المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)
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Jordan Journal of Biological Sciences (JJBS) (ISSN: 1995–6673 (Print); 2307-7166 (Online)): An International Peer- Reviewed Research Journal financed by the Scientific Research Support Fund, Ministry of Higher Education and Scientific Research, Jordan and published quarterly by the Deanship of Research and Graduate Studies, The Hashemite University, Jordan.

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Jordan Journal of Biological Sciences

An International Peer-Reviewed Scientific Journal
Financed by the Scientific Research Support Fund

ISSN 1995-6673

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EDITORIAL PREFACE

It is my pleasure to present the eighth volume of the *Jordan Journal of Biological Sciences* (JJBS) to the audience. JJBS is a refereed, peer reviewed quarterly international journal issued by the Jordanian Ministry of Higher Education and Scientific Research Support Fund in cooperation with The Hashemite University, Zarqa, Jordan. This journal publishes papers in Biological Sciences encompassing all the branches at molecular, cellular and organismal levels.

A group of distinguished scholars have agreed to serve on the Editorial Board. Without the service and dedication of these eminent scholars, JJBS would have never existed. Now, the Editorial Board is encouraged by the continuous growth of the journal and its formation into a true multidisciplinary publication. I am also honored to have the privilege of working with all members of the international advisory board served by a team of highly reputable researchers from different countries across the globe. I am also delighted with our team of national and international reviewers who are actively involved in research in different biological sciences and who provide authors with high quality reviews and helpful comments to improve their manuscripts.

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I would like to reaffirm that the success of the journal depends on the quality of reviewing and, equally, the quality of the research papers published. Five articles from the International Symposium of International Humboldt Conference "Building International Network for Enhancement of Research in Jordan" at Princess Summaya University for Technology in April 2014 appear in this issue. This Conference was chaired by HE Prof. Khaled Toukan, run by eight Jordanian Universities, Jordan Atomic Energy Commission, Jordanian Club of Humboldt Fellows. This Conference was mainly supported by the Alexander von Humboldt Foundation, Jordanian Scientific Research Support Fund, King Abdullah II for Development and Abdul-Hameed Shoman Foundation. The journal invites submission of high quality manuscripts for publication.

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At the end of this preface, I would like to thank our readers and authors for their continuing interest in JJBS, and each member of our editorial and review boards for their continued hard work, support and dedication, which made it possible to bring another new issue of JJBS to the multidisciplinary international audience. My thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous support to Jordan Journal of Biological Sciences. I very much appreciate your support as we strive to make JJBS one of the most leading and authoritative journals in the field of Biological Sciences.

March, 2015

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
The Hashemite University, Zarqa, Jordan

JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673

Jordan Journal of Biological Sciences

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Review

Influencing the Contents of Secondary Metabolites in Spice and Medicinal Plants by Deliberately Applying Drought Stress during their Cultivation[1]

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Received: September 9, 2014 Revised: September 30, 2014 Accepted: October 25, 2014

Abstract

Medicinal plants grown under semi-arid conditions generally reveal significantly higher concentrations of relevant natural products than identical plants of the same species, which are cultivated in moderate climates. So far, there is very limited information on this well-known phenomenon. In the present study, corresponding data are compiled and relevant aspects are discussed. It turns out that metabolic reactions triggered by drought stress are responsible for the higher natural product accumulation in plants grown in semi-arid regions. The related plant physiological and biochemical background can be summarized as follows: In plants suffering drought stress, the water shortage caused by limited water supply triggers stomata closure. As a result, the uptake of CO_2 is markedly decreased and the consumption of reduction equivalents ($NADPH+H^+$) consumed in the course of CO_2-fixation via Calvin cycle declines considerably, generating a massive oversupply of $NADPH+H^+$. Consequently, all metabolic processes are pushed towards the synthesis of highly reduced compounds, such as isoprenoids, phenols or alkaloids. Based on these considerations, impulses for novel practical approaches for enhancing the product quality by deliberately applying drought stress during the cultivation of medicinal plants are outlined. However, as drought stress concomitantly also leads to massive reductions in biomass production, special emphasis must be put on the interference of these stress related effects.

Keywords: Drought stress, Secondary metabolites, Over reduced state, Surplus of energy, Medicinal plants.

1. Introduction

It is well known that plants grown under Mediterranean or semi-arid climate conditions have a much more pronounced taste and are more aromatic than those obtained from similar plants of the same species cultivated in areas with a moderate climate, such as Central Europe. Obviously, under semi-arid conditions the concentrations of aroma relevant natural products are enhanced. Analogous quality differences are observed with regard to medicinal plants, i.e., the content of the corresponding relevant secondary plant products in general is less in plants grown in a moderate Atlantic climate than in those cultivated in semi-arid regions. In the past, this phenomenon was frequently explained by the unsophisticated statement that plants grown in Southern Europe "*are exposed to much more sunlight, resulting in enhanced rates of biosynthesis*". Indeed – on the first sight – such an assertion seems to be consistent; however, we have to bear in mind that the sunlight, generally, is not at all a limiting factor for plant growth. In consequence, even in Central Europe, most plants, growing in open areas without any shading, absorb much more light energy than what they require and what they utilize for photosynthetic CO_2-fixation (Wilhelm and Selmar, 2011). Nonetheless, we have to take into account that - at least in the subtropics - high irradiation often co-occurs with water deficiencies. In consequence, under semi-arid conditions, plants are frequently exposed to drought stress, which affects the entire metabolism. Accordingly, the synthesis and accumulation of secondary metabolites should also be affected. Unfortunately, in the past, these coherences were neglected and had never been given due adequate consideration (Selmar, 2008). Recently, Selmar and Kleinwächter (2013a) have compiled the relevant literature in order to get a comprehensive picture of this

[1] This article summarizes a lecture, on this topic, held by Dirk Selmar at the International Humboldt conference "Building International Network for Enhancement of Research in Jordan" in Amman at Princess Sumaya University for Technology (PSUT) in April 2014; it is also based on a synopsis of three relevant review articles: Selmar and Kleinwächter (2013a), Selmar and Kleinwächter (2013b), Kleinwächter and Selmar (2014).

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issue; and, for the first time, the metabolic background for the stress related enhancement of natural product synthesis was outlined (Selmar and Kleinwächter, 2013b; see Figure 1). The drought stress related water shortage leads to stomata closure and, thus, the uptake of CO_2 is markedly decreased. In consequence, the consumption of reduction equivalents ($NADPH+H^+$) for CO_2-fixation via Calvin cycle declines considerably, generating a massive oversupply of $NADPH+H^+$. According to the law of mass action, any enhancement of reductive power shifts all reactions consuming $NADPH+ H^+$ towards the reduction side of the equilibrium. We have to take into account that these processes might be overlaid by complex regulatory mechanisms. However, even without any change in the enzyme activity, the rate of synthesis of the highly reduced secondary plant products, such as isoprenoids, phenols or alkaloids, should rise under this highly reduced metabolic status.

The main objective and intention of this contribution is to illustrate how these insights could be used for elaborating new practical approaches. By intentionally applying drought stress during the cultivation of spice and medicinal plants, the product quality of the related commodities could be enhanced. However, as drought stress concurrently leads to massive reductions in biomass production, special emphasis must be put on the interference of these two stress related effects.

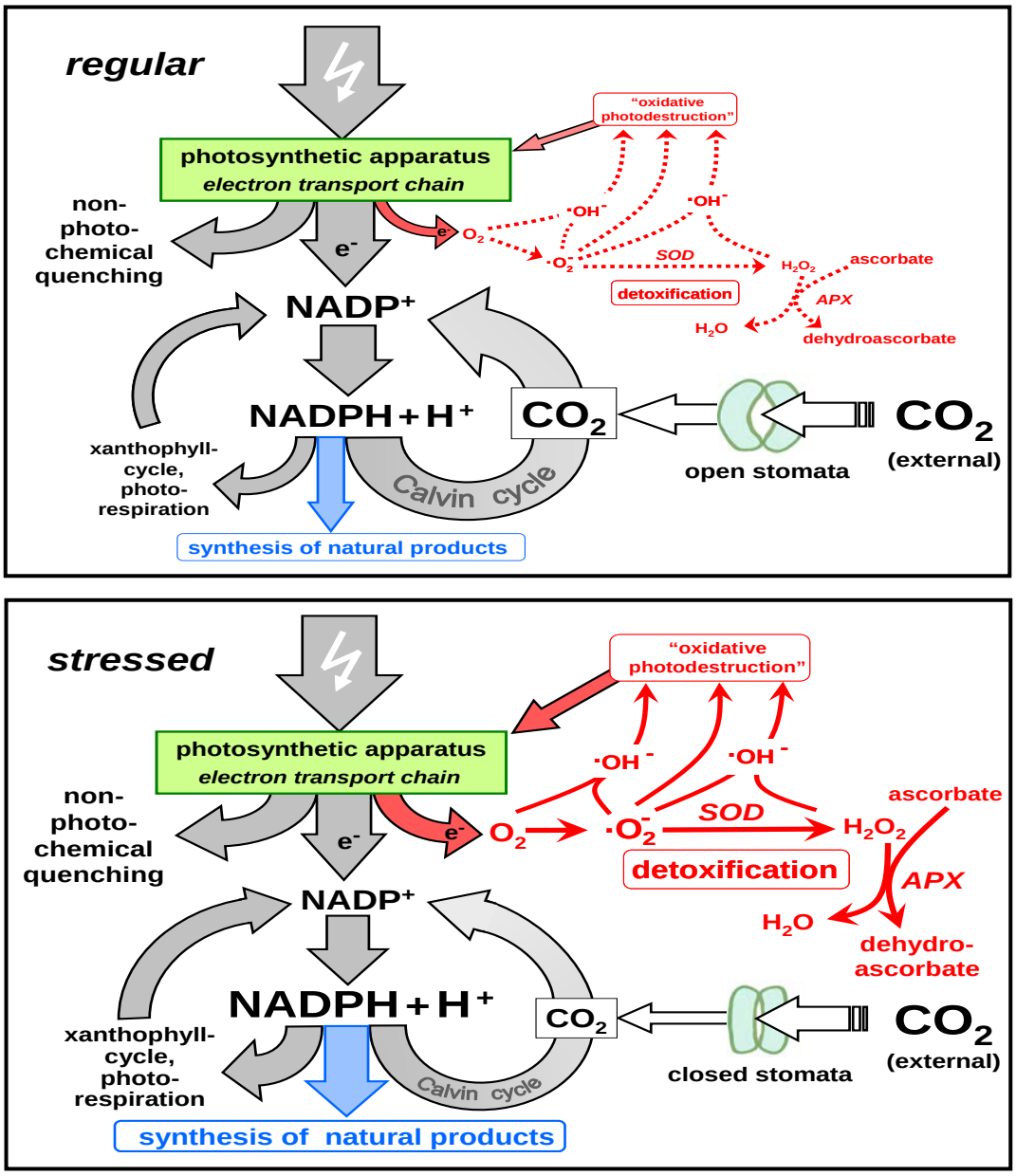

Figure 1. Model scheme energy dissipation and the drought stress related increase of natural product biosynthesis according to Selmar and Kleinwächter (2013b).

2. Synthesis and Accumulation of Natural Products is Enhanced in Drought Stressed Plants

It is well known that the synthesis and accumulation of secondary plant products strongly depends on growth conditions, e.g., on the temperature, the light regime, the nutrient supply, etc. (Gershenzon, 1984; Falk *et al*., 2007). Consequently, much more severe environmental influences, such as the various stress situations, which are known to drastically influence general metabolism (Bohnert *et al*., 1995), should also impact on secondary metabolism, i.e., on the biosynthesis and accumulation of natural products. Astonishingly, there is a tremendous lot of information dealing with the impact of biological stress (e.g., pathogen or herbivore attack) on the synthesis of secondary plant products (Harborne, 1988; Hartmann, 2007; Wink, 2010). Yet, corresponding information on how abiotic stress changes the secondary metabolism is rare. This especially accounts for the knowledge on the related physiological background (Ramakrishna and Ravishankar, 2011; Selmar and Kleinwächter, 2013b). A putative reason for these discrepancies might be due to the fact that host-pathogen and host-herbivore interactions are in general quite assessable, since only one distinct factor is involved, whereas the situation related to abiotic stress is frequently much more complex, since various interferences between numerous factors co-occur, e.g., the increase in light intensity mostly correlates with elevated temperatures, or lower water availability, inducing drought stress, often entails higher salt concentrations in the soil. As a consequence, the results of numerous studies on the impact of a certain abiotic stress on secondary metabolism lack the distinction of influences to other putative stress factors. Notwithstanding, a thorough reviewing of the relevant literature allows to evaluate and to filter out the putative impact of one certain factor on the accumulation of natural products, and thereby enabling decisive deductions. This treatise focuses on the effects of drought stress.

Up till now, numerous studies have shown that plants exposed to drought stress indeed reveal higher concentrations of secondary metabolites than those cultivated under well watered conditions (Table 1). Actually, the increase of this drought stress related concentration seems to be a common feature of all different classes of natural products and corresponding enhancements are reported to occur in the case of simple as well as complex phenols, and also for the various classes of terpenes (Table 1). Similarly, nitrogen containing substances, such as alkaloids, cyanogenic glucosides and glucosinolates, are influenced by drought stress (Table 1). As a general statement, it could be concluded that drought stress frequently enhances the concentration of secondary plant products. In this context, however, we have to consider that drought generally reduces the growth of stressed plants. Thus, due to the reduction in biomass – even without any increase in the overall amount of natural products – their concentration on dry or fresh weight basis could, simply, be enhanced. Accordingly, corresponding explanations are frequently adduced in the literature. As in most of the studies published, solid data on the overall biomass of the plants are lacking, only limited information is available on whether or not the observed concentration increase is also correlated with an enhancement of the total amount of related natural products. Certainly, one reason for this lack of information seems to be due the fact that mostly only certain plant parts or organs, e.g., roots, leaves or seeds, had been the focus of study and investigation, whereas the overall content of natural products on whole plant basis was not of interest. Notwithstanding, at least in some studies, the total contents of secondary plants products are given, or could be calculated from the data presented.

Table 1. Drought stress related concentration increase of natural products - Compilation of literature

Simple Phenols			
Helianthus annuus	chlorogenic acid	massive increase (tenfold)	del Moral, 1972
Prunus persica	total phenols	higher contents	Kubota *et al*., 1988
Thymus capitatus	phenolics	higher contents	Delitala *et al*., 1986
Echinacea purpurea	total phenols	strong increase (67 %)	Gray *et al*., 2003
Crataegus spp.	chlorogenic acid	massive increase (2 - 6fold)	Kirakosyan *et al*., 2004
Hypericum brasiliense	total phenols	strong increase (over 80 %)	de Abreu *et al*., 2005
Trachyspermum ammi	total phenols	strong increase (100 %)	Azhar *et al*., 2011
Labisia pumila	total phenols	significant increase (50 %)	Jaafar *et al*., 2012
Complex Phenols			
Pisum sativum	flavonoids	strong increase (45 %)	Nogués *et al*., 1998
Pisum sativum	anthocyanins	strong increase (over 80 %)	Nogués *et al*., 1998
Crataegus spp.	catechins / epicatechins	massive increase (2 - 12fold)	Kirakosyan *et al*., 2004
Hypericum brasiliense	rutine / quercetin	massive increase (fourfold)	de Abreu *et al*., 2005
Hypericum brasiliense	xanthones	strong increase (over 80 %)	de Abreu *et al*., 2005
Camellia sinensis	epicathecins	higher contents	Hernández *et al*., 2006
Salvia miltiorrhiza	furoquinones	significant increase	Liu *et al*., 2011
Prunella vulgaris	rosmarinic acid	slight increase	Chen *et al*., 2011
Labisia pumila	anthocyane / flavonoids	significant increase	Jaafar *et al*., 2012

Monoterpenes / Essential Oils			
Mentha x piperita ssp.	essential oils	significant increase	Charles *et al*., 1990
Cymbopogon pendulus	geraniol & citral	strong increase	Singh-Sangwan *et al*.,1994
Pinus halepensis	α-pinen, carene	strong increase	Llusià and Peňuelas, 1998
Cistus monospeliensis	caryophyllene	enormous increase	Llusià and Peňuelas, 1998
Satureja hortensis	essential oils	increase	Baher *et al*., 2002
Picea abies	monoterpenes	strong increase	Turtola *et al*., 2003
Pinus silvestris	monoterpenes	strong increase	Turtola *et al*., 2003
Petroselinum crispum	essential oils	strong increase (double)	Petropoulos *et al*., 2008
Salvia officinalis	essential oils	massive increase (2 - 4fold)	Beattaieb *et al*., 2009
Salvia officinalis	monoterpenes	strong increase	Nowak *et al*., 2010
Scrophularia ningpoen.	iridoid glycosides	increase	Wang *et al*., 2010
Nepeta cataria	essential oils	significant increase	Manukyan, 2011
Ocimum basilicum	essential oils	significant increase	Forouzandeh *et al*., 2012
Di- and Triterpenes			
Solanum tuberosum	steroid alkaloids	strong increase	Bejarano *et al*., 2000
Hypericum brasiliense	betulinic acid	strong increase	de Abreu *et al*., 2005
Bupleurum chinense	saikosaponin	significant increase	Zhu *et al*., 2009
Prunella vulgaris	triterpenes	slight increase	Chen *et al*., 2011
Alkaloids			
Senecio longilobus	pyrrolizidine alkaloids	strong increase	Briske and Camp, 1982
Lupinus angustifolius	quinolizidin alkaloids	strong increase	Christiansen *et al*., 1997
Solanum tuberosum	steroid alkaloids	strong increase	Bejarano *et al*., 2000
Glycine max	trigonelline	higher contents	Cho *et al*., 2003
Papaver somniferum	morphine alkaloids	strong increase	Szabó *et al*., 2003
Catharanthus roseus	indole alkaloids	strong increase (with Ca^{2+})	Jaleel *et al*., 2007
Phellodend. amurense	benzylisoquinolines	strong increase	Xia *et al*., 2007
Senecio jacobaea	pyrrolizidine alkaloids	massive increase	Kirk *et al*., 2010
Nicotiana tabacum	nicotiana-alkaloids	strong increase	Çakir and Çebi, 2010
Various Classes			
Manihot esculenta	cyanogenic glucosides	strong increase	de Bruijn, 1973
Triglochin maritima	cyanogenic glucosides	strong increase	Majak *et al*., 1980
Brassica napus	glucosinolates	massive increase	Jensen *et al*., 1996
Coffea arabica	γ-aminobutyric acid	massive increase (tenfold)	Bytof *et al*., 2005
Brassica oleracea	glucosinolates	significant increase	Radovich *et al*., 2005
Brassica carinata	glucosinolates	significant increase	Schreiner *et al*., 2009
Phaseolus lunatus	cyanogenic glucosides	higher content in stressed plants	Ballhorn *et al*., 2011
Tropaeolum majus	glucotropaeolin	significant increase	Bloem *et al*., 2014

In stressed peas (*Pisum sativum*), the massive stress related increase in the concentration of phenolic compounds reported by Nogués *et al*. (1998) resulted in a 25% higher overall amount of anthocyanins (product of biomass and anthocyanin concentration), despite the fact that the total biomass of the pea plants grown under drought stress is just about one third of those cultivated under standard conditions. In the same manner, in *Hypericum brasiliense*, not only the concentration but also the total content of the phenolic compounds is drastically enhanced under drought stress in comparison to the control plants (de Abreu and Mazzafera, 2005). Although the stressed *H. brasiliense* plants had been quite smaller, the product of biomass and concentration of the related phenolics yields in a 10% higher total amount of these compounds. Similarly, in *Labisia pumila*, not only the concentration but also the overall production of total phenolics and flavonoids per plant is enhanced in plants suffering from drought stress (Jaafar *et al*., 2012).

Also, when evaluating the literature on terpenoids, it becomes obvious that, in some studies, an increase of the overall content of these natural products is reported, i.e., the total amount per plant. In sage (*Salvia officinalis*), drought stress induces a massive increase of monoterpenes, which over-compensates the reduction in

biomass (Nowak *et al*., 2010). As a result, the total amount of monoterpenes accumulated in sage is significantly higher in plants suffering from moderate drought stress as compared to well watered controls. In contrast, in catmint and lemon balm plants the slight drought stress related increases in the concentrations of monoterpenes do not compensate the stress related detriment of growth. Consequently, the overall content of terpenoids is lower in the drought stressed plants of *Melissa officinalis* and *Nepeta cataria* than in the corresponding controls (Manukyan, 2011). Obviously, depending on the plant species and the stress conditions applied, the drought stress related concentration enhancement of essential oils does not always result in a corresponding increase in the total amount of natural products, but is compensated or even overcompensated by the related retards in growth. Nonetheless, as a cardinal assertion, it could be stated that in nearly all plants analyzed, the concentrations of secondary plant products are significantly enhanced under drought stress conditions. Only in few cases, apart from the stress induced concentration increase, an enhancement of the total content of corresponding natural compounds is reported. This could be either due to the lack of data on the biomass of the corresponding plants, or to the fact that the stress related decrease in biomass generally over-compensates the increase in the concentration of relevant natural products.

3. Metabolic Background: Higher Reduction Capacity in Drought Stressed Plants

Based on our recurring experience in daily life, we all have internalized that energy saving represents one of the most crucial issues in our subsistence, and corresponding statements have become fundamental. Indeed, on the first sight, it seems appropriate to transfer these considerations into plant biology, and even in reputable textbooks and in scientific publications; corresponding claims and statements can frequently be found. Despite the fact that we are aware of the fact that light energy, in general, is not the limiting factor of photosynthesis in plants (Wilhelm and Selmar, 2011), it is generally stated: "*In order to save energy, plants have evolved a certain mechanism…*" or "*Due to cost-benefit equations, the energy costs for a certain metabolic process must be minimized*". However, when considering this more closely, it becomes obvious that in contrast to heterotrophic organisms, quite other cardinal coherences are crucial for autotrophic plants. In general, plants absorb much more energy than that being required for photosynthetic CO_2-fixation. This is easily demonstrated by the massive enhancement of photosynthesis, when CO_2 concentration is elevated (for details see Wilhelm and Selmar, 2011). Under ambient environmental conditions, the surplus of energy is dissipated effectively by various mechanisms, i.e., non-photochemical quenching, photorespiration, or xanthophyll cycle (Figure 1). However, under drought stress the situation changes markedly. Due to water shortage, stomata are closed, and thus the CO_2-influx is diminished. As a result, far less reduction equivalents are consumed (re-oxidized) within the Calvin cycle. Thus, although the energy dissipating mechanisms are enhanced, they are overstretched. Therefore, electrons are transferred to molecular oxygen and superoxide radicals are generated (Figure 1), which are generally detoxified by superoxide dismutase (SOD) and ascorbate peroxidase (APX). Nonetheless, the chloroplastic reduction status increases and the ratio of $NADPH+H^+$ to $NADP^+$ is enhanced. In consequence, all reactions consuming $NADPH+H^+$, such as the biosynthesis of highly reduced secondary plant products, will be favoured without the need for any change in enzyme activity (Figure 1). As a result of the stress related increase in the biosynthesis rate of highly reduced natural products, large amounts of $NADPH + H^+$ are consumed and the over-reduced state is mitigated. Accordingly, this process could be evaluated as a further and supplementary mechanism for energy dissipation. The extent of this $NADPH + H^+$ re-oxidation is impressively demonstrated by the strong isoprene emission of various drought stressed plants. Several authors postulated that isoprene emission could significantly contribute to the dissipation of the excess of photosynthetic energy (Fall, 1999; Sharkey and Yeh, 2001). A calculation by Magel *et al*. (2006) revealed that under standard conditions, the energy consumption for isoprene biosynthesis accounts for less than 1%. However, at elevated temperatures, when isoprene emission massively increases, the amount of energy dissipated by isoprene biosynthesis could rise up to 25% of the energy supply for net photosynthesis. These considerations demonstrate that the biosynthesis of natural products, indeed, may represent a relevant system to dissipate a surplus of energy. Accordingly, secondary metabolites, apart from their ecological significance, could also be crucial as a part of the supplemental energy dissipation machinery (Grace and Logan, 2000; Wilhelm and Selmar, 2011).

4. Interferences and Interactions with other Factors

In principle, there are two quite different explanations for why the concentrations of natural products are generally enhanced in plants suffering drought stress. This effect could either be due to a stress related change in the benchmark, i.e., the dry or fresh weight used as reference value, or to a real increase in biosynthesis. In the first case, the drought stress related decline in biomass production is associated with a more or less unchanged rate of biosynthesis of natural products. Accordingly, when calculated on a dry or fresh weight base, in the case of the stressed plants, the corresponding concentration is increased. In the second case, stress does result in an authentic increase in the total content of secondary plant products caused by an enhanced biosynthesis. This increase is putatively due to the stress related over-reduction, boosting the biosynthesis of highly reduced compounds. Unfortunately, these simplified causal coherences become much more intransparent, since both issues are frequently overlaid and interfere with numerous factors and side effects.

It is well established that secondary plant products reveal a high significance for the plants by accomplishing various ecological functions within the complex

interactions of plants with their environment, e.g., to repel herbivores, to protect against pathogens, or to attract pollinators (Harborne, 1988; Hartmann, 2007; Wink, 2010). Furthermore, with respect to abiotic stress, various putative functions for secondary plant products are described, e.g., protection against UV-light or too high light intensities, action as compatible solutes, radical scavenging, or reduction of transpiration (Edreva1 *et al*., 2008; Wink, 2010). Due to the tremendous progress in molecular biology, a lot of information is available on how the synthesis of the secondary metabolites is induced, modulated and regulated by various biotic and abiotic factors, respectively (e.g. Hahlbrock *et al*., 2003; Saunders and O´Neill, 2004; Ferry *et al*., 2004). Consequently, the actual synthesis and accumulation of a certain natural product is influenced and determined by numerous factors. Moreover, a particular stress situation, in general, influences several factors, e.g., a h igh irradiation is frequently accompanied by elevated temperatures, a h igh irradiation often parallels the UV-radiation, and elevated temperatures co-occur with higher evaporation rates. As drought also impacts on t he entire ecosystem, it might be associated with a higher herbivore pressure, but a lesser number of pathogens. Consequently, we have to be aware that complex interferences of numerous factors occur, influencing the metabolism of plants exposed to drought stress, and thus, the synthesis and accumulation of natural products. Therefore, we have to consider that complex interferences of numerous factors occur, additionally influencing the metabolism of plants exposed to drought stress, and thus, the synthesis and accumulation of natural products.

A comprehensive and solid scientific investigation of drought stress requires an explicit differentiation between those effects, which are due to the osmotic potential, i.e., the reduced water availability within the cell, and those which are induced by the shift in redox potential caused by the decrease in CO_2 influx, when stomata are closed. Upon first sight, with respect to the decrease in water availability, the actual water potential seems to be an appropriate parameter. However, in response to drought, many plants produce and accumulate osmotic active substances. These substances, denoted as compatible solutes, significantly reduce the water potential of the cell without changing the actual water content. Accordingly, the water content seems to be a better option to describe the impact of drought stress on the water household. Just recently, in addition to the classical gravimetrical methods, a new methodology based on t erahertz technology has been presented to determine the actual water content (Breitenstein *et al*., 2011).

The most appropriate marker for the stress related shift in the redox potential would be the enhanced ratio of $NADPH + H^+$ to $NADP^+$. Unfortunately, the *in situ* ratio of the reduction equivalents could not be quantified without great efforts and inappropriate expenditures. The next choice for the estimation of the over-reduced status in the chloroplastic electron transport chain would be the quantification of oxygen radicals generated. However, due to the extremely high reactivity of these oxygen species, such approach is even more problematic. Alternatively, the enzymes responsible for detoxification of toxic reactive oxygen species (ROS) generated, the superoxide dismutase (SOD) and the ascorbate peroxidase (APX), are frequently estimated as characteristic stress related enzymes. Unfortunately, these enzymes occur in various isoforms and are also part of various signal transduction chains. Accordingly, they do not reliably indicate a specific stress situation. A further alternative is the evaluation of the glutathione system, which is also part of the antioxidative defence against ROS. Yet, the interrelation between the glutathione concentrations and the redox status does not seem to be consistent (Tausz *et al*., 2004). To follow another approach, the occurrence of characteristic stress-metabolites, which are synthesized and accumulated more or less specifically in response to a particular stress situation, is analysed. In this context, proline is in the centre of focus. This amino acid is accumulated as compatible solute in plants suffering from drought stress (Rhodes *et al*., 1999). Unfortunately, the drought stress induced proline accumulation does not occur in all plant species. Alternatively, γ-amino butyric acid (GABA) is quantified, a stress metabolite produced by decarboxylation of glutamic acid (Kinnersley and Turano, 2000). GABA is accumulated in response to drought stress, but, unfortunately, it is also produced, to a high extent, under other various stress conditions (Satya Narayan and Nair, 1990; Bown and Shelp, 1997). Thus, further markers are required; the most promising complementation to the classical markers mentioned so far, is the determination of the abundance of dehydrins. These small protective proteins were first discovered in maturing seeds in the course of late embryogenesis. Moreover, it is well known that dehydrins are also frequently synthesized in plant cells suffering from drought stress (Close, 1997; Allagulova *et al*., 2003; Bouché and Fromm, 2004). These small hydrophilic proteins are thought to reveal various protective functions in desiccating cells (Hara, 2010). Meanwhile, the expression of dehydrins is well established to monitor the impact of drought stress on the metabolism. However, the complexity of such stress reactions was in general vividly demonstrated by Kramer *et al*. (2010), who demonstrated that the expression of dehydrins and the accumulation of the stress metabolite GABA follow different time patterns in coffee seeds whilst drying. Clearly, various metabolic responses occur in parallel. So, we have to consider (as outlined above) the fact that in leaves exposed to drought stress, apart from the impact of the decrease in water availability, the overreduction due to stomata closure entails numerous metabolic responses. Therefore, the elucidation of the entire metabolic syndrome, induced in drought stressed medicinal plants, requires a combination of several markers.

5. Practical Applications

Indeed, the stress related impacts on metabolism are multilayered. However, the corresponding effects with respect to the secondary metabolism are even more multifarious, since many mechanisms of elicitation and induction are overlaid, and numerous effects and processes are counteracting. This especially accounts for drought stress. In addition to the direct influence on

secondary metabolism outlined above, we have, first of all, to consider the consequences and aftereffects of drought stress on g eneral metabolism, which causes severe repercussions, such as losses in biomass, retards in development, or changes in growth behaviour. Moreover, marked changes in the general metabolic status regularly shift the ratio between generative and vegetative characteristics of tissues and organs (Houter and Nederhoff, 2007). This means that the drought stress might also change the *source-sink* properties of the entire plant. This – in addition to the inducing factors already mentioned – also impacts on the overall performance of the biosynthesis, translocation and accumulation of secondary plant products. Consequently, apart from the enhanced biosynthesis due to the stress related over-reduction, drought stress also impacts on the allocation and accumulation of natural products. In consequence, the overall effects of drought stress on secondary metabolism, and, thus, on the quality of the medicinal plants are multi-layered and very complex. Indeed, as outlined above, a deliberate application of drought stress during the cultivation of medicinal plants should principally result in an increase in the biosynthesis of secondary metabolites. However, due to the other co-occurring impacts and the various effects mentioned above, this enhancement is frequently compensated or even over-compensated. Thus, a general recommendation for the deliberate application of drought stress to increase the quality of medicinal plants cannot be made. Notwithstanding, in many cases, such approach undoubtedly could be successful. However, we always have to sound out the advantages and the drawbacks of a corresponding approach. To facilitate corresponding assessments, Selmar and Kleinwächter (2013a; 2014) have proposed to answer some simple questions:

- What kind and which level of stress enhance the accumulation of the desired compounds without causing too high losses in biomass?
- What is required, a high concentration or a large bulk? (total amount of natural products versus high concentrations in the drug)
- Are the substances synthesized and accumulated in source or in sink tissues?
- Are the substances synthesized and accumulated in generative or vegetative organs?
- Could the accumulation also be increased by phytohormone treatments (e.g., methyl jasmonate, salicylic acid)?
- Should the stress be applied only within a certain phase of cultivation or during a special developmental phase in order to obtain maximal quality?

In forthcoming approaches, we have to elaborate how these objectives could be transferred into appropriate agricultural applications. Indeed, most simple approaches should be based on altering the irrigation regime (Radovich *et al*., 2005); but this seems to be restricted to semi-arid regions, where supplemental watering is required. In moderate climates, the water supplied by rainfall cannot be diminished. However, the moisture content of the soil could be influenced by the choice of the cultivation area or by some simple measures, e.g., fields with slopes will retain markedly less water than flat plains consisting of soils with the same water holding capacity. Even in plains, the drainage effect could be realized artificially by certain cultivation measures. The creation of a furrow and ridge system, which is frequently used for surface irrigation in arid regions is also appropriate for generating drought stress situations by establishing significant gradients of soil moisture contents. Without any irrigation, the soil water content in the furrows will nearly be the same as in untreated fields, but the moisture content in the ridges strongly decreases due to an enhanced drainage effect. Moreover, elevated drainage could be achieved by increasing the proportion of sand in the soil. However, such an approach would irreversibly change the character of the soil and should only be applied in exceptional cases.

Alternative strategies to influence the product quality are based on the application of phytohormones or growth regulators to induce and modulate the stress related signal transduction chain for secondary metabolite synthesis. In this context, jasmonic acid is of special interest. Due to its physico-chemical properties, in general, the active growth regulator is not applied; what is actually applied is its volatile methyl ester. After uptake into the cells, it is hydrolysed, and the active jasmonic acid is generated. Corresponding approaches had been successfully employed in numerous tissue and cell culture systems to enhance the concentration of secondary metabolites (Namdeo, 2007). Meanwhile, methyl jasmonate was also used for effective elicitation of natural products in intact plants, e.g., to increase the synthesis of indole alkaloids in seedlings of *Catharanthus roseus* and *Chinona ledgerina*, respectively (Aerts *et al*., 1994). The application of methyl jasmonate also enhanced the contents of phenols and monoterpenes in *Ocimum basilicum* (Kim *et al*., 2006) and the concentration of glucosinolates in *Brassica rapa* (Loiavmäki *et al*., 2004). These promising approaches indicate that the usage of methyl jasmonate seems to be an encouraging alternative for quality improvement of medicinal and spice plants instead of a direct drought treatment. Analogously, salicylic acid, another endogenous signal substance, was also used for impacting on the synthesis and accumulation of secondary metabolites. The application of this signal transducer, which is involved in systemic resistance of plants (Durrant and Dong, 2004), strongly increased the content of phenolics in *Echinacea purpurea* plants (Kuzel *et al*., 2009) and the accumulation of glucosinolates in oilseed rape (*Brassica napus*) (Kiddle *et al*., 1994). However, apart from their effects on secondary metabolism, salicylic acid and methyl jasmonate also impact on primary metabolism and developmental processes, e.g., by inducing senescence and thereby causing retardation of growth. Thus, in the same manner as outlined for the deliberate application of drought stress, when the application of growth regulators and phytohormones is considered to increase the product quality, a thorough balancing of pros and cons is required. This however, necessitates a sound and comprehensive knowledge of the related metabolic background.

6. Conclusion

The metabolic background for the stress related enhancement of natural product synthesis is outlined: Drought stress leads to stomata closure, and thereby generating a h ighly over-reduced metabolic status. According to the law of mass action, the enhancement of reductive power (NADPH+ H^+) causes an increase in the synthesis of all highly reduced secondary plant products, such as isoprenoids, phenols or alkaloids. By intentionally applying drought stress during the cultivation of spice and medicinal plants, the concentration of natural products, and thus the product quality of the related commodities, could be enhanced. However, due to numerous interactions, the related increase could be compensated by other metabolic responses, such as growth reduction, decrease in biomass production, or the onset of senescence. Thus, for each particular case, a t horough balancing of pros and cons is necessary, which, of course, requires much more research in order to comprehensively elucidate the entire issue.

References

Aerts RJ, Gisi D, de Carolis E, de Luca V and Baumann TW. 1994. Methyl jasmonate vapor increases the developmentally controlled synthesis of alkaloids in *Catharanthus* and *Cinchona* seedlings. *Plant J,* **5**: 635-643.

Allagulova CR, Gimalov FR, Shakirova FM and Vakhitov VA. 2003. The plant dehydrins: structure and putative functions. *Biochem*, **68**: 945–951.

Azhar N, Hussain B, Ashraf YM and Abbasi KY. 2011. Water stress mediated changes in growth, physiology and secondary metabolites of desi ajwain (*Trachyspermum ammi*). *Pak J Bot,* **43**: 15-19.

Baher ZF, Mirza M, Ghorbanli M and Rezaii MB. 2002. The influence of water stress on plant height, herbal and essential oil yield and composition in *Satureja hortensis* L. *Flavour Frag J,* **17**: 275–277.

Ballhorn DJ, Kautz S, Jensen M, Schmitt S, Heil M and Hegeman AD. 2011. Genetic and environmental interactions determine plant defences against herbivores. *J Ecol,* **99**: 313–326.

Bejarano L, Mignolet E, Devaux A, Espinola N, Carrasco E and Larondelle Y. 2000. Glycoalkaloids in potato tubers: the effect of variety and drought stress on the α-solanine and α-chaconine contents of potatoes. *J Sci Food Agric,* **80**: 2096–2100.

Bettaieb I, Zakhama N, Aidi Wannes W, Kchouk ME and Marzouk B. 2009. Water deficit effects on *Salvia officinalis* fatty acids and essential oils composition. *Sci Hortic,* **120**: 271-275.

Bloem E, Haneklaus S, Kleinwächter M, Paulsen J, Schnug E and Selmar D. 2014. Stress-induced changes of bioactive compounds in *Tropaeolum majus* L. *Ind Crops Products,* **60:** 349–359.

Bohnert HJ, Nelson DE and Jensen RG. 1995. Adaptations to environmental stresses. *Plant Cell*, **7**: 1099-1111.

Bouché N and Fromm H. 2004. GABA in plants: just a metabolite. *Trends Plant Sci,* **9**: 100–115.

Bown AW and Shelp BJ. 1997. The metabolism and functions of γ-aminobutyric acid. *Plant Physiol*, **115**: 1–5.

Breitenstein B, Scheller M, Shakfa MK, Kinder T, Müller-Wirts T, Koch M and Selmar D. 2011. Introducing terahertz technology into plant biology: A novel method to monitor changes in leaf water status. *J Appl Bot Food Qual,* **82**: 158-161.

Briske DD and Camp BJ. 1982. Water stress increases alkaloid concentrations in threadleaf groundsel (*Senecio longilobus*). *Weed Sci,* **30**: 106-108.

Bytof G, Knopp S-E, Schieberle P, Teutsch I and Selmar D. 2005. Influence of processing on the generation of γ-aminobutyric acid in green coffee beans. *Eur Food Res Technol*, **220**: 245–250.

Çakir R and Çebi U. 2010. The effect of irrigation scheduling and water stress on the maturity and chemical composition of Virginia tobacco leaf. *Field Crop Res,* **119**: 269-276.

Charles DJ, Joly RJ and Simon JE. 1990. Effects of osmotic stress on the essential oil content and composition of peppermint. *Phytochemistry*, **29**: 2837-2840.

Chen Y, Guo Q, Liu L, Liao L and Zaibiao Z. 2011. Influence of fertilization and drought stress on the growth and production of secondary metabolites in *Prunella vulgaris* L. *J Med Plants Res,* **5**: 1749-1755.

Cho Y, Njitiv N, Chen X, Lightfood DA and Wood AJ. 2003. Trigonelline concentration in field-grown soybean in response to irrigation. *Biol Plantarum,* **46**: 405-410.

Christiansen JL, Jørnsgard B, Buskov S and Olsen CE. 1997. Effect of drought stress on content and composition of seed alkaloids in narrow-leafed lupin, *Lupinus angustifolius* L. *Eur J Agron,* **7**: 307-314.

Close TJ. 1997. Dehydrins: a commonality in the response of plants to dehydration and low temperature. *Physiol Plantarum*, **100**: 291–296.

de Abreu IN and Mazzafera P. 2005. Effect of water and temperature stress on the content of active constituents of *Hypericum brasiliense* Choisy. *Plant Physiol Bioch,* **43**: 241–248.

de Bruijn GH. 1973. The cyanogenic character of cassava (*Manihot esculenta*). In: Nestel B and MacIntyre R (Eds), **Chronic Cassava Toxicity**. International Development Research Centre, Ottawa, pp. 43-48.

Delitala I-F, Gessa C and Solinas V. 1986. Water stress and flexibility of phenolic metabolism in *Thymus capitatus. Fitoterapia,* **57**: 401–408.

del Moral R. 1972. On the variability of chlorogenic acid concentration. *Oecologia,* **9**: 289–300.

Durrant WE and Dong X. 2004. Systemic aquired resistance. *Annu Rev Phytopathol,* **42**: 185-209.

