriers, affecting the conservation of the medicinal plants specially *Hypericum sinaicum* within the rich areas of SKP, and (2) compare and reassess documented by Assi (2007) in the same area. 237 circles with diameter 10 m were established to cover all environmental gradients with equal distance between each other. At each point we recorded all factors within the field that may be become Threat to the plant community, to medicinal plants. Results found that drought, feral donkeys and over collection are the most harmful threats for *Hypericum sinaicum* in Saint Katherine Protectorate, and most of root causes come from lack of awareness, weak law enforcement, lack of suitable strategies, weak financial support and lack of stakeholders' cooperation.

Keywords: Threat Assessment, *Hypericum sinanicum*, Conservation Challenges, Saint Katherine Protectorate, Threat Levels.

1. Introduction

It is clear that the loss of biodiversity has serious economic and social costs. The genes, species, ecosystems and human knowledge that are being lost represent a living library of options available for adapting to local and global change (UNEP, 1995). Environmental deterioration in arid ecosystems due to unmanaged human activities including harvesting of vegetation for fuel and medicine, overgrazing, urbanization and quarrying is evident in a decrease of plant cover, deterioration of soil productivity, and aggravation of soil erosion (Batanouny, 1983). Damage to vegetation and the soil surface and in arid lands is not easily repaired (Milton *et al*., 1994). These activities can impact the sustainable production of food, fiber, and fuel from these lands.

The Saint Katherine (SK) region is situated in the southern part of Sinai and is a part of the upper Sinai massif. It is located between 33˚ 55' to 34˚ 30' East and *28*° 30' to 28° 35' North. The Saint Katherine Protectorate (SKP) is one of Egypt's largest protected areas and includes the country's highest mountains. This arid, mountainous ecosystem supports a surprising biodiversity and a high proportion of rare and endemic plants. The flora of the mountains differs from other areas in Sinai, due to unique geology, morphology and climate (Hatab, 2009).

H. sinaicum is one of the near endemic species in SKP, as its distribution is limited to Sinai and Northwest Saudi Arabia (Boulos, 2002). *H. sinaicum* is listed as a rare species (IUCN, 1994), and it has high medicinal importance. Extraction from aerial parts produce substances like hypericin, protohypericin, pseudohypericin, protopseudohypericin, and hyperforin which have been shown to inhibit the growth of retroviruses in animals in addition to the treatment of depression (Rezanka and Sigler, 2007).

The vegetation in Saint Katherine Protectorate has been subjected to disturbance through human activities including "overgrazing, uprooting, tourism, quarrying and over-exploitation". For example, rarity of these species may be due to slow regeneration (e.g. *Thymus decussates*), or drought (e.g. *Hypericum sinaicum*) **(**Mosallam, 2007). Also *H. sinaicum* showed medium affect by tourism, construction and goat (*Capra hircus*) grazing (Assi, 2007).

The threat from feral donkeys (*Equus asinus*) is aggravated by the fact they cause destruction to a variety of plant species through trampling (Khafaja *et al*., 2006). Bedouins consume many plants in SKP (mainly as herbal infusion) (Khafaja *et al*., 2006). However, overharvesting is to a large extent due to commercial collecting and not collecting for personal use. The quantities collected for personal use are minor compared to those collected for trade (Assi, 2007).

Successful tourism attracts migrant labor, aggravating pressure on infrastructure and environmental resources. Even ecotourism can degrade the environment, because many of the places visited by ecotourists support fragile

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ecosystems (Budowski, 1976). Structures in ecologically fragile areas destroy habitats. Access roads impact a greater area of habitat than the tourist projects themselves.

Bedouins used to have their own system of protection (Hilf): An agreement to close an area to grazing for a certain period to allow for recovery. Now because of Bedouins' settlement, overgrazing has become a threat because of the limited area for grazing around the settlements, this is in spite of a reduction in average herd size from 50 prior to settlement to 10 currently (Assi, 2007). Some collecting has stemmed from an increased interest at the national and international level in research on the active ingredients of medicinal plants (MPs) particularly rare and endemic species. Researchers are interested in conducting their studies on sources obtained from the wild and not from cultivation (Assi, 2007).

The aim of this study is to: (1) identify and rank the different threats, identify their underlying root causes, and identify the barriers affecting the conservation of the medicinal plants, especially *Hypericum sinaicum* within SKP and (2) compare and reassess populations documented by Assi (2007) in SKP.

2. Materials and Methods

From the previous surveys we extracted 22 locations where *Hypericum sinaicum* was present (Shak Itlah, Wadi Tenia, Farsh Messila, Elmaein, Shak Sakr, Abo Tweita, Kahf Elghola, Elmsirdi, Wadi Eltalaa, Sherage, Ain Shekaia, Tobok, Elzawitin, Elgalt Elazrak, Abu Hebeik, Eltibk, Farsh Elromana, Abu Kasaba, Abu Walei, Elgabal Elahmar, Shak Mosa, Wadi Elrotk) within Saint Katherine Protectorate. We used a systematic sampling approach to capture local environmental gradients, placing 237 circles with 10 m diameters at equal distances apart. Within each circle we recorded within the field that may be a threat to the plant community. These factors included the following: feral donkeys, plant over collection, tourist intrusions (e.g. trespassing beyond trail borders and collection of firewood during camping), overgrazing, collection for scientific research, urbanization and settlement expansion and quarries as they were recorded by Assi (2007). This study taken place in the period from March 2012 to September 2012.

At each site a GPS fix was recorded in decimal degrees and datum WGS84 using Garmin 12 XL receiver. The fix was recorded to the fifth decimal digit. Arc View GIS 9.2 was used to plot the study sites. Wadi boundaries were digitized from 1:50,000 topographic maps with Egyptian Transverse Mercator projection (Blue belt). The Natural Neighbor tool from GIS 9.2 software (Spatial Interpolation) was used to make hot spots analysis by use x and y coordinates.

2.1. Identifying Threats

From Assi (2007), we extracted 7 main threats affecting the distribution of wild plants within SKP. Each threat was evaluated as follows:

2.1.1. Feral Donkeys

Using the methods of Alqamy (2005), Hatab (2009), and Omar *et al.* (2012), numbers of dung (droppings) of donkeys were counted at each circle to frequency of animal presence.

2.1.2. Overcollection

At each circle we recorded any sign of plant collection for the purposes of trade as medicinal plants, fuel or any economic value. Also assessment through meetings and interviews with the relevant stakeholders (collectors, traders and cco guides) will cover the medicinal plants rich sites within SKP and identify the hot spots (Assi, 2007).

2.1.3. Tourist Intrusions

At each site we recorded any tourism activity (paths, camping, rest points and wastes) and ranked each point by density level (How much area it cover) (Very low 20%, Low 40%, Medium 60%, High 80% and Very high >80%).

2.1.4. Overgrazing

Level of grazing was measured by dung abundance and ranked each point by density level (How much area it cover) (Very low 20%, Low 40%, Medium 60%, High 80% and Very high >80%).) Mammal dung was surveyed by recording the species concerned (mainly camel, goat, ibex, and fox) and the number of droppings (Alqamy, 2005, and Omar et al., 2012).

2.1.5. Collection for Scientific Research

We recorded all sites and target species of scientific interest by universities, research centers and scientific scholarships within SKP by reviewing reports and notifications from EIA (Environmental Impact Assessment) created by SKP staff.

2.1.6. Urbanization and Settlement Expansion

In this factor we used several approaches. First, using satellite images available in Google Earth 6.0.1.2032 (beta) with build date 2010, we observed settlements, roads and gardens and characterized them according to boundaries and density. Second, we carried out field assessment to detect any expansion (buildings, dams, wells and roads). Third, we carried out meeting with stockholders (City committee) to identify the hot spot sites defined as high impact area.

2.1.7. Quarries

We recorded all quarrying sites within SKP and created maps describing the exposed area for its impact of each site.

2.2. Underlying threat root causes, barriers and solutions

For each threat, we assigned the root causes, barriers, area, intensity, urgency, total ranking and categorical threat level. The above terms will describe as follows: **Root causes:** These are the underlying factors, usually social, economic, political, institutional, or cultural in nature, which enable or otherwise contribute to the occurrence and/or persistence of direct threats (IUCN definition). There is typically a chain of underlying causes behind any given direct threat.

Barriers: These are constraints (institutional, legal, technical, knowledge), which limit effective conservation of MPs.

A = Area: Approximate proportion of the overall area of a site likely to be affected by a threat under current circumstances (i.e. given the continuation of the existing situation). *Since there are 8 direct threats, the highest

ranked threat for "Area" receives a score of 8, and the lowest ranked threat receives a score of 1 (Assi, 2007).

I = Intensity: refers to the impact of the threat within a micro-site. Will the threat completely destroy the habitat in a small locality, or will it only cause minor changes (i.e. given the continuation of the existing situation). Since there are 8 direct threats, the highest ranked threat for "Intensity" receives a score of 8, and the lowest ranked threat receives a score of 1 (Assi, 2007).

U = Urgency: The importance of taking immediate action to counter the threat. Since there are 8 direct threats, the highest ranked threat for "Urgency" receives a score of 8, and the lowest ranked threat receives a score of 1.

TR = Total Ranking: Sum of Area + Intensity + Urgency (Assi, 2007).

2.3. Threat Level

Threat levels have been arranged as:

1. **Very High (rating above 20):** The threat is likely to be very widespread or pervasive in its scope, is likely to destroy or eliminate the conservation target over some portion of the target's occurrence at the site and seriously affect the conservation target throughout the target's occurrence at the site. The threat must be countered immediately or limited action today will likely mitigate much more intensive action in the future.
2. **High (rating between 15 and 20):** The threat is likely to be widespread in its scope, to seriously degrade and affect the conservation target at many of its locations at the site. The threat must be countered in the next 5 years OR limited action in the next 5 years will likely mitigate much more intensive action in the future.
3. **Medium (rating between 9 and 14):** The threat is likely to be localized in its scope, to moderately degrade and affect the conservation target at some of the target's locations at the site. The threat probably will need to be countered in the next 5-10 years.
4. **Low (below 9):** The threat is likely to be very localized in its scope, is likely to only slightly impair and affect the conservation target within a limited portion of the target's location at the site. The threat does not need to be countered in the next 10 years (Assi, 2007).

3. Results and Discussion

A total of 237 circles with average 10 per location were studied and output results can surmised as follows:

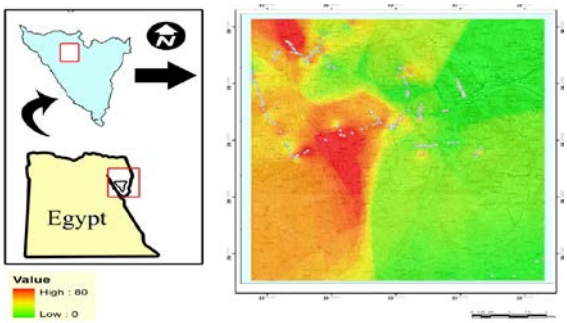
3.1. Threats hotspots and effect

3.1.1. Feral Donkeys

A total of 129 (54.4%) sites out of 237 were affected by feral donkeys. Twenty five (10.5%) sites had a high frequency of donkey occurrence, 45 (18.9%) had a medium level, 45 (18.9%) had a low level, 14 (5.9%) had a very low level and 108 (45.6%) sites had no donkey droppings.

It was observed that donkey's distribution affected by vegetation cover (donkeys concentrated on areas with high vegetation cover) which affecting by good water supply and showed negative relation with Bedouin community distribution (distributed away from human presence). Sites located within elevations ranging from 1800 m to 2000 m such as Abu Tweita, Wadi Gebal, Farsh Elromana and Farsh Messila recorded the highest presence for donkeys (Map 1). Grazing by these usually causes uprooting of the plants as indicated by Bedouins and field observations and this prevents plant regrowth. Soil compaction is associated with use by these animals and causes destruction to a variety of plant species through continuous trampling (Khafaja *et al*., 2006).

The field observations showed that feral donkeys grazed on a very wide spectrum of plant species compared with goats and camels; however, the numbers of feral donkeys have decreased sharply compared with the results of Assi (2007). The local community explained this was due to the sharp decrease in water supply.

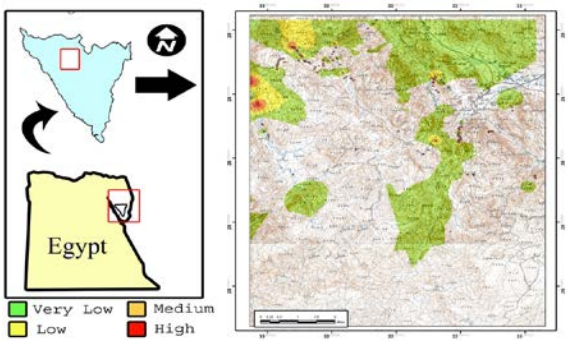


Map 1. Distribution of feral donkeys within study area

3.1.2. Over collection

Fifty-eight sites (24.4%) of 237 were affected by plant collection. One site 0.4%) had a high level of plant collection, 10 (4.2%) had a medium level, 16 (6.7%) had a low level, 32 (13.5%) had very low collection pressure and 178 (75%) sites showed no observations for plant collection.

Locations like Abo Hebik, Elgalt Elazrak, Abu Tweita, Sherige, Shak Musa, Elmesirdi and wadi Eltalaa are most targeted for collection (Map 2). These sites are characterized by high plant productivity and water supply; however, the collecting of plants increased with precipitation and was concentrated between March and December each year (flowering season). It was observed that collecting of plants may be affected by economic factors. In other words, when tourism levels fall, Bedouin start to collect plants for income. Results obtained from local communities showed that women are the most common collectors of plants, and they collect 5 times per season. Although the reasons for collecting these plants are always for trade or personal use as fuel, the use of plants as fuel has decreased sharply with the advent of butagaz.



Map 2. Hotspots of plant collection within study area

Results showed that *Origanum syriacum*, *Mentha longifolia*, *Salvia multicaulis, Chiliadenus montanus*, *Crataegus x sinaica* and *Thymus decussatus* are the most collected species for trade within the study area because of their medicinal value (Table 1), and these results confirm those of Assi, (2007). *Cotoneaster orbicularis, Phlomis aurea, Crataegus x sinaica*, *Ziziphus spina-christi*, *Rhamnus dispermus* and *Globularia arabica* are the most common species used as fuel (Table 1).

Table 1. Economic importance of some plant species within the study area

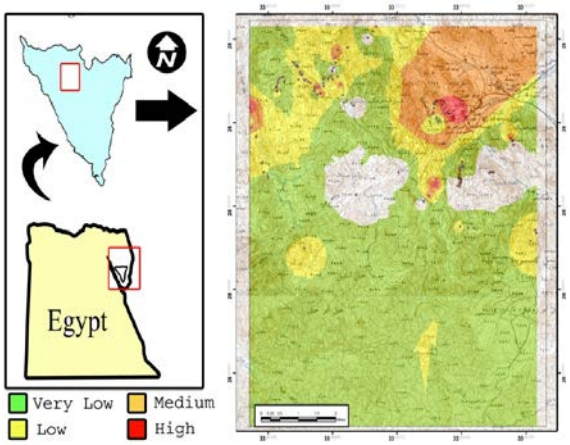
	Economic use		
Species	**Medicinal**	**Fuel**	**Food**
Ballota undulata (Fresen.) Benth.	Low		
Foeniculum vulgare (Ucria) Cout.	Low		
Globularia arabica Jaub. & Spach.	Medium	High	
Hyoscyamus boveanus (Dunal) Asch. & Schweinf.	Medium		
Plantago sinaica (Barneoud) Decne.	Medium		
Rhamnus dispermus Boiss.	Medium	High	
Rosa arabica Crep.	High	Medium	
Stachys aegyptiaca Pers.	High		
Tanacetum sinaicum (fresen.) Delile ex Bremer & humphries.	High		
Teucrium polium L.	High		
Verbascum sinaiticum Benth.	High		
Ziziphus spina-christi (L.)	High	High	High
Achillea fragrantissima (Forssk.) Sch. Bip.	High		
Serphedium herba-alba Asso.	High		
Artemisia judaica L.	High		
Chiliadenus montanus (Vahl) Brullo.	High		
Crataegus x sinaica Boiss.	High	High	
Mentha longifolia (L.) Huds.	High		
Origanum syriacum (Boiss.) Greater & Burdet.	High		
Pulicaria undulata (L.) C. A. Mey.	Medium		
Salvia multicaulis Vahl.	High		
Thymus decussatus Benth.	High		
Cotoneaster orbicularis Schltdl.		High	
Phlomis aurea Decne.		High	
Ficus carica L.			High

Hand picking of plant species is widely practiced as indicated by stakeholders, particularly collectors, which increases the rate of uprooting instead of using pruning shears. Collections of some species, such as *Origanum syriacum* and *Salvia multicaulis*, are limited to flowers. This could impact a plant species' life cycle and decrease the population size with time. It was observed that most collectors collect species with medicinal or economic value for personal consumption; however, the amount collected for this purpose is small compared with amounts collected for trade. Bedouin mentioned that *Hypericum sinaicum* with its great medicinal importance is still unused by local communities for folk medicine in SKP.

3.1.3. Tourist Intrusions

Two hundred one sites (84.8%) out of 237 were affected by tourism. Thirty eight sites (16%) had a high level of tourism, 47 (19.9%) had a medium level, 72 (30.3%) had a low level, 44 (18.5%) had a very low level and 36 (15%) sites showed no observations of tourism. Wadi Gebal, Farsh Elromana, Elgalt Elazrak, Abu Tweita, Wadi Tenia, Wadi Sherige and Wady Eltalaa were the sites with the highest levels of tourism (Map 3). About 3 million people from 51 nationalities visited SKP from 2003 to 2011 with an average 335.000 people per year. Most of them focused on the northern part of SKP, a world heritage site (Map 3). Many of the tourists do safari and camp in remote areas; usually safaris extend for many days using different camping sites; the most camping sites are in Firsh Elromana, Wadi Tenia, and Wadi Gebal.

Some of the negative impacts associated with tourists include collecting medicinal plants as souvenirs from the SKP and plant collection for fuel. Soil compactions due to trespassing leads to poor vegetation cover and results from trampling. Camping takes place in sheltered sites which provide water sources for tourists.



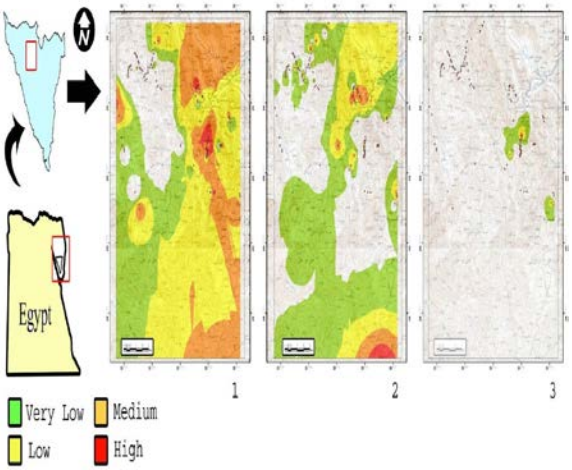
Map 3. Hotspots of tourism within study area

3.1.4. Grazing analysis

Results showed that the animals recorded within the greatest number of sites in the study area were goats followed by camels. A total of 158 sites (66.6%) out of 237 were affected by goats. Twenty eight sites (11.8%) had a high level of goat presence, 21 (8.8%) had a medium level, 69 (29.1%) had a low level, 39 (16.4%) had a very low level and 79 (33%) sites showed no observations for goat dung. Elmesirdi, Sheiage, Elahmar and Shak Musa had the most sites with goats presence which can be explained by their proximity to local community settlements (Map 4).

A total of 103 sites (43.5%) out of 237 were affected by camels. There were 14 sites (5.9%) with a high level of camel presence, 26 (10.9%) with a medium level, 42 (17.7%) with a low level, 21 (8.8%) with very low level and 134 (56.5%) sites with no observations for camel dung. Elawitein, Wadi Gebal, Wadi Tenia, Abu Tweita and Farsh Elromana had the most sites with recorded presence of camels. This can be explained by the easy access and heavy use by tourists for camping supported by camels (Map 4).

Ibex dung was found in low quantities at specific locations like Emesirdi, Shak Musa and Elahmar. The presence of these animals depended on the presence of water. Eleven sites (4.6%) sites out of 237 were affected by Ibex. Five (2.1%) had low presence, 6 (2.5%) recorded very low presence and 226 (95.3%) sites showed no observation for Ibex dung. There was significantly more domestic mammal dung (goats (58%) and camels (39%)) encountered than native mammal dung and this agrees with results observed by Guenther *et al*. (2005) and Omar *et al.* (2012).



Map 4. Hotspots of grazing animals within study area; 1- Goat, 2- Camel and 3- Ibex.

There were 18 plant families that showed heavy grazing; Asteraceae (33.3%), Lamiaceae (22.2%), Brassicaceae (16.6%) and Caryophyllaceae (16.6%) were the predominant families among grazed plants. It was observed that the following species were the most frequently grazed: *Juncus rigidus, Hypericum sinaicum, Galium sinaicum, Zilla spinosa, Mentha longifolia, Anarrhinum pubescens* and *Scrophularia libanotica*, See Tables (2, 3).

Table 2. Recorded families affected by grazing and number of species in each family

Family	Number of grazed species	Grazed species %
Asteraceae	6	33.3
Lamiaceae	4	22.2
Brassicaceae	3	16.7
Caryophyllaceae	3	16.7
Moraceae	2	11.1
Schrophulariaceae	2	11.1
Juncaceae	1	5.6
Asclepiadoideae	1	5.6
Dipsacaceae	1	5.6
Globulariaceae	1	5.6
Hypericaceae	1	5.6
Papaveraceae	1	5.6
Plantaginaceae	1	5.6
Poaceae	1	5.6
Resedaceae	1	5.6
Rosaceae	1	5.6
Umbelliferae	1	5.6
Zygophyllaceae	1	5.6

Table 3. Number of grazed stands by species within the study area and their frequency

Species	No of Grazed Stands
Juncus rigidus Desf.	25
Hypericum sinaicum Hochst.&Steud.	20
Galium sinaicum (Delile ex Decne.) Boiss.	14
Zilla spinosa Prantl.	13
Mentha longifolia (L.) Huds.	10
Anarrhinum pubescens Fresen.	10
Scrophularia libanotica Boiss.	9
Crataegus x sinaica Boiss.	9
Ficus palmata Forssk.	8
Diplotaxis harra. (Forssk.) Boiss	6
Centaurea eryngioides Lam.	6
Pterocephalus sanctus Decne.	6
Teucrium polium L.	5
Origanum syriacum (Boiss.) Greater & Burdet.	5
Achillea fragrantissima (Forssk.) Sch. Bip.	4
Silene schimperiana Boiss.	4
Phlomis aurea Decne.	3
Plantago sinaica (Barneoud) Decne.	3
Matthiola arabica Boiss.	3
Echinops spinosus L.	2
Seriphidium herba-album (Asso) Sojak.	2
Globularia Arabica Jaub. & Spach.	1
Arenaria deflexa Decne.	1
Fagonia mollis Delile	1
Ficus carica L.	1
Gymnocarpos decandrus Forssk	1
Bufonia multiceps Decne	1
Cynodon dactylon (L.)Pers.	1
Launaea spinosa (Forssk.) Sch. Bip. ex Kuntze	1
Caylusea hexagyna (Forssk.) M. L. Green.	1
Deverra triradiata Poir.	1
Launaea nudicaulis (L.) Hook. F.	1

Results showed that Tebok, Abo Twita, Ain Shekia, Shak Sakr and Elmesirdy had the highest number of grazed plants among the different locations (Table 4). These locations are having high levels of tourism and other human activities which are compounded by the presence of camels and donkeys used as transportation to and from historical sites. Bedouin communities are also settled beside these locations and this increases goat presence in these locations.

Table 4. Average no. of grazed individuals among different locations

Location	Average No. of Grazed Individuals
Tobok	9
Abu Tweita	7
Ain Shekaia	7
Shak Sakr	7
Abu Hebeik	6
Elahmar	6
Elgalt	6

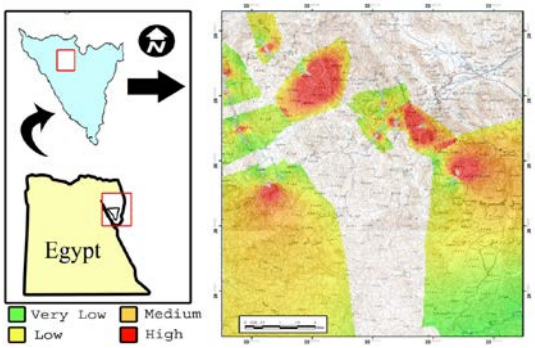
Elazrak	
Elmesirdy	6
Farsh Messila	5
Shak Itlah	5
Shak Musa	5
Sherage	5
Abu Kasaba	4
Elmaein	4
Wadi Eltalaa	4
Elzawitein	4
Farsh Elromana	4
Eltebk	3
Wadi Tenia	1
Abu Walei	0
Kahf Elghola	0

There is high grazing pressure on *Hypericum sinaicum* especially by goats which find *Hypericum* is a good source of moisture. Species like *Echinops spinosus and Hypericum sinaicum* with different morphological traits (High, width, leaf shape and size index) often differ in their responses to grazing (Landsberg *et al.*, 1999; McIntyre *et al.*, 1999; Bullock *et al.*, 2001; Diaz *et al.*, 2001; Dupre & Diekmann, 2001; McIntyre & Lavorel, 2001; Cingolani *et al.*, 2005; De Bello *et al.*, 2005; Louault *et al.*, 2005; and Omar *et al.*, 2012).

3.1.5. Collection for Scientific Research

A very low number of sites were affected by collection for scientific research (e.g., for the purpose of herbarium specimens, phytochemistry or genetics). The research that affected the most sites was the collection of specimens for herbaria. The collectors sometimes collected a big amount of plants complete with flowering parts and roots. Also, collection for phytochemistry requires more than a kilo for good extraction (traditional knowledge). Results showed that the most affected sites were Wadi Tennia, Abu Tweita, Elmesirdi, Abu Kasaba, Shak Musa and Elgalt Elazrak (Map 5).

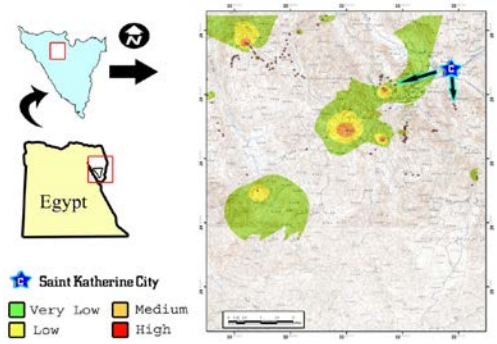
The organizations most associated with collecting for purposes of research were Egyptian research centers (Desert research center and National Research Center), Egyptian Universities (Cairo Univ. and Ain Shams) and foreign Universities (Nottingham Uni.).



Map 5. Hotspots of sites used for scientific research

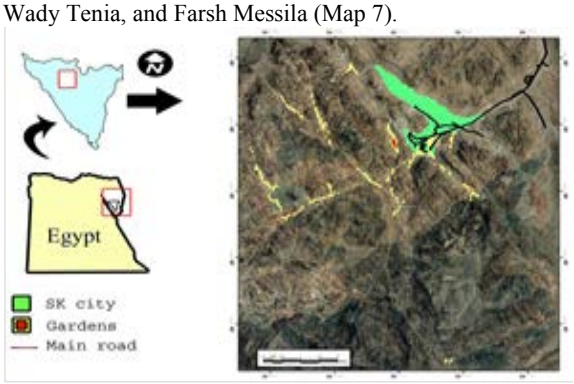
3.1.6. Urbanization and Settlements Expansion

The entire study area is located within a high mountain area, which is far from cities and Bedouin settlements. Within our study area, we recorded human activities, including destruction of rocks for building gardens and digging wells; the sites with the most frequent effects of human settlement were Abu Twita and Zawitein (Map 6).



Map 6. Hotspots of human activity within the study area

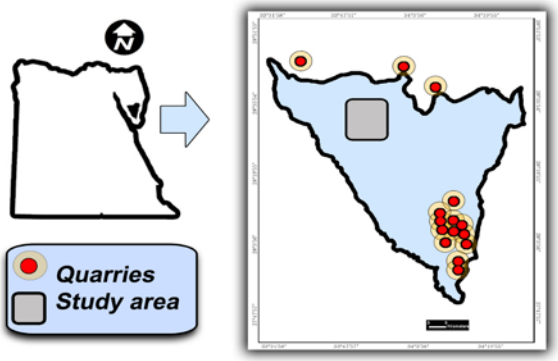
Main roads (asphalt roads) located and ending in SK City are also far from the study area. Bedouin gardens were distributed at all sites within the study area but had the highest frequency at Wadi Gebal, Farsh Elromana, Wady Tenia, and Farsh Messila (Map 7).



Map 7. Distribution of gardens within the study area

3.1.7. Quarries

No quarries were recorded within study area; all quarries are concentrated at the southern part of SKP (Wadi Elkabila, Wadi Elsamaa, Wadi Om Adawy and Al-Nheid (Map 8).



Map 8. Distribution of quarries within SKP

3.2. Threat ranking and level of effect

Results derived from current threat analysis were compared with those obtained by Assi (2007) (Table 5). The results showed that, some threats decreased such as feral donkeys (42%), over collection (29%) and quarries (25%) and others increased such as tourist intrusions (27%), overgrazing (20%), urbanization & settlement expansion (80%), and collection for scientific research (30%) this can be resulting from the change in climate conditions, it was recorded that the rainfall amount in 2007 was 17.60 mm comparing with 12.30 mm in 2012.

Table 5. Threat Analysis (TA) comparison between 2007 and 2012

Threats	Year	Criteria Rankings			Total Ranking	Threat level	change
		Area	Intensity	Urgency			
Feral Donkeys	2007	5	7	7	19	High	+
Feral Donkeys	2012	3	5	3	11	Medium	-
Over collection	2007	7	5	5	17	High	+
Over collection	2012	5	4	3	12	Medium	-
Tourist Intrusions	2007	3	4	4	11	Medium	-
Tourist Intrusions	2012	5	6	3	14	Medium	+
Overgrazing	2007	4	3	3	10	Medium	-
Overgrazing	2012	4	4	4	12	Medium	+
Collection for Scientific research	2007	2	2	2	6	Low	-
Collection for Scientificresearch	2012	3	3	2	8	Low	+
Urbanization & Settlements Expansion	2007	2	1	2	5	Low	-
Urbanization & Settlements Expansion	2012	3	3	3	9	Medium	+
Quarries	2007	1	2	1	4	Low	+
Quarries	2012	1	1	1	3	Low	-
Total 2007		24	24	24	72		+
Total 2012		23	25	19	69		-

3.3. Underlying threat root causes, barriers and solutions

From the previous results and from data collected from local comunities we can conclode and disscuss all threats, root causes, barriers and solutions in the following table (Table 6).

Table 6. Different threats root causes, barriers and solutions

Threat	**Root causes**	**Barriers**	**Solutions**
Feral Donkeys:	• Bedouins, after recent settlement around SK City, have left the donkeys neglected in the mountains. • Those animals require high amount of feeding and were largely replaced by camels. • The recent use of trucks for water transport.	• Lack of strategy to deal with invasive species. • Insufficient awareness on possible damages resulting from invasive species. • Loss of sufficient funding for addressing feral animal abundance.	• Use conventional methods of control including soft catch traps and hunting. • Increase awareness of Bedouins about the impacts (and potential impacts) of feral species on their environment and their culture emphasizing the importance of eradication and management. • Establish a comprehensive strategy, using a participatory approach with the local Bedouins, to deal with possible future colonization. • Establish a strategy to prevent and control invasive species.
Over collection:	• Quick economic gain. • Increased market demand for medicinal plants at the national level. • Firewood gathering for heating and cooking. • Bedouins recent settlements in "Wadis" around SK increased over collection around those settlements. • Poverty encourages intensive use of natural resources including medicinal plants. • Cheap prices offered per bag in the absence of added value and proper market linkages.	• Week enforcement of regulations. • Lack of awareness on plant values, endemism, and ecological role. • Most collectors are not organized in an association or cooperatives, etc. • Limited accessibility to firewood alternatives in remote settlements. • Land tenure: "Open access" system. • Inadequate alternative sources of income. • Cultivation areas insufficient to meet demand. • Cultivation programmers do not involve the wild collectors. • Lack of fund source to encourage local community to use other methods for gaining money.	• Develop species-specific regulations regarding harvesting quotas, rotation of collecting areas, etc. • Cultivation reduces the pressure on Medicinal Plants (MP) wild population and decreases overharvesting. The project should continue the cultivation program; however, there should be a focus to involve wild collectors in cultivation of MPs. • Increase awareness and capacities for the law enforcement cycle. • Enhance MP association (Association located at SK City) capacities for marketing of conservation friendly MP products. • Strengthen technical and capacities of the MP association for value-added process and product improvement. • Conduct extensive trainings for local collectors

	• It's away for money gain when tourism falls down.		on time of harvesting, suitable manners of transporting, and storing of medicinal plants to avoid loss in quality and quantity. • Increase consumer sensitivity towards biodiversity friendly MP-derived products. • Promote regeneration or reinforcement of populations by re-seeding or other ways of propagation as appropriate for each species. • Rehaplitation proses must take place for rare species affected by over collection. • Finding continues source for money to those who haven't any source for living except collection of medicinal plants.
Tourist Intrusions:	*Trespassing beyond trails borders:* • Negligence and saving time. *Collection of firewood during camping:* • Negligence. • Guide saving money (instead of buying the firewood from the city).	• Week enforcement of regulations. • Low level of awareness among tourists on plant values, endemism, and ecological role. • Insufficient awareness among tour operators and tour guides with respect to Protectorate's regulations. • Insufficient number of protectorate's staffing.	• Increase awareness regarding the regulations on firewood among stakeholders engaged in tourism businesses. • Increase awareness among tourists on plant values, endemism, and ecological role. • Produce awareness materials on the threat of firewood collection on biodiversity including MPs to be distributed in the protectorate visitor's center. • The appointment of people to work in order to monitor the activities of park visitors and provide environmental services and information to them.
Overgrazing:	•Bedouins recent settlements in "Wadis" around SK resulted in limited land available for grazing around those settlements. •Collapse of the traditional grazing system (Hilf).	• Lack of efficient and sustainable implementation of alternatives to grazing. • Limited access of the Bedouins to, and high cost, of supplementary animal feed. • Land tenure: "Open access" system. • Lack of extension and veterinary services for herds. • The Agricultural unit in SK is not active.	• Investigate local Feed block Unit feasibility. Promoting feed blocks as supplemental feeding may be a sustainable solution that could be investigated. Feed blocks make use of non-conventional local feed resources including olive and fruit remnants, etc. This is a cheap alternative and can alleviate some pressure on grazing resources. • Continue efforts in reviving the traditional grazing system "Hilf". • Increase the public awareness about the importance of MPs and endemism and the way they can select the most appropriate places for grazing.
Collection for Scientific Research:	•Increased interest at the national and international levels in studying the active ingredients and other characteristics of MPs species (particularly endemic and rare species). • Researchers are interested in conducting their studies on sources obtained from the wild rather than from cultivation. •Low of researcher awareness about the importance of MPs and the actual quantities they want.	• Week enforcement of regulations. • Low level of awareness on Good Harvesting Practices. • Insufficient number of rangers. • Most laboratories are using old equipment which requires large amount of plant material for extraction and detection of active ingredients. • Lack of communication between SKP and universities. • Low levels of trust between SK protectorate and universities which lead some researchers to collect plants without permission from SKP.	• Increase awareness in universities and research institutions on good harvesting practices when collecting for research studies. • Enforce regulations concerning collection permits signed by EEAA and universities and research institutes within and outside Egypt. • The appointment of new researchers to work within SKP in order to monitor the activities of park visitors and provide environmental services and information to them.
Urbanization and Settlements Expansion:	•More Bedouins are involved in tourism activities concentrated around SK City. •Expanding population. •Access to schools and other modern facilities. •Recently advices to reconstruction of Sinai by government to encourage youth to migrate to Sinai.	• Institutional planning deficiencies. • Lack of socio-economic development and adequate/essential services in remote areas. • Lack of cooperation between SKP and city council and the lack of trust between two organizations. • Encroachment of land by force from Bedouins.	• Increase the public awareness about how they can choose the places for gardens, dams, wells and houses. • Strength the cooperation between SKP and city council in planning and site management by sharing data about places and its importance.
Quarries:	• Meet high demand for construction in South Sinai. • Lucrative trade in granite, cement, limestone and sandstone inside and outside Egypt. • Part of Sinai re-construction by encourages peoples to work in this rich field.	• Institutional planning deficiencies. • Weak law enforcements.	• Increase public awareness about the importance of MPs and historical sites. • Select suitable sites far from valuable sites. • Raising the price of quarrying to reduce demand by Bedouins and people who interest in business.

