# المجلة الأردنية للعلوم الحياتية
# Jordan Journal of Biological Sciences (JJBS)
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# Jordan Journal of Biological Sciences

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## EDITORIAL PREFACE

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

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

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

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

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
Hashemite University
Zarqa, Jordan
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# Use of Anabolic Androgenic Steroids in Jordan: Mini- Review

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**Abstract**

Until recently, there has been a complete lack of prevalence data regarding anabolic androgenic steroid (AAS) abuse locally in Jordan and the Arab world at large. Encouraged by their trainers, many Jordanian athletes are abusing AAS. The sale of anabolic steroids in Jordan is still unregulated; selling AAS without a prescription is not banned. The Jordanian Anti-Doping Organization (JADO) was founded in 2007, and as a response to the increasing concern about steroid abuse among Jordanian youth JADO accepted the World Anti-Doping Code late 2009, committing Jordan to fighting all types of doping including AAS abuse. However, most Jordanians are not aware of the adverse side effects of AAS abuse. Promoting public awareness about the harmful consequences of AAS abuse, in addition to the role of the law enforcement authorities will hopefully curb the abuse of AAS in Jordan.

**Keywords: Doping, the Code, Bodybuilder.**

## 1. Introduction

Research about the use of anabolic androgenic steroids (AAS) in Jordan and in the Arab world at large is scarce. In addition to two prevalence studies (Al-Falasi *et al.*, 2008; Tahtamouni *et al.*, 2009), few reports from the Arab world came out after the year 2000 where only case reports of AAS abuse were discussed (Alaraj *et al.*, 2005; Samaha *et al.*, 2008), However, more attention has been drawn to this issue as a result to the growing concern of steroid abuse among Jordanian youth and athletes (Tahtamouni *et al.*, 2009).

As a political response to this concern, the Jordanian Anti-Doping Organization (JADO) was founded in 2007 (www.jado.jo). Late that year, government and national Olympic committee officials from Jordan and four other Arab countries met under the supervision of the West Asia Regional Anti-Doping Organization (West Asia RADO), which is a part of the World Anti-Doping Agency (WADA), and established the West Asia Anti-Doping Organization (WAADO). The members of WAADO (located in Amman, the capital of Jordan) have agreed to pull resources together to fight doping throughout the region (www.wada-ama.org).

JADO acts as an independent anti-doping organization for Jordan. It has the necessary authority and responsibility for implementing and advocating improvements in the doping control such as testing for AAS abuse among athletes, promoting anti-doping research and planning and monitoring information and education programs (www.jado.jo). Late 2009, JADO accepted the revised 2009 World Anti-Doping Code (Jordan Olympic Committee Anti-Doping Rules, 2009) (the "Code") (World Anti-Doping Code, 2012). Simultaneously, co-operation between various public authorities have led to new strategies which aim at reducing the supply of steroids and as such limiting their use within the general population.

### 1.1. The Anabolic Steroid Users

Despite signs of public concern and disapproval of anabolic steroid use, some athletes – competing as well as recreational – are using these drugs. In fact, competing bodybuilders, weightlifting athletes and students at Jordanian colleges have been identified as specific AAS-using groups (Tahtamouni *et al.,* 2009). Extractions from interviews with trainers/trainees in various gyms across this region - taken from newspapers - indicate that the motives behind the use of steroids are:

A salesman: "People want to build strength, lose fat and have the perfect bodies they see on TV" (Luck, 2009).

Iraqi gym owner: "So many people now want to work out. They want to look good" (Luck, 2009).

A-17-year old Jordanian weightlifting-trainee admitted using "supplements." He said: "I used to be 60 kilos and weak. Now, with the new products and a good coach, I'm going to be better than Rambo" (Luck, 2009).

A Kuwaiti male in his 30's said: "I wanted to bulk up, everyone at my gym had a lot of muscle, and I wanted to be like them. My friends started telling me about the courses [of steroids] they take, injections they use to get

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bigger. So I tried it, they showed me how" (Kolarov, 2009).

Competing bodybuilders and weightlifters that were surveyed by the author (Tahtamouni *et al.,* 2009) described how trainers were advocating "the wonderful properties" of anabolic steroids. As one trainer explained: "One month of hormonal injections and you will be a clone of Sylvester Stallone" and: "Injections work much quicker than pills in blowing the muscles" (Personal Communication). Despite the general disapproval of the use of performance enhancing drugs (AAS), still some seem quite willing to use them in order to obtain the desired body look they want to have.

## 2. The Public Health Concern

Some examples of the users' behavior when training and while using steroids indicate that there is a real cause for a public health concern. In a correspondence with an MD who is an expert in substance abuse and who is employed at a major hospital in Jordan, he explained how almost every week he was called upon for a consultation with a patient who was admitted to the hospital due to complications resulting from anabolic steroid overdosing. Even autopsies where the cause of death (apparently) was found to be excessive use of steroids are not uncommon (Personal Communication). However, exact numbers of such patients, numbers of autopsies conducted on steroids users and case reports demonstrating a direct relationship between use of steroids and cause of death are unavailable. The information provided in the correspondence should be viewed as an indication that some users are using steroids to an extent where they can be fatal or can cause serious health complications.

In general, very little information about the side effects associated with steroid use has been available in Jordan. It seems that many coaches and gym owners dealing anabolic steroids in gyms are in fact unaware of the potential harmful side effects of these drugs (Büttner and Thieme, 2010); and if they are, these effects are rarely discussed with the users (e.g. competing bodybuilders and weightlifters) (Tahtamouni *et al.*, 2009; Personal Communication). The quality of advice may therefore vary greatly. Some may even disregard the potential risks of using AAS, as expressed by one weightlifting trainer: "If I can bench press hundreds of pounds and have low fat, then in my opinion I'm healthy" (Luck, 2008). These available examples do point to the conclusion that something needs to be done.

Some abusers do not have the nerve to administer the injectable AAS for themselves, so they ask a friend to do it for them who in turn might be inexperienced (Personal Communication). Also, Jordan currently does not have a syringe exchange program (Syringe Exchange Programs Around the World, 2012), and therefore it is possible that the same syringe is used again and again, or is circulated among users, in which case, a high risk of infection will arise. It is worth mentioning that injection equipments, syringes and needles, are readily available over-the-counter in most Jordanian pharmacies; a pack of 100-1ml syringes cost less than 5$ (www.jomid.net).

## 3. The Drug Supply

The official sale of anabolic steroids in Jordan is still unregulated. Selling anabolic steroids at pharmacies - without a prescription - is not banned in Jordan and these drugs are so far unmentioned in any Jordanian law or any medicine regulation act (www.moh.gov.jo). Many pharmacists provide AAS as an over-the-counter drug (www.moh.gov.jo). Many AAS drugs are not registered in the Jordanian Pharmacist Association (www.jpa.org.jo). There are only three AAS that are prescribed in Jordan, namely Deca-durabolin, Andriol and Sustanon. Besides their use by athletes, these drugs are prescribed in Jordan as a treatment for reproductive dysfunction and breast cancer (Smith, 2009).

However, most of the steroids used in the gyms surveyed by the author (Tahtamouni *et al.*, 2009) are smuggled into the country, mainly from Pakistan, Egypt and India, which is against the customs law and regulations. The customs law regulates all types of smuggling into Jordan. Customs and police authorities prosecute violators and the Ministry of Health and the Food and Drug Administration handles any drugs being confiscated (www.customs.gov.jo). The drugs that are being smuggled into Jordan are supposedly of low quality (e.g. contaminated or less active compound than stated) (Evans-Brown and McVeigh, 2009). Still, to our knowledge no analysis of drugs confiscated at the borders has been published. Despite the supposedly low quality, these products are being used, presumably because they are sold at a cheaper price than steroids available from pharmacies.

### *3.1. The health policy answer*

In a rather conservative society, such as that of Jordan, drug use is negatively conceived of, especially when it comes to drugs such as cocaine and cannabis. With anabolic steroids, however, there seems to be public confusion and some people are unaware of the adverse side effects of these drugs (e.g. think anabolic steroids are vitamins) (based on field work by Tahtamouni, 2009-2011). It is a priority for health authorities to inform the public since "people are using all these products (steroids) without knowing the side effects and this is very dangerous" as Jordan's WHO representative explained (Luck, 2008). Can the formation of JADO increase the amount of available information about use of anabolic steroids?

With the establishment of JADO, competing athletes will be tested for use of performance enhancing drugs. However, in every gym which is licensed under the Higher Council of Youth – in all likelihood some non-competing athletes are found among the gym members – testing may be carried out. Does this mean that recreational athletes with no intention of competing may be subjected to testing regardless of whether or not they intend to compete as bodybuilders or weightlifters at competitions? Is this an unintended consequence of the testing system or does JADO deliberately seek to test recreational athletes?

Will doping tests have a deterrent effect on recreational athletes in these gyms?

There are gyms in Jordan which are not member of any kind of sports federation thus precluded from the testing system set up by JADO. The effects of this testing scheme remain to be seen and evaluated.

Alliances between public authorities - which could enforce and further implement new anti drug strategies – have been formed and institutions such as the Ministry of Health, Ministry of Education, Ministry of Higher Education, Higher Council of Youth, Department of Customs and Jordan Anti-Narcotics Police Department have been working together under the supervision and coordination of JADO. Between these co-operating alliances new legislation which will prohibit pharmacy sale of anabolic steroids without a prescription and introduce severe punishment for smuggling of steroids are currently in the process of being formulated. The upcoming law is still in working progress and sanctions for illegal sale or smuggling into the country remains undecided upon.

## 4. Conclusion

Possession of anabolic steroids without prescription in Jordan is currently legal, and new legislation attempts to limit supply rather than criminalize the individual user. However, so far use of anabolic steroids in Jordan has to a great extent been unregulated.

## Acknowledgements

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## Conflicts of interest

None declared.

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# Development of *Dermestes maculatus* (DeGeer, 1774) (Coleoptera, Dermestidae) on Different Fish Substrates

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**Abstract**

The development of *Dermestes maculatus* (DeGeer) on four smoked fish substrates [Trigger fish (*Balistes capriscus)*, Catfish (*Synodontis sp.*), African catfish (*Clarias gariepinus*) and Nile Tilapia (*Oreochromis niloticus*)] were investigated as completely randomized design under laboratory temperature of 30$^{o}$C, relative humidity of 65±5% and a light: darkness regimen of 12:12 hours. Females laid eggs within 24 hours of copulation. The numbers of eggs laid and the period between larval instars were not significantly different ($P > 0.05$). Mean total egg laying period varied from 18 days on *B. capriscus* to 30 days on *Synodontis sp.* with about 75% of eggs laid on days 13, 15, 15 and 17 for *O. niloticus*, *B. Capriscus*, *C. gariepinus*, and *Synodontis sp*., respectively. Hatching started 48 hours after copulation on all fish substrates. The mixed fish substrate (comprising all species) gave the longest ($P < 0.05$) developmental period of 42.75 days. Except *C. gariepinus* which recorded five larval instars, all others gave six instars. The total development period of *D. maculatus* from egg → larva → pre-pupa → pupa → adult emergence on the fish substrates decreased in the order *Synodontis* > *O. niloticus* > Mixed > *C. gariepinus* > *B. capriscus.*

***Keywords****: Dermestes Maculatus*, Developmental Period, Emergence Pattern, Fish Substrates, Copulation.

## 1. Introduction

Fish has remained an important source of food and income to many people in the developing world including Africa where as much as 25% of the population depend on it (Essuman, 1992). It is a very rich source of good quality protein in diets of man (Amusan and Okorie, 2001; Fasakin and Aberejo, 2002; Azam *et al*., 2004; Aderolu and Akpabio, 2009). Don-Pedro (1989) concurs that during storage, transportation and marketing, dried fish is readily attacked by several species of insects notably *D. maculatus, D. frischii, D. ater* and *Necrobia rufipes*. FAO (1990) reported that *Dermestes spp.* and *N. rufipes* were major pests of smoked fish, poultry products (Geden and Hogsette, 2001), museums (Linnie and Keatinge, 2000), Egyptian mummies (Adams, 1990) and stored cocoons of silk-worm *Bombyx mori* (Sahaf, 2007). Lale and Sastawa (1996) and Odeyemi *et al.* (2000) recorded about 50% losses during the storage of smoked fish products due to deterioration. The losses have been attributed to net reductions in the amount of nutrients available to the consumer (nutritive quality) resulting to declining consumer acceptability and market prices (economic losses) or both quantitative and qualitative losses (Odeyemi *et al*., 2000; Atijegbe, 2004). Thus, the experiment was designed to investigate the developmental processes of *D. maculatus* on substrates from four species of smoked fish with the aim of understanding the biology of the pest for effective and efficient management measures against losses caused by the pests in stored fish products.

## 2. Materials and Methods

The studies were carried out between October 2004 and May 2005 under controlled temperature (30ºC), relative humidity (65±5 %) and light-to-darkness regimen of 12:12 hours. Smoked fish from four species of fish – the Trigger fish (*Balistes capriscus* Gmelin*)*, Catfish (*Synodontis* sp.), African catfish (*Clarias gariepinus* Burchel) and Nile tilapia (*Oreochromis niloticus* Linnaeus) – were purchased from Madina and Makola local Markets in Accra, Ghana and used for the experiment. Treatments were arranged as completely randomized design (CRD), replicated four times and kept on open air shelves. The life cycle of the pest was determined on each food medium and appropriate records taken as outlined below.

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*2.1. Rearing of D. Maculatus*

Several unsexed adults of *D. maculatus* obtained from naturally infested smoked fish materials served as sources of the pests. The glass jars and cured fish species- *B. capriscus* (Trigger fish), *Synodontis sp.* (Catfish), *C. gariepinus* (African catfish) and *O. niloticus* (Nile Tilapia) used for the experiment were heat-sterilized at 60ºC for one hour in a hot-air Gallenkamp oven in the laboratory to kill all insect pests that may be present. The experimental bottles had their lids opened and sealed with 4 cm diameter mesh to facilitate aeration of the culture and placed on inverted Petri dishes submerged in white oil on shallow trays to keep out mites and other insect pests. Adult *D. maculatus* were then transferred into sterilized jars containing the disinfested smoked fish from *B. capriscus*, *Synodontis sp.*, *C. gariepinus* and *O. niloticus* species to initiate new colonies of the parent stock for rearing the pests. The insect pests were fed on the four different fish substrates.

About 1000 g of the sterilized fish substrates were then conditioned for 2 days under ambient laboratory conditions with about 600 g of each substrate later poured into a series of 1-litre jars. About 20 unsexed adults of *D. maculatus* were introduced into each jar and their offspring allowed developing up to the pupa stage. The adults were sieved out after fourteen days of oviposition to ensure that offspring of relatively same age were obtained as pure $F_1$ *D. maculatus*. The pupae were then transferred from each of the substrates into separate sub-culture bottles containing each pure fish substrates. The rearing cultures were left undisturbed over a long period of time but pupae were isolated from each substrate at 7-day intervals and introduced into separate test tubes prior to adult emergence to ensure that adults were 47 days old before sexing them and kept unmated until required. The males are distinguished from the females by their possession of a deep depression and brush of hairs on the 4th abdominal sternite (Imai *et al*., 1990). On emergence, the adults were placed in test tubes containing similar fish substrates to the ones on which they were bred and maintained under same conditions.

*2.2. Egg Laying Bioassay*

The experimental bottles were sterilized in a Gallenkamp oven, as described above, to obtain the number of egg(s) laid per female per day. Each fish species was then carefully dissected using entomological scissors and compacted with rubber band as in whole fish substrates. A male and a female adult *D. maculatus* (each 47-day old) were then introduced into separate tubes containing each fish species to serve both as food and oviposition medium and incubated for 30 days. Water was provided as soaked cotton wool and insects were allowed to drink for five minutes while egg count was done. The jars were monitored twice daily for the presence of eggs using hand lens and any egg seen was counted and removed using soft brush after which the medium was returned to its original position.

*2.3. Larval Development*

Larval instars were examined after collecting and placing eggs laid on each fish substrate from each of the experimental jars into glass tubes for incubation. On hatching, the larvae were separated into individual tubes of 2.2 cm × 15 cm dimensions containing 10 g of each fish species and kept under observation for their development. Duration of each larval instar was determined on each substrate by the presence of exuviate after each moult. All larvae were derived from eggs laid by individuals maintained on smoked fish substrates used in determining the number of eggs laid.

*2.4. Pupal Period*

The pre-pupal stage occurs when the last instar larva becomes almost C-shaped, shortened and remains non-motile for some days, while the pupal stage is what follows immediately. Pupae were removed and placed individually in clear tubes and held under laboratory conditions until adult emergence. The sexes, length and pupal periods were recorded for each emerged adult.

Data collected on eggs laid, developmental pattern, larval instars, prepupal and pupal durations were transformed using square roots of $\sqrt{(x+1)}$ and analysed using Genstat software version 5 Release 3.2 (Lawes Agricultural Trust, 1995) and subjected to analysis of variance at 95% level of significance and significant means were separated using LSD at 0.05 error limit.

## 3. Results

*3.1. Pattern of oviposition by D. maculatus on four different fish substrates*

Eggs laid on each fish species were random but gradually increased in number during the first week and subsequently declined with time. About 75% of eggs were laid on *Synodontis* by day 17 while the same level was reached on *C. gariepinus* and *B. capriscus* by day 15 and on *O. niloticus* by day 13 (Figure 1). Maximum egg laying periods were recorded on *Balistes sp.* and *Synodontis sp.* in 30 days while the minimum egg laying period recorded on *O. niloticus* was 18 days. The maximum recorded number of eggs laid in a batch per day was 28 on *O. niloticus* and a minimum of a single egg per day was recorded on all the fish substrates.

*3.2. Total Eggs Laid By D. Maculatus Within 30 Days on Different Fish Substrates*

The total number of eggs laid on the different fish substrates over 30 days was not statistically significant ($P > 0.05$, F Prb. = 0.275). The highest mean number of eggs was, however, laid on *O. niloticus* (151±33.67) and the lowest were on *B. capriscus* (103±43.5) with *Synodontis sp.* (132±31.11) and *C. gariepinus* (117±14.39) as intermediates (Table 1).

**Table 1.** Total number of egg laid on different fish substrates within 30 days

| Fish substrates | Total eggs laid ±SE* | Range (eggs/day) |
|---|---|---|
| *O. niloticus* | 151±33.67 | 1-28 |
| *Synodontis* sp | 132±31.11 | 1-20 |
| *C. gariepinus* | 117±14.39 | 1-19 |
| *B. capriscus* | 103±43.51 | 1-18 |

### *3.3. Larval Instars of D. Maculatus on Different Fish Substrates*

There were 6 larval instars of *D. maculatus* on all the fish substrates except on *C. gariepinus* substrates where only 5 instars were recorded (Table 2). The mean duration (in days) of the various larval instars on the different fish substrates was not statistically significant ($P > 0.05$, F Prb. = 0.287) without a clear trend in larval developmental periods on the substrates over 50 days. While larval development on *O. niloticus* had uniform periods except in the 6th larval instar, those on *Synodontis* and *B. capriscus* had the highest number of days recorded in the 2nd larval instar and least in the 6th instar. However, on *C. gariepinus* the highest period was recorded in the 5th instar. When placed on mixed substrates, there was generally a progressive increase in duration in each subsequent larval instar (Table 2). The mean duration of larval instars was highest in the 2nd instar (range: 6-12 days) followed by the 5th instar (range: 4-11 days), and the least duration was recorded on the 6th larval instar.

**Table 2.** Mean duration (days) of larval instars of *D. maculatus* on the different fish substrates

| Fish species | Duration (days) of larval instars ±S.E* | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Total |
| *O. niloticus* | 5.75±0.48 | 5.75±0.48 | 5.75±0.48 | 5.75±1.03 | 5.25±0.48 | 7±0.00 | 35.25 |
| *Synodontis* sp | 6.75±0.75 | 10±0.82 | 5.25±0.48 | 6.00±0.91 | 6.5±0.65 | 5±0.00 | 35.75 |
| *C. gariepinus* | 5.00±0.00 | 7.25±0.25 | 5.75±0.25 | 5.00±0.00 | 8.00±0.00 | - | 31.00 |
| *B. capriscus* | 5.75±0.48 | 9.00±0.41 | 5.75±0.48 | 6.00±0.71 | 5.25±0.48 | 5.5±0.35 | 37.25 |
| Mixed subs. | 5.00±0.00 | 6.50±0.29 | 7.25±0.48 | 6.50±0.65 | 9.00±1.35 | 8.5±0.35 | 42.75 |

*Values are means of four replicates ± SE. (Standard error)

### *3.4. Developmental Periods of Pre-Pupa and Pupal Stages of D. Maculatus*

Statistical analysis showed significant differences ($P < 0.05$, F Prb. = 0.083) in the mean period between pre-pupa and the emergence of external adults. *O. niloticus* recorded higher pre-pupal period while the least pre-pupal period was recorded on mixed substrates. The results further showed significant difference ($P < 0.05$, F Prb. = 0.083) in pupal period with *B. capriscus* recording the least from the other fish substrates (Table 3).

**Table 3.** Mean period of development from pre-pupa to adult emergence of *D. maculatus* on different fish substrates

| Fish species | Developmental period ± SE* | | | |
|---|---|---|---|---|
| | Pre-Pupa | Range | Pupa | Range |
| *O. niloticus* | $12.75^{a}$±1.65 | 9-16 | $9.00^{a}$±1.29 | 6-10 |
| *Synodontis sp* | $11.00^{ab}$±1.08 | 8-13 | $9.50^{a}$±0.96 | 8-12 |
| *C. gariepinus* | $8.75^{bc}$±1.11 | 6-9 | $7.50^{a}$±0.65 | 6-9 |
| *B. capriscus* | $8.00^{c}$±0.82 | 9-12 | $6.75^{b}$±1.38 | 5-10 |
| Mixed substrates | $7.25^{c}$±0.48 | 6-8 | $8.00^{a}$±1.08 | 6-11 |
| LSD 2.73 | | | | |

Means with the same superscripts in the same column are not significantly ($P > 0.05$) different.