Edreva1 A, Velikova V, Tsonev T, Dagnon S, Gürel A, Aktaş L and Gesheva E. 2008. Stress-protective role of secondary metabolites: Diversity of functions and mechanisms. *Gen Appl Plant Physiol,* **34**: 67-78.

Falk KL, Tokuhisa JG and Gershenzon J. 2007. The effect of sulfur nutrition on plant glucosinolate content: Physiology and molecular mechanisms. *Plant Biol,* **9**: 573-581.

Fall R. 1999. Biogenic emissions of volatile organic compounds from higher plants. In: Hewitt CN (Ed), **Reactive hydrocarbons in the atmosphere**. Academic Press, pp. 41-95.

Ferry N, Edwards MG, Gatehouse JA and Gatehouse AMR. 2004. Plant–insect interactions: Molecular approaches to insect resistance. *Curr Opin Biotech,* **15**: 155-161.

Forouzandeh M, Fanoudi M, Arazmjou E and Tabiei H. 2012. Effect of drought stress and types of fertilizers on the quantity and quality of medicinal plant Basil (*Ocimum basilicum* L.). *Ind J Innov Develop,* **1**: 734-737.

Gershenzon J. 1984. Changes in the levels of plant secondary metabolites under water and nutrient stress. *Recent Adv Phytochemistry,* **18**: 273-320.

Grace SC and Logan BA. 2000. Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. *Philos T Roy Soc B,* **355**: 1499-1510.

Gray DE, Pallardy SG, Garrett HE and Rottinghaus G. 2003. Acute drought stress and plant age effects on alkamide and phenolic acid content in purple coneflower roots. *Planta Med,* **69**: 50-55.

Hahlbrock K, Bednarek P, Ciolkowski I, Hamberger B, Heise A, Liedgens H, Logemann E, Nürnberger T, Schmelzer E and Somssich IE. 2003. Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. *P Natl A Sci USA*, **100**: 14569-14576.

Hara M. 2010. The multifunctionality of dehydrins - An overview. *Plant Signaling Behav,* **5**: 1-6.

Harborne JB. 1988. **Introduction to Ecological Biochemistry**, third ed. Academic Press, London.

Hartmann T. 2007. From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochem.,* **68**: 2831–2846.

Hernández I, Alegre L and Munné-Bosch S. 2006. Enhanced oxidation of flavan-3-ols and proanthocyanidin accumulation in water-stressed tea plants. *Phytochem.,* **67**: 1120–1126.

Houter B and Nederhoff E. 2007. Controlling plant growth - Part 3. Plant balance: 'vegetative' or 'generative'. *Grower*, **62**: 42-44.

Jaafar HZ, Ibrahim MH and Mohamad Fakri NF. 2012. Impact of soil field water capacity on secondary metabolites, phenylalanine ammonia-lyase (PAL), maliondialdehyde (MDA) and photosynthetic responses of Malaysian kacip fatimah (*Labisia pumila* Benth). *Molecules,* **17**: 7305-7322.

Jaleel CA, Manivannan P, Sankar B, Kishorekumar A, Gopi R, Somasundaram R and Panneerselvam R. 2007. Induction of drought stress tolerance by ketoconazole in *Catharanthus roseus* is mediated by enhanced antioxidant potentials and secondary metabolite accumulation. *Colloid Surface B,* **60**: 201-206.

Jensen CR, Mogensen VO, Mortensen G, Fieldsend JK, Milford GFJ, Andersen MN and Thage JH. 1996. Seed glucosinolate, oil and protein contents of field-grown rape (*Brassica napus* L.) affected by soil drying and evaporative demand. *Field Crop Res,* **47**: 93-105.

Kiddle GA, Doughty KJ and Wallsgrove RM. 1994. Salicylic acid-induced accumulation of glucosinolates in oilseed rape (*Brassica napus* L.) leaves. *J Exp Bot,* **45**: 1343-1346.

Kim HJ, Chen F, Wang X and Rajapakse NC. 2006. Effect of methyl jasmonate on secondary metabolites of sweet basil (*Ocimum basilicum* L.). *J Agric Food Chem,* **54**: 2327-2332.

Kinnersley AM and Turano FJ. 2000. Gamma aminobutyric acid (GABA) and plant responses to stress. *Cr Rev Plant Sci,* **19**: 479–509.

Kirakosyan A, Kaufman P, Warber S, Zick S, Aaronson K, Bolling S and Chul Chang S. 2004. Applied environmental stresses to enhance the levels of polyphenolics in leaves of hawthorn plants. *Physiol Plantarum,* **121**: 182-186.

Kirk H, Vrieling K, van der Meijden E and Klinkhamer PGL. 2010. Species by environment interactions affect pyrrolizidine alkaloid expression in *Senecio jacobaea*, *Senecio aquaticus*, and their hybrids. *J Chem Ecol,* **36**: 378-387.

Kleinwächter M and Selmar D. 2013. Influencing the product quality by applying drought stress during the cultivation of medicinal plants, In: Wani, M.R. (Ed.), **Physiological Mechanism and Adaptation Strategies in Plants Under Changing Environment**. Springer Verlag, pp. 57-73.

Kleinwächter M and Selmar D. 2014. Drought stress to enhance the quality of medicinal plants. A review. *Agron Sustainable Dev*, - *in press*

Kramer D, Breitenstein B, Kleinwächter M and Selmar D. 2010. Stress metabolism in green coffee beans (*Coffea arabica* L.): Expression of dehydrins and accumulation of GABA during drying. *Plant Cell Physiol,* **51**: 546–553.

Kubota N, Mimura H and Shimamura K. 1988. The effects of drought and flooding on the phenolic compounds in peach fruits. *Scientific Reports of the Faculty of Agriculture, Okayama University,* **171**: 17-21.

Kuzel S, Vydra J, Triska J, Vrchotova N, Hruby Mand Cigler P. 2009. Elicitation of pharmacologically active substances in an intact medical plant. *J Agric Food Chem, 57*: 7907-7911.

Liu H, Wang X, Wang D, Zou Z and Lianga Z. 2011. Effect of drought stress on growth and accumulation of active constituents in *Salvia miltiorrhiza* Bunge. *Ind Crop Prod,* **33** :84-88.

Llusià J and Peñuelas J. 1998. Changes in terpene content and emission in potted Mediterranean woody plants under severe drought. *Can J Bot,* **76**: 1366-1373.

Loivamäki M, Holopainen JK and Nerg AM . 2004. Chemical changes induced by methyl jasmonate in oilseed rape grown in the laboratory and in the field. *J Agric Food Chem,* **52**: 7607-7613.

Magel E, Mayrhofer S, Müller A, Zimmer I, Hampp R and Schnitzler JP. 2006. Photosynthesis and substrate supply for isoprene biosynthesis in poplar leaves. *Atmos Environ*, **40**: 138-151.

Majak W, McDiarmid R E, Hall JW and van Ryswyk AL. 1980. Seasonal variation in the cyanide potential of arrowgrass (*Triglochin maritima*). *Can J Plant Sci,* **60**: 1235-1241.

Manukyan A. 2011. Effect of growing factors on productivity and quality of lemon catmint, lemon balm and sage under soilless greenhouse production: I. Drought stress. *Medicinal Aromatic Plant Sci Biotech,* **5**: 119-125.

Namdeo AG. 2007. Plant cell elicitation for production of secondary metabolites: A review. *Pharmacogn Rev,* **1**: 69-79.

Nogués S, Allen DJ, Morison JIL and Baker NR. 1998. Ultraviolet-B radiation effects on water relations, leaf development, and photosynthesis in droughted pea plants. *Plant Physiol,* **117**: 173–181.

Nowak M, Manderscheid R, Weigel H-J, Kleinwächter M and Selmar D. 2010. Drought stress increases the accumulation of monoterpenes in sage (*Salvia officinalis*), an effect that is compensated by elevated carbon dioxide concentration. *J Appl Bot Food Qual,* **83**: 133-136.

Petropoulos SA, Daferera D, Polissiou MG and Passam HC. 2008. The effect of water deficit stress on the growth, yield and composition of essential oils of parsley. *Sci. Hortic,* **115**: 393-397.

Radovich TJK, Kleinhenz MD and Streeter, JG. 2005. Irrigation timing relative to head development influences yield components, sugar levels, and glucosinolate concentrations in cabbage. *J Amer Soc Hort Sci,* **130**: 943-949.

Ramakrishna A and Ravishankar GA. 2011. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal Behav,* **6**: 1720-1731.

Rhodes D, Verslues PE and Sharp RE. 1999. Role of amino acids in abiotic stress resistance. In: Singh BK (Ed.), **Plant Amino Acids: Biochemistry and Biotechnology.** Marcel Dekker Inc, New York, Basel, Hong Kong, pp. 319-356.

Satya Narayan V and Nair PM. 1990. Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. *Phytochem.,* **29**: 367–375.

Saunders J and O´Neill NR. 2004. The characterization of defense responses to fungal infection in alfalfa. *Biocontrol,* **49**: 715-728.

Schreiner M, Beyene B, Krumbein A and Stützel H. 2009. Ontogenetic changes of 2-propenyl and 3-indolylmethyl glucosinolates in *Brassica carinata* leaves as affected by water supply. *J Agric Food Chem,* **57**: 7259-7263.

Selmar D. 2008. Potential of salt and drought stress to increase pharmaceutical significant secondary compounds in plants. *Landbauforsch Völk,* **58**: 139–144.

Selmar D and Kleinwächter M. 2013a. Influencing the product quality by deliberately applying drought stress during the cultivation of medicinal plants. *Ind Crop Prod,* **42**: 558-566.

Selmar, D and Kleinwächter, M. 2013b. Stress enhances the synthesis of secondary plant products: The impact of the stress-related over-reduction on the accumulation of natural products. *Plant Cell Physiol,* **54**: 817-826.

Sharkey TD and Yeh S. 2001. Isoprene emission from plants. *Annu Rev Plant Phys,* **52**: 407-436.

Singh-Sangwan N, Abad Farooqi AH and Sangwan RS. 1994. Effect of drought stress on growth and essential oil metabolism in lemongrasses. *New Phytol,* **128**: 173-179.

Smirnoff N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytol,* **125**: 27-58.

Szabó B, Tyihák E, Szabó LG and Botz L. 2003. Mycotoxin and drought stress induced change of alkaloid content of *Papaver somniferum* plantlets. *Acta Bot Hung,* **45**: 409-417.

Tausz M, Šircelj H and Grill D. 2004. The glutathione system a stress marker in plant ecophysiology: is a stress-response concept valid? *J Exp Bot,* **55**: 1955-1962.

Turtola S, Manninen A-M, Rikala R and Kainulainen P. 2003. Drought stress alters the concentration of wood terpenoids in Scots pine and Norway spruce seedlings. *J Chem Ecol,* **29**: 1981-1995.

Wang DH, Du F, Liu HY and Liang ZS. 2010. Drought stress increases iridoid glycosides biosynthesis in the roots of *Scrophularia ningpoensis* seedlings. *J Med Plants Res,* **4**: 2691-2699.

Wilhelm C and Selmar D. 2011. Energy dissipation is an essential mechanism to sustain the viability of plants: the physiological limits of improved photosynthesis. *J Plant Physiol,* **168**: 79-87.

Wink M. 2010. Introduction: Biochemistry, Physiology and Ecological Functions of Secondary Metabolites. In: Wink M (Ed.), **Biochemistry of Plant Secondary Metabolism**. Wiley-Blackwell, pp. 1-19.

Xia L, Yang W and Xiufeng Y. 2007. Effects of water stress on berberine, jatrorrhizine and palmatine contents in amur corktree seedlings. *Acta Ecol Sin,* **27**: 58-64.

Zhu Z, Liang Z, Han R and Wang X. 2009. Impact of fertilization an drought response in the medicinal herb *Bupleurum chinense* DC.: Growth and saikosaponin production. *Ind Crop Prod* **29**: 629–63.

JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 11 - 15

Jordan Journal of Biological Sciences

The Association between Anti-Oxidant/Redox Status and Sister Chromatid Exchanges in Down Syndrome Individuals

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Received: October 7, 2014 Revised: November 2, 2014 Accepted: November 7, 2014

Abstract

The main objective of this project is to study the possible association between anti-oxidant/redox status and DNA instability in Down syndrome. The activities of 5 antioxidant enzymes were studied in 19 Down syndrome (DS) cases and in age- and sex-matched normal controls. Sister Chromatid Exchanges (SCE) were measured in lymphocyte cultures derived from all DS and control subjects. All DS and control individuals had normal hematological parameters, but the proliferation and mitotic indices were significantly lower in the DS- than in the controls-derived lymphocyte cultures, while the average generation time was higher than that in the controls. The specific activity of superoxide dismutase in the DS individuals was 40% higher than that in the controls, while the specific activity of glutathione S-transferase in the DS group was significantly lower than that in the controls ($P \leq 0.05$). Catalase and glutathione peroxides' activities were not different between the two groups ($P > 0.05$). SCE rate in the DS derived cultures was significantly higher ($P < 0.001$) than that of the controls. DS individuals have a higher oxidative stress, higher superoxide dismutase activities and higher rates of SCE in their derived lymphocyte cultures compared to those of the controls. We claim that such differences may have resulted from the over expression of superoxide dismutase gene, leading to imbalanced cellular antioxidant mechanisms and, consequently, resulted in a high concentration of free radicals that destabilized the DNA as expressed by the high rate of SCE.

Key Words: Down syndrome, Antioxidant enzymes, Catalase, Glutathione peroxidase, Glutathione S- transferase, Oxidative stress, Sister chromatid exchanges (SCE).

1. Introduction

Trisomy 21, known as Down Syndrome (DS), is one of the most common human chromosomal disorders in live born children (1 in 800–1000) (Capone, 2004), leading to the characteristic phenotypes of DS individuals, which include a constant common feature of early mental decline and premature aging. DS affected individuals may also suffer from developmental abnormalities, specific cardiac and gastrointestinal congenital malformations, thyroid dysfunction and increased risk for various types of leukemia (Antonarakis and Epstein, 2006; Roizen and Patterson, 2003; Shaw *et. al*, 2006). The characteristic phenotypes of DS were suggested to be the ultimate results of the triplication of the Down Syndrome Critical Region (DSCR) 21q22.1 - 21q22.3 (Vesa *et. al*, 2005), which includes the gene coding for the enzyme, copper zinc superoxide dismutase (SOD1), implicated in the conversion of the harmful superoxide anion radical ($O_2^{\cdot-}$) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Gardiner and Davisson, 2000; Sherman *et. al*, 1983). H_2O_2 can be converted by catalase (CAT) and by (selenium-containing) glutathione peroxidase (GPx) to water. The triplication of this DSCR leads to imbalance in the ratio of SOD1 to CAT and GPx, located on ot her chromosomes which results in excess H_2O_2 in the cells. Consequently, DS individuals are expected to have oxidative stress (OS), due to the imbalance between the generation and the removal of the reactive oxygen species (ROS) and result in harmful effects on different cellular macromolecules including DNA, where they cause DNA instability and induce single and double strand DNA breaks (Devasagayam *et. al*, 2004); they were suggested to be the cause of the high rate of cancer appearance in DS individuals (Wiseman and Halliwell, 1996).

In the present study, the association of the catalytic activities of the red blood cell antioxidant enzymes superoxide dismutase (EC 1.15.1.1) (SOD1), catalase (EC 1.11.1.6) (CAT), glutathione peroxidases (EC 1.11.1.9) (GPx), glutathione S-transferases (EC 2.5.1.18) (GST) were investigated along with the rate of sister chromatid

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exchanges (SCE) in lymphocyte cultures derived from DS individuals and sex and age matched controls.

2. Materials and Methods

2.1. Subjects and Blood Samples

Nineteen DS affected children (5 to 16 years old) were recruited from a special school for children with special needs in Jordan, and another group of 19 age- and sex-matched normal healthy children were used as controls. Blood sample of 5mL were collected from each participating child in heparinized tubes. An informed consent was obtained in all cases from the children's parents in accordance with the Helsinki declaration (Helsinki, 2000). Direct Complete Blood Count (CBC) was performed using an automated blood counter (COPAS Micros, France).

2.2. Determination of Enzymes Activities

All enzymes' activities were assayed in a hemolysate of the red blood cells by the addition of (5: 3) mixture of absolute ethyl alcohol and trichloromethane, followed by centrifugation at 18000 Xg for 60 minutes to precipitate Hb (Alkaraki *et. al*, 2010). All enzyme assays were measured spectrophtometrically through using UNICAM UV/Visible Spectrophotometer. Negative control reactions were prepared by substituting the hemolysate fraction by distilled water.

SOD1 activity was measured based upon pyrogallol auto-oxidation as reported earlier (Marklund and Marklund, 1974; Sun *et. al*, 1988), while CAT activity was assayed as reported previously (Aebi, 1984), GPx and GST activities were measured according to earlier reports (Habig *et. al*, 1974; Paglia and Valentine, 1967).

2.3. Preparation of Lymphocyte Cultures and Stained Metaphase Chromosomes

All cultures were prepared in duplicates using 25 cm^2 culture flasks. Each culture was initiated as reported earlier (Verma and Babu, 1989) with minor modifications. For each culture, fresh heparinized whole blood (0.8 mL) was added to 9.2 mL of RPMI 1640 culture media (Euroclone, Europe) supplemented with 10% fetal bovine serum (Euroclone, Europe), 1% penicillin-streptomycin (1000 U/µl, 10000 µg/µL) (Euroclone, Europe), 1% of 200 mM L-Glutamine (Sigma), 7.5 µg/mL phyto-haemagglutinin (Sigma) and 25 µg/mL 5′-Bromodeoxy-Uridine (BrdU) from Sigma Company. The culture flasks were incubated in the dark at 37°C in an upright position for 72 hours in a humidified 5% CO2 atmosphere incubator. Two hours prior to cell harvesting, cultures were treated with 100 µL of 10 µg/mL colcemid (Gibco, USA). Lymphocytes, from each culture, were collected by centrifugation at 275 Xg for 8 minutes using MPW–350R centrifuge. The lymphocytes were then used to prepare metaphase chromosomes that were differentially stained according to the fluorescence plus Giemsa method (Ishii and Bender, 1980). Permanent chromosomal preparations were made by mounting in D.P.X. mounting medium (WWR International Ltd., England). All slides were coded independently and scored by a blind study using a 100x oil-immersion objective mounted on a Labrolux 11 bright-field microscope (Zeiss, Germany).

2.4. Analysis of Sister Chromatid Exchanges and Cell Kinetics

The Mitotic Index (MI) and the Proliferative Index (PI) of each culture were measured to reflect the cytotoxicity of the conditions in the culture. The MI was calculated as the percentages of metaphases, providing that at least 1000 well spread cells were examined from each culture (Preston *et. al*, 1987). PI was determined by classifying 100 cells that passed through the replication cycle once (M1), twice (M2), three (M3) or more (>M3) using the following formula: PI = [M1%+ (2× M2%) + (3 × M3%)] / 100, while the Average Generation Mean time (AGT) was estimated as the number of hours cells incubated in BrdU divided by PI (Lamberti *et. al*, 1983; Preston *et. al*, 1987). SCE were scored for each culture using at least 50 clearly spread second division metaphase cells (M2) containing 46 ±1 chromosomes in presence of BrdU (Carrano *et al*., 1979).

2.5. Statistical Analysis

The average mean values of the hematological and cytological parameters and enzyme activities were expressed as mean values ± standard error (S.E.) of the mean. Based on normality assumption validation, comparisons between groups were accomplished using either the independent t test, when the normality assumption was valid, or, otherwise, the Mann-Whitney (MW) test. The differences were considered significant when the P value was ≤ 0.05. All statistical analyses were accomplished using the SPSS software version 11.5.

3. Results

Complete Blood Count (CBC), including Red Blood Cell count (RBC), hemoglobin (Hb), hematocrit (HCT), Mean Cell Volume (MCV), Mean Cell Hemoglobin (MCH), Mean Cell Hemoglobin Concentration (MCHC) and Red cell Distribution Width (RDW) were measured for all individuals in the DS and control groups. The average mean values of all the CBC values for all individuals, in both the DS and control groups, were within the standard normal ranges, as shown in Table 1. However, DS cases had significantly higher ($P \leq 0.05$) Mean of Corpuscular Volume (MCV) (84.1 μm^3) than those of the control (78.1 μm^3), while the DS group had significantly ($P \leq 0.05$) lower mean of MCHC (32.5 g/dl) than that of the control (31.7 g/dl).

Table 1. Average mean values (Mean ±S E) of the hematological parameters in the Down syndrome and the control groups

Hematological parameters	Controls	Down syndrome	*P – value* (Statistical Test)
RBC (10^6/mm^3)	5.0 ± 0.1	4.7 ± 0.1	0.061 (*t*)
Hb (g/dl)	12.7 ± 0.2	12.5 ± 0.3	0.686 (*t*)
HCT (%)	39.1 ± 0.6	39.7 ± 0.9	0.608 (*t*)
MCV (μm^3)	78.1 ± 1.0	84.1 ± 1.8	0.006 ^{S} (*t*)
MCH (pg)	26.9 ± 1.4	26.7 ± 0.7	0.064 (MW)
MCHC (g/dl)	32.5± 0.1	31.7± 0.2	0.002^S (*t*)
RDW (%)	14.9 ± 1.0	14.9 ± 0.3	0.077 (MW)

^{S}: Significant difference between DS and control groups at 0.05; MW: Mann-Whitney test; t: t-test; RBC: red blood cell count; Hb: hemoglobin; HCT: hematocrit; MCV: mean cell volume; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration; RDW: red cell distribution width.

The average mean values of the specific activities of the antioxidant enzymes in the DS and the control groups are summarized in Table 2. The mean value of the activity of SOD1 in DS group was significantly ($P \leq 0.05$) higher (140%), than that of the control group, while the specific activity of GST was statistically lower in the DS group (59.8%) than that of the control group ($P \leq 0.05$). Contrary to the differences in SOD1 and GST activities between the DS and control groups, the average mean values of CAT and GPx specific activities were not significantly different between the DS cases and the controls.

Table 2. Average values of the enzyme activities in DS and control groups

Enzyme	Enzyme activities Means ± S E in the studied groups		*P – value*
	DS individuals	Controls	(t test)
SOD1	2.247 ± 0.077	1.605 ± 0.083	0.042^S
CAT^a	195.3 ± 11.09	170.8 ± 7.03	0.070
GPX^b	51.3 ± 5.57	58.2 ± 9.87	0.546
GST^b	5.2 ± 0.65	8.8 ± 1.05	0.007 ^{S}

^{a}Unit /g Hb; ^{b} U/ mg Hb; ^{S} Significant difference at 0.05; SOD1: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidases; GST: glutathione S-transferases.

Chromosomal DNA stability was investigated by examining the average rate of SCE in all individuals within the DS and the control groups. SCE test is known as a highly sensitive indicator for DNA instability (Wolff *et. al*, 1977). The average mean values of SCE and the rates of cell cycle parameters for the lymphocytes cultures derived from DS and control groups are summarized in Table 3. The average mean values of SCE rates per cell in the DS and the control groups were 7.9 and 5.1 exchanges, respectively. Using the t test, this difference was statistically significant ($P < 0.01$) and showed that SCE rates in the lymphocyte cultures derived from DS group are higher than SCE rates in the cultures derived from the control group. Also, as shown in Table 3, the lymphocytes derived from the DS individuals exhibited significantly longer average mean value of AGT and lower values of MI and PI compared to the lymphocytes derived from the control group.

Table 3. Average mean values (Mean ±S E) of the genotoxicity and cytotoxicity parameters in lymphocyte cultures derived from Down syndrome and control subjects.

Cytological parameters	Mean ± S E		Ratio of Parameters In DS /control	*P – values* (test)
	Down syndrome	Controls		
SCE	7.993 ± 0.200	5.132± 0.0579	155.9	0.000 ^{S} (*t*)
MI	1.458 ± 0.04597	2.549± 0.05006	57.2	0.000 ^{S} (*t*)
PI	1.493 ± 0.05952	2.489 ± 0.04817	59.9	0.000 ^{S} (*t*)
AGT	49.539± 1.866	29.100 ± 0.570	170.2	0.000 ^{S} MW

^{S} Significant difference between DS and control groups at 0.05; SCE; sister chromatid exchanges; MI: mitotic index; PI: proliferative index; AGT: average mean time; MW: Mann-Whitney test.

4. Discussion

The normal ranges for the average mean values of all hematological parameters in both studied DS and control groups indicated that all DS and control individuals participated in this study were hematologically healthy, free of any of the common hemoglobinopathies such as thalassemia, sickle cell anemia and microspherocytosis, which, if present, would interfere with the results.

In a previous study of the same DS and control groups for aminothiols including total cysteine, cysteinyl-glycine, homocystiene and glutathione we found that these low molecular weight thiol-containing amino acids were not significantly different between the DS and the control group, suggesting that these molecules were not associated with DS syndrome phenotype (Alkaraki *et. al*, 2010). These same groups of DS and controls were further investigated in this study for the association between Down syndrome and the catalytic activities of the oxidative enzymes SOD1, CAT, GPx and GST in the red blood cells. In addition, the DNA stability in both groups was investigated by measuring the frequencies of the spontaneous rate of SCE among the Down Syndrome group and the healthy controls, since the changes of SCE rates were reported to be highly sensitive indicator for DNA instability (Wolff *et. al*, 1977). Our findings were in agreement with the results reported earlier (Sinet, 1982), which showed that SOD1 activity in DS could reach about 150% of that in the controls. It is well known that SOD1 works in conjunction with the two enzymes CAT and GPx, which neutralize the hydrogen peroxide produced in normal human cells (Gardiner and Davisson, 2000; Sherman *et. al*, 1983).

In this study, the lower GST activity in DS group compared to those in the controls indicated that there is an oxidative stress in DS subjects associated by the increase in SOD1 activity. Such association can be explained by the accumulation of genotoxic hydroxyl radicals as a result of the high SOD1 activity, which may contribute to the modulation of the second defense line against the generated free radicals provided by GST that functions as a detoxicant by catalyzing the conjunction of reduced glutathione with a variety of exogenous and endogenous harmful electrophiles. It is clear that the oxidative stress in DS individuals is due to the increase of SOD1 activity that resulted in excess production of H_2O_2 but not due to any adaptive increase in the catalytic activities of the CAT or GPx as suggested earlier (Mattei *et. al*, 1982; Sinet *et. al*, 1975). H_2O_2 is a potent oxidative agent that can generate highly reactive hydroxyl radicals in the presence of metal cations via the Fenton reaction (Emerit *et. al*, 2001; Urbañski and Berêsewicz, 2000). Such imbalance in the production of large amounts of reactive oxygen free radicals has harmful effects on the different cellular macromolecules including DNA, proteins, lipids and carbohydrates (Sies, 1993).

However, contrary to SOD1 activity in the DS group, the results of this study showed that CAT and GPx activities in the DS group are not significantly different from those in the control group, supporting the fact that the enhanced generation of ROS in DS individuals is a result of the increase in the SOD1 catalytic activity, which is not accompanied by an equivalent increase in the CAT and GPx activities that fail to neutralize the excess hydrogen peroxide produced in the trisomic cells. The elevated ratio of SOD1/CAT plus SOD1/GPX in DS group with respect to controls is a sign of oxidative imbalance in DS. This notion is in agreement with the recently reported results of Garlet *et al.* (2013) and Paristto *et al*. (2014); however the results of this study were different from previous reports, which showed an increase in the CAT and GPx activities in DS individuals compared to the normal controls (Mattei *et. al*, 1982; Sinet *et. al*, 1975).

The lower values of MI and PI observed in the lymphocyte cultures derived from DS individuals in parallel with the extension of AGT compared to those derived from the normal control group, indicated that DS lymphocytes cultures have a slower growth rate than those of the control cultures, which can be related to their higher oxidative stress. Similarly, the higher induced rate of SCE in the DS lymphocyte cultures compared to those derived from the controls confirmed the instability of DNA in DS cells compared to those of the controls. It is well reported that the elevation of SCE rate is a highly sensitive indicator for DNA instability (Wolff *et al*., 1977). Therefore, our results indicated that such DNA instability is most likely due to the oxidative stress status found in DS-derived lymphocytes compared to the controls due to their triplication of chromosome 21 and consequently their triplication of *SOD1* gene. However, the unexpected reduced activity of GST in DS cultures may be contributed to the down regulation of the *GST* gene by the over expression of the SOD1 enzyme. Further studies are required to clarify this notion.

In conclusion, this study showed that DS condition is associated with DNA instability that may have resulted from oxidative imbalance in the patients. Further studies related to the correlation between oxidative stress and DNA instability in DS should be stimulated by our results since recent studies in this area are very scarce.

Acknowledgments

The authors thank the families and children involved in the study. This study was fully supported by grant 34/2001 from the Deanship of Scientific Research and Graduate studies at Yarmouk University, Irbid, Jordan.

References

Aebi H. 1984. Catalase *in vitro*. *Methods in Enzymol*., **105**:121-126.

Alkaraki A, Hunaiti A and Sadiq M. 2010. Simultaneous aminothiol determination in Down syndrome individuals using a modified HPLC method. *Inter J Integrative Biol.,* **10(2)**:90-93.

Antonarakis SE and Epstein CJ. 2006. The challenge of Down syndrome. *Trends in Molecular Med.,* **12(10)**:473-479.

Capone GT. 2004. Down Syndrome: Genetic insights and thoughts on early intervention. *Infants and Young Children*. **17(1)**:45-58.

Carrano AV, Thompson LH, Stetca DG, Minkler JL, Mazrimas JA and Fong S. 1979. DNA crosslinking, sister-chromatid exchange and specific-locus mutations.*Mutat Res.,* **63 (1)**: 175-188.

Devasagayam TP, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS and Lele RD. 2004. Free radicals and antioxidants in human health: current status and future prospects. *J Association of Physicians of India*, **52**:794-804.

Emerit J, Beaumont C and Trivin F. 2001. Iron metabolism, free radicals, and oxidative injury. *Biomed and Pharmacother.,* **55(6)**:333-339.

Gardiner K and Davisson M. 2000. The sequence of human chromosome 21 and implications for research into Down syndrome. *Genome Biol.*, **1(2)**:1-9.

Garlet TR, Parisotto EB, de Medeiros GdS, Pereira LC, Moreira EA, Dalmarco EM, Dalmarco JB and Wilhelm Filho D. 2013. Systemic oxidative stress in children and teenagers with Down syndrome. *Life Sci*., **93(16)**:558-563.

Habig WH, Pabst MJ and Jakoby WB. 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem.,*. **249(22)**:7130-7139.

Helsinki. 2000. World Medical Association Declaration of Helsinki.

Ishii Y and Bender MA. 1980. Effects of inhibitors of DNA synthesis on spontaneous and ultraviolet light-induced sister-chromatid exchanges in Chinese hamster cells. *Mutation Res\ Genetic Toxicol*., **79(1)**:19-32.

Lamberti L, Ponzetto PB and Ardito G. 1983. Cell kinetics and sister-chromatid-exchange frequency in human lymphocytes. *Mutation Res Letters.* **120:**193-9.

Marklund S and Marklund G. 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem.,* **47(3)**:469-674.

Mattei JF, Baeteman MA, Baret A, Ardissone JP, Rebuffel P and Giraud F. 1982. Erythrocyte superoxide dismutase and redox enzymes in trisomy 21. *Acta Paediatrica,* **71(4)**:589-591.

Paglia DE and Valentine WN. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Medicine.* **70**:158-169.

Parisotto EB, Garlet TR, Cavalli VL, Zamoner A, da Rosa JS, Bastos J, Micke GA, FrÖde TS, Pedrosa RC and Wilhelm Filho D. 2014. Antioxidant intervention attenuates oxidative stress in children and teenagers with Down syndrome. *Res Developmental Disabilities*, **35(6)**:1228-1236.

Preston RJ, San Sebastian JR and McFee AF. 1987. The in vitro human lymphocyte assay for assessing the clastogenicity of chemical agents. *Mutation Res\ Genetic Toxicol*., **189(2)**:175-183.

Roizen N and Patterson D. 2003. Down's syndrome. The Lancet. 361(9365):1281-1289.

Shaw C, Thapalial A, Nanda S and Shaw P. 2006. Thyroid dysfunction in Down syndrome. *Kathmandu University Medical J*., **4(2)**:182-186.

Sherman L, Dafni N, Lieman-Hurwitz J and Groner Y. 1983. Nucleotide sequence and expression of human chromosome 21-encoded superoxide dismutase mRNA. P*roc National Academy of Sci.,* **80(18)**:5465-4569.

Sies H. 1993. Strategies of antioxidant defense. *Eur J Biochem.,* **215(2)**:213-219.

Sinet P, Michelson A, Bazin A, lejeune J and Jerome H. 1975. Increase in glutathione peroxidase activity in erthrythrocytes from trisomy 21 subjects. *Biochem. Biophys. Res. Commun.*, **67(3)**:910-915.

Sinet PM. 1982. Metabolism of Oxygen Derivatives in Down's Syndrome. *Annals of the New York Academy of Sci.,* **396(1)**:83-94.

Sun V, Oberley L and Ving U. 1988. A simple method for clinical assay of superoxide dismutase. *Clin Chem*., **34(3)**:497-500.

Urbañski NK and Berêsewicz A. 2000. Generation of *OH initiated by interaction of Fe^{2+} and Cu+ with dioxygen; comparison with the Fenton chemistry. *Acta Biochimica Polonica,* **47(4)**:951-962.

Verma RS and Babu A. 1989. **Tissue Culture Techniques and Chromosome Preparation. In: Human Chromosomes: Manual of Basic Techniques**. California: Pergamon Press, p. 4-10.

Vesa J, Brown Y, Greenfield D and Korenberg JR. 2005. Molecular and cellular characterization of the Down syndrome critical region protein 2. *Biochem Biophys Res Commun.,* **328(1)**:235-242.

Wiseman H and Halliwell B. 1996. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J*., **313**:17-28.

Wolff S, Rodin B and Cleaver JE. 1977. Sister chromatid exchanges induced by mutagenic carcinogens in normal and xeroderma pigmentosum cells. *Nature.* **265**(5592):347-349.

JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 17 - 21

Jordan Journal of Biological Sciences

Immunodominant Semen Proteins III: IgG_1 and IgG_4 Linkage in Female Immune Infertility

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Received: October 19, 2014 Revised: November 8, 2014 Accepted: November 17, 2014

Abstract

Active immune mechanism in the female reproductive tract may produce high levels of anti-seminal/sperm antibodies. Generated antibodies in the process of isoimmunization seem to be associated with female immune infertility. The aim of our study consists in the profiling of specific serum immunoglobulin classes and subclasses in infertile women. We focus on the distribution of serum seminal/sperm-specific antibodies in order to find those apparently related to female isoimmunization. Immunoglobulins G_{1-4}, $A_{1,2}$, M and E were measured by ELISA in serum from 30 infertile and 10 fertile females. Anti-seminal/sperm IgG_1 and IgG_4 fractions were predominantly detected. Anti-seminal IgG_1 and IgG_4 were observed approximately in the 2:1 ratio, anti-sperm fraction in the 1:2 ratio. Strikingly, the approximate ratio between IgG_1 and IgG_2 was 3:1 in seminal specific and 2:1 in sperm specific antibodies. Surprisingly, IgG_3 antibodies were nearly negative for both antigen fractions, seminal and sperm. Concerning our results, the proportionality does exist between seminal and sperm antibody fractions. Based on the poorly detectable levels of semen specific IgE, M, $A_{1,2}$, G_3, the markers of pathologic female isoimmunization appear to be the serum IgG_1 and IgG_4. These preliminary findings may contribute to a detailed patient diagnosis and an improved therapy.

Keywords: Antibodies, ELISA, Seminal fluid, Sperm

1. Introduction

Human semen, that is defined as a co mplex fluid containing sperm, cellular vesicles and other cells and components (e.g., leucocytes, environmental antigens of microbial origin), could provoke the immune reaction of the female genital tract (Moldoveanu *et al.*, 2005; Brazdova *et al.*, 2013). Semen immunoregulatory factors as well as immunogenic agents are, thus, the potential targets of activated inflammatory cytokines, initiate leukocyte infiltration and complement cascade in the female genital tract. The active local immunoregulatory mechanism of the female reproductive tract is related to the fertility potential (Wirth, 2007; Brazdova, 2014). Sperm is able to induce the production of sperm-reactive T-cells in men as well as in women. Anti-sperm antibodies interfere with the antibody-mediated infertility through various pre/post-fertilization processes (Kurpisz and Kamieniczna, 2009; Brazdova *et al.*, 2013). Seminal antibody-binding proteins contribute to sperm metabolism, passage in the female reproductive tract and block an interaction with immune effectors. Seminal Fluid (SF) induces pro-inflammatory cytokines and suppresses the cell-mediated cytotoxicity (Chiu and Chamley, 2002; Brazdova *et al.*, 2012a). In primary response to some allergens/antigens, IgE antibodies might be prevalent. The so-called switch into IgG and IgA antibodies is induced at the late phase of primary immune response and/or after the repeated exposure to the same antigen (Batard *et al.*, 1993). After the chronic antigen exposure, IgG_1 and IgG_4 become the predominantly produced subclasses of IgG isotype. In addition, IgG_4 is unable to activate the classical complement pathway and is then known as an anti-inflammatory immunoglobulin and a blocking antibody towards IgE antibodies. Still, it remains unclear whether IgG_4 is a protective or rather sensitizing antibody

List of abbreviations :AP: Alkaline Phosphatase; ELISA: Enzyme-Linked Immunosorbent Assay; F: control sera of Fertile women; IF: sera of Infertile Female patients; L: sperm Lysate; ND: Non Detectable levels; SD: Standard Deviation; SF: Seminal Fluid.

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(Aalberse and Schuurman, 2002; Guma and Firestein, 2012).

In the present paper, the profile of antibody-based immune response to seminal/sperm proteins in infertile and fertile women is studied to document which class or subclass of serum immunoglobulin might be mostly involved in the immune rejection of sperm associated with female immune infertility.

2. Materials and Methods

2.1. Sample preparation

Semen samples from 8 normozoospermic (WHO, 2010) donors (aged 25-30) were treated as previously described (Brazdova *et al.*, 2012a; Brazdova *et al.*, 2013). Sperm-free SF was processed untreated; the sperm disintegration was processed with Triton X-100. To increase the amount of potential antigens in the protein extracts and to eliminate individual variations, the obtained individual sperm Lysates (L) were pooled as well as individual SF. The samples were stored at -20 °C until assayed. All experiments were performed after obtaining informed written consent.

2.2. Patients

This study was approved by the institutional ethical committees and informed written consent was returned by patients. Sera were obtained from 30 w omen with a fertility disorder (patients with repeated in vitro fertilization failure, aged 29-38) and from 10 w omen (control group, aged 28-37) with proven fertility (1-2 healthy children). The serum samples were frozen at -20 °C until assayed.

2.3. Quantification of Serum Anti-Seminal/Sperm Immunoglobulins

Serum anti-seminal/sperm IgG_{1-4}, $IgA_{1,2}$, IgM, IgE and then the SF/L reactivity of patient and control sera were tested by ELISA developed in our laboratory.

In the first protocol, to obtain the standard calibration curves, the micro-plates (MaxiSorp^{TM}, Denmark) were coated with either mouse anti-human IgG_{1-4} (Calbiochem, United States), $IgA_{1,2}$ (Abcam, United Kingdom), goat anti-human IgM (Sigma-Aldrich, United States) or IgE (Sigma-Aldrich, United States) in a 50 mM carbonate-bicarbonate buffer (Friguet *et al.*, 1985) overnight at 4 °C. The plates were saturated with 0.5% gelatine (Sigma-Aldrich, United States) in PBS-Tw 0.1% (0.01 M Phosphate Buffered Saline with NaCl 0.15 M, pH 7.4 supplemented with Tween; Brazdova *et al.*, 2012a,b) for 2 h at room temperature. The wells were incubated with the individual patient or control sera or human IgG_{1-4}, $IgA_{1,2}$, IgM, IgE (Calbiochem, United States) as standards of known concentration in serial dilution for 2 h at 37 °C in PBS-Tw 0.1% and then with alkaline phosphatase (AP)-conjugated, either mouse anti-human IgG_{1-4} (Calbiochem, United States), $IgA_{1,2}$ (Beckman Coulter, United States), goat anti-human IgM or IgE (Sigma-Aldrich, United States) for 2 h at 37 °C. The AP activity was detected by p-nitrophenyl phosphate disodium (Sigma-Aldrich, United States). Optical density was measured at 405 nm versus 630 nm with a multichannel spectrophotometer (Tecan Group Ltd., Switzerland).

In the second protocol, to quantify the serum anti-seminal/sperm IgG_{1-4}, $IgA_{1,2}$, IgM, IgE, the micro-plates were coated with SF/L in a 50 mM carbonate-bicarbonate buffer overnight at 4 °C. The following procedure was identical to the first protocol. The anti-seminal/sperm immunoglobulin concentrations were obtained by linear regression in comparison with the standard calibration curve obtained in the first protocol.