Natural resource policies aim to provide people the opportunity to enjoy and benefit from natural environments evolving by natural processes with minimal influence by human actions. The National Park Service (NPS) will ensure that lands are protected within park boundaries. Where parks contain nonfederal lands, the NPS uses cost-effective protection methods. Preservation of character and resources of wilderness areas designated within a park, while providing for the appropriate use, represent the primary management responsibility. The National Parks and Conservation Association is a national nonprofit membership organization dedicated to defending, promoting, and enhancing our national parks, and educating the public about the NPS.

Hypericum sinaicum is an important target for scientific research. There is a current project at Egyptian National Research Center that is focused on *Hypericum* as the main source for deriving the substance hypercin, a primary substance in the manufacture of depression medicine. They use seeds

and leaves from SKP as the only site in Egypt for *Hypericum* tissue culture. Another human modification was the extent of water cannons relocating water from elevated wadies rich in water supply to low wadies. This activity leads to consume and loss of water from wells which directly affect the plant community health. This was observed clearly at Wadi Gebal, Farsh Elromana, Wady Tenia and Abu Tweita.

When Bedouin do not use their gardens for tourist camping, they can be a tool for in-situ conservation because they exclude grazers and provide a continuous water supply. Results showed that gardens provide good shelter for *Hypericum* where the water shades are present. We found that *H. sinaicum* cover and number of individuals increased inside gardens compared with outside. However drought is still the major factor (threat) affecting the distribution of *Hypericum sinaicum* within the study area. With drought, the effects of overgrazing, over collection and feral donkeys are compounded and may be lead to a decrease in the population size with time. It was showed that drought is the major factor controlling the distribution of feral donkeys. Results showed that these animals decreased from 2007 with about 40% of total frequency without achieving any of the above.

The results of evaluation of monitoring data will help to pinpoint where, and how, a plan should be remodeled. Restructuring or redesign of plan elements based on the results of this study, will contribute to adaptive management, i.e. management which is responsive to changing conditions and project objectives. The plan should set out the time intervals (mid-term, terminal) between evaluations and should state who (individual, organization, or agency) will carry out evaluations and who will be the recipients of reports. For the evaluation to have some practical effect in improving conservation management, there should be specific mechanisms for feeding the results of evaluation back into the management process, and assigned responsibilities for follow-up. As with monitoring, evaluation should be an ongoing part of biodiversity conservation management, rather than a project-based activity.

From the previous threat results we can conclude that most root causes of threats come from lack of awareness, weak law enforcement, lack of suitable strategies, weak financial support and lack of stakeholders cooperation. These results confirm the results of Assi (2007). Most of the presented solutions were obtained by Assi (2007); however, threats persist and some of them have increased due to lack of enforcement. Drought, feral donkeys and over collection are the most harmful threats for *Hypericum sinaicum* in SKP. To achieve these solutions and decrease the effect of previous threats within study area, Egyptian Environmental Affairs Agency must improve the annual budget of SKP and insert new departments within SKP organization.

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References

Alqamy H. 2005. Developing and assessing a population monitoring program for the Dorcas Gazelle *Gazella dorcas* using Distance Sampling in South Sinai, Egypt. MSc thesis, University of Saint Andrewsa, Scotland.

Assi R. 2007. MP Threat Analysis and Threat Reduction Assessment Report. Conservation and sustainable use of medicinal plants in arid and semi-arid ecosystems project.

Batanouny KH. 1983. Human impact on desert vegetation. In: Holzner W, Werger M J A and Ikusima I. (Eds**.) Man's Impact on Vegetation,** Dr. W. Junk Publishers, London.

Boulos L. 2002. **Flora of Egypt**, Al hadara publishing, Cairo, Egypt, Vol. 3: 373pp.

Budowski G. 1976. 'Tourism and environmental conservation: conflict, coexistence or symbiosis?' *Environmental Conservation* ,**3(1):**27-31.

Bullock JM, Franklin J, Stevenson MJ, Silvertown J, Coulson SJ, Gregory SJ. and Tofts R. 2001. A plant trait analysis of responses to grazing in a long-term experiment. *J Appl Ecol*., **38**:253-267.

Cingolani AM, Posse G and Collantes MB. 2005. Plant functional traits, herbivore selectivity and response to sheep grazing in Patagonian steppe grasslands. *J Appl Ecol*., **42**:50–59

De Bello F, Lepš J and Sebastia MT. 2005. Predictive value of plant traits to grazing along a climatic gradient in the Mediterranean. *J Appl Ecol.*, **42**:824-833.

Diaz S, Noy-Meir I and Cabido M. 2001. Can grazing response of herbaceous plants be predicted from simple vegetative traits. *J Appl Ecol.* ,**38**:497-508.

Dupre C and Diekmann M. 2001. Differences in species richness and life-history traits between grazed and abandoned grasslands in southern Sweden. - *Ecography* , **24**:275-286.

Guenther R, Gilbert F, Zalat S, Selimk and The volunteers of Operation Wallacea in Egypt 2005. Vegetation and grazing in the St Katherine Protectorate, South Sinai, Egypt. *Egyptian J Biol*., **7**: 55-65.

Hatab EE. 2009 Ecological studies on the *Acacia* Species and Ecosystem Restoration in the Saint Katherine Protectorate, South Sinai, Egypt. Ph.D., Thesis, Fac. Sci., Al-Azhar Univ.

IUCN 1994. IUCN Redlist Categories. Prepared by the Special Survival Commission. IUCN, Gland, Switzerland.

Khafaja T, Hatab A and Dsouki A. 2006. Report on the current situation of the plant vegetation cover in the high mountain area of Saint Katherine. Plant Conservation and Monitoring Programme, Saint Katherine Protectorate.

Landsberg J, O'Connor T and Freudenberger D. 1999. The impacts of livestock grazing on biodiversity in natural ecosystems. In: Jung, HJ and Fahey GC (Eds.) **Nutritional Ecology of Herbivores**. American Society of Animal Science, Savoy, IL, pp 752–777.

Louault F, Pillar VD, Aufrere J, Garnier E and Soussana JF. 2005. Plant traits and functional types in response to reduced disturbance in a semi-natural grassland. *J Veg Sci.*, **16**:150-160.

McIntyre S and Lavorel S. 2001. Livestock grazing in subtropical pastures: steps in the analysis of attribute response and plant functional types. *J Ecol.,* **89**:209-226.

McIntyre S, Lavorel S, Landsberg J and Forbes TDA. 1999. Disturbance response in vegetation - towards a global perspective on functional traits. *J Veg Sci.,* **10**:621-630.

Milton SJ, Dean WRJ, Duplessis MA and Siegfri WR. 1994. A conceptual model of arid rangeland degradation. *Bioscience***, 44**:70–76.

Mosallam HAM. 2007. Assessment of target species in Saint Katherine Protectorate, Sinai, Egypt. *J Appl Sci Res.,* **3(6):** 456-469.

Omar K, Khafagi O and Elkholy M. 2012. Eco-geographical analysis on mountain plants: A case study of Nepeta septemcrenata in South Sinai, Egypt. LAP LAMBERT Academic Publishing, Germany.

Řezanka T and Sigler K. 2007. Sinaicinone, a complex adamantanyl derivative from Hypericum sinaicum. *Phytochem.,* **68**(9): 1272–1276.

United Nations Environment Programme (UNEP). 1995. **Global Biodiversity Assessment**. Cambridge University Press. New York, NY.

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Molecular Analysis of Intracultivar Polymorphism of 'Panchadarakalasa' Mango by Microsatellite Markers

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Abstract

Juicy mangoes (sucking type/*rasaalu*) are one of the most significant mango *(Mangifera indica* L.) businesses in Andhra Pradesh state, India. The mango orchardists cultivate a great number of juicy cultivars for which genetic homogeneity has never been demonstrated.'Panchadarakalasa' is one of the choicest juicy cultivars of mango in the state, where certain level of intracultivar variability in fruit morphology has been observed among trees. In this study, fruit and leaf samples of 16 trees of 'Panchadarakalasa' (PK Acc-l to PK Acc-16) spread over the three eco-geographical regions (Coastal Andhra, Rayalaseema and Telangana) of the state were collected during summer 2009, which were subjected to *in-situ* morphological and *ex-situ* microsatellite analysis, respectively to identify whether there is variability in the plants grown in the state. Characterization and evaluation of fruit samples based on 9 quantitative and 7 qualitative traits revealed phenotypic variations among accessions under study. Twenty out of 109 mango-specific microsatellite markers validated, were amplified. Of the 20 microsatellites amplified, only 4 were polymorphic with a total of 11 alleles ranging from 130 bp to 245 bp. The polymorphic information content of the polymorphic alleles ranged from 0.25-0.56, whereas the Jaccard's similarity coefficient values ranged from 0.9-1.0. The pair-wise genetic dissimilarities ranged from 0.00-0.10 with a mean value of 0.05. Dendrogram based on unweighted pair group method of arithmetic means algorithm indicated that the accessions were not grouped as per geographic separation. Microsatellite analysis revealed smaller intracultivar variability of 10% in *in-situ* conditions and a genetic divergence between trees attesting that 'Panchadarakalasa' whatsoever cultivated throughout the state is not pure clone. The traditional nursery practices are likely to be responsible for the intracultivar polymorphism since the 'Panchadarakalasa' not propagated exclusively vegetatively. Highly polymorphic microsatellites like SSR-83, MngSSR-24 and MngSSR-26 were more useful in differentiating the 'Panchadarakalasa' accessions. The results generated with microsatellite markers will be helpful in intracultivar improvement as well as in the application of breeder rights in the country.

Keywords: Fruit Morphology, Fruit Quality, Intravarietal Diversity, Molecular Analysis, Simple Sequence Repeats

1. Introduction

Mango is botanically *Mangifera indica* L., belonging to the family Anacardiaceae. It is believed to have originated in Eastern India (Knight, 1980). It is one of the most economically grown fruits in the tropical and subtropical areas around the world (Rajwana *et al.,* 2011) both for fresh (table/juicy) and industrial consumption. The global consumption of mango has increased significantly because of its nutritional and bioactive properties (Poovarodom *et al.,* 2010). Since mango has been under cultivation in India since antiquity and is highly cross-pollinated, there are thousands of varieties of mango arising from natural cross-pollination or mutation (Karihaloo *et al.,* 2003). Juicy cultivars of mango (juicy mangoes/sucking type), popularly known as '*rasaalu*' are the specialty mangoes of Andhra Pradesh state, India. The juicy mango business is one of the most relevant in the state mango business with a leading role in the socio-economic situation of the Krishna-Godavari zone in the Coastal Andhra region, as well as in other production areas in Telangana and Rayalaseema regions. 'Panchadarakalasa' is one of the choicest juicy mangoes among millions of people in the state. Although 'Panchadarakalasa' originated in Krishna-Godavari zone, it is well adapted and produced in all the three eco-geographical regions (Coastal Andhra, Telangana and

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Rayalaseema) of the state. Although it is not distantly marketed and currently exported but an important demand exists in the local markets.

In India, most of the mango cultivars including juicy mangoes were selected from native mango populations 100 to 150 years ago. Since their initial selection, the identities and trueness-to-type of many cultivars have been obscured due to various biological and cultural factors. The mango peasants cultivate a great number of mango varieties including juicy mangoes for which genetic homogeneity has never been demonstrated. In India, a large proportion of the commercial mango orchards have been established asexually from grafts. In spite of this, there was a wide variation in fruit morphology, production and quality within and between the orchards of a cultivar. There were very few reports on the intracultivar variability of certain mango cultivars on the basis of morphological traits (Naik, 1948; Oppenheinmer, 1956; Naik, 1971) and molecular markers (Bally *et al.,* 1996; de Souza and Lima, 2004; Diaz-Matallana *et al.,* 2009; Singh *et al.,* 2009; Rocha *et al.,* 2012). This intracultivar variability may have serious economic ramifications for mango orchardists who unknowingly establish mango orchards with less productive genotypes of a cultivar. In addition, this intracultivar variability within cultivar mother blocks will further aggravate the variability within and between orchards of a cultivar. It has also been observed that there was locality dependent variation in the fruit appearance and quality of 'Panchadarakalasa' across the state (personal communication), which is somewhat troublesome for mango growers. Since fruit morphology and quality traits are quantitative in inheritance, this variation could probably be due to the influence of environment and/or genotype. To determine this both morphological and molecular analysis is necessary.

Several procedures for the identification and characterization of intracultivar variability in mango have been developed based on morphological or genetic traits. Traditionally, in India, intracultivar variability in mango has been characterized based particularly on fruit characteristics like size, shape and color (Naik, 1948; Oppenheinmer, 1956; Naik, 1971; Singh *et al.,* 2009). Morphological traits, being influenced by environmental parameters, are unreliable. Recently, intracultivar variability in mango has been characterized based on molecular markers like randomly amplified polymorphic DNA-RAPDs (Bally *et al.,* 1996; de Souza and Lima, 2004; Diaz-Matallana *et al.,* 2009) and inter simple sequence repeats-ISSRs (Singh *et al.,* 2009; Rocha *et al.,* 2012). From all these studies, different estimates for the degree of intracultivar genetic variation were obtained, reflecting the differences in the selected sets of accessions of cultivars of mango or marker systems. These researches suggest that there was considerable genetic variability within the cultivars of mango, which offer good scope for breeding within the cultivar for intracultivar improvement. Simple sequence repeats (SSRs) or microsatellites are widely used as a versatile tool in plant breeding programs because of their high ability for showing diversity among the genotypes. In mango, although microsatellites have been successfully used for intercultivar genetic diversity analysis (Duval *et al.*, 2005; Honsho *et al.,* 2005; Schnell *et al.,* 2005; Viruel *et al.,* 2005; Schnell *et al.,* 2006; Lopez *et al.,* 2009; Hirano *et al.,* 2010; Wahdan *et al.*, 2011; Begum *et al*., 2012; Vasugi *et al*., 2012), no attempt has been made on intracultivar genetic diversity analysis. Morphological traits combined with molecular characterization are essential for better understanding of genetic diversity in mango (Singh *et al*., 2009; Begum *et al.,* 2012). Morphological descriptors and molecular markers were used to determine the intra-varietal diversity of oca (*Oxalis tuberosa* Mol.) varieties (Pissard *et al*., 2008).

The objective of this study was to assess the intracultivar genetic diversity of 'Panchadarakalasa' mango trees cultivated in the three eco-geographical regions of Andhra Pradesh, using morphological traits and SSR markers to identify whether there is variability in the plants grown in the state.

2. Materials and Methods

2.1. Eco-Geographic Survey

An eco-geographic survey was conducted by a team of scientists during May-June 2009. Following simple random sampling strategy, 3 mango mother blocks and 12 mango orchards possessing 'Panchadarakalasa' trees, spread over all the three eco-geographical regions and covering 7 districts, were selected. One 'Panchadarakalasa' tree was sampled from each of the 12 mango orchards and two mango mother blocks and two trees were sampled from one mango mother block (accession IDs starting with PK; PK Acc-1 to PK Acc-16) through simple random sampling (Table 1).

Table 1. Collection sites of 'Panchadarakalasa' mango accessions

Accession	Sampling unit	Collection site		
		Village	District	Region
PK Acc-1	Mother block, ARI	Rajendranagar	Rangareddy	Telangana
PK Acc-2	Mother block, FRS	Sangareddy	Medak	Telangana
PK Acc-3	Mother block, FRS	Sangareddy	Medak	Telangana
PK Acc-4	Orchard	Kathipudi	East Godavari	Coastal Andhra
PK Acc-5	Orchard	Kathipudi	East Godavari	Coastal Andhra
PK Acc-6	Orchard	Pithapuram	East Godavari	Coastal Andhra
PK Acc-7	Orchard	Pithapuram	East Godavari	Coastal Andhra
PK Acc-8	Orchard	Pithapuram	East Godavari	Coastal Andhra
PK Acc-9	Orchard	Bobbili	Vizainagaram	Coastal Andhra
PK Acc-10	Orchard	Bobbili	Vizainagaram	Coastal Andhra
PK Acc-11	Orchard	Bobbili	Vizainagaram	Coastal Andhra
PK Acc-12	Orchard	Anakapalli	Visakhapatnam	Coastal Andhra
PK Acc-13	Orchard	Anakapalli	Visakhapatnam	Coastal Andhra
PK Acc-14	Orchard	Errakoneru	East Godavari	Coastal Andhra
PK Acc-15	Orchard	Pondugala	Prakasam	Coastal Andhra
PK Acc-16	Mother block, HRS	Anantharajupeta	Kadapa	Rayalaseema

PK Acc = Panchadarakalasa accession; ARI= Agricultural Research Institute

FRS= Fruit Research Station; HRS= Horticultural Research Station

2.2. Fruit Sampling, Characterization, Evaluation and Analysis

Following simple random sampling strategy, ten tree-ripe fruits spread over all the sides of the tree canopy were collected from each of the 16 selected trees (accessions) of 'Panchadarakalasa'. Morpho-physiol-ogical characters of fruit samples were recorded following descriptors of mango (IPGRI, 2006). Fruit samples were evaluated for 9 quantitative traits like fruit length (cm), fruit width (cm), fruit thickness (cm), fruit weight (g) fiber length (mm), peel (%), pulp (%), stone (%), total soluble solids (TSS) (°Brix) and shelf life (days) and for 7 qualitative traits like fruit shape, skin colour of mature fruit, skin thickness, skin texture, quantity of fiber, pulp colour and eating quality. The mean data of each of the random fruit sample of 16 'Panchadarakalasa' accessions was analysed for 9 quantitative traits following 'Descriptive Statistics' for mean, standard error, standard deviation and coefficient of variation.

2.3. Leaf Sampling

Mature leaves of each of the sampled 'Panchadarakalasa' accession were collected for genetic characterization. The leaves were identified and maintained in styrofoam boxes with ice to be transported from the collection sites to the Institute of Biotechnology, Acharya N. G. Ranga Agricultural University (ANG RAU), Rajendranagar where they were frozen in liquid nitrogen. Then, they were stored in a freezer at -80 °C, until the time of the extraction of the genomic DNA.

2.4. DNA Extraction

The Genomic DNA from leaf samples was extracted by a modified Cetryl Trimethyl Ammonium Bromide (CTAB) method (Porebski *et.al.,* 1997).

2.5. Microsatellite Screening and Amplification

Polymerase chain reaction (PCR) amplification was performed in a Perkin Elmer Thermocycler (PCR-Gene Amp PCR System 9700) as per the protocol suggested by Williams *et al.* (1990) using 109 mango-specific microsatellite markers. Amplified products were separated by electrophoresis in a 3% metaphor-agarose gel using Tris-acetate EDTA (TAE) buffer at pH 8.0. The amplified fragments were observed and photographed under UV light in Gel Doc System (Syngene, Cambridge, United Kingdom). Eleven alleles generated by the four polymorphic microsatellites (SSR-36, SSR-83, MngSSR-24 and MngSSR-26) were chosen for their clear pattern and high allele numbers to study diversity within the whole sample.

2.6. Data Analysis

The simple sequence repeat (SSR) bands were scored visually on the basis of their presence (1) or absence (0), separately for each accession of 'Panchadarakalasa' mango and each SSR primer. The sizes of fragments (molecular weight in base pairs) were estimated by using 100-bp ladder marker, which was run along with the amplified products. The scores obtained using all four polymorphic primers in the SSR analysis were then used for constructing a single matrix. Pair-wise difference matrix between accessions was determined using Jaccard's similarity coefficient. Data analysis was performed using the numerical taxonomy and multivariate analysis system (NTSYS)-pc version 2.1 computer program package (Rohlf, 2000). The coefficients were utilized to construct a dendrogram using the unweighted pair group of arithmetic means algorithm (UPGMA).

3. Results

3.1. Morphological Variability

From the results of mean, standard error, standard deviation and coefficient of variation (Table 2), it is evident that there was significant variation in 9 quantitative fruit traits among 16 accessions of 'Panchadarakalasa' under study.

Fruit length, width and thickness ranged from 7.20 to 10.00, 5.80 to 8.00 and 4.40 to 7.60 cm, respectively. Fruit weight and fiber length ranged from 154.00 to 350.00 g and 5.00 to 9.00 cm, respectively. The peel, pulp and stone contents ranged from 18.20 to 23.80%, 56.30 to 64.60% and 17.10 to 23.10%, respectively. Total soluble solids ranged from 15.00 to 19.80 °Brix. These ranges, refereed to evident morpho-physiological variation in fruit size and weight, fiber length, peel, pulp and stone contents and total soluble solids among 16 'Panchadarakalasa' accessions (Table 2).

There were some differences among 16 accessions of 'Panchadarakalasa' with respect to certain qualitative traits (Table 3) like fruit shape, color of skin of mature fruit, skin thickness, pulp color and eating quality.

3.2. Microsatellite Polymorphism

Of the 109 SSRs validated with total sample of 16 'Panchadarakalasa' accessions, only 20 SSRs could amplify and produce distinct and clear bands, while the remaining 89 SSRs could not amplify. Of these 20 amplified SSRs, 16 SSRs were monomorphic (Table 4), while the remaining 4 SSRs (SSR-36, SSR-83, MngSSR-24 and MngSSR-26) were polymorphic (Table 5). These four polymorphic SSR primers produced 11 bands in the total sample of 16 accessions. Four bands were common in all the cultivars, while the other 7 bands were polymorphic (63.63%). The allele size of polymorphic bands ranged from 130 to 245 bp. There was a wide variation in the range of polymorphic bands produced by the primers. The level of polymorphism present in the microsatellites was variable ranging from 2 to 4 alleles per SSR with an average of 2.75 alleles per SSR. Primers SSR-36 and SSR-83 produced the lowest number of polymorphic bands (2 bands), while primer MngSSR-26 produced the highest number of polymorphic bands (4 bands). In this study, the polymorphic information content (PIC) values ranged from 0.25 to 0.56 with moderate mean value of 0.42 for all loci indicating the moderate discriminatory power of the 4 polymorphic SSRs. Markers with high PIC values such as SSR-83 and SSR-36 could be effectively used in intracultivar genetic diversity studies of 'Panchadarakalasa'. Agarose gel showing SSR amplification profile of 'Panchadarakalasa' accessions by SSR-83 primer is depicted in Figure 1. The

Jaccard's similarity coefficient values calculated from SSR data ranged from 0.9-1.0. The pair-wise genetic dissimilarities ranged from 0.00 to 0.10 with a mean value of 0.05. The largest genetic distance calculated by the Jaccard's similarity coefficient was 0.10. Genetic similarity between accessions was in the range of 90-100%.

Table 2. Quantitative fruit characteristics of 'Panchadarakalasa' accessions

Accession	Fruit length (cm)	Fruit width (cm)	Fruit thickness (cm)	Fruit weight (g)	Fibre length (mm)	Peel (%)	Pulp (%)	Stone (%)	Total soluble solids(°Brix)
PK Acc-1	7.50	5.80	5.50	190.00	5.00	20.50	58.40	21.10	15.00
PK Acc-2	8.00	6.50	6.50	172.00	8.00	19.80	58.10	22.10	17.00
PK Acc-3	10.00	6.00	5.80	255.00	5.00	22.40	58.80	18.80	16.00
PK Acc-4	9.00	6.50	5.80	260.00	7.00	19.20	59.20	21.50	18.00
PK Acc-5	8.00	6.20	6.00	228.00	9.00	19.70	62.70	17.50	19.80
PK Acc-6	9.50	7.20	6.40	286.00	8.00	18.20	64.60	17.10	16.00
PK Acc-7	8.00	6.80	5.00	208.00	5.00	22.10	56.30	21.60	15.00
PK Acc-8	8.30	6.00	5.60	212.00	5.00	19.80	57.10	23.10	16.00
PK Acc-9	8.50	8.00	7.00	240.00	9.00	22.50	60.00	17.50	19.60
PK Acc-10	8.00	6.50	4.40	154.00	5.00	20.10	59.10	20.80	17.00
PK Acc-11	7.80	6.10	4.80	160.00	6.00	20.00	58.10	21.90	16.00
PK Acc-12	9.70	6.30	5.60	220.00	9.00	22.70	59.10	18.20	16.40
PK Acc-13	8.50	7.60	5.20	200.00	5.00	20.00	62.50	17.50	17.00
PK Acc-14	8.00	6.50	5.00	190.00	6.00	20.60	56.80	22.60	16.20
PK Acc-15	7.20	6.50	5.20	168.00	6.00	23.80	58.30	17.90	15.60
PK Acc-16	8.90	7.50	7.60	350.00	5.00	22.90	60.00	17.10	18.50
Mean	8.43	6.63	5.71	218.31	6.44	20.89	59.32	19.77	16.82
Standard Error	0.20	0.16	0.21	12.89	0.41	0.40	0.56	0.56	0.37
Standard Deviation	0.80	0.64	0.84	51.56	1.63	1.60	2.25	2.23	1.47
CV (%)	2.36	2.40	3.68	5.90	6.34	1.92	0.95	2.83	2.18

PK Acc = Panchadarakalasa accession

Table 3. Qualitative fruit characteristics of 'Panchadarakalasa' accessions

Genotype	Shape	Color of skin of matured fruit	Skin thickness	Skin texture	Quantity of fibre	Pulp color	Eating quality
PK Acc-1	Ovate	Yellowish green	Thick	Smooth	Abundant	Yellow	Good
PK Acc-2	Ovate	Yellowish green	Thin	Smooth	Abundant	Yellow	Good
PK Acc-3	Ovate	Yellowish green	Thin	Smooth	Abundant	Yellow	Good
PK Acc-4	Ovate	Yellowish green	Thin	Smooth	Abundant	Yellow	Good
PK Acc-5	Ovate	Yellowish green	Thin	Smooth	Abundant	Yellow	Good
PK Acc-6	Round	Yellowish green	Thin	Smooth	Abundant	Yellow	Good
PK Acc-7	Ovate	Yellowish green	Thin	Smooth	Abundant	Yellow	Good
PK Acc-8	Ovate	Dark yellow	Thin	Smooth	Abundant	Yellow	Good
PK Acc-9	Ovate	Dark yellow	Thin	Smooth	Abundant	Yellow	Good
PK Acc-10	Round	Yellow	Thin	Smooth	Abundant	Yellow	Good
PK Acc-11	Ovate	Yellow	Thin	Smooth	Abundant	Yellow	Good
PK Acc-12	Ovate	Yellow	Thin	Smooth	Abundant	Yellow	Good
PK Acc-13	Round	Yellow	Thin	Smooth	Abundant	Yellow	Good
PK Acc-14	Ovate	Yellow	Thin	Smooth	Abundant	Golden yellow	Good
PK Acc-15	Ovate	Yellowish green	Thin	Smooth	Abundant	Yellow	Good
PK Acc-16	Ovate	Green	Thin	Smooth	Abundant	Yellow	Excellent

PK Acc = Panchadarakalasa accession

Table 4. Characteristics of monomorphic microsatellite markers used in this study

Primer	Sequence 5'-3'	Annealing temperature (°C)	Size range of alleles (bp)
SSR-8	F: TTGATGCAACTTTCTGCC	53	200-224
	R: ATGTGATTGTTAGAATGAACTT		
SSR-15	F: TTTACCAAGCTAGGGTCA	52	201-226
	R: CACTCTTAAACTATTCAACCA		
SSR-16	F: GCTTTATCCACATCAATATCC	54	160-170
	R: TCCTACAATAACTTGCC		
SSR-20	F: CGCTCTGTGAGAATCAAATGGT	58	295-310
	R: GGACTCTTATTAGCCAATGGGATG		
SSR-39	F: TGTCTACCATCAAGTTCG	53	150-190
	R: GCTGTTGTTGCTTTACTG		
SSR-46	F: TCATTGCTGTCCCTTTTC	54	154–210
	R: ATCGCTCAAACAATCC		
SSR-52	F: AAAAACCTTACATAAGTGAATC	52	207
	R: CAGTTAACCTGTTACCTTTTT		
SSR-59	F: TTCTTTAGACTAAGAGCACATT	56	191
	R: AGTTACAGATCTTCTCCAATT		
SSR-61	F: AAAGATAGCATTTAATTAAGGA	52	206
	R: GTAAGTATCGCTGTTTGTTATT		
SSR-65	F: ATAGATTCATATCTTCTTGCAT	53	233
	R: TATAAATTATCATCTTCACTGC		
SSR-82	F: TCTGACCCAACAAAGAACCA	57	108-155
	R: TCCTCCTCGTCCTCATCATC		
SSR-84	F: TCTATAAGTGCCCCCTCACG	58	210-250
	R: ACTGCCACCGTGGAAAGTAG		
SSR-85	F: GCTTGCTTCCAACTGAGACC	58	229-269
	R: GCAAAATGCTCGGAGAAGAC		
SSR-88	F: CTGAGTTTGGCAAGGGAGAG	55	222-244
	R:TTGATCCTTCACCACCATCA		
MngSSR-14	F: TCATTAAGCTGTGGCAACCA	59	160-192
	R: CATTGCATAGATGTGGTCATT		
MngSSR-27	F: CGAAACCGACTGCCTATTTT	57	158-172
	R: CCATTAATAAAGTTGTGGCCA		

bp= Base pair

Table 5. Characteristics of polymorphic microsatellite markers used in molecular analysis of intracultivar variability in 'Panchadarakalasa' mango

Primer	Sequence (5'-3')	Annealing temperature(°C)	Size range of alleles(bp)	No. of alleles	PICvalues
SSR-36	F: CCTCAATCTCACTCAACA	55	215-245	2	0.43
	R: ACCCCACAATCAAACTAC				
SSR-83	F: AGCTATCGCCACAGCAAATC	57	190-213	2	0.56
	R: GTCTTCTTCTGGCTGCCAAC				
MngSSR-24	F: CGATGGACTTCATAAGAAGAG	58	150	3	0.45
	R: GCTAGCAGAATCACCTTGGTC				
MngSSR-26	F: ACCTTGGTCAGGACAAAATCC	60	135-150	4	0.25
	R: GACTTCATAAGAAGAGGCGTC				

PIC= Polymorphic information content ; bp= Base pair
Source: eurofins mwg/operon (www.Eurofinsdna.com)

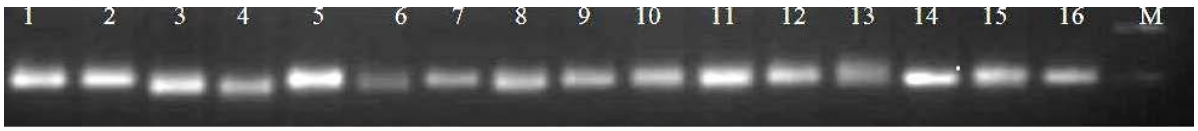

Figure 1. SSR profile of 'Panchadarakalasa' accessions by primer SSR-83. Lane 1 to16 denote 'Panchadarakalasa' accessions PK Acc-1to PK Acc-16 serially; M= 100 bp standard marker

3.3. Cluster analysis, genetic diversity, genetic similarity and geographical diversity

Pair-wise comparison was performed among all the accessions included in this study. The genetic relationship achieved by applying SSR markers is shown in Figure 2. The first major bifurcation in the dendrogram (Figure 2) separated the 16 accessions into four major clusters (cluster-I to cluster-IV). Clusters III and IV could be further divided into two sub clusters. Clusters I and II were solitary; consisting of only one accession each. PKAcc-14 from Errakoneru, PKAcc-11 from Bobbili branched out from the base, were found the most unique and divergent. Of these two divergent accessions, the accession PKAcc-14 occupied a unique position and was most diverse from rest of the accessions. 'Panchadarakalasa' accessions from one site of collection were more or less clustered together with few exceptions. The multiple accessions collected from certain collection sites like Sangareddy (PKAcc-2 and PKAcc-3), Pithapuram (PKAcc-7 and PKAcc-8) and Bobbili (PKAcc-9, PKAcc-10 and PKAcc-11) did not form separate groups or subgroups. Of the 2 accessions (PKAcc-2 and PKAcc-3) from the Fruit Research Station, Sangareddy, the first one (PKAcc-2) was grouped in sub cluster IVB along with three accessions PKAcc-1 from Rajendranagar, PKAcc-15 from Pondugala and PKAcc-16 from Anantharajupeta, while the later (PKAcc-3) was grouped solitarily in sub cluster IVA. Of the 3 accessions (PKAcc-6, PKAcc-7 and PKAcc-8) from Pithapuram, PKAcc-6 and PKAcc-8 grouped in subcluster IIIB, while PKAcc-8 grouped in a distinct solitary subcluster IIIA. Further, certain 'Panchadarakalasa' accessions from different collection sites were clustered together. The multiple accessions collected from different collection sites like Kathipudi (PKAcc-4 and PKAcc-5) and Anakapalli (PKAcc-12 and PKAcc-13) grouped together in the same subcluster IIIB.