However, the cumulative developmental periods of *D. maculatus* from pre-pupa to pupa on the various fish substrates did not show any significant differences ($P > 0.05$, F Prb. = 0.135) (Table 4). The total development period of *D. maculatus* from egg → larva → pre-pupa → pupa → adult emergence on the fish substrates decreased in the order *Synodontis* > *B. capriscus* > *O. niloticus* > *C. gariepinus*.

**Table 4**. Mean development period (days) of pre-pupa and pupa of D. maculatus on different fish substrates

| Developmental stages | Days± SE | Range |
|---|---|---|
| Pre-pupa | 9.55±1.14 | 7.25-12.75 |
| Pre-pupa | 8.15±0.56 | 6.75-9.50 |
| LSD | 1.74 | |
| Values are means of four replicates ±SE | | |

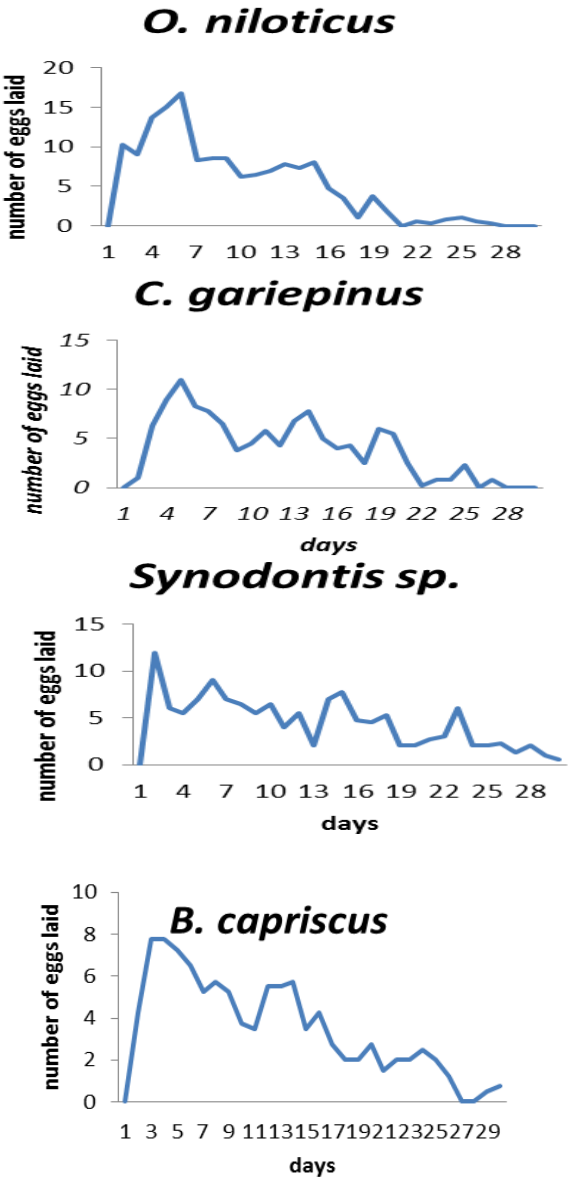


**Figure 1.** Oviposition pattern of *D. maculatus* on different fish substrates

## 4. Discussion

### *4.1. Oviposition in D. maculatus on fish substrates*

The study revealed that in *D. maculatus* copulation occurred immediately the adults were paired and this could be explained as reported that both male and female *D. maculatus* produce sex pheromone (Rakoswki and Cymborowski, 1986; Jaskulska *et al*., 1987) which enhances communication and within 48 hours creamy white eggs were laid. The eggs laid were oval in shape and bluntly pointed at both ends as earlier reported (Archer and Elgar, 1999; Jones and Elgar, 2004; Ezenwaji and Obayi, 2004). The results of this study confirm the works of various researchers that *D. maculatus* females copulate within 30 minutes of pairing with the males initiating copulation (Archer and Elgar, 1999; Jones and Elgar, 2004).

Egg laying in *D. maculatus* per female within 30 days on each of the different fish substrates was random and varied. The egg laying capacity of the pest tested on different fish substrates followed the descending order: *O. niloticus* > *Synodontis* > *C. gariepinus* > *B. capriscus*. *O. niloticus* was thus the most suitable medium for egg laying on the four substrates tested and expected to carry the heaviest infestation in the field. These differences may be attributed to the fact that oviposition in insects on a specific host is determined by various factors that may determine its suitability or otherwise as a breeding medium, such as nutritional quality, host abundance (Jansen and Nylin, 1997; Barros and Zucoloto, 1999), morphology, environmental conditions, age and size of individual (Stejskal and Kucerova, 1996; Johnson and Kistler, 1987) and competition (Siemens *et al.,* 1991). The study showed that none of the fish substrates deterred egg laying, though some of the media proved to be better than others as more suitable for oviposition by *D. maculatus*.

Generally there was an initial increase in total number of eggs laid during the first week of oviposition on all the fish substrates but subsequently there was reduction in numbers as the days progressed and insects got older (Ezenwaji and Obayi, 2004). Oviposition behaviour in insects is an important contributor to the fitness of insects because of the consequent effect on the number and quality of offspring (Honek, 1993; Stejskal and Kucerova, 1996). The study further showed that 75% of eggs were laid between the 13th and 17th day on all the fish substrates. The peak laying period agrees with the results of Ezenwaji and Obayi (2004) who indicated that full oviposition in *D. maculatus* is attained during the first 6-8 days, becoming fairly uniform in about 16 days, indicating reduction in rate of oviposition with time as sperm viability also declined with age (Kidd *et al*., 2001; Oakes *et al.*, 2003; Szczesnny *et al.*, 2003).

Eggs were laid in different batches, ranging from 2-6 batches with 28 eggs per batch as the highest and this contradicts the results of (Osuji, 1975) who recorded upto 38 eggs in a batch. Maximum number of eggs recorded on *O. niloticus* was 151 within 30 days confirming the findings by previous workers (Amusan and Okorie, 2001), but differs from the findings of (Seal and Tilton, 1985 and Ezenwaji and Obayi, 2004) who recorded 407 and 598 eggs respectively.

The maximum egg laying period of 30 days recorded contradicts those of Taylor (1964) and Osuji (1975) who found maximum egg laying period in *D. maculatus* to be 14 and 189 days, but similar to the works of Coombs (1978). These may be attributed to the differences in temperature, relative humidity, age of the insects and the amount and kind of food supplied.

### *4.2. Larval instars in D. maculatus reared on different fish substrates*

Observations made during the study showed that the hairy creamy larva on emergence darkens to light grey within a few hours. Although there were no significant differences in the duration of development of the larvae in the various fish species, larval development was shorter on *C. gariepinus* than on the other fish substrates. Assuming that a short development time on a certain fish species is an indication of good host suitability then, *C. gariepinus* may be marginally more suitable for development of *D. maculatus* which suggests higher level of infestation on *C. gariepinus.* Five larval instars were recorded on *C. gariepinus* but six on each of the other substrates. These differences could be attributed to the nutritional composition of the fish species as reported by Samish *et al*. (1992) that *D. maculatus* larva prefers substrates with high protein content. The second instar was longer on *Synodontis sp.* and *B. capriscus,* while it was longer for the fifth instar on *C. gariepinus* and the mixed substrates. This conforms with the findings of Osuji (1975) and Rustin and Munro (1984), but different from Lale *et al* (2000) who observed no differences in the numbers of larval instars on different fish species.

Larval development in *D. maculatus* does not involve any visible morphological change but only an increase in size from the previous instars (Osuji, 1975). The total development period of 31 days observed on *C. gariepinus* to 42.75 days on mixed substrates greatly differed from 91 days by Scoggin and Tauber (1951) and 16 days by Kreyenberg (1928). No differences in total developmental period between males and females were observed as was reported by (Kreyenberg, 1928).

### *4.3. Pre-pupal periods*

In each of the fish substrates a quiescent period was observed at the end of the last larval instar where it became almost C-shaped, thickened and reduced in length from 10.99 to 6.54 mm on *O. niloticus*, 12.50 to 9.38 mm on *Synodontis sp*., 12.95 to 8.42 mm on *C. gariepinus*, 11.38 to 8.34 mm on *B. capriscus* and 11.97 to 9.22 mm on mixed substrates. The observation of a non-motile nature of the pre-pupa agrees with those of Osuji (1975), Anonymous (1980) and Cloud and Collison (1986). However, the pre-pupal and pupal duration differed from those of Ezenwaji and Obayi (2004) and Rustin and Munro (1984). It was also observed that pupal duration was not sex dependent as reported by (Kreyenberg, 1928).

### *4.4. Adult emergence*

Comparing developmental period from eggs to adult emergence of the four fish substrates indicated that *C.*

*gariepinus* proved to be the most suitable for *D. maculatus* development because this fish substrate recorded the shortest period, even though it did not differ statistically from the others. These differences recorded may be due to evolutionary trend, physical form of the fish or its nutritional composition as reported by (Zakka *et al*., 2009). It is not clear why *D. maculatus* females would prefer one fish substrate for oviposition and a different fish for feeding, since more eggs were laid on *O. niloticus*.

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# Composition and Larvicidal Activity of *Artemisia vulgaris* L. Stem Essential Oil Against *Aedes aegypti*

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**Abstract**

This study investigates the composition and potential larvicidal activity of the essential oils isolated from the elite plant, *Artemisia vulgaris* L. which is commonly known as mugwort. Essential oils were hydrodistilled from *in vitro* raised stems and analysed by GC-MS. The major components of this oil were camphor, camphene, α-thujone, 1,8-cineole, γ-muurolene and β-caryophyllene. Mosquito larvicidal assays were carried out to test the efficacy of the oil against the dengue vector, *Aedes aegypti*. Laboratory reared third instar larvae were exposed to different concentrations of the oil solution and activity was assessed at different exposure times according to standard WHO procedure. Results showed that 100% larval mortality was achieved when the larvae were treated with 500 ppm oil solution with an exposure time of 8 h. The present study has shown that mugwort oil is a potential larvicide against *Aedes aegypti* even in low doses of the test solution. The results indicate that the natural insecticides could be used in the place of synthetic insecticides and save our environment from chemical hazards.

***Keywords**: Artemisia vulgaris*, stem essential oil, larvicidal, *Aedes aegypti*, exposure time.

## 1. Introduction

The mosquito species, *Aedes aegypti* L. is a vector of major diseases such as dengue haemorrhagic fever, chikungunya and yellow fever. *A. aegypti* is reported to infect more than hundred million people every year in more than 110 countries in the tropics (Halstead, 2000). The present resurgence of these diseases is due to the higher number of breeding places in today's throwaway society (Ravikiran *et al*., 2006). Further, the indiscriminate use of synthetic insecticides is creating multifarious problems like environmental pollution, insecticide resistance, and toxic hazards to humans. Globally, there have been conscientious efforts to overcome these problems, and great emphasis has been placed recently on enviro-friendly and economically viable methodologies for pest control.

Phytochemicals obtained from the huge diversity of plant species are important source for safe and biodegradable chemicals, which can be screened for mosquito repellent, larvicidal, and insecticidal activities; and tested for mammalian toxicity. A large number of plant products have been reported to have mosquito larvicidal and/or repellent activity against adult mosquitoes. The discovery of insecticide activity in Asteraceae species has stimulated interest in this plant family as part of the search for new plant derived insecticides (Rawls, 1986; Prashant *et al*., 2006; Masotti *et al.,* 2012). In recent years, essential oils have received much attention as potent bioactive compounds against various mosquito species (Tripathi *et al.,* 2009).

*Artemisia vulgaris* L. (mugwort) is a member of the Asteraceae family. It is a tall (0.8-1.4 m), aromatic, threatened perennial herb distributed throughout the northern temperate regions of Africa, Asia, Europe, India, and North America. In traditional medicine, this plant is widely used for the treatment of diabetes and extracts of the whole plant is used for epilepsy and in combination for psychoneurosis, depression, irritability, insomnia and anxiety states (Lewis and Elwin-Lewis, 2003). Numerous medicinally active components of *A. vulgaris* have been identified, including coumarins, essential oils, flavonoids, polyacetylenes, sesquiterpene lactones, and sterols (USDA-ARS-NGRL, 2004). Essential oils make a major contribution to the plant's biological activity (Judzentiene and Buzelyte, 2006).

Mugwort essential oil is used in India for its insecticidal, antimicrobial and antiparasitical properties (Judzentiene and Buzelyte, 2006). It was reported to exhibit 90% repellence against *Aedes aegypti*, a mosquito that transmits yellow fever (Hwang *et al.,* 1985). Repellent and fumigant activity of *A. vulgaris* essential oil against *Musca domestica* L. and the stored-product

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insect pest *Tribolium castaneum* (Herbst) were also reported (Judzentiene and Buzelyte, 2006; Wang *et al*., 2006). The essential oil exhibits many other biological activities such as analgesic, anaesthetic, antidiarrheic, antineuralgic, antiseptic, antispasmodic, antiasthmatic, anti-inflammatory,sedative,CNS-stimulant, decongestant, expectorant, antiacne, larvicidal, nematicide, pesticide, antibacterial, and it is also used in the flavour and perfumery industry (Teixiera da Silva, 2004). To date there are no published reports on larvicidal activity of *in vitro* grown stem essential oils of *A. vulgaris*. Therefore, we herein present a brief report on the composition of *A. vulgaris* stem essential oil and its larvicidal activity against *Aedes aegypti*.

## 2. Materials and Methods

### *2.1. Plant material and essential oil extraction*

*A. vulgaris* seeds were collected from National Medicinal Plants Board, India and cultures were raised *in vitro* as previously reported (Sujatha and Ranjitha Kumari, 2007). Stem material was collected from *in vitro* raised plants before the onset of flowering and subjected to essential oil analyses. A 100 g sample of the air-dried stems was mixed with 1000 ml distilled water and subjected to hydrodistillation in a Clevenger-type distilling apparatus for 2 h. The resulting oil was dried over anhydrous sodium sulphate and stored in airtight fuscous glassware in a refrigerator at 4 ºC until analysis.

### *2.2. Gas chromatography – mass spectrometry (GC-MS)*

GC/EIMS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures at 220° and 240° C respectively; oven temperature was programmed from 60° C to 240° C at 3° C/min; carrier gas helium at 1 ml/min; injection of 0.2 µl (10% hexane solution); split ratio 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their Linear Retention Indices relative to the series of *n*-hydrocarbons, and by computer matching against commercial (NIST 98 and ADAMS 95) and home-made library mass spectra built up from pure substances and components of known essential oils and MS literature data (Stenhagen *et al*., 1974; Jennings and Shibamoto, 1980; Adams, 1995). Moreover, the molecular weights of all the identified substances were confirmed by GC/CIMS, using MeOH and CI ionizing gas.

### *2.3. Procurement of eggs and rearing of Aedes Aegypti mosquito larvae*

*Aedes aegypti* eggs (Fig. 1a) were obtained from a colony maintained at the Center for Medical Entomology, Indian Council for Medical Research, Madurai, Tamil Nadu, India. The eggs of *A. aegypti* were obtained as egg rafts on a filter paper. The egg rafts were brought to the laboratory and kept in a tray containing tap water (as culture medium) at laboratory conditions (29 ± 1°C; RH 70–75%; photoperiod 14 : 10 (light : dark)). On the next day, the eggs were observed to hatch out into first instar larvae. Appropriate amounts of nutrients (yeast powder and dog biscuit (1:2 w/w) were added to the culture medium. On the third day after hatching, the first instar larvae moulted into second instar larvae. On the fifth day, third instar larvae were observed. The third instar larvae of *A. aegypti* were used for the larvicidal experiments in the present study (Fig. 1b).

### *2.4. Preparation of the oil solution*

A sufficient amount of target (TR) oil was dissolved in tap water using 2 ml of 100 % acetone to produce a stock solution of 500 ppm. This solution was used to prepare other serial dilutions of target oil in concentrations of 500 – 1 ppm through dilution of stock with tap water. Five replicates of each concentration were made, in addition to five control replicates containing 0 ppm of oil, 2 ml of 100 % acetone and tap water (WHO, 1981).

### *2.5. Larvicidal bioassay*

The tests were conducted at room temperature. The oil dilutions were tested against the third instar larvae of *A. aegypti* mosquitoes (WHO [World Health Organization], 1981; Ansari *et al*., 2000; Rey *et al*., 2001; Amer and Mehlhorn, 2006) to detect their toxicity on mosquito larvae. Five replicates of each oil dilution (1-500 ppm) were prepared. Each replicate containing 200 ml of the described oil solution was placed in a 500 ml glass beaker. Ten third-instar larvae of target mosquito were transferred into each beaker (Mohtar et *al*., 1999). After that, the beakers were left on the laboratory table for 24 h. The number of dead larvae in each beaker was counted after 1, 8, 16, 20 and 24 h.

### *2.6. Statistical analysis*

Experiments were set up in a complete randomized block design and each experiment was repeated five times. Data were recorded on the percentage of larval mortality. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means, and the treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5 % level of significance (Gomez and Gomez, 1976). The results were analyzed statistically using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) for windows.

## 3. Results and Discussion

### *3.1. Essential oil analyses*

The oil was a fluid liquid, slightly greenish in colour and possessed powerful fresh-camphoraceous aroma with sweet bitter taste. The intense odour could be due to the presence of a large number of oxygenated mono- and sesquiterpene components in the stem oil. Fifty one compounds were identified, accounting for 91.0% of the essential oil. The identified constituents and corresponding percentage of the essential oil composition are listed in Table (1).

**Table 1.** Composition of *in vitro* stem essential oils of *A. vulgaris*

| Compound | Rt | % MS | RI[#] | identification[Φ] |
|---|---|---|---|---|
| *cis*-salvene | 3.39 | trace | 846 | MS, RI |
| santolinatriene | 4.44 | 0.1 | 906 | MS, RI |
| tricyclene | 4.86 | 0.2 | 920 | MS, RI |
| artemisiatriene | 5.17 | 0.8 | 930 | MS, RI |
| alpha-fenchene | 5.57 | 3.9 | 943 | MS, RI |
| camphene | 5.61 | 6.0 | 944 | MS, RI, co-GC |
| sabinene | 6.37 | 2.4 | 969 | MS, RI, co-GC |
| beta-pinene | 6.47 | 0.8 | 973 | MS, RI , co-GC |
| 2,3-dehydro-1, 8-cineole | 6.94 | 0.1 | 988 | MS, RI |
| 2-carene | 7.92 | 0.1 | 1012 | MS, RI |
| p-cymene | 8.28 | 0.8 | 1020 | MS, RI, co-GC |
| 1,8-cineole | 8.58 | 5.1 | 1026 | MS, RI, co-GC |
| arbusculone | 10.24 | 0.4 | 1061 | MS, RI |
| alpha-thujone | 12.52 | 10.7 | 1105 | MS, RI, co-GC |
| beta-thujone | 13.04 | 2.8 | 1114 | MS, RI, co-GC |
| camphor | 14.88 | 17.3 | 1140 | MS, RI, co-GC |
| isoborneol | 15.39 | trace | 1147 | MS, RI, co-GC |
| borneol | 16.16 | 3.7 | 1158 | MS, RI, co-GC |
| 3-thujanol | 16.80 | 0.6 | 1167 | MS, RI |
| myrtenal | 18.00 | 0.6 | 1185 | MS, RI |
| myrtenol | 18.10 | trace | 1186 | MS, RI |
| verbenone | 18.85 | trace | 1197 | MS, RI |
| *trans*-carveol | 19.66 | trace | 1210 | MS, RI |
| cumin aldehyde | 21.05 | 0.2 | 1232 | MS, RI |
| perilla aldehyde | 23.55 | 0.2 | 1272 | MS, RI |
| bornyl acetate | 24.58 | 0.6 | 1288 | MS, RI |
| iso-3-thujyl acetate | 25.38 | 0.8 | 1301 | MS, RI |
| delta-elemene | 28.35 | 0.4 | 1334 | MS, RI |
| alpha-copaene | 31.26 | 0.8 | 1366 | MS, RI |
| beta-cubebene | 32.46 | 0.6 | 1379 | MS, RI |
| beta-elemene | 32.64 | 0.3 | 1381 | MS, RI |
| alpha-gurjunene | 33.83 | 1.3 | 1394 | MS, RI |
| beta-caryophyllene | 34.71 | 5.8 | 1405 | MS, RI |
| beta-gurjunene | 35.30 | 0.3 | 1413 | MS, RI |
| alpha-humulene | 37.17 | 1.9 | 1438 | MS, RI |
| allo-aromadendrene | 37.64 | 0.3 | 1444 | MS, RI |
| gamma-muurolene | 39.58 | 9.0 | 1469 | MS, RI |
| bicyclogermacrene | 40.58 | 1.6 | 1482 | MS, RI |
| alpha-muurolene | 41.00 | 0.6 | 1488 | MS, RI |
| gamma-cadinene | 41.90 | 0.6 | 1499 | MS, RI |
| davana ether isomer [*] | 42.42 | 0.4 | 1506 | MS, RI |
| delta-cadinene | 42.85 | 2.5 | 1512 | MS, RI |
| davana ether isomer [*] | 43.87 | 0.2 | 1525 | MS, RI |
| germacrene D-4-ol | 46.60 | trace | 1560 | MS, RI |
| spathulenol | 46.65 | 1.1 | 1561 | MS, RI |
| caryophyllene oxide | 46.88 | 1.2 | 1564 | MS, RI |
| *cis*-davanone | 47.94 | 2.5 | 1577 | MS, RI, co-GC |
| cadinol-epi-alpha | 51.32 | 0.4 | 1623 | MS, RI |
| alpha-cadinol | 52.29 | 0.5 | 1637 | MS, RI |
| davanone-2-ol-beta | 57.50 | 0.4 | 1712 | MS, RI |
| cadinene-14-hydroxy-delta | 62.67 | 0.1 | 1789 | MS, RI |

Rt = Retention time expressed in minutes; Trace: <0.05; [#] the retention index was calculated using a homologous series of n-alkanes C8-C18; [*] correct isomer not determined; [Φ]Co-GC: co-injection with an authentic sample.