3. Results

Figure 1 s hows the concentration of seminal/sperm-specific IgG_{1-4} in the individual sera of infertile/fertile females. In the sera of infertile women, the predominant anti-SF IgG subclasses were IgG_1 and IgG_4, reaching maximum concentration of 45 µg/ml (IgG_1) and 38 µg/ml (IgG_4). In particular, anti-SF IgG_1 antibodies were detected in 100% of sera tested with the mean of 22 µg/ml (SD 9), IgG_2 in 97% with the mean of 8 µg/ml (SD 6), IgG_3 in 6% with the mean of 0.1 µg/ml (SD 0.05), IgG_4 in 90% with the mean of 12 µg/ml (SD 9). In the sera of infertile women, the prevalent anti-sperm IgG subclass was IgG_4 ranging from 9 to 45 µg/ml with the mean of 23 µg/ml (SD 10). Anti-sperm IgG_{1-4} antibodies were detected within all patient sera processed with the mean of 11 (SD 5, IgG_1), 7.5 (SD 5, IgG_2), 0.3 (SD 0.1, IgG_3), 23 µg/ml (SD 10, IgG_4). Twenty percent of control sera contained seminal-specific IgG_1 and 30% contained seminal-specific IgG_2, both of which ranged from 0.2 to 0.5 µg/ml. Sperm-specific $IgG_{1,2}$ were detected in 30% of control sera reaching the top value of 0.5 µg/ml. None of the control sera had the detectable levels of anti-seminal/sperm $IgG_{3,4}$.

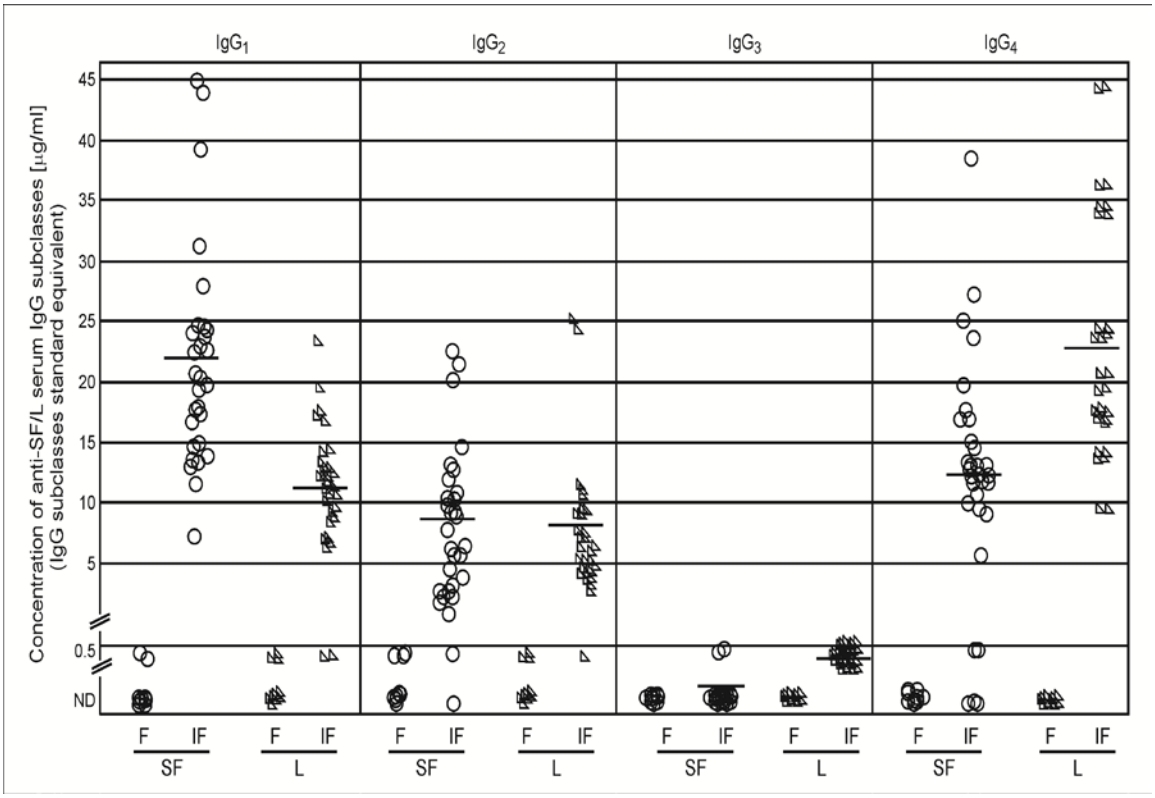

Figure 1. Quantified female serum anti-seminal/sperm $IgG_{1,2,3,4}$. IF: sera of infertile patients (30), F: control sera of fertile women (10), ND: nondetectable level, ○: anti-seminal antibodies, ◺ : anti-sperm antibodies, bars: arithmetic means.

Figure 2 s hows seminal/sperm-specific $IgA_{1,2}$, IgM, IgE concentrations in the individual sera of infertile/fertile women. Anti-seminal/sperm IgE, IgM and IgA_1 were not detected in the patient sera. Only 1 patient serum out of 30 contained anti-sperm IgA_2 (0.18 µg/ml). Then, 1 control serum out of 10 contained anti-seminal IgA_1 (1.2 µg/ml), IgA_2 (1.8 µg/ml) and anti-sperm IgA_1 (1.9 µg/ml), IgA_2 (2.1µg/ ml).

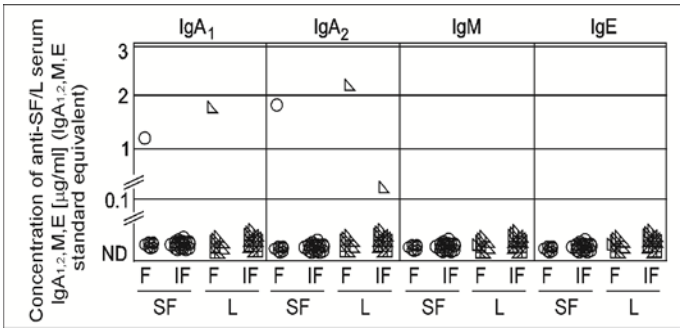

Figure 2. Quantified female serum anti-seminal/sperm $IgA_{1,2}$, IgM, IgE. IF: sera of infertile patients (30), F: control sera of fertile women (10), ND: nondetectable level, ○: anti-seminal antibodies, ◺ : anti-sperm antibodies.

4. Discussion

We present our essential preliminary findings concerning the distribution of serum anti-SF/L $IgG_{1,2,3,4}$, $IgA_{1,2}$, IgM and IgE as a co nsequence of pathophysiological female isoimmunization. We show IgG_1/IgG_4 predominance depending on the values themselves, 2:1 in anti-seminal specific antibodies, also valid in a reverse statement between anti-sperm IgG_1 and IgG_4 levels. The IgG_1:IgG_4 ratio in anti-sperm antibodies is in agreement with the working hypothesis of Batard *et al.* (1993) who proved that the prolonged exposure to immunogenic agents, such as grass pollen allergens, generates IgG_1 further shifting into IgG_4. Then, we speculate that the detected antigens might be the same since the distribution of tested antibodies follows the similar trend of $IgG_1>IgG_2>IgG_3<<IgG_4$. Based on barely detectable IgG_3 within the patient group, we assume that IgG_3 does not have any protective or inflammatory role in female immune infertility. Among the four IgG subclasses, $IgG_{1,3}$ activate complement cascade (Jefferis, 2012). Since patients do not suffer from the inflammatory symptoms, we suggest that complement cascade may not be activated by IgG_3 in the semen rejection. It was specified (Tamayo *et al.*, 2012) that the response to protein antigens is primarily mediated by IgG_1 and IgG_3, whereas IgG_2 especially and IgG_4 are induced in response to polysaccharide antigens. Since seminal/sperm-specific IgG_1 and IgG_4 were inversely correlated, IgG_1 could theoretically bind to seminal antigens of a protein character and IgG_4 to sperm structures of an oligosaccharide nature. Whether or not serum IgG_4 is a blocking or sensitizing antibody in infertile females remains unexplained, unlike in other studies dealing with other pathologies (Aalberse and Schuurman, 2002; Guma and Firestein, 2012). The absence of data following patients over time prevented us to better understand the role of IgG_4. Concerning male autoimmunity, IgA antibodies were proved to be associated with a s ystematic factor in male immune infertility (Kutteh *et al.*, 1994). The nondetectable levels of $IgA_{1,2}$ in 97% of female patient sera tested imply that either IgA_1 or IgA_2 do not correlate with systemic isoimmunization. However, we are able to affirm that only IgA_1 is not involved in the disease as it predominates in the sera (Woof and Kerr, 2006) examined in our study. Immunoglobulin M contributes to opsonization and is involved in the primary response to antigen exposure (Schoeder and Cavacini, 2010). Since the patient sera are neither collected nor tested immediately after the sensitization, the nondetectable level reflects the potential secondary immune response where IgM does not play a significant role. However, we cannot refute that IgM is not involved either in primary or secondary antibody response to isoimmunization. Not a single patient was diagnosed with semen hypersensitivity, which is in agreement with an absence of anti-seminal/sperm IgE, usually related to the pathophysiology of allergy (Brazdova *et al.*, 2012a,b).

5. Conclusion

The described distribution of seminal/sperm-specific $IgG_{1,2,3,4}$, $IgA_{1,2}$, IgM, IgE indicates that female iso-immunization seems to be IgG-dependent as immunoglobulins E, M, $A_{1,2}$ are not involved in this pathophysiological process. Specific IgG_1 predominates in anti-seminal serum antibody fraction; on a contrary, specific IgG_4 in anti-sperm serum antibody fraction. We believe that the determination of serum seminal/sperm-specific immunoglobulin G subclasses might make patient profiling more precise and complete the information for diagnosis. Anti-seminal/sperm IgG_1 and IgG_4 could be of interest for further therapy targets.

Acknowledgement

We thank Prof. Corinne Mitchell (Pierre and Marie Curie University, Paris, France) for the language proofreading of our article. This study was supported by a specific university (University of Chemistry and Technology, Prague, Czech Republic) research (MSMT No. 20/2014), by the Charles University (Prague, Czech Republic) research fund (project No 36) and by Grant Agency of the Czech Republic (GACR 13-09685S).

References

Aalberse RC and Schuurman J. 2002. IgG_4 breaking the rules. *Immunol.,* **105**: 9-19.

Batard T, Basuyaux B, Lambin P, Bremard-Oury C, Hamilton RG, David B and Peltre G. 1993. Isotypic analysis of grass-pollen-specific immunoglobulins in human plasma. 1. Specialization of certain classes and subclasses in the immune response. *Int Arch Allergy Immunol.,* **100**: 68-78.

Brazdova A. 2014. Antiseminal and antisperm antibodies in female immune infertility. Characterization of targeted proteins. PhD dissertation, Pierre and Marie Curie University, Paris, France.

Brazdova A, Vermachova M, Zidkova J, Ulcova-Gallova Z and Peltre G. 2013. Immunodominant semen proteins I: New patterns of sperm proteins related to female immune infertility. *Cent Eur J Biol*, **8**: 813-818.

Brazdova A, Zidkova J, Senechal H, Peltre G, Cibulka J and Ulcova-Gallova Z. 2012a. Female serum immunoglobulins G, A, E and their immunological reactions to seminal fluid antigens. *Folia Biol (Praha)*, **58**: 251-255.

Brazdova A, Zidkova J, Peltre G and Ulcova-Gallova Z. 2012b. IgG, IgA and IgE reactivities to sperm antigens in infertile women. *Jordan J Biol Sci.*, **5**: 85-89.

Chiu W and Chamley L. 2002. Antibody-binding proteins in human seminal plasma. *Am J Reprod Immunol.,* **48**: 269-274.

Friguet B, Chaffotte AF, Djavadi-Ohaniance L and Goldberg ME. 1985. Measurements of the true affinity constant in solution of antigen complexes by enzyme-linked immunosorbent assay. *J Immunol Methods*, **77**: 305-319.

Guma M and Firestein GS. 2012. IgG4-related diseases. *Best Pract Res Clin Rheumatol.,* **26**: 425-438.

Jefferis R. 2012. Isotype and glucoform selection for antibody therapeutics. *Arch Biochem Biophys.*, **526**: 159-166.

Kurpisz M and Kamieniczna M. 2009. Immune chemistry of ASA. In: Krause W and Naz RK (Eds.), **Immune Infertility: The impact of immune reaction on human infertility.** Springer-Verlag Berlin Heidelberg, Germany, pp. 79-90.

Kutteh WH, Kilian M, Ermel LD, Byrd EW and Mestecky J. 1994. Antisperm antibodies (ASAs) in infertile males: Subclass distribution of IgA antibodies and the effect of an IgA1 protease on sperm-bound antibodies. *Am J Reprod Immunol.,* **31**: 77-83.

Moldoveanu Z, Huang WQ, Kulhavy R, Pate MS and Mestecky J. 2005. Human male genital tract secretions: both mucosal and systemic immune compartments contribute to the humoral immunity. *J Immunol*., **175**: 4127-4136.

Schroeder Jr HW and Cavacini L. 2010. Structure and function of immunoglobulins. *J Allergy Clin Immunol.*, **125**: 41-52.

Tamayo E, Fernandez A, Almansa R, Carrasco E, Goncalves L, Heredia M, Andaluz-Ojeda D, March G, Rico L, Gomez-Herreras JI, de Lejarazu RO and Bermejo-Martin JF. 2012. Beneficial role of endogenous immunoglobulin subclasses and isotypes in septic shock. *J Crit Care*, **27**: 616-622.

Wirth M. 2007. Immunology of the genital tract. PhD dissertation, Ludwig-Maximilians-Universität zu München, Munich, Germany.

Woof JM and Kerr MA. 2006. The function of immunoglobulin A in immunity. *J Pathol*., **208**: 270-282.

World Health Organization. 2010. **WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction**, fifth ed. Cambridge University Press, Cambridge.

JJBS

Jordan Journal of Biological Sciences

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 23 - 30

Application of Fermentation Technology to Use Slaughterhouse Blood as Potential Protein Supplement in Fish Feed

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Received: September 29, 2014 Revised: November 11, 2014 Accepted: November 17, 2014

Abstract

Slaughterhouse Blood (SHB) is a rich source of protein, but it requires a suitable processing before its use as an ingredient in animal feed formulations. In this study, SHB was fermented using whey or pure culture of *Lactobacillus acidophilus* as the fermentation inoculums. Mixtures of SHB, fermentation inoculums and molasses were incubated at 35°C and microbiological and biochemical changes were determined every day until the end of fermentation. The results revealed that the fermentation of SHB by both the inoculums required 4 days to complete. The harmful microorganisms were removed or reduced to a negligible level after fermentation. Both the fermented products were also aerobically stable. Protein, lipid and essential amino acid contents of SHB were almost completely recovered after fermentation. The *L. acidophilus* fermented SHB (LFSHB) exhibited a better microbiological quality than the whey fermented SHB (WFSHB), while WFSHB exhibited a higher amount of free amino acid than the LFSHB. The present study concludes that fermentation is an appropriate technique to convert SHB into a nutritionally sound and microbiologically safe ingredient for the use in fish feed formulation. Whey can be used as an easily available and cost effective inoculum to carry out the fermentation.

Keywords: Organic Wastes, Fermentation, Lactic Acid Bacteria, Feed, Crude Protein, Amino Acids

1. Introduction

Disposal and management of organic wastes generated from slaughterhouses are tough jobs because of the huge volume in which these wastes are generated everyday in solid and liquid forms (Bergleiter, 2011). In India, there are 2,702 registered slaughterhouses which generate about 21 lakh tones of wastes per annum (Jayathilakan *et al*., 2012). The solid slaughterhouse wastes consist mainly of inedible parts of animal while the liquids comprise blood, urine, water, dissolved solids and gut contents. Fearon *et al*. (2014) observed that about 0.7 ton blood was produced from slaughtering of 125 livestock comprising 55 cattle, 50 sheep and 20 goats. In India, cattle, sheep and goat slaughtering is the major sources of slaughterhouse blood (SHB). Most of the SHB generated from these slaughterhouses is discarded as wastes and disposed into the environment in an unorganized manner. Otherwise, these wastes can be used as protein supplement in the formulation of feed for fish. However, nutrient compositions, recovery of the nutrients and microbiological status of SHB generated in India are poorly documented. The idea of recycling these wastes through agriculture and animal husbandry is gaining importance because of multiple benefits like reduction of environmental pollution arising out of disposal of the wastes, prevention of loss of huge nutrients contained in these wastes and curtailing in production cost in agriculture and animal husbandry. Since the past decade, many studies have actively been engaged in developing suitable techniques to recycle animal wastes in the formulation of feed for fish because of an urge to find suitable cost effective alternative of fishmeal, the most dependable but a costly source of protein in aquaculture feed (De Silva and Anderson, 2009; Zhu *et al*., 2011).

Slaughterhouse blood (SHB) is a rich source of protein. However, SHB contains a high amount of moisture and huge load of unwanted microbes, the two most important factors responsible for the breakdown of protein and the generation of unpleasant odors (Jayathilakan *et al*., 2012). Therefore, processing of SHB is essential before its use as an ingredient in aquaculture feed formulation. In spite of supplying a high quality protein in the feed, inclusion of unprocessed SHB exhibited lower digestibility in some fish (Zhu *et al*., 2011), while processed SHB or Blood Meal (BM) was always considered as a quality protein source for the use in aquaculture feed industry (Agbedi *et al*., 2009). Therefore, a suitable technique to produce a cost effective and a nutritionally improved SHB to be used as an ingredient in aquaculture feed is in demand. Spray dried or oven dried blood meals are frequently used in the formulation of feed for fish (Ribeiro *et al*., 2011;

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Aladetohun and Sogbesan, 2013). However, heat treatment often results in the loss of nutrients of the ingredients.

The success of converting organic wastes into feedstuff depends on the treatment used to process the wastes. Fermentation has been used as a reliable biotechnological tool in processing nutrient rich organic wastes to reduce the inherent problems contained in animal wastes and to make them nutritionally sound feedstuff (Kader *et al.,* 2012). Lactic Acid Bacteria (LAB) serve as the main players in the fermentation and preservation of such wastes (Pyar *et al.,* 2011). Improvisation of a fermentation technique to produce a cost effective, microbiologically safe and nutritionally sound fermented product suitable for use as feed ingredient in the formulation of feed has been given importance in recent years (Samaddar and Kaviraj, 2014). The objective of the present study is to develop an eco-friendly and efficient fermentation technique acceptable to aquaculturists to process SHB and to use it as a low cost protein supplement in the formulation of feed for fish without compromising nutrient quality of the feed.

2. Materials and Methods

2.1. Collection of Sample

Fresh Slaughterhouse Blood (SHB) was collected from a few selected slaughterhouses immediately after slaughtering sheep and goats. The blood was allowed to clot and only the clotted portion of the blood was collected and brought to the laboratory in an ice bucket for fermentation. Two inoculums were used for the fermentation of the SHB: One is a pure culture of *Lactobacillus acidophilus*,a representative microorganism of Lactic Acid Bacteria (LAB) and the other is whey, a byproduct of milk industry and a cheap source of LAB. Samaddar and Kaviraj (2014) discussed in detail the potential of whey and pure culture of *L. acidophilus* as fermentation inoculums and their procurement. Cane molasses was used, as discussed previously (Samaddar and Kaviraj, 2014), as a source of fermentable carbohydrate for microorganisms involved in the fermentation and was collected from the local market.

2.2. Fermentation Process

Fermentation of SHB was carried out in 1 L Erlenmeyer flasks, each containing 400 g of fermentation mixture. The mixture contained 80% of SHB, 15% of cane molasses and 5% of fermentation inoculum containing either whey or actively growing culture [109 colony forming units (CFU)/ml] of *Lactobacillus acidophilus* (NCIM 2903) procured from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The pre-fermented mixture was subjected to biochemical and microbiological analyses before the incubation was started. All analyses were made following standard analytical methods described below. The fermentation mixture (mean moisture level = 72 %) was then incubated at 35°C. Seven replicates were maintained for each of the fermentation groups. Every day one replicate from each group was opened and pH, Water Soluble Carbohydrates (WSC) and quantity of Lactic Acid (LA) and Acetic Acid (AA) produced in the fermentation mixture were determined. Determinations were continued till the mixture attained a pH value in between 4.0 and 4.2, after which the fermentation was discontinued. The entire experiment was repeated thrice. The fermented products were subjected to microbiological and biochemical analyses. Detailed analytical methods are described below.

2.3. Microbiological Analyses

Determinations of Standard Plate Count (SPC), population of LAB, yeast and hazardous microorganisms such as total and faecal coliform, *Staphylococcus* and *Clostridium* were made in the pre-fermented mixture and in the fermented products, Whey-Fermented Slaughterhouse Blood (WFSHB) and *L. acidophilus*-Fermented Slaughterhouse Blood (LFSHB). Ten g of each sample was blended with 90 ml of saline water (NaCl, 0.85 % w/v), diluted serially (10-folds), plated with 1 ml of the suspension in appropriate media using pour plating technique (Cappuccino and Sherman, 2007) and incubated at the appropriate temperature. The colonies that appeared on the selected plates after incubation were enumerated as CFU/g or ml of sample. Samples were incubated in Plate Count Agar (Standard Methods Aagar) (HiMedia, Mumbai, India) at 30° C for 2–3 days for enumeration of SPC, in Potato Dextrose Agar (PDA) (HiMedia, Mumbai, India) acidified by LA solution to pH 3.5 at 30°C for 72 h for enumeration of yeast, in *Lactobacillus* MRS agar (HiMedia, Mumbai, India) at 30°C for 48 h for enumeration of LAB, in MacConkey broth (HiMedia, Mumbai, India) supplemented with agar, at 37°C for 24 h for enumeration of total coliform bacteria and again in the same media at 44°C for 24 h for determination of faecal coliform bacteria. For enumeration of *Staphylococcus*, the samples were incubated in Mannitol salt agar (HiMedia, Mumbai, india) at 37°C for 24 h. Yellow colonies were enumerated and checked by Gram stain and catalase reactions. For enumeration of *Clostridium* colonies, the samples were heated at 80°C for 10 min, immediately followed by cooling on ice water. The cooled samples were serially diluted, plated and incubated at 44°C for 24 h in Reinforced clostridial agar (HiMedia, Mumbai, India).

Aerobic stability against the growth of moulds and yeast on the pre-fermented and fermented products was checked by a short-term experiment. Ten grams of dry sample were taken in a plate of 90 mm diameter, spread in a very thin layer and kept in open air or covered by a lid in the laboratory. Two control sets, one containing Potato Dextrose Agar (PDA) and another containing PDA acidified by LA to pH 3.5 (acidified PDA) in similar plates, were also maintained. The temperature and humidity were recorded during the experiments. Samples of the prefermented and fermented products and the control media were taken out from the respective plates after 1 h and 5 days of incubation, diluted serially (1:10) in saline water (NaCl, 0.85 % w/v), plated following spread plating technique and incubated for 3 days at 30°C separately in two media: One containing PDA and another containing acidified PDA to grow a mixture of mould and

yeast and only yeast respectively. CFU were enumerated in the plates after 3 days.

2.4. Biochemical Analyses

The samples were diluted with water (1:10) for determination of pH in a direct reading digital pH meter. Water-Soluble Carbohydrate (WSC) was determined by modified spectrophotometric method described by Murphy *et al.* (2007). For the determination of Lactic Acid (LA) and Acetic Acid (AA), samples were diluted with water (1:10), homogenized and filtered through Whatman-2 filter paper. The filtrate was centrifuged at 14,400 g for 15 min and the supernatant was further filtered through 0.45 µm membrane filter. LA in the filtrate was determined by the method of McHugh *et al*. (2006) in an HPLC system (Waters) using a 4.6 9 25 0 mm, 5 µm, column (ZORBAX Rx-C18; Agilent Technologies), a Waters 2996 photodiode array UV detector p rogrammed at 210 nm and a m ixture of acetonitrile:buffer (3:97) as the mobile phase with a flow rate of 1.2 ml/min. The buffer used was 25 mM KH_2PO_4 (adjusted to pH 2.5 by H_3PO_4). AA in the filtrate was determined in the same HPLC system, but 50 m M KH_2PO_4 (adjusted to pH 2.5 by H_3PO_4) buffer alone was used as the mobile phase with a flow rate of 1 ml/min (Dremetsika et al., 2005). Retention time of the standard LA and AA was found at 2.2 and 2.9 min., respectively. The quantity of both LA and AA in the samples was determined using Empower software associated with the HPLC system. Proximate compositions were determined by AOAC methods (Helrich, 1990). Samples were oven dried at 55–60° C for 18–20 h before analysis. Moisture was determined by oven drying at 105° C for 24 h; crude protein (nitrogen × 6.25) was determined by Kjeldahl method after acid hydrolysis; lipid was extracted by petroleum ether (boiling point 40–60° C) for 7–8 h in a Soxhlet apparatus followed by determination of lipid gravimetrically; crude fiber was determined as loss on ignition of dried lipid-free residues after digestion with 1.25 % H_2SO_4 and 1.25 % NaOH and ash was determined by combustion at 550° C in a muffle furnace till a constant weight was achieved. Nitrogen-free extract (NFE) was calculated by taking the sum of values for crude protein, crude fiber, crude lipid, moisture and ash and subtracting this from 100. Essential and non-essential amino acids were determined in the pre-fermented mixture and in fermented products by the method of Kwanyuen and Burton (2010) in a HPLC system (Waters, USA) equipped with UV detector programmed at 254 nm. The analysis was performed by using similar column and software as discussed above. Analytical grade amino acid standard (AAS18, Fluka) was purchased from Sigma-Aldrich (St. Louis, USA). During acid hydrolysis tryptophan was partially destroyed. Therefore, the level of tryptophan was not determined in the samples. Free amino acid was determined by the method of Moore and Stein (1948) using ninhydrin reagent dissolved in methyl cellosolve (2-methoxyethanol).

2.5. Statistical Analyses

Single factor analysis of variance and then the least significant difference test (Gomez and Gomez, 1984) were employed to test the significant difference in pH, WSC, LA and AA content of the two fermentation mixtures between initial and terminal days of fermentation as well as between the two final fermented products (WFSHB and LFSHB). Significant differences in other biochemical compositions between dried WFSHB and LFSHB as well as between pre-fermentation mixture and the final product of each category (WFSHB and LFSHB) were also determined.

3. Results

3.1. Microbiological Status

Standard Plate Count (SPC) and population of Lactic Acid Bacteria (LAB), yeast and hazardous microorganisms (total and faecal coliform bacteria, *Staphylococcus* and *Clostridium*) determined in pre-fermented mixture, WFSHB and LFSHB have been presented in Table 1. A high degree of contamination of hazardous microorganisms was found in the pre-fermented SHB mixture. Interesting microbiological changes occurred during fermentation. As a r esult, SPC, LAB and yeast population increased and *Staphylococcus, Clostridium* and coliform population decreased after fermentation in both WFSHB and LFSHB. *Clostridium* and feacal coliforms were totally removed from both the fermented products, while other coliforms were also totally removed from LFSHB and reduced to a negligible level in WFSHB. *Staphylococcus* was also reduced to a negligible level in both the fermented products. LAB became the dominating microflora due to fermentation in both the fermented products. The aerobic stability test showed that both moulds and yeast grew profusely in the control and moderately in pre-fermented SHB, but failed to grow on the fermented products (Table 2).

Table 1. Microbiological profile[a] of the pre-fermented and fermented slaughterhouse blood (SHB).

	Pre-fermented SHB (log CFU per g)	LFSHB (log CFU per g)	WFSHB (log CFU per g)
Standard plate count	7.13 ± 0.07	7.71 ± 0.05	7.73 ± 0.06
Total coliform	5.32 ± 0.03	ND[b]	1.2 ± 0.07
Faecal coliform	3.87 ± 0.03	ND	ND
Staphylococcus	4.61 ± 0.02	0.84 ± 0.01	1.5 ± 0.02
Clostridium	3.78 ± 0.02	ND	ND
Lactic acid bacteria	6.91 ± 0.02	7.57 ± 0.02	7.65 ± 0.06
Yeast	1.06 ± 0.05	6.04 ± 0.04	6.88 ± 0.03

[a]Data are mean of three replications and presented as number of colony forming units (CFU) per unit w/vol of the sample with ±SD; [b]Not detected

Table 2. Mould and yeast population (log CFU/g)[a] in the fresh and fermented waste.

Treatments	After 1 hour exposure to air		After 5 days of incubation	
	Mixture of moulds and yeast	Yeast	Mixture of moulds and yeast	Yeast
Prefermented SHB	0.60±0.03	ND[b]	2.71±0.1	2.42±0.06
LFSHB[c]	ND	ND	ND	ND
WFSHB[d]	ND	ND	ND	ND

[a]Data are mean of three replications and presented as number of colony forming units (CFU) per unit w/vol of the sample with ±SD; [b] ND=Not detected; [c] *L. acidophilus* fermented slaughter house blood; [d] Whey fermented slaughter house blood.

3.2. Biochemical Status

Changes observed in the level of pH, Water Soluble Carbohydrate (WSC), Lactic Acid (LA) and Acetic Acid (AA) contents during fermentation have been presented in Figure 1. pH of the fermentation mixture reduced to the range indicative of completion of fermentation (4.0-4.2) within 4 days of incubation. Reduction of pH was accompanied by an increase in LA and AA concentration and a reduction of WSC in both the treatments. There was no change in biochemical parameters between Whey Fermented SHB (WFSHB) and *L. acidophilus* Fermented SHB (LFSHB), except for a higher AA level in WFSHB at the end of incubation as compared to LFSHB.

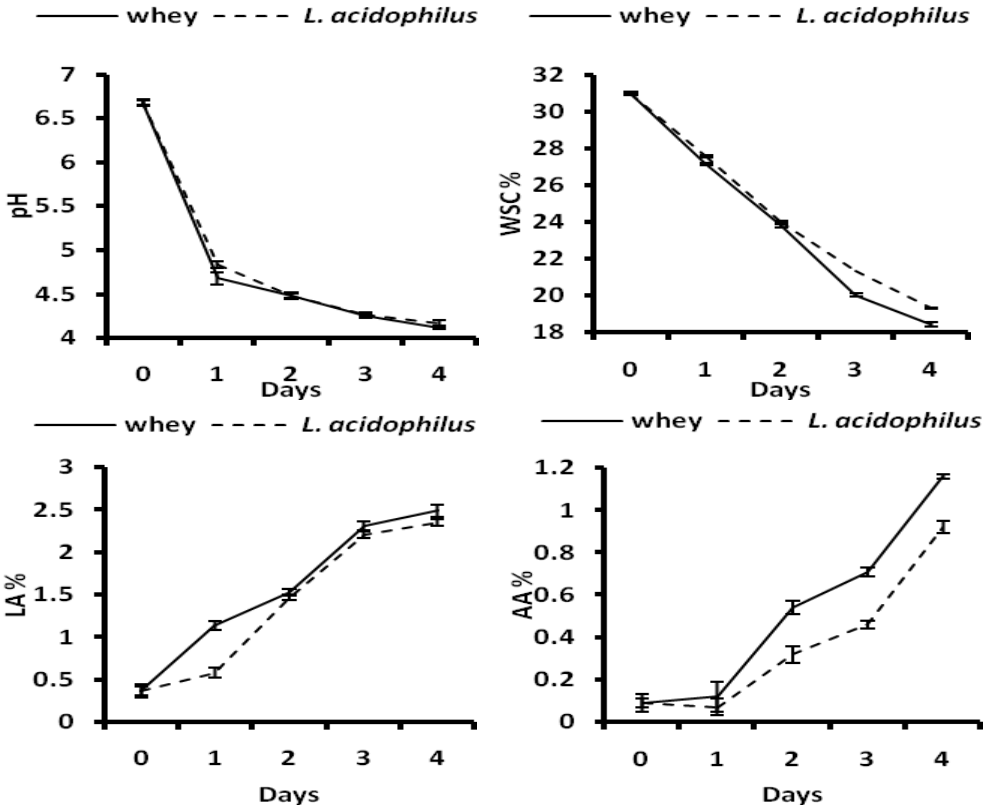

Figure 1. Biochemical changes during fermentation of slaughter house blood mixture.

Table 3 shows a proximate composition of the pre-fermented mixture containing SHB and changes in these parameters after fermentation with *L. acidophilus* and whey. The pre-fermented mixture showed an excellent content of crude protein, leucine and lysine. The level of arginine, phenylalanine, threonine and valine was moderate and the percentage of other essential amino acids was lower. Among non-essential amino acids, amount of alanine, aspartic acid and glutamic acid were higher in comparison to others.

Crude protein level decreased slightly in both LFSHB and WFSHB as compared to pre-fermented mixture.

Crude lipid level remained similar, but crude fiber level significantly reduced in both LFSHB and WFSHB after fermentation. Higher amount of free amino acid was found in WFSHB as compared to LFSHB. Among essential amino acids, arginine, histidine, phenylalanine and threonine contents reduced significantly in both groups, while leucine and lysine contents reduced significantly in WFSHB and valine content reduced significantly only in LFSHB.

Table 3. Proximate composition* of pre-fermented and fermented slaughter house blood.

Parameters (percent dry matter)	Pre-fermented SHB	LFSHB	WFSHB
Crude protein	46.72±0.04[a]	46.18±0.04[b]	46.02±0.11[c]
Crude lipid	2.09±0.03[a]	2.14±0.02[a]	2.13±0.03[a]
Crude fibre	1.16±0.02[a]	0.94±0.03[b]	0.81±0.01[c]
Moisture	13.14±0.18[b]	14.52±0.04[a]	14.68±0.04[a]
Ash	4.53±0.05[a]	4.33±0.03[b]	4.28±0.13[b]
Nitrogen free extract	29.34±0.12[b]	29.66±0.23[a]	29.88±0.11[a]
Free amino acid	0.13±0.04[a]	0.73±0.03[b]	1.16±0.02[c]
Gross energy (kJ g^{-1})	10.53±0.02[a]	10.31±0.01[b]	10.27±0.01[c]
Essential amino acids			
Arginine	2.62 ± 0.03[a]	2.37 ± 0.01[b]	2.32 ± 0.02[b]
Histidine	1.65 ± 0.02[a]	1.44 ± 0.02[b]	1.46 ± 0.02[b]
Isoleucine	0.71 ± 0.03[a]	0.7 ± 0.02[a]	0.74 ± 0.01[a]
Leucine	6.43 ± 0.03[a]	6.42 ± 0.03[a]	6.34 ± 0.03[b]
Lysine	4.65 ± 0.07[a]	4.66 ± 0.05[a]	4.5 ± 0.03[b]
Methionine	0.93 ± 0.05[a]	0.92 ± 0.005[a]	0.93 ± 0.03[a]
Phenylalanine	3.02 ± 0.04[a]	2.93 ± 0.03[b]	2.81 ± 0.04[c]
Threonine	2.22 ± 0.02[a]	2.19 ± 0.01[ab]	2.16 ± 0.01[b]
Valine	2.2 ± 0.03[a]	2.14 ± 0.03[b]	2.2 ± 0.02[a]
Non essential amino acids			
Alanine	4.11 ± 0.05[a]	3.95 ± 0.01[b]	3.95 ± 0.03[b]
Aspartic acid	5.58 ± 0.05[a]	5.43 ± 0.07[b]	5.5 ± 0.02[b]
Cystine	0.32 ±0.01[b]	0.4 ± 0.01[a]	0.37 ± 0.02[a]
Glutamic acid	4.77 ± 0.07[a]	4.17 ± 0.04[b]	4.15 ± 0.02[b]
Glycine	0.95 ± 0.03[a]	0.85 ± 0.01[b]	0.91 ± 0.02[a]
Proline	1.21 ±0.01[b]	1.29 ± 0.01[a]	1.23 ± 0.04[b]
Serine	0.94 ± 0.03[a]	0.93 ± 0.02[a]	0.97 ± 0.01[a]
Tyrosine	0.35 ±0.02[a]	0.24 ± 0.02[b]	0.24 ± 0.02[b]

*Data are mean of three replications. Dissimilar superscripts in a row indicate significant difference (P<0.05).

4. Discussion

4.1. Effects on Microbiological Profile

The pre-fermented SHB exhibited a high degree of contamination by hazardous microorganisms, like faecal coliform bacteria, *Staphylococcus* and *Clostridium.* The presence of such microorganisms in drinking water or food is of great concern for public health (Prescott *et al*., 2002). Irrespective of treatments, LAB became the dominant microorganisms at the end of fermentation, an obvious outcome of successful fermentation process (Faid *et al*., 1994; Burns and Bagley, 1996). Acid tolerant yeast also grew in the medium as observed during the fermentation of other organic wastes (Oude Elferink *et al*., 2000), but failed to inhibit the growth of LAB. Results of the present study indicate that fermentation by whey or *L. acidophilus* could substantially reduce the population of *Staphylococcus*, *Clostridium* and coliform bacteria. Faecal coliforms were totally removed from all groups of fermented products, while in LFSHB there was no trace of any kind of coliform. The increase in the population of LAB and subsequent production of organic acids created an acidic environment during fermentation, which probably resulted in conditions unfavorable to the hazardous microorganisms mentioned above (Nwad *et al*., 2008). El Jalila (2001) also observed a net decrease in the number of the hazardous microorganisms (enterobacteria, enterococci, *Clostridium*, *Salmonella*) during fermentation of poultry wastes manure with *Lactobacillus plantarum* and *Pediococcus acidilactici* as inoculum. Ndaw *et al*. (2008) did not either observe any growth of *Staphylococcus aureus*, *Salmonella* sp. and sulfite-reducing *Clostridium* after two weeks of fermentation of the fish *Sardina pilchardus* with *Lactobacillus delbrueckii*.

A prolonged exposure of non-fermented product to open air also showed aerobically unstable condition, because moulds and yeast grew moderately in them. Such a microbial growth does not only reduce the nutritional value of the product, but it also increases the risk of the proliferation of potential pathogenic and undesirable microorganisms (Driehuis and Oude Elferink, 2000). Yeast is the principal spoiling organism that oxidizes preserving acids, increases pH and, finally, results in the growth of moulds (Woolford, 1990; Pahlow *et al*., 2003). Such growth of moulds and yeast reduces the feed value and the palatability of the fermented products and produces a n egative effect on animal health (Oude Elferink *et al*., 2000). In the present study, both of the fermented products showed an aerobically stable condition due to the inhibition of the growth of yeast and mould and, thus, indicated enhanced shelf life of the SHB after fermentation. Shelf life of the fermented products depends, to a l arge extent, on WSC content left after fermentation, because WSC acts as a substrate for growth of moulds and yeast when exposed to air. Accumulation of LA and AA in sufficient quantity in both the fermented products probably produced antagonistic effects on t he growth of moulds and yeast (Moon, 1983) rendering aerobic stability to the two fermented products. Danner *et al*. (2003) observed that, during ensilaging, the main factor behind the aerobic stability of the fermented products was the accumulation of AA. Driehuis *et al.* (2001) also observed a similar aerobic stability of the product after the fermentation of grass silage.

4.2. Effects on Biochemical Parameters

The desired level of pH indicative of completion of fermentation is 4.0 to 4.2. Within this pH range the fermented products are safe from spoilage and fit for the use as feed stuff for animals (Lee *et al*., 2004; Yashoda *et al*., 2001). Fermentation of SHB was completed within 4 days by both the inoculums. LA and AA are the major metabolites responsible for the reduction of pH in a fermentation mixture. In the present study, LA was generated in a h igher quantity than AA because of the capacity of LAB to produce LA as the major metabolite (Piard and Desmazeaud, 1991). This is also a n ormal phenomenon in silage fermentation process (Kung and

Shaver, 2001). While comparing with the fermentation of fish offal (FO) by *L. acidophilus* and whey (Samaddar and Kaviraj, 2014), the present study revealed less amount of LA generation during fermentation, even though the amount of AA generated and the amount of WSC reduced were similar in the fermentation of FO and SHB. In the fermentation of FO, *L. acidophilus* was found to complete fermentation within 3 days as compared to 5 days by whey. In the present study fermentation was completed within 4 days in both the inoculums. LABs are known to require complex nitrogenous sources for their metabolism during fermentation (Narayanan *et al*., 2004). FO appeared, as a better source of nitrogen for *L. acidophilus* than SHB, while SNB served as a better source of nitrogen than FO for the complex group of fermentation microorganisms supplied by whey. Whey, being a by-product of dairy industry, is expected to carry homo-fermentative group of LAB (Moreira *et al*., 2000; Ng *et al*., 2011). Since AA was also detected in all fermentation mixtures, the presence of some hetero-fermentative LAB or other AA producing microorganism in these mixtures could not be ruled out. A probable source of these microorganisms was either SHB or molasses. The accumulation of AA in a relatively higher amount in WFSHB, as compared to LFSHB, is probably helpful to protect spoilage under aerobic condition (Filya, 2003; Kung *et al*., 2003). There was no significant difference in WSC content between the two fermentation groups, indicating that the carbohydrate utilization rate was the same in these two groups.