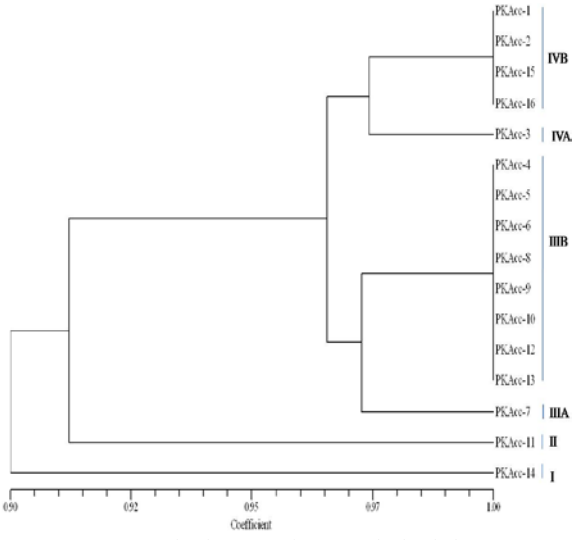

Figure 2. UPGMA dendrogram of 16 'Panchadarakalasa' accessions based on 4 SSR markers

A narrow range (0.9-1.0) of Jaccard's similarity coefficient was observed between the pairs of 16 accessions. The pair-wise genetic similarity between accessions was in the range of 90-100%; the lowest (90%) being observed between PKAcc-11 and PKAcc-14. Highest (100%) similarity was found between accessions PKAcc-1 (Rajendranagar), PKAcc-2 (Sangareddy), PKAcc-15 (Pondugala) and PKAcc-16 (Anantharajupeta). There was a pair-wise dissimilarity of only 2.85% between the two accessions (PKAcc-2 and PKAcc-3) collected from the same mother block, Fruit Research Station, Sangareddy. Of the 3 accessions collected from Pithapuram (PKAcc-6, PKAcc-7 and PKAcc-8), PKAcc-6 and PKAcc-8 had 100% similarity, while PKAcc-7 had only 2.7% dissimilarity with the other two accessions (PKAcc-6 and PKAcc-8). PKAcc-1, PKAcc-2, PKAcc-15 and PKAcc-16 were the accessions from different collection sites (Rajendranagar, Sangareddy, Pondugala and Anantharajupeta, respectively), which had 100% similarity. Two accessions each from Kathipudi (PKAcc-4 and PKAcc-5), Pithapuram (PKAcc-6 and PKAcc-8), Bobbili (PKAcc-9 and PKAcc-10) and Anakapalli (PKAcc-12 and PKAcc-13) had 100% similarity with the 4 highly polymorphic SSRs. There was a dissimilarity of 3.8% between the two distinct sub groups IVB (PKAcc-,1 PKAcc-2, PKAcc-15 and PKAcc-16) and IIIB (PKAcc-4, PKAcc-5, PKAcc-6, PKAcc-8, PKAcc-9, PKAcc-10, PKAcc-12 and PKAcc-13), exhibiting 100% pair-wise similarity within the groups.

3.4. Identification and differentiation of accessions

There were some loci which were present only in one accession; such loci may also be of use in the 'Panchadarakalasa' accessions differentiation. Here, comparison of the 7 alleles which were detected using 4 polymorphic SSRs revealed distinct banding patterns (alleles) to discriminate and identify the 'Panchadarakalasa' accessions. Microsatellites producing unique alleles for specific accessions of 'Panchadarakalasa' are given in Table 6. SSR-83 generated a unique allele of 200 bp for PKAcc-14. MngSSR-24 generated a unique allele of 150 bp for PKAcc-11. MngSSR-26 generated a unique allele of 140 bp for PKAcc-14.

Table 6. Microsatellites producing unique alleles for specific accessions of 'Panchadarakalasa'

Accession	SSR producing specific band	Size of the specific bands (bp)
PK Acc-11	MngSSR-24	150
PK Acc-14	SSR-83	200
PK Acc-14	MngSSR-26	140

4. Discussion

The peasants cultivate a great number of juicy cultivars and the juicy mangoes are one of the most significant mango businesses in Andhra Pradesh state, India. Juicy mango business in this state is based largely on 'Panchadarakalasa', one of the choicest juicy cultivars of mango with the great appreciation for fresh consumption (sucking type) due to its superior characteristics, such as sweet taste, pulp color and flavor. It has been under cultivation for more than a century in this state, which stands out as a major producer and supplier of this cultivar. In spite of possessing so many

virtues, this cultivar is troublesome for peasants because of its locality dependent variation in the fruit size, shape and quality resulting in heterogeneity in production and quality. Data on the regional polymorphism of this cultivar is scarce or non-existent. In this study, the genetic diversity of 'Panchadarakalasa' mango trees cultivated in all the three eco-geographical regions of the state was assessed based on morphological traits and microsatellite markers, to identify whether there is variability in the plants grown in the state.

Assessment of intracultivar diversity of mango has traditionally been made through morphological traits by several researchers (Naik, 1948; Oppenheinmer, 1956; Naik, 1971; Singh *et al*., 2009), where in intracultivar variability was found. Here also, analysis of 9 quantitative fruit traits following descriptive statistics indicated significant variability in fruit morpho-physiology among 16 accessions of 'Panchadarakalasa' under study. In addition, the data on 7 qualitative fruit traits also revealed considerable variation among total sample under study. On the whole, morphological analysis indicated considerable variability among the 'Panchadarakalasa' trees grown across the state. However, assessment of genetic variability based on phenotype has certain limitations, since most of the morphological characters of economic importance are often limited in number; have complex inheritance and dramatically influenced by environmental factors (Bernatzky and Tanksley, 1989). These results are suggesting both to focus our attention on the effects of the environment on the genotype and to consider, as a practical consequence, the importance of preserving these accessions found in different areas to truly preserve the richness of the germplasm of a cultivar.

In contrast, molecular markers based on DNA sequence polymorphisms are independent of environmental conditions and show a higher level of polymorphism. To confirm whether the phenotypic variability in this study is due to the influence of environment or genotype, molecular analysis with microsatellites was undertaken. In the present study, relatively small number of SSR bands (11) was amplified in a set of 16 'Panchadarakalasa' accessions using 4 primers. The pair-wise genetic dissimilarities ranged from 0.00 to 0.10 with a mean value of 0.05, thus showing a small degree of intercultivar genetic diversity at the DNA level. This dissimilarity value of 0.10 is much higher to that calculated from RAPD (10 primers) data among 15 accessions of 'Kensington Pride' cultivar of mango (0.05) by Bally *et al*. (1996) but much lower than that calculated from RAPD data (32 primers) among 25 accessions of 'Rosa' cultivar of mango (0.45) by de Souza and Lima (2004), indicating that the level of intracultivar genetic diversity in different cultivars of mango is dependent on the total sample size and number of molecular markers used. Amplified fragment length polymorphism (AFLP) analysis of the 160 phenotypically divergent 'Plavac Mali' vines (*Vitis vinifera* L.) has also revealed significantly lower polymorphism (Zdunic *et al*., 2009). In the present study, the genetic dissimilarity of 0.10 among accessions although small, the genetic differences among the sampled materials may affect some phenotypic character that is useful for the culture. This intracultivar variability in 'Panchadarakalasa' as evident from both the morphological and microsatellite analysis could probably be due to poor diffusion of uniform quality planting material to mango growers in the state. In many rural areas, where mango cultivation has a high potential, no fruit tree nurseries are available. Even the surveyed governmental nurseries could not meet the high demand of farmers for grafts. Although the use of homogenous, well documented plant material for mother blocks is highly recommended, some of the surveyed nurseries still use heterogeneous scions from commercial orchards for the development of grafts. This will definitely result in variable quality planting material.

In this study, the extent of diversity among accessions was studied in relation to their location and set of accessions with narrow genetic base developed from particular location were identified. From the perusal of the geographical locality of each accession (Table 1) and their clustering pattern (Figure 2), it could be inferred that the grouping of the accessions is not associated with their geographical location. The four groups formed do not present clear cut separation from the accessions related to the sampling locations, which also indicates low variability. In the present study, from the highest value of Jaccard's similarity coefficient of 1.00, it is evident that accessions with 100% of similarity were found, indicating the presence of duplicates. The presence of duplicates among the accessions studied explains the utilization of same scions to the formation of grafted trees. The accessions with a common ancestry and/or multiplied clonally from a single mother plant exhibited highest (100%) genetic similarity. The presence of 50% of the accessions in the same group (IIIB) may be explained by the prevalence of this accession in the Coastal Andhra region of the state and by the fact that this cultivar has been almost exclusively propagated clonally, which may be favoring multiplication of genetically similar plants, thus reducing genetic variability.

'Panchadarakalasa' mango is a clonally propagated fruit crop in Andhra Pradesh. Although the pair-wise genetic similarity between the accessions within the two distinct sub groups IIIB and IVB is 100% (Figure 2), there was a genetic dissimilarity of 3.8% between the two distinct sub groups IIIB and IVB, indicating the distinctness of two sub groups. The accessions PKAcc-1, PKAcc-2, PKAcc-15 and PKAcc-16 of sub group IVB could have been multiplied clonally from one mother tree and could be regarded as one distinct clone. Similarly, the accessions PKAcc-4, PKAcc-5, PKAcc-6, PKAcc-8, PKAcc-9, PKAcc-10, PKAcc-12 and PKAcc-13 of sub group IIIB might have been multiplied clonally from another mother tree and could be regarded as another distinct clone. Bally *et al*. (1996) observed absolutely low level of genetic dissimilarity coefficient of 0.05 among 15 accessions of 'Kensington Pride', a polyembryonic cultivar of mango using 10 RAPD markers and concluded that they are pure clones. de Souza and Lima (2004) also observed a genetic dissimilarity coefficient of 0.45 among 25 accessions of 'Rosa' cultivar of mango using RAPD markers and concluded that they are not pure clones. Rocha *et al*. (2012) while studying intravarietal heterogeneity in 'Uba', a polyembryonic cultivar of

mango using ISSR markers, no duplicates (clones) were found among the 102 accessions at the Zona da Mata of Minas Gerais state, Brazil. Molecular analysis with ISSR markers also revealed an intra-varietal genetic diversity attesting that oca (*Oxalis tuberosa* Mol.) varieties are not pure clones (Pissard *et al*., 2008). Our microsatellite data here also strongly suggest that the 'Panchadarakalasa' samples collected in Andhra Pradesh state do not belong to the same clone. Hence, it is proposed to use the term 'variety' instead of 'clone'.

When considering intracultivar breeding programs of 'Panchadarakalasa' mango, SSR profiling studies dealing with the structure of intracultivar diversity may give some insights about the selection of genetically distinct and elite accessions of this cultivar. Here, the pair-wise genetic dissimilarity of 0.00-0.10 among accessions revealed that some of the accessions like PK Acc-3, PK Acc-7, PK Acc-11 and PK Acc-14 could be considered as distinct genotypes exhibiting varied level of pair-wise dissimilarity with the rest of the accessions under study (Figure 2). It could be possible to select these accessions of 'Panchadarakalasa' adapted to the agro-climatic conditions of the cultivation region after further yield trials. Hence, it is necessary to test these genetically distinct new accessions *viz*., PK Acc-3, PK Acc-7, PK Acc-11 and PK Acc-14 under replicated yield trials to compare them against standard commercial juicy varieties (Peddarasam, Chinnarasam, Cherukurasam and Panchadarakalasa) to confirm their distinctiveness and superiority. There were no reports on the utilization of intracultivar selection for improvement of this 'Panchadarakalasa' mango. However, exploitation of natural variability through selection of superior clones of other commercial mango cultivars has been undertaken by several workers. Singh and Chadha (1981) located four superior clones from orchards of 'Dashehri', while Singh *et al*. (1985) isolated high yielding clones from 'Langra' orchards. Pandey (1998) studied different clones of this cultivar, *viz*., 'Alphonso of Behat' in Saharanpur (Uttar Pradesh), 'Alphonso Batli' of Kirkee, Pune (Maharashtra), 'Alphonso Punjab', 'Alphonso White' of North Kanara district of Karnataka, and observed that they vary from one another in more than one character. Therefore, it is also possible to select accession of 'Panchadarakalasa' mango tree adapted to the conditions of soil and climate of the cultivation region. Rocha *et al*. (2012) also studied intravarietal heterogeneity among the 102 accessions of 'Uba' mango using ISSR markers at the Zona da Mata of Minas Gerais state, Brazil and concluded that it is possible to select elite accession of 'Uba' mango tree adapted to the conditions of soil and climate of the cultivation region.

The identification of accessions of a cultivar is extremely important both for cultivation and intracultivar improvement of fruit crops. Accession identification based on morphological characteristics can be difficult and complicated. PCR technologies, such as microsatellite analysis, can readily and quickly identify accessions of a cultivar using young leaves. In this study, the markers SSR-83 and SSR-36 with high PIC values (Table 5) provide an opportunity for direct comparison and identification of different accessions independent of any influences. Some specific bands/loci observed in the 'Panchadarakalasa' accessions studied (Table 6) may be useful for accession identification. Further, the presence of specific loci indicates the genetic distinctness of the accessions under study. For example MngSSR-24 and MngSSR-26 produced a unique allele of 150 bp and 140 bp, respectively with PKAcc-11, while SSR-83 generated a unique allele of 200 bp with PKAcc-14. Presence of one particular band is possibly due to genomic recombination and may be of use in accession discrimination. This study has shown that even though the genome of mango is allotetraploid and relatively large, the microsatellite allelic patterns generated through PCR are capable of individualizing accessions.

A great proportion of the commercial orchards of mango in the state are raised asexually through grafts. The development and supply of mango grafts in the state is undertaken through clonal propagagation by the Private, Government and University nurseries. The variability (heterogeneity) and purity (homogeneity) of mother blocks of nurseries and commercial orchards can be analyzed through the utilization of fingerprints based on microsatellite markers. In the present study, microsatellite analysis revealed a pair-wise dissimilarity of only 2.85% between the two accessions PKAcc-2 and PKAcc-3 collected from the mother block, Fruit Research Station, Sangareddy indicating heterogeneity in mother block itself. Of the 12 individual accessions (PKAcc-4 to PKAcc-15), collected from the 12 individual orchards, the accessions PKAcc-4 to PKAcc-6, PKAcc-8 to PKAcc-10 and PKAcc-12 to PKAcc-13 of sub group IIIB, PKAcc-15 of sub group IVB, PKAcc-7 of sub group IIIA, PKAcc-11 of group II and PKAcc-14 of group I, indicate that there was heterogeneity among the orchards sampled. However, this study could not give any insight about the homogeneity and/or heterogeneity within the orchards sampled because of the fact that only one tree (accession) per orchard was sampled. For having better insight about the homogeneity and/or heterogeneity of orchards, multiple trees must be sampled from the individual orchards to be sampled. Further, more accurate DNA studies involving multiple samples from the individual mother blocks of nurseries across the state give correct information about the homogeneity of mother blocks. This information can be extremely useful to the mango nurseries for the correct choice (i.e., supported by more accurate intravarietal variability analysis) of the mango multiplication materials. This process can harmonize both quantity and quality of fruit production across the state. Development of an efficient and sustainable system for supplying interested farmers with high quality uniform planting material of the most elite form of the variety together with information on good management practices is urgently needed to harmonize 'Panchadarakalasa' mango production and quality in the state.

5. Conclusion

Classical analysis based on morphological fruit traits revealed considerable phenotypic variations among 16 accessions of 'Panchadarakalasa'. PCR-based SSR analysis confirmed genomic polymorphism in this

cultivar. Morphological markers combined with molecular characterisation are essential for better understanding of intravarietal heterogeneity of 'Panchadarakalasa' mango. Our results here strongly suggest that the 'Panchadarakalasa' samples collected in Andhra Pradesh state do not belong to the same clone. Hence, it is proposed to use the term 'variety' instead of 'clone'. Microsatellite markers have proven to be a valuable tool for identifying intracultivar heterogeneity in mango. If SSR diversity is combined with fruit and other important agronomic characteristics, performing the similar studies on the other 'Panchadarakalasa' accessions may lead to planning of a better intracultivar breeding program in the state.

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References

Bally ISE, Graham GC and Henry RJ. 1996. Genetic diversity of Kensington mango in Australia. *Aust J Exp Agric*, **36**: 243-247.

Begum H, Reddy MT, Malathi S, Reddy BP, Arcahk S, Nagaraju J and Siddiq EA. 2012. Molecular analysis for genetic distinctiveness and relationships of indigenous landraces with popular cultivars of mango (*Mangifera indica* L) in Andhra Pradesh, India. *Asian Aust J Plant Sci Biotechnol***, 6 (1):** 24-37.

Bernatzky R and Tanksley SD. 1989. Restriction fragments as molecular markers for germplasm evaluation and utilization. In: Brown AHD, Frankel OH, Marshel DR and Williams JT (Eds.), **The Use of Plant Genetic Resources**, Cambridge University Press, New York, pp. 353-362.

de Souza VAB and Lima PSC. 2004. Genetic variability in mango genotypes detected by RAPD markers. *Acta Hort***, 645**: 303-310.

Diaz-Matallana M, Schuler-Garcia I, Ruiz-Garcia M and Hodson-de-Jaramillo E. 2009. Analysis of diversity among six populations of Colombian mango (*Mangifera indica* L cv. Hilacha) using RAPDs markers. *Electron J Biotechnol*, **12**: 1-8.

Duval MF, Bunel J, Sitbon C and Risterucci AM. 2005. Development of microsatellite markers for mango (*Mangifera indica* L). *Mol Ecol Notes*, **4**: 824-826.

Hirano R, Htun-Oo T and Watanabe KN. 2010. Myanmar mango landraces reveal genetic uniqueness over common cultivars from Florida, India, and Southeast Asia. *Genome*, **53**: 321-330.

Honsho C, Hishiyama K, Eiadthong S and Yonemori K. 2005. Isolation and characterization of new microsatellite markers in mango (Mangifera indica). *Mol Ecol Notes*, **5**: 152-154.

IPGRI. 2006. Descriptors for Mango (Mangifera indica L). International Plant Genetic Resources Institute, Rome, Italy.

Karihaloo JL, Dwivedi YK, Archak S and Gaikwad AB. 2003. Analysis of genetic diversity of Indian mango cultivars using RAPD markers. *J Hortic Sci Biotech*, **78**: 285-289.

Knight RJR. 1980. Origin and world importance of tropical and subtropical fruit crops. In: Nagy S and Shaw PE (Eds.), **Tropical and Subtropical Fruits,** AVI, Westport, CT, USA.

Lopez DG, Delgado SH, Paz MG, Leor ENZ, Figueroa MS and Perez NM. 2009. Genetic analysis of mango landraces from Mexico based on molecular markers. *Plant Gen Res*, **7**: 244-251.

Naik KC. 1948. Improvement of mango (Mangifera indica L) by selection and hybridization. *Indian J Agric Sci*, **18 (1):** 35-41.

Naik KC. 1971. Mango improvement. *Andhra Agric J*, **18 (6):** 221-222.

Oppenheimer C. 1956. Study tour report on subtropical fruit growing and research in India and Ceylon Special Bulletin No. 3, State of Israel, Ministry of Agriculture, Agicultural Research Station, Rehovat, Israel, October.

Pandey SN. 1998. Mango cultivars. In: Srivastav RP (Ed.), **Mango Cultivation**, International Book Distributing Company, Lucknow, India, pp. 39-99.

Pissard A, Rojas-Beltran JA, Faux A-M, Paulet S and Bertin P. 2008. Evidence of intra-varietal genetic variability in the vegetatively propagated crop oca (*Oxalis tuberosa* Mol.) in the Andean traditional farming system. *Plant Syst Evol*, **270 (1-2):** 59-74

Poovarodom S, Haruenkit R, Vearasilp S, Namiesnik J, Cvikrova M, Martinincova O, Ezra A, Suhaj M, Ruamsuke P and Gorinstein S. 2010. Comparative characterization of durian, mango and avocado. *Food Sci Technol*, **45**: 921-929.

Porebski S, Bailey G and Baum BR. 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol Biol Rep*, **15**: 8-15.

Rajwana IA, Khan IA, Malik AU, Saleem BA, Khan AS, Ziaf K, Anwar R and Amin M. 2011. Morphological and biochemical markers for varietal characterization and quality assessment of potential indigenous mango (Mangifera indica L) germplasm. *International J Agric Biol*, **13**: 151-158.

Rocha A, Salomao LCC, Salomao TMF, Cruz CD and de Siqueira DL. 2012. Genetic diversity of 'Uba' mango tree using ISSR markers. *Mol Biotechnol*, **50 (2):** 108-113.

Rohlf FJ. 2000. NTSYS-pc: Numerical taxonomy and multivariate analysis system, version 2.1 Exeter Software, Setauket, New York, USA.

Schnell RJ, Brown SJ, Olano CT, Meerow AW, Campbell RJ and Kuhn DN. 2006. Mango genetic diversity analysiand pedigree inferences for Florida cultivars using microsatellite markers*. J Am Soc Hortic Sci***, 13**: 214-224.

Schnell RJ, Olano CT, Quintanilla E and Meerow AW. 2005. Isolation and characterization of 15 microsatellite loci from mango (Mangifera indica L) and cross-species amplification in closely related taxa. *Mol Ecol Notes*, **5**: 625-627.

Singh H and Chadha KL. 1981. Improvement of Dashehari by clonal selection National Symposium on Tropical and Sub-tropical Fruit crops. Horticultural Society of India, Bangalore, p 5 (Abstr).

Singh RN, Gorakh S, Rao OP and Mishra JS. 1985. Improvement of Banarsi Langra through clonal selection. *Prog Hort,* **17**: 273-277.

Singh S, Gaikwad AB and Karihaloo JL. 2009. Morphological and molecular analysis of intracultivar variation in Indian mango (Mangifera indica L) cultivars. *Acta Hortic*, **829**: 205-212.

Vasugi C, Dinesh MR, Sekar K, Shivashankara KS, Padmakar B and Ravishankar KV. 2012. Genetic diversity in unique

indigenous mango accessions (Appemidi) of the Western Ghats for certain fruit characteristics. *Curr Sci*, **103 (2):** 199-207.

Viruel MA, Escribano P, Barbieri M, Ferri M and Hormaza JI. 2005. Fingerprinting, embryo type and geographic differentiation in mango (*Mangifera indica* L, Anacardiaceae) with microsatellites. *Mol Breed*, **15**: 383-393.

Wahdan MT, Abdelsalam AZ, El Naggar AA and Hussein MA. 2011. Preliminary horticultural studies to describe and identify two new Egyptian mango strains using DNA fingerprint. *J Am Sci*, **7 (2):** 641-650.

Williams JGK, Kubbelik A, Livak KJ, Rafiski JA and Tinjey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*, **18**: 6531-6535.

Zdunic G, Maletic E, Vokurka A, Kontic JK, Pezo I and Pejic I. 2009. Intravarietal variability of the cultivar 'Plavac Mali' (V*itis vinifera* L.). *Acta Hort***, 827**: 203-206.

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An Initial *In vitro* Investigation into the Potential Therapeutic Use Of *Lucilia sericata* Maggot to Control Superficial Fungal Infections

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Abstract

In this work an attempt was performed to investigate the in vitro ability *of Lucilia sericata* maggots to control fungi involved in superficial fungal infections. A novel GFP-modified yeast culture to enable direct visualization of the ingestion of yeast cells by maggot larvae as a method of control was used. The obtained results showed that the GFP-modified yeasts were successfully ingested by *Lucilia sericata* maggots and 1mg/ml of *Lucilia sericata* maggots excretions/ secretions (ES) showed a considerable anti-fungal activity against the growth of *Trichophyton terrestre* mycelium, the radial growth inhibition after 10 days of incubation reached 41.2 ±1.8 % in relation to the control, these results could lead to the possible application of maggot therapy in the treatment of wounds undergoing fungal infection.

Keywords: *Lucilia sericata,* Maggot Therapy, Superficial Fungal Infections And *Trichophyton Terrestre.*

1. Introduction

Biosurgical debridement or "maggot therapy" is defined as the use of live, sterile maggots of certain type of flies to remove the necrotic tissue from non-healing tissue or wounds and thereby promote healing of the remaining healthy tissue (Zacur and Kirsner, 2002; Graninger *et al*., 2002). The approach involves applying sterile larvae of *Lucilia sericata*, commonly called the green bottle fly (Borak, 2008; Chan *et al.*, 2007) to a wound, while a bandage is applied to keep them in place, the number of maggots used varies according to the type and the size of the wound and to the amount of necrotic tissue. The maggots are replaced regularly until the wound is healed (Borak, 2008).

The use of larvae to improve wound healing has long been recognized in ancient cultures, including the Chinese. During the 1930s, maggot therapy became popular In Europe and North America to treat some chronic or infected wounds. Not surprisingly however, the use of maggot therapy declined immediately after the widespread introduction of penicillin (Chan *et al*., 2007). The increase in the emergence of multi antibiotic resistance in the late 1990s however, has led to a revival in interest in bio-surgical debridement therapy and it is now being used in many countries to treat surface wounds (Sherman *et al*., 2000), including diabetic foot ulcers (Sherman, 2003), malignant adenocarcinoma (Sealby, 2004), and for venous stasis ulcers (Sherman, 2009); it is also used to combat infection after breast-conservation surgery (Church 2005).

The beneficial effects of maggots on wounds have been attributed to various mechanisms notably the debridement (degradation) of necrotic tissue. It was originally believed that this debriding action of maggots was restricted only to their mechanical wriggling, but recently many proteolytic enzyme classes have been isolated from maggot excretions and secretions (ES) which are able to specifically dissolve the laminin and fibronectin of the extracellular matrix in the necrotic tissue. This liquefies the dead tissues enabling the maggot to take it up by suction (Chan *et al.*, 2007; Graninger *et al.*, 2002). Several recent studies have demonstrated that the maggots ES from aseptically-raised *Lucilia sericata* larvae (figure 1) exhibit antibacterial actions against *both* Gram-positive and Gram-negative bacteria, including MRSA, *Escherichia coli* and *Pseudomonas aeruginosa* (Bexfield *et al.*, 2004; Kerridge *et al.*, 2005; Thomas *et al.*, 1999; Jaklic *et al.*, 2008; Jukema *et al.*, 2008). In addition, maggots can ingest bacteria as part of their normal feeding process (Zacur and Kirsner, 2002; Chan *et al*., 2007; Bowler *et al*., 2001; Mumcuoglu *et al*., 2001). Finally, maggots promote wound healing, stimulate

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granulation and promote the formation of human fibroblasts (Zacur and Kirsner, 2002).

Figure 1. Green bottle fly larva (*Lucilia sericata)*

2. Materials and Methods

2.1. Trichophyton terrestre Strain

Trichophyton terrestre (IMI 277732) was maintained on PDA (Potato dextrose agar).

2.2. Filamentous Fungi Feeding Experiments

Five-ten larvae of *L. sericata* were transferred either onto PDA (Potato dextrose agar) or PDA plates inoculated with *Trichophyton terrestre*. Larvae fed on only PDA (Potato dextrose agar) were used as controls. After being incubated for 60 minutes at 25°C, larvae were removed from the plate and washed twice with PBS. Larvae were then surface sterilized for 2 min in 70% ethanol and rinsed in sterile water. The larval posterior and anterior ends were removed, and then mild pressure was applied at the middle of the larval body to release the entire digestive tract.

2.3. Filamentous Fungi Ingestion Confirmation

Collected digestive tract were mixed with 50 ml of sterile distilled water; vigorously vortexed for 3 minutes and 100 ul of gut suspension were plated on PDA medium supplemented with chloramphenicol (50 mg/l) and cycloheximide (500 mg/l). After incubation for two weeks, plates were checked for any *Trichophyton terrestre* growth (Deshmukh, 2004).

2.4. Yeast Strain

S. cerevisiae (BY4742) GFP was labelled with green fluorescent protein (GFP) using transformation plasmid Pex3p-GFP. *S. cerevisiae* Pex3p-GFP was maintained on YPD Agar (Sigma).

2.5. Yeast Feeding Experiments

Ten well washed larvae of *L. sericata* were transferred onto agar plate inoculated with *S. cerevisiae* Pex3p-GFP. Unfed larvae were used as controls. After being incubated for 60 minutes at 25 °C, larvae were removed from the plate and washed twice with PBS. Larvae were then surface sterilized for 2 min in 70% ethanol and rinsed in sterile water. The larval posterior and anterior ends were removed, and then a pressure was applied at the middle of the larval body to release the entire whole digestive tract. (Lerch *et al*., 2000).

2.6. Detection of Fluorescence

To check that the yeast were ingested by the larvae, the digestive tract contents were fixed and then examined using a Nikon Eclipse E400 fluorescent microscope. Results were documented by photography.

2.7. Collection of Excretion/secretion (ES) from Lucilia sericata Larvae

In order to collect *Lucilia sericata* larva secretions the following protocol was followed:10 g of *Lucilia sericata* larvae were placed in individual, sterile 50 ml universal falcon tube containing 4 ml of sterile Milli-Q ultrapure water and incubated over night at 25°C in the dark. The resulting ES was collected from the larvae with a sterile syringe or pipette and centrifuged at 4000rpm for 10 minutes to remove particulate material, after which the supernatant was filter-sterilised (0.20 um) and lyophilized. Prior to use, freeze-dried ES was resuspended in sterile Milli-Q ultrapure water at a final concentration of 40 mg/ml (Bexfield *et al.*, 2004).

2.8. Preparation of Trichophyton terrestre Inocula

A standard sized inoculum of *T. terrestre* was prepared from 7- to 14-day old cultures grown on PDA at 25°C. Mature colonies were covered with approximately 5 ml of sterile PBS (pH 7.4), PBS was then gently rubbed over the surface with a sterile spreader. The resulting mixture of conidia and hyphal fragments was drawn off with a pipette and transferred to sterile tubes. Heavy particles of the suspension were allowed to settle for 10 to 15 min at room temperature, and the upper homogeneous suspension was used for further testing. The optical densities of the suspensions were read at 530 nm and adjusted to 0.15 to 0.17 to yield 0.6×10^6 to 1.4×10^6 to spores/ml of strains. The suspensions containing conidia and hyphal fragments were further diluted to obtain the final desired inoculum size of approximately 0.4×10^4 to 5×10^4 spores/ml (Karaca and Nedret Ko, 2004). The spore suspension (5 µl) was mixed with 20 µl of various concentrations of extracts, fractions (or active compounds) and transferred to Petri dishes containing SDA (Sabouraud Dextrose Agar). After incubation at 28 °C for 7 days the plates were photographed.

2.9. Anti-Trichophyton terrestre Activity of Lucilia sericata ES

The agar dilution method was used to assess the activity of *L. sericata* ES on *T. terrestre*, *Trichophyton terrestre* was inoculated onto PDA plates and incubated at 25°C for 7-10 days to obtain young, actively growing cultures consisting of mycelia and conidia. Sterilised ES was incorporated into PDA sterilised pre-poured medium to give a range of final concentrations (µg $ml^{-1)}$, the medium poured and the agar in the plates allowed to set. A mycelial disc, 8 mm in diameter, cut from the periphery of the 7-10-day-old cultures, was aseptically inoculated onto the medium. The inoculated plates were then incubated at 25 °C and the colony diameter measured and measured after 5, 10, 15 days. The percentage of mycelial inhibition was calculated as follows: % mycelial inhibition = $[(d_c-d_t)/d_c] \times 100$; d_c= colony diameter in control, d_t = colony diameter in treatment, three replicate plates were used for each treatment.

3. .Results

As shown in Figure 2, after placing the *Lucilia sericata* maggots into the agar plates inoculated with *S. cerevisiae* (BY4742) GFP for 60 minutes at 25 °C, under fluorescent microscope a significant fluorescence was observed in the gut contents of ten different maggots out of ten examined (i.e.100%), whereas no fluorescence was seen in maggots fed on only agar. This clearly shows that the *Lucilia sericata* maggots successfully ingested yeasts.