There are no previous reports on the essential oil composition of *in vitro* raised *A. vulgaris* stems. Monoterpenes reached about 70% of the whole essential oil. The main ones were camphene (6.0%) and α-fenchene (3.9%). Oxygenated monoterpenes constituted the main chemical class of the oil (51.3%) and they were represented with camphor (17.3%) and α-thujone (10.7%) as principal chemicals. Sesquiterpenes constituted about 26% of the whole oil. Among them, hydrocarbons (27 compounds) reached 21.5%, whereas oxygenated derivatives (12 compounds) represented 4.4% of the oil. Among sesquiterpene hydrocarbons, β-caryophyllene (5.8%) was the main constituent. In the case of oxygenated sesquiterpenes, the principal one was *cis*-davanone (2.5%). Thus, the isolated essential oil was characterized by a high content of oxygenated monoterpenes [camphor (17.3 %), α-thujone (10.7 %), and 1,8-cineole (5.1%)]; the monoterpene hydrocarbon camphene (6.0%); and by the sesquiterpene derivatives, β-caryophyllene (5.8%) and γ-muurolene (9.0%).

It has been demonstrated that *A. vulgaris* grown in different countries possessed qualitative and quantitative differences in their essential oil composition. The oil from Italy were rich in camphor (47.7%), camphene (9.1%) and verbenone (8.6%) (Mucciarely *et al*., 1995). The oil from the Republic of Bashkortostan was found to contain high amounts of α-pinene (53.7 %), trans-chrysanthenol (13.1%), β-myrcene (8.8%) and β-pinene (7.4%) (Khalilov *et al*., 2001). Whereas in Croatia, the chief components reported were β-thujone (20.8%), α-pinene (15.1%) and 1,8-cineole (11.7%) (Jerkovic *et al*., 2003). The oil isolated from North Lithuania was high in amounts of sabinene, β-pinene, 1,8-cineole, artemisia ketone, cis- and trans- thujone, chrysanthenyl acetate, germacrene D, and β-caryophyllene (Judzentiene and Buzelyte, 2006). The oil isolated from Indian grown plants was characterized with high amount of camphor (38.7%), isoborneol (8.2%) and artemisia alcohol (4.5%) (Haider *et al*., 2003). α-thujone was stated as the main constituents of *A. vulgaris* oil (Misra and Singh, 1986), while we found an average value of 10.7% in this study. Thus the oils isolated from plants native to different countries significantly differ in their composition. This clearly depicts that variations in population genetics, environmental conditions, and the stress factors the plant faces during its survival and growth influence the accumulation of essential oils.

### *3.2. Larvicidal activity of stem essential oil*

Different concentrations (1-500 ppm) of stem essential oil solutions were bioassayed against the third instar larvae of *Aedes aegypti*. The results were recorded after 1, 8, 16, 20 and 24 h of treatment (Table 2). In control treatments, no larvicidal effect was observed; the larvae remained alive, and they moulted into fourth instar larvae. Whereas, when the different oil concentrations were tested, different mortality rates were recorded with respect to exposure time.

At 1 ppm oil solution, the larvae remained immobile after 24 h of treatment. When 10 ppm oil solution was

tested, 5.0 % and 12.3 % larval mortality was recorded after 20 h and 24 h of treatment respectively. When 100 ppm oil solution was tested, 78.2 % mortality was recorded after 24 h (Table 2).

**Table 2.** Effect of different concentrations of oil solution and exposure time on larvicidal bioassay of third instar larvae of *Aedes aegypti*

| Oil Solution (ppm) | Larval Mortality Rate (%) After | | | | |
|---|---|---|---|---|---|
| | 1 h | 8 h | 16 h | 20 h | 24 h |
| Control (0) | - | - | - | - | - |
| 1 | - | - | - | - | - |
| 10 | - | - | - | 5 | 12.3 d |
| 100 | 10.4 e | 45.9 e | 64.8 d | 70.1 c | 78.2 c |
| 200 | 20.1 d | 52.7 d | 72.9 c | 85.3 b | 91.5 b |
| 300 | 59.4 c | 71.2 c | 89.5 b | 100 a | 100 a |
| 400 | 65.1 b | 83.2 b | 100 a | 100 a | 100 a |
| 500 | 89.7 a | 100 a | 100 a | 100 a | 100 a |

* Treatment means followed by different letters are significantly different from each other ($p$<0.05) according to Duncan's Multiple Range Test

At 300 ppm, complete mortality was recorded after 20 h of exposure, and at 400 ppm, 100 % mortality was recorded after 16 h (Table 2). The maximum result (100 %) was recorded with 500 ppm oil concentration after 8 h exposure (Fig. 1c). Lethal concentration (50 % larvicidal activity) was observed from 100 ppm oil concentration onwards. The exposure time is very important for 50 % larvicidal activity of the oil solution. Very low concentrations of the oil led to high mortality rates.

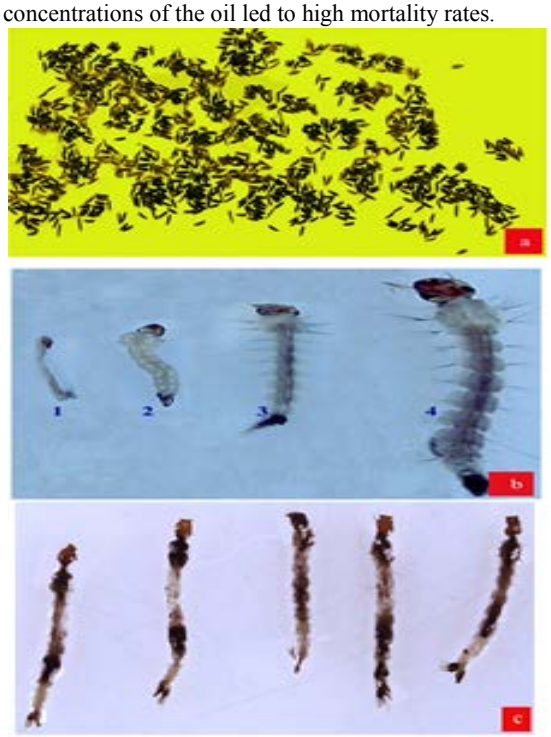

**Figure 1.** Larvicidal Activity of *Artemisia vulgaris* stems essential oil solution. a – Eggs of *Aedes aegypti* b – Different stages of larval development (1, 2, 3 & 4 instars) c – Treated larvae (at 500 ppm test solution) exhibiting complete mortalit (after 8 h)exposure time on larvicidal activity was also previously reported (Amer and Mehlhorn, 2006).

The present study has shown that *in vitro* produced *A. vulgaris* stem essential oil is a potential larvicide against *Aedes aegypti* in low concentrations (100 ppm) of the oil solution. High doses of the oil solution will be required for large breeding habitats, to be effective. However, the oil might be used as a selective larvicide in small breeding places where water is stagnant, such as in domestic containers and desert coolers. *A. vulgaris* essential oils were previously reported as potent larvicidals against *Aedes aegypti* (Ram and Mehrotra, 1995). But this is the first larvicidal bioassay carried out using essential oil extracted from *in vitro* propagated stems. *In vitro* production of source material helps in conserving the wild resources and thus this protocol can be used for the steady production of *A. vulgaris* plants for essential oil production.

This study indicates that the essential oil of *in vitro* propagated *A. vulgaris* stems has larvicidal properties and its use as a larvicide against mosquitoes should be explored. It is worthwhile to study extensively the larvicidal properties of the plant's essential oil by isolating and identifying the active components responsible for larval mortality, and then test them in field trials in order to assess their potential as an alternative to synthetic chemical larvicides.

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# Susceptibility of the Hymenopteran Parasitoid, *Habrobracon hebetor* (Say) (Braconidae) to the Entomopathogenic Fungi *Beauveria bassiana* Vuillemin and *Metarhizium anisopliae* Sorokin

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**Abstract**

*Habrobracon hebetor* (Say) is an ectoparasitoid and has been studied as a biocontrol agent of various lepidopteran pests such as cotton bollworm *Helicoverpa armigera* (Hübner). With regards to the negative effects of common pesticides used in cotton fields on the parasitoids, in this study the effects of different isolates of entomopathogenic fungi (*Beauveria bassiana* and *Metarhizium anisopliae*) were evaluated on *H. hebetor.* Bioassay experiments were performed by the immersion method. For each of the treatments 15 immature individuals of the parasitoid were used. After recording the results, data were analyzed using SAS software. Bioassay results of fungi isolates on larval stages showed that the value of $LC_{50}$ for IRAN187C isolate of *B. bassiana* was $1.46 \times 10^9$ conidia/ml. Because of the low mortality caused by the other isolates, the value of $LC_{50}$ was not set for them. Also, bioassay of fungal isolates showed that none of the isolates had any effect on the parasitoids pupal stage. According to the obtained results, it can be concluded that various fungal isolates of *B. bassiana* and *M. anisopliae* had little adverse impact on the parasitoid wasp, thus after doing field tests, the microbial control agents may be used along with these parasitoids in integrated pest management programs (IPM) in cotton.

***Keywords:*** *Habrobracon hebetor*, entomopathogenic fungi, IPM, bioassay, natural enemies..

## 1. Introduction

Cotton (*Gossypium hirsutum*) is the most important economic and fiber crop worldwide (Chen *et al.*, 2002). This crop is also a major agricultural product in Iran and the area cultivated with cotton is about 91019 hectare for the years 2009-2010 (Anonymous, 2011). Insect pests are limiting factors for healthy growth of cultivated plants (Ramzan Asi *et al.*, 2009). Among insect pests, *Helicoverpa armigera* (Hübner) (Lep.: Noctuidae) is one of the most important arthropod pests of cotton crop (Matthews, 1999). This pest is a polyphagous agricultural pest which attacks a wide variety of agricultural crops including cotton, corn, tomatoes, sorghum, soybeans and groundnuts (Fitt, 1989). Early instars are foliar feeders and later instars attack seeds, fruits and bolls leading to economic loss (Fitt, 1989) and their infestations cause severe economic losses as a result of crop yield reduction (Soomro *et al.*, 1992), and the pest causes economic losses up to 30% of the total production (Yazdanpanah *et al.*, 2009). Different strategies have been employed for control of this notorious pest (Ramzan Asi *et al.*, 2009). Farmers mostly prefer chemical pesticide application for its control because it is quicker, however, indiscriminate application of broad spectrum chemical pesticides exterminates these susceptible natural enemies and leaves behind the pests that are more resistant to pesticides (Feng *et al.*, 1994) as well as these compounds can cause serious problems such as pest outbreaks (Luck *et al.*, 1977; Metcalf, 1986). In order to reduce crop losses, the use of microbial control agents which have a lower risk on the environment and humans is recommended (Hull and Beers, 1985). Among the microbial control agents are entomophathogenic fungi. Entomopathogenic fungi have a considerable potential for efficacious suppression of a variety of arthropod pests. *Beauveria bassiana* (Balsamo) Vullemin is one of the most important entomopathogenic fungi (Leland *et al.,* 2005; Quesada-Moraga *et al*., 2006; Al-maza *et al.*, 2006). This fungus is widely distributed in the world (St.-Leger *et al.*, 1986) and has the potential to control over 70 insect pest species (Hung and Boucias, 1992; Alizadeh *et al*., 2007).

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Another fungus effective in controlling insect pests is *Metarhizium anisopliae* Sorokin that is able to control a wide range of pests (Zimmermann, 1993).

On the other hand, one of the important methods to control pests is the use of natural enemies. Among these natural enemies is the parasitoid wasp *Habrobracon hebetor* (Say) (=*Bracon hebetor*) (Haeselbarth, 1983; Amir-Maafi and Chi, 2006) (Hymenoptera: Braconidae). *H. hebetor* is a valuable biocontrol agent of lepidopteran pests attacking crop plants and stored products, including *H. armigera* (Magro and Para, 2001). In Iran, mass rearing of *H. hebetor* is done on Mediterranean flour moth, *Ephestia* (*Anagasta*) *kuehniella* Zeller (Mudd and Corbet, 1982) and the adult wasps are released to parasitize *H. armigera* larvae in cotton fields in Ardabil and Golestan provinces in the northern parts of the country (Attaran, 1996; Navaei *et al.*, 2002).

Since the strategy of IPM includes the simultaneous use of different methods of control, different methods of control must be examined together to finally be able to utilize them for pest control. Potential effects of microbial control agents on the parasitoids must be studied (Hajek and St. Leger, 1994). In particular, *Metarhizium anisopliae* Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin have been isolated from diverse species of parasitoids (Thungrabeab and Tongma, 2007).

In the present study, the effects of different isolates of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* were evaluated on the immature stages of *H. hebetor* in the laboratory, in order to evaluate the possibility of simultaneous application of entomopathogenic fungi and the parasitoid *H. hebetor* in the field.

## 2. .Materials and Methods

### *2.1. Insect Rearing*

The *H. hebetor* colony was obtained from an insectarium maintained by the Plant Protection Bureau of Kaleibar, Iran in 2010. The colony was maintained in the laboratory at 26 ± 1°C, 60 ± 5% RH and a photoperiod of 16:8 (L: D) on larval *E. kuehniella*, that was reared on flour in a growth chamber at the above mentioned environmental conditions. Parasitoid wasps were reared on 5th instar larvae of *E. kuehniella* for five generations and used for all experiments. Honey was provided as food for the adult parasitoids on 5 × 30 mm strips of paper (Sarmadi, 2008).

### *2.2. Fungal Isolates*

Fungi isolates used in this study are shown in Table 1.

**Table 1**. Isolates of fungi used in this study and origin within Iran

| Fungi | Isolates | Host | Location area |
|---|---|---|---|
| *Beauveria bassiana* | IRAN 187C | *Leptinotarsa decemlineata* | Ardabil |
| *Beauveria bassiana* | EUT116 | Lepidopteran larvae | Tehran |
| *Beauveria bassiana* | EUT105 | Soil | Fashand-Karaj |
| *Metarhizium anisopliae* | M-115 | *Parandra caspica* | Sari |
| *Metarhizium anisopliae* | M-396 | *Parandra caspica* | Sari |

### *2.3. Culture of Fungi*

Fungi were cultured on Saboraud's Dextrose Agar Yeast extract (SDAY) in Petri dishes at 25±1°C, 80±5% RH and a photoperiod 16:8 h (L:D). After preparing the medium, piece of the culture medium containing conidial fungi to be removed by a sterile scalpel were transferred to Petri dishes containing fresh medium. After 15 days the stages were full of germinated fungi. Petri dishes containing conidial of entomopathogenic fungi were used for experiments. Since strains maintained in the laboratory, after preparing them for about 2-3 weeks.

### *2.4. Production of Suspension*

For producing fungi suspension, conidia were transferred into tubes with lid consist of sterile distilled water. For screening mycelium and medium, this suspension was passed through mesh fabric. A haemocytometer (Paul Marienfeld GmbH and Co. KG, Germany) was used to determine the concentration of conidia in the initial suspension. The hemocytometer is a device originally designed for the counting of blood cells. It is now also used to count other types of cells as well as other microscopic particles for example the entomopathogenic fungi conidia. After counting conidia using haemocytometer, the main concentration was determined using the formula $Y= 5X\times10^4$ (X= number of conidia in five squares) (Erwin, 2002). Subsequent concentrations were determined using the logarithmic distant.

### *2.5. Bioassays*

The immature stages of the parasitoid were dipped in fungi solutions at the 4th or 8th day for 10 s. These days correspond to larval (without cocoon) and pupal (with cocoon) stages of the parasitoid, respectively (Rafiee-Dastjerdi *et al.*, 2008). Initial dose-setting experiments were carried out to determine the highest and lowest concentrations causing 80% and 20% mortality for both isolates (Robertson *et al.*, 2007). Concentration ranges were $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ and $10^{11}$ conidia/ml. Tween 80 (Merck, Darmstadt, Germany) was used at a concentration of 200 ppm in all dilutions as a spreader (Rosenheim and Hoy, 1988). Our previous experiments showed that Tween 80 (200 ppm) has no effects on bioassays. The control plates were sprayed with distilled water plus Tween 80. After immersion, Petri dishes containing filter paper and immature stages were transferred to growth chamber with 26±1°C and 80% RH. Data analysis was performed by SAS program (SAS Institute, 2002).

## 3. Results and Discussion

Effects of *B. bassiana* and *M. anisopliae* isolates showed that the isolates EUT105 (*B. bassiana*) and M-396 (*M. anisopliae*) didn't cause mortality in the *H. hebetor* larval stage at all concentrations tested. The EUT116 and IRAN187C isolates of *B. bassiana* at the concentration $10^{10}$ conidia/ml had 6.67 and 51.11% mortality on parasitoid larvae, respectively. Also, M-115 isolates of *M. anisopliae* at $10^{10}$ conidia/ml/ml caused 2.22% mortality on larval stage of *H. hebetor* (Table 3). The above results showed that, $LC_{50}$ values didn't apply for the listed

isolates. The only isolate for which the value of $LC_{50}$ was determined was IRAN187C isolate of *B. bassiana* (Table 2).

**Table 2**. Probit analysis of the fungal isolates IRAN187C (*B. bassiana*) tested on parasitoid larval stage

| Isolate | *N | Slope ± SE | $^2\chi$ | $LC_{50}$ Conidia/ml | **FL (95%) |
|---|---|---|---|---|---|
| IRAN187C *B. bassiana* | 270 | 0.6 ±0.08 | 3.4 | $1.46 \times 10^9$ | $(6.5 \times 10^8 - 3.2 \times 10^9)$ |

* Number of used insect

** Fiducial limits

According to our results, the isolate of IRAN187C (*B. bassiana*) had an adverse influence on the larval stage of the parasitoid followed by EUT116 (*B. bassiana*) and M-115 (*M. anisopliae*). Different isolates of *Beauveria* and *Metarhizium* did not show any effect on the pupal stage of the parasitoid (Table 3). Probably, the cocoon around the parasitoid pupa was responsible for the lack of effectiveness of the fungal treatments on this developmental stage. The results showed that in the control treatment (normal conditions) in the larval and pupal stages, no losses were observed (Table 3).

**Table 3**. Mortality (± SE) of immature stages parasitoid treated with concentration of $10^{10}$ conidia/ml of fungal isolates tested and control treatment

| Isolates of fungi | Stages of parasitoid | |
|---|---|---|
| | Larval | Pupal |
| IRAN187C *(B. bassiana)* | 51.11 ± 4.01 | 0 ± 0 |
| EUT116 *(B. bassiana)* | 6.67 ± 3.85 | 0 ± 0 |
| EUT105 *(B. bassiana)* | 0 ± 0 | 0 ± 0 |
| M-115 *(M. anisopliae)* | 2.22 ± 2.22 | 0 ± 0 |
| M-396 *(M. anisopliae)* | 0 ± 0 | 0 ± 0 |
| Control | 0 ± 0 | 0 ± 0 |

Means in column followed by different small letters are significantly different. ANOVA with Tukey post hoc test ($\alpha$<0.05)

Before this study, no investigation has been conducted on the impact of entomopathogenic fungi on *H. hebetor*, but the impact of *B. bassiana* and *M. anisopliae* on other parasitoids was studied. Rashki *et al.* (2009), in studying the effect of *B. bassiana* on *Aphidius matricariae* and its host *Myzus persicae*, showed that this pathogen had no effect on biological parameters of the parasitoid and concluded that *B. bassiana* and the parasitoid *A. matricariae* can be successfully combined for biological control of *M. persicae*. These reports are in line with the results of this study. The results of this study indicate very little effect of the entomopathogenic fungi on the parasitoid which is consistent with the results of Stolz *et al.* (2002). In evaluating the susceptibility of the parasitoids *Apoanagyrus lopezi* and *Phanerotoma sp.* to the entomophatogenic fungus *M. anisopliae*, they reported that different isolates of this fungus had very little risk on parasitoids. Also, Rosa *et al.* (2000) studing the effect of *Beauveria* and *Metarhizium* on the parasitoid *Prorops nasuta* reported that various isolates of the fungus have little negative impact on the parasitoid and can be used as a component compatible with natural enemies. Also, the effects of fungal isolates on the host field (*Helicoverpa armigera* Hübner) showed that mentioned isolates had a good control on the *H. armigera* (Vojoudi, 2011). According to the obtained results, it can be concluded that different isolates of the fungi *B. bassiana* and *M. anisopliae* had few adverse effects on *H. hebetor*, and therefore these microbial control agents can be used of along with the parasitoid in integrated pest management (IPM) programs.

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# Accumulation of Copper in Different Tissues and Changes in Oxygen Consumption Rate in Indian Flying Barb, *Esomus danricus* (Hamilton-Buchanan) Exposed to Sub-lethal Concentrations of Copper

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**Abstract**

In the present work, the accumulation pattern of sub-lethal doses (0.005, 0.0025 and 0.001 $\mu gl^{-1}$) of copper (Cu) in different tissues of Indian flying barb, *Esomus danricus* and its influence on the rate of oxygen consumption of this fish at the end of 28 days of exposure were studied. It was found that the pattern of accumulation changed with concentration. Cu concentration increased in all the tissues except bone and brain and the rate of uptake of Cu increased with time in the gill, liver and flesh but remained same in kidney. Treatment of 0.005 and 0.0025 $\mu gl^{-1}$ of Cu produced significant decline in the rates of oxygen consumption of the fish at all exposure duration, while treatment of 0.001 $\mu gl^{-1}$ produced similar decline only after 14 days of exposure when compared to control. Higher doses of exposure had more severe effects.