Significant variations were observed in the proximate compositions between the pre-fermentation mixture and the fermented SHB except crude lipid and ash content. The crude protein level reduced significantly in all fermented products, as compared to the pre-fermented category, but the reduction was restricted up to 1.15 % in LFSHB and 1.49 % in WFSHB. Reduction in the level of crude protein in the fermented products was probably due to the utilization of a part of the protein by microorganisms as a nutrient source in their metabolism through extracellular proteolytic activity. As a result, there was a breakdown of a part of the protein along with the increase in the Free Amino Acid (FAA) level in both fermented products (Savijoki *et al*., 2006; Haq and Mukhtar, 2006). The breakdown of the protein and the subsequent increase in FAA contents increase the digestibility of the food ingredient and the excess FAA can also act as potential chemo-attractants for fish (Ramírez-Ramírez *et al*., 2008; Lian *et al*., 2005). Among the essential amino acids, the reduction of arginine, histidine, phenylalanine and threonine contents in both groups indicated their need for the microorganisms irrespective of the inoculums used, while leucine and lysine reduced significantly in WFSHB and valin reduced significantly in LFSHB, indicating selective requirements of these amino acids by different groups of microorganisms grown from these two inoculums. However, only a slight variation was observed in the level of crude protein and essential amino acids between the two fermented products and both products contained adequate amount of crude protein and essential amino acids required for formulating balanced aquaculture feed. A significant reduction of crude fiber level of the SHB mixture due to fermentation is another advantage of using fermented SHB as feed ingredient. The decrease in crude fiber level in the fermented products was due to cellulolytic microbial action during fermentation (Shi *et al*., 2006). El Jalil *et al.* (2001) also observed a depletion of crude protein and crude fiber level along with unchanged crude lipid value, as observed in the present study, during fermentation of poultry wastes manure with *L. plantarum* and *Pediococcus acidolactici* as inoculum.

5. Conclusion

Fermented Slaughterhouse Blood (SHB) showed an excellent protein quality with the availability of some essential amino acids like, arginine, leucine, lysine, phenylalanine, threonine and valine, essential for a product to establish itself in aquaculture feed industry as a good quality protein supplement. Fermentation renders a microbiological safety, an enhanced shelf life and a minimum loss of nutrients (especially crude protein and essential amino acids). Besides, commercially available *L.acidophilus* as fermentation inoculums, whey, a byproduct of milk industry and an easily available source of LAB, appeared to make technology a more eco-friendly and feasible one to the small scale aquaculturists.

Acknowledgements

The financial support received from UGC, New Delhi, and DST PURSE, Govt. of India, for this research is gratefully acknowledged.

References

Agbedi OT, Outbusin SO and Ogunleye FO. 2009. Effect of different levels of substitution of fishmeal with blood meal in pelleted feeds on catfish *Clarias gariepinus* (Burchell 1822) culture in net cages. *Eur J Sci Res*., **31**: 6-10.

Aladetohun NF and Sogbesan OA. 2013. Utilization of blood meal as a protein ingredient from animal waste product in the diet of *Oreochromis niloticus. Int J Fish Aquacult*., **5(9)**:234-237.

Bergleiter S. 2011. **Organic Aquaculture – From a "Nice Niche" to the "Whole Cake" to Increase the Organic Share of the World Aquaculture**. EAF special publication, pp 114-115.

Burns JC and Bagley CP. 1996. Cool-season grasses for pasture. In: Moser L.E., Buxton D.R., Casler M.D. (eds.), **Cool Season Forage Grasses**. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison pp. 321–355.

Cappuccino J and Sherman N. 2007. **Microbiology: a Laboratory Manual**. Pearson Education, New Delhi, p 14.

Danner H, Holzer M, Mayrhuber E and Braun R. 2003. Acetic acid increases stability of silage under aerobic conditions. *Appl Environ Microbiol*., **69(1)**:562.

De Silva SS and Anderson TA. 2009. **Fish Nutrition in Aquaculture.** Springer International Edition, Indian reprint, New Delhi.

Driehuis F and Oude Elferink SJWH. 2000. The impact of quality of silage on animal health and food safety: a review. *Vet Q*., **22**: 212–217.

Driehuis F, Oude Elferink SJWH, Van Wikselaar PG. 2001. Fermentation characteristics and aerobic stability of grass silage

inoculated with *Lactobacillus buchneri,* with or without homofermentative lactic acid bacteria. *Grass Forage Sci*., **56**: 330-343.

Dremetsika AV, Siskos PA and Bakeas EB. 2005. Determination of formic and acetic acid in the in terior atmosphere of display cases and cabinets in Athens museums by teverse phase high performance liquid chromatography. *Indoor Built Environ*., **14**: 51–58.

El Jalila MH, Faid M and Elyachioui M. 2001. A biotechnological process for treatment and recycling poultry wastes manure as a feed ingredient. *Biomass Bioenerg*., **21**: 301–309.

Faid M, Karani H, Elmarrakchi A and Achkari-Begdouri A. 1994. A biotechnological process for the valorization of fish waste. *Bioresour Technol*., **49**: 237–241.

Fearon J, Mensah SB and Boateng V. 2014. Abattoir operations, waste generation and management in the Tamale metropolis: Case study of the Tamale slaughterhouse. *J. Public Health Epidemiol*., **6 (11)**:14-19.

Filya I. 2003. The effect of *Lactobacillus buchneri* and *Lactobacillus plantarum* on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. *J Dairy Sci*., **86**: 3575–3581.

Gomez KA and Gomez AA. 1984. **Statistical Procedures for Agricultural Research**. John Wiley and Sons.

Haq IU and Mukhtar H. 2006. Biosynthesis of protease from *Lactobacillus paracasei*: kinetic analysis of fermentation parameters. *Indian J Biochem Biophys*., **43**: 377–381.

Helrich W. 1990. **Official Methods of Analyses. Association of Official Analytical Chemists**. Washington DC. USA.

Jayathilakan K., Sultana K., Radhakrishna K. and Bawa A. S. (2012) Utilization of byproducts and waste materials from meat, poultry and fish processing industries: a review. *J Food Sci Tech*., **49(3)**: 278–293.

Kader A, S hunsuke K, Manabu I, Saichiro Y, Mahbuba B, Nguyen B, Gao J and Laining A. 2012. Can fermented soybean meal and squid by-product blend be used as fishmeal replacements for Japanese flounder (*Paralichthys olivaceus*). *Aquacult Res*., **43**: 1427–1438.

Kung L and Shaver R. 2001. **Interpretation and use of silage fermentation analyses reports**. In: Focus on forage, Forage management fact sheet, University of Wisconsin, Madison, USA, Vol. 3, No. 13. Available via http:// http://www.uwex.edu/ces/crops/uwforage/Fermentaion.html. Accessed 21 Sept, 2013.

Kung L, Taylor CC Jr, Lynch MP and Neylon JM. 2003. The effect of treating alfalfa with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for lactating dairy cows. *J Dairy Sci*., **86**: 243–336.

Kwanyuen P and Burton JW. 2010. A Modified amino acid analysis using pitc derivatization for soybeans with accurate determination of cysteine and half-cystine. *J Am Oil Chem Soc*., **87**: 127–132.

Lee KS, Lee KY, Oh CS, Lee DG, Kim YJ. 2004. Effect of aeration for the probiotic feed production from food wastes by *Lactobacillus acidophilus* and *Saccharomyces cerevisiae. J Kowrec*., **11(4)**: 114– 119.

Lian P.Z., Lee C.M. and Park E. (2005) Characterization of squid-processing by-product hydrolysate and its potential as aquaculture feed ingredient. *J Agric Food Chem*., **53**: 5587–5592.

McHugh J, Dumont SN, Julie P, Nguyen AL, Levesque S and Carrier A. 2006. New and simple HPLC method for the determination of lactic acid content in ciprofloxacin injection. *J Liq Chromatogr Related Technol*., **29**: 1905– 1916.

Moore S and Stein WH. 1948. Photometric ninhydrin method for use in the chromatography of a mino acid. *J Biol Chem*., **176**: 367-388.

Moon NJ. 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. *J Appl Bacteriol*., **55**: 453–460

Moreira M, Abraham A and De' Antoni G. 2000. Technological properties of milks fermented with thermophilic lactic acid bacteria at suboptimal temperature. *J Dairy Sci*., **83**: 395–400.

Murphy PT, Moore KJ, Richard TL, and Bern CP. 2007. Enzyme enhanced solid- state fermentation of kenaf core fiber for storage and pretreatment. *Bioresour Technol*., **98**: 3106-3111.

Narayanan N, Roychoudhury PK and Srivastava A. 2004. L (+) lactic acid fermentation and its product polymerization. *Electron J Biotechn*., **2**: 0717-3458.

Ng E, Yeung M and Tong P. 2011. Effect of starter cultures on the survival of *Lactobacillus acidophilus*. *Int J Food Microbiol*., **145(1)**: 169–175.

Nwad AD, Faid M, Bouseta A, Zinedine. 2008. Effects of controlled lactic a cid fermentation on the microbiological and chemical quality of Moroccan sardines (*Sardina pilchardus*). *Int J Agr Biol*., **10(1)**: 21-27.

Oude Elferink SJWH, Driehuis F, Gottschal JC and Spoelstra SF. 2000. Silage fermentation process and their manipulation, in: Mannetje L. (eds.), **Silage Making in the Tropics with Particular Emphasis on Smallholders.** Proceedings of the FAO electronic conference on tropical silage, FAO, Rome, pp. 17-30.

Pahlow G, Muck RE, Driehuis F, Oude Elferink SJWH, Spoelstra SF and Madison HJH. 2003. Microbiology of ensiling, in: Buxton D.R., Muck R.E. (eds.), **Silage Science and Technology**. American Society of Agronomy, Madison, WI, USA, pp. 31-93.

Prescott LM, Harley JP and Klein DA. 2002. **Microbiology**, 5th edition. McGraw-Hill, London, UK.

Piard JC and Desmazeaud M .1991. Inhibiting factors produced by lactic acid bacteria: 1. Oxygen metabolites and catabolism end products. *Lait*., **71**: 525- 541.

Pyar H, Peh KK and Liong M. 2011. Inhibitory effects of metabolites from probiotics *Lactobacillus acidophilus* strains on growth of pathogenic bacteria. *J Pharmacol Toxicol*., **6(5)**: 533-540.

Ramírez-Ramírez JC, Huerta S, Arias L, Prado A. and Shirai K. 2008. Utilization of fisheries by-catch and processing wastes for lactic acid fermented silage and evaluation of degree of protein hydrolysis and in vitro digestibility. *Rev Mex Ing Quim*., **7:** 195-204.

Ribeiro RA, Ozóório RODA, Batista SMG , Pereira-Filho M , Ono EA and Roubach R. 2011. Use of spray-dried blood meal as an alternative protein source in Pirarucu (*Arapaima gigas*) diets. *J Appl Aquacult*., **23(3):**238-249.

Samaddar A, Kaviraj A. 2014. Processing of fish offal waste through fermentation utilizing whey as inoculum. *Int J Recycl Org Waste Agricult*., **3**: 45.

Savijoki K, Ingmer H and Varmanen P. 2006. Proteolytic systems of lactic acid bacteria. *Appl* Microbiol *Biotechnol.,* **71**: 394–406.

Shi J.G., Zeng G.M., Yuan X.Z., Dai F., Liu J. and Wu X.H. (2006) The stimulatory effects of surfactants on composting of wastes rich with cellulose. *World J Microb Biot*., **22**: 1121-1127.

Woolford MK. 1990. The detrimental effects pf air on silage. *J Appl Bacteriol*., **68**: 101-116.

Yashoda KP, Sachindra NM, Rao D and Mahendrakar NS. 2001. Changes in microbial population during fermentation of silk worm pupae. *Sericologia*., **41(2)**: 253-261.

Zhu H, Gong G, Wang J, Wu X, xue M, Niu C, Guo L and Yu Y. 2011. Replacement of fishmeal with blend of rendered animal protein in diets for Siberian sturgeon (*Acipenser baerii* Brandt), results in performance equal to fish meal fed fish. *Aquacult Nutr*., **17**: 389-395.

JJBS
Jordan Journal of Biological Sciences

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 31 - 36

Mangrove Health Card: A Case Study on Indian Sundarbans

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Received: November 9, 2014 Revised: December 3, 2014 Accepted: December 9, 2014

Abstract

Relative abundance and Shannon-Weiner species diversity index of true mangrove species were estimated in 10 sampling stations of Indian Sundarbans during 2013. The mangrove patches in the selected stations were categorized into a 4-point scale depending on the values of Shannon-Weiner species diversity index. The health of the mangrove forest as per our constructed 4-point scale was found to be regulated primarily by anthropogenic factors, although in station like Sagar South, the natural erosion may be the key player in determining the mangrove floral diversity.

Keywords: Indian Sundarbans, Relative abundance, Shannon-Weiner species diversity index, 4-Point scale, Anthropogenic pressure, Erosion.

1. Introduction

Mangrove forests are among the world's most productive ecosystems. The real ecosystem services of mangroves have come in the forefront only after the outbreak of Tsunami on 26th December 2004. Mangroves protect coastal lands by absorbing and diverting the energy of tidal currents and storm driven wind and wave action, creating natural break water that retards the process of erosion. The mangrove community also provides a buffer between a terrestrial and nearby marine environment; trapping and stabilizing sediments, nutrients and several types of conservative pollutants (Mitra, 2013; Chakraborty *et al*., 2014a; Chakraborty *et al*., 2014b), hence helping to maintain the water quality. Evaluation of such ecosystem services of mangroves and their economic evaluation are yet unrevealed, even though several studies are available on the use of mangroves as nursery and breeding ground of fishes, sources of timber, honey, wax, firewood, etc. (Naskar, 1993; Ewel *et al*., 1998; Hogarth, 2007; Naskar and Mandal, 2000; Saenger, 2002; Naskar, 2004; Nybakken and Bertness, 2005; Raman *et al*., 2007; Walters *et al*., 2008; Spalding *et al*., 2010). Considering the importance of mangroves as primary service providers to coastal populations and island dwellers, it is extremely important to assess the biodiversity and relate the same with the anthropogenic and natural threats existing in and around the habitats. The present study is an approach in this direction considering 500 m sampling width from the Low Tide Line (LTL) existing in different sampling sites of Indian Sundarbans. The significance of taking such a band width of 500 m is the niche preference of different mangrove species (Duke and Kleine, 2007) as shown in Figure 1.

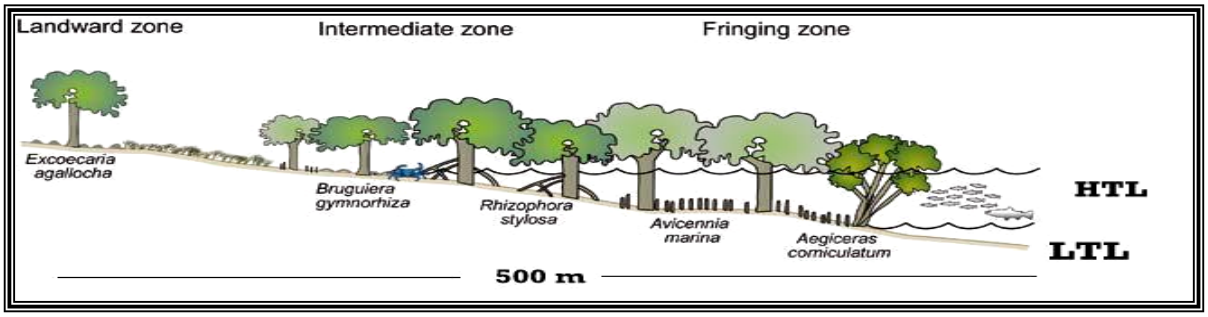

Figure 1. Distribution and preference of mangrove species in the intertidal mudflats of a model mangrove forest in the tropical estuary. (Source: modified after Duke and Kleine, 2007; www.dpi.nsw.gov.au/__data/assets/pdf_file/0020/.../mangroves.pdf)

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2. Materials and methods

2.1. Site selection and Sampling

The present study was conducted in the Indian Sundarbans mangrove ecosystem during 2013. Several field trips were made to select the field stations (Table 1) and study the distribution of mangrove vegetation, geographic nature of riverine system, salinity, environmental quality and anthropogenic pressure. While selecting the sampling sites, we focused on two broad issues namely natural threats (like erosion, sea level rise, salinity fluctuation, etc.) and human induced factors (like industrialization, urbanization, tourism, aquacultural practice, etc.). These criteria have been selected to observe the factors regulating the survival/degradation of mangroves in and around the selected sampling stations.

Table 1. Selected sampling stations with coordinates

Stations	Latitude	Longitude
Sagar South	21°38'54.37"N	88°03'06.17"E
Canning	22°19'03.20"N	88°41'04.43"E
Kakdwip	21°52'26.50"N	88°08'04.43"E
Chemaguri	21°39'58.15"N	88°10'07.03" E
Lothian island	21°39'01.58"N	88°22'13.99"E
Prentice island	21°42'40.97"N	88°17'10.04"E
Bonnie Camp	21°49'42.90"N	88°37'13.70"E
Sajnekhali	22°05'13.40"N	88°46'10.80"E

Quadrates of 10m × 10m were laid randomly up to 500 m from LTL and data from each one were recorded from 15 such quadrates. Plant materials collected during the sampling were identified from Naskar (1993) and confirmed from Botanical Survey of India, Kolkata (India).

2.2. Enumeration of mangrove health

Relative abundance of the species was estimated as per the expression: RA = Abundance of a particular species/sum of the abundance of all species × 100; where RA represents relative abundance.

Shannon Weiner index for diversity was calculated based on the abundance value of plant species in different categories as per the following expression:

$$H = -\sum_{i=1}^{s} \text{Pi} \log \text{Pi}$$

Where, H = Shannon-Weiner species diversity index; $P_i = n_i/N$ (n_i = Number of individuals of i^{th} species and N= total number of individuals of all the species in the quadrate).

The values of Shannon Weiner index were calibrated on 4-point scale to assign a status to health of the mangrove diversity in each region. Four distinct groups were derived from the 4-point scale on the basis of the values of Shannon-Weiner Species Diversity Index. These groups are designated as follows:

< 1: Worst Health;

1-2: Moderate Health;

2-3: Good Health;

3-4: Excellent Health

3. Results

A total of 25 mangrove species (*Acanthus ilicifolius, Acrostichum aureum, Aegiceras corniculatum, Aegialites rotundifolia, Avicennia alba, Avicennia marina, Avicennia officinalis, Bruguiera cylindrical, Bruguiera gymnorhiza, Bruguiera hexangular, Bruguiera parviflora, Ceriops decandra, Ceriops tagal, Exocoecaria agallocha, Heritiera fomes, Kandelia candel, Lumnitzera racemosa, Nypa fruticans, Phoenix paludosa, Rhizophora apiculata, Rhizophora mucronata, Sonneratia apetala, Sonneratia caseolaris, Xylocarpus granatum, Xylocarpus mekongensis*) was recorded from the selected sites (Table 2).

The total number of individuals of all the documented species, which represents the dense growth of the trees, exhibits a pronounced variation due to which the Shannon Weiner species diversity is affected. Highest numbers of individuals are observed in the quadrate of Lothian island (165/100m^2), a pristine forest patch almost with no human intervention, whereas lowest value (37/100m^2) is observed in Kakdwip, the sampling station with maximum anthropogenic stress (Figure 2).

The results of Shannon-Weiner index are shown in Figure 3. The spatial order of the index is Prentice island (3.052) > Lothian island (3.006) > Sajnekhali (2.984) > Bonnie camp (2.737) > Chemaguri (2.563) > Sagar south (2.441) > Canning (2.192) > Kakdwip (1.719).

The variation in the value of the index reflects (i) the degree of stress (both natural and anthropogenic) (ii) conditions of the ambient environment (in terms of hydrological parameters and soil quality). Greater value of the index represents a more congenial environment which usually occurs due to the survival of more number of species or even distribution of the number of individuals amongst different species in the quadrate.

Table 2: Selected sampling species with their family

Species	Family
Acanthus ilicifolius	Acanthaceae
Acrostichum aureum	Pteridaceae
Aegiceras corniculatum	Myrsinaceae
Aegialites rotundifolia	Plumbaginaceae
Avicennia alba	Avicenniaceae
Avicennia marina	Avicenniaceae
Avicennia officnalis	Avicenniaceae
Bruguiera cylindrical	Rhizophoraceae
Bruguiera gymnorhiza	Rhizophoraceae
Bruguiera hexangular	Rhizophoraceae
Bruguiera parviflora	Rhizophoraceae
Ceriops decandra	Rhizophoraceae
Ceriops taga,	Rhizophoraceae
Exocoecaria agallocha	Euphorbiaceae
Heritiera fomes	Sterculiaceae
Kandelia candel	Rhizophoraceae
Lumnitzera racemosa	Combretaceae
Nypa fruticans	Palmae
Phoenix paludosa	Palmae
Rhizophora apiculata	Rhizophoraceae
Rhizophora mucronata	Rhizophoraceae
Sonneratia apetala	Sonneratiaceae
Sonneratia caseolaris	Sonneratiaceae
Xylocarpus granatum	Meliaceae
Xylocarpus mekongensis	Meliaceae

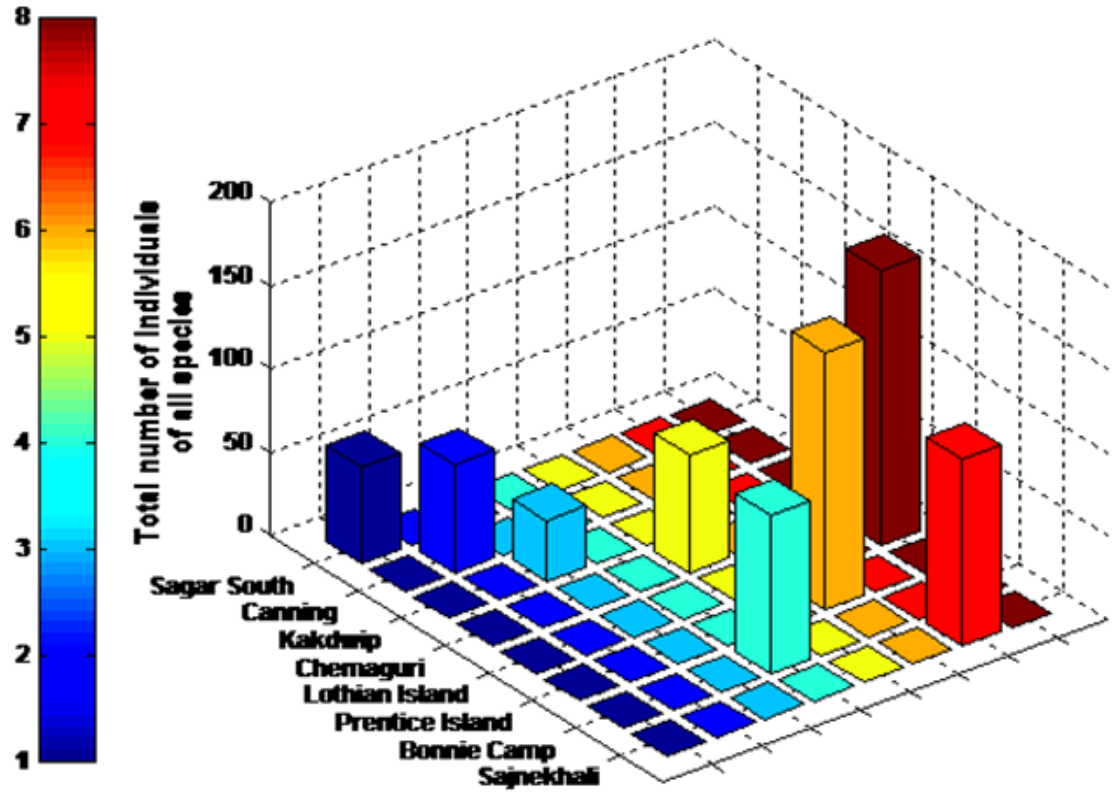

Figure 2. Spatial variation of total number of individuals of all species (N)

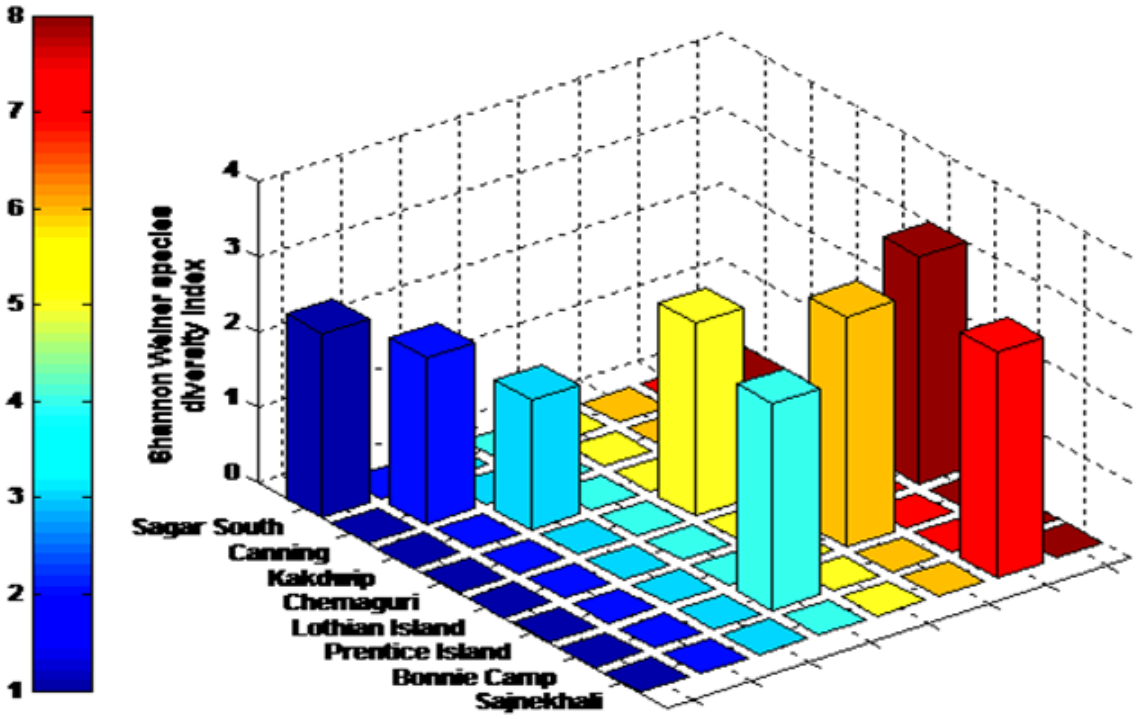

Figure 3. Spatial variation of Shannon Weiner species diversity index.

4. Discussion

Mangroves are salt tolerant plant communities that grow luxuriantly in the intertidal zone in the tropics and sub-tropics. A total of 52 species have been identified by Giesen *et al*. (2007) as true mangroves in south-east Asia. Mangroves that are dominant and typify the flora in most locations are considered as core species (Spalding *et al*., 2010; ITTO, 2012). Globally, a total of 38 core species have been identified in the Indo-West Pacific and Atlantic East Pacific regions. In Indian Sundarbans, a total of 34 true mangrove species have been documented (Chaudhuri and Choudhury, 1994; Mitra, 2000; Mitra *et al*., 2004; Mitra and Banerjee, 2005) of which, in the present study, only 25 species were documented during the sampling period (Table 2). On the basis of our calibration (as stated in the materials and method section) it is observed that Prentice island and Lothian island fall within the category of 'Excellent health'. The category of 'Good health' encompasses 5 stations in our study area namely Sajnekhali, Bonnie camp, Chemaguri, Sagar south and Canning, whereas Kakdwip with H value of 1.719 is under the category of 'Moderate health' as per our 4-point calibration scale. The variation in health may be attributed to degree and magnitude of threats to which these sampling stations are exposed. Prentice island and Lothian island showing 'Excellent health' fall within the protected area of the Reserve Forest due to which minimum human interference occur in these stations (Mitra and Banerjee, 2005). Moreover, the rich diversity of mangroves in these two stations may also be related to optimum salinity of the ambient aquatic phase due to inundation of the forest with the water of the Hooghly riverine system (Mitra *et al*., 2009; Mitra *et al*., 2011; Banerjee *et al*., 2012; Sengupta *et al*., 2013; Chakraborty *et al*., 2014a; Ray Choudhury *et al*., 2014) that receive the freshwater through Farakka discharge.

The stations exhibiting 'Good health' (like Bonnie camp and Sajnekhali) are hypersaline in nature due to complete blockage of the freshwater flow on account of Bidyadhari siltation since the late 15th century (Chaudhuri and Choudhury, 1994). These two stations also witness minimum human interference except seasonal tourism (preferably during the winter season prevailing from December to February). The other three stations (Canning, Chemaguri and Sagar South) within the category of 'Good health' experience threats like tourism, fish-landing, pilgrims and sporadic presence of shrimp farms. Inspite of these multifarious threats, appropriate dilution of the sampling sites with fresh water has provided the mangroves of these sites a congenial environment. It is to be noted, at this point, that mangroves are halophytes, but their luxuriant growth in brackish water (~ 5 psu to 15 psu) and stunted growth and extinction in high saline water have been observed by several researchers in the present geographical locale (Mitra *et al*., 2011; Banerjee *et al*., 2012; Sengupta *et al*., 2013). It is also observed, in the present study area, that species like *Heritiera fomes* is highly sensitive to salinity and prefers a low saline condition around 2-4 psu (Figure 4). The gradual vanishing of this species from the Indian Sundarbans is a confirmatory test of the preference of some mangrove species to hyposaline environment (Zaman *et al*., 2013; Mitra and Zaman, 2014).

Figure 4. *Heritiera fomes*: gradually vanishing from Indian Sundarbans

Kakdwip, located in the western Indian Sundarbans, exhibits 'Moderate health' due to high degree of anthropogenic pressure arising from passenger vessel jetties, fish-landing activities, shrimp farms, brick kilns, busy market related activities etc.

In addition to this, natural factors, like erosion due to wave action, also play a crucial role in modifying the health of the mangrove forest as seen in case of Sagar south. The gradual erosion of the island makes the substratum unstable and washes out the nutrient and organic matter from the intertidal mudflats on which the growth and survival of mangrove flora depend (Sengupta *et al*., 2013). The types of threats exerted on the mangroves inhabiting the selected stations are highlighted in Table 3.

Table 3. Nature of threats on the mangroves of selected sampling stations

Stations	Natural threat	Anthropogenic threat
Sagar South	Erosion, sea level rise	Fish landing, fish drying, tourism, pilgrim related pressure, navigational channel
Canning	Siltation and complete blockage of fresh water	Fish landing stations, boat/fishing vessels/trawlers mending and repairing units
Kakdwip	Erosion	Fishing harbour, market and fish selling points, heavy metal pollution from antifouling paints used for conditioning fishing vessels and trawlers
Chemaguri	Erosion	Fish landing units, shrimp farms
Lothian island	Erosion	Minimum because of its location within the Reserve Forest
Prentice island	Erosion	Minimum and adjacent to the Reserve Forest
Bonnie Camp	Erosion	Minimum and adjacent to the Reserve Forest, tourism pressure particularly during the postmonsoon season
Sajnekhali	Siltation and hypersalinity	Minimum because of its location within the Reserve Forest

5. Conclusion

The overall discussion, thus, leads to the conclusion that human interference is the major factor in the lower Gangetic delta that plays the key role in determining the health of the mangrove forest in terms of diversity. A more serious intervention of the Government and local NGOs is necessary to restore the degraded health of the mangrove forest in areas like Kakdwip, which otherwise may face complete degradation in near future.

References

Banerjee K, Roy Chowdhury M, Sengupta K, Sett S and Mitra A. 2012. A. Influence of anthropogenic and natural factors on the mangrove soil of Indian Sundarban Wetland. *Arch. Environ Sci*., **6,** 80-91.

Chakraborty S, Trivedi S, Fazli P, Zaman S and Mitra A. 2014a. *Avicennia alba*: an indicator of heavy metal pollution in Indian Sundarban estuaries. *J of Environ. Sci., Computer Science and Engineering & Technology*, **3(4):** 1796-1807.

Chakraborty S, Zaman S, Fazli P and Mitra A. 2014b. Bioaccumulation pattern of heavy metals in three mangrove species of *Avicennia* inhabiting lower Gangetic delta. *J of Chem., Biol. and Phy. Sci.*, **4(4)**: 3884-3896.

Chaudhuri AB, and Choudhury A. 1994. *Mangroves of the Sundarbans. Volume 1: India*. International Union for Conservation of Nature and Natural Resources (IUCN).

Duke N. and Kleine, D. University of Queensland, 2007. www.dpi.nsw.gov.au/__data/assets/pdf_file/0020/.../ mangroves.pdf.

Ewel K, Twilley R and Ong J. 1998. Different kinds of mangrove forests provide different goods and services. *Glob. Ecol. and Biogeo. Letts.*, **7**: 83–94.

Giesen W, Wulfraat S, Zieren M and Scholten L. 2007. **Mangrove Guidebook for Southeast Asia**. FAO, Bangkok, Thailand and Wetlands International, Wageningen, Netherlands.

Hogarth PJ. 2007. **The Biology of Mangroves and Seagrasses**. Oxford University Press.

ITTO. 2012. Special edition summarizing the findings of the 2010 World Atlas of Mangroves. *Tropical Forest Update*, **21**: 1–24.

Mitra A. 2000. The Northeast coast of the Bay of Bengal and deltaic Sundarbans. In: Sheppard C. (Ed): **Seas at the Millennium – An Environmental Evaluation**, Chapter 62, University of Warwick, Coventry, UK), Elsevier Science, 143-157.

Mitra, A., 2013. **Sensitivity of Mangrove Ecosystem to Changing Climate,** Springer, **62**: 143- 157.

Mitra A, Banerjee K and Bhattacharya DP. 2004. **The Other Face of Mangroves,** Published by Department of Environment, Govt. of West Bengal, India.

Mitra A and Banerjee K. 2005. Living resources of the Sea: Focus Indian Sundarbans. WWF-India, Sunderbans Landscape Project, Canning Field Office, West Bengal, India.

Mitra A, Banerjee K, Sengupta K and Gangopadhyay A. 2009. Pulse of climate change in Indian Sundarbans: A myth or reality? *Natl. Acad. Sci. Letts.*, **32(1/2):** 19-25.

Mitra A, Sengupta K and Banerjee K. 2011. Standing biomass and carbon storage of above-ground structures in dominant mangrove trees in the Sundarbans, *For. Ecol. and Manage.* (ELSEVIER DOI:10.1016/j.foreco.2011.01.012), **261(7)**: 1325 - 1335.

Mitra A and Zaman S. 2014. **Carbon Sequestration by Coastal Floral Community;** published by The Energy and Resources Institute (TERI) TERI Press, India.

Naskar K. 1993. 'Plant wealth of the lower Ganges', In: **Eco-Taxonomical Approach,** Hard Cover, 1-836, Published from Daya publishing house, ISBN: 13-978-81-7035-117-7.

Naskar K and Mandal R. 2000. **Ecology and Biodiversity of Indian Mangroves,** published from Daya publishing house, India. 1-783, ISBN: 81-7035-190-1.

Naskar K. 2004. **Manual of Indian Mangroves**, published from Daya publishing house, India. XVI: 220 (ISBN: 978-81-7035-303-4).

Nybakken JW and Bertness MD. 2005. Tropical Communities, Mangrove Forests. **Marine Biology, An Ecological Approach**. Edition 6. Pearson Education Inc., publishing as Benjamin Cummings, San Francisco, 454 pp.

Raman DJ, Jonathan MP, Srinivasalu S, Altrin JS, Mohan ASP and Mohan VR. 2007. Trace metal enrichments in core sediments in Muthupet mangroves, S.E coast of India: application of acid leachable technique, *Environ. Poll.*, **145:** 245-257.

Ray Choudhury M, Zaman S, Jha CS, Sengupta K and Mitra A. 2014. Mangrove biomass and stored carbon in relation to soil properties: A case study from Indian Sundarbans, *Intl. Jr. for Pharma. Res. Schos.*, **3(1-2):** 58-69.

Saenger P. 2002. **Mangrove Ecology**, **Silviculture and Conservation.** Kluwer Academic Publishers, 360.

Sengupta K, Roy Chowdhury M, Bhattacharyya SB, Raha A, Zaman S and Mitra A. 2013. Spatial variation of stored carbon in *Avicennia alba* of Indian Sundarbans. *Discov. Nat.,* (ISSN: 2319-5703), **3(8)**: 19 -24.

Spalding M, Kainuma M and Collins L. 2010. **World Atlas of Mangroves. Earthscan**, London, UK and Washington, DC, USA.

Walters BB, Rönnbäck P, Kovacs J, Crona B, Hussain S, Badola R, Primavera J, Barbier EB and Dahdouh-Guebas F. 2008. Ethnobiology, socio-economic and adaptive management of mangroves: A review. *Aquat. Bot.*, **89**: 220–236.

Zaman S**,** Bhattacharyya SB, Pramanick P, Raha AK, Chakraborty S and Mitra A. 2013. Rising water salinity: A threat to mangroves of Indian Sundarbans. In: **Water Insecurity: A Social Dilemma Community, Environment and Disaster Risk Management,13**:97-113.

JJBS

Jordan Journal of Biological Sciences

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 37 - 43

A Specific Haplotype Framework Surrounds the Omani Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Mutation S549R

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Received: October 19, 2014 Revised: December 3, 2014 Accepted: December 9, 2014

Abstract

Cystic Fibrosis (CF) is an autosomal recessive disorder affecting the chloride transport in mucus-producing epithelial cells. The disease is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), which is responsible for trans-epithelial chloride transport. Approximately 1900 mutations and gene variants of the CFTR have been described. The spectrum of major White-European mutations includes F508del, G542X, G551D and N1303K. F508del is the most common CF-causing mutation, found in approximately 70% of all CF patients worldwide. The spectrum of CF mutations of Arab populations is under-investigated. However, initial molecular-epidemiological studies indicate the existence of specific CF mutation clusters within geographical regions in the Middle East, suggesting specific distributions of CF mutation carrying chromosomes in this part of the world. We showed that the world-wide rare CF mutation S549R is the predominant disease causing mutation in the Omani population. We reported that S549R, together with two other identified mutations, F508del and the rare private mutation V392G, are genetically linked to the exonic methionine polymorphism c.1408A>G; p.Met470Val at exon 10 and the intronic dimorphic 4-bp GATT 6-repeat at intron 6, c.744_33GATT[6_8]. We detected three haplotypes in 28 alleles of the Omani CF cohort and 408 alleles of our control cohort of unrelated and unaffected Omani volunteers. The CF disease associated haplotype consisting of an M allele and a 6-repeat expansion, occurred with an allele frequency of only 0.174 in the normal Omani population. The discriminative power of the haplotype was attributed to the intronic dimorphic 4-bp GATT 6-repeat. Furthermore, we found only one mutation, c.1733_1734delTA in the Omani CF cohort which deviated from the rule and shared the most common haplotype, a V allele and a 7-repeat extension, with the normal population.

Key words: Arab populations, Mutations, Cystic Fibrosis, Cystic Fibrosis Transmembrane Conductance Regulator, S549R, Oman, Haplotypes, Diplotypes, M470V

1. Introduction

Cystic fibrosis (CF) (OMIM 602421) is the most common autosomal recessive inherited disorder in White-European populations. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), which is a chloride channel at the apical membrane of mucus producing epithelial cells (Kerem *et al*., 1989; Riordan *et al*., 1989; Rommens *et al*., 1989). The CFTR protein is predominantly expressed in epithelial cells of the lung, pancreas and gastro-intestinal tract. Mutations in the CFTR gene and their differential impact on the number of ion channels reaching the plasma membrane, on the passage of chloride ions through the ion-conducting pathway or the amount of intrinsic ATP-driven activity of CFTR, result in altered chloride conductance and lead consequently to a multiple organ involvement in CF or CF-related disorders (Sheppard *et al*., 1996; Wang *et al*., 2014). The classical clinical CF phenotype is characterized by repeated obstruction and infection of the lung (Ciofu *et al*., 2013), a failure to thrive as a consequence of mal-digestion (pancreatic involvement) and mal-absorption (gastro-intestinal involvement) (Gelfond *et al*., 2013).

Moreover, specific mutations in this gene can contribute to a form of male infertility known as Congenital Bilateral Absence of the Vas Deference

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(CBAVD) (Daudin *et al*., 2000), idiopathic and chronic pancreatitis (Cohn, 2005; Sharer *et al*., 1998; Witt, 2003) and bronchiectasis (Feldman, 2011).