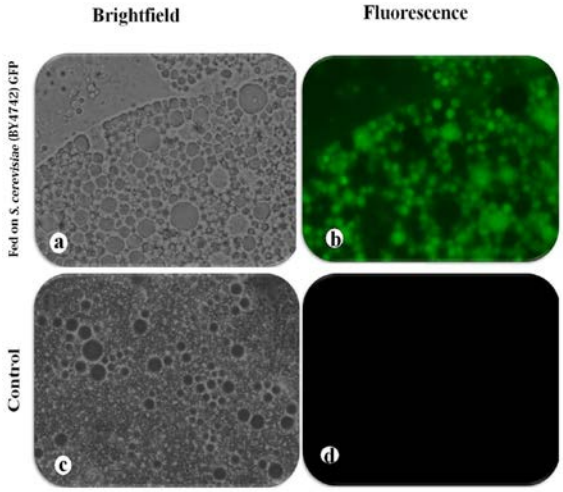

Figure 2. Left and Right microscopy images correspond to bright field and GFP fluorescence, respectively. (a) bright field microscopy image of the digestive tract of maggots fed on *S. cerevisiae* (BY4742) GFP (b) GFP fluorescence microscopy image of the gut content of maggots fed on *S. cerevisiae* (BY4742) GFP (c) and (d) bright field and GFP fluorescence microscopy images of the gut content of control maggots

When excretions/secretions (ES) of *Lucilia sericata* maggots were tested against *Trichophyton terrestre* by the agar dilution method, 1mg/ml of ES showed a considerable inhibition of growth of mycelium as illustrated in Figure 3. The radial growth inhibition after 10 days of incubation reached 41.2 ±1.8 % in relation to the control. This appears to be the first time that the antifungal activity (i.e filamentous) of maggot's excretions/secretions has been both tested and observed.

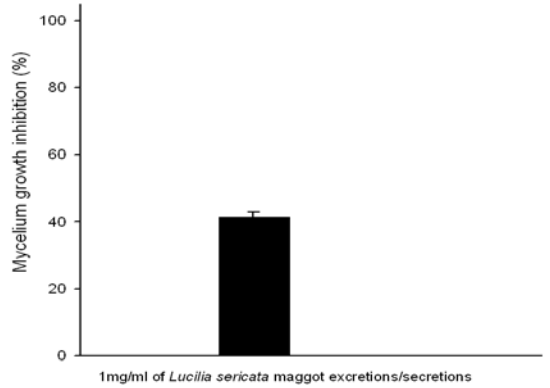

Figure 3. Percent inhibition of mycelial growth of Trichophyton terrestre in presence of 1 mg/ml Lucilia sericata maggot excretions/secretions. Percentage of mycelial growth inhibition respective to the control (no ES added). Means of three replicates ±SD.

In investigation of the ability of *L. sericata* maggots to ingest filamentous fungi, no growth of *Trichophyton terrestre* was observed in 16 different samples after 10 days of incubation of a subcultures of the maggot's gut suspensions fed on *Trichophyton terrestre.* This observation could indicate that *L. sericata* maggots are unable to ingest *Trichophyton terrestre* mycelium and spores, or that the fungus is killed as the result of ingestion.

4. Discussion

Fungal infections are considered as an important cause of morbidity and death of patients with burn wounds (Horvath *et al*., 2007; Murray *et al*., 2008; Becker *et al*., 1991). According to Rode *et al*. (2008) fungal infections complications that delay the healing process are associated with 30% of burn wounds.

Dermatomycoses, such as ringworm or tinea (Lakshmipathy and Kannabiran, 2010; Weitzman and Summerbell, 1995), are infections of the keratinized layers of skin, hair and nail. Dermatomycoses are among the most widespread infectious diseases in the world, mainly in the tropical and subtropical countries (Brasch and Graser, 2005; Lakshmipathy and Kannabiran, 2010). Such infections are difficult to control and expensive to treat (Bokhari, 2009). It has been estimated that the worldwide annual cost of dermatophytosis drug development is over USD $ 0.5 billion (Gräser *et al*., 2008). The prolonged systemic use of antifungal drug treatment of dermatomycoses is highly linked to fungal resistance, drug toxicity and interactions (Koroishi *et al.*, 2008) although positive outcomes in dermatophytosis control are generally associated with topical antifungal therapy (Karaca and Nedret Ko, 2004) .

The introduction of maggot therapy to treat heavily fungal colonized burn wounds and dermatomycoses such as athlete's foot might prove a viable alternative to the use of conventional antibiotics, especially where antibiotic resistance is found.

In order to evaluate the use of *Lucilia sericata* maggots in fungal wound infections and superficial fungal infections management, two questions need to be answered; firstly, do the *Lucilia sericata* maggot excretions/secretions have antifungal activity ?- and secondary, can *Lucilia sericata* maggots ingest pathogenic fungi ? To the best of our knowledge there are no previous reports on the antifungal activity of *Lucilia sericata* maggots against filamentous fungi, but as the insect larvae are subjected to entomopathogenic fungus invasion, the cuticle layer of the larvae is expected to have antifungal compounds (Gołębiowski *et al*., 2012a). Insect cuticular fatty acids inhibited the hyphal growth and spore germination of the entomopathogenic fungus *Conidiobolus coronatus* (Boguś *et al*., 2010). Recently, a mixtures of alcohols present in cuticular lipids of *M. domestica* insect and larvae has exhibited a considerable antifungal activity against some yeast and filamentous fungi (Gołębiowski *et al*., 2012b). In regarding to the yeast fungi, complete lysis of *Candida albicans* has been shown *in vitro* after 24 h of maggot application (Margolin and Gialanella (2010) and Jarczyk *et al.* (2008) have

reported the elimination of *Candida* spp. from chronic foot ulcerations after treatment with maggots.

In this study attempts were made to: a) investigate the ability of *Lucilia sericata* maggots to ingest filamentous fungi and yeasts, b) investigate the antifungal activity of *Lucilia sericata* maggot excretions/secretions against filamentous fungi only (as it has already been demonstrated against yeasts). *Trichophyton terrestre* was chosen as a model dermatophyte filamentous fungus.

Results obtained here show that maggots are able to ingest yeasts and as mentioned above, it has been shown that maggots application led to a completed lysis and elimination of *Candida* spp. (Jarczyk *et al.*, 2008; Margolin and Gialanella, 2010). With regarding to filamentous fungi, the results show that the maggot ES have moderate antifungal activity, E/S contain a lot of alkaline compounds like ammonium carbonate, allantoin and urea (Gołębiowski *et al.*, 2012c), which might therefore be partially responsible for this antifungal activity. In 2010, Čeřovský and his co-workers succeed to purify a novel antimicrobial defensin, named lucifensin, from the (*Lucilia sericata*) larvae excretions/secretion that may provide protection to larvae when they are exposed to the highly infected wounds during the maggot therapy (Čeřovský *et al.*, 2010). The results failed to provide evidence about whether the maggots are able to ingest filamentous fungi. However, this does not necessarily mean that maggots are unable to deal with such fungi as maggots are mainly feed through extracorporeal digestion where a mixture of digestive enzymes (such as tryptase, peptidase, and lipase) are continuously produced by larval salivary glands into the surroundings (Lerch *et al.*, 2000; Andersen *et al.*, 2010), the secreted digestive enzymes could lead to the destruction and lysis of the fungal mycelium, to be subsequently adsorbed by the maggot's powerful suction apparatus (Andersen *et al*., 2010) especially since maggots are known to have an ability to ingest as much as half of their body weight within a few minutes (Fleischmann *et al*., 2004).

5. Conclusion

The moderate antifungal activity (yeast and mould) of ES of *Lucilia sericata* maggots and the ability of these maggots to ingest yeast and probably to destroy and lyse mould mycelium, could lead to the possible application of maggot therapy in the treatment of wounds undergoing fungal infection and with superficial fungal infections i.e. athlete's foot. Further studies are now needed to help confirm this possibility.

References

Andrews JM. 2001. Determination of minimum inhibitory concentrations. *J Antimicrob Chemoth*, **48:** 5-16.

Alnaimat S. 2011. A contribution to the study of biocontrol agents apitherapy and other potential alternative to antibiotics. Ph.D. thesis, University of Sheffield, Sheffield, UK.

Becker W, Cioffi Jr W, McManus A, Kim S, McManus W, Mason A and Pruitt Jr B. 1991. Fungal burn wound infection. A 10-year experience. *Arch Surg* , **126**: 44-48.

Bexfield A, Nigam Y, Thomas S and Ratcliffe NA. 2004. Detection and partial characterisation of two antibacterial factors from the excretions/secretions of the medicinal maggot Lucilia sericata and their activity against methicillin-resistant *Staphylococcus aureus* (MRSA). *Microbes Infect*, **6**: 1297-1304.

Boguś MI, Czygier M, Gołębiowski M, Kędra E, Kucińska J and Mazgajska J. 2010. Effects of insect cuticular fatty acids on *in vitro* growth and pathogenicity of the entomopathogenic fungus *Conidiobolus coronatus*. *Exp Parasitol*, **125:**400-408.

Bokhari FM. 2009 Antifungal activity of some medicinal plants used in Jeddah, Saudi Arabia. *Mycopath*, **7**: 51-57.

Borak T. 2008. Maggot Debridement. *Surg Tech*, **5**:553-555

Bowler P, Duerden B and Armstrong D. 2001. Wound microbiology and associated approaches to wound management. *Clin Microbiol Rev*, **14**: 244-249.

Brasch J and Graser, Y. 2005. *Trichophyton eboreum* sp. nov. isolated from human skin. *J Clin Microbiol*, **43**: 5230-5237.

Čeřovský V, Žďárek J, Fučík V, Monincová L, Voburka Z and Bém R. 2010. Lucifensin, the long-sought antimicrobial factor of medicinal maggots of the blowfly *Lucilia sericata*. *Cell Mol Life Sci,* **67**: 455-466.

Chan DC, Fong DH, Leung JY, Patil NG and Leung GK. 2007. Maggot debridement therapy in chronic wound care. *Hong Kong Med J,* **13**: 382-386.

Church JC. 2005. Re: Bleeding complications in patients treated with maggot debridement therapy, Steenvoorde P and Oskam J, IJLEW 2005;4:57-58. *Int J Low Extrem Wounds*, **4**: 59-60.

Fleischmann W, Grassberger M and Sherman R. 2004. **Maggot Therapy: A handbook of Maggot-Assisted Wound Healing**: George Thieme Verlag.

Gołębiowski M, Boguś MI, Paszkiewicz M, Wieloch W, Włóka E and Stepnowski P. 2012a. The composition of the cuticular and internal free fatty acids and alcohols from *Lucilia sericata* males and females. *Lipids*, **47**:613-622.

Gołębiowski M, Dawgul M, Kamysz W, Boguś MI, Wieloch W and Włóka E. 2012b. Antimicrobial activity of alcohols from *Musca domestica*. *J Exp Biol*, **215**: 3419-3428.

Gołębiowski M, Paszkiewicz M, Grubba A, Gąsiewska D, Boguś M and Włóka E. 2012c. Cuticular and internal n-alkane composition of *Lucilia sericata* larvae, pupae, male and female imagines: application of HPLC-LLSD and GC/MS-SIM. *Bull Entomol Res*, **102**: 453

Graninger M, Grassberger M, Galehr E, Huemer F, Gruschina E, Minar E and Graninger W. 2002. Biosurgical debridement facilitates healing of chronic skin ulcers. *Arch Intern Med*, **162**:1906-1907.

Gräser Y, Scott J and Summerbell R. 2008. The new species concept in dermatophytes a polyphasic approach. *Mycopathol.,* **166**: 239-256.

Horvath EE, Murray CK, Vaughan GM, Chung KK, Hospenthal DR, Wade CE, Holcomb JB, Wolf SE, Mason Jr AD and Cancio LC. .2007.Fungal wound infection (not colonization) is independently associated with mortality in burn patients. *Annals of Sur.*, **245**: 978-985.

Jaklic D, Lapanje A, Zupancic K, Smrke D and Gunde-Cimerman N. 2008. Selective antimicrobial activity of maggots against pathogenic bacteria. *J Med Microbiol*, **57**: 617-625.

Jarczyk G, Jackowski M, Szpila K, Boszek G, Kapelaty S, Skwarek E and Michalak M. .2008. Biosurgical treatment results in patients with chronic crural and foot ulcerations. *Pol J Surg*, **80**: 190-201.

Jukema GN, Wai SW, Lagendijk EL, Van Dissel JT, Bloemberg GV and Nibbering P.H. 2008. Maggot excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *J Antimicrob Chemoth*, **61**: 117–122.

Karaca N and Nedret Ko A. 2004. *In vitro* susceptibility testing of dermatophytes: comparison of disk diffusion and reference broth dilution methods. *Diagnostic Microbiol and Infect Dis*. **48**: 259-264.

Kerridge A, Lappin-Scott Hand Stevens, J. 2005. Antibacterial properties of larval secretions of the blowfly, *Lucilia sericata*. *Med Vet Entomol*, **19**: 333-337.

Koroishi AM, Foss SR, Cortez DAG, Ueda-Nakamura T, Nakamura CV and Dias Filho BP. 2008. *In vitro* antifungal activity of extracts and neolignans from *Piper regnellii* against dermatophytes. *J Ethnopharmacol*, **117**: 270-277.

Lakshmipathy DT and Kannabiran K. 2010. Review on dermatomycosis: pathogenesis and treatment. *Nat Sci,* **2**: 726-731.

Lerch K, Linde H, Lehn N and Grifka J. 2000. Bacteria ingestion by blowfly larvae: an *in vitro* study. *Dermatology*, **207**: 362-366.

Margolin L and Gialanella P. 2010. Assessment of the antimicrobial properties of maggots. *Int Wound J*, **7**: 202-204.

Mumcuoglu KY, Miller J, Mumcuoglu M, Friger M and Tarshis M. 2001. Destruction of bacteria in the digestive tract of the maggot of *Lucilia sericata* (Diptera: Calliphoridae). *J Med Entomol*, **38**: 161-166.

Murray CK, Loo FL, Hospenthal DR, Cancio LC, Jones JA, Kim SH, Holcomb JB, Wade CE and Wolf SE. 2008. Incidence of systemic fungal infection and related mortality following severe burns. *Burns* **34:** 1108-1112.

Rode H, Vale ID and Millar, A.J.W. 2008. Burn wound infection. *Cont Medi Educ*, **26**: 440-444.

Sealby N. 2004. The use of maggot therapy in the treatment of a malignant foot wound. *Br J Community Nurs***, 9**: S16-19.

Sherman RA. 2003. Maggot therapy for treating diabetic foot ulcers unresponsive to conventional therapy. *Diabetes Care*, **26**: 446-451.

Sherman RA. 2009. Maggot therapy takes us back to the future of wound care: new and improved maggot therapy for the 21st century. *J Diabetes Sci Technol*, **3**: 336-344.

Thomas S, Andrews A, Hay N and Bourgoise S. 1999. The anti-microbial activity of maggot secretions: results of a preliminary study. *J Tissue Viab*, **9**: 127-132.

Weitzman I and Summerbell RC. 1995. The dermatophytes. *Clin Microbiol Rev* **8**: 240-259.

Zacur H and Kirsner RS. 2002. Debridement: rationale and therapeutic options. *Wounds 14*. Published online in September 2008. ONLINE REF.

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Trehalose Accumulation in Wheat Plant Promotes Sucrose and Starch Biosynthesis

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Abstract

Seeds of *Triticum aestivum* L. (cv. Sakha 93) were sown in pots and grown under controlled conditions in growth chamber. The plants were irrigated with half strength of Hoaglad solution without or with 10 or 30 µM validamycin A, a potent inhibitor of trehalase. Plants were collected at three different stages of growth (17, 24 and 31 DAP). Validamycin A decreased the activity of trehalase which leads to the accumulation of trehalose in shoot and root of wheat plants. Raising trehalose level in the plant tissues was accompanied by increase in the sucrose content and starch content of the shoot. The increased contents in sucrose and starch were mainly attributed to the increased levels of trehalose. The effect of trehalose on the sucrose degrading enzymes (alkaline and acid invertases and sucrose synthase) showed stimulation of alkaline invertase activity and inhibition of acid invertase and sucrose synthase. The opposite behavior of sucrose degrading enzymes suggests a regulation mechanism controlling the sucrose pool.

Keywords: Trehalose, Trehalose -6-phosphate, Carbohydrate, *Triticum aestivum*, Trehalase, Invertase, Sucrose Synthase, Validamycin.

1. Introduction

Trehalose is a non-reducing disaccharide, formed of two α, D glucose molecules linked by an α,α -1,1 glycosidic linkage. Although there are three possible anomers of trehalose: α, α- trehalose; α, β (neotrehalose) and β, β (isotrehalose); yet only the α, α 1-1 configuration has been isolated from and biosynthesized in living organisms (Elbein *et al*., 2003). Trehalose is widespread through the biological world. It is found in a number of different bacteria (Kaasen *et al*., 1994; Shimakata and Minatagawa, 2000) and fungi (Nwaka and Holzer, 1998). Trehalose was also present in many plants, it has been detected in the resurrection plant *Selaginella lepidophylla* (Adams *et al*., 1990), *Glycine max* (Müller *et al*., 1992), *Arabidopsis thaliana* (Müller *et al*., 2001), *Triticum aestivum* (El-Bashiti *et al*., 2005), *Phaseolus vulgaris* (Gracía *et al*., 2005), *Lotus japonicus* and *Medicago truncatula* (Lopéz *et al*., 2006; 2009).

There are five known naturally occurring trehalose biosynthetic pathways. Only one of these, the *OtsA–OtsB* (TPS –TPP) pathway which involves the intermediate trehalose -6- phosphate (T6P). The recent discovery of trehalose pathway in plants and T6P in particular has a powerful function in metabolic regulation. A direct action of T6P as a signal is the redox activation of ADP-glucose pyrophosphorylase (AGPase), the key enzyme of starch synthesis (Kolbe *et al*., 2005). T6P was reported to inhibit SnRK1 which is a protein kinase involved in the regulation of carbohydrate metabolism (Zhan *et al*., 2009). Altering the trehalose pathway exerts numerous effects on metabolism and development. These effects include embryo development (Eastmond *et al*., 2002), starch metabolism (Kolbe *et al*., 2005), sucrose utilization (Schluepmann *et al*., 2003) and tolerance of abiotic stresses (Almeida *et al*., 2005; Karim *et al*., 2007 and Pilon-Smits *et al*., 1998). Over-expression of genes encoding TPS and TPP is reported to be effective for improving abiotic stress tolerance in tobacco (Holmström *et al*., 1996), potato (Yeo *et al*., 2000), tomato (Cortina and Culiăñez-Maciă, 2005), *Arabidopsis* (Miranda *et al*., 2007), rice (Ge *et al*., 2008) and maize (Jiang *et al*., 2010).

The photosynthetic assimilation of atmospheric CO_2 by leaves yields sucrose and starch as end products of two separated pathways namely sucrose in cytosol and starch in chloroplasts. Whereas starch is an insoluble polyglucose formed in the plastids, fructan is soluble polyfructoses synthesized and stored in the vacuole. Wheat, barley and many other grasses from temperate climates store fructan (Taiz and Zeiger, 2006).

In higher plants, two enzymes are able to catalyze sucrose degradation: invertase (E.C. 3.2.1.26) and sucrose synthase (E.C. 2.4.1.13). Invertases are usually divided into acid, neutral or alkaline groups according to the optimum pH requirements and subcellular localization (Schroeven *et al*., 2008; Tao *et al*., 2010).

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Abbreviations :AGPase (ADP-glucose pyrophosphorylase),**T6P** (Trehalose -6-phosphate),**TPS** (Trehalose phosphate synthase),**TPP** (Trehalose phosphate phosphatase),**DAP** (Days after planting),**SnRK1** (Sucrose nonfermenting related protein kinase).

Sucrose synthase activity has been shown to play a major role in energy metabolism, controlling the mobilization of sucrose into various pathways important for the metabolic, structural, and storage functions of the plant cell (Hesse and Willmitzer, 1996; Tang *et al*., 1999).

Trehalase (EC 3.2.1.28) is a glycosyl hydrolase that hydrolyzes trehalose. It is detected in many prokaryotic and eukaryotic cells. It is the only known pathway of utilization of trehalose (Reguera *et al*., 2011). Trehalase has been detected in various organisms such as *Saccharomyces cerevisiae* (Alizadeh and Klionsky, 1996), *Lentinula edodes* (Murata *et al*., 2001) and *Acidobacterium capsulatum* (Inagaki *et al*., 2001). It is also present in soybean (Aeschbacher *et al*., 1999), *Medicago sativa* (Wolska-Mitaszko *et al*., 2005), *Phaseolus vulgaris* (Gracía *et al*., 2005) and *Triticum aestivum* (Kord *et al*., 2012). This enzyme plays an important role in trehalose metabolism, as it is either directly involved in the assimilation of exogenous trehalose, or it controls its level in the cell.

Validamycin A ($C_{20}H_{35}NO_{13}$) is a non-systemic antibiotic with fungicide action. It is produced from fermentation of *Streptomyces hygroscopicus* variety limoneus. It is most effective against soil borne diseases and is used for the control of *Rhizoctonia solani* in rice, potato and other vegetables (Asano *et al*., 1987). It is a specific competitive inhibitor of trehalase. Treatment with validamycin raised trehalose in plant tissue (Müller *et al*., 1995).

The present work is allotted to study the effect of trehalose on the metabolism of carbohydrates in wheat plant grown under different levels of validamycin A.

2. Materials and Methods

2.1. Plant Material

Seeds of *Triticum aestivum* L. (cv. Sakha 93) was purchased from Agriculture Research Center, Giza, Egypt.

2.2. Design of the Experiment

Seeds of wheat were sterilized by immersing in sodium hypochloride (20 %) (v/v) for 20 min, rinsed with sterilized distilled water, and sown in plastic pots (12 cm × 12 cm), containing 500 g sterilized mixture of perlite and vermiculate (1:1 w/w). Eighteen pots were used. The pots were kept in growth chamber at 25-18 °C with 16 h light and 8 h dark photocycle (5000 Lux) at 70 % relative humidity. Seedlings were grown up to 10 days and irrigated with sterilized tap water. The seedlings were thinned to ten seedlings in each pot and irrigated with half strength of Hoagland solution. The pots were grouped into three sets. The first set of pots irrigated with half strength of Hoagland solution and designated as control. The second and the third sets were irrigated with half strength of Hoagland solution containing 10 or 30 µM validamycin A, respectively. Plants were irrigated twice every week keeping the field capacity at 70 %. Plant samples were collected after 17 days of planting (DAP) when two leaves were fully expanded, after 24 DAP when three leaves were fully expanded and after 31 DAP when four leaves were fully expanded.

The harvested plants of three pots of each set were divided into shoots and roots, ground in liquid nitrogen and used for the determination of carbohydrates (trehalose, sucrose, starch and fructan). The remaining three pots of each set were used in the determination of enzymes (trehalase, alkaline invertase, acid invertase and sucrose synthase).

2.3. Determination of Trehalase Activity

Crude extracts of shoots and roots were prepared according to the method of Müller *et al*. (1992). Extracts were prepared by homogenized 0.1 g of frozen tissue in a mortar with 10 mg/g fresh weight PVP and 1 mL of 100 mM cold sodium citrate buffer pH 5.5, containing 1 mM PMSF, 2 mM EDTA. The crude extract was centrifuged for 15 min at 10,000 g, the supernatant was used to determine enzyme activity.

Trehalase activity was measured by estimating the glucose produced by hydrolysis of trehalose with the glucose oxidase-peroxidase kit (Spainreact) as described by Bergmeyer and Bernt (1974). The reaction mixture contained 100 mM trehalose (Sigma Aldrich Co.), 50 mM sodium citrate buffer (pH 5.5) and 0.25 mL crude extract in a final volume of 1.5 mL. After incubation at 55 °C for 30 min, the reaction was stopped by boiling for 3 min and then the reaction mixture was centrifuged at 5000 g for 10 min. For the analysis, 10 µL of the supernatant was mixed with 1 mL of glucose oxidase-peroxidase kit solution, mixed by vortex and then the mixture was incubated at 37 °C for 15 min. The absorbance of the sample was measured at 470 nm. Enzyme and substrate blanks were subtracted. One unit (nkat) of trehalase activity is defined as the amount of enzyme that hydrolyzes nmol trehalose per second at pH 5.5.

The total soluble protein was determined according to Lowry *et al*. (1951). The specific activity of trehalase was expressed as nmol/mg protein/sec.

2.4. Extraction and Determination of Carbohydrates

2.4.1. Extraction and Determination of Trehalose

Trehalose was extracted according to Ferreira *et al*. (1997). 0.1 g of frozen tissue (shoots or roots) was boiled in 2 mL ethanol, evaporated at 60 ºC, the residue was dissolved in 5 mL of 5 mM H_2SO_4, centrifuged at 10,000 g for 10 min and filtered. Filtrate was heated in boiling water bath for 60 min to hydrolyze sucrose in the extract, since the sucrose retention time is the same as that of trehalose. The pH was adjusted to 7.0, the solution was evaporated and the residue was dissolved in distilled water. The content of trehalose was determined by the method described by Cizmarik *et al*. (2004) using HPLC (Hewlett Packard, HP 1090 liquid chromatograph), Hypersil, 100 × 3 mm, 3 µm column. The separation was carried out at 32 °C using a flow rate of 0.8 ml/ min with acetonitrile: H_2O (85: 15) as mobile phase. The elution was detected with a Diode Array Detector (DAD).

2.4.2. Extraction and Determination of Sucrose

Sucrose was extracted by grinding 0.1 g of tissue, mixing with 10 mg PVP, 80% ethanol. The homogenate

has been incubated at 70 °C for 20 min. After centrifugation at 4,000 g for 10 min, the supernatant was separated and the pellet was re-extracted twice with 80 % ethanol at 70 °C for 30 min. The supernatant was pooled and evaporated. The residue was dissolved in 1 mLof deionized H_2O. The content of sucrose was determined using previously mentioned HPLC method (Cizmarik *et al*., 2004).

2.4.3. Extraction and Determination of Starch

Starch was extracted according to the method of Grotelueschen and Smith (1967). The released glucose from hydrolysis of starch was determined in the supernatant by glucose oxidase peroxidase kit according to the method described by Bergmeyer and Bernt (1974).

2.4.4. Extraction and Determination of Fructan

Fructan was extracted by adding 250 µL HCl (0.2 M) to 250 µL of sugar extract, heated at 80 °C for 10 min. Samples were neutralized by adding 250 µL of 0.2 M NaOH and diluted by deionized H_2O (Sprenger *et al*., 1995). The released fructose was determined by Nelson's method (Clark and Switzer, 1977).

2.5. Extraction and Determination of Invertase Activity

One gram of tissue was homogenized in 10 mL of extraction buffer (50 mM phosphate buffer (pH 7.5), 1 mM 2-mercaptoethanol and 5 mM $MnSO_4$. After centrifugation at 20,000g for 10 min, alkaline invertase was measured according to the method described by Morell and Copeland (1984).

Acid invertase was assayed according to Schellenbaum *et al*. (1998). The specific activity of alkaline invertase and acid invertase were expressed as µmole sucrose/ mg protein/min.

2.6. Extraction and Determination of Sucrose Synthase Activity

Plant extract was prepared by homogenizing 0.2 g of tissue in a mortar with 33% PVP and 1.5 mL of 50 mM potassium phosphate buffer (pH 8.0) containing 1mM EDTA and 20 % (v/v) ethylene glycol (López *et al*., 2009). The plant extract was centrifuged at 20,000 g for 10 min. The supernatant was used for determining enzyme activity. Sucrose synthase activity was measured according to Morell and Copeland (1985). Specific activity of sucrose synthase is expressed as µmole sucrose/ mg protein/min.

2.7. Statistical Analysis

The data of the experiment were subjected to statistical analysis of variance, (One way ANOVA) using SPSS 10.0 software. The significant differences were considered at $P < 0.05$ (Snedecor and Cochran 1989).

3. Results and Discussion

As shown in Table (1), treatment with validamycin A (10 or 30 µM) decreased the activity of trehalase in the shoot and root of the wheat cultivar at the different growth stages as compared to the control. This decrease was more pronounced with the higher validamycin A concentration (30 µM). The drop in the specific activity of trehalase was accompanied by accumulation of trehalose in the treated plants; the level of trehalose was higher with the higher validamycin A concentration.

Several researchers have used validamycin A to raise the level of trehalose in plants. Thus, Müller *et al*. (1995) reported that trehalose increased significantly upon validamycin A treatment in nodules of *Glycine max* and *Vigna uniguiculata*. López *et al*. (2006; 2009) reported that validamycin A was able to increase the level of trehalose in nodules of *Lotus japonicas* and *Medicago truncatula*. Trehalose significantly increased in rice seedling by using validamycin A (Garg *et al*., 2007). Validamycin A was used safely to raise the level of trehalose in plant tissues, since it proved to have no effect on growth (Müller *et al*., 2001; Gracia *et al*., 2005; Qaid, 2010). Validamycin A does not affect nitrogen fixation in nodules and does not cause any visible damage or growth reduction in soybean and common bean (Müller *et al*., 1995; Gracia *et al*., 2005). Pellny *et al*. (2004) and Gomez *et al*. (2006) who reported that trehalose pathway and in particular T6P exerts numerous effects on plant growth and development.

It is clear from the present work that the accumulation of trehalose in the wheat plant is accompanied by increase in the level of sucrose in shoot and root (Figure 1). These results are in agreement with those reported by Bae *et al*. (2005) using *Arabidopsis thaliana* seedlings and by Garg *et al*. (2007) using rice seedlings. However, reduction in sucrose content in response of trehalose accumulation was reported by Müller *et al*. (1998) in nodules of soybean; Wingler *et al*. (2000) in shoots of *Arabidopsis thaliana*; Müller *et al*. (2001) in flowers, leaves and stems of *Arabidopsis* and in nodules of *Lotus japonicas* and *Medicago truncatula* (Lopéz *et al*., 2006; 2009).

From the literature, it is clear that trehalose and T6P accumulate in plants treated with the potent trehalase inhibitor "validamycin A". When the cytoplasmic trehalose level increases, its feedback inhibition of trehalose phosphate phosphatase (TPP) activity enhanced the level of T6P (Schluepmann *et al*., 2004; Brohmann, 2006).

Trehalose and its intermediate (T6P), both have indispensable effect on plant growth and development. T6P was reported to inhibit SnRK1 (sucrose nonfermenting related protein kinase). Zhang *et al*. (2009) found that genes normally induced by SnRK1 were repressed by T6P and those normally repressed by SnRK1 were induced strongly supporting a role of T6P as inhibitor of SnRK1 in vivo. The inhibition of SnRK1 by T6P stops the phosphorylation of sucrose -6-phosphate synthase (SPS) and keeps the enzyme SPS in the active form which leads to increase sucrose synthesis (see Diagram 1).

Table 1. Mean values for specific activity of trehalase and the content of trehalose in shoot and root of *Triticum aestivum* (cv.Sakha 93) at 17, 24 and 31 DAP treated with 10 µM and 30 µM validamycin A. Specific activity (S.A): nmol/mg protein / sec. Trehalose content: (mg/g dry weight). Values are means of three replicates. (*) Significantly different from control (P <0.05).

Treatments	Shoot					
	17 DAP		24 DAP		31 DAP	
	S.A	**Trehalose**	**S.A**	**Trehalose**	**S.A**	**Trehalose**
Control	0.186 ±0.007	0.097 ±0.005	0.299 ±0.006	0.238 ±0.002	0.272 ±0.003	0.346 ±0.015
10 µM	0.130 * ±0.003	0.143* ±0.006	0.178* ±0.005	0.887* ±0.030	0.094* ±0.003	1.057* ±0.042
30 µM	0.054* ±0.002	0.487* ±0.009	0.099* ±0.005	1.777* ±0.090	0.062* ±0.002	4.799* ±0.230
	Root					
Treatments	17 DAP		24 DAP		31 DAP	
	S.A	**Trehalose**	**S.A**	**Trehalose**	**S.A**	**Trehalose**
Control	0.366 ±0.006	0.342 ±0.015	0.310 ±0.006	0.431 ±0.025	0.659 ±0.007	0.564 ±0.030
10 µM	0.278* ±0.005	1.242* ±0.028	0.209* ±0.004	1.554 ±0.042	0.313* ±0.008	1.748* ±0.180
30 µM	0.199* ±0.005	2.976* ±0.090	0.162* ±0.004	3.568* ±0.056	0.254* ±0.005	3.628* ±0.190

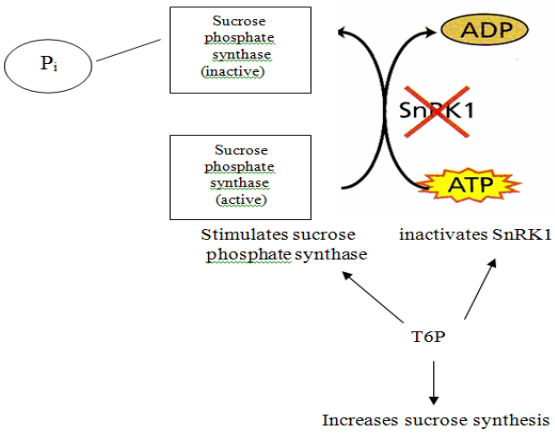

Diagram 1. Effect of T6P on regulation of sucrose synthesis. T6P inhibits SnRK1, thus precludes the phosphorylation of sucrose -6- phosphate synthase thus keeping it in the active form, which means increase in sucrose synthesis. Modified from Taiz and Zeiger (2006).

In the present work, the starch content increased in the shoot of wheat by almost 30% to 50 % over the control in response to trehalose accumulation. However, it decreased in the root of the plant (Figure 1). Our results are in agreement with those reported by other authors. Wingler *et al*. (2000) reported that starch was accumulated in the shoot of *Arabidopsis*, Fritzius *et al*. (2001) found that starch accumulated in shoot of *Arabidopsis* seedlings grown on trehalose. Bae *et al*. (2005) reported that starch was 3-fold greater in the trehalose treated samples than in the control of *A. thaliana* seedlings.

It is interesting to mention here that T6P which acts as a sugar signal is likely generated in the cytosol and possibly transported to the chloroplasts where it could produce trehalose by TPP enzymes in the chloroplasts. A direct action of T6P as a signal is the redox activation of ADP-glucose pyrophosphorylase (AGPase), the key enzyme of starch synthesis (Kolbe *et al.*, 2005; Lunn *et al.*, 2006).