***Keywords:*** Teleost, copper, accumulation, oxygen consumption.

## 1. Introduction

Copper (Cu) is an essential heavy metal that possess the ability to enter and concentrate in various tissues many times higher than the ambient levels and alter oxygen consumption rates of fish (Farkas *et al.*, 2002; De Boeck *et al.*, 1995). Free Cu may catalyze the formation of highly reactive hydroxyl radicals, which can result in oxidative damage to cells (Gaetke and Chow, 2003). Major sources of Cu in aquatic environment are sewage, industrial effluents and pesticides (Palacios and Risbourg, 2006). Fishes have shown to concentrate Cu in their tissues but the metal accumulation capacity is dependent on the assimilation and excretion capacities of species concerned (Rao and Patnaik, 1999). In a study, Gupta (1998) had shown that wetlands like floodplain lakes, marshes and swamps of Barak Valley, in Assam state of India, serve as sinks for heavy metals including Cu. Indian flying barb, *Esomus danricus* (Hamilton-Buchanan), a minnow having food and ornamental value, commonly inhabits such water bodies and are susceptible to Cu which target gill, liver, kidney and other tissues. It would, thus, be interesting to study the accumulation pattern of sublethal doses of Cu in different tissues of *E danricus* and its influence on the rate of oxygen consumption of this fish.

## 2. Materials and Methods

### *2.1. Fish and experimental system*

Fish of similar length (46.77 ± 4.30 mm) and weight (0.86 ± 0.16 g) were collected from unpolluted, freshwater ponds near Assam University campus, Barak valley, South Assam, India (Das and Gupta, 2009). They were acclimatized under laboratory conditions seven days prior to experimentation and commercially available fish food was given *ad libitum* twice daily. Temperature, pH, hardness and dissolved oxygen under laboratory condition were 29°C, 6.8, 30 mg $l^{-1}$ and 5.5 mg $l^{-1}$ respectively. Stock solution of Cu was prepared from $CuCl_2.2H_2O$ (Merck, Germany) and serial dilutions were prepared using chlorine free tap water as per dilution techniques (APHA, 2005). Three sub-lethal concentrations (0.001, 0.0025 and 0.005 $\mu g.l^{-1}$) of Cu were selected based on 96 hours $LC_{50}$ value of Cu for *E. dandricus* (0.01 $\mu gl^{-1}$) determined in a prior study (Das and Gupta, 2010).

### *2.2. Metal accumulation study*

Two hundred fish were randomly selected into four groups of 50 fish each. The 50 fish in each group were housed in five bowls, each containing ten animals. Each of the bowls contained 2 liters of water. Bowls of group I, II, III and IV contained 0.005, 0.0025 and 0.001 $\mu gl^{-1}$ and

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control (tap water without Cu) respectively. 5 bowls of each group were marked as 0, 7, 14, 21 and 28d corresponding to the days of exposure to that particular concentration of Cu. For example, bowl of group I marked '7 d' had 10 fish exposed to 0.005 $\mu gl^{-1}$ that were sacrificed after 7 days of exposure and so on. Cu treatment was carried out twice daily, and on each time, fresh water was used to avoid accumulation of waste and to ensure constant metal concentration. During the study period, dead fish (if any) were removed. During exposure periods, the fish were fed twice daily. Feeding was, however, stopped 24 h before each sacrifice. After the specified duration of exposure, fish were sacrificed and the gill, kidney, liver, bone, flesh and brain were excised, dried, weighed and digested in 5ml concentrated $HNO_3$ to dryness in oven and dissolved in 10 ml distilled water. Analysis for Cu was carried out in a Perkin-Elmer 3110 atomic absorption spectrophotometer (AAS). The detection limit of the instrument was 0.01$\mu gl^{-1}$. The readings were checked with those of standard solutions, and contamination errors were minimised by using blanks, acid washed glass wares, analytical grade reagents and double distilled water. Digestion of samples was based on a modification of the method of Jayakumar and Paul (2006). Statistical significance of the differences in rate of Cu incorporation between 7$^{th}$ day and subsequent intervals (14, 21 and 28$^{th}$ day) of exposure at different Cu concentrations were made by one-way ANOVA and Tukey Test using SYSTAT 13 software for Windows.

*2.3 Oxygen Consumption*

Three test chambers (each of 3 litre capacity) were marked A, B and C containing 0.005, 0.0025 and 0.001 $\mu gl^{-1}$ of Cu respectively. Each test chamber contained ten fish. At the beginning (0 day), each fish of test chamber A was transferred to respiratory chamber, which was also numbered in accordance with the test chamber and the experiment was run for a period of 1h. After the experiment, the fish was weighed and replaced in its respective test chamber. The same process was repeated for other fishes of the test chamber A (ten replicates) and for 7d, 14d, 21d and 28day. Controls were also run simultaneously in chlorine-free tap water to obtain information on the oxygen consumption of the fish in normal state. Similarly, the process was repeated for fish in test chambers B and C. Respiratory measurements were made by the closed chamber method (Fitch, 1975) and the dissolved oxygen was estimated adopting Winkler's method. Rate of oxygen consumption was measured in ml/hr/100g tissue. Statistical significance of the differences in oxygen consumption between control and exposed fish at different Cu concentrations were made by one-way ANOVA and Tukey Test using SYSTAT 13 software for Windows.

## 3. Results

The initial level (on 0 day) of Cu for all tissues was below detection limit. However, the level of Cu accumulated by each tissue after 28 days of exposure was directly proportional to the exposure concentration of Cu. Cu, irrespective of exposure level, was never detected in the flesh within7 days of exposure and in bone and brain within 28 days of exposure (Table 1).

**Table 1**. Accumulation ($\mu g\ g^{-1}$ dry weight) of Cu in selected tissues of *Esomus danricus* exposed to sub-lethal concentrations over time

| Exposure concentration of Cu ($\mu g\ l^{-1}$) | Tissue | Concentration (µg.g-1) after days of exposure (d) | | | |
|---|---|---|---|---|---|
| | | 7d | 14d | 21d | 28d |
| Control | Gill | BDL | BDL | BDL | BDL |
| | Kidney | BDL | BDL | BDL | BDL |
| | Liver | BDL | BDL | BDL | BDL |
| | Flesh | BDL | BDL | BDL | BDL |
| | Bone | BDL | BDL | BDL | BDL |
| | Brain | BDL | BDL | BDL | BDL |
| 0.001 | Gill | BDL | BDL | 0.0003± 0.0001 | 0.0008± 0.0001 |
| | Kidney | BDL | BDL | BDL | BDL |
| | Liver | BDL | 0.0007± 0.0002 | 0.0015± 0.0001 | 0.0026± 0.002 |
| | Flesh | BDL | BDL | BDL | BDL |
| | Bone | BDL | BDL | BDL | BDL |
| | Brain | BDL | BDL | BDL | BDL |
| 0.0025 | Gill | 0.0004± 0.0006 | 0.0019± 0.001 | 0.004± 0.002 | 0.007± 0.002 |
| | Kidney | BDL | BDL | BDL | 0.0013± 0.001 |
| | Liver | 0.0008± 0.0001 | 0.0027± 0.0003 | 0.006± 0.002 | 0.015± 0.002 |
| | Flesh | BDL | BDL | BDL | 0.001± 0.0004 |
| | Bone | BDL | BDL | BDL | BDL |
| | Brain | BDL | BDL | BDL | BDL |
| 0.005 | Gill | 0.0035± 0.0008 | 0.008± 0.001 | 0.015± 0.003 | 0.021± 0.004 |
| | Kidney | 0.0015± 0.0006 | 0.0029± 0.001 | 0.004± 0.002 | 0.008± 0.002 |
| | Liver | 0.005± 0.002 | 0.016± 0.002 | 0.03± 0.014 | 0.05± 0.016 |
| | Flesh | BDL | BDL | 0.0005± 0.0002 | 0.004± 0.001 |
| | Bone | BDL | BDL | BDL | BDL |
| | Brain | BDL | BDL | BDL | BDL |

BDL – Below detection limit

At the end of 28 days of exposure, the total tissue Cu concentration followed the pattern liver>gill>kidney>flesh>bone=brain for exposure to 0.005 and 0.0025 $\mu gl^{-1}$ Cu and liver>gill; kidney for 0.001 $\mu gl^{-1}$ Cu, bone, flesh and brain showing no detectable values. The study reveals that the uptake of Cu is tissue specific. Evaluating the rate of accumulation (tissue concentration / days of exposure) it was revealed that for exposure to 0.005 $\mu gl^{-1}$ Cu, the accumulation of Cu in gill and liver was similar up to 14 days but increased thereafter up to 28 days ($p<0.05$). Kidney, on the other hand, showed similar rate of accumulation for all exposure durations for the same concentration ($p<0.05$). For 0.0025 $\mu gl^{-1}$, the rate of accumulation of Cu in gill up to 14 days was similar but increased thereafter, up to 28 days. Kidney and liver, on the other hand, showed similar rate of accumulation for all exposure durations for the same concentration ($p<0.05$). For exposure to 0.001 $\mu gl^{-1}$Cu, the rate of accumulation of Cu in gill, kidney and liver showed similar pattern for all exposure durations ($p<0.05$). However, the rate of accumulation increased significantly with increase in concentration. Besides, bone and brain did not show Cu accumulation at any dose and flesh did not show any accumulation at the lowest dose up to 28 days of exposure.

The present study revealed that the flying barb responded to Cu by reducing the rate of oxygen consumption. It was observed that the oxygen

consumption rate of flying barb decreased with the increase in concentration of Cu, exposure duration also played a crucial role and oxygen consumption rate was found to decline with the increase in exposure period at 0.005 and 0.0025 µg $l^{-1}$ of Cu, whereas, 0. 001 µ$gl^{-1}$ Cu showed no significant changes in oxygen consumption upto14 days of exposure, but declined significantly thereafter up to 28 days of exposure (Table 2).

**Table 2.** Effect of Copper on rate of oxygen consumption in *Esomus danricus*

| Cu conc. (µg $l^{-1}$) | Oxygen Consumption (ml/hr/100g tissue) | | | |
|---|---|---|---|---|
| | 7 d | 14 d | 21 d | 28 d |
| Control | 38.94± 0.46 | 39.19± 0.29 | 39.03± 0.24 | 39.06± 0.15 |
| 0.001 | 38.39± 0.61** | 38.01± 0.64** | 30.31± 1.25* | 24.11± 1.76* |
| 0.0025 | 29.24± 1.15* | 24.87± 1.64* | 21.70± 1.43* | 19.20± 1.70* |
| 0.005 | 27.27± 1.0* | 21.67± 1.25* | 19.70± 1.93* | 14.84± 1.94* |

*Significant;

** Not significant at $p<0.05$

## 4. Discussion

Gill is the primary route for Cu uptake in fish due to its direct exposure to toxicants in water (Jayakumar and Paul, 2006; Kamunde *et al.*, 2002). In flying barb, Cu accumulated progressively in gills in concentration dependent manner, reaching4.4 fold increase after 28 days of exposure to 0.005 µ$gl^{-1}$ of Cu and only 0.8 fold magnification observed at 0.001 µ$gl^{-1}$ of Cu. Though both liver and kidney are typically important for metal accumulation and storage in fish (Gigue`re *et al.*, 2004), Cu metabolism is controlled chiefly by the liver. The liver not only tends to accumulate Cu from medium, but also plays an important role in Cu homeostasis (Grosell *et al.*, 1997). In flying barb, Cu concentration was 10 fold in liver after 28 days of exposure to 0.005 µ$gl^{-1}$ (Table 1). In rainbow trout exposed to radioactive Cu, the liver was shown to be the major target organ while the kidney was less important for Cu accumulation (Clearwater *et al*., 2000). Similarly, Cu concentrations were seven times higher in the liver than the kidney after 70 day of exposure in yellow perch *Perca flavescens* (Kraemer *et al.*, 2005). Thus, Cu was predominantly removed from the body and accumulated in the liver over time. Apart from the bone and brain, which had no detectable level of Cu throughout the study, the flesh accumulated the lowest level of Cu, even after 28 days of exposure (Table 1). This may be connected with the fact that the flesh and bone are not concerned with detoxification and therefore the transportation of Cu from other tissues to flesh and bone may not arise. Lack of a detectable level of Cu in the brain can be due to blood–brain barrier that prevents the entry of Cu into the brain (Crowe and Morgan, 1997).

The measurement of oxygen consumption of fish in presence of pollutant is the best index of its activity (Delhaye and Cornett, 1975). In the present study, flying barb responded to Cu by reducing the rate of oxygen consumption. Adverse effects of Cu on respiratory capabilities, as seen in the present study, were also studied in *Tilapia sparrmanii* (Van Aardt and Hough, 2006) and in *Esomus danricus* (Vutukuru *et al.*, 2005). Cu had a depressing effect on oxygen consumption in *Cyprinus carpio* (De Boeck *et al*., 1995) and in *Labeo capensis* and *Micropteris salmoides* (Van Aardt and Hough, 2007). Cu reduced oxygen consumption by common carp and rainbow trout larvae in a concentration-dependent way (Jezierska and Sarnowski, 2002). In this study, all the sublethal doses of Cu induced excessive mucous secretion in gills. This phenomenon of mucous secretion can also impair gas exchange across the secondary lamellae epithelium (Handy and Eddy, 1989).

In conclusion, the present study indicates that Cu is accumulated at alarming level in gill and liver in Indian flying barb when exposed to level of Cu above 0.001 µ$gl^{-1}$. The fish responds to such high accumulation by lowering oxygen uptake.

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# Modification of the Mechanical Properties of Red Blood Cell Membrane by Spent *Plasmodium falciparum* Culture Supernatant

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**Abstract**

The effect of spent culture supernatant on RBC membrane mechanical properties, RBC membrane deformability and stability, were assayed by ektacytometry after treating RBC ghost membranes with spent *Plasmodium falciparum* (*P. falciparum*) malaria culture supernatant fluid (Pf(+)), malaria culture medium which had not been exposed to *P. falciparum*-infected RBCs Pf(-), and malaria culture medium from an *in vitro* culture system containing uninfected RBCs (M). All treated RBC membranes were less deformable, and more stable than control, untreated RBC membranes. However, compared to controls, Pf(+)-treated RBC membranes were more deformable than both Pf(-)-treated and M-treated RBC membranes. Similarly, Pf(+)-treated RBC membranes were more stable than membranes treated with both Pf(-) and M. The deformability and stability of Pf(+)-treated RBC membranes were significantly different from membranes treated with both Pf(-) and M ($P \leq 0.05$). However, there was no significant difference between deformability and membrane stability for Pf(-)-treated and M-treated RBC membranes. Our results suggest that spent *P. falciparum* culture supernatant may significantly modify RBC membrane mechanical properties.

**Keywords:** RBC membrane, deformability, stability, *P. falciparum*, parasite culture supernatant.

## 1. Introduction

The red blood cell (RBC) membrane has been well described in terms of its structure and composition (Snyder and Sheafor, 1999). It is composed of a lipid bilayer, integral proteins, a sub-membranous skeletal protein network of spectrin, and peripheral proteins (Chasis and Mohandas, 1986). Deformability and stability are two essential qualities of the RBC membrane that play a crucial role in the maintenance of normal blood flow and supply to tissues (Arai *et al.,* 1990; Chien, 1987; Mohandas and Chasis, 1993). Consequently, any variation in RBC membrane deformability, as in the case of malaria (Miller, Baruch, Marsh & Doumbo, 2002) or sickle cell diseases (Platt, 1995) can potentially compromise the micro-circulatory function. Studies have demonstrated that the protein network underlying the RBC membrane, together with the membrane bilayer and the network of membrane-associated proteins, play a key role in regulating RBC membrane deformability and stability (Takakuwa, 2001). Considerable effort and time have been invested in the *in vitro* cultivation of the erythrocytic stages of *Plasmodium*, the stages most often associated with the pathogenesis of malaria.

A major accomplishment in this area was defining ideal *in vitro* conditions for continuous cultivation of *P. falciparum* strains from different geographical areas, using HEPES-buffered RPMI 1640 culture medium that is supplemented with human serum, RBCs, and sodium bicarbonate (Trager and Jensen, 1976; Trager and Jensen, 1977; Trager and Jensen, 1980). RBCs are an obligatory requirement in this *in vitro* medium. Indeed, fresh and stored erythrocytes appear to be equally suitable for continuous *in vitro* cultivation provided that adenosine triphosphate (ATP) levels of RBCs are within the normal range (Capps and Jensen, 1983, Schuster, 2002). While this unique medium has been shown to be suitable for *in vitro* cultivation of *P. falciparum* malaria stages, the effect of malaria culture medium which has not been exposed to *P. falciparum*-infected RBCs (iRBCs) and spent malaria parasite culture supernatant on RBC membrane mechanical properties has not been fully explored. *In vitro* cultivation of *P. falciparum* is usually carried out through

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the continuous preservation of iRBCs deposited in static thin layers of settled infected cells. However, not all aspects regarding how the culturing methodology affects the development and growth of the parasite are fully understood.

Although some agents have been shown to alter the RBC membrane mechanical properties (Beder *et al*., 2002), it is uncertain how *P. falciparum* spent parasite culture supernatant will affect both RBC membrane deformability and stability. The aim of this study was to assess the effect of spent parasite culture, and malaria culture medium which has not been exposed to iRBCs, on membrane deformability and stability of RBC.

## 2. Materials and Methods

### *2.1. In vitro cultivation of malaria parasites*

*P. falciparum* (F32 strain) used for this study was a gift from Prof. Akira Kaneko of Tokyo Women's Medical University. *P. falciparum* F32 was maintained in a continuous culture as previously described by Trager & Jensen (1976). The culture contains type $O^+$ RBCs at 2% hematocrit in malaria culture medium, Pf(-) which consists of RPMI 1640 medium buffered with 25mM HEPES and 24 mM $NaHCO_3$ and supplemented with 10 % heat-inactivated human $O^+$ serum. Culture media were kept under a standard gas mixture of 5% $O_2$, 5%$CO_2$ and 90% $N_2$ at 37$^o$C. From a culture system containing mixed stage *P. falciparum*-infected RBCs at 10 % parasitemia, the supernatant was harvested and centrifuged for 15 minutes at ×500 g (GS-6KR: Beckman, Fullerton, California). This supernatant (Pf(+)), typically described as "spent", was used for subsequent experiments. Similarly, supernatant from malaria culture medium incubated with uninfected RBCs (Pf(-)), under conditions similar to that of spent culture system, was harvested and treated in the same manner described for spent *P. falciparum* culture supernatant. All solvents and chemicals were of analytical grade and were purchased from Wako Chemicals (Osaka, Japan). Water was purified using the milliQ pore system (Millipore, Bedford, MA, USA).

### *2.2. RBC lysis and 5T5K ghost preparation*

Fresh type $O^+$ venous blood was drawn from healthy volunteers, collected in heparin-coated tubes and processed within minutes after collection. RBCs were separated from leukocytes by filtration through polyurethane filter kit (Terumo), and washed three times with 10 mM Tris-HCl buffer (pH 7.4) that contains 120 mM KCl to remove cytosolic components. RBCs were lysed and washed once in ice-cold 1:35 diluted 5T5K buffer (5T5K buffer: 5mM Tris-HCl, pH 7.4, containing 5mM KCl) by centrifugation at ×15,000 g to obtain 5T5K RBC ghost cells. Ghost-cell suspensions were incubated at 37°C for 40 min for membrane resealing.

### *2.3. RBC lysis and MgATP ghost preparation*

MgATP ghost-cells were prepared in the same manner described for 5T5K ghost-cells with slight modifications, as described previously by Mohandas & Chasis (1993). The 5T5K buffer (lysing buffer) was supplemented with 10 mM ATP and 1 mM $MgCl_2$. To restore isotonicity, 100 µl of a mixture of 150 mM KCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol was added to RBC ghost suspensions and incubated at 37°C for 40 min for membrane resealing. MgATP-ghosts were prepared in the presence of MgATP throughout the process, whereas 5T5K ghosts were prepared in the absence of MgATP. The morphology of unfixed resealed ghosts was examined by dark-field light microscopy using a Nikon microscope.

### *2.4. Pre-treatment of ghost membranes*

MgATP ghost suspensions were re-suspended in nine volumes of either spent culture supernatant (Pf(+)), malaria culture medium (Pf(-)), or a medium from *in vitro* culture containing uninfected static RBCs (M). MgATP ghost re-suspensions were incubated for 30 minutes at 37$^o$C. Untreated MgATP ghosts served as control. After incubation, all RBC ghosts were centrifuged once at ×500 g for 10 minutes and assayed by ektacytometry for changes in membrane deformability and stability.

### *2.5. RBC membrane stability assay*

RBC membrane resistance to shear-induced fragmentation was measured by subjecting MgATP ghosts to a constant high shear stress of 750 dynes $cm^{-2}$ in the ektacytometer, as described by Hardeman *et al*. (1994). For the present study, 500 µl of each test sample (treated and untreated ghosts) was thoroughly mixed with 3 ml of 50 % dextran (wt./vol.) in 10 Mm TBS buffer (pH 7.4), resuspended in the ektacytometer chamber, and rotated at 100 rpm to generate the desired shear stress. When the shear stress is first applied, RBC ghosts are maximally deformed into ellipsoids and produce a narrow elliptical pattern that generates a maximal value of the deformability index (DI). RBC ghosts are unable to withstand the large value of applied shear stress, and subsequently begin to fragment, with DI gradually decaying under the constant high shear stress. A decrease in the DI value reflects fragmentation of the intact membranes into small, undeformable spherical particles. $T_{50}$ is the time required for DI to decay to half its maximum value. A decrease in the $T_{50}$ value reflects a decrease in RBC membrane stability, and vice versa. Suspending RBC ghosts in a viscous medium is necessary for the ektacytometric method as cells suspended in a low viscosity fluid (e.g. Tris-buffered saline, TBS) tumble, and do not deform in response to the applied shear stress (Johnson, 1989). It has been reported that the use of dextran, a neutral polysaccharide, to increase the viscosity of the suspending medium, does not affect the integrity of RBC membrane or alter its mechanical behavior (Nash & Meiselman, 1984).