Following the cloning of the CFTR gene, detailed studies of CF causing mutations and the elucidation of mutational panels for CF in the White-European populations emerged (Bobadilla *et al*., 2002; Grody *et al*., 2001; Watson *et al*., 2004). CF has an overall prevalence of approximately 1:2500 and a carrier frequency of 1:25 in White-European populations (Bobadilla *et al*., 2002). Currently more than 1900 CF mutations and sequence variations have been reported and documented (Cystic Fibrosis Mutation Database; accessed June 2014). The major mutation in White-European populations is F508del, a deletion of phenylalanine at c.1521_1523delCTT in exon 10 which has an average frequency of approximately 70% (Bobadilla *et al*., 2002). Only four other CFTR mutations, G542X, N1303K, G551D and W1282X, have worldwide allele frequencies above 1% in White-European populations (Estivill *et al*., 1997). Therefore the majority of CFTR mutations must be considered as rare or private and their regional occurrence can be attributed to subpopulations either geographically (e.g., G551D) or religiously (e.g., W1282X) (Estivill *et al*., 1997; Bobadilla *et al*., 2002).

This situation appears to be different among Arab populations. The spectrum of CF mutations, incidence and prevalence of the disease are largely unknown in the Arab populations (Wei *et al*., 2006). However, emerging studies suggest that the major and predominant CF mutations of Arab populations are different from the known CF mutational patterns in White-European populations. To date, a predominant major CF mutation, like the F508del CF mutation in White-European populations, was not described in Arab populations. Disease causing mutations for the majority of CF cases in Arab populations are considered as rare or private in White-European and other populations. For instance, the mutation I1234V is a major mutation in Qatar (Wahab *et al*., 2001), whereas in Saudi Arabia 3120+1G>A appears to be the most common and predominant CFTR mutation (El-Harith *et al*., 1997). The CFTR mutation E1140X was reported with 40% as the most common mutation in Libya (Fredj *et al*., 2011). Another specific and distinct mutational pattern was reported for Oman (Fass *et al*., 2014) and the neighboring country, the United Arab Emirates (UAE) (Frossard *et al*., 1997). The CFTR mutation S549R, a mutation altering the signature motif of the CFTR protein, is disease causing for more than 75% of all CF cases in both Gulf countries (Fass *et al*., 2014; Frossard *et al*., 1997; Lestringant *et al*., 1999).

The CF mutational panel of Oman is defined by two major mutations S549R and F508del with frequencies in the patient cohort of 0.75 and 0.14, respectively. Other mutations, which account for the remaining allele frequency of 0.11, were private and occurred in single patients or families (Fass *et al*., 2014). Carrier screening for all identified Omani mutations of a student population at Oman Medical College confirmed clinical CF incidence data and allowed the estimation of a CF prevalence of 1:8,264 in the country (Fass *et al*., 2014).

The absence of genetic evidence for an accumulation of CFTR mutations at a specific region in the CFTR gene, a so-called hot spot, suggests different reasons for the occurrence of the large number of CFTR mutations in various populations. One of the reasons might be a selective advantage of specific variants of the CFTR gene (Pompei *et al*., 2006). It was known even before the cloning of the CFTR gene that DNA polymorphisms such as short tandem repeats and single-nucleotide polymorphisms (SNP) co-segregate with disease causing alleles (Cutting *et al*., 1984). These various polymorphic markers were used for genetic counseling, risk assessment and carrier predictions (Beaudet *et al*., 1989), and served as tools for the identification of the origin, age and evolution of CFTR mutations (Morral *et al*., 1994). Haplotype frameworks are known for common White-European CFTR mutations but remained almost completely unexplored within the specific mutation patterns of Arab populations.

Two characteristic polymorphic markers in the CFTR gene are c.1408A>G (p.Met470Val; M470V), and c.744-33GATT[6_8], which will be abbreviated henceforth as M/V and 6/7 polymorphism, respectively (Kerem *et al*., 1990; Chehab *et al*., 1991; Gasparini *et al*., 1991). The M/V polymorphism is an exonic diallelic marker in exon 10 of the CFTR gene. The M allele is known to be associated with the CFTR mutation F508del (Cuppens *et al*., 1994; Pompei *et al*., 2006). Physiological differences between the wild type (wt) V and M allelic CFTR variant exist. It has been reported that the M allele of wt-CFTR gene exerts a higher chloride conductance (Cuppens *et al*., 1998). Another more recent study by Kosova *et al*. (2010) reports that the M allele is associated with a lower birth rate in fertile man. The 6/7 polymorphism is a sequence alteration in intron 6 of the CFTR gene (Chehab *et al*., 1991; Gasparini *et al*.,1991). The 6-GATT repeat structure was reported to be associated with F508del but have remained under-explored in other non-F508del White-European CFTR mutations. A potential physiological consequence has not been reported and seems unlikely because of the distance of this sequence alteration to the splice site.

The aim of this study is to classify the initial haplotype patterns of the M/V and 6/7 polymorphisms for the recently identified Omani mutations in an Omani CF cohort and a corresponding Omani control cohort. An analysis of haplotype patterns has the potential to identify the origin of the mutation and specify the genetic risk assessment in clinical practice.

2. Materials and Methods

2.1. Omani CF Patient and Volunteer Cohort

The Omani CF patient and volunteer cohorts were described elsewhere (Fass *et al*., 2014). An informed consent was taken from the 14 unrelated CF patients and the 204 unrelated student volunteers from Oman Medical College in Sohar. Consanguinity is high in the Omani population (Rajab and Patton, 2000). We ensured, by a questionnaire, that the enrolled volunteers are not related to each other. Furthermore, we inquired about the geographic origin of the parents and the grandparents of

the volunteer. The ancestry of 172 (84.35%) volunteers could be traced over three generations to the territory of Oman. DNA was isolated from ethylene-diamine-tetra-acidic acid (EDTA) buffered blood using the Wizard® genomic DNA Purification Kit (Promega Corp., Madison, Wisconsin, USA) according to the manufactures protocol. The disease causing mutations were identified in the Omani CF cohort by analysis of the entire exonic region and the flanking introns of the CFTR gene (Fass *et al*., 2014). The following CFTR mutations occur in the CF cohort in this study: (1) S549R (c.1647T>G, p.Ser549Arg), (2) F508del (c.1521_1523delCTT, p.Phe 508del), (3) V392G (c.1175T > G, p.Val392Gly), and (4) c.1733_1734delTA with allele frequencies of 0.75, 0.14, 0.035 and 0.075, respectively. The clinical characteristics and the severity of CF for those mutations have been recently reported (Fass *et al*., 2014).

2.2. Determination of Polymorphic Loci

Two polymorphic loci M/V and 6/7 were investigated in 28 CF patients' alleles and 408 alleles of the volunteer control cohort.

The M/V polymorphism was analyzed by restriction digest with HphI (New England Bio Labs Inc, Ipswich, Massachusetts, USA) (Kerem *et al*., 1990). Briefly, PCR primer and amplification conditions were applied as described previously (Fanen *et al*., 1992). For the restriction digest 10 µL of amplification product was digested with four units of HphI in a total volume of 15 µL overnight. The restriction fragments were separated and visualized by electrophoresis of a 3% agarose gel at 90 volts for 30 m inutes (Figure 1A). The 6/7 polymorphism at intron 6 was analyzed by DNA shift assay using polyacrylamide gel electrophoresis (PAGE) (Chehab *et al*., 1991). For the amplification of the 4-bp GATT repeat structure at intron 6 r eported primers and PCR conditions were used (Zielinsky *et al*., 1991). 3 µL amplification products were mixed with 7µL 6X bromophenol blue / xylene cyanol-loading dye. Electro-phoresis was conducted on 10% PAGE gels (32cm x 18cm x 0.75mm), in 1X Tris-acetate-ETDA buffer (TAE) at 250 volt for 6-8 hours. The DNA shift was visualized by silver staining (Fass *et al*., 2014) (Figures. 1B and C).

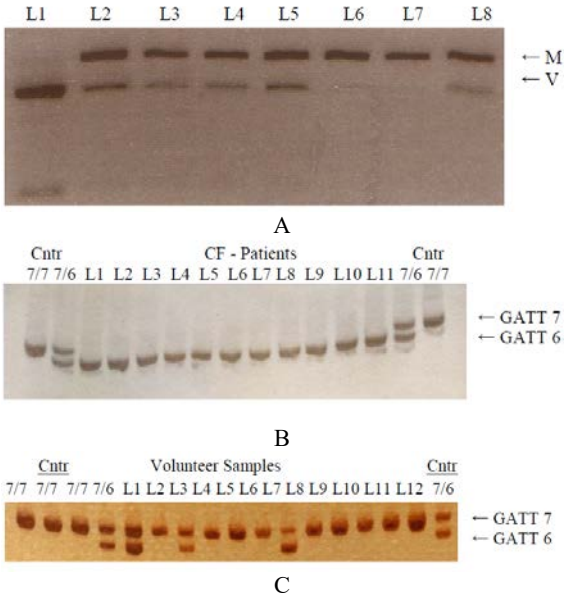


Figure 1. Representative **a**nalysis of the polymorphic loci in Omani CF patients (CF-Pts) and volunteers of the Omani Control Cohort (Vol): (A) Polymorphism M470V (M/V) of exon 10: Restriction digest with HphI on 3% agarose; The restriction site is abolished in the M allele of the M/V polymorphism. L 1: Vol= V/V (homozygote V/V); L 2-4+8: Vol=M/V (heterozygote M/V); L 6-7: Pts=M/M (homozygote M/M); (B) Dimorphic 4-bp GATT repeat polymorphism (6/7) of intron 6: DNA shift assay, 10% PAGE, silver staining: CF-Pts samples L1-9: S549R/S549R and L10-11: F508del/F508del. CF-Pts carry the 6 allele. (C) Dimorphic 4-bp GATT repeat polymorphism (6/7) of intron 6: DNA shift assay, 10% PAGE, silver staining: Vol Samples L 2, 4-6, 8-12: Vol=7/7 (homozygote 7/7), L 1,3 and 7: Vol=6/7 (heterozygote 6/7)

2.3. Analysis of Allele Frequencies, Haplotypes and Diplotypes

The allele frequencies of the polymorphic loci were established by direct allele counting. The two investigated polymorphic markers (M/V and 6/7) result in 4 theoretically possible haplotypes (M6, M7, V6, V7) and, consequently, 10 t heoretically possible diplotypes (M6M6, M6V7, M6M7, M7M7, M7V7, V7V7, V7V6, V6V6, V6M7, V6M6). The Chi-square test (χ^2) was applied to compare the allele frequency differences between the Omani CF patient cohort and the Omani volunteer cohort.

3. Results

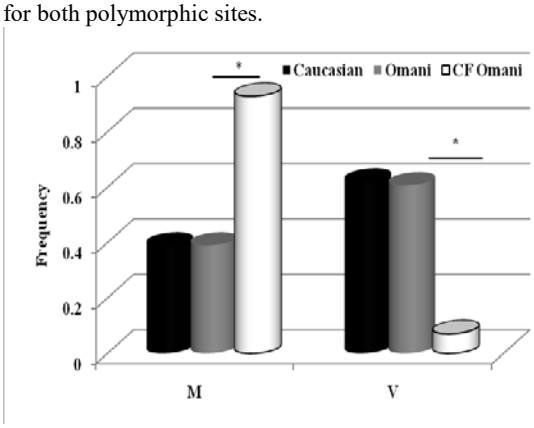
3.1. Allele frequency of M/V and 6/7 polymorphisms in the cohorts of unaffected Omani Volunteers and Omani CF patients

The analysis of M/V polymorphism and 6/7 polymorphism of the representative samples from the Omani volunteer cohort and the Omani CF patient samples are shown in Figure 1. Homozygote patients for the major Omani CF mutation S549R (allele frequency 0.75) carry a M allele at the M/V locus which results in an abolished restriction site of HphI at c.1408A (Figure 1A).

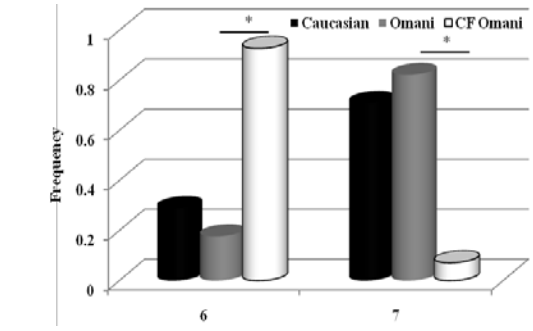
A similar pattern for the mutation S549R was observed for the 6/7 polymorphism. Patients, with the S549R CFTR mutation, are completely homozygote for the 6-GATT repeat (Figure 1B). In contrast, the 6 allele of the 6/7 polymorphism was not the most abundant allele in the Omani volunteer cohort. Figure 1C illustrates the predominance of the 7 a llele in a representative electrophoresis of 12 samples from the Omani volunteer cohort.

The M/V polymorphism at exon 10 occurred in the unaffected Omani population with allele frequencies of 0.392 and 0.607, respectively (Figure 2A). In contrast, the 6/7 polymorphism at intron 6 was less polymorphic and was detected in the control cohort with allele frequencies of 0.177 and 0.822, respectively (Figure 2B).

However, the 28 investigated alleles of the CF patients revealed an almost completely different distribution of the M/V and 6/7 polymorphism. The M and 6 polymorphic makers were both associated with the Omani mutations S549R, F508del and V392G. The allele frequency for each the M and 6 polymorphism was 0.928 (Figure 2). In contrast, only the alleles of the mutation c.1733_1734delAT were associated with the V and 7 polymorphisms and were found with a frequency of 0.071 for both polymorphic sites.



A

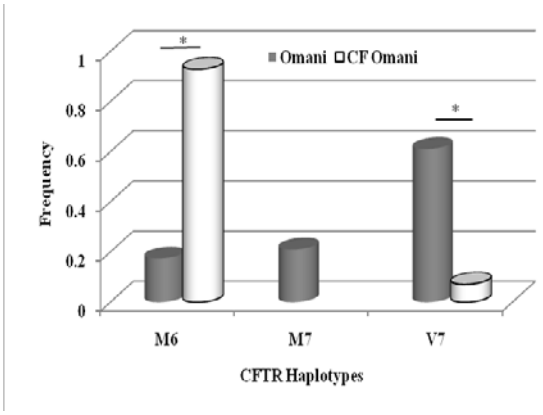


B

Figure 2. Allele frequencies of (A) exonic polymorphism M470V (M/V) of exon 10 and (B) intragenic, intronic dimorphic 4-bp GATT repeat (6/7) of intron 6 in White-European (Pompei F. *et al* 2006; Chehab FF. *et al.* 1991), Omani Control Cohort, and Omani CF patient population: The Omani mutations S549R, F508del and c.1175T>G are associated with the M ($\chi^2 = 3.1x10^{-8}$) and 6 ($\chi^2 = 2.1 \times 10^{-15}$) allele. Only the muation c.1733-1734 del AT is associated with V ($\chi^2 = 2.8 \times 10^{-4}$) and 7 ($\chi^2 = 1.2 \times 10^{-5}$).

3.2. Haplotypes and Diplotypes of the Omani CF and control cohorts

Out of four theoretically predicted haplotypes (M6, M7, V6, V7), only three were observed in the Omani population. The haplotype V6 was neither found in 408 alleles of the volunteer cohort nor in 24 alleles of the CF patient cohort. Therefore, only the haplotypes M6, M7 and V7 appeared to exist in the Omani population. The allele frequencies of the haplotypes M6, M7 and V7 in the volunteer cohort were 0.174, 0.214 and 0.611, respectively (Figure 3A).



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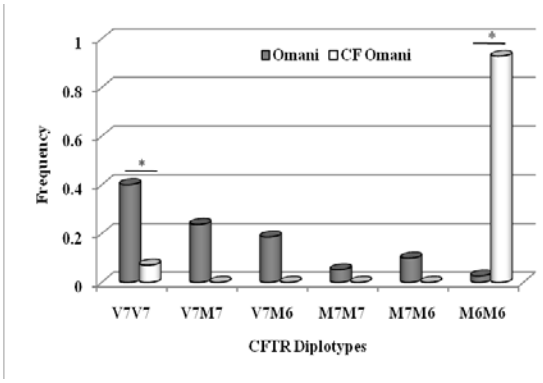

B

Figure 3.(A) Haplotypes and (B) Diplotypes frequencies of exonic polymorphism M470V (M/V) of exon 10 and intragenic, intronic dimorphic 4-bp GATT repeat (6/7) of intron 6 in the Omani Control Cohort, and Omani CF patient population: The Omani mutations S549R, F508del and c.1175T>G are associated with the haplotype M6 ($\chi^2 = 2.8 \times 10^{-11}$) and the diplotype M6M6 ($\chi^2 = 2.0 \times 10^{-3}$). The mutation c.1733-1734 del AT appears as exception and is associated with the haplotype V7 ($\chi^2 = 2.4x10^{-5}$) and diplotype V7V7 ($\chi^2 = 4.3 \times 10^{-12}$).

Interestingly, the Omani CF mutations S549R, F508del and V392G were completely associated with the haplotype M6 ($\chi^2 = 2.8 \times 10^{-11}$). This high haplotype frequency of M6 for the mutated alleles stands in a significant contrast to the haplotype frequency of M6 in the volunteer cohort, where it was detected with an allele frequency of only 0.174 ($\chi^2 = 2.8 \times 10^{-15}$) (Figure 3A). The association of M6 implies a specific genomic polymorphic pattern surrounding the most common Omani CF mutation S549R and the other two mutations, F508del and V392G. In contrast, only the two alleles of

the mutation c.1733_1734delAT are associated with a V7 haplotype. The V7 haplotype is the most common haplotype in the Omani population (Figure 3A). Caused by the absence of the V6 haplotype in the control and patient cohort, only 6 diplotypes out of 10 possible combinations were detected. The diplotypes V7V7, V7M7 and V7M6 were with 82.5% predominance in the volunteer cohort and occurred with relative frequencies of 0.401, 0.238 and 0.186, respectively (Figure 3B). The diplotypes, M7M6, M7M7, M6M6 occurred with 17.7%, were less frequent and had relative frequencies of 0.098, 0.052 and 0.026, respectively. However, the Omani CF patients with the mutation S549R, F508del and V392G were homozygote for M and 6 and form a M6M6 diplotype. The M6M6 diplotype in the patient cohort had a relative frequency of 0.928. The test of the association between the control cohort and the patient cohort revealed a strong association of S549R, F508del and V392G with the diplotype M6M6 ($\chi^2 = 2 \times 10^{-3}$). Surprisingly, only the private mutation c.1733_1734delAT was linked to the V7V7 diplotype and had a relative frequency of 0.071. The very strong association of S549R to the haplotype M6 and, consequently, to the diplotype M6M6 revealed a core characteristic of the mutation associated polymorphic pattern in the CFTR gene which seems rare in the normal Omani population.

4. Discussion

We have previously observed, during routine diagnostic gene wide screening of all exons and intronic borders of CFTR in suspected CF-patients that the predominant Omani mutation S549R occurred together with specific polymorphisms. This initial observation prompted us to study the two polymorphic loci M/V and 6/7 in 28 alleles of Omani CF patient cohort and in 408 alleles of unrelated and unaffected Omani healthy volunteers. Our initial observation about the genetic association of specific polymorphisms with alleles carrying S549R was immediately confirmed by the analysis of the M/V and 6/ 7 loci for representative samples (Figure 1). The M and 6 allele of the M/V and 6/7 polymorphism were linked to the Omani mutation S549R. In addition, we observed that this linkage occurred to F508del and V392G (Figure 2). An exception was the private mutation c.1733_1734delTA, a recently reported CFTR mutation from Oman which caused a frameshift and a formation of a premature stop codon (Fass *et al*., 2014). This mutation was associated with a V and 7 allele. A V7 haplotype is the predominant haplotype in the volunteer cohort with an allele frequency of 0.611 (Figure 3).

The occurrence of the allele frequencies of the M/V polymorphism in the unaffected Omani population was 0.392 and 0.607, respectively. This observation is similar to the reported M/V allele frequencies in the Caucasian population where M/V was reported with allele frequencies of 0.39 and 0.62, respectively (Pompei *et al*., 2006).

It was reported earlier that the M allele of the M/V polymorphism is associated with the CF mutation F508del (Cuppens *et al*., 1994; Dork *et al*., 1994; Ciminelli *et al*., 2007; Pompei *et al*., 2006). Therefore, the association of F508del with the M allele of the M/V polymorphism in our Omani CF cohort confirms this observation. One plausible reason for the association of F508del and other CF mutations might be a selective advantage of the V over the M allele in the CFTR gene (Pompei *et al*., 2006). Although the M/V sequence change in the CFTR gene is considered as a polymorphism, the physiological responses of both alleles appear different, which indirectly supports the notion of a selective advantage of the V allele (Cuppens *et al*., 1998; Kosova *et al*. 2010).

Furthermore, it has been reported that the association of non-F508del mutations is correlated to the genetic distance from the M allele (Ciminelli *et al*., 2007). The Omani mutation S549R in exon 11 occurs 28 kb away from the M470 site and, therefore, within a distance where a genetic association is expected (Ciminelli *et al*., 2007). However, we observed that the private mutation V392G shares similar associations with the M polymorphism. This mutation occurs in exon 8 and, therefore, is far from the M470 site.

The intronic 6-GATT repeat structure of the 6/7 polymorphism was linked to the reported Omani CF mutations S549R, F508del and V392G and had an allele frequency in the patient population of 0.928. In contrast, the allele frequency of 0.177 of the 6 polymorphism in the volunteer cohort was a rare genetic manifestation. This means that the 6 polymorphism is by 42% less frequent in the Omani volunteers in comparison to the reported frequency of 0.29 in White-European populations (Chehab *et al*., 1991) (Figure 2). Its low allele frequency provides a discriminative power for a potential risk assessment of the genetic predisposition of a disease causing CFTR mutation within the Omani population. Due to the absence of a V6 haplotype, the M6 haplotype appears to define a CF disease predisposition in Oman. The allele frequency of 0.17 for M6 represents 35 unaffected healthy individuals of the Omani control cohort. This means that those 35 individuals (70 alleles) are on higher risk of being CF carriers, whereas other volunteers have a reduced risk. We found two CF carriers in 204 individuals, one carrier with S549R and another with F508del (Fass *et al*., 2014). Both CF carriers had a M6 haplotype which highlights the clinical genetic significance of haplotypes as additional CFTR screening tools. It is possible that other, yet unidentified polymorphisms or haplotypes are associated with an even larger discriminative power to S549R and other Omani mutations. A further exploration and extension of haplotype pattern in the Omani population would clarify the origin and character of the rare mutation c.1733_1734delTA which shared the V7 haplotype.

5. Conclusion

We demonstrated that the major Omani CFTR mutation S549R, together with two other identified CFTR mutations in the Omani population, F508del and V392G, are associated with the haplotype M6 of the polymorphic markers M/V and 6/7. M6 is a rare haplotype in the Omani population, suggesting a migration of this

haplotype into the population. A fourth, recently identified CFTR mutation in Oman, c.1733_1734delTA, was associated with the haplotype V7. The highest haplotype frequency in the unaffected Omani population is V7. Although the association of the haplotype V7 with CF appeared like an exception, there is the possibility that this rare mutation originates from Oman.

Acknowledgement

This research was funded by a grant from the Oman Research Council (TRC Grant #ORG OMC HSS 100008). The research was part of a plenary talk during the International Conference of the Humboldt Kolleg, "Networking for the enhancement of Research in Jordan," in Amman, Jordan (April 4-6, 2014). The authors would like to express their deep appreciation to Prof. Dr. Marwan Mousa for his insightful planning and organization of this beneficial and interactive meeting. In addition, we express our thanks to Dr. A. Rodney, Head of the Department, and the rest of our colleagues of the Department of Pediatrics of Sohar Regional Teaching Hospital for their discussion of potential clinical aspects of this genetic research work. We thank the students of Oman Medical College Sohar for their volunteerism in providing blood for the population based aspects of the study.

References

Beaudet AL, Feldman GL, Fernbach SD, Buffone GJ and Brien WE. 1989. Linkage disequilibrium, cystic fibrosis and genetic counseling. *Am J Hum Genet*,**44:** 319-326

Bobadilla JL, Macek M, Fine JP and Farrell PM. 2002. Cystic fibrosis: A worldwide analysis of CFTR mutations: Correlation with incidence data and application to screening. *Hum Mutat*,**19:** 575-606.

Ciminelli BM, Bonizzato A, Bombieri C, Pompei F, Gabaldo M, Ciccacci C, Begnini A, Holubova A, Zorzi P, Piskackova T, Macek M, Catellani C, Modiano G and Pignatti PF. 2007. Highly preferential association of NonF508del CF mutations with the M470 allele. *J Cyst Fibr*, **6:**15-22

Chehab FF, Johnson J, Louie E, Goossens M, Kawasaki E and Erlich H. 1991. A dimorphic 4-bp repeat in the cystic fibrosis gene is in absolute linkage disequilibrium with the ΔF508 mutation: implication for prenatal diagnosis and mutation origin. *Am J Hum Gent*, **48:** 223-226

Ciofu O, Hansen CR and Høiby N. 2013. Respiratory bacterial infections in cystic fibrosis. *Curr Opin Pulm Med*, **19:** 251-8

Cohn JA. 2005. Reduced CFTR function and the pathobiology of idiopathic pancreatitis. *J Clin Gastroenterol*, **39:** (4 Suppl.2) S70-77

Cuppens H, Teng H, Raeymaeckers P, De Bock C and Cassiman JJ. 1994. CFTR haplotype backgrounds on normal and mutant CFTR genes. *Hum Mol Genet*, **3:** 607-614

Cuppens H, Lin W, Jaspers M, Costes B, Teng H, Vankeerberghen A, Jorissen M, Droogmans G, Reynaert J, Goosens M, Nilius B, and Cassiman JJ. 1998. Polyvariant mutant cystic fibrosis transmembrane conductance regulator genes. The polymorphic $(TG)_m$ locus explains the partial penetrance of the T5 polymorphism as a disease mutation. *J Clin Invest*, **101(2):** 487-496

Cutting GR, Antonarakis SE, Buetow KH, Kasch LM, Rosenstein BJ and Kazazian HH. 1984. Analysis of DNA polymorphism haplotypes linked to the cystic fibrosis locus in North American Black and Caucasian Families supports the existence of multiple mutations of the cystic fibrosis gene. *Am J Hum Gent*, **44:** 307-318

Cystic Fibrosis Mutation Database. www.genet.sickkids.on.ca accessed: June 2014

Daudin M, Bieth E, Bujan L, Massat G, Pontonnier F and Mierusset R. 2000. Congenital bilateral absence of the vas deference: Clinical characteristics, biological parameters, cystic fibrosis transmembrane conductance regulator gene mutations and implications for genetic screening. *Fertil Steril,* **74:** 1164-1174

Dork T, Fislage R, Neumann T, Wulf B and Tummler B. 1994. Exon 9 of the CFTR gene: splice site haplotypes and cystic fibrosis mutations. *Hum Genet,* **93:** 67-73

El-Harith EA, Dörk T, Stuhrmann M, Abu-Srair H, Al-Shahari A, Keller KM, Lentze MJ and Schmidtke J. 1997. Novel and characteristic CFTR mutations in Saudi Arab children with severe cystic fibrosis. *J Med Genet*, **34:** 996-999

Estivill X, Bancells C, Ramos C and The Biomed CF Mutation Analysis Consortium.1997. Geographic distribution and regional origin of 272 cystic fibrosis mutation in European populations. *Hum Mutat,* **10:** 135-154

Fanen P, Ghanem N, Vidand M, Besmond C, Martin J, Costes B, Plassa F and Goossens M. 1992.Molecular characterization of cystic fibrosis: 16 novel mutations identified by analysis of the whole cystic fibrosis conductance transmembrane regulator (CFTR) coding regions and splice site junctions. *Genomics,* **13:** 770-776

Fass U, Al-Salmani M, Bendahhou S, Shivalingam G, Norrish C, Kallesh H, Clark F, Heming T and Al-Khusaiby S. 2014. Defining a Mutational Panel and Predicting the Prevalence of Cystic Fibrosis in Oman. *Sultan Quaboos Univ Med J*, **14(3)** 294-299

Feldman C. 2011. Bronchiectasis. New approaches to diagnosis and management. *Clin Chest Med*, **32(3):** 535-546

Fredj SH, Fattoum S, Chabchoub A and Messaoud T. 2011. First report of cystic fibrosis mutations in Libyan cystic fibrosis patients. *Ann Hum Biol*, **38(5):** 561-563

Frossard PM, Girodon E, Dawson KP, Ghanem N, Plassa F and Lestringant GG. 1997. Identification of cystic fibrosis mutations in the United Arab Emirates. *Hum Mut*, **11:** 412-13 On-line Mutation-in-Brief #133

Lestringant G., Girondo E, Goossens M and Dawson KP. 1999. Determination of the prevalence of cystic fibrosis in the United Arab Emirates by genetic carrier screening. *Clin Genet*, **55:** 496-497

Gasparini P, Dognini M, Bonizzato A, Rignatti F, Morral N and EstvillX.1991. A tetranucleotide repeat polymorphism in the cystic fibrosis gene. *Hum Genet*, **86:** 625

Gelfond D and Borowitz D. 2013. Gastrointestinal complications of cystic fibrosis. *Clin Gastroenterol Hepatol*,**11:** 333-342

Grody WW, Cutting GR, Klinger KW, Richards CS, Watson MS and Desnick RJ, Subcommittee on cystic fibrosis, Accreditation of genetic services committee, ACMG, American College of Medical Genetics. 2001. Laboratory standards and guidelines for population-based cystic fibrosis carrier screening. *Genet Med*, **3(2):** 149-154

Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M and Tsui LC. 1989. Identification of

the cystic fibrosis gene: genetic analysis. *Science*, **245(4922):**1073-1080

Kerem B, Zielenski J, Markiewicz D, Bozon D, Gazit E, Yahaf J, Kennedy D, Riordon JR, Collins FS, Rommens JM and Tsui LC.1990.Identification of mutations in regions corresponding to the two putative nucleotide(ATP)-binding folds of the cystic fibrosis gene. *Proc Natl Acad Sci USA*, **87:** 8447-8451

Kosova G,Pickrell JK, Kelley JL, McArdle PF, Shuldiner AR, Abney M and Ober C. 2010. The CFTR Met470 allele is associated with lower birth rates in fertile men from a population isolate. *PLoS Genet*, **6(6):** e1000974

Morral N, Bertranpetit J, Estivill X, Nunes V, Casals T, Gimenez J, Reis A and et al.. 1994. The origin of the major cystic fibrosis mutation F508del in European populations. *Nat Genet*,**7:** 169-175

Pompei F, Ciminelli BM, Bombieri C, Ciccacci C, Koudova M, Giorgi S, Belpinatti F, Begnini A, Cerny M, Des Georges M, ClaustresM, Ferec C, Macek M, Modiano G and Pignatti PF. 2006. Haplotype block structure study of the CFTR gene. Most variants are associated with the M470 allele in several European populations. *Eur J Hum Genet*,**14:** 85-93

Riordan JR, Rommens JM, Kerem B, Alon N,Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, Drumm ML, Iannuzzi MC, Collins FS and Tsui LC. 1989. Identification of the Cystic Fibrosis gene: cloning and characterization of complementary cDNA. *Science*, **245(4922):**1066-1073

Rajab A and Patton MA. 2000. A study of consanguinity in the Sultante of Oman. *Ann Hum Biol*, **27**: 321-326

Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, Rozmahel R, Cole JL, Kennedy D, Hidaka N, Zsiga M, Buchwald M, Riordon JR, Tsui LC and Collins FS. 1989. Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science*, **245(4922):**1059-1065

Sharer N, Schwarz M, Malone G, Howarth A, Painter J, Super M and Braganza J. 1998. Mutations of the cystic fibrosis gene in patients with chronic pancreatitis. *N Engl J Med*,**339:** 645-652

Sheppard DN and Ostedgaard LS. 1996.Understanding how cystic fibrosis mutations cause a loss of Cl- channel function. *Mol Med Today*, **2(7):** 290-297

Wahab AA, Al-Thani G., Dawod ST, Kamboursi M and Al-Hamed M. 2001. Heterogenity of the cystic fibrosis phenotype in a large Kindred family in Quatar with cystic fibrosis mutation (I1234V). *J. Trop Pediatr*, **47:** 110-112

Wang Y, Wrennal JA, Cai Z, Li H and Sheppard DN. 2014. Understanding how cystic fibrosis mutations disrupt CFTR function: From single molecules to animal models. *Int J Biochem Cell Biol*, **52:** 47-57

Watson MS, Cutting GR, Desnick RJ, Driscoll DA, Klinger K, Mennuti M, Palomaki GE, Popovich BW, Pratt VM, Rohlfs EM, Storm CM, Richards CS, Witt DR and Grody WW. 2004. Cystic fibrosis population carrier screening 2004 revision of American College of medical Genetics mutation panel. *Genet Med*, **6(5):** 387-391

Wei S, Feldman GI and Monaghan KG. 2006. Cystic fibrosis testing among Arab-Americans. *Genet Med*, **8:** 255-258

Witt H. 2003. Chronic pancreatitis and cystic fibrosis. *Gut*, **52:** Suppl. 2: ii 31-41

Zielinski J, Rozmahel R, Bozon D, Keren B, Grzelczak Z, Riordan JR, Rommens J and Tsui LC. 1991. Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics*, **10:** 214-28

JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 45 - 50

Jordan Journal of Biological Sciences

An Evaluation of the Nutrients and Some Anti-nutrients in Silkworm, *Bombyxmori* L. (Bombycidae: Lepidoptera)

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Received: October 10, 2014 Revised: December 19, 2014 Accepted: December 22, 2014

Abstract

Insects are important as items of aesthetic values, pests and as food. *Bombyx mori* L. is an economically important insect. It is an edible insect that is eaten in the tropics. The larvae of silkworm, *B. mori*, are popular for silk production. Silk is produced when the larvae are ready to pupate. Both the larval and the pupal stages of *B. mori* were analyzed for their nutrient composition, protein solubility, mineral, functional and anti-nutritional factors. The pupal stage had a higher protein content (21.59%) than the larval stage which had 20.79%. The fat content of the larva was 17.57% while that of the pupa was 19.90%. The ash content was higher in the larva (6.34%) than that in the pupal stage (5.50%). The proteins in the larval and pupal stage have two iso-electric points. The results of the mineral analyses showed that mineral salts are persistently higher in the pupal stage than in the larval stage. The functional analyses revealed that the larval stage is highly desirable for chopped meat or powdered food production than the pupal stage. The anti-nutritional content of both stages fall within the tolerance levels with the exception of phytin phosphorus and phytate where higher values were obtained.

Key words: Larval stage, Pupal stage, *Bombyx mori,* Silkworm, Solubility, Tannin, Phytate.

1. Introduction

Arthropods have established intimate relationships with man and his valuables since time immemorial (Banjo *et al.,* 2006; Omotoso and Afolabi, 2007). They are the most diverse of all organisms on earth. While some of them are foes, an appreciable percentage of them are friends in that they are edible, useful for research, and some are used in controlling other organisms, pests, for example.

Members of the class Insecta are important sources of food to many animal species and are also a v eritable source of dietary protein and nutrients to man (DeFoliart, 1992). The Silkworm, *Bombyx mori*, is a popular moth which belongs to the order Lepidoptera. This moth is remarkable in producing silk which is used in producing cloths and wigs that are of immense value to man. The rearing of silkworms was reported to have started around 2650 B.C. in China by the Queen of Huang-Di Empire (Satoshi, 2003). The rearing of silkworms consists of a series of tasks which includes the planting of mulberry plants, supplying of mulberry leaves to growing silkworm larvae, cleaning of the rearing beds, mounting of the larvae so that they will spin cocoons and collection and transporting of cocoons. Silkworm rearing requires relatively high temperatures (26 ^{o}C-28 ^{o}C) and high humidity (75%-90%) (Satoshi, 2003; Rahmathulla and Suresh, 2012).

The quests for animal proteins and nutrients have led to the investigation of alternative sources such as insects. Insects are reported to be rich in animal proteins, minerals and fats (Banjo *et al.,* 2006; Omotoso, 2006; Omotoso and Adedire, 2007, 2008). Some of the more popular insects eaten around the world include crickets, grasshoppers, ants, stick insects, beetles and various species of caterpillar. There are 1,417 known species of arthropods that are edible to humans (Yen, 2009). Edible insects are important sources of high protein to rural dwellers and many city dwellers in Nigeria (Fasoranti and Ajiboye, 1993). Among the most important orders of insects consumed in Nigeria are Coleoptera, Hymenoptera, Isoptera, Lepidoptera, Odonata, Orthoptera and they are highly priced (Fasoranti and Ajiboye, 1993). There has been a few studies on the nutrient composition of silkworm *B. mori* (Frye and Calvert, 1989; Finke 2002; Tom *et al.,* 2013). The present paper is carried out to determine the nutrient composition and the nutritive value of the insect as an alternative source of nutrients to man and his livestock.

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2. Materials and Methods

2.1. Collection and Preparation of Bombyx mori Larvae and Pupae

The larvae and the pupae of silkworm *B. mori*, used for this work, were obtained from the Ministry of Agriculture, Sericulture Department in Ado-Ekiti, Ekiti State of Nigeria. Ado-Ekiti is a town situated in the tropical humid region of Nigeria. The town lies on latitude 7° 38' 0' North of the Equator and longitude 5° 13' 0' East of Greenwich Meridian and 456 meters above the sea level. The larvae were three weeks old and they were handpicked into a plastic container which was used to convey them to the laboratory. They were allowed to stay for 3 h. in the laboratory. The larvae were asphyxiated in a refrigerator for 3 h. and later oven-dried at 60 °C for 4 h. The pupae were handpicked with their cocoons. The pupae were a week old from the day they completed their cocoon formation. The pupae were oven-dried together with their cocoons at 60 °C for 4 h. The cocoons were later cut open with a razor blade and the pupae were poured into a container. The dried larvae and the pupae were separately pulverized with a blender and kept in air-tight bottles and put in refrigerator until needed for analysis.

2.2. Nutrient Composition Analysis

The moisture content, ash content, fat content and the mineral content were determined by employing the method explained by AOAC (1990). Sodium and potassium were determined by flame photometric method while phosphorus was determined by the phosphovanado-molybdate reagent method reported by AOAC (2005) using Spectronic 20 Colorimeter. Other minerals, such as magnesium, iron, calcium, zinc, manganese, lead, nickel, copper and cobalt, were determined with Alpha 4 Atomic absorption spectrophotometer. Carbohydrate was determined by difference. The protein content was determined by the method reported by Pearson (1970). Joslyn (1970) method was employed in the determination of the crude fiber content. The protein solubility was determined by Biuret method, using Standard Bovine Serum (BSA).

2.3. Determination of Anti-nutrient Composition

Young and Greaves (1940) method was employed in phytin determination. Oxalate was determined by the method reported by Day and Underwood (1986). Tannin was determined by the method reported by Markkar and Goodchild (1996). Alkaloid was determined by the method reported by Harbone (1973) while the Bohm and Kocipal-Abyazan (1994) method was adopted in flavonoid determination.

2.4. Determination of Functional Properties of Silkworm Protein

The saponin content was determined by adopting the method reported by Obadoni and Ochuko (2001). The methods reported by Coffman and Garcia (1977) were employed in the determination of the least gelation concentration, foaming capacity and foaming stability. Water and oil absorption capacity were determined by Beuchat (1977) methods while emulsion capacity and emulsion stability were determined by Yasumatsu *et al.* (1972) method.

The data collected were subjected to Analysis Of Variance (ANOVA) and where significant differences existed, treatment means were compared at 0.05 significant level using Tukey Test.

3. Results

The result of the nutrient analysis revealed that the ash content in the larval stage was 6.34% while it was 5.50% in the pupal stage. Protein was the second most prominent class of food in silkworm, in which 20.79% and 21.59% were obtained in the larval and pupal stages, respectively (Table 1). The moisture content ranged between 7.92% (in the larva) and 8.26% (in the pupa). Carbohydrate forms the main source of food found in silkworm. The proteins in silkworm are more soluble in acidic medium. The highest solubility obtained in the larval stage was 84.13% in pH 1 and pH 12. In the pupal stage, the highest solubility occurred in pH 1 and pH 11. Each of the developmental stages has two iso-electric points (Figure 1).

Table 1. Nutrient composition of Silkworm, *Bombyx mori*

Parameters (%)	*B. mori*larvae	*B. mori*pupal
Ash content	6.34±0.84	5.50±0.51
Moisture content	7.92±0.98	8.26±0.66
Fat content	17.57±1.51	19.90±1.80
Crude fibre content	6.46±0.21	6.30±0.12
Crude protein content	20.79±2.22	21.59±2.91
Carbohydrate content	40.93±3.20	38.47±4.24

Each value is a mean ± Standard deviation of three replicates (Tukey Test)

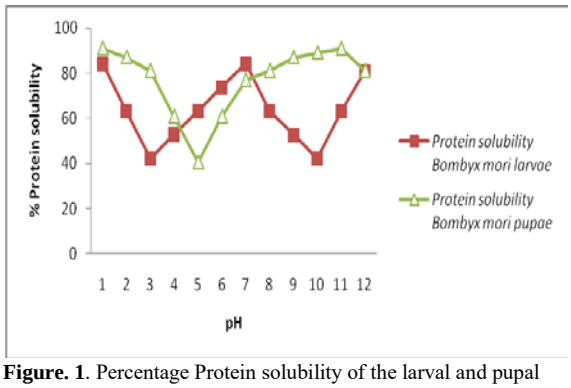

Figure. 1. Percentage Protein solubility of the larval and pupal stages of Silkworm, *B. mori.*

Table 2 shows the results of mineral analysis in the larval and the pupal stages. All the essential minerals are present in silkworm. Phosphorus was the highest mineral obtained in both stages. Copper was not discovered in the larval stage but a negligible quantity (0.10%) was obtained in the pupal stage. Iron, zinc, magnesium, calcium, potassium and sodium were obtained in different quantities in both larval and pupal stages. The quantities of trace mineral salts such as cobalt, nickel, copper, manganese and lead are negligible in both stages.