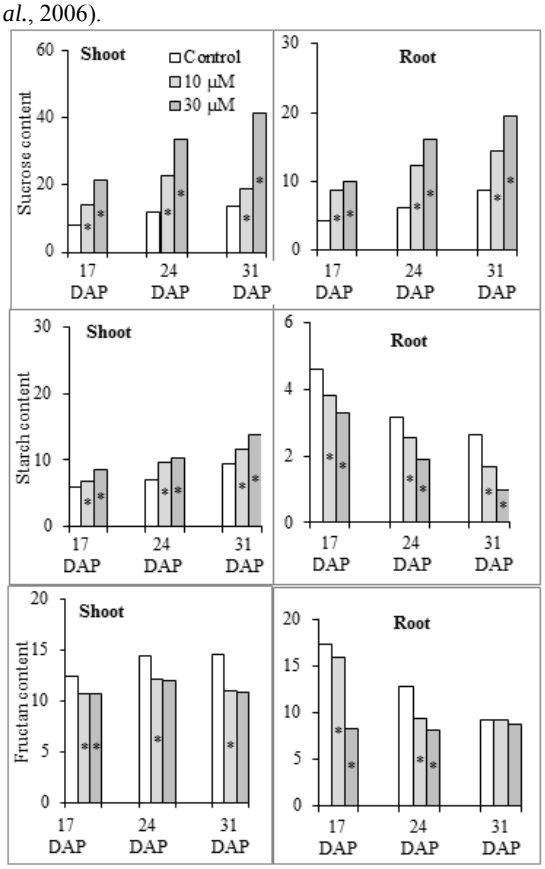

Figure 1. The contents of sucrose, starch and fructan in shoot and root of *Triticum aestivum* (cv.Sakha 93) at 17, 24 and 31 DAP treated with 10 µM and 30 µM validamycin A. (*) Significantly different from control (P <0.05).

Our results show that accumulation of trehalose was accompanied by increases in sucrose and starch contents. The increase was more pronounced with the higher trehalose concentration which was achieved by using the higher validamycin A concentration and also by the increase in plant age.

In the present study, the fructan content did not show remarkable changes in the shoot of wheat cultivar (Figure 1). However, the fructan content of the roots showed significant decreases particularly with the higher concentration of validamycin A (30 µM). As previously mentioned, the accumulation of trehalose raised the sucrose level in the roots, but decreased the starch content. Such decrease is due to the hydrolysis of starch to maintain soluble sugar supply. The same trend happened with the fructan fraction.

In the present work, the analysis of sucrose degrading enzymes showed that the specific activity of alkaline invertase increased in response of increasing the level of trehalose (Figure 2). This takes place in shoot and root of wheat plant using the two concentrations of validamycin and at the three stages of growth. On the other hand decrease in specific activity of acid invertase as well as sucrose synthase were found. It is possible that the activation of some and the inhibition of other sucrose degrading enzymes may have significance in sucrose pool regulation. So that, we can conclude that low amount of trehalose accumulation can alter the soluble carbohydrate pool of the plant.

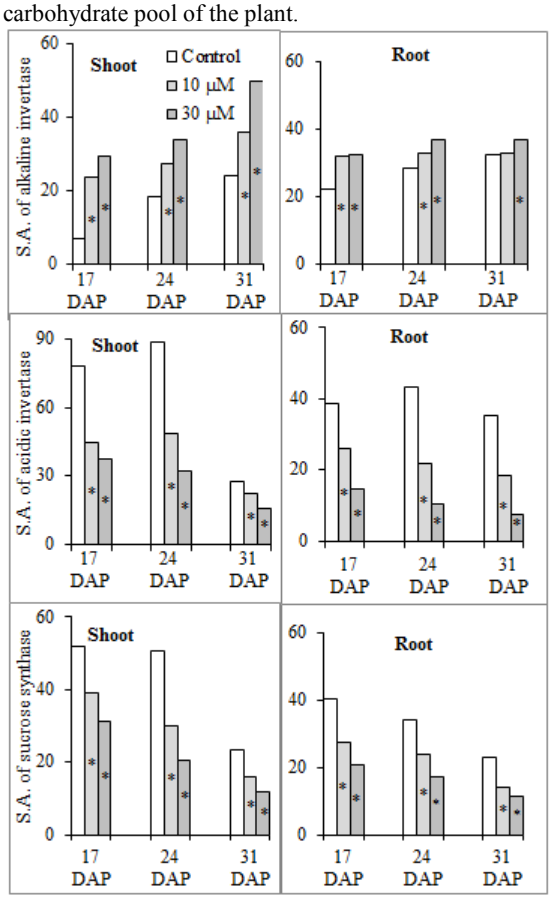

Figure 2. Specific activities of alkaline invertase, acidic invertase and sucrose synthase in shoot and root of *Triticum aestivum* (cv.Sakha 93) at 17, 24 and 31 DAP treated with 10 µM and 30 µM validamycin A. (*) Significantly different from control (P <0.05).

4. Conclusion

This work demonstrates that accumulation of trehalose in wheat plant has considerable potential for raising sucrose and starch contents in shoots. Moreover, the elevation of level of a sucrose degrading enzyme (alkaline inveratse) and decreasing of others (acid invertase and sucrose synthase) suggest that trehalose accumulation plays a role in regulating the sucrose pool.

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References

Adams RP, Kendall E and Kartha KK. 1990. Comparison of free sugars in growing and desiccated plants of *Selaginella lepidophylla*. *Biochem Syst Ecol*. **18**: 107-110.

Aeschbacher RA, Müller J, Boller T and Wiemken A. 1999. Purification of the trehalase *GMTRE1* from soybean nodules and cloning of its cDNA. *GMTRE1* is expressed at a low level in multiple tissues. *Plant Physiol*. **119**: 489-495.

Alizadeh P and Klionsky DJ. 1996. Purification and biochemical characterization of the *ATH1* gene product, vacuolar acid trehalase, from *Saccharomyces cerevisiae*. *FEBS Lett*. **391**: 273-278.

Almeida AM, Villalobos E, Araújo SS, Leyman B, van Dijck P, Alfaro-Cardoso L, Fevereiro PS, Torné JM and Santos DM. 2005. Transformation of tobacco with an *Arabidopsis thaliana* gene involved in trehalose biosynthesis increases tolerance to several abiotic stresses. *Euphytica* **146**: 165-176.

Asano N, Yamaguchi T, Kameda Y and Matsui K. 1987. Effect of validamycins on glycohydrolase of *Rhizoctonia solani.* *J Antibiot*. **40**: 526-532.

Bae H, Herman E and Sicher R. 2005. Exogenous trehalose promotes non-structural carbohydrate accumulation and induced chemical detoxification and stress response proteins in *Arabidopsis thaliana* grown in liquid culture. *Plant Sci*. **168**: 1293-1301.

Bergmeyer HU and Bernt EM. 1974. **Methods of Enzymatic Analysis.** Second ed. HU Bergmeyer. Academic Press. New York.

Brohmann D. 2006. Effects of trehalose on gene expression in *Arabidopsis thaliana* seedlings a genome–wide analysis. PhD thesis. Basel University.

Čižmárilk J, Hroboňová K and Lehotay J. 2004. Determination of monosaccharides and disaccharides in honey by ion-exchange high performance chromatography. *Acta Facultatis Pharmaceuticae Universitatis Comenianae.* **51**: 73-78.

Clark JM and Switzer RL. 1977. **Experimental Biochemistry**. Freeman and Company. New York.

Cortina C and Culiáñez-Macià FA. 2005. Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. *Plant Sci*. **169**: 75-82.

Eastmond PJ, van Dijken A, Spielman M, Kerr A, Tissier A, Dickinson HG, Jones JD, Smeekens SC and Graham IA. 2002. Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. *Plant J*. **29**: 225-235.

El-Bashiti T, Hamamci H, Öktem HA and Yücel M. 2005. Biochemical analysis of trehalose and its metabolizing enzymes in wheat under abiotic stress conditions. *Plant Sci*. **169**: 47-54.

Elbein AD, Pan YT, Pastuszak I and Carroll D. 2003. New insights on trehalose: a multifunctional molecule. *Glycobiology*. **13**: 17R-27R.

Ferreira JC, Paschoalin VMF, PAnek AD and Trugo LC. 1997. Comparison of three different methods for trehalose determination in yeast extracts. *Food Chem*. **60**: 251-254.

Fritzius T, Aeschbacher R and Wiemken A. 2001. Induction of ApL3 expression by trehalose complements the starch-deficient *Arabidopsis* mutant adg2-1 lacking ApL1, the large subunit of ADP-glucose pyrophosphorylase. *Plant Physiol*. **126**. 883-889.

Garg AK, Ranwala AP, Miller WB and WU RJ. 2007. HPLC analysis of trehalose and other soluble carbohydrates from the leaf tissue of *Indica and Japonica* rice varieties. *Rice Genetics Newsletter*. **24**: 44-47.

Ge LF, Chao DY, Shi M, Zhu MZ, Gao JP and Lin HX. 2008. Overexpression of the trehalose-6-phosphate phosphatase gene *OsTPP1* confers stress tolerance in rice and results in the activation of stress responsive genes. *Planta*. **228**: 191-201.

Gomez LD, Baud S, Gilday A, Li Y and Graham I. 2006. Delayed embryo development in the *Arabidopsis* trehalose 6-phosphate synthase 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. *Plant J*. **46**:69-84.

Gracía NAT, Tribarne C, López M, Herrera-Cervera JA and Liuch C. 2005. Physiological implications of trehalase from *Phaseolus vulgaris* root nodules: partial purification and characterization. *Plant Physiol Bioch*. **43**: 355-361.

Grotelueschen RD and Smith D. 1967. Determination and identification of nonstructural carbohydrates removed from grass and legume tissue by various sulfuric acid concentrations, takadiastase, and water. *J Agr Food Chem.* **15**: 1048-1051.

Hesse H and Willmitzer L. 1996. Expression analysis of a sucrose synthase gene from sugar beet (*Beta vulgaris* L.). *Plant Mol Biol*. **30:** 863-72.

Holmström KO, Mäntylä E, Welin B, Mandal A, Palva ET, Tunnela OE and Londesborough J. 1996. Drought tolerance in tobacco. *Nature*. **379**: 683-684.

Inagaki K, Ueno N, Tamura T and Tanaka H. 2001. Purification and characterization of an acid trehalase from *Acidobacterium capsulatum*. *J Biosci Bioeng*. **91**: 141-146.

Jiang W, Fu FL, Zhang SZ, Wu L and Li WC. 2010. Cloning and characterization of functional trehalose -6- phosphate synthase gene in maize. *J Plant Biol*. **53**: 134-141.

Kaasen I, McDougall J and Strøm AR. 1994. Analysis of the *otsBA* operon for osmoregulatory trehalose synthesis in *Escherichia coli* and homology of the *OtsA* and *OtsB* proteins to the yeast trehalose-6-phosphate synthase/phosphatase complex. *Gene*. **145**: 9-15.

Karim S, Aronsson H, Ericson H, Pirhonen M, Leyman B, Welin B, Mäntylä E, Palva ET, van Dijck P and Holmström K. 2007. Improved drought tolerance without undesired side effects in transgenic plants producing trehalose. *Plant Mol Biol*. **64**: 371-386.

Kolbe A, Tiessen A, Schluepmann H, Paul M, Ulrich S and Geigenberger P. 2005. Trehalose 6-phosphate regulates starch synthesis via post-translational redox activationof ADP-glucose pyrophosphorylase. *Pntal Acad Sci USA.* **102**:11118-11123.

Kord MAE, Youssef EA, Ahmed HE and Qaid EA. 2012. Purification and some properties of trehalase from *Triticum aestivum* seeds. Proceedings of the Second international conference of PMEPS. University of Mina. Egypt.

López M, Herrera-Cervera JA, Liuch C and Tejera NA. 2006. Trehalose metabolism in root nodules of the model legume *Lotus japonicus* in response to salt stress. *Physiol Plant*. **128**: 701-709.

López M, Tejera NA and Liuch C. 2009. Validamycin A improves the response of *Medicago truncatula* plants to salt stress by inducing trehalose accumulation in the root nodules. *J Plant Physiol*. **166**: 1218-1222.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951. Protein measurement with the folin phenol reagent. *J Biol Chem.* **193**: 265-275.

Lunn JE, Feil R, Hendriks JH, Gibon Y, Morcuende R, Osuna D, Scheible WR, Carillo P, Hajirezaei MR and Stitt M. 2006. Sugar induced increases in trehalose 6-phosphate are correlated with redox activation of ADP-glucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *Biochem J*. **397**: 139-148.

Miranda JA, Avonce N, Suárez R, Thevelein JM, Van Dijck P and Iturriaga G. 2007. A bifunctional TPS-TPP enzyme from yeast confers tolerance to multiple and extreme abiotic-stress conditions in transgenic *Arabidopsis*. *Planta*. **226**: 1411-1421.

Morell M and Copeland L. 1984. Enzymes of sucrose breakdown in soybean nodules. *Plant Physiol*. **74**: 1030-1034.

Morell M and Copeland L. 1985. Sucrose synthase of soybean nodules. *Plant Physiol*. **78**: 149-154.

Müller J, Aeschbacher RA, Wingler A, Boller T and Wiemken A. 2001. Trehalose and trehalase in *Arabidopsis*. *Plant Physiol.* **125:** 1086-1093.

Müller J, Boller T and Wiemken A. 1995. Effects of validamycin A, a potent trehalase inhibitor, and phytohormones on trehalose metabolism in roots and root nodules of soybean and cowpea. *Planta*. **197**: 362-368.

Müller J, Boller T and Wiemken A. 1998. Trehalose affects sucrose synthase and invertase activities in soybean (*Glycine max* L. Merr.) roots. *J Plant Physiol*. **153**: 255-257.

Müller J, Staehelin C, Mellor RB, Boller T and Wiemken A. 1992. Partial purification and characterization of trehalase from soybean nodules. *J Plant Physiol*. **140**: 8-13.

Murata M, Nagai M, Takao M, Suzuki A, Sakai T and Terashita T. 2001. Purification and characterization of an extracellular acid trehalase from *Lentinula edodes*. *Mycoscience*. **42**: 479 - 482.

Nwaka S and Holzer H. 1998. Molecular biology of trehalose and trehalases in the yeast, *Saccharomyces cerevcesiae*. *Prog Nucleic Acid Res*. **58**: 197-237.

Pellny TK, Ghannoum O, Conroy JP, Schluepmann H, Smeekens S, Andralojc J, Krause KP, Goddijn O and Paul MJ. 2004. Genetic modification of photosynthesis with *E. coli* genes for trehalose synthesis. *Plant Biotechnol J*. **2**: 71-82.

Pilon-Smits EAH, Terry N, Sears T, Kim H, Zayed A, Hwang S, Van Dun K, Voogd E, Verwoerd TC, Krutwagen RWHH and Goddijn O J. 1998. Trehalose-producing transgenic tobacco plants show improved growth performance under drought stress. *J Plant Physiol*. **152**: 525-532.

Qaid EA. 2010. Physiological and biochemical studies for trehalose and trehalse in some higher plants. MSc dissertation. Cairo University, Giza, Egypt.

Reguera M, Peleg Z and Blumwald E. 2012. Targeting metabolic pathways for genetic engineering abiotic stress-tolerance in crops. *Biochim Biophys Acta.* **1819**: 186-194.

Schellenbaum BL, Müller J, Boller T, Wiemken A and Schüepp H. 1998. Effects of drought on non-mycorrhizal and mycorrhizal maize: changes in the pools of non-structural carbohydrates, in the activities of invertase and trehalase, and in the pools of amino acids and imino acids. *New Phytol*. **138**: 59-66.

Schluepmann H, Pellny T, van Dijken A, Smeekens S and Paul M. 2003. Trehalose-6-phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. *Pntal Acad Sci USA.*. **100**: 6849-6854.

Schluepmann H, van Dijken A, Aghdasi M, Wobbes B, Paul M and Smeekens S. 2004. Trehalose mediated growth inhibition of *Arabidopsis* seedlings is due to trehalose 6-phosphate accumulation. *Plant Physiol*. **135**: 879-890.

Schroeven L, Lammens W, Van Laere A and Van den Ende W. 2008. Transforming wheat vacuolar invertase into a high affinity sucrose: sucrose 1-fructosyltransferase. *New Phytol*. **180**: 822-831.

Shimakata T and Minatagawa Y. 2000. Essential role of trehalose in the synthesis and subsequent metabolism of corynomycolic acid in *Corynebacterium matruchotii*. *Acta Biochim Biophys*. **380**: 331-338.

Snedecor GM and Cochran WG. 1989. Statistical Methods. Eight ed. Iowa State University Press.Sprenger N, Bortlik K, Brandt A, Boller T and Wiemken A. 1995. Purification, cloning, and functional expression of sucrose: fructan 6-fructosyltransferase, a key enzyme of fructan synthesis in barley. *Pntal Acad Sci USA*. **92**: 11652-11656.

Taiz L and Zeiger E. 2006. **Plant Physiology**. Fourth ed. Sinauer Associates, USA.

Tang GQ, Lüscher M and Sturm A. 1999. Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. *Plant Cell*. **11**:177-89.

Tao J, Xu XY, Yu J, Cao CY and Liang GH. 2010. Cloning and expression of cell wall acid invertase gene fragment from poinsettia (*Euphorbia pulcherrima* wild). *African J Biotechnology*. **9**: 443- 448.

Wingler A, Fritzius T, Wiemken A, Boller T and Aeschbacher RA. 2000. Trehalose induces the ADP-glucose pyrophosphorylase gene APL3, and starch synthesis in *Arabidopsis*. *Plant Physiol*. **124**: 105-114.

Wolska-Mitaszko B, Molestak E and Malek W. 2005. Properties of trehalase from different organs of alfalfa, *Medicago sativa*. *Acta Physiol Plant*. **27**: 53-60.

Yeo ET, Kwon HB, Han SE, Lee JT, Ryu JC and Byu MO. 2000. Genetic engineenering of drought resistant potato plants by introduction of the trehalose-6-phosphate synthase (*TPS1*) gene from *Saccharomyces cerevisiae*. *Mol Cell*. **10:** 263–268.

Zhang Y, Primavsei LF, Jhurreea D, Andralojc PJ, Mitchell RA, Powers SJ, Schluepmann H, Delatte T, Wingler A and Paul MJ. 2009. Inhibition of SNF-1-related protein kinase 1 activity and regulation of metabolic pathways by trehalose-6-phosphate. *Plant Physiol*. **149**: 1860-1871.

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Response of Three Accessions of Jordanian *Aegilops crassa* Boiss. and Durum Wheat to Controlled Drought

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Abstract

Drought is a major abiotic stress that is threatening the production and the survival of many crops such as cereals. The response of three *Aegilops crassa* accessions (C1, C2 and C3) that inhabit areas with different rates of rainfall and durum wheat to controlled drought (with soil moisture of 50% field capacity) was tested in terms of changes in: relative water content, chlorophyll content, chlorophyll fluorescence and biomass accumulation. At the end of drought treatment, a slightly significant decrease in relative water content (RWC) was shown in two *Ae. crassa* accessions (C2 and C3). RWC of C1 accession and durum wheat showed no change. In all *Aegilops* accessions and durum wheat, the effect of drought on chlorophyll content and chlorophyll fluorescence was minimal. A differential response to drought in terms of biomass accumulation was revealed. *Ae. crassa* C2 and C3 accessions that are adapted to semiarid and arid areas, respectively, showed no significant difference in their biomass under drought stress. The biomass of C1 accession that is adapted to well-watered area was significantly decreased. A highly significant decrease in biomass was also shown in durum wheat. Hence, C2 and C3 accessions of *Ae. crassa* are promising genetic sources for the genetic engineering of drought tolerant wheat plants. Future understanding the molecular basis of how drought-tolerant *Aegilops* species respond to drought stress, can be one of the approaches to improve drought tolerance in wheat.

Keywords: Controlled drought, *Ae. crassa*, durum wheat, biomass, acclimation.

1. Introduction

Being sessile, plants are susceptible to environmental changes. Drought is a major abiotic stress, which challenge crop production and plant survival. Large body of information has been gained from studies of abiotic stress in the model plants Arabidopsis and rice at physiological, biochemical and molecular levels (Ingram and Bartels, 1996; Bartels and Sunkar, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007; Nakashima *et al.,* 2009). However, still a lot need to be learned about the complexity of plant interaction with the surrounding environment.

Drought can be chronic in semiarid and arid areas with low water availability, or random and unpredictable due to weather changes during the growing season. Drought problem is exacerbating, due to global warming and climate change, in addition to the increasing demands of water for many purposes including agriculture. Therefore, studying the response of crop plants to drought will enhance our understanding of this problem. In addition, the screening of natural variations is a promising approach to harness traits that fit the changing environments (Nevo and Chen, 2010).

Wild relatives of cultivated crops that are distributed in a wide climatic range are valuable genetic sources for the improvement of economic crop plants. *Aegilops* (goatgrass) belongs to Poaceae and is well known as the wild relative of wheat. Indeed, bread wheat (*Triticum aestivum*) resulted from the hybridization between wheat and *Aegilops* (Dvorak *et al.,* 1998; Hedge *et al.,* 2000; Faris *et al.,* 2002; Petersen *et al.,* 2006). Hence, *Aegilops* is considered as the progenitor of wheat. Twenty-three *Aegilops* species were identified (Kilian *et al.,* 2011). Eleven species are diploids and 12 are allopolyploids. *Aegilops* plants exist with different types of genomes: A, B, D and G (Kimber and Feldman, 1987). *Aegilops* species are distributed in southwestern Asia. *Aegilops* is considered as a Mediterranean plant (Hegde *et al.,* 2002). It was shown that *Aegilops* species inhabit warm areas with short winter and hot and dry summer (Baalbaki *et al.,* 2006).

Ae. crassa is distributed in different parts of Asia: Iran, Iraq, Afghanistan, Kazakhstan, Kyrgyzstan, Syria, Turkmenisatn, Usbekistan, Tajikistan, Turkey, Jordan and Lebanon (Kilian *et al.,* 2011). *Ae. crassa* is considered

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drought tolerant species because it grows in areas with 150 - 350 mm rainfall (Kilian *et al.,* 2011).

At all stages of their development, plants are exposed to different environmental stresses. Both the developmental stage and the severity of drought were found to play crucial role in plant response to stress (Blum *et al.,* 1980; Harb *et al.,* 2010). No correlation was found between the response of wheat grains and the photosynthesizing seedling when exposing these stages to osmotic stress induced by polyethylene glycol (PEG) (Blum *et al.,* 1980). Therefore, one cannot extrapolate results about stress response from one developmental stage to another, which necessitates independent studies of stress response for each developmental stage.

The performance of *Aegilops* species under soil drought were evaluated in different areas (Farooq and Azam, 2001; Baalbaki *et al.,* 2006; Colmer *et al.,* 2006). Response of some Lebanese *Aegilops* species and accessions to drought was tested; *Ae. geniculata* and *Ae. markgrafii* were found to be the most drought tolerant species (Baalbaki *et al.,* 2006). In another study, utilizing gas exchange parameters (photosynthesis and stomatal conductance) to evaluate drought tolerance in some Hungarian *Aegilops* species indicated that *Ae. tauschi* and *Ae. speltoides* were shown to be drought tolerant. These two species showed high stomatal conductance and CO_2 fixation at low water potential, which was measured in terms of plant relative water content (RWC) (Molnar *et al.,* 2004). Biochemical evaluation of drought response of two accessions of *Ae. biuncialis* revealed different strategies of drought tolerance (Czovek *et al.,* 2006). All previous studies demonstrate the potential of *Aegilops* species and accessions as rich reservoir for stress resistance genes towards wheat improvement (Schneider *et al.,* 2008). Indeed, screening *Aegilops* plants for abiotic stress resistance is the first step in the mining process for stress responsive genes towards the improvement of wheat for high yield under stress conditions.

In the present study, acclimation efficiency of *Ae. crassa* accessions collected from areas that differ in the rate of rainfall under controlled drought compared to that of durum wheat was tested. Acclimation efficiency was evaluated in terms of biomass accumulation at the end of drought treatment.

2. Materials and Methods

2.1. Plant Material

Grains of three *Ae. crassa* accessions were collected from areas with different rainfall rate in Jordan during the summer of 2011 (Table 1). One accession (C1) is grown in area that is considered well-watered with rainfall 300 - 600 mm. The second one (C2) is grown in a semiarid area with rainfall 150 - 300 mm. The third accession (C3) is grown in an arid area with rainfall 50 - 200 mm. Grains were harvested and properly stored. In addition, grains of durum wheat (*Triticum durum* Desf. cv. Haurani 27) were provided by the National Center for Agriculture Research and Extension (NCARE)/Jordan.

2.2. Plant Growth Conditions

Grains of *Ae. crassa* and durum wheat were surface sterilized by 5% Chlorox and washed with distilled water for several times. Sterile grains were kept in 9 cm plates on wet filter paper. Plates were wrapped with aluminum foil and kept for 5 days at 4ºC for dormancy breakage. After that, grains were kept to germinate at 25ºC. After 2 days of germination, seedlings were transferred to pots of capacity of 150 g filled with soil mix of 1:1:2 (loam: peatmoss: sand). Seedlings were kept to grow under six white fluorescent lamps for 16 hrs and temperature of 20 ± 2ºC.

2.3. Drought Treatment

At 2-leaf stage, plants were grouped into 8 plants of the control (well-watered 100% Field capacity (FC)) and 8 plants of the drought-treated (50% FC). Watering was withheld for the drought treated group. Soil water moisture was monitored until it reached 50% FC. After that, pots were weighed daily and soil water content was adjusted to final weight based on calculation using EXCEL software. Final weight that gives 50% FC was calculated as follows:

FC = (Wet soil wt - Dry soil wt)/ (Dry soil wt - pot wt)*100

50% FC = 0.5 * FC

Wt of soil and pot at 50% FC = ((1 + 50 % FC)* Dry soil wt) - (50% FC * pot wt)) (Harb *et al*., 2010)

Table 1. Areas of collection of *Ae. crassa* with their geographic location and bioclimate

Ae. crassa accession	Area	Longitude (East)	Latitude (North)	Bioclimate
C1	Nuya'ma	42º 03' 74"	61º 12' 47"	Mediterranean; Alttidues range from 700 - 1750 m, rainfall ranges from 300 - 600 mm. The dominant soil types are: the red Mediterranean soil (terra rosa) and the yellow Mediterranean soil (rendzina). This region is the most fertile part of Jordan with 90% of the kingdom's poulation.
C2	Jordan University of Science and Technology (JUST)	40º 45' 77"	59º 61' 14"	Irano-Turanian; Alttidues range from 500 - 700 m, rainfall ranges from 150 - 300 mm. Soils are mostly calcareous.
C3	Almafraq	38º 18' 59.66"	32º 25' 6.96"	Saharo-Arabian; Altitude ranges between 500–700 m. The mean annual rainfall ranges from 50–200 mm. Soil is mostly poor, either clay, hammada, saline, sandy or calcareous.

2.4. Determination of Relative Water Content (RWC)

After 10 days of 50% FC drought treatment, segments of the third fully expanded leaves of 6 drought-treated and 6 well-watered plants were taken and their fresh weight (FW) was measured. After that, they were soaked in water and kept in water at 4ºC for 16 hrs. After soaking, segments were weighed to determine their turgid weight (TW). Then, segments were dried at 80ºC for 2 days and their dry weights (DW) were measured. RWC was calculated as follows:

RWC% = (FW- DW)/ (TW - DW)*100

2.5. Determination of Cut Leaf Water Loss (CLWL)

After 10 days of 50% FC drought treatment, the second leaves were excised from 6 drought-treated and 6 well-watered plants, and their fresh weight was measured. Leaves were kept to dehydrate under growth room conditions (Temperature of 20 ± 2ºC) for 2 hrs and then their weight was measured. Dry weight was measured after drying plant samples in oven at 80ºC for 2 days. CLWL was calculated as follows:

CLWL% = (W0-Wt)/ (W0-Wd)*100

W0: Initial weight (fresh weight)

Wt: Weight after dehydration period (2 hrs in this experiment)

Wd: Dry weight

2.6. Chlorophyll Quantification

Samples from the third fully expanded leaves of 5 drought-treated and 5 well-watered plants were used for the quantification of chlorophyll after 10 days of 50% FC drought treatment. Chlorophyll (Chl) was extracted in 80% acetone solution. Absorbance was determined at 663 nm and 645 nm and Chl a, Chl b and total Chl were calculated as shown in the following equations:

mg Chl a/ g fresh tissue wt = (12.7*A663 - 2.69 *A645)* V/1000 * wt

mg Chl b/ g fresh tissue wt = (22.9*A645 - 4.68*A663)* V/1000 * wt

mg total Chl / g fresh tissue wt = (20.2*A645 + 8.02 *A663)* V/1000 * wt

V: final volume of 80% acetone used in the extraction of chlorophyll.

2.7. Chlorophyll Fluorescence Measurements

The quantum efficiency of photosystem II (YII) was determined using OS1p chlorophyll fluorometer (Opti-Science, USA). Measurements were made on the youngest fully expanded leaves of the well-watered control and the drought-treated (50% FC) plants. YII was measured for 8 plants per treatment per accession.

2.8. Fresh Weight and Dry Weight Determination

After 10 days of 50% FC drought treatment, 3 – 8 plants of the treated and the control groups were harvested and their fresh weight was measured. After that, they were kept into an oven to dry at 80ºC for 2 days and their dry weights were measured.

2.9. Statistical Analysis

All data were analyzed by Student's - T test. Differences with *p*-value less than 0.05 were considered significant.

3. Results

3.1. Changes in Relative Water Content in Response to Drought

The effect of drought treatment (Fig. 1) on the water status of plants in terms of relative water content (RWC) was diagramed. No significant differences occurred in RWC between the drought-treated and the well-watered control in each of *Ae. crassa* accession C1 and durum wheat (P = 0.3 and 0.6, respectively) (Fig. 2A). However, a significant decrease (P=0.04) in RWC was shown in leaves of C2 and C3 of *Ae. crassa* accessions (Fig. 2A).

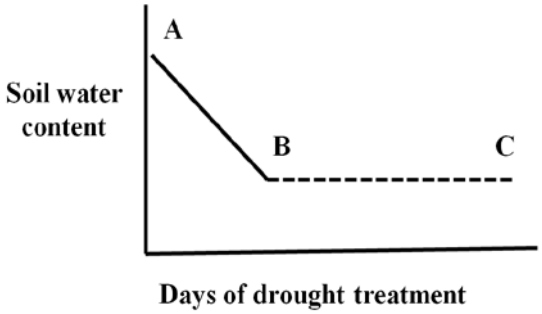

Figure 1. Schematic illustration of the drought treatment. A. Watering was withheld at two true leaf stages. B. Soil water content reached 50% of field capacity (FC). Then, pots were weighed daily and their weights were adjusted to keep soil water content 50% FC for 10 days. C. Drought treatment was terminated and plants were harvested for the physiological, biochemical and morphological analyses.

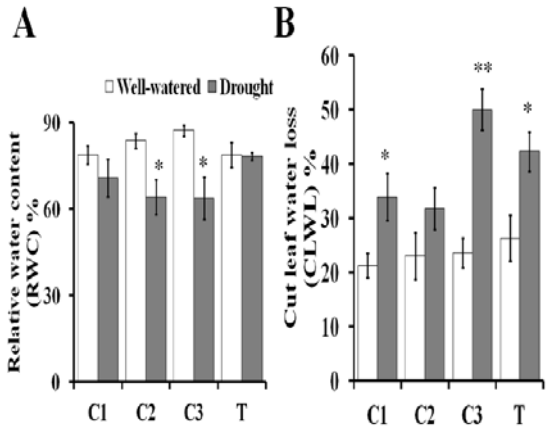

Figure 2. Physiological response of plants to drought. A. Relative water content (RWC) of *Ae. crassa* accessions (C1, C2 and C3) and durum wheat (T) under drought compared to the well-watered control. B. Cut leaf water loss (CLWL) of *Ae. crassa* accessions (C1, C2 and C3) and durum wheat (T) under drought compared to the well-watered control. * represents significant difference ≤ 0.05 and ** represents significant difference ≤ 0.01.

3.2. The Effect of Drought Pretreatment on the Cut Leaf Water Loss under Dehydration

To test the effect of controlled drought pretreatment on water loss under dehydration, The cut leaf water loss (CLWL) was determined for all accessions under two treatments (drought and well-watered). *Ae. crassa* accession C1 and durum wheat plants that were drought-treated have significantly higher water loss than the well-watered control (P =0.04 and 0.02, respectively) (Fig. 2B). Drought-treated plants of *Ae. crassa* accession C3 showed a highly significant water loss compared to the well-watered (P = 0.0007) (Fig. 2B). No significant change in CLWL appeared in *Ae. crassa* accession C2 (P = 0.2) (Fig. 2B).

3.3. Chlorophyll Content in Response to Drought

It is known that chlorophyll quantity decreases under drought treatment and this effect is correlated with the severity of drought. To test the effect of controlled moderate drought on chlorophyll quantity, chlorophyll content of the drought-treated and the well-watered plants was determined. For all *Ae. crassa* accessions and durum wheat, no significant differences in chlorophyll a and b and the total chlorophyll content between the drought-treated and the well-watered were shown (Table 2).

Table 2. Chlorophyll a, b and total chlorophyll content (µg/ g fresh weight) of *Ae. crassa* accessions (C1, C2 and C3) and durum wheat under drought compared to the well-watered control.

Plant Genotype *Ae. Crassa*	Control			Drought		
	Chl a	Chl b	Total Chl	Chl a	Chl b	Total Chl
C1	2.35 ± 0.53	1.41 ± 0.30	3.75 ± 0.82	2.23 ± 0.64	1.33 ± 0.42	3.55 ± 1.06
C2	1.98 ± 0.81	1.42 ± 0.60	2.10 ± 0.70	1.88 ± 0.16	1.33 ± 0.18	3.21 ± 0.31
C3	2.37 ± 0.54	1.24 ± 0.23	3.61 ± 0.78	1.41 ± 0.24	0.74 ± 0.12	2.16 ± 0.34
T. durum	2.00 ± 0.24	1.33 ± 0.30	3.33 ± 0.45	3.10 ± 0.80	1.45 ± 0.33	3.44 ± 0.33

Values are the means ± standard error (n = 4 – 5).