#### *2.5.1. RBC membrane stability assay*

The Maximum Deformability Index ($DI_{max}$) and initial rate of change of the deformability index ($DI_{initial}$) which is a direct measure of membrane deformability, were determined at various shear stress values by laser diffraction analysis using an ektacytometer (LORCA: RR Mechantronics; Hoorn, The Netherlands). Details of ektacytometry have been described elsewhere (Johnson, 1989; Hardeman *et al*., 1994). Briefly, 100 µl of each test sample was re-suspended in 3.0 ml of 50% dextran in 10 mM TBS (wt./vol.), pH 7.4, and subjected to increasing shear stress up to 200 dynes $cm^{-2}$. Changes in laser

diffraction patterns were analyzed to derive $DI_{max}$. A decrease in $DI_{initial}$ indicates a reduction in RBC membrane deformability. Suspending RBC ghosts in a viscous medium is necessary for the ektacytometric method as cells suspended in a low viscosity medium (e.g. TBS) tumble and do not deform in response to the applied shear stress (Johnson, 1989).

## 3. Results

### *3.1. Morphology of resealed ghosts*

Resealed ghosts prepared without MgATP were predominantly echinocytic in shape (Fig1.A). However a few discoid-shaped ghosts were observed. Ghosts prepared in the presence of MgATP were all discoid in shape (Fig. 1B), indicating that a minimum concentration of ATP is essential for maintenance of normal discoid shape. Pre-treatment with the various media did not alter the discoid shape of MgATP ghosts (Fig.1C, D and E).

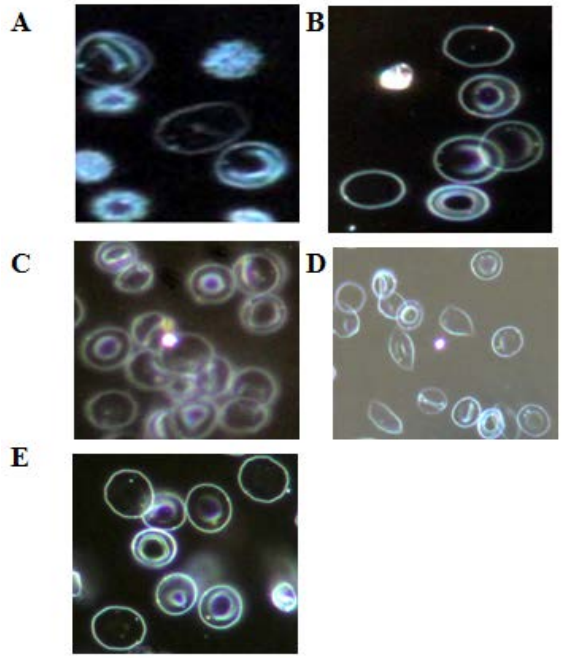


**Figure 1**. Dark-field light microscopy of ghosts. Panel A: 5T5K ghosts, Panel B: MgATP ghosts, Panel C: Pf(+)-treated MgATP ghosts, Panel D: Pf(-)-treated MgATP ghosts, Panel E: M- treated MgATP ghosts.

### *3.2. Membrane stability profile of MgATP and 5T5K RBC ghosts*

Membrane stability profile of 5T5K ghosts and MgATP ghosts is shown in Fig. 2. When ghosts were constantly subjected to a high shear stress of 750 dynes/cm$^2$, ghost membranes were maximally deformed and fragmented soon after attaining the maximum deformability index ($DI_{max}$), resulting in decreased DI value. The rate of decrease of DI is a direct measure of membrane mechanical stability. 5T5K ghosts underwent fragmentation faster than MgATP ghosts which resulted in the reduction of DI value.

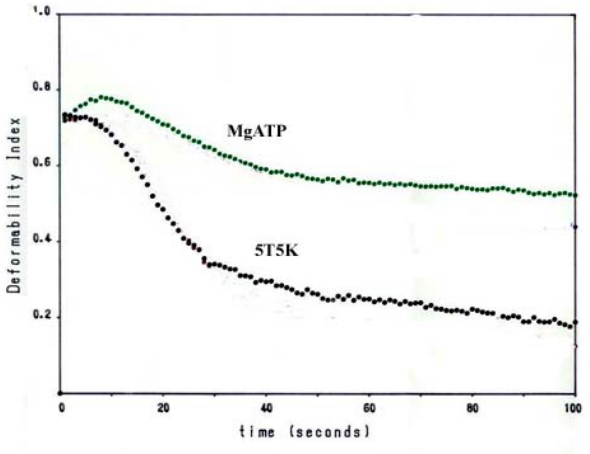


**Figure 2**. Deformability profile of 5T5K and MgATP ghosts

### *3.3. Duration for DI to reach half its maximum value ($T_{50}$)*

The Mean $T_{50}$ values for the various treatments were 96, 40, 31 and 28 seconds for control, Pf(+), Pf(-) and M-treated MgATP ghosts, respectively (Fig. 5). Control MgATP had the longest fragmentation time, whereas M-treated MgATP ghosts had the shortest. Treated RBC ghosts exhibited varying decline in $T_{50}$ values to about 58%, 68% and 71% of that of the control ghosts. These reductions correspond to Pf(+), Pf(-) and M-treated MgATP ghosts.

The $T_{50}$ for all treatments were significantly different from each other, except for Pf(-) and M-treated MgATP ghosts ($P \leq 0.05$).

### *3.4. Maximum deformability index ($Di_{max}$)*

MgATP ghosts attained higher $DI_{max}$ values than 5T5K ghosts (Fig. 3), and because of that they were used for subsequent experiments. When MgATP ghosts were subjected to increasing values of shear stress, the deformability index value increased, reaching a maximum of 0.79, 0.73, 0.77 and 0.74 for control, Pf(+), Pf(-) and M-treated ghosts, respectively (Fig.3). Although Pf(+)-treated ghosts recorded the lowest $DI_{max}$ value, the Newman-Keuls Multiple Comparison Test showed that there was no significant difference between the mean $DI_{max}$ values of Pf(+)- and M-treated ghosts ($P \leq 0.05$). Similarly, a comparison between untreated and Pf(-)-treated ghosts showed no significant difference between their mean $DI_{max}$ values ($P \leq 0.05$).

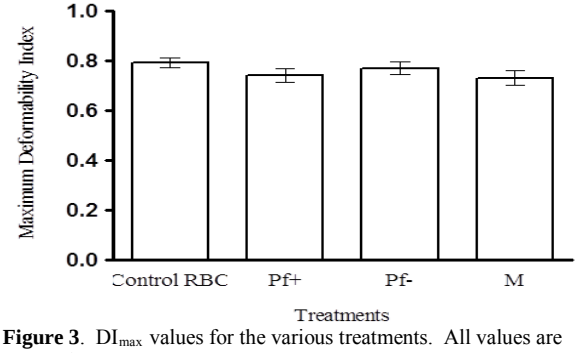


**Figure 3**. $DI_{max}$ values for the various treatments. All values are reported as Mean ± S.D.

### *3.5. Initial rate of change of the deformability index ($Di_{initial}$)*

$DI_{initial}$ values for the various treatments are shown in Fig. 4. Mean $DI_{initial}$ values obtained were 2.9, 2.5, 1.5 and 1.4 for control, Pf(+), Pf(-) and M-treated 5T5K ghosts, respectively. The mean $DI_{initial}$ value was highest for control RBCs, and lowest for M-treated RBCs ($P \leq 0.05$). Compared to control ghosts, treated RBCs exhibited reductions in $DI_{initial}$ values to about 14%, 48% and 52% of that of the control ghosts, corresponding to Pf(+), Pf(-) and M-treated MgATP ghosts, respectively. Stastical analysis indicates that the $DI_{initial}$ for Pf(-) and M-treated ghosts were not significantly different from each other. The mean $DI_{initial}$ values of all other treatments were significantly different from each other ($P \leq 0.05$).

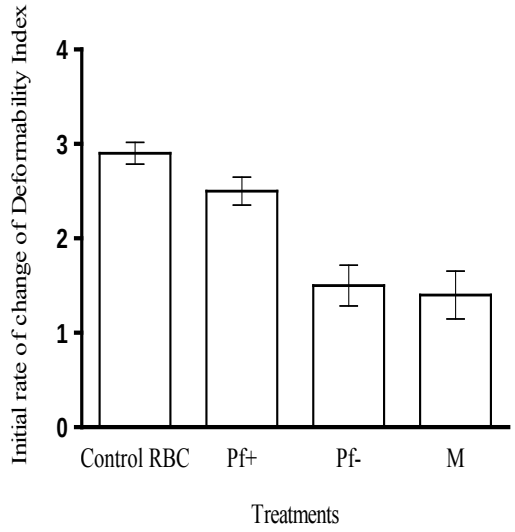


**Figure 4.** DIinitial values for the various treatments. All values are reported as Mean ± S.D

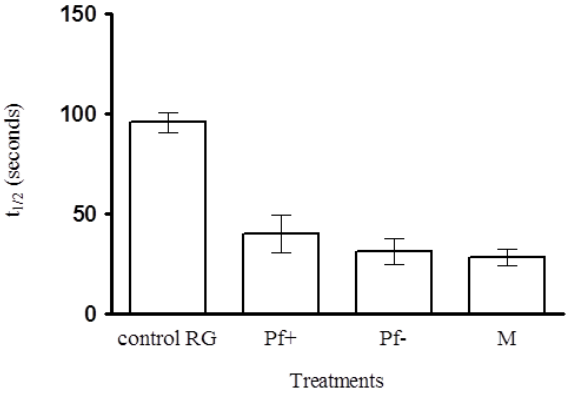


**Figure 5**. $T_{50}$ values for the various treatments. All values are reported as Mean with Range

## 4. Discussion

The effects of a variety of agents on RBC membrane deformability and stability have been studied previously (Hardeman & Ince, 1999; Beder *et al*., 2002; Tadesse *et al.,* 2004). However, the effect of *P. falciparum* culture supernatant on RBC membrane mechanical properties of deformability and stability, has not been established. The present study shows that RBC ghosts prepared in the presence of MgATP attained higher $DI_{max}$ values than ghosts prepared without MgATP, as indicated by the membrane stability profile (Fig. 2). This may possibly have to do with the ATP-dependent discocyte configuration, as ATP depletion of RBCs has been associated with discocyte-echinocyte transformation (Palek, Stewart, & Lionetti, 1974). The mechanism by which RBCs maintain their biconcave shape has been the subject of numerous studies. One of the critical factors for the maintenance of biconcave shape is the level of RBCs ATP (Weed *et al.* 1969; Szasz, 1970). We have used a laser diffraction method (ektacytometry) to directly measure changes in RBC membrane indices of deformability and stability. This technique was validated by subjecting resealed RBC ghosts to manipulations known to modify the membrane shear modulus (Heath *et al.*, 1982). Our results show that, compared to control untreated RBC membranes, ~~a~~ there was a reduction in RBC membrane deformability upon pretreatment with spent parasite culture supernatant (Pf(+)), malaria culture medium (Pf(-)), and malaria culture medium containing uninfected RBCs (M). The reduction in RBC membrane deformability by spent supernatant from *P. falciparum* cultures is consistent with the observation that in *falciparum* malaria, the deformability of the entire RBC population is reduced in proportion to disease severity (Nuchsongsin *et al.*, 2007). It has been found that in patients with severe *falciparum* malaria, the entire RBC mass, containing both uninfected and infected RBCs, becomes rigid (Cooke, Mohandas, & Coppel, 2004; Parker *et al.*, 2008; Nuchsongsin *et al.*, 2007). Several mechanisms such as hemin-induced oxidative damage of the RBC membrane, alterations in the phospholipid bilayer, and attached spectrin network have been proposed as being responsible for the increased rigidity and reduced deformability of the RBCs infected with *falciparum* malaria (Parker *et al.*, 2008; Nuchsongsin *et al.*, 2007). The cause of rigidity of RBCs under *in vitro* culture conditions, however, is not well-defined, but has been attributed, according to one study, to the discharge of a multitude of proteins from infected RBCs into the culture media (Naumann *et al.*, 1991), some of which could be involved in modulating the deformability of uninfected RBCs *in vitro*. Our results indicate that both Pf(-) and M-treated RBCs caused a greater reduction in RBC membrane deformability than Pf(+)-treated RBCs. This difference in membrane deformability is most likely due to the depletion of one or more of these components during parasite cultivation. As expected, our results reveal that the control RBC ghosts had the highest membrane stability. This is due to the intact membrane skeleton that is devoid of any modulation. However, the RBC membrane stability was reduced significantly after exposure to malaria culture medium, but was partially restored as time progressed. Data from both pathologic membranes and biochemically perturbed membranes have elucidated that RBC deformability and stability are not related to one another, as decreased deformability can be associated with either increased or decreased membrane stability. (Chasis and Mohandas, 1986). These findings imply that different skeletal protein interactions may regulate RBC membrane deformability and stability.

Although multiple parasite ligand-erythrocyte receptor interactions must occur for successful *Plasmodium* invasion of the human red cell (Montero, Rodriguez, Oksov & Lobo, 2009), it is probable that a transient change in RBC membrane stability is essential for *P. falciparum* invasion of the RBC or its survival within the micro-environment of the *in vitro* culture. This change may occur independently of changes in the RBC membrane deformability.

## 5. Conclusion

In this study, we have shown that malaria culture media and spent parasite culture supernatant can modify the stability and deformability of the RBC membrane. However, these two parameters do not seem to be related. Although medium change is essential for the sustenance of *P. falciparum in vitro*, the premise that modulation of RBC membrane mechanical properties may be indispensable for *P. falciparum* invasion, requires further elucidation.

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**Authors' Contributions**

NAA and YT designed the study. NAA was responsible for the study implementation and data collection. NAA and YT analyzed the data. NAA wrote the paper and all authors reviewed the manuscript.

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# The Antihyperglycaemic Effect of the Aqueous Extract of *Origanium vulgare* Leaves in Streptozotocin-Induced Diabetic Rats

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**Abstract**

The current study aimed to investigate the antihyperglycaemic effect of aqueous extract of *Origanum vulgare* (oregano, OV) leaves in streptozotocin (STZ) induced diabetic rat. Streptozotocin induced diabetic rats showed significant ($P < 0.05$) increase in the levels of blood glucose, glycosylated haemoglobin, pancreatic amylase, liver and kidney weights /body weight ratios, urea, uric acid, creatinine and significant decrease in the levels of plasma insulin, liver and muscle glycogen and body weight. Oral administration of the aqueous extract of OV leaves (20 mg/kg) produced a significant decrease in blood-glucose levels, glycosylated haemoglobin, pancreatic amylase in STZ diabetic rats ($P < 0.05$) in comparison with standard drug Glibenclamide (GB) (0.9 mg/kg body weight). Treatment with the aqueous extracts of OV leaves decreased liver weights/body weight ratios in diabetic rats, while kidney weight/body weight ratios, urea, uric acid, creatinine levels were partially improved. Oral administration of the aqueous extract of OV leaves (20 mg/kg) improved the reduction in serum insulin, liver and muscle glycogen contents and body weight in STZ diabetic rats. The data in the present study may support the use of *O. vulgare* plants as traditional remedies for the treatment of diabetes mellitus.

**Keywords**:*Origanum vulgare*, streptozotocin, glycosylated haemoglobin, glucose,insulin, rats**.**

## 1. Introduction

Diabetes mellitus is a chronic metabolic disease which now afflicts 10 % of the world population. It is considered a "modern-day epidemic" and is rightly recognized as a global public health issue (Gispen and Biessels, 2000; Burke *et al*., 2003). In recent years, there has been renewed interest in the treatment of diabetes using herbal drugs, which are generally non-toxic. World Health Organization has also recommended the evaluation of the effectiveness of plants in condition where we lack safe modern drugs. Plant derivatives with hypoglycaemic properties had been used in folk medicine and traditional healing systems around the world from very ancient times (Yeh *et al*., 2003).

*Origanum vulgare* (oregano, OV) is a member of the plant family Lamiaceae, the genus'*Origanum* is an annual, perennial and shrubby herb that is native to the Mediterranean, Euro-Siberian and Irano-Siberian regions (Aligiannis *et al*., 2001). Oregano contains oleanolic and ursolic acids, flavonoids and hydroquinones, caffeic, rosemarinic, and lithospermic acid, tannins, and phenolic glycosides. Phenolic compounds represent 71% of the total oil. The polar phenols' thymol and carvacrol are responsible for many of the properties of the essential oil, as well as p-cymene and terpinene (Dadalioglu and Evredlik, 2004; Giordani *et al*., 2004; Koukoulist *et al*., 2005; Tampieri *et al*., 2005; Bozin *et al*., 2006). The main known pharmacological activities of OV were antibacterial (Nazia *et al*., 2007) antifungal (Portillo-Ruiz *et al*., 2005) antiparasitic (Force *et al*., 2000) anti-thrombin (Goun *et al*., 2002) anti-oxidant ( Stashenko *et al*., 2002 ) and anti-inflammatory (Ocaña-Fuentes *et al*., 2010). There are also some reports regarding the antimutagenic and anticarcinogenic effect of oregano; representing an alternative for the potential treatment and/or prevention of certain chronic ailments, like cancer (Arcila-Lozano *et al*., 2004).

Oregano is a powerful antioxidant. It contains several potent antioxidants that may contribute to the findings in preliminary studies that oregano exhibits benefits towards the cardiovascular and nervous systems and relieves inflammation and modulates blood sugar and lipids (Singletary, 2010).

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The aim of this study was directed to determine the antihyperglycaemic effect of OV extract in streptozotocin diabetic rats. Glibenclamide (GB) was used as standard antihyperglycaemic drug.

## 2. Materials and Methods

Streptozotocin was purchased from Sigma (St. Louis, MO, U.S.A.) , glibenclamide (gift from Pharmacy, University hospital, Jeddah, Saudi Arabia). Dried leaves of *O.vulgare* were purchased from an authentic source at local market (Jeddah, Saudi Arabia) .

### *2.1. Preparation of aqueous extracts*

Plant material was prepared according to Eddouks *et al*., 2003: 1g of powdered leaves mixed with 100 ml distilled water was boiled for 10 min. and then cooled for 15 min.There after, the aquaeous extract was filtered using a Millipore filter (Millipore 0.2 mm) to remove particulate matter. The filterate was then freeze- dried and the desired dose ( mg of lyophilized aquaeous extract of OV leaves per kg body weight ) was prepared and reconstituted in 1.5 ml of distilled water. The aquaeous extracts were prepared daily, just before administration. The extracts obtained were then given orally to different groups of rats at a dose of 20 mg /kg body weight.

### *2.2. Experimental design*

The experimental animals were obtained from the Animal Unit of King Fahd Medical Research Center, King Abdul Aziz University, Jeddah, Saudi Arabia. Animals were housed under environmental conditions ( 23 ± 1ºC , 55 ± 5% humidity and 12-h light :12- h dark cycle ) and maintained with free access to water and a standard chow diet. Diabetes was induced by an intraperitoneal injection of streptozotocin at a dose of 45 mg/kg body weight dissolved in a citrate buffer (0.1M, pH 4.5) (Burcelin *et al*., 1995 ). After 3 days the rats with fasting blood-glucose levels more than 200 mg/dL were considered as diabetic and selected for the study. All the pharmacological experiments were carried out after obtaining approval of the Institutional animal ethics Committee of King Abdulaziz University, Saudi Arabia.

The preliminary studies of different doses (20,40,60 mg /kg) showed that the most effective dose of oregano is 20 mg /kg. The animals were randomly divided into five groups of 12 animals each. Group I (untreated controls): normal rats receiving water and fed *ad libitum* and served as a control group. Group II (untreated diabetics): diabetic rats receiving water and fed *ad libitum* and served as diabetic control rats. Group III (treated controls): normal rats receiving water and fed *ad libitum* and oregano at 20 mg /kg body weight (Lemhadri *et al*.,2004). Group IV (treated diabetics): diabetic rats receiving water and fed *ad libitum* and oregano 20mg /kg body weight. Group V ( treated diabetics ):diabetic rats receiving water and fed *ad libitum* and antidiabetic drug (glibenclamide). Rat equivalent dose of glibenclamide was calculated using conversion table devised by Paget and Barnes (1964) and was 0.9 mg/kg body weight.

The drug treatment solutions were administered orally by gastric intubation using a syringe once daily at 08:00 a.m. The effect of OV aqueous extracts or glibenclamide on blood glucose was determined in fasted rats, after 2 and 6 weeks of once daily repeated oral administration (20 mg/kg). The body, liver and kidney weights of all rats were measured at weeks 2 and 6.

### *2.3. . Biochemical analysis*

At the end of experiment, rats were anaesthetized and blood samples were collected from the tail vein. Fasting blood-glucose level was measured in the whole blood after 12 h fasting. Glycosylated haemoglobin (HbA1-test) was estimated in whole blood by fast ion – exchange resin separation method (Nuttall, 1998). Serum was separated, and insulin and pancreatic amylase were determined according to methods of Finlay and Dillard (2007) and Winn-Deen *et al*.(2008), respectively. Liver and muscle glycogen contents were determined by the method of Huijing (1970). Urea, uric acid and creatinine were estimated by using the F-200 fluorescence spectrophotometer in serum (Newman and Price, 2001).