Table 2. Minerals present in Silkworm, *B. mori*.

Parameters (mg/100g)	*B. mori*larvae	*B. mori*pupal
Na	10.52±1.31	11.66±1.22
K	18.65±1.42	22.45±1.72
Ca	20.31±2.78	26.65±3.49
Mg	31.24±3.61	27.53±3.76
Zn	35.63±4.98	37.50±4.64
Fe	5.31±0.72	6.33±0.81
Pb	0.01±0.00	0.02±0.00
Mn	0.02±0.00	0.01±0.00
Cu	ND	0.10±0.00
P	37.66±4.10	41.35±5.82
Ni	0.01±0.00	0.10±0.00
Co	0.33±0.01	0.36±0.01

Each value is a mean ± Standard deviation of three replicates (Tukey Test). ND = Not Detected

The results of the functional properties of the proteins of silkworm is shown in Table 3. Water absorption capacity was 175% in the larvae while it was 115% in the pupa. Oil absorption capacity was higher in the pupal (284.87%) than in the larva (252.18%). Foaming capacity and foaming stability were not detected in both stages. Least gelation in both stages was 6% while emulsion capacity and stability in the larval stage was 75% and 25%, respectively. In the pupal stage, the emulsion capacity was 75% while the emulsion stability was 23%.

Table 3. Functional properties of the proteins in Silkworm, *B. mori*.

Parameters (%)	*B. mori* larvae	*B. mori*pupal
Water absorption capacity	175±8.50	115.00±7.30
Oil absorption capacity	252.18±9.22	284.87±9.45
Foaming capacity	ND	ND
Foaming stability	ND	ND
Emulsion capacity	75.00±3.24	75.00±3.50
Emulsion stability	25.00±2.11	23.00±2.45
Least gelation	6.00±1.02	6.00±1.12

Each value is a mean ± Standard deviation of three replicates (Tukey Test). ND = Not Detected.

Table 4 shows the results of anti-nutrient composition of silkworm. Phytate was the highest anti-nutrient recorded in both the larval stage (72.89%) and pupal stage (110.16%). The amounts of phytin phosphorus in both stages were higher (20.54% in larva and 31.03% in pupa). The quantity of saponin present in the larval stage was lower (6.88%) than the quantity obtained in the pupal stage (7%). Oxalate recorded the lowest quantity of 0.91% in the larval while 1.22% was obtained in the pupal stage. The quantity of flavonoid obtained in the pupal stage (11.54%) was higher that the quantity obtained in the larval stage (11.33%). Alkaloids and tannic acids contents in the larval stage were 8.55% and 1.93% respectively. In the pupal stage, alkaloid was 8.61% while tannic acid was 2.04%.

Table 4. Anti-nutrient composition of Silkworm, *B. mori*.

Constituents	*B. mori*larvae	*B. mori*pupal
Tannic acid (%)	1.93±0.12	2.04±0.24
Saponin (%)	6.88±0.18	7.00±0.88
Alkaloids (%)	8.55±1.26	8.61±1.12
Flavonoid (%)	11.33±1.34	11.54±1.93
Oxalate (mg/g)	0.91±0.07	1.22±0.01
Phytate (mg/g)	72.89±3.72	110.16±9.67
Phytin phosphorus (mg/g)	20.54±2.98	31.03±3.33

Each value is a mean ± Standard deviation of three replicates (Tukey Test).

4. Discussion

This study revealed that the larval and the pupal stages of *Bombyx mori* had high nutritional qualities (Table 1). The protein content of the larval stage (20.79%) and the pupal stage (21.59%) were higher than the values reported in *B. mori* (93g/kg)by Finke (2002). Lower protein contents have been reported by Banjo *et al.* (2006) in *Analeptes trifasciata* F. (Cerambycidae: Coleoptera), *Rhynchophorus phoenicis* F (Curculionidae; Coleptera) and *Zonocerus variegatus* L. (Pyrgomorphidae: Orthoptera). Adeyeye and Awokunmi (2010) reported protein values of 258 g/kg and 324g/kg for female and male giant African crickets, *Brachytrypes membranaceus* L. (Gryllidae: Orthoptera), respectively. Protein content of 11.37% was reported in *Epiphora bauhiniae* (Guerin Meneville) (Saturniidae: Lepidoptera). Protein is essential for the development and repair of body tissues of animals. Both the larval and the pupal stages of *B. mori* can adequately supply this essential protein to growing animals. The results of the protein solubility also attested to the fact that the proteins present in each of the developmental stages of *B. mori* contained so many amino acids. There are two iso-electric points in the larval and the pupal stages. The proteins present in each of the stages were more soluble in acidic medium than in alkaline medium (Figure 1). The minimum and maximum solubility in the larval is 42.07% and 84.13%, respectively. However, in the pupal stage, 40.51% was the minimum while 91.15% was the maximum. The moisture content of the developmental stages of silkworm was lower and this indicates that they will have a better shelf-life. The values of moisture content in the stages of *B. mori* were lower than the value reported by Finke (2002) for *B. mori* (827g/kg) and *B. membranaceus* by Adeyeye and Awokunmi (2010). Fat was the second most prominent food component in silkworm stages (17.57% - 19.90%). Fat content of 27% was reported in *E. bauhiniae* by Tom *et al.* (2013). Omotoso and Adedire (2007) reported that fat was the highest food value in the developmental stages of *R. phoenicis* F. (52.40% and 61.45%). Crude fiber which is very important in bowel movement was lower in both stages of *B. mori*. Carbohydrate content is the main food content in *B. mori*. The value of carbohydrate in *B. mori* (38.47% -40.93%) was higher than the value reported in *B. mori* (44g/kg) by

Finke (2002) but lower than the values reported in *Macrotermes notalensis* (Hav.) (Termitidae: Isoptera) (42.8%), *Apismellifera* L. (Apidae: Hymenoptera) (73.6%) and *Brachytrypes spp* (85.3%) by Banjo *et al.* (2006). The ash content was higher in all the stages of development of *B. mori*. However, Finke (2002) reported a lesser quantity in *B. mori* (11g/kg). Ash content of 1% was reported in wild silk moth, *E. bauhiniae* by Tom *et al.* (2013). Ash content is an indication of the quantity of the minerals present in the insect. Both the larval and the pupal stages were rich in minerals which are essential for the normal development and growth of organisms.

The highest mineral salt obtained in the larval and pupal stages of *B.mori* was phosphorus (Table 2). The same trend was reported by Finke (2002) in *B. mori.* The value of 136.4 mg/100g was obtained in *A. trifasciata* by Banjo *et al.* (2006) while Adeyeye and Awokunmi (2010) reported the value of 10880 mg/kg and 10936 mg/kg in male and female *B. membranaceus*, respectively. The highest mineral salt obtained in *R. phoenicis* ranged between 372.50 - 457.50 mg/kg (Omotoso and Adedire, 2007) while in *Cirinaforda* Westwood (Saturniidae: Lepidoptera), the highest mineral salt obtained was phosphorus (215.54 mg/100g) (Omotoso, 2006). The larval and pupal stages of *B. mori* were rich in important mineral salts, such as sodium (Na), potassium (K), calcium (Ca), iron (Fe), magnesium (Mg) and zinc (Zn). The amount of minerals, such as lead (Pb), manganese (Mn), copper (Cu), nickel (Ni) and cobalt (Co), were negligible. The values of minerals reported by Finke (2002) in *B. mori* are consistently higher than all the values reported in this study. Incorporating the larval and pupal stages of *B. mori* in the diets of both adults and children will greatly promote the normal functioning of the systems in the body. Magnesium, copper, zinc, selenium, iron, manganese and molybdenum are important co-factors found in the structure of certain enzymes and are indispensable innumerous biochemical pathways (Soetan *et al.,* 2010). Iron (Fe) is important in blood formation and blood plays a crucial role as a means of communication between the foetus and the mother during pregnancy. A pregnant woman requires 23g of iron per day in her diet (NHMRC, 2005). A pregnant woman can get the required iron content per day (23g) by incorporating 433g of the larvae or 363g of the pupae of silkworm into her diet. Zinc is a component of various enzymes that help maintain structural integrity of proteins and regulate gene expression. Zinc metallo enzymes include ribonucleic acid polymerases, alcohol dehydrogenase, carbonic anhydrase and alkaline phosphatase. The biological function of zinc can be catalytic, structural or regulatory. More than 85% of total body zinc is found in skeletal muscle and bone (King and Keen, 1999). The zinc content of the larvae and the pupae of silkworm are 6 times more than the daily requirements of boys and girls of 9-13 years (6 mg/day).

The results of the functional properties of silkworm show that the meat of the insect will be good and useful in food industries (Table 3). Functional properties of proteins in food are the physic-chemical properties which govern the behavior of protein in foods. The insect can be easily incorporated into confectioneries and comminuted foods, such as pie, cake and buns. The higher water absorption capacity shows that the meat of the insect will be very useful in confectionery industries that deal with baking processes such as bread bakeries. The higher oil absorption capacity also shows that the meat of the insect will be useful in cake and pie making industries since they will easily bind to greater quantities of oil. There are no reported works on the functional properties of insects that this result can be compared with except the work of Omotoso and Adedire (2008) in which higher water absorption capacity (93.33%) and higher oil absorption capacity (112.33%) were reported in *R. Phoenicis*. The foaming capacity and stability were not detected. This shows that the oil derived from silkworm may not be useful in food industries where foaming is essential to cause the rising of the food. The values of emulsion capacity (75%), stability (23%-25%) and least gelation concentrations (6%) are encouraging, showing that the silkworm meats are good candidates for food industries. Forming gels at 6% shows that the meat of this insect will be very good in canned food as the food will stay together in the can.

The highest anti-nutrient content of silkworm was phytate (72.89 mg/g-110.16 mg/g), followed by phytin phosphorus (20.54-31.03mg/g) while oxalate was the least (0.91 mg/g-1.22mg/g). The low levels of oxalate, tannic acid, saponin, alkaloid and flavonoids obtained in the developmental stages of silkworm are indications that the insect is very safe for human and livestock consumption (Table 4). Oxalates are naturally occurring substances found in plants, animals and humans (Rahman *et al.,* 2013). Oxalates combine with calcium and magnesium to form insoluble Ca and Mg oxalates which lead to low serum Ca and Mg levels as well as to renal failure because of the precipitation of these salts in the kidneys (kidney stones) (Rahman *et al.,* 2013). There are a few relatively rare health conditions that require strict oxalate restrictions. These conditions include absorptive hypercalciuria type II, enteric hyperoxaluria and primary hyperoxaluria (Rahman *et al.,* 2013). Saponins are also other naturally occurring compounds that are widely distributed in all cells of legumes (Shi *et al.,* 2004). Saponin has some beneficial effects on hum an. Such effects include promoting the immune system so as to protect it against cancer, lowering cholesterol levels, lowering the risks of contacting cancer, lowering blood glucose response, inhibiting dental caries and inhibiting platelets aggregation in humans (Shi *et. l.,* 2004). Flavonoids are ubiquitous in plants and are recognized as the pigments responsible for the colors of leaves (Theoharis, 2000). Fruits and vegetables are the main dietary sources of flavonoids for humans, together with tea and wine (Yao *et al.,* 2004). Wink (2013) reported that legumes produce more nitrogen containing secondary metabolites, such as alkaloids and amines, non-protein amino acids, cyanogenic glucosides and peptides, than other plants. Flavonoids were reported to inhibit mast cell secretions in mammals (Theoharis, 2000). The importance of flavonoids in humans include antioxidative activity, free-radical scavenging capacity, coronary heart disease prevention and anti-cancer activity (Yao *et al.,* 2004). Silkworm has better nutritive values than pulses

and legumes which contain higher levels of tannins, oxalates and phytic acids that are known to affect protein digestibility and absorption. Similar observations of lower anti-nutrient values were reported by Omotoso and Adedire (2008) in *R. phoenicis*. All the anti-nutrients reported in the developmental stages of silkworm could have been from the leaves (Mullberry leaves) they eat. Mullaney *et al*. (2000) reported that non-ruminant animals lack the required digestive phytase which removes phosphate from phytic acid, phytate and phytin phosphorus. Thus, the anti-nutrients are generally not bioavailable to non-ruminant animals.

5. Conclusion

Both the larval and the pupal stages of silkworm *B. mori* are rich in proteins and other food nutrients that are of importance to man and his livestock. These developmental stages (larvae and pupae) are also rich sources of most essential mineral salts, which are needed for the normal development of humans. It is a fact that these developmental stages are good candidates of food processing industries where higher oil and water absorption capacities are required.

6. References

Adeyeye E I and Awokunmi E E. 2010.Chemical composition of female and male giant African crickets, *Brachytrypes membranaceus* L. *Inter J Pharma and Bio Sciences,* **1(4):** 125-136.

AOAC .1990. **Official Methods of Analysis**, Association of Official Analytical Chemists. Washington DC, 15thedt, pp1-87.

AOAC.2005.**Official Methods of Analysis,**Association of Official Analytical Chemists.Washington DC, USA, 18thedt, pp1 – 45.

Banjo A D, Lawal O A and Songonuga E A. 2006.The nutritional value of fourteen species of edibleinsects in southwestern Nigeria. *Afr J Biotechnol.,* **5 (3):** 298-301.

Beuchat L R. 1977.Functional and electrophoretic characteristics of succinylated peanut flour protein. *J Agriculture and Food Chem.,***25(2):** 258-261.

Bohm B A and Kocipal-Abyazan R. 1994.Flavonoids and condensed tannins from leaves of Hawaiian *Vaccinium reticulatum* and *V. calycinum* (Ericaceae). *Pac Sci* ., **48(4):** 458-463.

Coffman C W and Garcia V V. 1977. Functional properties and amino acid content of protein isolate from mung flour. *J Food Sci.*,**12**: 473-484.

Day R A and Underwood A L. 1986.**Qualitative Analysis**. 5thedt. Prentice-Hall Publication. 701pp.

DeFoliart G R. 1992. Insects as human food, some nutritional and economic aspects.*Crop Protection.***11**: 395-399.

Fasoranti J O and Ajiboye D O. 1993. Some edible insects of kwara State, Nigeria. *American Entomol.,***39**: 113-116.

Finke M D. 2002. Complete nutrient composition of commercially raised invertebrates used as food for insectivores. *Zoo Biology,* **21**:269–285.

Frye F L and Calvert C C. 1989.Preliminary information on the nutritional content of mulberry silkmoth (*Bombyxmori*) larvae. *J Zoo Wildl Med.,* **20**:73–75.

Harbone J B. 1973.**Phytochemical Methods**, London Chapman and Hall Ltd. pp 49-188.

Joslyn A M. 1970. **Methods in Food Analysis: Physical, Chemical and Instrumental Methods of Analysis.**2nd Edit. Academic Press, New York, San Francisco, pp1-3.

King J C and Keen C L. 1999.Zinc. In: Shils ME, Olsen JAS, Shike M and Ross AC (Eds). **Modern Nutrition in Health and Disease** 9th edition. Baltimore. Williams and Wilkins, pp 223–239.

Markkar A O S and Goodchild A V. 1996.Quantification of tannins. A laboratory manual International Centre for Agricultural Research in dry Areas (ICARDA). Aleppo, Syria. 1-25pp.

Mullaney E J, Daly C B and Ullah A H. 2000.Advances in Phytate Research. *Adv. Appl. Microbiol.,* **47**: 157-199.

NHMRC (National Health and Medical Research Council) (2005). Nutrient reference values for Australia and New Zealand. Australian Government Department of Health and ageing. pp 1-317.

Obadoni O B and Ochuko P O. 2001. Phytochemical studies and comparative efficacy of the crude extract of some homeostatic plants in Edo and Delta States of Nigeria. *Global J Pure Applied Sci.,***2**: 203-208.

Omotoso OT. 2006.Nutritional quality, functional properties and anti-nutrient composition of the larva of *Cirinaforda* (Westwood) (Lepidoptera: Saturniidae). *J Zhejiang University Sci B.,* **7(1):** 51-55.

Omotoso OT and Afolabi O. 2007. Nutritional evaluation, functional properties and anti-nutritional factors of *Macrobrachiumrosenbergii,* an underutilized animal .*Pak J Sci Industrial Res.,***50(2)**: 109-112.

Omotoso O T and Adedire C O. 2007. Nutrient composition, mineral content and the solubility of the proteins of palm weevil, *Rhynchophorus phoenicis* F. (Coleoptera: Curculionidae). *J Zhejiang University Sci B.,***8**: 318-322.

Omotoso O T and Adedire C O. 2008. Potential industrial uses and quality of oil of oilpal weevil, *Rhynchophorus phoenicis* F. (Coleoptera: Curculionidae). *Pak J Sci Industrial Res.,***51**: 93-97.

Pearson D. 1970. **Chemical Analysis of Foods**, 7thedt. London, Churchill Livingstone pp 7-11.

Rahman M M, Abdullah R B and Wan Khadijah W E.2013. A review of oxalate poisoning in domestic animals: tolerance and performance aspects. *J Animal Physiol, Animal Nutrition,* **97(4)**: 605-614.

Rahmathulla V K and Sureshi H M. 2012.Seasonal variation in food consumption, assimilation and conversion efficiency of Indian bivoltine hybrid Silkworm, *Bombyxmori. J Insect Sci.,***12(82)**: 1-15

Satoshi T. 2003. Sericulture .In: Vincent, H.R. and Ring, T.C (Eds): **Encyclopedia of Insects**. Academic Press, California, USA. pp 1027-1029.

Shi J, Arunasalam K, Yeung D, Kakuda Y, Mittal G and Jiang Y. 2004. Saponins from edible legumes: Chemistry, processing and health benefits. *J Medicinal Food,* **7(1)**: 67-78.

Soetan K O, Olaiya C O and Oyewole O E. 2010. The importance of mineral elements for humans, domestic animals and plants: A review. *Afr J Food Sci.,* **4(5)**: 200-222.

Theoharis C T. 2000. The effects of plant flavonoids on mammalian cells: Implications for inflammation , heart disease and cancer. *Pharmacol Rev.*,**52(4)**: 673-751.

Tom M,Talaat A E, Magid D A and Eldeen E B. 2013. Investigation of the nutritional constituents of the wild silk moth Epiphora Bauhiniae (Guerin Meneville) Lepidoptera: Saturniidae in Sudan. *J Forest Products and Industries,* **2(3):** 69-74.

Wink M. 2013. Evolution of secondary metabolites in legumes (Fabaceae).*South Afr J Botany,***89**: 164-175.

Yao L H, Jiang Y M, Shi J, Thomas-Barberan F A, Datta N, Singanusong F A and Chen S S. 2004. Flavonoids in foods and their health benefits. *Plant Foods for Human Nutr.,***59(3):** 113-122.

Yasumatsu K, Sawada K, Moritaka S, Misalei M, Toda J, Wada T and Ishii K. 1972. Whipping and emulsifying properties of soy bean products. *Agri Biol Chem.,* **36**: 719-727.

Yen A. 2009. Edible insects:Traditional knowledge or Western phobia. *Entomol Res.,* **39(5):** 289-298.

Young S M and Greaves J E. 1940. Influence of variety and treatment on phytin content of wheat. *J Food Sci*., **5**: 103-108.

JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 51 - 54

Jordan Journal of Biological Sciences

The Effect of Simulated Acid Rain on Plant Growth Component of Cowpea (*Vigna unguiculata*) L. Walps

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Received: October 25, 2014 Revised: December 18, 2014 Accepted: December 27, 2014

Abstract

The rain forest belt in Southern Nigeria is potentially susceptible to problems related to acid rain because of the increase in the consumption of petroleum oil products, such as diesel, gasoline and coal, used to produce energy for different sectors of the economy. The effect of simulated acid rain on the plant growth component of cowpea, *Vigna unguiculata* was studied. A greenhouse experiment was conducted at the Federal University of Technology, Akure, Ondo state, Nigeria. Simulated acid rain was prepared with a mixed concentrated sulphuric acid (H_2SO_4) and concentrated nitric acid (HNO_3) in a ratio 2:1 to get the desired pH using a Deluxe pH meter. The plant was exposed to simulated acid rain of pH 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 which was the control. Simulated acid rain induced morphological changes including chlorosis, early leaf senescence, necrosis, leaf abscission, leaf folding and death. Plant height, leaf area, fresh weight, relative growth rate, chlorophyll content of the leaf and harvest index were highest at 7.0 (control) but significantly (p<0.05) decreased with increasing the acidity levels. *V. unguiculata* was seriously affected by the simulated acid rain.

Keywords: Harvest index, Plant growth, Relative growth rate, Simulated acid rain, *V. unguiculata*

1. Introduction

Acid rain is a wet deposition that has been acidified when pollutants, such as oxides of sulphur and nitrogen, contained in power plant emission, factory smoke and car exhaust, react with the moisture in the atmosphere (Kita *et al*., 2004). In natural conditions atmospheric precipitation is slightly acidic due to the dissolution of atmospheric carbon dioxide (Nduka *et al*., 2008). Rain that presents a concentration of H^+ ions greater than 2.5μeq^{-1} and pH values lower than 5.6 is considered acid. Acid deposition may cause a decline in the health and growth of plants (Wyrwicha and Sklodowska, 2006; Liu *et al*., 2010). Several experiments have been carried out in the field and in greenhouses to investigate the effect of acid rain on plants. Acid rain exposure of plants result in a characteristic of foliar injury symptoms, modified leaf anatomy (Stoynora and Velikova, 1998; Park and Yanai, 2009), structural changes in the photosynthetic pigment apparatus and a decrease in the chlorophyll concentrations (Sant' Anna-Santos *et al*., 2006; Wang, 2010). Also, a reduction in plant growth and a yield of field corn (Banwart, *et al*.,1988), green pepper (Shripal *et al*., 2000), tomato (Dursun *et al*., 2002) were reported.

Cowpea, *Vigna unguiculata* (L.)Walp, is a leguminous plant belonging to the family Fabaceae. It is of immense benefit to mankind because it is useful as a rotational cover crop. It is also used to control erosion and to improve soil properties. Cowpea also suppresses weeds and can encourage populations of beneficial insects to defend cash crops from insect pests. It can also be used for the production of high quality hay or silage, when mixed with crops, such as corn or sorghum. Cowpea, being a stable food in Nigeria for millions of Nigerians, can potentially be affected by acid rain, and so, there is a need to examine the potential effects of acidic precipitation on cowpea. Acid rain is an issue in the study site and this experiment has been done on test species. With the increasing rate of population in Nigeria which is leading to the high demand for automobiles, it is evident that acidic rain is a reality in Nigeria. In view of the importance of this plant in human diet and the adverse effect of acid rain, the present study is carried out to assess the effect of simulated acid rain on this plant.

2. Materials and Methods

2.1. Planting Procedure

A greenhouse experiment was conducted in the Department of Biology of the Federal University of Technology, Akure, Ondo State, Nigeria. Disease free seeds were collected from the International Institute of Tropical Agriculture (IITA) Ibadan in Oyo State of Nigeria. The plants were not provided with nutrient solution.

The seeds were tested for viability before planting. Six seeds were planted in each experimental plastic pots. Each pH treatment had four replicates and was arranged in a

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Completely Randomized Design (CRD). The plants were watered every other day and grown for a week before the application of the simulated acid rain treatment. The plants were sprayed with 10ml of acidic solution according to their pH values of 2.0, 3.0, 4.0, 5.0, 6.0 and the control (7.0). The solutions were applied every three days using a medium size pressurized sprayer on t he plants. The plants were grown for fifteen weeks before the termination of the experiment.

2.2. Preparation of Simulated Acid Rain

The simulated acid rain was formed from a mixture of concentrated sulphuric acid (H_2SO_4) and concentrated nitric acid (HNO_3) in a ratio 2:1. The acidic solution was then calibrated using distilled water with a D eluxe pH meter to get the desired pH (2.0, 3.0. 4.0, 5.0 and 6.0) and cross checked with pH pen. The control pH (7.0) had distilled water.

Several parameters were used in assessing the growth of the plant. The height of the shoots was measured using a tape rule in (cm) from the soil level to the terminal bud. The measurements were taken in an interval of 2 weeks from the day the acid rain treatment commenced to the day of harvest. Leaf area was determined by the proportional method of weighing a cut-out of traced area of the leaves on g raph paper with standard paper of known weight to area ratio. The fresh weights and the Relative Growth Rate (RGR) were calculated following the methods of Hunt (1990) and the fresh weight of the whole plant was used to determine the relative growth rate:

$$RGR = (\log W_2 - \log W_1)/(T_2 - T_1)$$

Where: W_2 = final weight

W_1 =initial weight

T_2 =final time

T_1 = initial time.

The chlorophyll content of the leaves was determined by the method of Arnon (1949) and it is expressed on the fresh weight:

Chlorophyll a $=12.7_{D663}-2.69_{D645}$ X Vmg/1000W (mgg^{-1}).

Chlorophyll b$=22.9_{D645}- 4.68_{D663}$ X Vmg/1000W (mgg^{-1}).

Total chlorophyll (chlorophyll a & b) $= 20.2_{D645}- 8.02_{D663}$ X V/1000W.

Where D_x = absorbance of the extract at the wavelength X nm.

V = total volume of the chlorophyll solution (ml).

W = weight of the tissue extracted (g).

The harvest index was determined by the method of Ekanayake (1994).

2.3. Statistical Analysis

Data obtained were subjected to analysis using the Statistical Package for Social Sciences, Version 15.0 (SPSS, 2003). Treatment means were separated using the Duncan Multiple Range Test (Zar, 1984).

3. Results and Discussion

Morphological changes were observed through the 15 weeks after simulated acid rain treatment. The effects of simulated acid rain on t he morphology shows that the leaves turned brownish, with red with 70% leaf abscission. The dropping and eventual collapse of leaves stretched over a period of 12-15 weeks at pH 4.0 to pH 2.0. Leaf abscission started with the leaves at the base of the shoot droppings with long petiole at pH 4.0 treatment. Leaves were chlorotic and necrotic. At 2.0 pH treatment, plants died from the base of the shoot. Leaves had 60% leaf abscission.

Table 1. Effect of simulated acid rain (SAR) on the plant height (cm), leaf area (cm), fresh weight (g) of *V. unguiculata*

pH of SAR	Plant height (cm)	Leaf area (cm)	Fresh weight (g)
7.0 (Control)	54.74 ± 2.34 a	38.14 ± 2.27 a	192.11 ± 0.56 a
6.0	46.12 ± 2.06 b	26.08 ± 1.17 b	130.34 ± 0.44 b
5.0	40.10 ± 1.60 b	20.54 ± 1.08 b	122.12 ± 0.20 c
4.0	30.33 ± 1.48 b	15.20 ± 0.20b	94.21 ± 0.12 d
3.0	22.34 ± 0.31 c	11.26 ± 0.18c	64 .10 ± 0.07e
2.0	10.01 ± 0.10 d	06.32 ± 0.04c	42.23 ± 0.04 f

Each value is a mean of ± standard error of four replicates. Means within the same column followed by the same letter are not significantly different at (p>0.05) from each other using New Duncan Multiple Range Test.

The plant had the highest plant height, leaf area and fresh weight significantly higher (p< 0.05) at the control (pH 7.0) compared to the other acidity treatments.

Table 2. Effect of simulated acid rain (SAR) on the relative growth rate ($gg^{-1}d^{-1}$), chlorophyll content (mg/g) and harvest index of *V. unguiculata*

pH of SAR	Relative growth rate	Chlorophyll content	Harvest index
7.0 (Control)	2.6 ± 0.22 a	3.8 ± 0.20 a	0.5
6.0	2.0 ± 0.16 a	3.0 ± 0.16 a	0.4
5.0	1.6 ± 0.12 b	2.2 ± 0.12 b	0.3
4.0	1.2 ± 0.08 b	1.7 ± 0.10 b	0.2
3.0	0.8 ± 0.05 b	0.6 ± 0.05 c	0.1
2.0	0.4 ± 0.02 c	0.2 ± 0.01 c	0.1

Each value is a mean of ± standard error of four replicates. Means within the same column followed by the same letter are not significantly different at (p>0.05) from each other using New Duncan Multiple Range Test.

The effect of simulated acid rain on the Relative Growth Rate (RGR), the chlorophyll content and the harvest index of *V. unguiculata* are presented in Table 2. The plant had a relative growth rate, a chlorophyll content and a harvest index significantly higher (p< 0.05) at pH 7.0 compared to other acidity treatments. There was a significant reduction in relative growth rate, chlorophyll content and harvest index with decreasing pH level.

Symptoms of plants polluted with simulated acid rain include chlorosis, necrosis, stunted growth, lesion,

suppression of leaf production, leaf curling, withering of leaves, leaf abscission and even death of plants. Silva *et al*. (2006) and Wang (2010) found that plants exposed to low pH rain (pH 3.0) are generally retarded with leaf chlorosis, necrotic spot coupled with dehydration of the plants. Simulated acid rain exposure caused chlorosis, necrotic lesions and leaf tip injuries at the different pH levels of *V. unguiculata*. Marked chlorotic and marginal necrotic symptoms were observed at pH 4.0 and 5.0. However, this was less pronounced in comparison to pH 3.0 and pH 2.0. Similar symptoms were also observed by Johnston and Shriner (1985) on wheat at pH 4.3 and 2.3.

All the plant growth parameters that studied the plant height, leaf area and fresh weight of *V. unguiculata* were decreased significantly at all acidity levels with respect to the control set; the highest reductions were observed at pH 2.0 level (Table 1). The adverse effects of simulated acid rain on plant growth parameters on several crops were also observed by Evans *et al*. (1997), Banwart *et al*. (1990), Chevone *et al*. (1984) and Liu *et al*. (2010).

Photosynthetic pigments were also inhibited with respect to acidity levels. Chlorophyll content was significantly reduced by simulated acid rain treatment compared to the control at pH 2.0 and pH 3.0 (Table 2). The reduction was due to the removal of Mg^{+} from the tetrapyrol ring of the chlorophyll molecules by H^{+} (Foster, 1990) or due to the increase of transpiration by acid rain (Evans *et al*., 1997). Recently similar results have also been observed on many crops like mustard, radish, potato (Agrawal *et al*., 2005; Kausar *et al*., 2005; Khan and Devpura 2005; Varshney *et al*., 2005; Park and Yanai , 2009).

Relative growth rate and harvest index were lowest at pH 2.0 and pH 3.0 of *V. unguiculata* compared to the control plants (Table 2), and this have been reported by Seinfield *et al*. (1998). According to Iglesias *et al*. (1994), harvest index of 0.5- 0.6 is the optimum level for crops because at higher values of harvest index, root production decreases due to a reduced leaf area, light interception and photosynthesis. The present paper shows that all the levels of simulated acid rain have a negative effect on the growth and the yield component of cowpea *V. unguiculata* due to the reduction of photosynthesis as a result of chlorosis, necrosis and leaf abscission.

References

AgrawalS, Raghav D and Khan A A. 2005. An evaluation of the impact of simulated acid rain on the growth of mustard in pots. *Test of Agrochem Cultivars*, (Supplement), **21**: 25- 26.

Arnon D I. 1949. Copper enzymes in isolated chloroplast and polyperoxidase in *Phaseolus vulgaris. Plant Physiol.,* 24: 1-15.

Banwart WL, Porter PM, Ziegler EL and Hassett JJ. 1988. Growth parameters and yield component response of field corn to simulated acid rain. *Environ Experimental Bot.,* **28**: 43-51.

Banwart W L, Finke R L, Porter P M and Hassett J J. 1990. Sensitivity of twenty soybean cultivars to simulated acid rain. *J Environ Quality,***19**:339-346.

Chevone BI, Herzfeld K and Chappelka AH.1984. Effects of acid aerosol and ozone on yellowpoplar seedlings. *Can J Res.,* **14**: 820-891.

Dursun A,Kumlay AM,Yilderin E and Guvenc I. 2002. Effects of simulated acid rain on plant growth and yield of tomato. *Acta Hort*., **579**: 245-248.

Ekanayake I J. 1994.Terminology for growth analysis of cassava.*Tropical Root and Tuber Crops Bulletin,* **8(1):**2.

Evans LS, Gmor NF and Dacosta F 1997. Leaf surface and histological perturbations of leaves of *Phaseolus vulgaris* and *Helianthus annuus* after exposure to simulated acid rain. *Am J Bot*., **4**: 304-313.

Foster JR. 1990. Influence of pH and plant nutrients status onion fluxes between tomato plants and simulated acid mists. *New Phytologist.,***116** : 475-485.

Hunt R. 1990. **Basic Growth Analysis: Plant Growth Analysis for Beginners**. AcademicPress. London, UK. 41p.

Iglesias C A, Calle F, Hershey C, Jaramillo G and Mesa E. 1994. Sensitivity of cassava (*Manihot esculetna Crantz)* clones to environmental changes. *Field Crops Res.,* **36**: 213-220.

Johnston J W Jr. and Shriner D S.1985. Response of three wheat cultivars to simulated acid rain. *Environ Experimental Bot.,* **25**: 349-353.

Kausar S, Khan A A and Rahman F. 2005. Impact of simulated acid rain on potato.In :**Herbal Drug and Environmental Pollution**. A satellite Session of Third International Conference on Plants and Environmental Pollution, Jamia Hamdard, New Delhi, pp. 56.

Kita I, Sato T, Kase Y and Mitropoulos P. 2004. Neutral rains at Athens, Greece: a natural safeguard against acidification of rains. *Sci Total Environ.,* **327**:285-294.

Khan T I and Deopura S. 2005. Physiological and biochemical effects of simulated acid rain on *Phaseolus vulgaris* Var HUR 15. *The Environ.,* **24**:224-226.

Liu K H, Mansell R S and Rhue R D. 2010. Cation removal during application of acid solution into air dry soil columns. *Soil Sci Society J.,* **4**:1747 – 1753.

Nduka JKC, Oriskwe OE, Ezenweke LO, Ezenwa TE, Chendo MN and Ezeabasili NG.2008. Acid rain phenomenon in Niger Delta region of Nigeria: economic, biodiversity and public health concern. *The SciWorld J* **8**: 811-818.

Park B B and Yanai R D. 2009. Nutrient concentrations in roots, leaves and wood of seedling and mature sugar maple and American beech at two contrasting sites. *Forest Ecol Management,* **258:**1153 – 1160.

Sant' Anna-Santos B F, Silva LC, Azevedo A and Deque-Brasil R. 2006. Effects of simulated acid rain on photosynthesis, chlorophyll fluorescence and antioxidative enzymes in *Cucumis sativus* L. *Photosynthetica*, **40**: 331-335.

Shripal N, Pal KS and Kumar N. 2000. Effects of simulated acid rain on yield and carbohydrate contents of green pepper. *Advanced Plant Sci*., **13**: 85-88.

SPSS .2003. **Statistical Package for Social Sciences** (SPSS 15.0 Inc., Chicago, U.S.A).

Silva L C, Oliva M A, Azevedo A A and Araujo J M. 2006. Responses of resting plant species to pollution from an iron pelletization factory.*Water Air and Soil Pollution,* **175** (1-4): 241-256.

Seinfield J H, Pandis S N and Spyries N. 1998. **Atmospheric Chemistry and Physics from Air Pollution to Climate Damage.** AVI publishing Co, West Print Connecticut, pp 224.

Stoynora D and Velilova V. 1998. Effects of simulated acid rain on chloroplast ultrastructure of primary levels of *Phaseolus vulgaris. Biol Plant,* **40**:589-598.

Varshney P , Kausar S and Khan AA. 2005. An evaluation of the susceptibility of radish inpots to simulated acid rain. *Test of Agrochem Cultivar*, **27**: 29-30.

Wang CQ. 2010. Exogenous calcium alters activities of antioxidant enzymes in *Trifolium repens* L. leaves under peg-induced water deficit. *J Plant Nutr*., **33**: 1874-1885.

Wyrwicha A and Sklodowska M. 2006. Influence of repeated acid rain treatment on antiocidative enzyme activities and on lipid peroxidation in cucumber leaves. *Environ Experimental Bot.,* **56**: 198-204.

Zar J H. 1984. **Biostatatical Analysis**, 2nd edition, Prentice- Hall International, Englewood Cliffs, N.J.

JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 55 - 59

Jordan Journal of Biological Sciences

The Influence of Iron Oxide Nanoparticles on the Red Blood Cells Photohemolysis Sensitized with Photofrin: Temperature Effect

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Received: October 19, 2014 Revised: December 24, 2014 Accepted: January 4, 2015

Abstract

The present study aims to measure the temperature-dependence of *in vitro* continuous photohemolysis (CPH) photosensitized by Photofrin® in the presence or absence of Iron Oxide Nanoparticles (IONPs) and to evaluate the results using Gompertz function. Red Blood Cells (RBCs) were isolated from human healthy volunteers blood; they were then incubated with Photofrin® only or with IONPs for 45 minutes at 37 °C and then irradiated to a range of temperatures (4-41°C). The results show that Photosensitization of RBCs by Photofrin® with IONPs presence reduces the inhabitation effect of Photofrin® at the same irradiation temperature since the decreasing in activation energy and increment in t_{50} were obvious evidence for such result as well as the applicability of Gompertz function to the fractional photohemolysis ratio (a) and the rate of fractional photohemolysis (b), is found to be the most appropriate model to fit the experimental data with minimum parameters and minimum errors, Parameter (**a)** and the curves steepness were found to be temperature-independent. On the other hand, values of parameter (**b)** increased as irradiation temperature increased with or without IONPs presence. The apparent activation energy was found to be 18.85 ± 0.72 kcal/mol in the absence of IONPs and 17.29 ± 0.71 kcal/mol in the presence of IONPs. Our results indicated that Photofrin® incorporated with IONPs could be considered as a modality to improve cytotoxicity in photodynamic therapy and/or reduces the inhabitation effect.

Keywords: Continuous Photohemolysis (CPH), Gompartz parameters, Iron Oxide Nanoparticles (IONPs), Irradiation temperatures, Photodynamic Therapy (PDT)

1. Introduction

Photodynamic Therapy (PDT) is a promising treatment modality that has been successfully used in treating localized tumours, providing tumour selectivity and normal tissue sparing with almost no s erious side effects. PDT employs the combination of light and photosensitizers to damage localized cancer cells (Sternberg *et al*., 1997; Dougherty *et al*., 1998). As light applied to an area to be treated; it chemically altered the photosensitizer which undergoes internal reactions with substrate that finally creates cytotoxic Reactive Oxygen Species (ROS) which then attack the main structural entities in the target cells. Therefore, molecular Oxygen existence is a key point in PDT (Henderson *et al*., 2000; Sil *et al*., 2004). Human erythrocytes were shown to be a primary target in PDT because they have a relatively simple structured model which enables the compounds to create photooxidation process, and the released haemoglobin, due to membrane damage, can be easily measured through spectrophotometrey (Ben-hur *et al*., 1986). The photohemolysis of erythrocytes (lysis of erythrocytes when they exposed to light) was tested by using many different natural and chemical photosensitive drugs, such as Chinese Corolla (Alzoubi *et al*., 2014), Cichorium pumilum (Al-Akhras *et al*., 2007) and Photofrin® (Al-Akhras *et al*., 1996). *In vitro* studies on photohemolysis rate, measurements were investigated based on t wo techniques: lysis of erythrocytes during irradiation (Continuous Photohemolysis; CPH) and lysis of erythrocytes after being given a controlled light dose (Delayed Photohemolysis; DPH) (Al-Akhras *et al*., 1996). Photofrin® (Porfimer Sodium) is considered to be among the most efficient and vastly used photosensitizers in clinical PDT, especially for localized tumour that accumulates in cancerous tissues in a m uch higher rate than in normal ones (Allison *et al*., 2004).. On the other hand, some studies reported that RBCs photohemolysis curves showed a good agreement with Gompertz function module (Al-Akhras *et al*., 1996; Choe *et al*., 2013; Al-Akhras *et al*., 2006). The Gompertz function is defined as:

$$H = H_O e^{-a e^{-bt}} \quad \text{.....} \quad \text{(Eq.1)}$$

Where: H is the percentage of hemolysis during the lysis time t (the time measured from start of hemolysis the RBCs

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at dark incubation), H_o is the initial maximum number of cells, normalized to one, a is a fractional hemolysis ratio, and b is the rate of fractional hemolysis change.