3.4. The Effect of Drought on Chlorophyll Fluorescence

Under drought stress, the efficiency of photosystem II (PSII) is decreased depending on the nature and the severity of drought treatment. Therefore, the quantum efficiency of PSII (YII) of the drought-treated and the well-watered plants was measured. For all accessions, there was no significant difference in YII between the drought-treated and the well-watered control (Fig. 3). However, under drought stress YII of *Ae. crassa* accession C3 was significantly higher than that of C1 accession and durum wheat (P = 0.02 and 0.04, respectively) (Fig. 3).

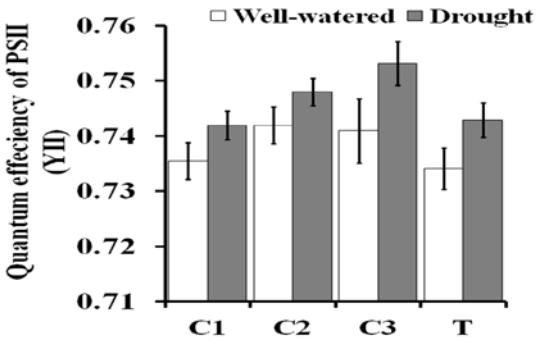

Figure 3. The effect of drought on the quantum efficiency of PSII (YII) of *Ae. crassa* accessions (C1, C2 and C3) and durum wheat (T) under drought compared to the well-watered control.

3.5. Changes in Fresh and Dry Weight under Drought

The outcome of drought stress will be manifested as a change in plant's biomass. Therefore, fresh and dry weights of plants were measured for the drought-treated and the well-watered plants. Both fresh weight and dry weight of *Ae. crassa* accession C1 were significantly reduced (P= 0.01 and 0.03, respectively). For C2 accession, the reduction in fresh weight in response to drought was highly significant, whereas no significant change was shown in the dry weight (P= 0.009 and 0.12, respectively). Plants of C3 accession showed no significant change in fresh and dry weight (P= 0.1 and 0.2, respectively). In durum wheat, the reduction in fresh and dry weight was highly significant in the drought-treated plants compared to the well-watered ones (P= 0.01 and 0.0008, respectively) (Fig. 4).

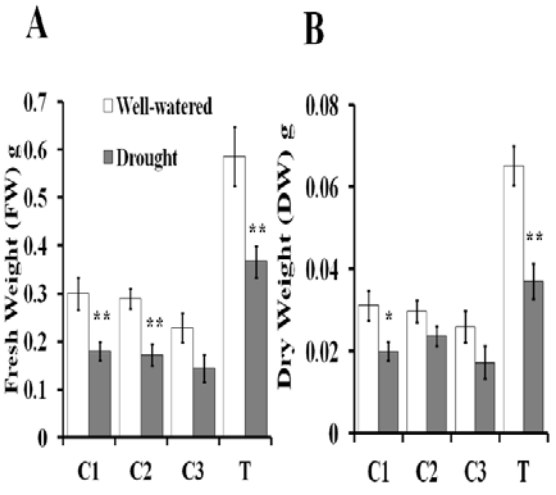

Figure 4. The effect of drought on plants growth. A. Fresh weight (FW) in grams of *Ae. crassa* accessions (C1, C2 and C3) and durum wheat (T) under drought compared to the well-watered control . B. Dry weight (DW) in grams of *Ae. crassa* accessions (C1, C2 and C3) and durum wheat (T) under drought compared to the well-watered control. * represents significant difference ≤ 0.05 and ** represents significant difference ≤ 0.01.

Discussion

Plant water status is a good indicator of plant performance under drought stress. In two maize hybrids, progressive drought for 9 days resulted in a significant decrease in RWC of the two hybrids (Medici *et al*., 2003). In four bread wheat cultivars, rainfed plants showed reduced RWC compared to the irrigated control (Sarker *et al*., 1999). In *Aegilops* and wheat, severe drought resulted in a significant reduction in RWC (Molnar *et al*., 2004; Keyvan, 2010). In one accession of *Ae. bicornis* adapted to area with rainfall of 75-275 mm, progressive drought resulted in slow decrease in RWC, which led to a fast stomatal closure and reduction of photosynthetic efficiency (Dulai *et al*., 2006). In the latter study, two other *Aegilops* species adapted to arid regions (*Ae. tauschii* and *Ae. speltoides)* showed differential response to progressive drought in terms of change in RWC. Their RWC was drastically decreased, but they kept high stomatal conductance even at 70% RWC. In the present study, *Aegilops* and wheat plants showed differential response to controlled moderate drought. Two *Ae. crassa* accessions C2 and C3 slightly decreased their RWC under moderate drought, whereas C1 accession and durum wheat showed no change in their RWC. Reduction of RWC under drought stress could be drought tolerance strategy to alleviate the detrimental effect of drought on plant's growth (Price *et al*., 2002). Reduction of RWC increases the internal osmotic potential, and consequently protects plants against water loss. This is consistent with the strategy adopted by one accession of *Ae. biuncialis* that inhabits areas with 550 mm annual rainfall (Molnar *et al*., 2004). In this accession of *Ae. biuncialis*, the reduction in RWC did not affect its performance under mild and severe osmotic stress. Indeed, it showed a better performance under stress conditions in terms of CO_2 assimilation and biomass compared to accessions adapted to areas with high rainfall rate. Moreover, progressive drought under greenhouse conditions resulted in the reduction of the RWC of this accession without affecting its yield (Molnar *et al*., 2004).

The response of plants to dehydration can be by reducing water loss through stomatal closure to avoid the detrimental effects of dehydration, or by tolerating low internal water content because of fast water loss (Levitt, 1980). In this study, CLWL of *Ae. crassa* accession C1 and C3 and durum wheat was significantly increased in the drought-treated plants compared to the well-watered ones. This is inconsistent with a study in natural accessions of *Arabidopsis* as for many accessions no change in CLWL between the two treatments was shown while other accessions reduced their CLWL under drought treatment as a strategy to avoid drought stress (Bouchabke *et al*., 2008).

The effect of drought on plant photosynthesis can be shown as a decrease of CO_2 diffusion due to stomatal closure, or by impeding metabolites regeneration during photosynthesis (Prasad *et al*., 2008). Chlorophyll fluorescence as a method of evaluation of plants response to drought stress is well known (Sayed, 2003). It was used for the evaluation of the performance of many crops: barley, oat, rice, sorghum and maize under different environmental stresses including drought (Sayed, 2003). Drought stress that led to RWC of 40% had no effect on dark and light adapted PSII activity in tomato and potato plants (Havaux, 1992). In two accessions of *Ae. biuncialis*, osmotic stress of -1.8 MPa resulted in a significant decrease in the quantum efficiency of PSII (YII) (Molnar *et al*., 2004). This decrease in YII did not affect the biomass of these accessions, whereas for the third accession the biomass was decreased without any change in its YII. In this study moderate controlled drought did not affect the quantum efficiency of PSII, but a significant increase in YII was shown in *Ae. crassa* accession C3 under drought compared to durum wheat. This might suggest a mechanism adopted by this accession to keep almost normal growth under drought stress.

Environmental stresses such as drought affect chlorophyll synthesis and consequently chlorophyll content in plants. In a study on 157 accessions of *Ae. geniculata*, some accessions reduced their chlorophyll content when exposed to drought stress in the field (Zaharieva *et al*., 2001). This was explained as a mechanism to alleviate the negative effect of high energy from sunlight. Moreover, a positive correlation was found between chlorophyll content and plant biomass (Zaharieva *et al*., 2001). Drought-treated plants of the three *Ae. crassa* accessions (C1, C2 and C3) and durum wheat showed no change in Chl a, Chl b and total chlorophyll content compared to the well-watered plants. This suggests that the drought treatment was not severe enough to inhibit chlorophyll synthesis. Indeed, exposing different cultivars of durum wheat to severe drought under field conditions resulted in the reduction of their chlorophyll content (Talebi, 2011). Moreover, progressive drought for 7 days led to a decrease in chlorophyll content of three cultivars of bread wheat (Nikolaeva *et al*., 2010). The effect of drought on chlorophyll content was found to be developmental stage dependent (Keyvan, 2010). In bread wheat, drought imposed at earlier reproductive stage resulted in higher decrease of chlorophyll content compared to that imposed at later stages (Keyvan, 2010).

Plant growth occurs by two processes: cell division and cell expansion. It was found that cell expansion is more sensitive to drought stress than cell division (Prasad *et al*., 2008). Cell division and cell expansion are sensitive to mild drought before any noticeable change in photosynthesis (Prasad *et al*., 2008). In plants, the effect of drought is manifested as a reduction in biomass. In different *Aegilops* species, two levels of drought stress (moderate and severe) resulted in a significant reduction in biomass (Baalbaki *et al.*, 2006).

Different drought regimes: progressive and controlled moderate drought showed differential morphological, physiological, biochemical and molecular changes in *Arabidopsis* plants (Harb *et al*., 2010). Multiphasic effect was shown when *Arabidopsis* plants were exposed to controlled moderate drought (Harb *et al*., 2010). At early priming phase, most of the physiological, biochemical and molecular changes take place. This phase is followed by intermediate phase during which plants are preparing to acclimatize to drought stress. At late acclimation stage, plants reach new homeostasis and are acclimated to

drought stress. In this study, *Ae. crassa* accessions and durum wheat were exposed to controlled moderate drought. At the end of drought treatment, morphological, physiological and biochemical parameters were evaluated. This phase might be the same as the late acclimation phase shown in *Arabidopsis*, at which plants are already acclimated to drought stress with new homeostasis. At this phase a few physiological, biochemical and molecular changes can be captured compared to the early priming phase. This might explain the minimal effect of drought treatment on RWC, chlorophyll content and chlorophyll fluorescence. The effect of drought on biomass is accumulative, so a substantial change in biomass will be shown at the end of drought treatment.

The two accessions of *Ae. crassa* (C2 and C3) are naturally adapted to semiarid and arid habitats, respectively. This may explain the minimum effect of drought stress obtained on these accessions compared to C1 accession that is adapted to well-watered habitat and durum wheat. Therefore, *Ae. crassa* C2 and C3 accessions are promising genetic sources for the genetic engineering of drought tolerant wheat plants.

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References

Baalbaki R, Hajj-Hassan N and Zurayk R. 2006. *Aegilops* species from semiarid areas of Lebanon: variation in quantitative attributes under water stress. *Crop Sci* **46**: 799-806.

Bartels D and Sunkar R. 2005. Drought and salt tolerance in plants. *Critical Reviews in Plant Sci* **24**:23-58.

Blum A, Sinemina B and Ziv D. 1980. An evaluation of seed and seedling drought tolerance screening tests in wheat. *Euphytica* **29**: 727-736.

Bouchabke O, Chang F, Simon M, Voisin R, Pelletier G and Durand-Tardif M. 2008. Natural variation in *Arabidopsis thaliana* as a tool for highlighting differential drought responses. *PLoS ONE* **3**: e1705.

Colmer T, Flowers T and Munns R. 2006. Use of wild relatives to improve salt tolerance in wheat. *J Experimental Bot*. **57**:1059-1078.

Czovek P, Kiraly I, Paldi E, Molnar I and Gaspar L. 2006. Comparative analysis of stress tolerance in *Aegilops accessions* and *Triticum* wheat varieties to detect different drought tolerance strategies. *Acta Agronomica Hungarica* **54**: 49-60.

Dulai S, Molnar I, Pronay J, Csernak A, Tarnai R and Molnar-Lang M. 2006. Effects of drought on photosynthetic parameters and heat stability of PSII in wheat and in *Aegilops* species originating from dry habitats. *Acta Biologica Szegediensis* **50**: 11-17.

Dvorak J, Luo M, Yang Z and Zhang H. 1998. The structure of the *Aegilops tauschii* gene pool and the evolution of hexaploid wheat. *Theoretical and Applied Genetics* **97**:657-670.

Faris J, Friebe B and Gill B. 2002. Wheat genomics: Exploring the polyploid model. *Current Genomics* **3**: 577-591.

Farooq S and Azam F. 2001. Co-existence of salt and drought tolerance in *Triticea*e. *Hereditas* **135**: 205-210.

Harb A, Krishnan A, Ambavaram M and Pereira A. 2010. Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. *Plant Physiol* **154**: 1254-1271.

Havaux M. 1992. Stress tolerance of photosystem II *in vivo*, antagonistic effects of water, heat and photoinhibition stresses. *Plant Physiol* **100**: 424-432.

Hedge S, Vaalkoun J and Waines J. 2000. Genetic diversity in wild wheats and goat grass. *Theoretical Applied Genetics* **101**: 309-316.

Hegde S, Valkoun J and Waines J. 2002. Genetic diversity in wild and weedy *Aegilops*, *Amblyopyrum* and *Secale* Species—A preliminary survey. *Crop Sci* **42**: 608-614.

Ingram J and Bartels D. 1996. The molecular basis of dehydration tolerance in plants. *Annual Reviews of Plant Physiol and Plant Molecular Biol* **47**: 377-403.

Keyvan S. 2010. The effects of drought stress on yield, relative water content, proline, soluble carbohydrates and chlorophyll of bread wheat cultivars. *J Animal and Plant Sci* **8**: 1051-1060.

Kilian B, Mammen K, Millet E, Sharma R and Graner A. 2011. *Aegilops*. In: Kole C (Ed). **Wild Crop Relatives: Genomics and Breeding Resources: Cereals**. Springer-Verlag, Berlin, Germany, p: 1-76.

Kimber G and Feldman M. 1987. **Wild Wheat: An Introduction**. College of Agriculture, University of Missouri-Columbia, USA.

Levitt J. 1980**. Responses of Plants to Environmental Stresses. Vol 2. Water, Radiation, Salt and other Stresses**. Academic Press, New York, USA.

Medici L, Machado A, Azevedo R and Pimemtel C. 2003. Glutamine synthetase activity, relative water content and water potential in maize submitted to drought. *Biologia Plantarum* **47**: 301-304.

Molnar I, Gaspar L, Sarvar E, Duli S, Huffmann B, Molnar-Lang M and Galiba G. 2004. Physiological and morphological responses to water stress in *Aegilops biuncialis* and *Triticum aestivum* genotypes with differing tolerance to drought. *Functional Plant Biol* **31**: 1149-1159.

Nakashima K, Ito Y and Yamaguchi-Shinozaki K. 2009. Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. *Plant Physiology* **149**: 88-95.

Nevo E and Chen G. 2010. Drought and salt tolerances in wild relatives for wheat and barley improvement. *Plant Cell Environment* **33**: 670-685.

Nikolaeva M, Maevskaya S, Shugaev A and Bukhov N. 2010. Effect of drought on chlorophyll content and antioxidant enzyme activities in leaves of three wheat cultivars varying in productivity. *Russian J Plant Physiol* **57**: 87-95.

Petersen G, Seberg O, Yde M and Berthelse K. 2006. Phylogenetic relationships of *Triticum* and *Aegilops* and evidence for the origin of the A, B and D genomes of common wheat (*Triticum aestivum*). *Molecular Phylogenetics and Evolution* **39**: 70-82.

Prasad P, Staggenborg S and Ristic Z. 2008. Impacts of drought and/or heat stress on physiological, developmental, growth and yield processes of crop plants. In: Ahuja L, Reddy V, Saseendran S and Yu Q (Eds). **Response of Crops to Limited Water: Understanding and Modeling Water Stress Effects on Plant**

Growth Processes, American Society of Agronomy (ASA), Crop Science Society of America (CSSA), Soil Science Society of America (SSSA), Madison, USA., p: 301-355.

Price AH, Cairns JE, Horton P, Jones HG and Griffiths H. 2002. Linking drought-resistance mechanisms to drought avoidance in upland riceusing a QTL approach: progress and new opportunities to integrate stomatal and mesophyll responses. *J Experimental Botany* **53**: 989–1004.

Sarker A, Rahman M and Paul N. 1999. Effect of soil moisture on relative leaf water content, chlorophyll, proline and sugar accumulation in wheat. *Crop Sci* **183**: 225-229.

Sayed O. 2003. Chlorophyll fluorescence as a tool in cereal crop research. *Photosynthetica* **41**: 321-330.

Schneider A, Molnar I and Molnar-Lang M. 2008. Utilisation of *Aegilops* (goatgrass) species to widen the genetic diversity of cultivated wheat. *Euphytica* **163**: 1-19.

Shinozaki K and Yamaguchi-Shinozaki K. 2007. Gene networks involved in drought stress response and tolerance. *J Experimental Botany* **58**: 221-227

Talebi R. 2011. Evaluation of chlorophyll content and canopy temperature as indicators for drought tolerance in durum wheat (*Triticum durum* Desf.). *Australian J Basic and Applied Sci* **5**: 1457-1462.

Zaharieva M, Gaulin E, Havaux M, Acevedo E and Monneveux P. 2001. Drought and heat responses in the wild wheat relative *Aegilops geniculata* Roth.: Potential interest for wheat improvement. *Crop Sci* **41**: 1321-1329.

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Effects of Cigarette Smoking on Some Immunological and Hematological Parameters in Male Smokers in Erbil City

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Abstract

The present study was done to investigate the effect of cigarette smoking (CS) on some immunological and hematological parameters in Erbil city. The study is carried out on fifty male smokers, who smoked at least 10 cigarettes per day for at least10 years. Depending on the age of the smokers, they were divided into two groups. The first group includes smokers with age range between 25-35 years, and the second group includes smokers with age range between 36-45 years. Two control (non-smokers) groups were collected with the same range of age for statistical comparison. The results of the study revealed a significant increase of interferon-gamma (IFN-γ) level in both age groups when compared with their controls. A significant decrease of the total immunoglobulin A (IgA) level was recorded in both age groups when compared with their control. Furthermore the level of malondialdehyde (MDA) which is an indicator of lipid peroxidation (LPO) and oxidative stress significantly increased in cigarette smokers in both age groups when compared with control groups. While the level of Carbohydrate Antigen 19-9 (CA 19-9 Ag) increased in the age group 25-35 years, but this increase is not significant when compared with its control group; the level of CA 19-9 Ag significantly increased in age group 36-45 years when compared with its control group. Moreover the results revealed a significant increase in the total white blood cells (WBC), Neutrophil, Eosinophil, Basophil, Monocyte, and Lymphocyte count in both age groups when compared with their control groups. However, the basophil in the first group (25-35 years), the increase was not significant. While the number of platelets count did not statistically change in both age groups.

Keywords: Cigarette Smoking, IFN-γ, IgA, Hematology

1. Introduction

Smoking is one of the most common addictions of modern times. It has been implicated as an etiological agent for various chronic diseases, including a variety of infections, cancers, heart diseases, and respiratory illnesses such as chronic obstructive pulmonary disease (COPD), that have impairment in the balance between cell growth and cell death, which, put together, are the leading causes of morbidity and mortality in today's society (Zhong *et al.,* 2008; Mehta *et al.,* 2008). Unless current smoking patterns are reversed, the World Health Organization (WHO) estimates that, by the decade 2020-2030, tobacco will be responsible for 10 million deaths per year, with 70% of them occurring in developing countries (WHO, 2001; Suriyaprom *et al.,* 2007).

Cigarette tobacco smoke contains over 4000 compounds, including at least 200 toxicants, 80 known or suspected carcinogens, large quantities of oxidants and free radicals that induce oxidative stress, oxidative lung injury and apoptosis (Zhong *et al*., 2008; Soldin *et al.,* 2011).

Smoking generates many toxic and carcinogenic compounds harmful to the health, such as nicotine, nitrogen oxides, carbon monoxide, hydrogen cyanide, and free radicals (Hoffmann *et al.,* 2001). Smoking is associated with increased oxidative stress and exerts an inflammatory stimulus on lung macrophages which may, like bacterial and viral infection, result in the production of free radicals and the inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), IFN-□ and tumor necrosis factor α (TNF-α); these may be the precursors to the diseases associated with smoking (Francus *et al.,* 1992; Takajo *et al.,* 2001).

Chronic inhalation of CS alters a wide range of immunological functions, including innate and adaptive immune responses. It has been speculated that many of the health consequences of chronic inhalation of CS might be due to its adverse effects on the immune system. The possibility that the increased prevalence of diseases that are associated with CS might, in part, be due to tobacco-smoke-induced changes in the immune and inflammatory processes, was recognized first in the 1960s (Sopori, 2002).

Cigarette smoke (CS) affects a wide range of immunological functions in humans and experimental

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animals, including both the humoral and cell-mediated immune responses. It is postulated that this increased susceptibility reflects cigarette smoke-induced impairment of the immune system (Sopori and Kozak, 1998; Kalra *et al.,* 2000).

The acute effects of CS on markers of oxidative stress have been analyzed in exhaled air, broncho-alveolar lavage fluid (BALF) and blood, even though that, in chronic smoking, the numbers of neutrophils are increased in the blood and BALF. In addition, CS causes an acute inflammatory reaction characterized by the accumulation of neutrophils and macrophages in the membranous bronchioles and alveoli of the lungs, leading to destruction of the peribronchiolar alveolar attachments and eventually pulmonary dysfunction (Sopori, 2002).

Interferon-gamma (IFN-γ) dimerized soluble cytokine that is the only member of the type II class of IFNs (Abbas and Andrew, 2005). This IFN was later called macrophage-activating factor, a term now used to describe a larger family of proteins to which IFN-γ belongs. IFN-γ is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. Aberrant IFN-γ expression is associated with a number of autoinflammatory and autoimmune diseases. The importance of IFN-γ in the immune system stems, in part, from its ability to inhibit viral replication directly, and most importantly from its immunostimulatory and immunomodulatory effects. IFN-γ is produced predominantly by natural killer (NK) as part of the innate immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once Ag-specific immunity develops (Schoenborn and Wilson, 2007).

Secretory IgA (sIgA) is the main Ig isotype mediating humoral immunity in human secretions at luminal sites including oral, gastrointestinal, respiratory passages as well as in the eye. sIgA production is triggered after translocation of Ag from the lumen to mucosa-associated lymphoid tissue. The presence of IgG Abs at the mucosal surface may be protective and it has been show that IgG2 subtype predominates. IgG in secretions is derived from serum or mucosa. Studies show tobacco smoke impacts both the systemic and mucosal immunity with changes demonstrated in mucosal Ab production and systemic Ab production. This includes passive smoking, which increases the risk of respiratory disease (Bouvet *et al.,* 2002).

CA 19-9 is biochemically identified as 36 kD sialyl derivative of lacto- N-fucopentaose II, hapten of human Lewis blood group carbohydrate antigenic determinant occurring as monosialoganglioside/glycolipid in tissue or as circulating mucin with a molecular weight >106 D in serum (Lamerz, 1999; Rottenberg *et al.,* 2009). This carbohydrate Ag is expressed in bronchiolar epithelial cells and found in bronchoalveolar lavage in patients with pulmonary fibrosis. Purified CA19-9 stimulated neutrophil chemotaxis to C5a and IL-8. Normally, CA19-9 may be found in healthy individuals and it increases in benign hepatobiliary disease, with the highest levels in excretory ductal pancreatic adenocarcinoma, biliary, hepatocellular and cholangiocarcinoma cancer. CA19-9 is associated as a tumor marker with metastatic disease in pancreatic cancer, colorectal cancer, biliary cancer, urothelial cancer and melanoma. Neoplasm transformation is induced by high expression of CA19-9. Extravasations of tumor cells from the bloodstream and formation of metastatic disease were associated with CA19-9. The mechanism is probably due to its interaction with E-selectin expressed on endothelial cells (Miki *et al.,* 1995; Rottenberg *et al*., 2009).

Oxidative stress is implicated in CS-induced airway diseases, such as COPD (Rahman and MacNee, 1999). It is suggested that oxidative stress is an important trigger in the up regulation of pro-inflammatory genes (Van den Berg *et al.,* 2001)

Cigarette smoke (CS) contains numerous oxidants that have the capability of interacting with various biomolecules to cause adverse biological effects. Some of the more prominent targets of CS include DNA, RNA, lipids, amino acids, proteins, dietary antioxidants and various endogenously synthesized biomolecules such as glutathione and α1-antiprotease (Traber *et al.,* 2000; Bruno, 2004).

Exposure to CS causes cellular oxidative stress, a key feature in smoking-induced lung inflammation (Rahman, 2003). Oxidative stress can enhance nuclear factor (NF-κB) DNA binding activity (Schreck *et al*., 1991). NF-κB is a critical transcription factor regulating many cytokines, including IL-8, IL-6, TNF-α, granulocyte–macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1, and macrophage chemotactic protein-1 (MCP-1). Enhanced NF-κB activation has been shown in bronchial biopsies from smokers and in guinea pigs exposed to CS, with a subsequent increase in IL-8 release. Activator protein (AP)-1, like NF-κB, regulates many of the inflammatory genes that are overexpressed in response to CS (Li *et al*., 2009).

The aim of the present study is to evaluate the effects of CS on some immunological parameters, like IFN-□, total IgA, oxidative stress (represented by MDA), and CA19-9 Ag, which is a tumor marker in different organs in the human body, and also to evaluate its effects on some hematological parameters in male smokers.

2. Materials and Methods

2.1. Design of the Study

Fifty male smokers were used in this study; they smoked at least 10 cigarettes per day for at least10 years. The smokers were collected in Erbil city during the period (May 2012 - July 2012).

Depending on the age of the smokers, they were divided into two groups. The first group includes smokers with age range between 25-35 years, and the second group includes smokers with age range between 36-45 years. Two control (non-smokers) groups were collected with the same range of age for statistical comparison.

2.2. Blood Sampling

Blood samples were taken by 5 cc syringe and put into chilled tubes with and without ethylene diamine tetra acetic acid (EDTA) (4.5mM) as anticoagulant and

centrifuged at 3000rpm at 4°C for 15 minute; then the sera were stored at -40C°.

2.3. Estimation of Interferon-Gamma (IFN-γ)

Estimation of interferon-gamma (IFN-γ) was done by enzyme-linked immuno sorbent assay (ELISA) technique, which obtained from Cusabio Company (China).

2.4. Detection of Serum Total IgA by Immune Precipitation Technique

The plate from its envelope was removed and left to stand at room temperature for few minutes so that any condensed water in the wells could evaporate. The wells were filled with 5 µl of sample and/or controls and a period of time of waiting was required till it was completely absorbed before handing the plate. The plate was closed and placed in a moist chamber. 72 h period of waiting was required for incubation. The concentration value corresponding to the precipitating ring diameter was read on the enclosed reference table.

2.5. Determination of Serum Malondialdehyde (MDA)

The assessment of the lipid peroxidation process is achieved via determining the end product MDA. The level of serum MDA was determined spectrophotometrically with a thiobarbituric acid (TBA) solution. In brief, to 150 µl serum sample the following were added: 1ml (17.5%) trichloroacetic acid (TCA) and 1ml of 0.66% TBA, mixed well by vortex, incubated in boiling water for 15 minutes, and then allowed to cool. One ml of 70% TCA was added and the mixture allowed to stand at room temperature for 20 minutes, centrifuged at 2000 rpm for 15 minutes, the supernatant was taken out for scanning spectrophotometrically at (532nm) (Muslih *et al.*, 2002).

The concentration of MDA calculated as follow:

$$\text{MDA } (\mu\text{mol/L}) = \frac{\text{Absorbance at532nm}}{\text{L}\times\text{E0}} \times D \times 10^6$$

L: light path (1cm)

E_0: Extinction coefficient 1.56×10^5 M^{-1} .Cm^{-1}

D: Dilution factor = 1 ml Vol. Used in ref./0.15 =6.7

2.6. Estimation of Carbohydrate Antigen 19-9 (CA 19-9 Ag)

Estimation CA 19-9 Ag was done by enzyme-linked immunesorbent assay (ELISA) technique, obtained from Human Gesellschaft Company (Germany).

2.7. Hematological Analysis

Blood parameters were immediately determined by using automated hematology analyzer (Coulter counter, Sysmex xt-2000i Japan) for determining total WBC count, deferential leukocyte, and platelet numbers.

2.8. Statistical Analysis

All data are expressed as mean± standard error (M±S.E), and statistical analysis was carried out using statistically available software (SPSS). Comparisons between groups were made using independent samples T test at (P<0.05).

3. Results

3.1. Serum Level of Interferon-γ (IFN-γ)

A significant increase of IFN-γ level was recorded in smokers 25-35 years group with mean value (48.73± 0.854) when compared with its control (37.81± 1.796) (Table 1). Furthermore, the level of IFN-γ significantly increased in smokers 36-45 years group with mean value (55.63± 0.997) when compared with its control (41.20± 0.734) (Table 2).

3.2. Serum Level of Total IgA

A significant decrease of total IgA level was recorded in smokers 25-35 years group with mean value (222.2± 13.79) when compared with its control (346.6± 21.53) (Table 1). Furthermore, the level of total IgA significantly decreased in smokers 36-45 years group with mean value (183.2± 18.71) when compared with its control (319.5± 14.34) (Table 2).

Table 1. Mean ± SE of effect of CS on IFN-γ and total IgA in male smokers (25-35 years)

Parameters	Control 25-35 years	Smoker 25-35 years	Statistical evaluation (*P*-value)
IFN-γ (pg/ml)	37.81± 1.796	48.73± 0.854	0.000
IgA (mg/dl)	346.6± 21.53	222.2± 13.79	0.000

P<0.05

Table 2. Mean ± SE of effect of CS on IFN-γ and total IgA in male smokers (36-45 years)

Parameters	Control 36-45 years	Smoker 36-45 years	Statistical evaluation (*P*-value)
IFN-γ (pg/ml)	41.20± 0.734	55.63± 0.997	0.000
IgA (mg/dl)	319.5± 14.34	183.2± 18.71	0.000

P<0.05

3.3. Serum Malondialdehyde (MDA) Level

The level of MDA in smokers 25-35 years group was significantly increased with mean value (3.982± 0.131) when compared with its control group (2.939± 0.153) (Table 3). Besides the level of MDA in smokers 36-45 years group was significantly increased with mean value (4.289± 0.318) when compared with its control group (3.196± 0.126) (Table 4).

3.4. Serum Level of Carbohydrate Antigen 19-9 (CA 19-9 Ag)

The level of CA 19-9 Ag in smokers 25-35 years group was increased with mean value (19.18± 3.434) when compared with its control group (11.87± 1.861) but this increasing in CA 19-9Ag level is not significant (Table 3), while the level of CA19-9 Ag in smokers 36-45 years group was increased significantly with mean value (23.97± 4.178) when compared with its control group (14.13± 1.276) (Table 4).

Table 3. Mean ± SE of effect of CS on MDA and CA 19-9 Ag in male smokers (25-35 years)

Parameters	Control 25 -35 years	Smoker 25-35 years	Statistical evaluation (*P*-value)
MDA (µmol/L)	2.939± 0.153	3.982± 0.131	0.000
Ca19-9 (U/ml)	11.87± 1.861	19.18± 3.434	0.068

P<0.05

Table 4 . Mean ± SE of effect of CS on MDA and CA 19-9 Ag in male smokers (36-45 years)

Parameters	Control 36-45 years	Smoker 36-45 years	Statistical evaluation (*P*-value)
MDA (µmol/L)	3.196± 0.126	4.289± 0.318	0.004
Ca19-9 (U/ml)	14.13± 1.276	23.97± 4.178	0.029

P<0.05

3.5. Haematological Analysis

A significant increase was recorded in most of haemtological parameters, in smokers 25-35 years group, the total WBC count increased significantly with mean value (9.177± 0.619) when compared with its control group (7.063± 0.468); the neutrophils increased significantly with mean value (5.372± 0.547), when compared with its control group (3.840± 0.284). Likewise, a significant increase was recorded in eosinophil numbers with mean value (0.216± 0.032) when compared with its control group (0.094± 0.013), while basophil numbers statistically did not change with mean value (0.056± 0.006) when compared with its control group (0.046± 0.006). On the other hand, a significant increase was observed in monocyte count with mean value (0.765± 0.036) when compared with its control group (0.551± 0.027). Moreover lymphocyte numbers significantly increased with mean value (2.761± 0.133) when compared with those in their control group (1.967± 0.096) (Table 5).

In smokers 36-45 years group, the total WBC count increased significantly with mean value (8.697± 0.302) when compared with its control group (6.550± 0.281), and neutrophils increased significantly with mean value (4.741± 0.291) when compared with its control group (3.948± 0.253). Likewise, a significant increase was recorded in eosinophil numbers with mean value (0.265± 0.020) when compared with its control group (0.132± 0.017). The basophil numbers significantly increased with mean value (0.072± 0.008) when compared with their control group (0.041± 0.006). On the other hand, a significant increase was observed in monocyte count with mean value (0.751± 0.025) when compared with those in their control group (0.534± 0.034). Moreover lymphocyte numbers significantly increased with mean value (2.698± 0.173) when compared with those in their control group (1.829± 0.078) (Table 6).

In smokers 25-35 years group, the numbers of platelets count statistically did not change with mean value (271.6 ±12.89) when compared with its control groups (245.8± 8.911) (Table 5).

Likewise, in smokers 36-45 years group, the numbers of platelets count statistically did not change with mean value (221.5±10.09) when compared with its control groups (241.0±10.34) (Table 6).