### *2.4. Statistical analysis*

Values reported are expressed as mean ± SE. Statistical significance of the difference between groups was determined by one-way analysis of variance test (ANOVA). The values were considered to be significantly different when the *P* value was less than 0.05 (Zar, 1996).

## 3. Results

In this study, the level of blood glucose, glycosylated haemoglobin (HbA1C), insulin after using a 45 mg/kg dose of streptozotocin, ensured induction of diabetes in rats (figures 1,2,3). The blood-glucose levels rose markedly after STZ administration, and the high glucose levels were maintained for 6 weeks (Fig 1). Oral administration of the aqueous OV extracts (20 mg/kg) produced a significant decrease (81.65±1.049) in blood-glucose levels in STZ diabetic rats (526.80 ±7.889 ) ($P < 0.05$). Treatment with glibenclamide (GB), showed reduced blood-glucose levels as compared to control group.

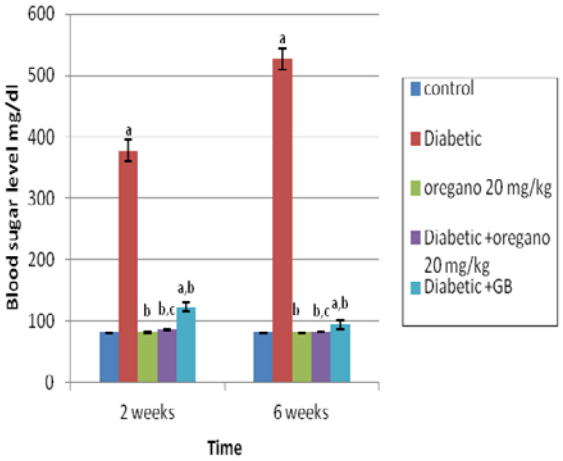


**Figure 1**. Effect of aqueous extracts of oregano leaves (20 mg/kg) on blood-sugar level (mg/dl) - Data are expressed as the mean ± SE. Each value corresponds to a mean of 6 animals. a, comparison of Group I vs Group II .b ,comparison of Group III, Group IV & Group V vs Group II. c, comparison of Group IV vs Group V at $P<0.05$.

In the untreated diabetic animals, the initial HbA1C value increased significantly (3.634 ±0.0093) compared to the control. In the oregano treated diabetic groups (20 mg/kg) the HbA1C value return to the normal value as compared to control group (Fig.2).

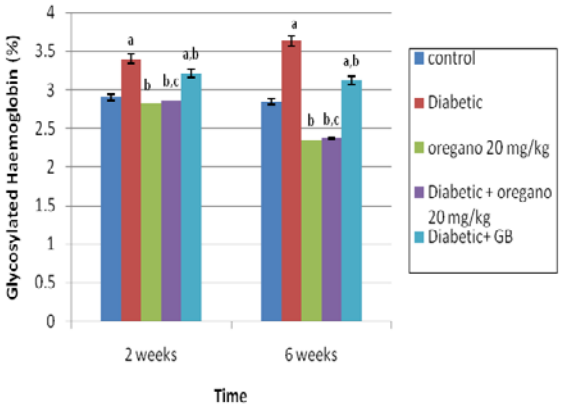


**Figure 2**. Effect of aqueous extracts of oregano leaves (20 mg/kg) on glycosylated haemoglobin (%) - Data are expressed as the mean ± SE. Each value corresponds to a mean of 6 animals. a, comparison of Group I vs Group II .b ,comparison of Group III, Group IV & Group V vs Group II. c , comparison of Group IV vs Group V at *P<0.05*.

On the other hand, the serum insulin levels decreased markedly after STZ administration. Oral administration of the aqueous OV extracts (20 mg/kg) or GB partially improved insulin levels in STZ diabetic rats ($P < 0.05$) after 2 weeks. After 6 weeks, oral administration of the aqueous OV extracts (20 mg/kg) significantly improved insulin levels in STZ diabetic rats ($P < 0.05$) in comparison with standard drug GB as shown in figure 3.

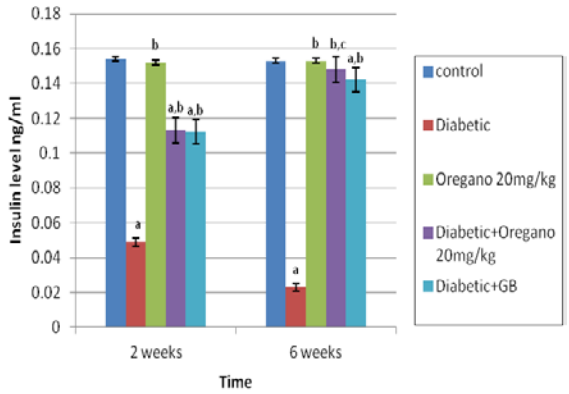


**Figure 3**. Effect of aqueous extracts of oregano leaves (20 mg/kg) on Insulin (ng/ml). Data are expressed as the mean ± SE. Each value corresponds to a mean of 6 animals. a , comparison of Group I vs Group II .b ,comparison of Group III, Group IV & Group V vs Group II. c , comparison of Group IV vs Group V at *P<0.05*.

Figures 4 and 5 revealed marked depletion in the liver and muscle glycogen contents ($P < 0.05$) in STZ-induced diabetic rats compared to control. The administration of OV (20 mg/kg) or GB for six weeks significantly ($P < 0.05$) increased the liver and muscle glycogen contents in diabetic rats compared to untreated diabetic and control groups. It was also noticed that OV (20 mg/kg) control groups showed a significant increase in liver and muscle glycogen contents (3.12 ± 0.174 and 0.594 ± 0.0209) throughout the experimental period.

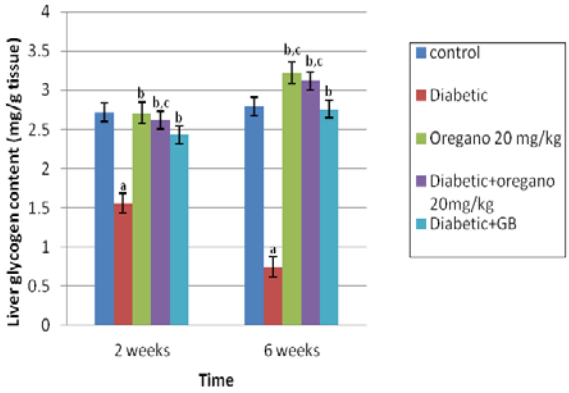


**Figure 4**. Effect of aqueous extracts of oregano leaves (20 mg/kg) on Liver glycogen content (mg/g tissue) - Data are expressed as the mean ± SE. Each value corresponds to a mean of 6 animals. a, comparison of Group I vs Group II .b, comparison of Group III, Group IV & Group V vs Group II. c, comparison of Group IV vs Group V at *P<0.05*.

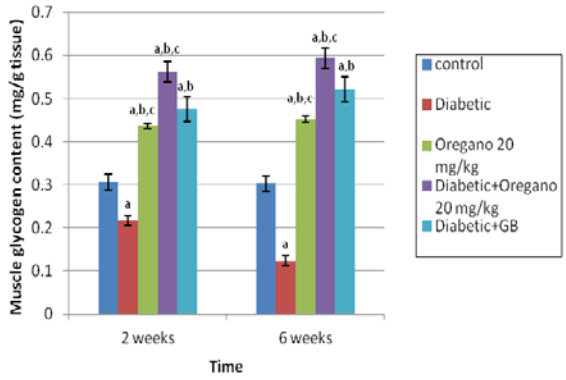


**Figure 5**. Effect of aqueous extracts of oregano leaves (20 mg/kg) on muscle glycogen content (mg/g tissue) - Data are expressed as the mean ± SE. Each value corresponds to a mean of 6 animals. a, comparison of Group I vs Group II .b, comparison of Group III, Group IV & Group V vs Group II. c, comparison of Group IV vs Group V at *P<0.05*.

In the STZ-treated groups, the pancreatic amylase level was significantly increased. Oral administration of the aqueous OV extracts (20 mg/kg) administration produced a significant decrease in pancreatic amylase levels in STZ diabetic rats ($P < 0.05$) as compared to control group (Table 1).

**Table1**. Effect of aqueous extracts of oregano leaves (20 mg/kg) on Pancreatic amylase.

| parameter / Group | Pancreatic amylase(U/L) | |
|---|---|---|
| | 2nd week | 6th week |
| Control | 243.80 ±0.707 | 242.80 ± 0.735 |
| Diabetic | 365.42 ± 2.315 [a] | 287.20 ± 0.583 [a] |
| Oregano(20 mg/kg) | 235.20 ±0.860 [a, b] | 237.60 ± 0.927 [b] |
| Diabetic + Oregano(20 mg/kg) | 232.20 ± 0.718 [a, b] | 246.00 ± 1.140 [b,c] |
| Diabetic + GB | 242.20 ± 1.772 [b] | 252.60 ±0.926 [a, b] |

- Data are expressed as the mean ± SE. Each value corresponds to a mean of 6 animals. a, comparison of Group I vs Group II .b, comparison of Group III, Group IV & Group V vs Group II. c, comparison of Group IV vs Group V at *P<0.05*.

The mean body weight of rats was shown in table 2. Results showed that weight of diabetic controls significantly decreased during the experimental period while normal controls and OV extract rats gained significant weight ($P < 0.05$). The diabetic group given oregano leaves extract (20 mg/kg) or GB administration maintained weight gain. The liver weight/body weight ratios have been shown to increase in diabetic rats significantly ($P < 0.05$) in comparison to control animals (Table 2). This increase was completely reversed by OV leaves extract (20 mg/kg) administration and partially improved by GB. STZ-induced diabetes caused a significant ($P < 0.05$) increase in kidney weight/body weight ratios in comparison to control. This enhancement was partially improved by OV (20 mg/kg) or GB (Table 2).

**Table 2**. Effect of aqueous extracts of oregano leaves on body weight ,liver and kidney weights/body weight ratios in rats

| parameter / Group | body weight (g) | | Liver weight /ody weight | | Kidney weight / body weight | |
|---|---|---|---|---|---|---|
| | 2nd week | 6th week | 2nd week | 6th week | 2nd week | 6th week |
| Control | 210.00 ± 1.303 | 235.00 ± 1.612 | 0.03268 ±0.00029 | 0.03278 ±0.00025 | 0.00420 ±0.00049 | 0.00419 ±0.00005 |
| iabetic | 186.00 ± 4.703 [a] | 172.00 ± 1.327 [a] | 0.03992 ±0.00062 [a] | 0.05273 ±0.00115 [a] | 0.00602 ±0.00022 [a] | 0.00612 ±0.00026 [a] |
| regano (20 mg/kg) | 205.00 ± 1.581 [b] | 236.00 ± 1.095 [b] | 0.03042 ±0.00013 [b] | 0.03321 ±0.00070 [b] | 0.00414 ±0.00004 [b] | 0.00412 ±0.00010 [b] |
| iabetic +Oregano (20 mg/kg) | 202.00 ± 1.140 [b,c] | 225.00 ± 2.509 [b,c] | 0.03290 ±0.00024 [b,c] | 0.03268 ±0.00738 [b,c] | 0.00519 ±0.00010 [a, b] | 0.00490 ±0.00043 [a, b] |
| iabetic + GB | 196.00 ± 1.673 [a] | 211.00 ±2.881 [b] | 0.03814 ±0.00153 [a, b] | 0.04267 ±0.02059 [a, b] | 0.00544 ±0.00021 [a, b] | 0.00493 ±0.00010 [a, b] |

- Data are expressed as the mean ± SE. Each value corresponds to a mean of 6 animals. a, comparison of Group I vs Group II .b, comparison of Group III, Group IV & Group V vs Group II. c, comparison of Group IV vs Group V at $P<0.05$.

The results in Table 3 showed significant increase ($P < 0.05$) in the level of urea, uric acid and creatinine, which are markers of renal dysfunction in the diabetic group compared to control group. This enhancement was partially improved by OV (20 mg/kg) or GB (Table 3).

**Table 3**. Effect of aqueous extracts of oregano leaves on urea , uric acid and creatinine in rats

| parameter / Group | Urea (mg/dl) | | Uric acid (mg/dl) | | Creatinine (mg/dl) | |
|---|---|---|---|---|---|---|
| | 2nd week | 6th week | 2nd week | 6th week | 2nd week | 6th week |
| Control | 30.40 ±2.461 | 30.60 ±2.021 | 1.24 ±0.040 | 1.30 ±0.032 | 0.30 ±0.010 | 0.31 ±0.020 |
| Diabetic | 59.21 ±6.406 [a] | 65.43 ±5.137 [a] | 1.94 ±0.323 [a] | 2.52 ±0.902 [a] | 0.72 ±0.096 [a] | 0.94 ±0.137 [a] |
| Oregano (20 mg/kg) | 32.00 ±3.284 | 33.00 ± 3.153 | 1.32 ±0.583 | 1.34 ±0.058 | 0.32 ±0.157 | 0.33 ±0.155 |
| Diabetic +Oregano (20 mg/kg) | 53.00 ±2.030 [a, b] | 49.00 ±2.144 [a, b] | 1.84 ±0.340 [a, b] | 1.60 ±0.045 [a, b] | 0.65 ±0.030 [a, b] | 0.56 ±0.144 [a, b] |
| Diabetic + GB | 55.00 ±3.112 [a, b] | 52.20 ±3.138 [a, b] | 1.86 ±0.082 [a, b] | 1.65 ±0.178 [a, b] | 0.67 ±0.112 [a,b] | 0.58 ±0.138 [a, b] |

- Data are expressed as the mean ± SE. Each value corresponds to a mean of 6 animals. a, comparison of Group I vs Group II .b, comparison of Group III , Group IV & Group V vs Group II. c, comparison of Group IV vs Group V at $P<0.05$.

## 4. Discussion

Inspite of the presence of known antidiabetic medicine in the pharmaceutical market, herbal drugs are frequently considered to be less toxic and also free from side effects, than synthetic ones. In the present study, the administration of streptozotocin (45mg/kg) induced hyperglycaemia in rats. Treatment of diabetic rats with oregano at 20 mg/kg showed a significant decrease in the blood-sugar level. This may be due to that oregano enhance the insulin sensitivity of the receptors on cells, leading to reduced levels of blood sugar and more energy production. The accumulating evidence suggests that modulation of insulin secretion and/or insulin action mechanisms could be involved in the antidiabetic effect of oregano. This evidence was confirmed with Talpur *et al*. (2005) who reported that extracts of oregano improve blood sugar levels by enhancing insulin sensitivity. Also,the hypoglycaemic effect of oregano may be due to the interference on absorption of dietary carbohydrates in small intestine or stimulation of glucose utilization by peripheral tissues. In line with this evidence of this study, Maghrani *et al*. (2003) and Ortiz-Andrade *et al*. (2007) reported that the hypoglcaemic action of medicinal plant may be due to a reduction in the intestinal absorption of glucose and/or inhibiton of tubular glucose reabsorption. Oregano leaves contain phenolic glucosides that help control blood sugar as reported by Takeda *et al*. (2008).

The level of glycosylated haemoglobin (HbA1C) has been shown to be an important parameter of chronic glycaemic control in patients with DM, an elevated HbAlc almost always indicates DM (The International Expert Committee, 2009). The present data showed that, the high levels of HbAlc in diabetic rats were significantly lowered by the treatment with aqueous extract of oregano leaves. Decreased HbA1C levels in the treated diabetic rats could be due to an improvement in insulin secretion from the remnant pancreatic β-cells in diabetic rats, consequently, resulting in improvement in glycemic control (Vinay *et al*., 2010).

Increased insulin level in diabetic rats after treatment with the oregano treatment (Fig. 3) this may be due to the bioactive molecules present in oregano leaf extract that may probably stimulate the β cells of the pancreas to produce insulin. Furthermore, this effect may be due to that oregano has been shown to have an insulin-like biological activity. This explanation agrees with that reported by Broadhurst *et al.* (2000) who showed that the positive effects of oregano extracts on insulin activity suggest a possible role of this plant in improving glucose and insulin metabolism. In contrast, Lemhadri *et al*. (2004) concluded that aqueous extract of OV (20 mg/kg) exhibits an anti-hyperglycaemic activity in STZ rats without affecting basal plasma insulin concentrations.

Glycogen is the primary intracellular storable form of glucose and its levels in various tissues, especially skeletal muscles are direct reflection of insulin activity. The observed decrease in hepatic and muscle glycogen may be due to insufficient insulin and inactivation of the glycogen synthetase system in the diabetic state (Vinay *et al*., 2010). However, after the treatment with oregano, there was a significant increase in the liver and muscle glycogen levels

in the diabetic rats. The increased hepatic glycogen level in the treated diabetic rats may be due to increased level of insulin, which has increased glycogenesis and decreased glycogenolysis and gluconeogenesis. Thus antihyperglycemic effect of OV may be due to protection of surviving pancreatic β cells, increase in insulin secretion and glycogen storage (Jagtap & Patil, 2009). Inhibitory effects on glycogenolysis have been reported for glibenclamide in the presence of insulin after stimulation of glycogenolysis by glucagon (Carvalho-Martini *et al*., 2006).

The increment in pancreatic amylase in diabetic rats was antagonized by oregano treatment. The inhibition of pancreatic amylase delay carbohydrate digestion and protract overall carbohydrate digestion time, resulting in the reduction in glucose absorption rate and consequently, dulling the postprandial plasma glucose rise. Several indigenous medicinal plants have a high potential in inhibiting pancreatic amylase enzyme activity (Valiathan, 1998). Mc Cue *et al*. (2004) reported that extracts of clonal oregano lines have strong inhibitory activity against porcine pancreatic amylase (PPA) *in vitro*. MC Cue and Shetty (2004) reported the ability of rosmarinic acid, one of the principal phenolic components of oregano, to inhibit porcine pancreatic amylase (PPA) activity. One of the potentially important components of anti-diabetic activity by oregano extracts is mild amylase inhibition by phenolic antioxidants that contribute management of hyperglycemia (Mc Cue *et al*., 2004).

Weight loss has been known to be one of the symptoms of DM. Similar observations were detected in many experimental studies (Al-Attar & Zari, 2007; Subash-Babu *et al*., 2008; Sellamuthu *et al*., 2009 and Salahuddin *et al*., 2010). In the diabetic control rats, deficiency of insulin led to decreased amino acids uptake by tissues with a consequent reduction in the level of protein synthesis. Also insulin deficiency results in lipolysis in adipose tissues and protein breakdown (Vasudenvan & Sreekumari, 2007). The increase in weight observed in the group treated with oregano, and normal control group may be a reflection of efficient insulin action. The liver and kidney weights /body weight ratios in the diabetic groups were higher than those of the normal groups (Table, 2) suggesting the occurrence of the edema and inflammation of these organs as reported by Kamath and Rajini (2006). High concentrations in serum urea, uric acid, and creatinine strongly suggested impairment of kidney function in diabetic rats. Similar effect was recorded previously by Alarcon *et al*. (2005) Jaya *et al*. (2010) and Manikandaselvi *et al*. (2012). The present data indicated that the oregano supplement has a partial renoprotective effect. These results are in agreement with other previous studies by Khan *et al*. (2011) who stated that oregano showed antiurolithic activity both *in vitro* and *in vivo* models in addition to its antioxidant, renal epithelial cell protective, antispasmodic and diuretic activities. These different activities observed in the crude extract might be due to the presence of flavonoids that were known to possess diuretic activities (Ramamoorthy *et al*., 2010).

Oregano has long been used in traditional medicine in the treatment of common ailments and have been potential for positive modulation of oxidation-linked diseases such as diabetes. Flavonoids are considered as active principles in many medicinal plants and natural products with a positive effect for human health (Wollenweber, 1988). These natural compounds could act separately or synergistically to cause the hypoglycaemic effect. This could not exclude the intervention of other phytochemical constituents as bioactive hypoglycaemic agents. Most of oregano's effects on the body are due to its high content of antioxidants, which play a role in destroying the production of free radicals (Spiridon *et al*., 2011) and modulates blood sugar and lipids (Singletary, 2010).

## 5. Conclusion

The obtained results may support the use of oregano as culturally adopted treatments for insulin resistance and hyperglycemia and support its inclusion as a natural, safe, anti-diabetic therapy for modulation of Type 2 diabetes mellitus.

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# Fecundity of Bigfin squid, *Sepioteuthis lessoniana* (Lesson, 1830) (Cephalopoda: Loliginidae)

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**Abstract**

A total of 25 mature female *Sepioteuthis lessoniana* (Lesson, 1830) specimens, with a dorsal mantle length between 120-196 mm, were investigated. Estimated potential fecundity varied from 180 to 1054 eggs with mean value of 497 eggs. The potential fecundity values of *S. lessoniana* in the Mandapam waters (Palk Bay) are found almost similar to those found in Tanzanian waters but higher than the ones in Sri Lankan lagoons. Larger females have higher potential fecundity. The correlation values showed that the fecundity is more closely related to the ovary weight and body weight than to the mantle length. Fecundity increased exponentially with mantle length. The Nidamental gland weight was also found more closely related to the ovary weight in logarithmic relationship. Fecundity vs. ovary weight (r = 0.899) and Nidamental gland weight vs. ovary weight (r = 0.942) exhibited a strong relationships while fecundity showed week correlations with mantle length (r = 0.658) and body weight (r = 0.663).

**Keywords:** *Sepioteuthis lessoniana*, fecundity, Mandapam Waters (Palk Bay).

## 1. Introduction

Cephalopods (squid, cuttlefish and octopus) form increasingly essential resources for human consumption and a chief food for many top predators (Lefkaditou *et al*., 2003). Adult cephalopods are voracious and active carnivores feeding mainly on fishes and crustaceans. Loliginid squids play an important role in near-shore ecosystems both as prey and as predators (Jackson and Pecl, 2003).