In recent years, there has been much research on the utilization of nanomaterials in biomedical applications. Because of their high surface ratio, small size (near to bio-macromolecules), fast diffusion that improved their chemical reactivity and multi-functioning for biomaterials, their ability to fit into dimensions of micron (Amirnasr *et al*., 2011), IONPs have become widely applied to biomedicine and biotechnology cell sorting and isolation, delivery vehicles and diagnostic agents (Yang *et al*., 2013), drug targeting, magnetic resonance imaging (Patrick *et al*., 2009; Morales *et al*., 2003), as some results showed that the interaction of IONPs with RBCs, which serves as main target in biomedical applications, do not affect some important physiological parameters such as the pH and Ca^{2+} content (Moersdorf *et al*., 2010). In the present study, the temperature dependence of *in vitro* CPH photosensitized by Photofrin® was measured in the absence and presence of IONPs and the results were evaluated using Gompertz function.

2. Materials and Methods

2.1. Materials

RBCs were isolated from human healthy male mature volunteers by repeated centrifugation at 4000 rpm and resuspension in pH7.4, 10 mM Phosphate Buffer Saline (PBS). IONPs stock with 0.75 mg/ml solution diluted to 0.15 mg/ml and ultrasonic wave's generator was used for 25 minutes to ensure dissolving. Photofrin® stock was prepared by dissolving 1mg of Photofrin® powder in 10 ml PBS, Iron oxide (Fe_3O_4) nanopowder, 98% with a radius of 50 nm purchased from Sigma-Aldrich (St Louis, MO, USA). Photofrin® powder (Porfimer Sodium) was purchased from Cyanamid (Pearl River, New York).

2.2. Methods

The washed RBCs was diluted in PBS to get Optical Density (OD) about 2 cm^{-2} at 680 nm, which corresponds to 7.86 x 10^6 cells/mm^3 erythrocytes concentration, measured using haemocytometer. CPH measurements were conducted onto two types of samples; RBCs incubated with 2 µg/ml Photofrin® and 50 µg/ml IONPs and RBCs incubated with 2 µg/ml Photofrin® only, both types were incubated at 37˚C for 45 minutes with shaking each 15 minutes then they continually irradiated to a range of irradiation temperatures (T_{irr}) for 4-41˚C until reaching absorbance stability. The irradiated sample was contained in a 2 cm X 2 cm cylindrical quartz cuvette and mounted in a stand which has a water circulation for controlling temperature set (AL-Akhras *et al*., 1996). The samples were located at 29 cm from light source and the output intensity the samples reached was about 60 W/cm^2 as measured by Filed Max II Laser Power/Energy Meter/Coherent/USA**.** The photohemolysis light source was 200 W high-pressure Hg/Xe arc lamp, housed in an Oriel Research Arc Lamp Housing model: 66903, with Oriel Digital Arc Lamp Power Supply model: 68907.

The absorbance rate was measured by calibrating the SHIMADZA, UV-Vis -2450 spectrophotometer to read 100% transmittance at 680 nm for PBS solution and comparing the intensity of transmitted beam through reference sample (PBS) with a transmitted beam through test sample (our sample), which is detected by photomultiplier for each sample, and, finally, the data were read by a designed computer connection through UV Probe-[Spectrum] computer program version 2.33.

3. Results and Discussion

Continuous Photohemolysis (CPH) curves for samples with and without IONPs irradiated at different temperatures were obtained by studying the relationship between fractional photohemolysis and time of exposure as shown in Figures 1 and 2, respectively.

In vitro study of RBCs photohemolysis is based on CPH, in which the suspension of RBCs along with Photofrin® and IONPs is exposed to a direct dose of light source with photohemolysis measurements in dark that may be assayed by hemoglobin release. Photohemolysis of RBCs is related to the generation of Reactive Oxygen Species (ROS) that accumulate with biomolecules of cell membrane, such as lipids and DNA proteins that lead to cells swelling and, finally, rupturing (Al-Akhras *et al*., 1996). Photosensitization of RBCs with Photofrin® conjugated with IONPs inhibits erythrocytes membrane lysis, the decreasing in activation energy and increment in t_{50} were obvious evidences for such a result. A similar result was reported by Reddy *et al*. (2006)**.** They noted that the rates sensitized with Photofrin® under irradiation causes death within 13 days but rates sensitized with Photofrin® in the presence of IONPs stayed 33 days. Experimental data were mathematically modelled using Gompertz function (see Table 1). For all curves, the points represent the experimental data while the solid lines show their best fitting with Gompertz function. Relative steepness for lysis curves (S) is defined as t_{80}/t_{30} where t_{80} is the time required to 80% lysis of cells and t_{30} is the time required to 30% lysis of cells and it does not seem to be affected by IONPs presence.

The irradiation temperature (T_{irr}) and the time required to lysis 50% of the cells (t_{50}) are clear to be inversely proportional. The value of t_{50} is larger at lower values of T_{irr} and much larger with the presence of IONPs at same T_{irr}. The applicability of Gompertz function to the fractional photohemolysis ratio, (**a)** and the rate of fractional photohemolysis, (**b)** is found to be the most appropriate model to fit the experimental data with minimum parameters and minimum errors. The parameter (**a)** was showed to be independent of temperature and the values of parameter (**b)** increases with increasing irradiation temperature for 2 µg/ml Photofrin® and 50 µg/ml IONPs. Similarly, the parameter (**a)** was showed to be independent of temperature, while the parameter (**b)** increases with increasing irradiation temperature for 2 µg/ml Photofrin® in the absence of IONPs.

The Arrhenius equation can thoroughly analyze the dependence of temperature and cells killing (Alzoubi *et al*., 2014). Arrhenius equation is defined as $1/t_{50} = A\, e^{-E/kT}$, where $1/t_{50}$ reciprocal to the time required to 50% lysis of cells that acts as the activation rate which is described by the steepness of the killing curve; A is

constant, E is the activation energy, k is the Boltzmann constant, and T is the absolute temperature. Hyperthermia measurements with human RBC led to no hemolysis after 30 h a t a temperature below 37°C (Gershfeld and Murayaman, 1988). The typical curves of $1/t_{50}$ Vs 1/T are shown in Figure 3. The curves were fitted with Arrhenius equation to calculate the activation energy. The apparent activation energy destabilization of the RBC membrane by photosensitization for 4 – 41°C were quantitatively calculated and found to be 18.85 ± 0.72 kcal/mol in the absence of IONPs and 17.29 ± 0.71 kcal/mol in the presence of IONPs. It was also noticed that the activation energy for the samples without IONPs are greater than the activation energy with IONPs. On the other hand, the presence of IONPs decreases the photohemolysis process and increases the t_{50}.

The generally accepted colloid-osmotic mechanism postulates that the photochemical damage to the RBC membrane leads to cation efflux, followed by cell swelling and rupture (Pooler, 1985). A speculative connection to colloid-osmotic lysis is that ***a*** and ***b*** are related to the rate of the damaged band 3 protein sites that act in concert to form a K^+ leak. An additional consideration is that singlet oxygen, generated by strong bound Photofrin® with IONPs, may react with membrane targets *via* the external medium. Figure 4 s howed microscopic images of RBCs with the presence and absence of IONPs: Figure 4 (a) the RBCs incubated with 2µg/ml Photofrin® only and Figure 4 (b) incubated with 2 µg/ml Photofrin® and 50µg /ml IONPs. The images were taken using NIKON inverted microscope. Strong bounded IONPs, on the cell surface with possible diffusions inside the membrane, are clearly shown in Figure 4 (b). In conclusion, our results indicate that Photofrin® incorporated with IONPs could be considered as a modality to improve cytotoxicity in photodynamic therapy or reduces the inhabitation effect.

Table 1. Effect of irradiation temperature on RBCs photosensitized by Photofrin® with presence and absence of IONPs. **Group I**: RBCs incubated with 2 µg/ml Photofrin® and 50 µg/ml IONPs**, Group II:** RBCs incubated with 2 µg/ml Photofrin® only

	$(t_{50})_{exp}$	$(t_{50})_{th}$	T_{irr}	*S*	Gompertz function parameters			R
Group	(min)	(min)	(°C)		H_o	*a*	*b* $(min)^{-1}$	%
I	47.23	45.93	4.0		1.10±0.03	52.46±12.65	0.09±0.01	0.997
	42.68	41.90	18.0	1.37	1.17±0.04	33.09±6.73	0.08±0.01	0.997
	37.86	37.08	25.0	±	1.18±0.05	50.89±15.29	0.12±0.01	0.996
	32.26	31.70	35.0	0.02	1.12±0.04	57.26±17.27	0.14±0.02	0.997
	25.75	25.23	41.0		1.14±0.04	38.85±10.21	0.15±0.01	0.998
II	39.04	39.04	4.0		1.25±0.07	43.05±13.05	0.10±0.01	0.996
	33.22	32.74	18.0	1.38	1.27±0.07	44.66±13.57	0.12±0.01	0.997
	28.53	28.21	25.0	±	1.15±0.04	59.88±17.41	0.15±0.01	0.998
	21.73	21.43	35.0	0.09	1.44±0.14	20.00±5.25	0.16±0.02	0.997
	15.54	14.98	41.0		1.21±0.09	45.90±23.40	0.26±0.04	0.996

Results are expressed as mean ±S.D. ***S:*** Curves Steepness defined as t_{80}/t_{30} Gompertz Function (Eq.1) Parameters: H_0 Initial maximum number of cells (normalized to one). ***a*** Fractional hemolysis ratio. ***b*** The rate of fractional hemolysis change. (t_{50})**exp:** the experimental value of time required to lysis 50% of cells (t_{50})**th:** the theoretical value of time required to lysis 50% of cells T_{irr}: Irradiation temperature **R:** correlation coefficient

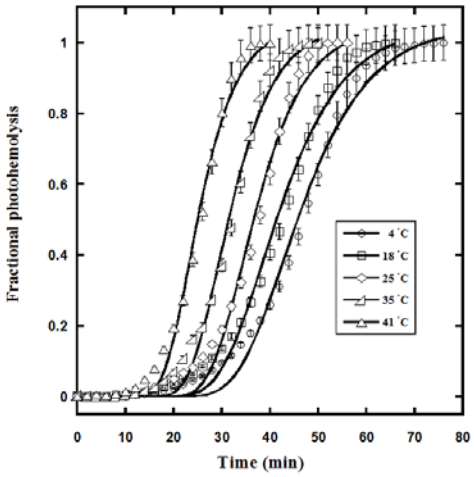
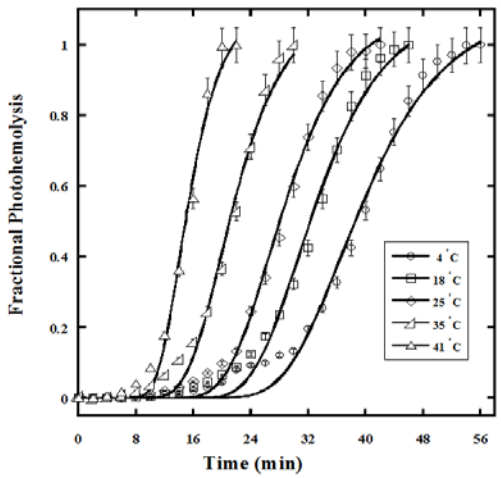


Figure 1. Photosenstization of CPH by 2 µg/ml Photofrin® with 50 µg/ml IONPs, irradiated to range of temperature at fixed incubation temperature. The solid lines are the Gompertz function fitting.



Figuer 2. Photosensitization of CPH by 2 µg/ml Photofrin®, irradiated to range of temperature at fixed incubation temperature. The solid lines are the Gompertz function fitting.

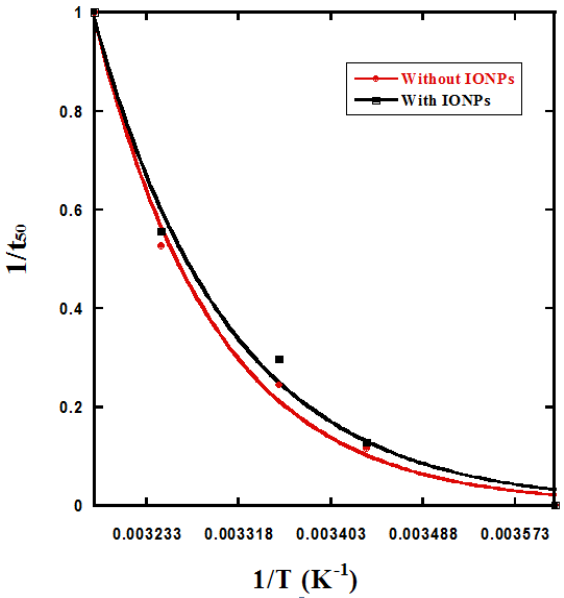
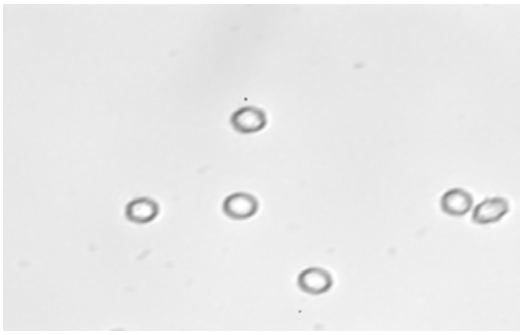
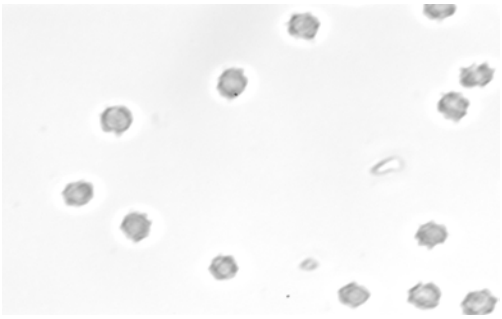


Figure 3. Arrhenius plot for the dependence of $1/t_{50}$ and temperatures corresponds to CPH curves with and without IONPs. The solid lines are the Arrhenius equation fitting. The apparent activation energy was found to be 18.85 ± 0.72 kcal/mol in the absence of IONPs and 17.29 ± 0.71 kcal/mol in the presence of IONPs



(a)



(b)

Figure 4. Microscopic images of RBCs with presence and absence of IONPs. (a) RBCs incubated with 2µg/ml Photofrin® only; (b) incubated with 2 µg/ml Photofrin® and 50µg /ml IONPs. The images were taken using NIKON inverted microscope.

4. Conclusion

Photosensitizations of RBCs with Photofrin® conjugated with IONPs are investigated. Gompertz function serves as a typical mathematical model for CPH that leads to suitable modelling parameters and is found to be in a v ery good agreement with the experimental parameter. The notable increase in the rate (***b)*** suggests that additional membrane targets are accessible to singlet oxygen generated in the external medium or might be attributed to membrane lysis which combines the effects of cell swelling induced by a damage to the anion transport protein and a thermally activated photochemical damage to structural membrane proteins.

Acknowledgements

This work was supported by a grant from the Deanship of Scientific Research at Jordan University of Science and Technology (JUST): Grants number 113/2013.

References

Al-Akhras MA and Grossweiner LI 1996. Sensitization of photohemolysis by hypericin and photofrin, *J Photochem Photobiol B*. **34(2-3):**169-75.

Al-Akhras M A, Amin A, Mohammad K, Al-Haddad Fand Allaaeldin H. 2007. *In vitro* studies on the effect of phototoxicity of a new photosensitizer extracted from flowers and aerial parts of *Cichorium pumilum*. *Am J Pharmacol Toxicol* **2 (2)**: 39-45

Al-Akhras M A. 2006. A new application of Gompertz function in photohemolysis: the effect of temperature on red blood cell hemolysis photosensitized by protoporphyrin IX. *Med Bio Eng Comput*. **44**:703–710

Allison, R R, Downie G H, Cuenca R, Hu, XH, Child, C JH and Sibata C H. 2004. Photosensitizers in clinical PDT, *Photodiagnosis and Photodyn Ther.*, **1***:* 27-42.

Alzoubi, F Y, AL-Akhras M A, Al-Omari H A and Aljarrh K. 2014. Evaluating the photosensitivity of the Chinese Chlorella on Red Blood Cells. *J Mol Pharm Org Process Res*. **2**:2.

Amirnasr A, Emtiazi G, Abasi S and Yaaghoobi M. 2011. Adsorption of hemoglobin, fatty acid and glucose to iron nanoparticles as a mean for drug delivery, *J Biochem Tech,* **3(2)***:*280-283.

Ben-hur E and Rosenthala I. 1986. Photohemolysis of Human Erythrocytes Induced by Aluminum Ph'i'halocyanine Tetrasulfonate, *Cancer Letters,* **30**: 321-327.

Choe S-W , Terman D S, Rivers A E, Rivera J, Lottenberg R and Sorg B. S. 2013. Drug-loaded sickle cells programmedex vivofor delayed hemolysis target hypoxic tumor microvessels and augment tumor drug delivery. *J Control Release*. **171**: 184–192.

Dougherty T J. Gomer C J, Henderson B W, Jori G, Kessel D, Korbelik M, Moan J and Peng Q. 1998. Photodynamic Therapy, *J Natl Cancer Inst,* **90**: 889–905.

Gershfeld, N. L. and Murayama M.1988. Thermal instability of red blood cell membrane bilayer: temperature dependence of hemolysis. *J Membr Biol.* **101**: 67-72

Henderson B W, Busch T M, Vaughan L A, Frawley N P, Babich D, Sosa T A, Zollo J D, Cooper M T, Bellnier D A, Greco W R and Oseroff A R, 2000. Photofrin photodynamic therapy can significantly deplete or preserve oxygenation in human basal cell carcinomas during treatment, depending on fluence rate, *Cancer Res.*, **60**: 525-529.

Morales, P. Verdaguer, S. Teresita, G. and Serna, C. (2003). The preparation of magnetic nanoparticles for applications in biomedicine, *J. Phys. D: Appl. Phys.* 36: 182–197.

Moersdorf D, Hugounenq P, Truonc Phuoc L, Mamlouk-Chaouachi H, Felder-Flesch D, Begin-Colin S, Pourroy G and Bernhardt I. 2010. Influence of magnetic iron oxide nanoparticles on red blood cells and Caco-2 cells. *Adv Biosci Biotechnol*. **1**:439-443.

Patrick A L, Qian Y, Shao R, Guo N L, Schwegler-Berry D, Pacurari M and Flynn D C. 2009. Iron oxide nanoparticles induce human microvascular endothelial cell permeability through reactive oxygen species production and microtubule remodeling. *Particle Fibre Toxicol.,* **6(1)**: 1.

Pooler J. 1985. The kinetics of colloid osmotic hemolysis. II. Photohemolysis, *Biochim Biophys Acta*, **812:** 199-205.

Tartaj P, Morales M P, Veintemillas-Verdaguer S, Alez-Carre˜ T G and Serna C J. 2003. The preparation of magnetic nanoparticles for applications in biomedicine. *Phys D: Appl Phys.*,**36**: 182–197.

Reddy G. R, Bhojani M S. McConville P, Moody, J, Moffat B A, Hall, D E, Kim G, Koo, Y-E, Woolliscroft M J, Sugai, J V, Johnson T D, Philbert M A, Kopelman R, Rehemtulla A and Ross B D. 2006. Vascular targeted nanoparticles for imaging and treatment of brain tumors. *Clin Cancer Res.,* **12 (22)**: 6677-6686

Renno R Zand M iller J W. 2001. Photosensitizer delivery for photodynamic therapy of choroidal neovascularization, *Advanced Drug Delivery Rev.,* **52**: 63–78.

Sil S, Bose T, Roy D and Chakraborti A S. 2004. Protoporphyrin IX-induced structural and functional changes in human red blood cells haemoglobin and myoglobin. *J. Biosci.* **29**: 281–291.

Sternberg, Dolphin, David .1997. Porphyrin-based photosensitizers for use in photodynamic therapy, *Tetrahedron.* **54**: 4151-4202.

Yang C, Wang J, Chen D, Chen J, Xiong F, Zhang H, Zhang Y, Gu N, Dou J. 2013. Paclitaxel-Fe_3O_4 nanoparticles inhibit growth of $CD138^-$ $CD34^-$ tumor stem-like cells in multiple myeloma-bearing mice, *Int J Nanomedicine*, **8**: 1439–1449.

JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 61 - 64

Jordan Journal of Biological Sciences

Evaluating the Genetic Relatedness within *Lupinus pilosus* L. Species Based on RAPD Analysis

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Received: December 26, 2014 Revised: January 13, 2015 Accepted: January 17, 2015

Abstract

Nine Random Amplified Polymorphic DNA (RAPD) primers showing polymorphic bands were used to determine the genetic relatedness among and within single individual plants of *Lupinus pilosus* and used for the construction of the dendrogram and the similarity matrix. A total of 1112 bands were obtained, 219 of which were polymorphic. Similarity values among the studied single individuals of *Lupinus pilosus* ranged from 0.450 to 0.02. The cluster analysis obtained by Unweighted Pair-Group Method with arithmetic averages (UPGMA) grouped the tested individuals into three clusters. A genetic variation was found within *Lupinus pilosus* based on RAPD markers. The variability emphasis the presence of genetic diversity within *Lupinus pilosus* species.

Keywords: Lupine, Genetic diversity, Polymorphism, RAPD.

1. Introduction

Lupinus, commonly known as Lupine or lupin, is a genus including more than 200 species; it is one of the most diverse and widespread taxonomic groups of flowering plants. Its wild species occur naturally in the Mediterranean region, including areas of Northern Africa and in North and South America and used as a sources of food protein (Wolko *et al*., 2011; Drummond *et al*., 2012). Sienkiewicz *et al*. (2008) reported that wild lupins, including perennial and annual species, are distributed across climatic ranges from subarctic Alaska, Mediterranean and semi desert climates, Africa, Mexico and USA. The chromosome numbers were determined in 22 accessions of 16 *Lupinus* species from Bolivia, Ecuador and Peru, all had 2n=48 chromosome except *L. bandelierae* (Camillo *et al*., 2006). *Lupinus pilosus*, like the Old World (Africa) rough-seeded lupines, has chromosome number 2n=42 as described by (Naganowska *et al*., (2003). Lupine oil could also be a valuable source of phosphatidylcholine in the human diet; the content of fatty acids in lupine oil is similar to that of oils from other oilseed crops (Borek *et al*., 2009). Many techniques proved useful for both inter- and intra-specific studies of diversity in *Lupinus*. Random Amplified Polymorphic DNA (RAPD) was used to study the molecular taxonomy among *Mentha spicata*, *Mentha longifolia* and *Ziziphora tenuior* populations (Al-Rawashdeh, 2011); also RAPD and Inter Simple Sequence Repeat (ISSR) markers were used to determine the genetic relationships among 20 Old World lupin genotypes from 31 lupin species (*Lupinus albus, L. angustifolius, L. luteus*) using 15 primers in each case (Yorgancilar *et al*., 2009). Talhinhas *et al*. (2003) used AFLP, ISSR and RAPD techniques to evaluate the genetic diversity among *L. albus, L. angustifolius, L. cosentinii, L. hispanicus, L. luteus, L. mutabilis, L. pilosus* and *L. polyphyllus*; they found low levels of similarity that ranged from 0.205 to 0.432. Aïnouche and Bayer (1999) studied 44 taxa of *Lupinus* genus using Internal Transcribed Spacer (ITS). DNA Amplification Fingerprinting (DAF) techniques were used to evaluate the genetic relationships among 24 randomly selected white lupin (*Lupinus albus* L.), accessions originating from four endemic regions (Qiu *et al*., 1995). The genetic diversity was studied in 94 accessions of white lupin (*Lupinus albus* L.) (Raman *et al*., 2014) and among different taxa (Sienkiewicz *et al*., 2008) as well as among and within Moroccan lupine species, using ISSR and AFLP (Sbabou *et al*., 2010) and molecular phylogeny which was reconstructed using nucleotide sequences of *rbc*L and nuclear ITS regions of 54 lupine species (Wink *et al*., 1999). The 2C nuclear DNA content was estimated for 18 species of genus *Lupinus* using propidium iodide (Naganowska *et al*., 2003). To the best of our knowledge, there are few studies on the genetic diversity of *Lupinus pilosus* using RADP markers, particularly in Jordan. The present study was undertaken to assess the genetic diversity and relatedness within some single individual plants of *Lupinus pilosus* using RAPD markers.

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2. Materials and Methods

2.1. Plant Material and DNA Isolation

The leaves of a single plant were collected from the wild field at As-salt governorate in Jordan (Plate 1) and then transferred into a lab of NCARE for DNA analysis. The total cellular DNA was extracted from a single individual of each sample according to the Doyle and Doyle (1987), Yorgancilar *et al*. (2009) and Talhinhas *et al*. (2003) with minor modifications. Twenty mg of fresh leaves were grounded in the presence of liquid nitrogen and the homogenate resuspended in extraction buffer with 750 µl of freshly and preheated 2x CTAB solution with 0.8g PVPP in 2 ml tubes and then placed at 65ºC for 1 hr. The mixture was added to 750 µl of chlorophorm/ isoamyl alcohol (24:1), vortexed for few seconds, and then centrifuged at 14,000g for 20 min. The supernatant was placed in 2 ml tubes with 750 µl isopropanol, and then mixed gently until the thread of DNA appeared; it is then centrifuged for 20 min at 14000 g. The solution is poured in tubes and left to dry; then 750 µl of cooled 70% ethanol was added to the solution and placed in the refrigerator (–20ºC) overnight. The following day, the ethanol was poured in the dried tubes and 100 µl of TE was added and the whole mixture was placed at 65ºC for 1 hr. Four micolitters of RNAase (10mg/ml) were added per tube and left for 1hr at 37ºC. The DNA concentration was measured using S2100 UV/VIS DIODE-Array-Spectrophotometer, machine Version 1.7.

Plate 1. Wild *Lupinus pilosus* L. species grown at Wadishueib in Jordan

2.2. PCR Amplification

In order to select primers with high level of polymorphism, the RAPD analysis was carried out using 40 of 10-mer primers, corresponding to kits A, B, D, T, W and Z (Opern Technologies), as previously described by Williams *et al*. (1990); those analyzed for polymorphism used in 4 populations. Each reaction was repeated twice. Only repeatable fragments with strong and medium intensity were used in the analysis. Nine RAPD markers showed a high polymorphism and were used in genetic diversity analysis. The PCR reaction was performed as described by Williams *et al*. (1990) with 10-mer ologonucleotides synthesized by Operon technologies (Almeda, Calif.). The final volume of 25µl contained 10 x buffer with $MgCl_2$, 20 ng of total genomic DNA, 0.25 mM dNTPs (Promega), 12 pm ole of primers (Opern technologies, US), 1.5 mM $MgCl_2$ and 1U of *Taq* polymerase (Promega). Amplification was carried out in thermocycler (MJ Research, USA, Model PCT-200), one cycle of 1 min at 94°C followed by 44 cycles, each consisting of a denaturation step for 1min at 94°C, followed by an annealing step for 1min at 36°C and an extension step for 2 min at 72°C, followed by a further extension step for 5 min at 72°C. After the final cycle, the samples were cooled at 4°C. Samples of 10 µl RAPD-PCR product were analyzed by electrophoresis on 1.4% agarose gel and the amplified products were detected after staining by ethidium bromide.

2.3. Data Analysis

For subsequent statistical analysis, in order to obtain a binary matrix, polymorphic bands amplified by primers of RAPD were scored as present (1) or absent (0). Genetic similarities for RAPD markers were calculated by using the Jaccards' coefficient (Jaccard, 1908) and the dendrogram obtained by clustering according to the Unweighted Pair-Group Method with arithmetic averages (UPGMA) using the SPSS 2000 (v.11.0) software. The polymorphism percentage was estimated by dividing the number of polymorphic bands over the total number of bands.

3. Results

3.1. RAPD Markers Variations

A total of 1112 bands with an average of 124 polymorphic bands per primer were amplified by RAPD analysis (Table 1). Among those, 219 were polymorphic across the 40 single individual. The highest and lowest numbers of polymorphic bands per assay were 33 and 20 for (OPD14) and (OPB01 and OPB12) bands, respectively (Table 1). The highest percentage of polymorphism was obtained by OPD14, OPB12, OPT16 and OPB01, while OPA16 showed the lowest (%) of polymorphism.

Table 1: Total amplified bands, number of polymorphic bands and percent of polymorphic bands of RAPD primers used in *Lupinus pilosus* analysis.

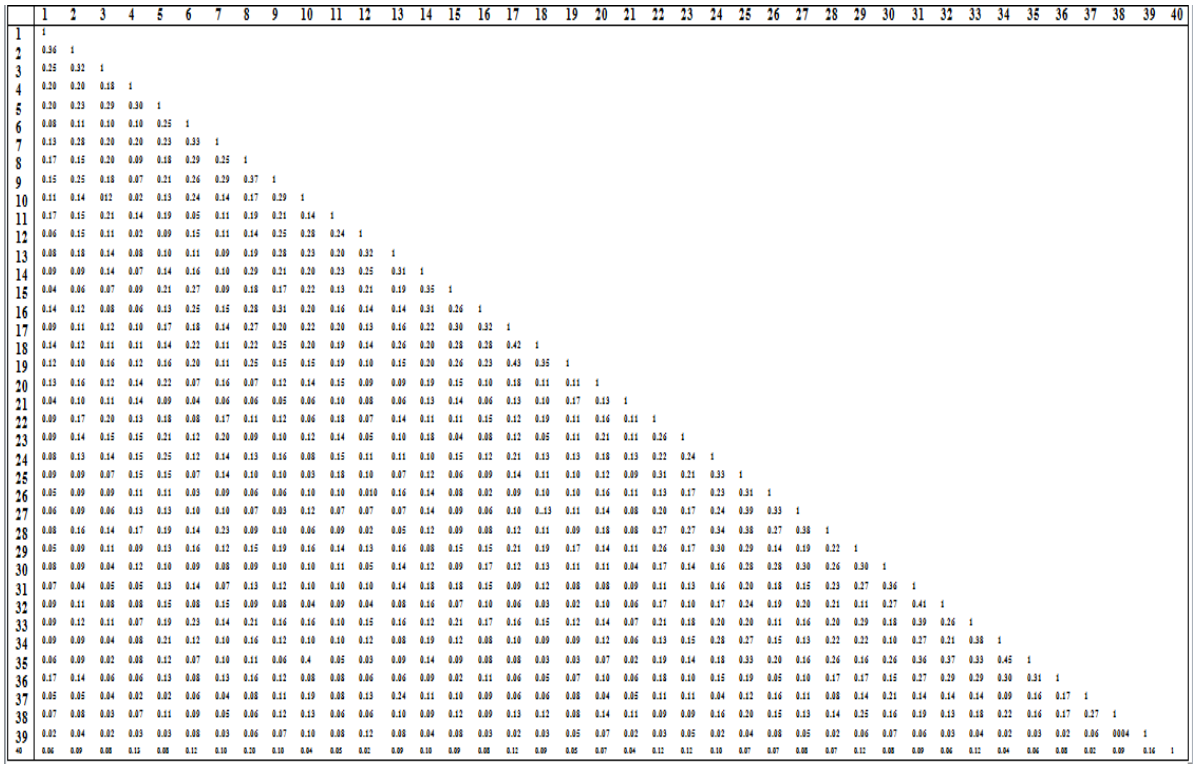
Primer name	Total bands/primer	Number of polymorphic bands	% of polymorphism
OPA16	153	22	14
OPB01	095	20	21
OPB06	150	23	15
OPB12	083	20	24
OPB10	123	24	20
OPB17	144	28	19
OPD14	117	33	28
OPT16	183	28	24
OPT19	068	21	31
Total over loci	**1112**	**219**	**19.6**
Mean per primer	**124**	**24**	-

3.2. Estimation of Genetic Similarity

High similarity values, recorded between pair of individuals of *Lupinus pilosus*, were (17 and 19), (34 and 35), (31 and 32), (25 and 27) and (1 and 2), 0.43, 0.45, 0.43, 0.39 and 0.36, respectively; while the lowest similarity value (0.02) was recorded between individuals (3 and 35; 12 and 4; and 4 and 10) (Figure 1 and Table 2).

A dendrogram was arbitrarily divided into three clusters. The first cluster was divided into two sub clusters; the first including 17 single individuals, the second cluster had 20 individuals (Figure 1). The second cluster included only one individual (number 21). The final cluster contained two individuals (number 39 a nd 40)

Table 2. Similarity matrix values among and within *Lupinus*



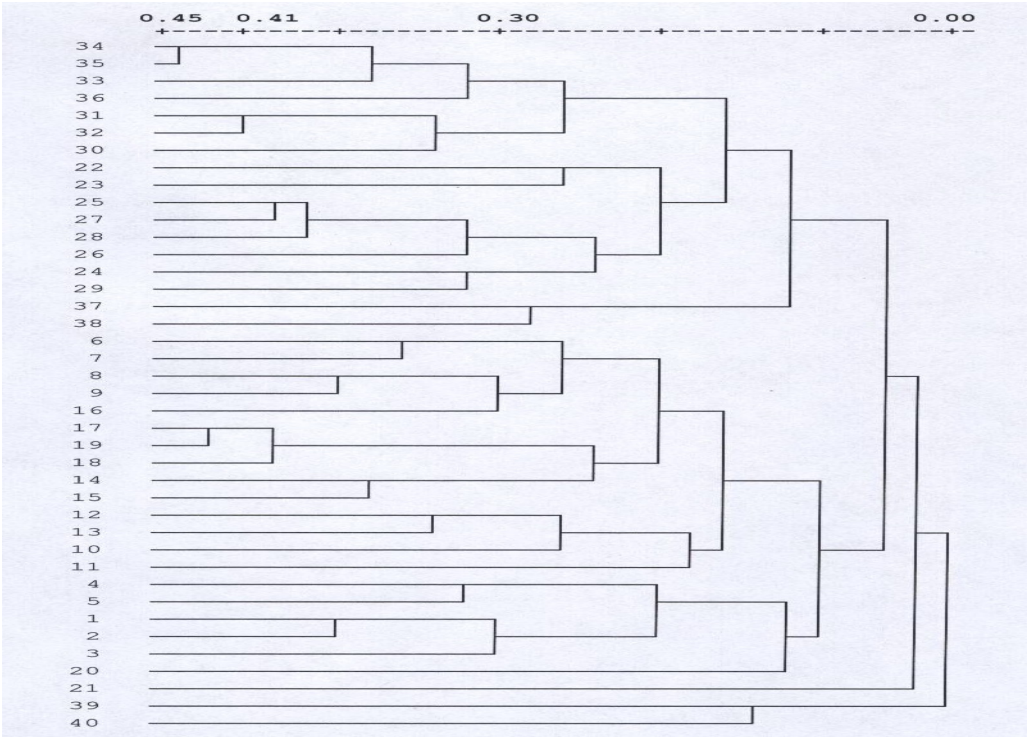


Figure 1: A dendrogram within *Lupinus pilosus* L. species using nine polymorphic RAPD primers based on Jaccards´ coefficient of similarit

4. Discussion

Knowing the genetic diversity between and within the populations of *Lupinus* species is a p rerequisite for the management, the monitoring and the conservation of genetic resources. The genetic diversity within and among the tested individuals of *Lupinus pilosus* was present under the present study. The forming separate cluster with one single individual confirmed that each individual had a unique DNA sequence, which could be due to the differences in the fatty acid compositions, protein content as well other gene expressions. Borek *et al*. (2009) reported that the main fatty acid in yellow lupine cotyledons was linoleic acid; in white lupine it was oleic acid, and, in Andean lupine, it was both linoleic and oleic acids. On the other hand, a low percentage of similarity could be due to a high, long diverging process concerning non-coding regions. Talhinhas *et al*. (2003) reported that the low genetic similarity among *Lupinus* spp. is most unlikely to be due to the differences in coding sequences. In this investigation, a high level of polymorphisms was found within single tested individuals of *Lupinus pilosus* L. This is due to its ploidy level. *Lupinus pilosus*, as the Old World (Africa) rough-seeded lupines, has chromosome number 2n=42 as described by Naganowska *et al*. (2003). A high level of genome diversity was found among the lupine accessions, but several others, from the Middle East and West Africa, tended to cluster together; the results support the future use of DAF markers for the characterization and identification of white lupine germplasm (Qiu *et al*., 1995). Sienkiewicz *et al*. (2008) indicated that the American species were characterized by the widest diversity with respect to ecological distribution and adaptive abilities. Pezhmanmehr *et al*. (2009) stated that a high level of polymorphisms (86%) was obtained by RAPD analysis, indicating the effectiveness of this marker for the evaluation of genetic diversity in Black cumin. Knowledge of wide genetic diversity observed in the *Lupinus pilosus* L. populations, using molecular markers, provides important information for the management of germplasm resources with regard to future domestication and breeding programs, in addition to selecting markers linked to a gene conferring disease resistance within *Lupinus* genus. Further molecular markers, such as ISSR, AFLP and SSRs, are needed to test the genetic relatedness between other *Lupinus* species (native or exotic) and compared to *Lupinus pilosus* in the future. Also, the individuals that showed diverse traits can be selected by breeders to use in the breeding programs and they can be invested in establishing propagate working for *Lupinus* species in Jordan.

References

Al-Rawashdeh IM. 2011. Molecular taxonomy among *Mentha spicata*, *Mentha longifolia* and *Ziziphora tenuior* populations using RAPD technique. *Jordan J Biol Sci*, **4 (2)**: 63-70

Aïnouche A-K and Bayer RJ. 1999. Phylogenetic relationships in *Lupinus* (Fabaceae: papilionoideae) based on internal transcribed spacer sequences (ITS) of nuclear ribosomal DNA. *Amer J Bot,* **86(4)**: 590-607

Borek S, Pukacka S, Michalski K and Ratajczak L. 2009. Lipid and protein accumulation in developing seeds of three lupine species: *Lupinus luteus* L., *Lupinus albus* L., and *Lupinus mutabilis* Sweet. *J Exp Bot,* **60 (12)**: 3453–3466

Camillo MF, Pozzobon MT and Schifino-Wittmann MT. 2006.Chromosomes numbers in South American Andean species of *Lupinus* (Leguminosae). *Bonpl,* **15(3-4):**113-119.

Doyle JJ and Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phyto Bull,* **19**: 11–15

Drummond CS, Eastwood RJ, Miotto STS and Hughes CE. 2012. Multiple continental radiation and correlates of diversification in lupines (Leguminosae): testing for key innovations with incomplete sampling. *Syst Biol,* **61(3)**: 443-460

Jaccard P. 1908. Nouvelles reserches sur la distribution florale. *Bull Soc Vaud Des Sci Natu,***44**: 223-270

Naganowska B, Wolko B, Sliwińska E and Kaczmarek Z. 2003. Nuclear DNA content variation and species relationships n the genus *Lupinus* (Fabaceae). *Annu Bot,* **92**:349-355

Pezhmanmehr M, Hassani ME, Jahansooz F, Najafi AA, Sefidkon F, Mardi M and Pirseiedi M. 2009. Assessment of genetic diversity in some Iranian populations of *Bunium persicum* using RAPD and AFLP markers. *Iran J Biot,***7 (2)**: 93-100

Qiu J, van Santen E and Tuzun S. 1995. Optimization of DNA amplification fingerprinting techniques to study the genetic relationships of white lupin germplasm. *Pla Breed,* **114(6)**: 525-529

Raman R, Cowley RB, Raman H and Luckett DJ. 2014. Analyses using SSR and DArT molecular markers reveal that Ethiopian accessions of white lupin (*Lupinus albus* L.) represent a unique gene pool. *Ope J Genes,* **4**: 87-98

Sbabou L, Brhada F, Alami IT and Maltouf AF. 2010. Genetic diversity of Moroccan *Lupinus* germplasm investigated using ISSR and AFLP. *Inter J Agri Biol,* **12(1)**: 26-32

Sienkiewicz SE, Galek R, Clements JC and Wilson J. 2008. Difficulties with interspecific hybridization in the genus *Lupinus. In* : J. A. Palta and J.B. Berger(eds). 2008. ***Lupinus* for Health and Wealth,** proceedings of the 12th International Lupin Conference, 12-14 Sept.2008, Fremantle, Western Australia. International Lupin Association, Canterbury, New Zealand. ISBN0-8676-153-8.

SPSS, 2002. **Advanced Statistical Analysis using SPSS** (v.11.0). Chicago, Illinois.