Table 5 . Mean ± SE of effect of CS on hematological parameters in male smokers (25-35 years)

Parameters	Control 25-35 years $x10^3/\mu l$	Smokers 25-35 years $x10^3/\mu l$	Statistical evaluation (*P*-value)
WBC	7.063± 0.468	9.177± 0.619	0.009
Neutrophil	3.840± 0.284	5.372± 0.547	0.016
Eosinophil	0.094± 0.013	0.216± 0.032	0.001
Basophil	0.046± 0.006	0.056± 0.006	0.239
Monocyte	0.551± 0.027	0.765± 0.036	0.000
Lymphocyte	1.967± 0.096	2.761± 0.133	0.000
Platelets	245.8± 8.911	271.6 ± 12.89	0.105

P<0.05

Table 6. Mean ± SE of effect of CS on hematological parameters in male smokers (36-45 years)

Parameters	Control 36-45 years $x10^3/\mu l$	Smokers 36-45 years $x10^3/\mu l$	Statistical evaluation (*P*-value)
WBC	6.550± 0.281	8.697± 0.302	0.000
Neutrophil	3.948± 0.253	4.741± 0.291	0.045
Eosinophil	0.132± 0.017	0.265± 0.020	0.000
Basophil	0.041± 0.006	0.072± 0.008	0.002
Monocyte	0.534± 0.034	0.751± 0.025	0.000
Lymphocyte	1.829± 0.078	2.698± 0.173	0.000
Platelets	241.0± 10.34	221.5± 10.09	0.183

P<0.05

4. Discussion

4.1. Serum Level of Interferon-γ (IFN-γ)

The results of the present study showed a significant increase in the level of IFN-γ in both age groups. The results of previous study showed dissimilar results about the level of IFN-γ when compared with other studies.

With regard to CS and immune function, *in vitro* and *in vivo* studies on the effects of CS toxins and nicotine on both IFN-γ are inconclusive. While some *in vitro* rodent studies suggest that nicotine may decrease IFN-γ (Hallquist *et al.*, 2000; Nouri-Shirazi and Guinet, 2003), other studies suggest just the opposite; exposure to tobacco smoke also appears to increase IFN-γ (Petro *et al.*, 1992) and IL-10 secretion (Zhang and Petro, 1996).

Although IFN-γ secreting cells were decreased in smokers' airways compared to nonsmokers' (Hagiwara *et al.*, 2001), no statistically significant difference in IFN-γ levels were found in smoking vs. nonsmoking (Cozen *et al.*, 2004; Zavitz *et al.*, 2008). Further, Zeidel *et al.* (2002) reported an increase in IFN-γ without an increase in the balancing effect of IL-10 in the peripheral blood of cigarette smokers.

Whetzel *et al.* (2007) reported that IFN-γ levels were higher among female smoker. Ouyang *et al.* (2000) reported a significant down regulation of IFN-γ and TNF-α in cultured peripheral blood mononuclear cells treated with CS extract, while Nordskog *et al.* (2005) revealed that the secretion of IL-6, IL-8, IL-4, IL-2, IFN-γ and GM-CSF was stimulated in response to CS condensate.

Increasing evidence suggests that macrophages and lymphocytes are important immunocytes in the smoke induced chronic inflammatory process. Activated T cells could release IFN-γ, which acts as macrophage- activating factor. Activated macrophages secrete many inflammatory proteins that may orchestrate the inflammatory process. Macrophages have the capacity to release the chemokines IFN-γ inducible protein (IP-10), IFN-inducible T-cell α-chemoattractant (I-TAC), and monokine induced by IFN-γ (Mig), which may be chemotactic for CD8+ T cells via interaction with the CCR5(markers of T helper 1 cells) receptor- CCL5 in smokers (Costa *et al.,* 2008).

4.2. Serum Level of Total IgA

The present study indicates a significant decrease in total IgA in both age groups. This result is analogous with other studies on the effects of smoking on the immune system (Schwartz and Weiss, 1994; Sopori and Kozak, 1998; Arnson *et al.*, 2010).

Griesel *et al.* (1999) determined sIgA levels in people who stopped smoking for at least 2 weeks. Transient decrease in sIgA occurred followed by a return to normal values within 2 weeks of stopping smoking.

Several studies have found that smokers had serum Ig levels (IgA, IgG, and IgM) up to 10e20% lower than those of non-smokers (Ussher *et al.*, 2004).

There is increasing evidence that chronic nicotine treatment leads to inhibition of the Ab response indicating that nicotine is a major immunosuppressive component in CS (Geng *et al.*, 1996; Sopori and Kozak, 1998).

4.3. Serum MDA Level

The results of the present study show a significant increase in the level of MDA in both studied age groups. This result is similar to the previous studies like Schmid *et al.* (1996) and Durak *et al.* (2002).

Cigarette smoke (CS) is known to contain a large number of oxidants; it has been hypothesized that many of the adverse effects of smoking may result from oxidative damage to critical biologic substances (Skurnik and Shoenfeld, 1998). Two major phases were identified in CS: a tar phase and a gas phase; both phases are rich in oxygen-centered, carbon-centered and nitrogen-centered free radicals as well as non-radical oxidants. From the analysis of each phase, it was estimated that a single cigarette puff contains approximately 10^{14} free radicals in the tar phase, and 10^{15} radicals in the gas phase. These include various compounds, which are capable of causing an increase in the generation of various ROS like superoxide ($O_2^{\bullet}-$) hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals. These ROS, in turn, are capable of initiating and promoting oxidative damage in the form of lipid peroxidation (Pasupathi *et al.*, 2009). Moreover, copper and iron elements in CS have been found to promote hydroxyl radical ($OH^{\bullet}$) formation by Fenton-type reactions in activated cells (Durak *et al.*, 2002).

Durak *et al.* (2002) suggested that smoking creates a significant oxidant load in the erythrocytes. As a result, toxic free radicals and other oxidant substances in CS damages unsaturated fatty acids and some other oxidation- sensitive structures in the erythrocytes leading to MDA level increase.

Oxidative stress plays a critical role in the inflammatory response to CS through up regulation of redox-sensitive transcription factors and subsequently pro-inflammatory gene expression (Gilks *et al.*, 1998; Keating *et al.*, 1999). Inflammation itself also induces oxidative stress in the lungs and polymorphisms of genes for inflammatory mediators or antioxidant genes may have a role in individual susceptibility to the effects of CS (Smith and Harrison, 1997).

The redox-sensitive transcription factor NF-κB, which can be activated by oxidants and inhibited by antioxidants (Sen and Packer, 1996), plays an important role in the coordinated expression of inflammatory genes induced by CS (van den Berg, 2001).

Exposure to CS causes a cellular oxidative stress, a key feature in smoking-induced lung inflammation (Rahman and MacNee, 1999; Rahman, 2003). Oxidative stress (particularly hydrogen peroxide) can enhance NF-κB DNA binding activity (Schreck *et al.*, 1991). NF-κB is a critical transcription factor regulating many cytokines, including IL-8, IL-6, TNF-α, GM-CSF, macrophage inflammatory protein (MIP)-1, and MCP-1(Di Stefano *et al.*, 2002).

4.4. Serum Level of Carbohydrate Antigen 19-9 (CA 19-9 Ag)

The results of this study show that the level of CA19-9 in age group 25-35 years smokers increased but this increase is not significant, while the level of CA 19-9 increased significantly in age group 36-45 years smokers. This result is similar to what is obtained by Kawai *et al.* (2008), Rottenberg *et al.* (2009), and Wang *et al.* (2012). Lee *et al.* (1998) reported a significant association between CA19-9 and average number of cigarette consumed per day.

CA 19-9 is a carbohydrate antigen expressed in bronchiolar epithelial cells and found in bronchoalveolar lavage in patients with pulmonary fibrosis. Purified CA19-9 stimulated neutrophil chemotaxis to C5a, and IL-8. Normally, CA19-9 may be found in healthy individuals and it increases in benign hepatobiliary disease, with the highest levels in excretory ductal pancreatic adenocarcinoma, biliary, hepatocellular and cholangiocarcinoma cancer (Rottenberg *et al.*, 2009).

The presence of elevated CA19-9 levels in patients with lung cancer has been described in Japan. Most of these patients were diagnosed with adenocarcinoma. In

addition, cell lines from poorly differentiated adenocarcinoma of the lung may produce this tumor marker (Nagami *et al.*, 1988).

Correlation between CA19-9 and the size of the pancreatic adenocarcinoma was reported by Ferrone *et al.* (2006). CA19-9 is associated as a tumor marker with metastatic disease in pancreatic cancer, colorectal cancer, urothelial cancer and melanoma. Neoplasm transformation is induced by high expression of CA19-9. Extravasations of tumor cells from the bloodstream and formation of metastatic disease were associated with CA19-9. The mechanism is probably due to its interaction with E-selection expressed on endothelial cells.

Rottenberg *et al.* (2009) concluded that CA19-9 may be elevated not only in the case of pancreatic cancer, but also in patients with non-small cell lung cancer. And due to the similar demographic and environmental risk factors for both conditions, it is important to interpret the CA19-9 results in light of the clinical presentation as well as chest and abdomen imaging.

4.5. Haematological Parameters:

The results show a significant increase in the total WBC, neutrophil, eosinophil, basophil, monocyte, and the lymphocyte count in both groups, except basophil which statistically did not show any change in group 25-36 years; this result is similar to what is obtained by Schwartz and Weiss (1994), Freedman *et al.* (1996).

Cigarette smoking (CS) has been shown to be associated with an elevated peripheral blood leucocyte count (Schwartz and Weiss, 1994). One of the possible mechanisms of increasing of total WBC may be due to the glycoprotein from the tobacco leaf which can stimulate lymphocyte proliferation and differentiation by interacting with a specific membrane component, as occurs in antigenic response (Freedman *et al.*, 1996).

Cigarette smoke (CS) can induce cyclooxygenase-2 (COX-2) expression and lead to prostaglandin-E3 (PGE2) release from many cell types, such as fibroblasts, blood monocytes, AMs, lung dendritic cells and neutrophils, most of which are immune cells. In addition, the ability of CS and its carcinogens, nicotine in particular, to promote the production of COX-2-derived PGE2 has also been demonstrated in various types of human cancer cells (Badawi *et al.*, 2002).

Arachidonic acid (AA) is liberated from cell membrane phospholipids and converted by COXs to unstable PGG2 and PGH2, which are further metabolized by cell specific prostanoid synthases to biologically active prostanoids, mainly including PGE2, TxA2 and PGI2. TxA2 can bind to its receptor to activate COX-2 gene regulators NF-κB which induce pro- inflammatory cytokines that cause increasing in WBC count (Huang and Chen, 2011)

The eosinophil is likely to be primarily important in terms of IgE-mediated airway injury. Eosinophils are attracted to the lung by elevations in serum IgE, which increases in cigarette smokers. Cytokine activation, particularly IL-3 and-5, may be important in attracting eosinophils to injured airways. CS was shown to activate IL- 2 and may activate other cytokines. In addition, CS is known to be associated with protean immunologic effects, such as increased CD4 cells in light smokers, increased CD8 cells in heavy smokers, and decreased IgG, IgM, and IgA. Thus, other cells and immunologic mechanisms may also be important in airway inflammation due to CS (Schwartz and Weiss, 1994).

It has often been hypothesized that AMs, a key innate immune cell in the lungs, are the orchestrators of CS-induced inflammation. Work by D'Hulst (2005), and Botelho *et al*. (2009) demonstrate that mechanisms that drive CS-induced inflammation are associated with innate immunity. It is acceptable that innate immune mechanisms initially drive CS induced inflammatory processes, and that the engagement of adaptive immunity occurs in a more chronic setting (Nikota and Stämpfli 2012).

The net effect of CS on neutrophils is an elevation of the neutrophils count and a reduction of their functionality. The systemic inflammatory response triggered by exposure to CS is characterized by the stimulation of the hematopoietic system, specifically the bone marrow, which results in the release of leukocytes and platelets into the circulation (Arnson *et al.*, 2010).

Nicotine has been shown to inhibit formation of free oxygen radicals in PMN. Thus, it is likely that nicotine also has the capacity to suppress several neutrophil-mediated inflammatory actions. PMN from the peripheral blood of smokers also exhibits depressed migration and chemotaxis compared with PMN from non-smokers (Nguyen *et al.*, 2001).

The results of the present study show that there is no significant change in the platelets count PDW% and PLCR % between the control and smokers in both groups. This result is in agreement with previous results by Butkiewicz *et al.* (2006), who reported that there is no statistically significant difference in platelet count between male smokers and non-smokers. Suwansaksri *et al.* (2004) observes no alterations in platelets in male smokers and non-smokers. According to Blann *et al.* (1998), smoking two cigarettes a day by chronic smokers of both sexes do not affect the platelet count; Hawkins (1972) also appears to have substantiated the findings of the present study. She observed no significant difference between the platelet counts of nonsmokers, light smokers, and heavy smokers. Various reports have focused on the influence of smoking on platelets because of a possible association between smoking and alteration of blood platelets. Some of these results showed an increase of platelets turnover and a decrease of platelet survival in smokers; increased destruction of platelets, however, was not sufficient to reduce the number of circulating platelets (Fuster *et al.*, 1981).

5. Conclusion

From the present study, we can concluded that cigarette smoking increases the risk of cancer in different organs by the evidence of increasing of level of CA 19-9 and free radicals which have an important role in cancer. Also cigarette smoking increases inflammation responses which are represented by increasing the level of IFN-γ and WBC count.

References

Abbas A K and Andrew H L 2005. **Cellular and Molecular Immunology**. 5th edition. Philadelphia, Pennsylvania. Elsevier saunders.

Arnson Y, Shoenfeld Y and Amital H 2010. Effects of tobacco smoke on immunity, inflammation and autoimmunity. *J. Autoimm***, 34**: 258-265.

Badawi A F, Habib S L, Mohammed M A, Abadi A A and Michael M S 2002. Influence of cigarette smoking on prostaglandin synthesis and cyclooxygenase-2 gene expression in human urinary bladder cancer. *Cancer Invest.,* **20**: 651-6.

Blann A D, Kirkpatrick U, Devine C, Naser S and McCollum C N 1998. The influence of acute smoking on leucocytes, platelets and the endothelium. *Atherosclerosis*. **141**: 133-139.

Botelho F M, Gaschler G J, Kianpour S, Zavitz C C, Trimble N J, Nikota J K, Bauer C M and Stämpfli M R 2010. Innate immune processes are sufficient for driving cigarette smoke-induced inflammation in mice. *Am. J. Respir. Cell Mol. Biol.,*. **42**:394-403.

Bouvet J, Decroix N and Pamonsinlapatham P 2002. Stimulation of local antibody production: parenteral or mucosal vaccination. *Trends Immunol*. **23**:209–213.

Bruno R S 2004. The Role of Oxidative Stress and Vitamin C on Vitamin E Utilization in Humans. Ph.D. Thesis, School of the Ohio State University. USA.

Butkiewicz A M, Kemona-Chętnik I, Dymicka-Piekarska V, Matowicka-Karna J, Kemona H and Radziwon P 2006. Does smoking affect thrombocytopoiesis and platelet activation in women and men? *Advances Medical Sci.,* **51**:123-126.

Costa C, Rufino R, Traves S L, Lapa E, Silva J R, Barnes P J and Donnelly L E 2008. CXCR3 and CCR5 chemokines in induced sputum from patients with COPD. *Chest* **133**:26-33.

Cozen W, Diaz-Sanchez D, James Gauderman W, Zadnick J, Cockburn M G, Gill P S, Masood R, Hamilton A S, Jyrala M and Mack T M 2004. Th1 and Th2 cytokines and IgE levels in identicals twins with varying levels of cigarette consumption. *J Clin Immunol*., **24**: 617–622.

D'hulst A I, Maes T, Bracke K R, Demedts I K, Tournoy K G, Joos G F and Brusselle G G. 2005. Cigarette smoke-induced pulmonary emphysema in scid-mice. Is the acquired immune system required? *Respir Res.,* **6**: 147.

Di Stefano A, Caramori G, Oates T, Capelli A, Lusuardi M, Gnemmi I, Ioli F, Chung K F, Donner C F, Barnes P J and Adcock I M. 2002. Increased expression of nuclear factor-kappaB in bronchial biopsies from smokers and patients with COPD. *Eur Respir J.,* **20**:556–563.

Durak I, Elgün S, Kemal Bingöl N, Burak Cimen M Y, Kaçmaz, M, Büyükkoçak, S and Serdar Oztürk, H. 2002 . Effects of cigarette smoking with different tar content on erythrocyte oxidant/antioxidant status. *Addict Biol.*, **7**:255-258.

Ferrone C R, Finkelstein D M, Thayer S P, Muzikansky A Fernandez-delCastillo C and Warshaw A L. 2006. Perioperative CA19-9 levels can predict stage and survival in patients with resectable pancreatic adenocarcinoma. *J Clin Oncol*.,**24**:2897-2902.

Francus T, Romano P M, Manzo G, Fonacier L, Arango N and Szabo P. 1992. IL-1, IL-6, and PDGF mRNA expression in alveolar cells following stimulation with a tobacco-derived antigen. *Cell Immunol*., **145**: 156-174.

Freedman S, Dana Flanders W, Barboriak J J, Malarcher M and Gates L. 1996. Cigarette Smoking and Leukocyte Subpopulations in Men. *AEP***. 6**: 299-306.

Fuster V, Chesebro J H, Frye R L and Elivebach L R 1981. Platelet survival and the development of coronary artery disease in the young adult: effects of cigarette smoking, strong family history and medical therapy. *Circula.* **63**:546-551.

Geng Y, Savage S M, Razani-Boroujerdi S and Sopori M L 1996. Effects of nicotine on the immune response. II. Chronic nicotine treatment induces T cell anergy. *J Immunol.* ,**156**:2384-2390.

Gilks C B, Price K, Wright J L and Churg A. 1998. Antioxidant gene expression in rat lung after exposure to cigarette smoke. *Am. J. Pathol.*, **152**: 269–278.

Griesel A G and Germishuys J. 1999. Salivary immunoglobulin A levels of persons who have stopped smoking. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.*, **87**:170-173.

Hagiwara E, Takahashi K I, Okubo T, Ohno S, Ueda A, Aoki A, Odagiri S and Ishigatsubo Y. 2001. Cigarette smoking depletes cells spontaneously secreting Th1) cytokines in the human airway. *Cytokine*. **14**:121-126.

Hallquist N, Hakki A, Wecker L, Friedman H. and Pross S. 2000. Differential Effects of nicotine and aging on splenocyte proliferation and the production of Th1-vs. Th2-type cytokines. *Proceedings of the Society for Experimental Biology and Medicine*. **224**: 141–146.

Hawkins R I. 1972. Smoking, platelets and thrombosis. *Nature* **236**: 450-452.

Hoffmann D, Hoffmann I and El-Bayoumy K. 2001. The less harmful cigarette: a controversial issue. *Chem. Res. Toxicol.*, **14**: 767-790.

Huang Z S, Chien K L, Yang C Y, Tsai K S, Wang C H. 2001. Peripheral differential leukocyte counts in humans vary with hyperlipidemia, smoking, and body mass index. *Lipids*,.**36**:237-245.

Kalra R, Singh S P, Savage S, Finch G L and Sopori M L. 2000. Effects of cigarette smoke on immune response: chronic exposure to cigarette smoke impairs antigen-mediated signaling in T cells and depletes IP3-sensitive Ca^{2+} stores. *J Pharmacol Exp Ther.*, **293**:166-171.

Kawai S, Suzuki K, Nishio K, Ishida Y, Okada R, Goto Y, Naito M, Wakai K, Ito Y and Hamajima N. 2008. Smoking and serum CA19-9 levels according to Lewis and secretor genotypes. *Int J Cancer*, **123**:2880-2884.

Keating V M, Collins P D, Scott D M and Barnes P J. 1999. Differences in interleukin-8 and tumor necrosis factor-induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am. J. Respir. Crit. Care Med.*, **153:** 530–534.

Lamerz R. 1999. Role of tumour markers, cytogenetics. *Ann Oncol Suppl* **4**:145-149.

Lee M C, Lee S K, Yoo K Y, Park S K, Kang D H, Kim J Q and Chung J K. 1998. Factors Related to Serum Level of Carbohydrate Antigen 19-9 and Cancer Antigen 125 in Healthy Rural Populaions in Korea. *Korean J Nucl Med.*, **32**:71-80.

Li Y, He B and Wang Y. 2009. Exposure to cigarette smokes upregulates AP-1 activity and induces TNF-alpha overexpression in mouse lungs. *Inhal. Toxico*., **21**: 641–647.

Mehta H, Nazzal K and Sadikot R T. 2008. Cigarette smoking and innate immunity. *Inflamm Res.*, **57**:497-503.

Miki M, Isawa T, Teshima T, Anazawa Y, Mahmud A M and Nukiwa T. 1995. Establishment of a cell line from an adenocarcinoma of the lung producing carcinoembryonic antigen CEA) and CA19-9. *Tohoku J Exp Med.* ,**175**:269-270.

Muslih R D, Al-Nimer MA and Al-Zamely O M. 2002. The level of Malondialdehyde after activation with H_2O_2 and $CuSO_4$) and inhibited by Desferoxamine and Molsidomine in the serum of patients with acute myocardial infection. *Nat. J. Chem*. **5**:148-149.

Nagami H, Nohara T, Yamauchi M, Yamada K, Nakase A. 1988. A case of two patients with primary lung cancer secreting CA19-9. *Nihon Geka Gakkai Zasshi.* **89**:292-296.

Nguyen H, Finkelstein E, Reznick A, Cross C and van der Vliet A. 2001. Cigarette smoke impairs neutrophil respiratory burst activation by aldehyde-induced thiol modifications. *Toxicology.* **7**:207-217.

Nikota J K and Stämpfli M R. 2012. Cigarette smoke-induced inflammation and respiratory host defense: Insights from animal models. *Pulm Pharmacol Ther.* **25:**257-262.

Nordskog B K, Fields W R and Hellmann G M. 2005. Kinetic analysis of cytokine response to cigarette smoke condensate by human endothelial and monocytic cells. *Toxicol.* **1**:87-97.

Nouri-Shirazi M and Guinet E. 2003. Evidence for the immunosuppressive role of nicotine on human dendritic cell functions. *Imm.* **109**: 365–373.

Ouyang Y, Virasch N, Hao P, Aubrey M T, Mukerjee N, Bierer B E and Freed B M. 2000. Suppression of human IL-1beta, IL-2, IFN-gamma, and TNF-alpha production by cigarette smoke extracts. *J Allergy Clin Immunol.* **106**: 280-287.

Pasupathi P, Bakthavathsalam G Y, Rao Y and Farook J. 2009. Cigarette smoking-Effect of metabolic health risk: A review. Diabetes and Metabolic Syndrome: *Clin. Res. and Rev*. **3**: 120–127.

Petro T M, Peterson D S and Fung Y K. 1992. Nicotine enhances interleukin production of rat splenic T lymphocytes. *Immunopharmacology and Immunotoxicology*. **14**: 463–475.

Rahman I. 2003. Oxidative stress, chromatin remodelling and gene transcription in inflammation and chronic lung disease. *J. Biochem. Mol. Biol.* **36**:95–109.

Rahman I and MacNee W. 1999. Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *Am. J. Physiol. Lung Cell Mol. Physiol.* **277**: L1067–L1088.

Rottenberg Y, Nisman B and Peretz T. 2009. Extreme high levels of CA19-9 associated with adenocarcinoma of the lung. *Isr Med Assoc J.* **11**:116-117.

Schmid P, Karanikas G, Kritz H, Pirich C, Stamatopoulos Y, Peskar B A and Sinzinger H. 1996. Passive Smoking and Platelet Thromboxane. *Thrombosis Research.* **81**: 451-460.

Schoenborn J R and Wilson C B. 2007. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv. Immunol*. **96**: 41–101.

Schreck R, Rieber P and Baeuerle P A. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF- κB transcription factor and HIV-1. *EMBO J.* **10**:2247–2258.

Schwartz J and Weiss S T. 1994. Cigarette Smoking and Peripheral Blood Leukocyte Differentials. *AEP*. **4**: 236-242.

Sen C K and Packer L. 1996. Antioxidants and redox regulation of gene transcription. *FASEB J.* **10**:709–720.

Skurnik Y and Shoenfeld Y. 1998. Health effects of cigarette smoking. *Clin Dermatol.* **16**:545-556.

Smith C A D and Harrison D J. 1997. Association between polymorphism in gene for microsomal epoxide hydrolase and susceptibility to emphysema. *Lancet*. **350**: 630–633.

Soldin O P, Makambi K H, Soldin S J and O'Mara D M. 2011. Steroid hormone levels associated with passive and active smoking. *Steroids.* **76**:653-659.

Sopori M L. 2002. Effects of cigarette smoke on the immune system. *Nat Rev Immunol*. **2**:372-377.

Sopori M L and Kozak W. 1998. Immunomodulatory effects of cigarette smoke. *Journal of Neuroimmunology*. **83**: 148-156.

Suriyaprom K, Harnroongroj T, Namjuntra P, Chantaranipapong Y and Tungtrongchitr R. 2007. Effects of tobacco smoking on alpha-2-macroglobulin and some biochemical parameters in Thai males. *Southeast Asian J Trop Med Public Health*. **38**:918-926.

Suwansaksri J, Wiwanitkit V, Soogarun S. 2004. Effect of smoking on platelet count and platelet parameters: an observation. *Clin Appl Thromb Hemost*. **10**: 287-288.

Takajo Y, Ikeda H, Haramaki N, Murohara T and Imaizumi T. 2001. Augmented oxidative stress of platelets in chronic smokers. Mechanisms of impaired platelet-derived nitric oxide bioactivity and augmented platelet agreeability. *J Am Coll Cardiol*. **38**: 1320-1327.

Traber M G, van der Vliet A, Reznick A Z and Cross C E. 2000. Tobacco-related diseases. Is there a role for antioxidant micronutrient supplementation? *Clin Chest Med*. **21**: 173-187.

Ussher M, West R, Evans P, Steptoe A, McEwen A, Clow A and Hucklebridge F. 2004. Acute reduction in secretory immunoglobulin A following smoking cessation. *Psychoneuroendocrinology*. **29**:1335-1340.

Van den Berg R, Haenen G R M M, Berg H and Bast A. 2001. Nuclear factor-kB activation is higher in peripheral blood mononuclear cells of male smokers. *Environmental Toxicology and Pharmacology*. **9**: 147–151.

Wang D S, Wang Z Q, Zhang L, Qiu M Z, Luo H Y, Ren C, Zhang D S, Wang F H, Li Y H and Xu R H. 2012. Are risk factors associated with outcomes in pancreatic cancer? *PLoS One.***7**:41984.

Whetzel C A, Corwin E J and Klein L C. 2007. Disruption in Th1/Th2 immune response in young adult smokers. *Addict Behav*. **32**:1-8.

World Health Organization (WHO). 2001. Guidelines for controlling and monitoring the tobacco epidemic. Geneva; World Health Organization, 2-7.

Zavitz CC, Gaschler G J, Robbins C S, Botelho F M, Cox P G and Stampfli M R. 2008. Impact of cigarette smoke on T and B cell responsiveness. Cell Immunol. **253**:38-44.

Zeidel A, Beilin B, Yarkeni I, Mayburg E, Smirnov G and Bessler H. 2002. Immune response in asymptomatic smokers. *Acta Anaesthesiologica Scandinavica*. **46**: 959–964.

Zhang S and Petro T M. 1996. The effect of nicotine on murine CD4 T cell responses. *International Journal of Immunopharmacology*. **18**: 467–478.

Zhong C Y, Zhou Y M and Pinkerton K E. 2008. NF-kappaB inhibition is involved in tobacco smoke-induced apoptosis in the lungs of rats. *Toxicol Appl Pharmacol.* **230**:150-158.

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Knowledge of the Use and Benefits of Applying Biotechnology and Cell Based Therapy in Orthopaedics in Jordan: Questionnaire Survey and Regulation Assessment

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Abstract

New techniques have been developed, many derived from biotechnology that enhance and expand the use of human cells and tissues as therapeutic products. These new techniques hold the promise that some day they will provide therapies for many serious medical conditions. With the increasing numbers of trauma, cancer cases and diabetes mellitus cases every year in The Hashemite Kingdom of Jordan (Jordan), it is important to explore and introduce the use of new therapeutic technologies in the Jordanian health care system, i.e., biotechnology, tissue engineering, and cell based therapy. Before introducing these therapy modalities to the Jordanian market, assessing the knowledge of these new therapeutic modalities is worth performing. The current study aimed to assess the knowledge of biological growth factors and mesenchymal stem cells (MSCs) use and benefits in orthopaedics in Jordan. The assessment was made by analyzing questionnaire results filled by a sample of Jordanian orthopaedics surgeons and senior medical school students from the Hashemite University, Zarqa, Jordan. The questionnaire results reflected good knowledge and appreciation of the use and benefits of biological growth factors and MSCs in orthopaedics applications and treatments among participants. Surprisingly, higher percentages of positive answers were reported about the knowledge of mesenchymal stem cells benefits compared to biological growth factors in the year 2012. The results also showed statistically significant lower percentages of positive answers to the question of thinking that using these new technologies will reduce the treatment cost (≤36%), compared to the percentages of positive answers to the other questionnaire questions. Still, higher percentages of positive answers were reported to whether participants will agree to use biological growth factors to accelerate the healing process on themselves (82% in 2009 & 74% in 2012). The current study also presented an assessment of the Jordanian regulations and their ability to define and regulate the use of these new therapeutic technologies and their products in Jordan. The questionnaire results and the legislations assessment highlighted the necessity to reform the Jordanian laws to build a legal framework that encourage healthcare suppliers and providers to explore and evaluate new technologies that might reduce treatment cost and hospitalization time in Jordan.

Keywords: Biotechnology, Mesenchymal Stem Cells, Medical Education, Orthopaedics, Jordanian Medical Regulations.

1. Introduction

In Jordan, orthopaedics surgeons are challenged every day to accelerate healing, reduce cost, and reduce hospitalization time. In recent years, Jordan is experiencing an increase in trauma incidents due to automobile accidents, as well as an increase in life expectancy resulting in an increase in the number of diabetes mellitus and other hormonal deficiency cases among Jordanian population. These factors resulted in a high number of orthopaedics patients with medical conditions experiencing impaired or delayed bone healing/regeneration (Brighton and Shaman *et al.*, 1995) (Stuart and Morrey, 1990; Papa *et al.*, 1993; Tisdel *et al.*, 1995; Perlman and Thordarson, 1999).

Orthopaedics patients with impaired or delayed bone healing/regeneration are stressing the healthcare system with their long hospitalization time and the extra care they need in their treatment (Cozen, 1972; Loder, 1988). All these facts require orthopaedics surgeons and healthcare professionals in Jordan to consider using new technologies, such as the use of biotechnology and cell based therapy in orthopaedics applications in Jordan to reduce treatment cost and hospitalization time.

With the advances made in molecular medicine and molecular biology, several molecules have been identified that regulate the cascade of events in a time-dependent fashion leading to repair of bone tissue (Dimitriou *et al.*, 2005). This knowledge has led to a great interest in the application of these molecules in the clinical setting, especially for treatment of impaired

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fracture healing. The key growth factors characterized as being present in the fracture site are TGF-β1, TGF-β2, BMP-2, BMP-3, BMP-4, and BMP-7 (OP-1), PDGF, and acidic and basic FGF (FGF-1 and FGF-2) (Bolander, 1992; Linkhart *et al.*, 1996; Einhorn, 1998).

Several approaches have been utilized to elicit the formation of bone in segmental defects and facilitate the healing process (Flemming, 2002). These approaches have included the implantation of osteoconductive extracellular scaffolds (Holmes, 1987; Martin, 1989; Zardiackas, 1994; Johnson, 1996; Hollinger *et al.*, 2008) and the implantation of bone morphogenetic proteins in various matrices (Brighton and Hunt, 1991; Gehart, 1993; Cook, 1994; Stevenson, 1994; Wolff, 1994; Nurminskaya and Linsenmayer, 1996; Schmitz *et al.*, 1999; Haynesworth *et al.*, 2002). Another concept is based upon *ex vivo* expansion of pluripotent mesenchymal stem cells (MSC) loaded onto a carrier system.

The MSC are self renewing pluripotent progenitor cells that have been isolated from the whole marrow of chicks, mice, rats, rabbits, goats, and humans (Goshima, 1991; Haynesworth, 1992; Haynesworth *et al.*, 2002). These cells have the capability of differentiating into osteoblasts, chondrocytes, adipocytes, tenocytes, and myoblasts. Potential advantages of this strategy consist of decreased need for massive cellular proliferation and osteoblast progenitor cell chemotaxis into the defect as well as development of appropriate signaling for early bone formation in the graft site. This osteogenic potential of marrow derived MSC has been well defined *in vivo* (Mardon, 1987; Owens, 1988; Ohgishi, 1989; Goshima, 1991).

The role of MSC in bone regeneration and formation continues to be defined, and manipulation of MSC has resulted in new therapeutic strategies (Goshima *et al.*, 1991; Lennon *et al.*, 2001; Khan, 2005; Kraus, 2006). While this concept (application of MSC) has demonstrated its potential role for surgical reconstruction and arthrodesis procedures, significant questions exist regarding the use of this technology in developing countries including Jordan. This project aimed to assess the knowledge of biological growth factors and mesenchymal stem cells use and benefits in orthopaedics applications among a selected sample of Jordan's orthopaedics surgeons and senior medical school students.

We hypothesized that orthopaedics surgeons and the senior medical school students in Jordan are aware of the benefits, cost, applicability, and the advantages over the classical treatment modalities of using biotechnology and cell based therapy in orthopaedics applications and treatments in Jordan. Furthermore, it is hypothesized that orthopaedics surgeons and the senior medical school students in Jordan are willing to use this cutting edge technologies on themselves to accelerate the bone healing process.

2. Materials and Methods

2.1. The Questionnaire

The questionnaire was prepared by the investigation team and it was designed to investigate if the doctors and students participating had the knowledge about the use and benefits of biological growth factors (BGF) and mesenchymal stem cells (MSCs) in orthopaedics. The questionnaire consisted of two parts; the first part was designed to investigate the knowledge of BGF use and benefits in orthopaedics in Jordan, and it consisted of nine "Yes/No/I don't know" questions asking the participants [*(1) Do you have any information or any idea about using the biological growth factors in orthopaedics applications and treatments? ; (2) Do you know the benefits of its use?; (3) Do you recommend using it?; (4) Do you think that it will reduce the treatment cost?; (5) Do you think that it will reduce hospitalization time?; (6) If you are to choose between the available "old" technology and using the biological growth factors technology, will you use the biological growth factors technology?; (7) Do you think it is an expensive technology?; (8) Do you believe that it can be used in Jordan?; and, (9) If you got an injury in your bone, do you agree to use this technology to accelerate the healing process?*], successively.