The squids and cuttlefish have fewer eggs production compared to the finfishes and prawns (Nabhitabhata, 1995). There are very few reports of fecundity estimates of Indian Cephalopods (Asokan, 2000). With the increasing trawling activity and targeting the cephalopod, the cephalopods need regular monitoring to avoid a possible collapse of the fishery. In general, fecundity is low in cephalopods because of the absence of larval stage and hatchlings are virtually in miniature adults.

There are controversies in estimating cephalopod fecundity because of the different spawning strategies of cephalopods: single synchronous terminal spawning, or multiple spawning in which several batches are spawned. Therefore, it is better to use potential fecundity where the maximum number of eggs prior to spawning is counted (Boyle and Rodhouse, 2005).

In the past, many studies were conducted on the fecundity of other squids elsewhere (Macewicz *et al*., 2004; Salman and Onsoy, 2004; Laptikhovsky and Nigmatullin, 2005; Salman and Laptikhovsky, 2005; Nigmatullin and Markaida, 2009; Salman and Onsoy, 2010). Previous fecundity studies on *S. lessoniana* have been done in tropical regions such as Tanzania (Mhitu *et al*., 2001), Sri Lanka (Sivashanthini *et al*., 2010). No data on the fecundity of *S. lessoniana* in the Palk Bay waters is currently available. Hence, the objective of the work reported here is to study potential fecundity and various logarithmic relationships of fecundity in mature females of squid, *S. lessoniana* in this region.

## 2. Materials and Methods

A total of 25 mature females with dorsal manltle length (DML) between 120-196 mm and body weight (BW) between 118.3-406.1g were used for estimation of potential fecundity.

Samples were collected from commercial bottom trawlers of Mandapam landing centre (Palk Bay) between 2009-2010 (Figure 1).

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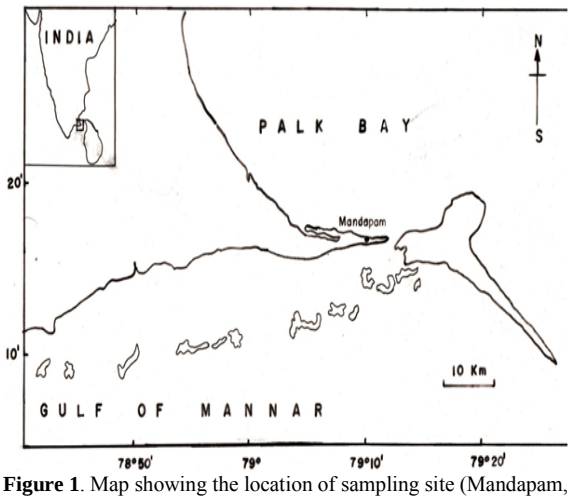


**Figure 1**. Map showing the location of sampling site (Mandapam, Palk Bay).

Collected specimens were kept in ice box and transferred to laboratory for the detailed study. For each animal, the DML was measured to the nearest 1mm and BW to the nearest 0.1g. After dissecting, gonad and nidamental glands were weighed to the nearest mg. The ovary comprises the proximal and distal oviducts where the former is chiefly a store for mature oocytes and the latter a store for immature and maturing oocytes (Lipinski and Underhill, 1995). Potential fecundity of mature squid was estimated by sub-sampling the eggs from both the proximal and distal oviducts throughout the postmonsoon sampling period i.e. January - June 2010. Eggs from both the oviducts were sub-sampled to about 0.1 - 0.5 g with eggs extracted in three regions (anterior, middle and posterior position) to minimize the variation due to the different stages of development of oocytes. Two or more subsamples were taken from each ovary, and the numbers of eggs in the samples were counted in a large petriplate by adding Gilson fluids with the help of stereo zoom microscope. Gilson fluid was prepared by mixing 10 ml 60% alcohol, 15 ml 80% nitric acid, 18 ml glacial acetic acid and 20 g mercuric acid with 880 ml of distilled water.

The total number of eggs from the known weight of the samples and the full ovary is calculated by the following formula;

Fecundity = (N X TOW)/SOW

Where, N = number of eggs in the sub-sample, TOW = Total ovary weight and SOW = sample ovary weight. The mean estimate of the number of eggs obtained in all individuals comprises the fecundity estimate of the species.

Regression analysis was used to estimate the relationship between fecundity (F) and the DML, BW, TOW and nidamental glands weight (NGW).

## 3. Results

Fecundity varied from 180 to 1054 in the individuals of DML of 120 to 196 mm (Table 1). The mean fecundity of *S. lessoniana* estimated from these samples was 497. Larger females have higher potential fecundity.

**Table 1**. Observations of potential fecundity in mature females of *S. lessoniana*.

| Sl. No. | DML (mm) | BW (g) | TOW (g) | F (Nos) |
|---|---|---|---|---|
| 1 | 120 | 118.3 | 7.45 | 309 |
| 2 | 125 | 126.1 | 1.982 | 187 |
| 3 | 126 | 140.8 | 6.44 | 342 |
| 4 | 127 | 143.5 | 2.185 | 180 |
| 5 | 132 | 142.2 | 5.463 | 221 |
| 6 | 133 | 183.0 | 13.279 | 744 |
| 7 | 134 | 175.7 | 8.24 | 477 |
| 8 | 135 | 158.2 | 8.21 | 316 |
| 9 | 136 | 170.1 | 16.72 | 912 |
| 10 | 138 | 166.9 | 1.41 | 167 |
| 11 | 141 | 166.4 | 3.975 | 300 |
| 12 | 142 | 199.3 | 8.05 | 403 |
| 13 | 144 | 200.4 | 8.15 | 411 |
| 14 | 145 | 196.2 | 5.04 | 347 |
| 15 | 148 | 204.6 | 5.14 | 328 |
| 16 | 148 | 240.3 | 16.9 | 357 |
| 17 | 152 | 230.2 | 8.89 | 470 |
| 18 | 153 | 190.7 | 17.254 | 805 |
| 19 | 166 | 270.6 | 9.88 | 400 |
| 20 | 170 | 271.7 | 10.68 | 441 |
| 21 | 170 | 240.4 | 13.794 | 904 |
| 22 | 170 | 270.2 | 27.2 | 918 |
| 23 | 174 | 300.5 | 13.7 | 521 |
| 24 | 194 | 378.5 | 18.44 | 918 |
| 25 | 196 | 406.1 | 21.9 | 1054 |

F, DML, BW, TOW, and NGW were transformed to logarithms (base 10) and by least square method, the following relations were obtained.

### *3.1. Relation between F and DML*

A linear relationship between the F and the DML of the squid existed (r = 0.658) (Figure 2) and regression equation is

Log F = log (-3.1757) + 2.6795 log DML

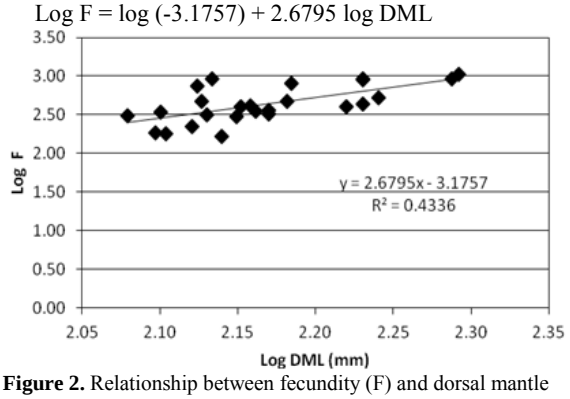


**Figure 2.** Relationship between fecundity (F) and dorsal mantle length (DML)

*3.2. Relation between F and BW*

The relation between the fecundity of squid and the weight of body was linear (r = 0.663) and the regression equation is

Log F = log (0.03) + 1.1315 log BW (Figure 3)

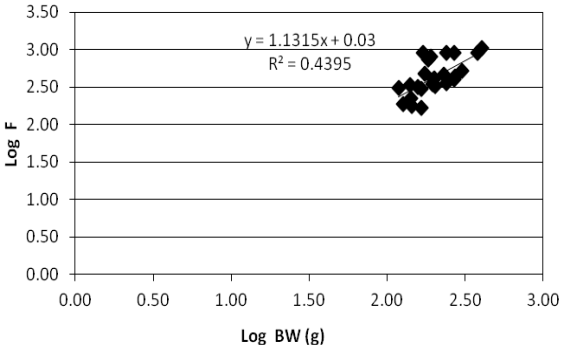


**Figure 3**. Relationship between fecundity (F) and body weight (BW)

*3.3. Relation between F and OW*

The relation between the fecundity of squid and the weight of ovary was linear (Figure 4) and the regression equation is

Log F = log (- 2.3453) + 1.2386 log OW (r = 0.899)

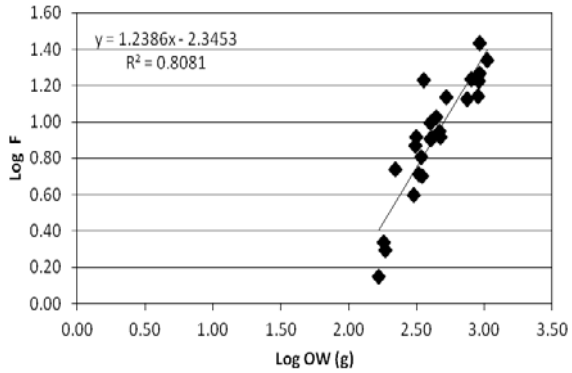


**Figure 4**. Relationship between fecundity (F) and ovary weight (OW)

*3.4. Relation between NGW and OW*

A linear relationship between the NGW and the OW of the squid existed (Figure 5) and regression equation is

Log NGW = log (0.1467) + 0.7657 log OW (r = 0.942)

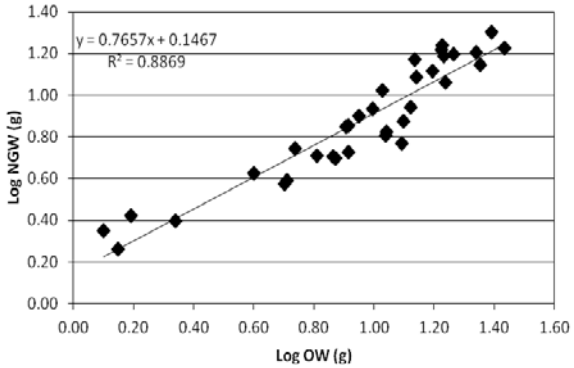


**Figure 5**. Relationship between nidamental gland weight (NGW) and ovary weight (OW)

## 4. Discussion

Knowledge on the fecundity is extremely important from the viewpoint of a successful management and exploitation of the fishery. The estimated fecundity for female *S. lessoniana* from Mandapam coastal waters ranged between 180 to 1054 for individual of 120-196 mm DML with a mean of 497 eggs. The fecundity values shows wide variations within the same species caught from different area. Fecundity for *S. lessoniana* in the Zanzibar coastal waters ranged from 180 to 1180 eggs for individuals of size range 140 –249 mm ML with a mean of 680 eggs (Mhitu *et al.,* 2001) which is higher than the present study. However, *S. lessoniana* of Mandapam water in this study found more fecund than those observed in Sri Lankan's lagoon waters by Sivashanthini *et al.,* (2010). Anon (1975) also estimated a low fecundity of 292 to 754 eggs on the same species in the Philippines and suggested that it may be due to multiple spawning. Tsuchiya (1981) studying in the same species in Okinawa, Japan estimated a lower fecundity of 86 to 728 eggs. However, Segawa (1987) noted the fecundity for the same species from Kominato, Japan ranged between 500 and 1000 eggs in one spawning for individuals of 200 to 250mm DML with a mean of 986 eggs. This lower and higher fecundity of *S. lessoniana* in different places may be due to the difference in short or long spawning period of the individuals in their respective places (Mhitu *et al.*, 2001). The variation in fecundity for the same species may be caused by varying environmental factors, food availability and different habitat (Sivashanthini *et al.*, 2010). In general, fecundity is low in cephalopods because of the absence of larval stage and the hatchlings are virtually miniature adults.

Fecundity increased with DML from 180 to 1054 (Figure 2) suggesting that larger individuals at maturity are likely to contribute more offspring to the next generation than smaller individuals. Nigmatullin and Markaida (2009) studying in jumbo squid *Dosidicus gigas* observed that the fecundity is closely related to female size. He also found that smaller sized female show lower fecundity compared to larger ones. A similar relationship was found in the squids *viz. Abraliopsis atlantica* (Laptikhovsky, 1999), *Loligo vulgaris* (Laptikhovsky, 2000), *L. duvaucelii* (Sang, 2007) and *S. lessoniana* (Mhitu *et al.*, 2001; Sivashanthini *et al.,* 2010). Different sizes of oocytes were observed in *S. lessoniana*, which means that the oocytes do not mature simultaneously. Lipinski and Underhill (1995) reported that there were always certain proportions of immature oocytes situated in distal portion of squid ovary. This indicates that *S. lessoniana* continue to produce eggs even after onset of spawning. This type of spawning pattern is also found in other loliginid squids, such as *L. vulgaris reynaudii* (Sauer and Lipinski, 1990) and *L. vulgaris* and *L. forbesi* (Rocha and Guerra, 1996). In Multiple spawning strategy, large eggs in the ovary causing the oviduct volume to be insufficient to accommodate all eggs. Hence, number of egg masses must be spawned in several batches (Rocha *et al.*, 2001).

There was high variation in oocyte numbers in each size (Table 1). This may be related to multiple spawning in which somatic growth occurs in between the separate batches of egg laying (Harman *et al.*, 1989) or it could be

due to different growth rates among individuals (Rocha and Guerra, 1996; Jackson *et al*., 1997) and environmental factors (Pecl *et al*., 2004).

In the present study the fecundity of *S. lessoniana* bore a logarithmic relationship with the total weight, mantle length and ovary weight. Fecundity exhibited a week relationship with DML (r = 0.658) and BW (r = 0.663). A similar week correlation was also obtained in squids *L. duvaucelii* (Sang, 2007) and *S. lessoniana* (Mhitu *et al*., 2001; Sivashanthini *et al*., 2010). These week correlations could be related to their spawning condition in which some females of similar size have already been laid different numbers of oocytes or multiple spawning strategies. A similar observation has also been reported by Pecl (2001) in *S. lessoniana* and *S. australis* of Australian waters. The correlation values showed that the fecundity is more closely related to the ovary weight and body weight than to the mantle length. The nidamental gland weight of *S. lessoniana* shown a strong logarithmic relationship with the ovary weight (r = 0.942) suggesting that the gland is more closely related to the ovary weight. A similar strong relationship between OW and NGW was also found by Nigmatullin and Markaida (2009). Asokan (2000) also observed the positive relationship between OW and NGW. He observed that the size of the nidamental gland increases with one set of maturation and becomes larger in the mature squids. Neethiselvan *et al*.(2001) studying in squid *Doryteuthis sibogae* found that the weight of gonad and nidamental gland increased during maturation and proved as good indices of maturation.

Maturation was size-related process in female *S. lessoniana* individuals, because OW vs. NGW and F vs. OW, BW, DML was highly correlated. Cephalopods produce lower number of eggs both in absolute numbers and per unit biomass and this reduction in numbers is compensated by reduced mortality during early stages of life cycle arising from greater parental care of the eggs and the shorter planktoinc stage of the life cycle.

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# Effect of Dredging on the Macrozoobenthos of Hazratbal Basin in the Dal Lake Srinagar Kashmir, India

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**Abstract**

In order to assess the effect of dredging on macrozoobenthos community, study was carried out in the Hazratbal Basin of Dal Lake from August 2010 to January 2011. The results showed strongly significant differences ($P<0.05$) between control site and the site which was sampled after dredging operation. The significant differences were also found between pre-dredging and post-dredging ($P = 0.004$), while as, there were no significant differences between control site and pre-dredged site ($P = 0.191$). Altogether 6 taxa were observed at post dredged site in comparison to the pre-dredged (9 taxa) and the control site (10 taxa). At control site, molluscans were found to be the dominant group (1255 ind/$m^2$) followed by annelida (1110 ind/$m^2$) and diptera (330 ind/$m^2$), There were six taxa were shared both sites (control, impact sites) and one taxa *Erpobdella octoculata* of annelida was only restricted to control site. The abundance of most taxa were significantly higher at control and pre-dredged site. Three species (*Lymnea stagnalis, Radix auricularia and Radix ovata)* were completely eliminated after dredging operation. A cluster analysis showed that macrozoobenthic communities revaealed 90% similarity levels between control site and pre-dredged site. After dredging similarity level was greatly declined and was recovered 76% after six months of dredging.

**Keywords:** Macrozoobenthos, dredging effects,Hazratbal Basin, Dal Lake.

## 1. Introduction

Disturbance in ecological term is an event that has profound impact on ecosystem and its resources (Pickett and White 1985). There are various disturbances which are continuously taking place at different levels in lake ecosystem. The dredging operations are long established human induced disturbances in lakes and rivers and are responsible for making change the environmental features of the water bodies (Pranovi *et al*., 1998). Previous studies show that species spectrum of aquatic biota including fish, shellfish and benthic invertebrates depict alteration after dredging processes (Taylor and Salomon 1968, Kenny and Rees, 1996; Lewis *et al*., 2001). In the past different researchers have investigated the impact of dredging on macrozoobenthos and have shown decreased diversity and density in macrobenthic community (Bemvenuti, *et al*., 2005, Johnson and Nelson, 1985; Palmer, *et al*., 2008). In addition, studies have pointed out lower diversity indices in the post dredging period in comparison to undisturbed area (Szymelfenig *et al*., 2006). Considering the important role of the benthic fauna in the secondary production of aquatic systems, any kind of impact upon the macrobenthos may seriously harm the ecology and economy of the aquatic ecosystem, limiting its ability to function as a nursary area (Bemvenuti *et al*., 2005).

In the last 50 years, there has been drastic changes in urban lakes of Kahmir Himalya including Dal Lake. Due to cultural eutrophication, the Dal has got silted up and consequently shrunk in size. A lake conservation plan was devised for the Dal Lake in 1980s and as per the plan dredging of the peripheral areas of the Hazratbal, Nishat and Gagribal basins was started in late 1990s' with the aim of improving the aesthetic value of the inshore area.

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In order to have a first hand information on the impact of dredging on different aquatic communities in this water body , a lake wide study has been initiated in 2010 and shall continue till 2013 . In the present communication an attempt has been made to assess the impact of the dredging programme in the lake on the macrobenthic community (important link in the food cain) living on the soft bottom in Hazratbal Basin of the Dal Lake based on the data procured from August, 2010 to January, 2011.We predicted that dredging will decrease the species diversity and density of macrozoobenthos in the areas where dredging operation was carried out. In addition, we assumed that there could be dominance of somes species after dredging operation (Figure 1-3).

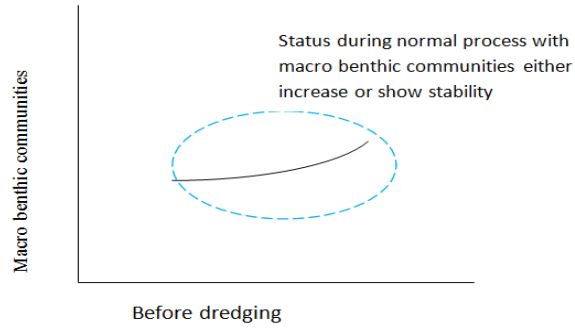


**Figure 1.** Hypothetical concept showing macro benthic growth before the start of dredging operation

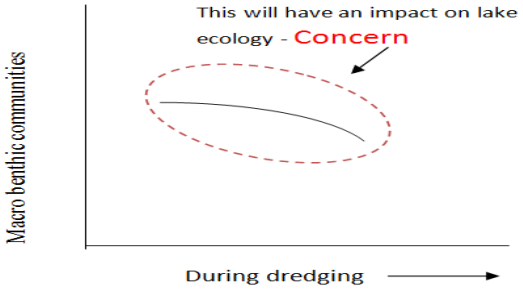


**Figure 2.**Hypothetical concept showing the impact of dredging on macro benthic communities

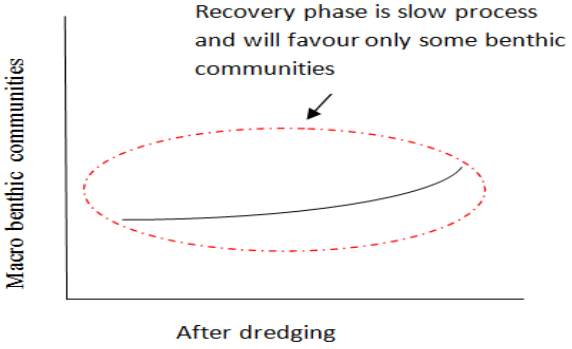


**Figure 3.** Hypothetical concept showing recovery phase after dredging

## 2. Materials and Methods

### *2.1. Study area*

Dal Lake is a Himalayan urban lake, located in the heart of Srinagar (34°18′ N latitude and 74°9′ 1E longitude) at an average altitude of 1583 m. Dal Lake comprises of four basins viz. Hazratbal Bod-Dal, Gagribal and Nageen. A perennial inflow channel known as Telbal Nallah enters the lake from the north in its Hazratbal basin. Another inlet, Bot Kol, also joins in the Hazratbal basin, just west to Telbal stream. The water from this catchment area inclusive of water input from a high altitude lake called Marsar Lake besides this the springs present within the lake basin are also a permanent water source to the lake.