Talhinhas P, Neves-Martins J and Leitao J. 2003. AFLP, ISSR and RAPD markers reveal high levels of genetic diversity among *Lupinus* spp. *Plan Breed,* **122**: 507–510

Williams J K, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl Acid Res,* **18**: 6531-6535

Wink M, Merino F and Këss E. 1999. Molecular evolution of *Lupinus* (Leguminosae: *Lupinus*). *In*: E. van Santeen, M.Wink, S. Weissrnann and P. Roemer (eds). **Lupin, an Ancient Crop for New Millennium**. Proceedings of the 9th International Lupin Conference Klink/Miritz. 20-24 June. 1999. international Lupin association, Caterbury, NewZealand.ISBN0-86476-126-6

Wolko B, Clements JC, Naganowska B, Nelson MN and Yang H. 2011. *Lupinus*. World Crop Relatives: *Geno and Breed- Reso,* pp. 153-206

Yorgancilar M, Babaoglu M, Hakki EE and Atalay E. 2009. Determination of the relationship among Old World Lupin (*Lupinus* sp.) species using RAPD and ISSR markers. *Afri J Biot,* **8 (15)**: 3524-3530

JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 65 - 70

Jordan Journal of Biological Sciences

A Comparison between the Anti-microbial Activity of Native Propolis and the Anti-microbial Activity of Imported Ones against Different Health Microbes

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Received: December 4, 2014 Revised: January 11, 2015 Accepted: January 21, 2015

Abstract

Propolis is a gum-like product gathered by bees from various plants. It is known that the propolis has anti-bacterial, anti-carcinogenic, and immune-stimulating biological activities. In the present study, we have investigated the anti-microbial activity of propolis against certain important human microbes. The paper disc diffusion method was used to investigate the propolis activity and the inhibition zones were measured. Results revealed that the ethanolic and water extracts of propolis have a strong inhibitory potential against *Aspergillus brasiliensis* Varga and *Escherichia coli* Migula strain (ATCC 0157:H7) regardless of the time of propolis harvesting. The propolis ethanolic and water extracts were ineffective against *Escherichia coli* strain (ATCC 29522) and *Proteus mirabilis* Hauser. Jordan propolis was the most effective in inhibiting the *Enterobacter aerogenes* 35029 Hormaeche and Edwards than the Chinese, Turkish and Tablet propolis samples. Furthermore, the Jordan propolis and the Chinese crude 2 propolis were the most effective against the *Candida albicans* mold. The Chinese propolis was the most effective against *A. brasiliensis* mold.

Key words: Anti-bacterial activity, Anti-fungal activity, Human microbes, Propolis extract

1. Introduction

Propolis is a sticky, rubbery, brown, thermoplastic resin collected by bees from buds of trees. Honey bees use propolis in their hives as a repairing crevice, and as a surface cover, hardener and preservative. Also, it is probably used as a repellent since it is applied inside the beehive and around its entrance (Burdock, 1998).

There are a number of studies documenting the bio-cidal functions of propolis, its extracts and constituents (Marcucci *et al*., 2008; Mello *et al*., 2010). Several biological activities have been described for propolis, including anti-bacterial, anti-fungal, anti-protozoal, anti-viral, anti-tumor, immune-modulation and anti-inflammatory activities, beside other activities (Gomes, 2007). Fernandes *et al.* (1995) demonstrated the antimicrobial activity of propolis against bacterial and yeast pathogens isolated from human infection. Park *et al.* (1998) reported that the growth of the *Streptococcus*, an oral pathogen, was inhibited by the ethanol extract of propolis from various regions in Brazil. Moreover, it was reported that propolis is active against Gram-positive bacteria, yet it showed a limited activity against Gram-negative bacteria (Li-Chang *et al*., 2005). The anti-microbial activity of propolis is reflected in its constituents that may differ from area to area and from season to season depending on its chemical composition (Hegazi *et al*., 2000; Lu *et al*., 2003). Propolis has bactericidal and fungicidal properties, and it is used as an alternative treatment for infections. The wide range of action of propolis on various microorganisms is the result of the combined activities of flavonoids and aromatic compounds (Hemändez and Bemal, 1990; Sforcin *et al.*, 2000; Ivanĉajiĉ *et al*., 2010). On the other hand, Li-Chang (2005) reported that the mechanism of anti-microbial activity is complicated and could be attributed to synergism between flavonoids hydroxyl acids and sesquiterpenes. Krol *et al.* (1993) also observed this effect.

In Jordan, although a widely distributed propolis flora is present, that are of common use in the folk medicine,

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and high number of honeybee colonies, there are no further studies on the type of the propolis and/or the chemical composition of each, and their anti-microbial activities. Based on these observations, the aim of this study is to investigate the anti-microbial activity of the ethanolic and the water extracts of the propolis from Jordan in comparison to the other sources of propolis produces widely against different species and strains of bacteria and fungi including: *Aspergillus brasiliensis, Candida albicans* strain (ATCC 18814), *Enterobacter aerogenes* 35029, *Escherichia coli* strain (ATCC 25922), *Escherichia coli* strain (ATCC 0157:H7), *Klebsiella oxytoca* 18182, *Klebsiella pneumonia* 13883, *Methicillin* resistant *Staphylococcus aureus* 29974, *Proteus mirabilis, Proteus vulgaris* 13315, *Pseudomonas aeruginosa* 27253, *Salmonella typhimurium* 19430, and *Staphylococcus aureus* 25923.

2. Material and methods

2.1. Propolis Extracts

Propolis was collected from the bee hives monthly for one year. The collection time of the local propolis was done in the periods from (February to April), (May to July), and (August to October) of 2012 to make sure that all propolis types were obtained during the whole year. The main plant sources of propolis during these periods were oak (in Badr, Naour and Salt areas), pine (in Badr, Naour and Salt areas) and almond trees (in Naour, North Ghour and Wadi Shuaib areas) where bee hives were located. The Jordan propolis was compared with other four non-Jordanian types. The imported Chinese propolis includes: the crude 1 propolis which is found in the local market in a liquid phase, and the crude 2 propolis which is found in a powder form. A tablet propolis form and a Turkish propolis (crude form) were also used in order to confirm the effect of area and environmental conditions on the propolis physiological activities.

Water extract of propolis (from different sources) was obtained as described by Suzuki (1990) with a slight modification according to Osman and Taha (2008), where 20.0g of propolis was suspended and extracted with 5 volumes of distilled water with shaking using shaker (GFL3005, Gesellschaft fur, D-30983, Germany) at 300 rpm. Then the mixture was boiled at 70°C for 7 hours and left at room temperature to cool down. The extract was centrifuged at 3000g for 15 minutes (Beckman Allegra 21R Refrigerated Bench Top Centrifuge, UK LABS Direct Ltd.), and the supernatant was taken. The obtained supernatant was then concentrated using a concentrator (Rotational vacuum concentrator for laboratory RVC 2-18 CD, CHARIST) at 45°C until the concentration reached 10 mg/100 microliter. Later, the extracts were collected and stored in tightly closed dark bottles at 4°C until being used in the assay. The ethanolic extract of propolis (from the different sources) was obtained as described by Valdes Gonzales *et al.* (1985), where 300g of finally grounded and dried propolis were placed in 700 ml of concentrated ethanol in a container to obtain a 30% of extract. The mixture was shaken for two weeks at room temperature in a dark place using a shaker at 450 rpm. The extract was filtered through a coffee filter and then filtered again with a filter paper (Whatman No. 1). The filtrate was concentrated by putting on glass Petri dishes and left at room temperature until all the ethanol is evaporated. The extract is then re-dissolved with ethanol and stored in tightly closed, dark bottles at 4°C until being used in the assay.

2.2. Test Organisms

Standardized pure cultures of fungi and bacterial species and strains, procured (Microbiologics Inc., Minnesota-USA) by the Department of Biotechnology, Al-Balqa Applied University, were used in the present study. The microbes were chosen according to the frequency in which they were used in previous studies and also according to the frequency of infections in human beings. Pure cultures of tested microorganisms were cultured in nutrient broth (Acumedia Manufacturers, Inc., Maryland) and stored at 4°C until being used. Each test microorganism was re-cultured in this manner three days in succession before the experiment.

2.3. Determination of Anti-microbial Activity

Stock cultures of bacteria were grown in nutrient broth at 26–27°C for 24 hours with shaking and enumerated using a serial dilution method. One ml of each of the microbes' cultures was separately poured in 9 ml of sterile distilled water, and hereafter 8-fold serial dilutions were made (Kango, 2010). Final cell concentrations were 10^7–10^8 cfu/ml. Disk diffusion assay was performed according to the protocol recommended by NCCLS (National Committee for Clinical Laboratory Standards, 2000) to detect the anti-microbial activity of the propolis extracts. Sterilized filter paper discs (5 mm in diameter) soaked with 15 ul of propolis extracts in absolute ethanol (Merck–Darmstadt, Germany) were put in the middle of Mueller-Hinton Agar plates inoculated with suspensions of cells adjusted to McFarland turbidity standards equals to 0.5 using Mueller-Hinton Broth. The absolute ethanol was used as a negative control and the penicillin (10 µg) as a positive control. The plates were incubated at 37°C and observed after 24 hours. A digital caliper was used to measure the diameters of the zones of inhibition.

2.4. Statistical Analysis

The mean values and the standard deviation were calculated from the data obtained from triplicate trials. Means were then compared using the least significant difference test (SAS, 2001). A probability level of 5% was considered statistically significant.

3. Results

Results revealed that both the ethanolic and the water extracts of the local propolis, harvested during different periods of the year, resulted in variable inhibitory effects on the tested microbes. The propolis ethanolic extracts were anti- *E. coli* strain (ATCC 0157:H7) and anti- *A. brasiliensis* regardless of the time of the propolis harvesting time ($F_{11,24}$= 33.8, P<0.0001). By the passage of time, however, the ethanolic extract activity extended to include *K. pneumonia* 13883 ($F_{11,24}$= 246.7, P<0.0001) (Table 1). The propolis water extracts were anti- *E. aerogenes,* anti- *E. coli* strain (ATCC 0157:H7), anti-

Methicillin resistant *S. aureus* 29974 and anti- *A. brasiliensis* ($F_{11,24}$= 192.3, P<0.0001). However, for the water extracts, their inhibition activities extended to include *P. vulgare* and *S. typhimurium* with the passage of time ($F_{11,24}$= 210.1, P<0.0001) (Table 1). *P. mirabilis, E. coli* strain (ATCC 29522) and *P. vulgare* were the most resistant microbes to the ethanolic extracts regardless of the time of the propolis harvesting time. *P. mirabilis, E. coli* strain (ATCC 29522) and *E. aerogenes* were the most resistant microbes to the water extracts (Table 1). Regarding the effect of propolis against microbes in respect to the time of propolis collection, the August-October propolis water extract was the most effective one against most of the microbes tested ($F_{11,24}$= 115.6, P<0.0001) followed by the August-October propolis ethanolic extract ($F_{11,24}$= 208.6, P<0.0001). The May-July propolis water extract was ranked the third in inhibiting the microbes ($F_{11,24}$= 115.6, P<0.0001) (Table 1). The standard antibiotic, penicillin, was superior over all the propolis extracts against 6 out of 12 microbes, these are *E. aerogenes, E. coli* strain (ATCC 29522), *E. coli* strain (ATCC 0157:H7), *Methicillin* resistant *S. aureus* 29974, *P. mirabilis, P. vulgare* and *S. aureus* 25923 (Table 1).

The impact of the Jordan propolis and the imported propolis on inhibiting different bacterial microbes is shown in (Table 2). The Jordan and Turkish propolis were effective against all microbes. The propolis crude 1 from China and the tablet propolis were effective against all tested microbes except against *P. mirabilis* (Table 2). Furthermore, the Chinese crude 2 propolis was effective only against two of the tested microbes; these are *E. coli* strain (ATCC 29522) and *E. coli* strain (ATCC 0157:H7) (Table 2). A comparison of the Jordan propolis with the others was done against each microbe.

Table 1. Effect of different local propolis ethanolic and water extracts against different microbes

	Propolis extract[2]	Local Feb -April ethanolic ext	Local May-July ethanolic ext	Local Aug – Oct. ethanolic ext	Local Feb. - April water ext	Local May - July water ext	Local Aug. – Oct. water ext	Penicillin
Inhibition Zone (mm) ± Standard Error	*Aspergillus brasiliensis*	30.0 ± 0.00 a A	30.0 ± 0.00 a A	30.0 ± 0.00 a A	30.0 ± 0.00 a A	30.0 ± 0.00 a A	30.0 ± 0.00 a A	-
	Enterobacter aerogenes 35029	14.3 ± 0.07 e A	11.0 ± 0.04 j B	13.0 ± 0.04 j A	10.8 ± 0.03 j B	12.5 ± 0.03 j A	13.5 ± 0.05 e A	5.0 ± 0.05 C
	Escherichia coli (ATCC 29522)	9.7 ± 0.03 e A	0.00 ± 0.00 k C	12.3 ± 0.03 j A	0.00 ± 0.00 j C	11.0 ± 0.07 j A	12.0 ± 0.07 e A	7.0 ± 0.04 B
	Escherichia coli (ATCC 0157:H7)	30.0 ± 0.00 a A	27.0 ± 0.07 b B	30.0 ± 0.00 a A	27.3 ± 0.06 b B	30.0 ± 0.00 a A	30.0 ± 0.00 a A	5.0 ± 0.04 C
	Klebsiella oxytoca 18182	17.0 ± 0.40 c A	20.0 ± 0.00 e A	24.3 ± 0.05 d A	15.5 ± 0.03 e A	25.0 ± 0.00 c A	12.3 ± 0.07 e A	0.0 B
	Klebsiella pneumonia 13883	15.0 ± 0.00 d C	27.0 ± 0.04 b A	26.8 ± 0.03 b A	17.8 ± 0.05 d B	25.5 ± 0.03 c A	25.3 ± 0.03 c A	0.0 D
	Methicillin resistant *Staphylococcus aureus* 29974	16.0 ± 0.00 c B	23.3 ± 0.12 d A	25.0 ± 0.00 c A	21.5 ± 0.10 c A	25.5 ± 0.03 b A	25.3 ± 0.03 b A	5.0 ± 0.03 C
	Proteus mirabilis	0.00 ± 0.00 f D	0.00 ± 0.00 k D	20.0 ± 0.0 e A	0.00 ± 0.0 j D	0.00 ±0.00 k D	13.3 ±0.03 e B	5.0 ± 0.04 C
	Proteus vulgaris 13315	12.0±0.00 e D	10.8 ±0.03 j D	13.3 ±0.05 f C	11.8 ± 0.03 f D	14.8 ± 0.03 e B	18.8 ± 0.0 d A	3.0 ± 0.03 E
	Pseudomonas aeruginosa 27253	25.0 ± 0.00 b A	20.5 ± 0.03 e B	21.0 ± 0.04 e B	20.5 ± 0.03 d B	23.8 ± 0.05 c A	23.5 ± 0.03 c A	0.0 C
	Salmonella typhimurium 19430	20.0 ± 0.00 c C	23.7 ± 0.06 c B	24.3 ± 0.05 d B	20.0 ± 0.00 d C	29.5 ± 0.05 a A	30.0 ± 0.00 a A	0.0 D
	Staphylococcus aureus 25923	19.3 ± 0.03 c A	14.8 ± 0.03 f A	19.0 ± 0.07 e A	18.8 ± 0.08 d A	20.5 ± 0.09 d A	17.0 ± 0.12 d A	10.0 ± 0.07 B

* Values with different small letters in the same row are significantly different (P < 0.05)

** Values with different capital letters in the same column are significantly different (P < 0.05) (effect of time of propolis collection)

Table 2. Effect of different local Jordan and imported (Chinese and Turkish) propolis ethanolic extracts against different microbes

	Propolis extract	Chinese Propolis (crude1)	Chinese Propolis (crude2)	Jordan Propolis	Tablet Propolis	Turkish Propolis	Penicillin
Inhibition Zone (mm) ± Standard Error	*Enterobacter aerogenes* 35029	20.0 ± 0.12 a A	21.7 ± 0.03 c B	26.3 ± 0.07 a A	15.7 ± 0.03 b C	10.3 ± 0.03 b D	5.0 ± 0.06 E
	Escherichia coli (ATCC 29522)	20.3 ± 0.09 a B	35.7 ± 0.19 a A	16.0 ± 0.06 b C	21.3 ± 0.09 a B	21.3 ± 0.09 a B	7.0 ± 0.05 D
	Escherichia coli (ATCC 0157:H7)	23.0± 0.12 a B	25.7± 0.09 b A	11.3 ± 0.09 b D	20.3 ± 0.03 a C	9.7 ± 0.03 b D	5.0 ± 0.04 E
	Klebsiella pneumonia 13883	14.3 ± 0.12 b D	20.3 ± 0.03 c A	13.7 ± 0.18 b B	14.7 ± 0.03 b B	9.0 ± 0.06 b C	0.0 E
	Methicillin resistant *Staphylococcus aureus* 29974	22.0 ± 0.06 a A	23.0 ± 0.06 c A	6.0 ± 0.06 b B	20.7 ± 0.03 a A	7.0 ± 0.06 b B	5.0 ± 0.05 C
	Proteus mirabilis	9.3 ± 0.00 c B	19.0 ± 0.00 c A	9.7 ± 0.07 b B	11.3 ± 0.07 c B	9.3 ± 0.03 b B	5.0 ± 0.04 C
	Proteus vulgaris 13315	19.3 ± 0.12 a A	20.3 ± 0.03 c A	10.3 ± 0.03 b B	18.7 ± 0.03 a A	6.3 ± 0.03 b C	3.0 ± 0.02 D
	Pseudomonas aeruginosa 27253	20.0 ± 0.07 a A	17.3 ± 0.07 c B	10.3 ± 0.00 b C	20.0 ± 0.07 a A	7.0 ± 0.07 b D	0.0 E
	Salmonella typhimurium 19430	21.3 ± 0.06 a A	17.7 ± 0.07 c B	7.7 ± 0.03 b C	17.3 ± 0.00 a B	8.7 ± 0.03 b C	0.0 D
	Staphylococcus aureus 25923	13.0 ± 0.12 b C	13.0 ± 0.06 d C	17.0 ± 0.03 b B	19.7 ± 0.07 a A	8.0 ± 0.05 b E	10.0±0.06 D

* Values with different capital letters in the same row are significantly different ($P < 0.05$)

** Values with different small letters in the same column are significantly different ($P < 0.05$)

Jordan propolis ranked the first as anti- *E. aerogenes* ($F_{4,10}$= 141.86, P<0.0001) and the second against *K. pneumonia* 13883 ($F_{4,10}$= 18.28, P<0.0001), Methicillin resistant *S. aureus* 29974 ($F_{4,10}$= 129.34, P<0.0001), *P. mirabilis* ($F_{4,10}$= 40.71, P<0.0001), *P. vulgare* ($F_{4,10}$= 129.34, P<0.0001) and *S. aureus* 25923 ($F_{4,10}$= 51.24, P<0.0001) (Table 2). The Turkish propolis was effective against only three of the microbes; it ranked the second against *E. coli* strain (ATCC 29522) ($F_{4,10}$= 45.43, P<0.0001), Methicillin resistant *S. aureus* 29974 and *P. mirabilis.* The Chinese crude 1 propolis was super anti-microbe against all microbes except for *K. pneumonia* 13883 and *S. aureus* 25923. The Chinese crude 2 propolis ranked the first or second against all microbes tested except for *S. aureus* 25923. The tablet propolis ranked the first as anti-Methicillin resistant *S. aureus* 29974, *P. vulgare, P. aeruginosa* 27253 ($F_{4,10}$= 69.07, P<0.0001) and *S. aureus* 25923. Penicillin was superior over the Jordan propolis and also superior over the other four types of propolis against all microbes except *K. pneumonia* 13883, *P. aeruginosa* 27253 and *S. typhimurium* (Table 2).

A comparison of the impact of the Jordan propolis and the imported propolis on inhibiting two different fungi species is shown in (Figure 1) based on the ethanolic extracts. The Chinese crude 1 propolis was the most effective against the *A. brasiliensis* and the Turkish propolis was the least effective (Figure 1). The Chinese crude 2 propolis was the most effective against the *C. albicans*, while the Turkish propolis was also the least effective. Moreover, the Jordan propolis ranked the second in its effectiveness against *C. albicans* among the tested propolis of different sources (Figure 1).

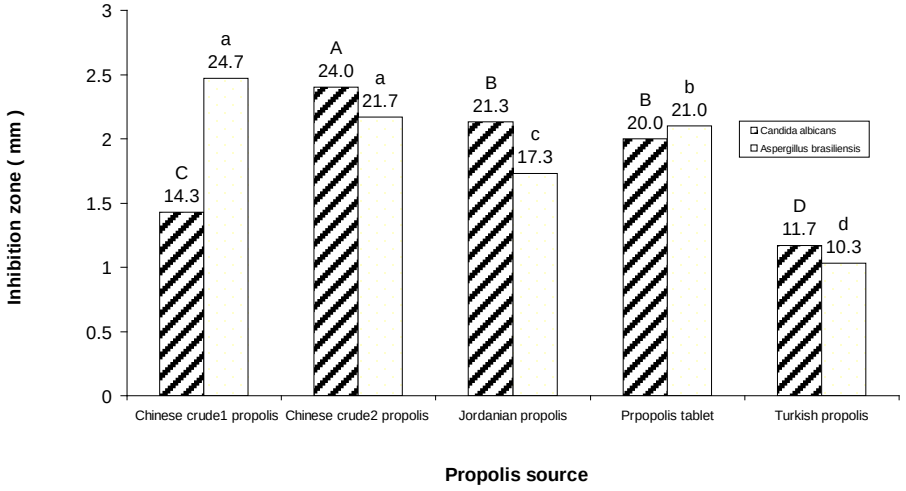

Figure 1. Effect of local Jordan ethanolic extracts and imported Chinese, Turkish and Tablet propolis against *Candida albicans* and *Aspergillus brasiliensis* molds. Means in columns for each fungus with same letter are not significantly different using LSD at 0.05.

4. Discussion

Over the last several years, a worldwide trend has been observed in the use of natural products like propolis due to its safe and its multidirectional biological properties (Topcuoglu *et al.*, 2012). It has been used commercially on the market as a component of toothpaste, mouth rinses, lozenges, and so forth. Both ethanolic and water extracts of the local propolis used in the present study demonstrated an anti-bacterial and anti-fungal activity against the tested human microbes. It inhibited the growth of ten out of the twelve microbes investigated; whereas *E. coli* strain (ATCC 29522) and *P. mirabilis* were not inhibited. Propolis composition is mentioned to be responsible for the anti-bacterial and anti-fungicidal biological properties (Marcucci, 1995). Chang *et al.* (2002) reported that the propolis samples collected in Taiwan contained various amounts of flavones, flavonols, flavanones, and isoflavones. Also, these constituents are generally regarded by Hemändez and Bemal (1990) and Sforcin *et al.* (2000) to be responsible for the anti-microbial activity of the propolis. Furthermore, Marcucci (1995) attributed the anti-bacterial effect of propolis mainly to the flavonoids.

The local propolis ethanolic extracts showed weak potential effects against the *S. aureus* 25923. This finding is in disagreement with the results of Ehsani *et al.* (2013) who found an anti-bacterial activity of the ethanolic extract against this microbe. On the other hand, our results confirmed the results of Ehsani *et al.* (2013) regarding the weak effect of the propolis aqueous extract against the *S. aureus* 25923. Moreover, Kashi *et al.* (2011) reported that the Iranian propolis ethanolic extract showed a bactericidal activity against *S. aureus* 25923. Lu *et al.* (2003) also observed that the ethanolic extracts of propolis samples from different regions in Taiwan also exerted various extents of anti-bacterial activities.

All the inhibited bacteria (*E. aerogenes, E. coli*, and *K. pneumonia* 13883) by the local Jordan propolis are gram negative bacteria. This type of propolis-bacteria relationship is demonstrated by Ozan *et al.* (2007). Furthermore, Ozan *et al.* (2007) found that these propolis solutions showed moderate effect on the fungus *Candida* strains. This finding is in full agreement with the results obtained in this study due to the Jordan propolis against the *C. albicans* in which the local propolis ranked the second when compared with the Chinese propolis, the Turkish propolis and the Tablet propolis. Same results were also obtained by Possamai *et al.* (2013) where the adsorbed Brazilian propolis to polyethylene glycol microspheres had a s timulatory effect on these cells to assist in combating the *C. albicans*. Moreover, Egyptian propolis ethanol extract in the concentration range 25 - 125 ng/µL were used to inhibit the adhesion of oral *Candida* and, therefore, preventing its colonization (Gomaa and Gaweesh, 2013). Moreover, the results of Khosravi *et al.* (2013) proved that the propolis inhibits the growth of pathogenic yeasts and confirmed the efficiency of propolis as an anti-*Candida* agent.

Jordan is mostly classified within the arid desert zone and the rainy season may extend from November to April at best (Al-Eisawi, 1996). The situation is largely different in China which is divided into tropical, subtropical and temperate zones. Most of China (www.warriortours.com/climate/) and Turkey (Weather Online, UK) lies in the warm temperate zone. Consequently, the vegetations and the biodiversity of China and Turkey flora are different from the Jordan flora. Hence, the chemical composition of the propolis from these countries is also different (Burdock, 1998). Therefore, their propolis efficacy is expected to be different. Results obtained here confirmed this assumption, while all types of propolis differed significantly in their inhibition ability to the investigated microbes (Table 2). The role of the geographic origin on the propolis anti-bacterial activity is supported by many researchers (Cheng and Wong, 1996; Kujumgiev *et al.*, 1999; Nieva Moreno *et al.*, 1999; Santos *et al.*, 1999). Identifying the active ingredient that could be responsible for the biological activities of the local propolis, revealed in the present study, is of vital importance.

In conclusion, the study showed a positive inhibitory influence of the local propolis ethanol and water extracts with respect to the *A. brasiliensis*, *E. coli* strain (ATCCO 157:H7), and *K. pneumonia* 13883. The Jordan propolis was superior to the Chinese propolis, the Turkish, and the Tablet propolis in its inhibitory effect against *E. aerogenes*. Moreover, Jordan propolis ranked second in inhibiting the *C. albicans* mold.

Acknowledgment

The authors would like to thank King Abdullah II Fund for Development for the financial support of this study and the beekeepers for their cooperation.

References

Al-Eisawi D. 1996. **Vegetation of Jordan**. UNESCO-Cairo Office, Regional Office for Science and Technology for the Arab States.

Burdock GA. 1998. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem Toxicol*., **36**: 347–363.

Chang CC, Yang MH, Wen HM and Chern JC. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal*., **10**: 178 – 182.

Cheng PC and Wong G. 1996. Honey bee propolis: prospects in medicine. *Bee World*, **77**: 8–15.

Ehsani M, Amin Marashi M, Zabihi E, Issazadeh M and Khafri S. 2013. A Comparison between antibacterial activity of propolis and aloe vera on *Enterococcus faecalis* (an *in vitro* study). *Int J Mol Cell Med*., **2 (3):** 110-116.

Fernandes A, Jr Sugizaki MF, Fogo ML, Funari SRC and Lopes CAM. 1995. In vitro activity of propolis against bacterial and yeast pathogens isolated from human infections. *J Venomous Anim Toxins*, **1**: 63–69.

Gomaa OM and Gaweesh AS. 2013. Variation in adhesion and germ tube formation of oral *Candida* using Egyptian propolis. *Can J Microbiol.*, **59 (3):** 197-203.

Gomes RT, Teixeira KIR, Cortés ME and Santos VR. 2007. Antimicrobial activity of a propolis adhesive formulation on different oral pathogens. *Braz J Oral Sci*., **6 (22):** 1387-1391.

Hemändez N M R and Bemal KC. 1990. Efecto antibio´tico del propo´leo frente a cepas de *Staphylococcus aureus* origen clinico humano. *Rev Cubana Farm*, **24**: 45-50.

Hegazi A G, Abd El-Hady FK and Abd Allah FAM. 2000. Chemical composition and antimicrobial activity of European propolis. *J Biosci*., **55**: 70-75.

Ivanĉajiĉ S, Mileusnic I and Ceniĉ-Milosevic D. 2010. In vitro antibacterial activity of propolis extracts on 12 different bacteria in conditions of 3 various PH values. *Arch Biol Sci,* Belgrade, **62 (4):** 915-934.

Kango N. 2010. **Textbook of Microbiology**. I. K. International Publishing House Pvt. Ltd., India.

Kashi TSJ, Kermanshahi RK, Erfan M, Dastjerdi EV, Rezaei Y and Tabatabaei FS. 2011. Evaluating the *in-vitro* antibacterial effect of Iranian propolis on oral microorganisms. *Iran J Pharm Res*., **10 (2):** 363-368.

Khosravi AR, Shokri H, Nikaein D, Mansouri P, Erfanmanesh A, Chalangari R and Katalin M. 2013. Yeasts as important agents of onychomycosis: *in vitro* activity of propolis against yeasts isolated from patients with nail infection. *J Altern Complement Med.,* **19 (1):** 57-62.

Krol W, Scheller S, Shani J, Pietsz G and Czuba Z. 1993. Synergistic effect of ethanolic extract of propolis and antibiotics on the growth of *Staphylococcus aureus*. *Drug Res*., **43 (5):** 607–609.

Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R and Popov S. 1999. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *J Ethnopharmacol*., **64**: 235–240.

Li-Chang L, Yue-Wen C and Cheng-Chun C. 2005. Antibacterial activity of propolis against *Staphylococcus aureus*. *Int J Food Microbiol*., **102**: 213– 220.

Lu LC, Chen YW and Chou CC. 2003. Antibacterial and DPPH free radical scavenging activities of the ethanol extract of propolis collected in Taiwan. *J Food Drug Anal*., **11**: 277-282.

Marcucci MC, Sawaya ACHF, Custodio AR, Paulino N and Eberlin MN. 2008. Scientific Evidence of the Use of Propolis in Ethnomedicine. In: Orsolic N and Basic I (Eds.). Transworld Res Network, Kerala, India, pp. 33-54.

Marcucci MC. 1995. Propolis: chemical composition, biological properties and therapeutic activity. *Apidologie*, **26**: 83–89.

Mello BCBS, Petrus JCC and Hubinger MD. 2010. Concentration of flavonoids and phenolic compounds in aqueous and ethanolic propolis extracts through nanofiltration. *J Food Eng*., **96**: 533-530.

NCCLS. 2000. National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Disk Susceptibility Tests-6th Ed. Approved Standard M2–A6. Wayne, PA

Nieva Moreno MI, Isla MI, Cudmani NG, Vattuone MA and Sampietro AR. 1999. Screening of antibacterial activity of Amaicha del Valle (Tucuman, Argentina) propolis. *J Ethnopharmacol*., **68 (1–3)**: 97–102.

Osman MF and Taha EA. 2008. Anti-oxidant activity of water extract of propolis from different regions in Kafr El-Sheikh Governorate. *Alexandria J Food Sci Technol*., **1**: 83–89.

Ozan F, Sümer Z, Polat ZA, Er K, Ozan U and Deer O. 2007. Effect of mouth rinse containing propolis on oral microorganisms and human gingival fibroblast. *Eur J Dentistry*, **11:** 195–200.

Park YK, Koo MH, Ikegaki M, Cury JA and Rosalen PL. 1998. Effects of propolis on *Streptococcus mutans*, *Actinomyces naeslundii* and *Staphylococcus aureus*. *Revista de Microbiol*., **29 (2):** 143–148.

Possamai MM, Honorio-Franca AC, Reinaque AP, Franca EL and Souto PC. 2013. Brazilian propolis: a natural product that improved the fungicidal activity by blood phagocytes. *Biomed Res Int.,* ID 541018, 9 pp.

Santos FA, Bastos EMAF, Uzedaˆ M, Carvalho MAR, Farias LM and Moreira ESA. 1999. Antibacterial activity of propolis produced in Brazil against *Actinobacillus actinomycetemcomitans*, *Fuscbacterium* spp. and bacteria from Bacteroides Fragilis group isolated from human and marmoset hosts. *Anaerobe*, **5**: 479-481.

SAS. 2001. A **Handbook of Statistical Analysis Using SAS**. 2nd edition, Chapman e Hall

Sforcin J, Jr Fernandes A, Lopes C, Bankova V and Funari S. 2000. Seasonal effect of Brazilian propolis antibacterial activity. *J Ethnopharmacol*., **73**: 243–249.

Suzuki I. 1990. In: Tanaka H, Yajima H, Fukuda H, Sezaki H, Koga K, Hirose M and Nakajima T (Eds.), **Pharmaceutical Research and Development**. Hirokawa Publishing Co., Tokyo, pp. 227 – 241.

Topcuoglu N, Ozan F, Ozyurt M and Kulekci G. 2012. In vitro antibacterial effects of glass-ionomer cement containing ethanolic extract of propolis on *Streptococcus mutans*. *Eur J Dentistry*, **6 (4):** 428–433.

www.warriortours.com/Chinaclimate/

www.weatheronline.co.uk/reports/climate/Turkey.htm

Valdes Gonzales G, Rojas Hernandez NM and Morales Vera C. 1985. Comparative study of the antimicrobial activity of propolis and that of antibiotics and conventional disinfectants. *Ciencia Y Tenica en la Agriculura, Apiculura*, **1**: 23-36.

JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 71 - 72

Jordan Journal of Biological Sciences

Case Report

Presumptive Secondary Ethylene Glycol (Antifreeze) Toxicity in a Turkey Vulture (A Case Report)

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Received: September 11, 2014 Revised: November 1, 2014 Accepted: December 7, 2014

Abstract

A presumptive diagnosis of secondary ethylene glycol toxicity in a female Turkey vulture (*Cathartes aura*) was made depending on the histopathological changes found in the renal tubules and presence of *Isospora spp*. of non-avian type within the colon. To the best of our knowledge, this is the first reported case of possible secondary ethylene glycol toxicity in a Turkey vulture in Louisiana State/USA.

Keywords*:* Ethylene glycol, oxalate nephrosis, kidneys, vulture

1. Introduction

Ethylene glycol is a sweet, clear and colorless fluid which is mostly found in antifreeze and hydraulic brake fluids. In addition, it is used as a solvent. However, ethylene glycol itself is relatively non-toxic; its metabolites are highly toxic causing renal failure secondary to calcium oxalate precipitate within the renal tubules and metabolic acidosis due to glycolic acid circulation (Brent, 2009).

2. Case Report

A 1.8 kg, adult female Turkey vulture, found neurologic at the front lawn of the Veterinary School, Louisiana State University, died shortly thereafter.

Upon necropsy on the next day, the vulture was in a good nutritional body condition and exhibited mild postmortem autolysis. No significant gross abnormalities were seen. Blood collected from the heart and brain were sent for bacterial culture and the colon was sent for parasitological examination. Representative tissue samples from lungs, heart, liver, kidneys, brain, skeletal muscles and intestines were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin, sectioned at 4µm, and stained with hematoxylin-eosin; and examined histopathologically.

3. Results

No significant histopathological findings were present throughout the examined tissues other than the kidneys. Both the cortical and medullary renal tubules were mildly to moderately dilated with degenerative, necrotic or desquamated epithelial linings and some renal tubules contained intraluminal crystals (Figure 1). Under polarized lenses, the crystals were birefringent either filling the renal tubules or tending to form rosette like structures (Figure 2).The renal blood vessels were congested.

E. coli and *Staphylococcus aureus* were isolated from the brain and heart blood. *Isospora spp.* was identified in the colon. However, the identified *Isospora spp.* was not consistent with those found in birds but was consistent with those found in canine/ feline host.

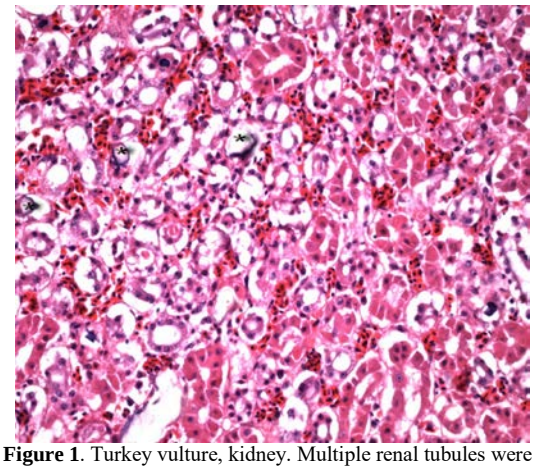

Figure 1. Turkey vulture, kidney. Multiple renal tubules were mildly to moderately dilated with degenerate, necrotic or desquamated epithelial linings with or without intraluminal crystals *. H&E. 400X

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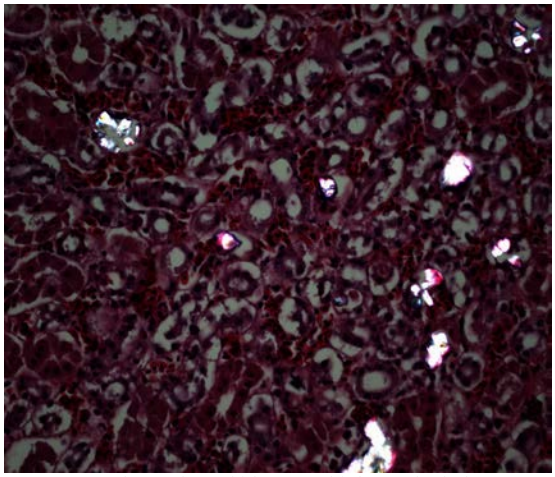

Figure 2. Turkey vulture, kidney. Multiple renal tubules exhibited birefringent shiny crystals under polarized lenses either filling the renal tubules or tending to form rosette like structures. H&E. 400x

4. Discussion

The findings mentioned above were similar to previously reported cases of ethylene glycol toxicity in birds **(**Stowe *et al.*, 1981; Hutchison and Dykeman, 1997**).** Not only had domestic birds and animals been intoxicated with ethylene glycol but wildlife birds and animals had been intoxicated too **(**Amstrup *et al.*, 1989**;** Murnane *et al.*, 1995; Foley and McBurney, 2002**).** Toxicity came mainly either through an accidental ingestion of antifreeze in animals or through an intentional ingestion of antifreeze, a method of suicide used in human beings **(**Hoffmann *et al.*, 2008**).** Also, for wild animals and birds, deliberate poisoning would be possible.

The present case is the first reporting presumptive secondary oxalate nephrosis in a Turkey vulture. The morphology and location of the birefringent crystals were consistent with oxalate nephrosis. The source of oxalate could not be determined, but toxicity has to be considered. Whether this bird drank water contaminated with antifreeze or possibly ate an animal that had been poisoned with antifreeze could not be determined. However, the latter possibility would be supported by the presence of *Isospora spp*. that were not consistent with an avian type. Furthermore, Campbell (2006) stated that whether wild animals are being exposed unintentionally, or whether wild animals may have, instead, been deliberately poisoned is questionable. The same author mentioned that The Canadian Peregrine Foundation recorded 19 different wild life cases that were poisoned by ethylene glycol. One of those records was a t urkey vulture. The same author considered that case as an unusual case of ethylene glycol poisoning. It is worth mentioning that the same foundation in 2002 recorded another case of ethylene glycol poisoning in a turkey vulture based on the presence of calcium oxalate crystals in renal tubules that were accompanied with damage to the tubules (The Canadian Peregrine Foundation). Those cases were limited to the Ontario region and they were only recorded in newsletters of the foundation. Moreover, those recorded cases were not described in detail in the literature.

The presence of *E. coli* and *Staphylococcus aureus* in the brain and heart blood culture is believed to be of no significant since no evidence of an infectious process was noted throughout the examined organs.

In summary, the present case report revealed that the Turkey vulture could be a v ictim of antifreeze toxicity either directly or indirectly. Furthermore, this case sheds light on the importance of proper antifreeze disposal.

5. Conflict of Interest Statement

The authors of this paper have neither financial nor personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper.

References

Amstrup SC, Gardner C, Myers KC, and Oehme FW. 1989. Ethylene glycol (antifreeze) poisoning in a free-ranging polar bear. *Vet Hum Toxicol.,* **31**: 317-319

Brent J. 2009. Fomepizole for Ethylene Glycol and Methanol Poisoning. *N Engl J Med.*, **360,** 2216-2223.

Foley P and McBurney S. 2002. Ethylene glycol toxicosis in a free-ranging raccoon (Procyonlotor) from Prince Edward Island. *Can Vet J.*,**43**: 291-292.

Hoffmann U, Abel P and Neurath H. 2008. Acute ethylene glycol poisoning after intentional ingestion. *Toxichem and Krimtech*., **75** (3), 130-133.

Hutchison T W and Dykeman J. C. 1997.Presumptive ethylene glycol poisoning in chickens. *Can Vet J.,* **38**: 647.

Murnane R D, Meerdink G, Rideout B and Anderson M. 1995. Ethylene glycol toxicosis in a captive – bred released California condor (gymnogyps californianus). *J Zoo Wildl Med.,* **26**: 306-310.

Stowe CM, Barnes DM and Arendt TD. 1981.Ethylene glycol intoxication in ducks. *Avian Dis.,* **25**: 538–541.

Campbell D. 2006.The Canadian Peregrine Foundation. Canadian Cooperative Wildlife Health Centre: Wildlife Health Centre Newsletter. Spring &Summer.Pp.11: Ontario Region.

The Canadian Peregrine Foundation. Canadian Cooperative Wildlife Health Centre: Wildlife Health Centre Newsletter. Winter 2001/2002.Pp. 14: Ontario Region.

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مجلة علمية عالمية محكمة

تصدر بدعم من صندوق دعم البحث العلمي

ISSN 1995-6673

المجلة الأردنية للعلوم الحياتية
مجلة علمية عالمية محكمة

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