The second part intended to investigate the knowledge of MSCs use and benefits in orthopaedics in Jordan, and it consisted of nine "Yes/No/I don't know" questions asking the participants [*(1) Do you have any information or any idea about using mesenchymal stem cells in orthopaedics applications and treatments? ; (2)Do you know the benefits of its use? ; (3)Do you recommend using it? ; (4) Do you think that it will reduce the treatment cost? ; (5) Do you think that it will reduce hospitalization time? ; (6) If you are to choose between the available "old" technology and using mesenchymal stem cells, will you use mesenchymal stem cells? ; (7) Do you think it is an expensive technology? ; (8) Do you believe that it can be used in Jordan? ; and (9) If you got an injury in your bone, do you agree to use this technology to accelerate the healing process?*].

The questionnaire was circulated to be filled at two different time points, the first time was in October 2009 and the second time was in March 2012. In 2009, the questionnaire was given to a sample of orthopaedics surgeons (n=19), whereas, in 2012, the questionnaire was given to another sample of orthopaedics surgeons (n=24) and to a sample of fourth year-senior medical school students (n=29). The questionnaire was written in English, then it was translated to Arabic by the investigation team. The questionnaire was presented to the participants in its Arabic version to be answered.

2.2. Statistical Analysis

Statistical analyses were performed using SigmaStat 3.0 (SPSS Inc., Chicago, Illinois). The amount of "yes" answers to each question in each questionnaire was analyzed. Analysis of variance (ANOVA) was performed followed by Holm-Sidak post hoc tests to identify differences between the percentages of positive "yes" answers among the questionnaire questions and the

differences between the overall additive percentages of the positive answers of the nine questions among the different groups of participants in all time points selected. *P* values less than 0.05 were considered statistically significant.

2.3. Evaluating Jordanian Regulations Related to Biotechnology and Cell Based Therapy Products

To investigate the Jordanian regulations regarding biotechnology and cell based therapy use, a review of the Jordanian regulations governing the definition, manufacturing, and circulation was made for those therapy modalities and their products.

3. Results

3.1. (2009) Questionnaire

In 2009, nineteen questionnaires in total were filled by orthopaedics surgeons; eight were filled by orthopaedics surgeons from the city of Amman in their private clinics, five were filled by orthopaedics surgeons from King Hussain medical city in Amman, two were filled by orthopaedics surgeons from the city of Zarqa in their private clinics, and four were filled by orthopaedics surgeons from the city of Irbid in King Abdullah-I hospital.

The percentages of positive "Yes" answers were determined for each question of the questionnaire, according to the medical institution they were filled in (Tables 1 & 2). There were no statistically significant differences among the percentages of positive answers in the MSCs part (Table 2).

Table 1. 2009 Orthopaedics surgeons knowledge of biological growth factors use and benefits questionnaire results according to each health care institution:

Question #	% of Positive answers of the knowledge of the biological growth factors use and benefits in orthopaedics				
	Amman private clinics (n=8)	King Hussain medical city (n=5)	Zarqa private clinics (n=2)	King Abdullah-I hospital (n=4)	Mean ± SD
1	87.5	100	100	75	90.6 ± 12
2	50	100	100	75	81.3 ± 24
3	87.5	60	50	100	74.4 ± 23
4	50	60	0	25	33.8 ± 27 [a]
5	62.5	80	100	50	73.1 ± 22
6	100	80	50	75	76.2 ± 21
7	75	100	50	50	68.8 ± 24
8	75	60	100	100	83.8 ± 20
9	75	80	100	75	82.5 ± 12

[a] : $P < 0.03$ versus all questions

Table 2. 2009 Orthopaedics surgeons knowledge of mesenchymal stem cells use and benefits questionnaire results according to each health care institution:

Question #	% of Positive answers of the knowledge of mesenchymal stem cells use and benefits in orthopaedics				
	Amman private clinics (n=8)	King Hussain medical city (n=5)	Zarqa private clinics (n=2)	King Abdullah-I hospital (n=4)	Mean ± SD
1	75	100	0	75	62.5 ± 43
2	75	100	50	100	81.3 ± 24
3	75	80	100	25	70 ± 32
4	62.5	60	0	25	36.9 ± 30
5	75	100	50	25	62.5 ± 32
6	87.5	80	100	75	85.6 ± 11
7	62.5	100	50	50	65.6 ± 24
8	62.5	80	100	50	73.1 ± 22
9	62.5	80	100	75	79.4 ± 16

However, in the BGF part, the percentages of the positive answers were significantly lower for the fourth question "*Do you think that it will reduce the treatment cost?*" as compared to all other questions in BGF part of the questionnaire (33.8 % with $P < 0.03$) (Table 1).

No differences in the percentage of the negative "No" answers nor in the percentages of the "I don't know" answers among the nine questions in both parts of the questionnaire (Data not shown).

3.2. (2012) Questionnaire

In 2012, fifty three questionnaires in total were filled; twenty four by orthopaedics surgeons from six different medical institutions and twenty nine by fourth year-senior medical school students (MD students) from The Hashemite University, Zarqa, Jordan. The twenty four orthopaedics surgeons' questionnaires were filled in the following medical institutions; King Hussain medical city in Amman (n=4), Jordan University Hospital in Amman (n=3), AlBasheer Hospital in Amman (n=5), Prince Hamza Hospital in Amman (n=3), the city of Salt public hospital (n=3), AlIsraa Private Hospital in Amman (n=6).

The percentages of positive "Yes" answers were determined for each question according to the medical institution they were filled in (Surgeons n=24), and for the senior medical school students (MD students n=29) (**Tables 3 & 4**). Furthermore, the percentages of positive "Yes" answers were determined for the entire sample participated of both the orthopaedics surgeons and the medical school students (Total n=53) (Table 5).

In the BGF part of the questionnaire, the percentages of the positive answers for the entire sample of participants (Total n=53) were significantly lower for the fourth question "*Do you think that it will reduce the treatment cost?*" as compared to the six other questions (18% with $P < 0.02$), and strongly trending lower as compared to the remaining two questions ($P = 0.078$).

Furthermore, the BGF results showed significantly lower percentages of the positive answers for the fifth question "*Do you think that it will reduce hospitalization time?*" as compared to three questions (35% with $P < 0.05$). On the other hand, the percentages of the positive "Yes" answer for the ninth question "*If you got an injury in your bone, do you agree to use this technology to accelerate the healing process?*" were significantly greater (74% with $P < 0.02$) as compared to two other questions (Table 3).

In the MSCs part of the questionnaire, the percentages of the positive answers for the entire sample of participants (Total n=53) were significantly lower for the fourth question "*Do you think that it will reduce the treatment cost?*" as compared all other questions (17 % with $P < 0.03$). Furthermore, the percentages of the positive answers of the sixth "*If you are to choose between the available "old" technology and using the biological growth factors technology, will you use the biological growth factors technology?"* and eighth "*Do you believe that it can be used in Jordan?*" questions were significantly lower as compared to the percentages of questions one and two (46% & 47% respectively with $P < 0.05$) (Table 4).

In the BGF part of the questionnaire, the overall percentage of the positive answers for the nine questions answered by the orthopaedics surgeons (Surgeons n=24) (54%) was significantly greater ($P < 0.01$) than the overall percentage of the positive answers for the nine questions answered by the senior medical school students (MD students n=29) (32%). A similar trend was found in the MSCs part of the questionnaire, where the overall percentage of the positive answers orthopaedics surgeons was significantly greater as compared to senior medical school students' answers.

The overall additive percentages of positive "Yes" answers in the 2009 questionnaire were significantly higher as compared to all groups of participants in the year 2012. Furthermore, the overall additive percentages of the 2012 questionnaire filled by the orthopaedics surgeons group results showed significantly higher positive "Yes" answers percentages in its both parts (BGF & MSCs) (54% & 57%) as compared to the 2012 questionnaire filled by the 4th year medical school students group results (32% & 31%) (Table 5).

3.3. Jordanian Regulations Related to Biotechnology and Cell Based Therapy Products

Drugs are regulated in Jordan by the Drug and Pharmacy Provisional Law No. (80) of the year 2001. In this law the term "drug" was defined as "*any substance or group of substances used to diagnose the diseases affecting human being or cure the same, lessen the pain or protect human body from diseases, or a group of substances other than foodstuff which has certain effect on human body or any of its functions*."

Moreover, section (B) of article (3) of this law states "It shall be prohibited to circulate any infant milk formula and its special formula, and supplementary food, medical plants, natural products, disinfectant and detergents, medical equipment and supplies, pharmaceutical preparations containing vitamins and minerals, cosmetic preparations and any other substances related to treatment or cure of human beings from disease, unless they are licensed according to the Minister's Directions and Coordinating with the concerned official authorities".

Two committees were formed according to the Drug and Pharmacy Provisional Law No. (80) of the year 2001. The first is The Higher Committee for Medicine & Pharmacy, with one of its responsibilities stated as "*Controlling of any substances or preparations related to treatment of diseases, or any other substances the Minister may deem necessary to have them under control*" according to bulletin (14) of Section (A) of Article (4). The second committee is the Technical Committee for Registration of New Drugs, with one of its responsibilities stated as "*Study the new developments related to drugs, and their precautions and side effects, and take any proper decisions in that regard*" according to bulletin (3) of section (C) of Article (9).

Table 3: 2012 Knowledge of biological growth factors use and benefits questionnaire results according to each health care institution.

Question #	% of Positive answers of the knowledge of the biological growth factors use and benefits in orthopaedics									
	King Hussain (n=4)	Jordan Univ (n=3)	AlBasheer (n=5)	Prince Hamza (n=3)	Salt Hospital (n=3)	Alsraa Hospoital (n=6)	4th year MD students (n=29)	Surgeons (n=24) Mean ± SD	Total (n=53) Mean ± SD
1	50	33	100	67	100	83	31	72 ± 27	66 ± 29
2	100	33	60	67	67	83	24	68 ± 23	62 ± 27
3	50	0	60	33	33	83	27	43 ± 28	41 ± 27
4	0	0	40	33	0	33	17	18 ± 20[a]	18 ± 18 *
5	50	0	40	33	33	50	41	34 ± 19[b]	35 ± 17 **
6	50	33	60	67	33	67	38	52 ± 16	50 ± 15
7	25	100	60	33	67	33	38	53 ± 28	51 ± 27
8	100	33	40	100	67	67	31	68 ± 29	63 ± 30
9	100	33	60	100	100	83	45	79 ± 28[c]	74 ± 28***

[a , *] : P< 0.02 versus questions (1,2,6,7,8,9) ; [b] : $P < 0.03$ versus questions (1,2,8, 9) ; [**] : $P < 0.05$ versus questions (1,2,8) , [c , ***] : $P < 0.02$ vs questions (3,5)

Table 4 : 2012 knowledge of MSC use and benefits questionnaire results according to each health care institution

Question #	% of Positive answers of the knowledge of mesenchymal stem cells use and benefits in orthopaedics								
	King Hussain (n=4)	Jordan Univ (n=3)	AlBasheer (n=5)	Prince Hamza (n=3)	Salt Hospital (n=3)	AlIsraa Hospoital (n=6)	4th year MD students (n=29)	Mean ± SD Surgeons (n=24)	Mean ± SD Total (n=53)
1	50	67	80	100	100	83	28	80 ± 19	72 ± 26
2	50	67	80	100	100	83	31	80 ± 19	73 ± 26
3	50	33	60	67	67	50	31	55 ± 13	51 ± 15
4	0	33	20	33	0	16	17	17 ± 15[a]	17 ± 14[*]
5	50	33	40	100	33	50	31	51 ± 25[b]	48 ± 24
6	50	33	60	33	67	50	28	49 ± 14[c]	46 ± 15 [**]
7	0	100	60	100	100	50	48	68 ± 40	65 ± 38
8	50	33	20	67	100	33	24	51 ± 29[d]	47 ± 29 [***]
9	75	33	60	67	67	67	45	62 ± 15	59 ± 15

[a ,*]: $P < 0.03$ versus all questions ; [b , c , d , **] : $P < 0.04$ versus questions (1,2) ; [***] : $P < 0.05$ versus questions (1,2)

Table 5: The overall additive percentages of the positive answers among all groups of participants in all time points selected:

Group #	Group Name	# of Participants (n)	Over all additive % of Positive answers (Mean ± SD)
1	2009 Orthopaedics Surgeons Biological growth factors Questionnaire Results	19	74 ± 16[a]
2	2009 Orthopaedics Surgeons Mesenchymal Stem Cells Questionnaire Results	19	69 ± 15[b]
3	2012 Orthopaedics Surgeons Biological growth factors Questionnaire Results	24	54 ± 20[c]
4	2012 Orthopaedics Surgeons Mesenchymal Stem Cells Questionnaire Results	24	57 ± 20[d]
5	2012 Medical School Students Biological growth factors Questionnaire Results	29	32 ± 9
6	2012 Medical School Students Mesenchymal Stem Cells Questionnaire Results	29	31 ± 10
7	2012 Total Number of Participants Biological growth factors Questionnaire Results	53	51 ± 18
8	2012 Total Number of Participants Mesenchymal Stem Cells Questionnaire Results	53	53 ± 17

[a] : $P < 0.03$ versus groups (4, 5, 6, 7, & 8); [b] : $P < 0.05$ versus groups (5, 6, 7, & 8); [c & d]: $P < 0.005$ versus groups (5 & 6)

4. Discussion

Using a simple questionnaire, this study assessed the knowledge of biological growth factors and mesenchymal stem cells use and benefits in orthopaedics among a sample of Jordanian orthopaedics surgeons and 4th year senior medical school students. The assessment aimed to test the hypothesis that Jordanian orthopaedics surgeons and the senior medical school students in Jordan are aware of the benefits, cost, applicability, and the advantages over the classical treatment modalities of using biotechnology and cell based therapy in orthopaedics applications and treatments.

When assessing the knowledge of biological growth factors (BGF) use and benefits among orthopaedics

surgeons, the questionnaire results showed that the percentages of the positive "Yes" answers to knowing the use and benefits of this technology (first & second questions) were notably high among participants in the year 2009 (90% & 81%) and moderate among participant in the year 2012 (72% & 68%) (Tables 1 & 3). The differences in percentages between the two time points might be attributed to the differences in sample size of participants, which was bigger in the 2012 time point, or due to the fact that participants were from different health care institutions at each time point. Still, the elevated positive percentage of knowledge indicates high level of education and interest in BGF and its applications among Jordanian orthopaedics surgeons.

In contrast, the BGF questionnaire part results showed that the percentages of the positive "Yes" answers to the forth question "*Do you think that it will reduce the treatment cost?*" were significantly lower compared to the other questions in the year 2009 (33% with $P<0.03$), and significantly lower compared to questions (1, 2, 6, 7, 8, & 9) in the year 2012 (18% with $P<0.02$). These findings reflect a negative view of inefficiency in cost reduction of using this treatment modality in orthopaedics applications in Jordan among the participants.

The negative view of inefficiency in cost reduction might be attributed to the lack of an exact estimate of the cost of these new technologies, especially that these technologies and their components are not frequently employed in Jordan's health care system providers and suppliers are considered emerging technology yet to be characterized and evaluated.

In the 2012 questionnaire, the BGF questionnaire part results of the orthopaedics surgeons group (n=24) showed lower percentages of positive "Yes" answers to the fifth question "*Do you think that it will reduce hospitalization time?*" as compared to the first, second, eighth, and ninth questions (%34 with P<0.03) (Table 3). This is consistent with the view of inefficiency among orthopaedics surgeons participated in the questionnaire, which was seen in the results of question four. Interestingly, the data showed that despite the negative view of inefficiency of the cost and hospitalization time reductions, the orthopaedics surgeons agreed on using this technology to accelerate the healing process on themselves in case they got injured (79%) (Table 3). This interesting finding might suggest that the participants are in favor of using this new technology on themselves but not on others, especially that the percentages of positive answers of question three "*Do you recommend using it?*" were low and comparable to the results of question four and five (Table 3).

When assessing the knowledge of mesenchymal stem cells (MSCs) use and benefits among orthopaedics surgeons, the questionnaire results obtained in the year 2009 showed no statistical significance among the percentages of positive "Yes" answers among the nine questions, reflecting no specific point of views adopted among the orthopaedics surgeons group (n=19) that participated about the use and benefits of this modality in orthopaedics in Jordan at that time point (Table 2).

On the other hand, when investigating the 2012 questionnaire results of the MSCs part filled by the orthopaedics surgeons group (n=24), the questionnaire results showed that the percentages of the positive "Yes" answers to knowing the use and benefits of this technology (First & Second questions) were significantly higher than the answers to questions (4, 5, 6, & 8) (80% with $P< 0.04$) (Table 4). Furthermore, questions one and two results were comparable to the results obtained in the year 2009 (Table 2) and relatively higher than the results reported in the BGF part in the year 2012 (Table 3). The high positive percentage of knowledge indicates high level of education and interest in mesenchymal stem cells and its applications among Jordanian orthopaedics surgeons, and it can be noticed in a higher extent when compared to the biological growth factors.

When investigating the 2012 questionnaire results for both BGF and MSCs filled by the 4th year medical school students group (n=29), the percentages of positive "Yes" answers were significantly lower as compared to the orthopaedics surgeons group in the year 2012 (n=24), except for question four where both percentages were low and comparable (Tables 3 & 4). A similar argument can be made when comparing the overall additive percentages of the positive "Yes" answers among the participated groups, where the 2012 orthopaedics surgeons group overall percentages were significantly higher compared to the medical school students percentages in both parts of the questionnaire (BGF & MSCs) ($P< 0.005$) Table (5).

These findings might raise concerns of the educational curricula presented to medical students which might be lacking the necessary material that explains new modalities and technologies and practices in medicine. It is considered vital to expose medical students to new modalities and technologies while they are attending their basic biomedical courses and before they start their clinical education, in order to avoid the elevated uncertainty and ignorance levels that were notably present in the medical students "I don't know" answers to the questions (Figure 3) as compared to the results obtained from the orthopaedics surgeons answers (Figures 1 & 2).

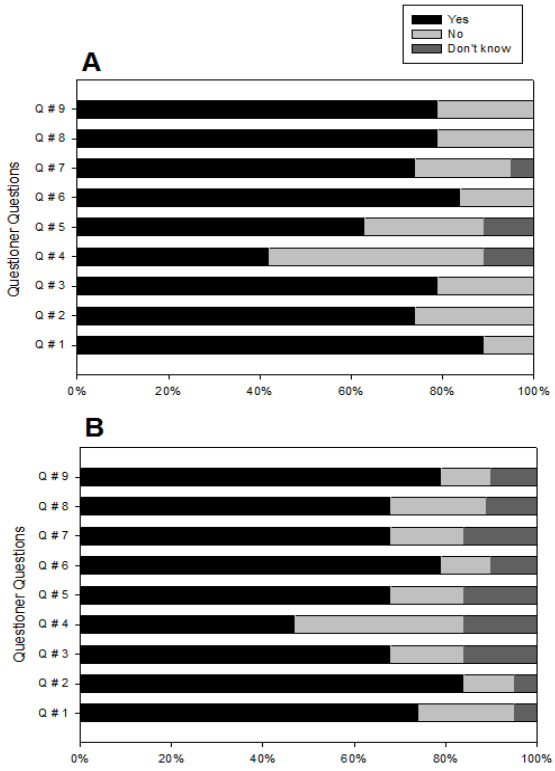

Figure 1: 2009 Questionnaire results obtained from orthopaedics surgeons. (A) Knowledge of biological growth factors use and benefits questionnaire results, (B) Knowledge of mesenchymal stem cells use and benefits questionnaire results. Values are Presented as percentages of the total sample obtained (n= 19).

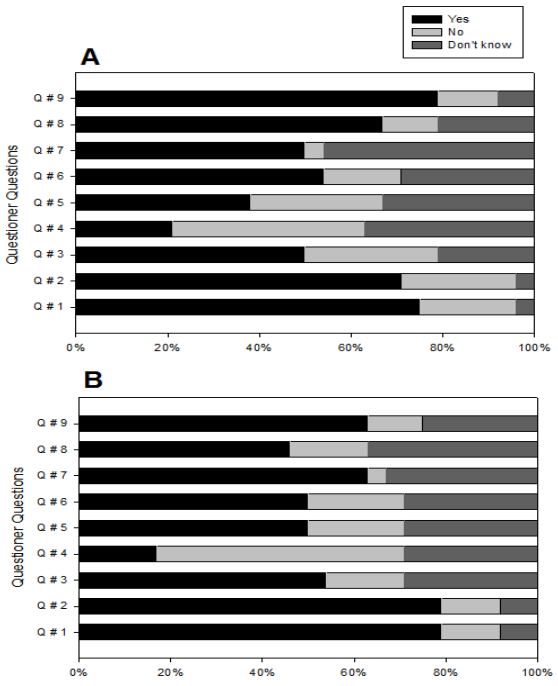

Figure 2: 2012 Questionnaire results obtained from orthopaedics surgeons. (A) Knowledge of biological growth factors use and benefits questionnaire results, (B) Knowledge of mesenchymal stem cells use and benefits questionnaire results. Values are presented as percentages of the total sample obtained (n= 24).

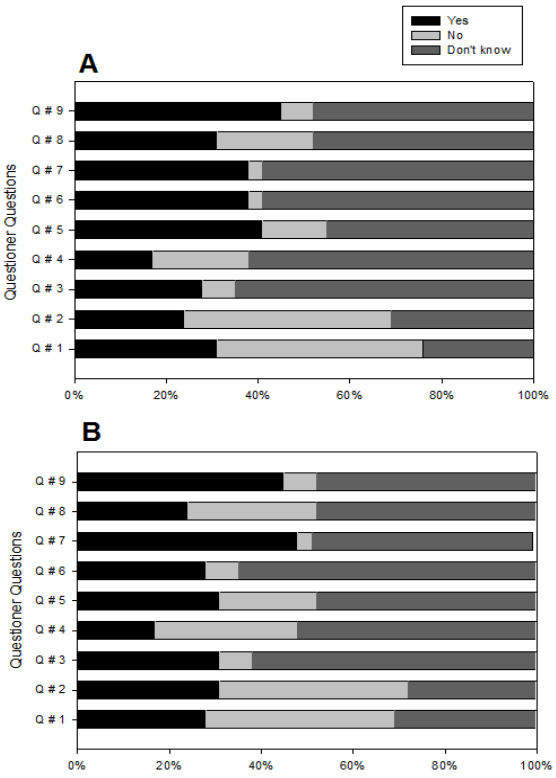

Figure 3: 2012 Questionnaire results obtained from 4th year medical school students. (A) Knowledge of biological growth factors use and benefits questionnaire results, (B) Knowledge of mesenchymal stem cells use and benefits questionnaire results. Values are presented as percentages of the total sample obtained (n= 29).

As of 2005, the value of the collective cell therapy market was estimated to be $26.6 billion. For 2010 and 2015 projections predicted the number to be $56.2 billion and $96.3 billion respectively (*Cell Therapy-Technologies, Markets, and Companies).* Considering the vast amount of resources invested in this market, it is clear from the questionnaire results obtained from this report, that accepting these technologies in Jordan requires clarifications to Jordan's healthcare providers of the financial efficiency of using biotechnology and cell based therapy products in orthopaedics treatments and applications. Furthermore, enhancements in Jordan's medical educational system are required to integrate biotechnology and cell based therapy sciences in Jordan's medical schools curriculum to improve the knowledge of these new technologies among medical school students and to build confidence in the efficacy and efficiency of using these therapy modalities in their future practices.

A vital aspect of accepting, acquiring, and using new biotechnologies and cell based therapy is having a clear unambiguous legal framework that governs manufacturing, importing, processing, and use of these new therapeutic products. The lack of such framework will prevent healthcare suppliers from manufacturing or importing the components of these new technologies,

preventing the healthcare providers from exposing themselves and their patients to these technologies to be able to evaluate its efficiency in reducing treatment cost and time.

In Jordan, the registration procedure for growth factors, cytokines, and their carriers is clear since it is well described in the "Jordanian Criteria of Registration of Drugs". On the other hand and to the best of our knowledge, there are no explicit regulations or rules that govern the manufacturing, circulation, inspection, or registration of biotechnology or cell based therapy products in Jordan.

In the term "Drug" definition, at the Drug and Pharmacy Provisional Law No. (80) of the year 2001, Some can argue that the last part of the definition, i.e. "or a group of substances other than foodstuff which has certain effect on human body or any of its functions.", might implicitly included biotechnology and cell based therapy in it. The argument will be mistaken because engineered-living cells are not considered "substances". Moreover, in section (B) of article (3) of the same law, the statement "and any other substances related to treatment or cure of human beings from disease" does not include engineered tissue and/or cell based therapy products, because again tissues and cells are not substances.

Despite the lack of proper definition that include engineered tissue and cell based therapy, the Drug and Pharmacy Provisional Law formed a committee with the name "The Higher Committee for Medicine & Pharmacy", which according to its responsibility listed in bulletin (14) of Section (A) of Article (4), one might consider engineered tissue and cell based therapy products under "*preparation related to treatment of disease*", however, it is more appropriate to have an explicit bulletin that aims to control tissue engineering and cell based therapy products.

The Drug and Pharmacy Provisional Law formed a committee called "The Technical Committee for Registration of New Drugs", which according to its responsibility listed in bulletin (3) of Section (C) of Article (9), this committee can study engineered tissue and cell based therapy products as "*new developments related to drugs*", however, the term "drug" need to include engineered tissue and cell based therapy products in its definition.

The Jordanian regulations need to be modified with explicit laws that regulate manufacturing, importing, and use of biotechnology and cell based therapy products to reduce bureaucratic committees' procedures, and to cope with the international standards and with the advances made in molecular medicine and molecular biology. These legal modifications will encourage healthcare suppliers and providers in Jordan to explore and evaluate new technologies in medicine that might reduce treatment cost and hospitalization time in Jordan, which will also lead to increasing the relatively low level of awareness of the use, benefits, and applications of biotechnology and cell based therapy products in orthopaedics in Jordan (Table 5).

The sample of orthopaedic surgeons who participated in questionnaire has not been classified according to specialty or experience in order to get a general assessment of the sample participating in the questionnaire, however, a classification of the participants specialty or experience is required in future studies to get a comprehensive and precise assessment. Moreover, the sample of fourth year medical school students is not a representative sample of all medical school students in Jordan; still it was selected to acquire a general assessment for later investigations among medical school and related field's students. The fourth year medical school students were selected to participate in the questionnaire due to the fact that they have just finished their basic biomedical course work and are just about to start their clinical education.

To the best of our knowledge this is the first study investigating the knowledge of growth factors and MSC use and benefits among a sample of Jordanian orthopaedics surgeons and senior medical school students. The investigation team realizes that the participating sample is far from being a representative sample, however, the scientific value lies in having a general assessment as a base line that will trigger further investigations aiming to enhance the educational practices in medical schools as well as the clinical practices in Jordan's health care centers and institutions.

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References

Bolander M E. 1992. Regulation of fracture repair by growth factors. *Proc Soc Exp Biol Med*., **200(2):** 165-170.

Brighton C T and Hunt R M.1991. Early histological and ultrastructural changes in medullary fracture callus. *J Bone Joint Surg Am*., **73(6):** 832-847.

Brighton C T, Shaman P, Heppenstall R B, Esterhai J L Jr., Pollack S R, Friedenberg Z B. 1995. Tibial nonunion treated with direct current, capacitive coupling, or bone graft. *Clin Orthop Relat Res*.,**321**: 223-234.

Cook S D, Baffes GC, Wolffe MW, Sampath TK and Rueger D C.1994. Recombinant human bone morphogenetic protein-7 induces canine long-bone segmental defect model. *Clin Orthop* **301**: 302-312.

Cozen L. 1972. Does diabetes delay fracture healing? *Clin Orthop*., **82**: 134-140.

Dimitriou R, Tsiridis E, Giannoudis P V. 2005. Current concepts of molecular aspects of bone healing. *Injury* **36(12):** 1392-1404.

Einhorn T A. 1998. The cell and molecular biology of fracture healing. *Clin Orthop*.,**355** (Suppl): S7-21.

Flemming, J E. 2002. The cell biology of bone tissue engineering. *Seminar of Arthoplasty* **13(3):** 143-157.

Gehart, T N, Kirker-Head CA, Kriz MJ, Holtrop ME, Hennig, GE, Hipp J, Schelling SH and Wang E. 1993. Healing segmental femoral defects in sheep using recombinant bone morphogenetic protein. *Clin Orthop* **293**: 317-326.

Goshima J V,. Goldberg M. and Caplan AI. 1991. Osteogenic potential of culture-expanded rat marrow cells as assayed *in vivo* with porous calcium phosphate ceramic. *Biomaterials* **12(2):** 253-258.

Goshima J, Goldberg VMand Caplan AI. 1991. The osteogenic potential of culture-expanded rat marrow mesenchymal cells assayed *in vivo* in calcium phosphate ceramic blocks. *Clin Orthop Rel Res* **262**: 298-311.

Haynesworth S E, Gishima J, Goldberg, VM and Caplan A I. 1992. Characterization of cells with osteogenic potential from human marrow. *Bone* **13**: 81-88.

Haynesworth S E, Kadiyala S. and Liang LN. 2002. Mitogenic Stimulation of Human Mesenchymal Stem Cells by Platelet Releasate Suggests a Mechanism for Enhancement of Bone Repair by Platelet Concentrate. Presented at the Orthopaedic Research Society, Dallas, TX.

Hollinger J O, Onikepe A O, MacKrell J, Einhorn T, Bradica G, Lynch S and Hart CE. 2008. Accelerated fracture healing in the geriatric, osteoporotic rat with recombinant human platelet-derived growth factor-BB and an injectable beta-tricalcium phosphate/collagen matrix. *J Orthop Res* **26(1):** 83-90.

Holmes R E, Bucholz RW and Mooney V. 1987. Porous hydroxyapatite as a bone graft aubstitute in a diaphyseal defect: a histometric study. *J Orthop Res* **5**: 114-121.

Johnson K D, Frierson KE , Keller TS, Cook C, Scheinberg R, Zerwekh J, Meyers L, and Sciadini MF. 1996. Porous ceramics as bone graft substitutes in long bone defects: a biomechanical, histological and radiographic analysis. *J Orthop Res* **14**: 351-369.

Khan S N, Cammisa FP, Sandhu HS, Diwan AD, Girardi FP, and Lane JM .2005). The biology of bone grafting. *J Am Acad Orthop Surg* **13**: 77-86.

Kraus K H and Kirker-Head C.2006. Mesenchymal stem cells and bone regeneration. *Vet Surgery* **35**: 232-242.

Lennon D P, Edmison JM and Caplan AI.2001. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. *J Cell Physiol* **187(3):** 345-355.

Linkhart T A, Mohan S and Baylink DJ. 1996. Growth factors for bone growth and repair: IGF, TGF beta and BMP. *Bone* **19(1 Suppl**): 1S-12S.

Loder R T. 1988. The influence of diabetes mellitus on the healing of closed fractures. *Clin Orthop* **232**: 210-216.

Mardon H F, Bee J, Mark VD and Owen ME. 1987. Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats. *Cell Tissue Res* **250**: 157-165.

Martin R B, Chapman M W, Holmes R.E, Sartoris DJ, Shors EC, Gordon JE, Heitter DO, Sharkey NA and Zissimos AG. 1989. Effects of bone ingrowth on the strength and non-invasive assessment of a coralline hydroxyapatite material. *Biomaterials* **10**: 481-488.

Nurminskaya M and Linsenmayer T F. 1996. Identification and characterization of up-regulated genes during chondrocyte hypertrophy. *Dev Dyn* **206(3):** 260-271.

Ohgishi H., Goldberg, VM and Caplan AI. 1989. Heterotopic osteogenesis in porous ceramics, induced by marrow cells. *J Orthop Res* **7**: 568-578.

Owens, M. E. 1988. Stromal stem cells: Marrow-derived osteogenic precursors. In: Evered DAS (Ed). **Cell and Molecular Biology of Vertebrate Hard Tissues** , Wiley: 42-60.

Papa J, Myerson M and Girard P.1993. Salvage, with arthrodesis, in intractable diabetic neuropathic arthropathy of the foot and ankle. *J Bone Joint Surg Am* **75(7):** 1056-1066.

Perlman M H and Thordarson D B.1999.Ankle fusion in a high risk population: an assessment of nonunion risk factors. *Foot Ankle Int* **20(8):** 491-496.

Schmitz M A, Finnegan M, Nataraian R and Champine J. 1999. Effect of smoking on tibial shaft fracture healing. *Clin Orthop Relat Res* **365**: 184-200.

Stevenson S, Cunningham N, Toth T, Davy D and Reddi A H. 1994. The effect of osteogenin (a bone morphogenetic protein) on the formation of bone in ortotopic segmental defects in rats. *J Bone Joint Surg* **76-A**: 1676-1687.

Stuart M J and Morrey B F. 1990. Arthrodesis of the diabetic neuropathic ankle joint. *Clin Orthop* **253**: 209-211.

Tisdel C L, Marcus R E and Heiple K G.1995. Triple arthrodesis for diabetic peritalar neuroarthropathy. *Foot Ankle Int* **16(6):** 332-338.

Wolff D, Goldberg VM and Stevenson S. 1994. Histomorphometric analysis of the repair of a segmental diaphyseal defect with ceramic and titanium fibermetal implants: effects of bone marrow. *J Orthop Res* **12**: 439-446.

Zardiackas L D, Teasdall RD, Black RJ, Jones GS, St. John KR, Dillon LD and Hughes J L. 1994. Torsional properties of healed canine diaphyseal defects grafted with a fibrillar collagen and hydroxyapatite/tricalcium phosphate composite. *J Appl Biomater* **5**: 277-283.

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