### *2.2. Sampling design*

In order to assess the impact of dredging operation on macrobenthos, two sites (Figure 4) were selected one in the area which was planned to be dredged (called site 1) and the other in the area which was not to get directly impacted by the dredging (called site 2). Ekman dredge (15 × 15 $cm^2$) was used to collect three replicate samples at each site. The unaffected site was sampled on monthly basis for the period August 2010 to January 2011. The site selected for dredging was first sampled before one week of dredging activity and then resampled after 1 day, 3 days, 6 days, 10 days, 20 days, 35 days, 50 days, 70 days, 3 months and 6 months of the dredging activity. The sampling continued till some macrobenthic recovery at the site was observed. In the field, samples were packed in polybags and immediately transferred to laboratory. In the laboratory the samples were carefully washed through 0.55mm mesh screen and preserved in 4% formalin. The sub-samples were stained with Rose Bengal, hand sorted under stereomicroscope and identified by using standard works on macrobenthos (Pennek, 1978; Milligan, 1997; Brinkhurst, 1971; Edmondson, 1959).

### *2.3. Univeriate Measures of Community Structure*

Univariate measures includeded: total anundance (N), number of taxa (S), dominance (D), Shannon-Wiener diversity indexwhich was calculated by using base-e logarithms (H'), Simpsons diversity index (1-D), d= Margalf's species richness index and J'= equitability, these measures were calculated by using PAST programme. The significance of differences between before/after dredging and control sites was tested using One-way analysis of variance (ANOVA).

### *2.4. Multivariate analysis of community structure*

Non-parametric multivariate techniques were used to measure similarity matrices. The similarity between the sites were calculated by means of Bray-Curtis index by using Bio Diversity Pro Satstical software.

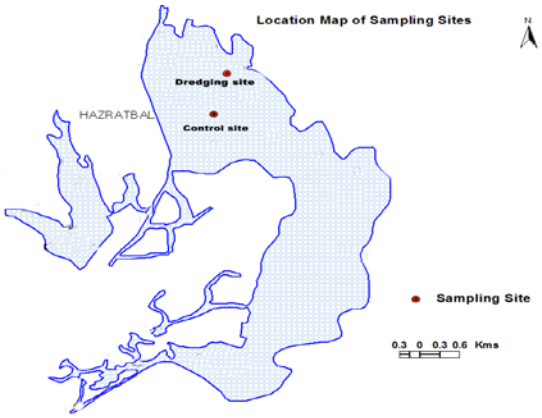


**Figure 4.** Map of Dal lake showing sampling sites in Hazratbal basin.

## 3. Results

A total of 10 taxa of macrozoobenthos were recorded at site 2, of which 4 were annelids, 5 molluscs and 1 dipteran. A similar species structure was recorded at site prior to dredging, there being a total of 9 taxa (3 belonged to annelida, 5 to mollusca and 1 to diptera). After the dredging of the site was performed only 6 taxa were observed in the samples from the area (3 belonged to annelida, 2 mollusca and 1 diptera). 3 species of mollusca (*Lymnea stagnalis, Radix auricularia* and *Radix ovate*) were completely missing from the area (site 1) after dredging (Table 1).

All the diversity indices were higher at site 2 throughout the study and at site 1 during pre-dredging period in in comparison to site 1 during post-dredged period (Table 2). No benthic organism was observed at site 1 during the dredging. This position continued 6 days of dredging. The early colonizers included annelids such as, *Tubifex tubifex* and *Limnodrillus hoffmiesteri* which reappeared in the area after 10th day of dredging. The same trend was observed up to one month after dredging. The total abundance and species richness showed increased recovery rate from 2nd month of dredging and reached to maximum richness on the 5th and 6th month after dredging (Figure 5). As per our observation 9 taxa were found before dredging operation and recovered to the level of 66.6% after 6 month of dredging. It can be presumed that total (100%) recovery will be attained after 1 year. Interestingly, three species of molluscan (*Lymnea stagnailis, Radix ovate* and *Lymnea auricularia*) were eliminated during the process of dredging and were not recovered even after 6 months of recovery phase.

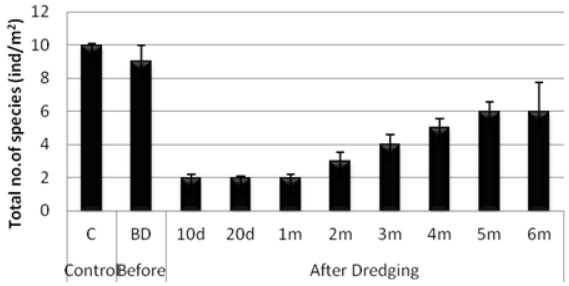


**Figure 5.** Graph depicts variation in species number at control, before dredging and decreasing trend after initial stages of dredging and then shows gradual increase in number of species (S) ind/$m^2$ along with standard errors.

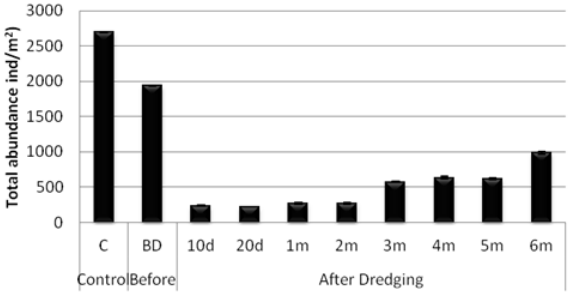


**Figure 6.** Graph depicts variation in total abundance at control site, before dredging and decreasing trend after initial stages of dredging and then shows gradual increase in abundance along with standard errors

It was observed during present study that the density of some species such as, *Tubifex tubifex, Limnodrillus hoffmiesteri* and especially *Chironomus* sp. increased after the dredging process, while, molluscs like *Lymnea stagnalis, Radix ovata* and *Lymnea auricularia* were affected by the dredging operation. Interestingly, *Sphaerium* sp. (belonging to Molluscan group) was observed after 4 months of dredging.

Significance of differences between before/after dredging and control sites were tested using One-way analysis of variance (ANOVA) was used to measure the significance of differences between before/after dredging and with control site. The results showed strongly significant ($P$<0.05) differences between control site and the site which was sampled after dredging operation. The significant differences were also found between pre-dredging and post-dredging ($P$ = 0.004), while as, there were no significant differences between control site and pre-dredged site ($P$ = 0.191). No significant differences (P >0.05) were found between Margalf's index with diversity (H') and equitability (J').

Cluster analysis revealed that after 5 months of dredging macrozoobenthos fauna showed maximum similarity with samples taken before dredging and with control site. As it is depicted in the dendrogram (Figure 7) that samples taken after 5th and 6th month of dredging were grouped in upper cluster with samples taken at pre-dredged and at the control site showed highest similarity level (76%). However, the similarity level between control site and pre-dredged site was 83%.The middle clusters contains samples which were taken after 10th day to 3rd month of dredging showed less similarity level in terms of macrozoobenthos fauna with control site and samples taken before dredging.

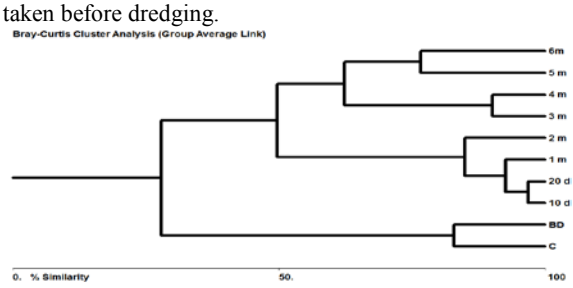


**Figure 7.** Cluster analysis of samplings of control site, before dredging, after 10, 20 days, 1, 2,3, 4,5 and 6 months of dredging.

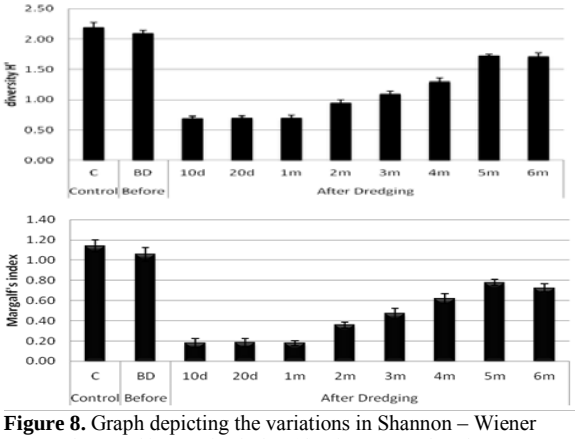


**Figure 8.** Graph depicting the variations in Shannon – Wiener (H') and Margalf's species index (d) of macrozoobenthos community with stanadard errors during study period in Hazratbal basin of Dal lake.

**Table 1**. Species composition per $m^2$ recorded during the present study at control site(C), before dredging (BD), during dredging (DD) and after 1, 3, 6, 10, 20 days and 1, 2, 3, 4, 5 and 6 months after dredging.

| | **Taxa/species** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **S.NO** | | **C** | **BD** | **DD** | **1 d** | **3 d** | **6 d** | **10 d** | **20 d** | **1 m** | **2 m** | **3 m** | **4 m** | **5 m** | **6m** |
| | **ANNELIDA** | | | | | | | | | | | | | | |
| 1 | *Tubifex tubifex* | 405 | 330 | 0 | 0 | 0 | 0 | 105 | 105 | 135 | 90 | 135 | 150 | 120 | 315 |
| 2 | *Limnodrillus hoffmeisteri* | 480 | 390 | 0 | 0 | 0 | 0 | 135 | 120 | 135 | 150 | 335 | 320 | 165 | 195 |
| 3 | *Branchiura sowerbyii* | 180 | 180 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 60 | 45 | 75 | 120 |
| 4 | *Erpobdella octoculata* | 45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **MOLLUSCA** | | | | | | | | | | | | | | |
| 5 | *Sphaerium sp.* | 330 | 120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 90 | 135 |
| 6 | *Lymnea columella* | 315 | 335 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 45 | 90 |
| 7 | *Lymnea stagnalis* | 225 | 90 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | *Radix ovata* | 180 | 150 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | *Radix auricularia* | 205 | 180 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **DITERA** | | | | | | | | | | | | | | |
| 10 | *Chironomus* sp. | 330 | 165 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 | 45 | 90 | 120 | 135 |

**Table 2**. The values of macrozoobenthos diversity indices at control, before dredging and after 10, 20 days, 1, 2, 3, 4, 5 and 6 months of dredging. S =No. of species, N = total abundance, D = Dominance, H'= Shannon diversity, 1 – D = Simpson index, d = Margalf's species richness index and J' = equitability.

| Indices | C | BD | 10 d | 20 d | 1 m | 2 m | 3 m | 4 m | 5 m | 6 m |
|---|---|---|---|---|---|---|---|---|---|---|
| Taxa_S | 10 | 9 | 2 | 2.00 | 2.00 | 3.00 | 4.00 | 5.00 | 6 | 6 |
| Individuals -N | 2695 | 1940 | 240 | 225.00 | 270.00 | 270.00 | 575.00 | 635.00 | 615 | 990 |
| Dominance_D | 0.12 | 0.14 | 0.51 | 0.50 | 0.50 | 0.43 | 0.41 | 0.34 | 0.19 | 0.20 |
| Shannon_H | 2.19 | 2.09 | 0.69 | 0.69 | 0.69 | 0.94 | 1.09 | 1.30 | 1.72 | 1.70 |
| Simpson_1-D | 0.88 | 0.86 | 0.49 | 0.50 | 0.50 | 0.57 | 0.59 | 0.66 | 0.81 | 0.80 |
| Margalef | 1.14 | 1.06 | 0.18 | 0.18 | 0.18 | 0.36 | 0.47 | 0.62 | 0.78 | 0.72 |
| Equitability_J | 0.95 | 0.95 | 0.99 | 1.00 | 1.00 | 0.85 | 0.79 | 0.80 | 0.96 | 0.95 |

**Table 3**. Detailed similarity matrix of Cluster analysis between different intervals of samplings with control site, pre and post dredging.

| | **C** | **BD** | **10 d** | **20 d** | **1 m** | **2 m** | **3 m** | **4 m** | **5 m** | **6m** |
|---|---|---|---|---|---|---|---|---|---|---|
| **C** | * | 82.87 | 16.35 | 15.41 | 18.21 | 18.21 | 35.17 | 38.14 | 37.16 | 53.73 |
| **BD** | * | * | 22.01 | 20.78 | 24.42 | 24.42 | 45.71 | 49.30 | 48.12 | 66.53 |
| **10 d** | * | * | * | 96.77 | 94.12 | 88.24 | 58.90 | 54.86 | 56.14 | 39.02 |
| **20 d** | * | * | * | * | 90.91 | 84.85 | 56.25 | 52.33 | 53.57 | 37.04 |
| **1 m** | * | * | * | * | * | 83.33 | 63.91 | 59.67 | 57.63 | 42.86 |
| **2 m** | * | * | * | * | * | * | 63.91 | 59.67 | 61.02 | 42.86 |
| **3 m** | * | * | * | * | * | * | * | 90.08 | 65.55 | 55.59 |
| **4 m** | * | * | * | * | * | * | * | * | 72.00 | 62.77 |
| **5 m** | * | * | * | * | * | * | * | * | * | 76.64 |
| **6 m** | * | * | * | * | * | * | * | * | * | * |

## 4. Discussion

The present study revealed significant changes in the macrozoobenthos community of Hazratbal basin of Dal lake with dredging activities. The diversity of macrozoobenthos was found to be greatest at control and pre-dredged site in comparison to the post-dredged site. A similar development after dredging process has been reported by Jamar and Mejuto (1988). The present data reveals that dredging operation leads to significant changes in macrozoobenthos in terms of species composition, density and diversity.

The main reason for reduction of abundance, diversity and species composition in benthos of Hazratbal Basin could be because of the fact that dredging operation has disturb the microhabitats of these communities. The findings of this study fully coincide with the observation of earlier researchers who studied the impacts of dredging on benthic communities (Oliver and Slattery, 1976 and Johnson and Nelson, 1985). We have noted during present study period that after dredging the sediment structure was fine as compared to before dredging, when sediment was mixed with a lot of organic matter and other material like wood debris etc. The fine sediments may become favourable for early colonizers such as *Tubifex tubifex* and *Limnodrillus hoffmeisteri*. These two species also showed increasing trend in species richness and diversity (H') (Table 1.). The colonization of two species of annelid group may be due to transportation of larvae and adults from the water column, and post-settlement movement of juvenile and adult life-stages across the sediment. The results from our study correspond very well with the findings of Guerra-Garcia *et al.* (2003). It has been also reported that earlier colonists generally have similar life history patterns which include similar kind of habitat and high larval availability (Bolam and Rees, 2003).

The data shows that the number of species starts recovering 20 days after dredging, but the diversity (H') and Margalf's index of richness (d) continue declining until 2 months of dredging (Figure 6) could be due to strong increase in abundance of some species such as, *Tubifex tubifex* and *Limnodrillus hoffmeisteri*. These species remain in open areas during dredging process where they grow up in large populations and recover immediately after dredging. When areas are depopulated through dredging operations, some opportunistic species usually have a good chance of building up large population in such 'open spaces (Guerra-Garcia, *et al.,*2003).This short-term change is reflected by the abundant increase of opportunistic species (Grassie and Sanders, 1973; Lopez-Jamer and Mejuto,1998; Van Dalfsen *et al*., 2000).

It was observed in the present study that *Tubifex tubifex*is the only species which shows 99% recovery after 6 months of dredging. Similar studies were also reported by Lu and Wu (1998). It was also observed from the present study that *Tubifex tubifex*, *Limnodrillus hoffmeisteri* and*Chironomus* sp. were more abundant after dredging than other taxa.

In connection with the molluscan community, the present study has shown that these communities are more impacted by dredging activity in Hazratbal basin. In addition most taxa of mollusca got eliminated after dredging. Further it was observed during present investigation that only two taxa such as, *Sphaerium* sp. and *Lymnea collumela* recovered after dredging. The recovery time of macrobenthic communities after this type of disturbance depends on the spatial scale, the hydrodynamic conditions, the bottom grain size and the structure of the community affected by the disturbance (Kaiser and Spencer, 1996; Pranovi *et al.*, 1998).

## 5. Conclusion

Present study reveals that dredging is responsible for short-term changes in macrozoobenthos community structure which include effects on species richness, diversity and reduction of benthic habitat. The Annelida group was showed rapid recovery followed by diptera and molluscs. In fact, dredging operation initially disturbs the macrozoobenthos of lake, but it should be carried at small-scale in selected areas. The dredging of whole lake bed is dangerous for lake biodiversity. So, the lake authorities should operate dredging operation in lake as per environmental goals and should, however, avoid operating it from engineering point view.

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# Evaluation of the Physicochemical Properties and Antimicrobial Activities of Bioactive Biodegradable Films

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**Abstract**

The use of edible films to release antimicrobial constituents in food packaging is a form of active packaging. Different extractions of *Myrtus communis*, *Urtica urens*, *Ziziphus spina-christi*, and *Zygophyllum coccineum* were tested for their antimicrobial activity against the food pathogenic microorganisms *Escherichia coli, Salmonella typhimurium* and *Aspergillus niger* by using agar diffusion assay method. Soy-starch and gelatin edible films were prepared and incorporated with *Myrtus communis* and *Ziziphus spina-christi* essential oils separately and as a mixture in different concentrations. The films were characterized for their antimicrobial activity by using agar diffusion assay method and their physico-chemical properties. The films were studied on different food applications (orange, apple, lemon, tomato, pizza dough, chicken salami, meat salami, artificial cheese, mayonnaise, yoghurt and skimmed cheese). The results showed that, the films extended the shelf-life of the food products depending on the effective chemical compounds of the essential oils α-pinene and limonene.

**Key Words:** *M. communis, Z. spina-christi, essential* oils, α –pinene, limonene, edible films.

## 1. Introduction

Researches on the microbial spoilage of food has become important for food safety and keeping qualities. In food industry, all the steps of food production usually occur under sterilized condition. But at the final step where the food packaged, it usually exposes to post process surface contamination, which leading to the reduction of shelf life.

At the same way, the using of extracts from aromatic plants particularly the essential oils as antimicrobial agents are in an increasing interest (Shahidi Bonjar *et al.*, 2003), because there were considered as a rich source of biologically active compounds. They have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Burt 2004, Kordali *et al.*, 2005) and have a wide range of possible applications ranging from the food industry to pharmaceuticals (Burt 2004, Holley and Patel, 2005).

There are many techniques that have been used for maintaining the quality of food products and in the recent years the edible films and coatings have received considerable amount of attention as antimicrobial packaging materials. The main advantage is that they can be consumed with the packaged products and even if the films are not consumed they could still help in the reduction of environmental pollution (Bourtoom, 2008).

Various antimicrobial edible films can serve as a carrier for antimicrobial compounds to reduce, inhibit or retard the growth of the food pathogenic microorganisms in packed foods and packaging material (Coma *et al.*, 2001, Rodrigues and Han, 2000). The film can helps preventing brown coloration, moisture loss during storage, reducing the rate of rancidity causing lipid oxidation and also restrict of the volatile flavor loss (Pérez-Pérez *et al.*, 2006).

In general; edible films have been made from several polysaccharides, lipids, and proteins (Cagri *et al.*, 2004). Several attempts have been made in developing active packaging systems in which antimicrobial agents are incorporated into the polymeric material and are slowly released on the food surface (Devlieghere *et al.*, 2004) by diffusion through partitions (Han, 2000). Finally, there is a need to explain the advantages of using edible films as an antimicrobial food packaging materials to the consumers in order to help the industry to replace the synthetic packaging materials with the environmental friendliness biomaterials (Sonti, 2003). This study aims at

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incorporating the naturally-derived essential oils with the edible films to use them as antimicrobial food packaging materials to extend the food shelf-life.

## 2. Materials and Method

### *2.1. Plant Materials.*

Both leaves and fruits of *Myrtus communis* and *Ziziphus spina-christi* were collected from Cairo arboretum; *Zygophyllum coccineum* was collected from Sinai (ras-sydr); and *Urtica urens* was collected from El – menofeya, while its seeds were purchased from Local herbal shop in Cairo. All the specimens were identified in the Herbaria of Botany Cairo University (CAI) and Ain-Shams University (CAIA).

A food grade soy protein (prolia) with a protein content of 55% was donated by "AWA" for food additives. Gelatin powder (animal gelatin) was purchased from a local herbal shop, bloom = (180-200) g. Corn starch was purchased from a local shop. Glycerol was obtained from Technogene Company, Dokki, Giza, Egypt. Ethanol was purchased from Sigma-Aldrich, Egypt. 1N sodium hydroxide purchased from El-gomhorea Company, Cairo, Egypt.

### *2.2. Organisms and Preparation of Cultures*

The food deterioration microorganisms *Aspergillus niger, Escherichia coli* and *Salmonella typhimurium* were used as test microorganisms.

Both *Aspergillus niger* and *Escherichia coli* were obtained from Department of Microbiology, Faculty of Science, Ain-shams University. The fungal and bacterial species were received on PDA medium and nutrient agar medium respectively. They were maintained on agar slants of both Czapek's Dox and nutrient media, respectively.

*Salmonella typhimurium* kindly provided from the Veterinary Serum & Vaccine Research Institute. The bacterial species was received on nutrient agar medium, and then cultured on *Salmonella – Shigella* agar slants.

Czapek's Dox agar and Nutrient agar media prepared according to the "Hand book of microbiological media" (Atlas, 1979). SS agar was obtained from Oxoid LTD Company, Basinoctoke, Hampshire, England.

### *2.3. Preparation of Plant Extracts*

Thirty six extracts of *Myrtus communis*, *Urtica urens*, *Ziziphus spina-christi* and *Zygophyllum coccineum* were used in this study. All the plants extractions (whether of air-dried or fresh parts or expressed Juice) prepared according to (Abo-Zaid, 2000), then stored at 4°C until use them in the experiments (not more than a week).

### *2.4. Extraction of Essential Oils*

*Myrtus communis* and *Ziziphus spina-christi* were used for extraction of essential oils. According to (Abo-Zaid, 2000), 100 g of the air-dried leaves of both plants were crushed, then hydrodistilled for 3 hours. The yields were on average 0.5 % (v/w dried weight) and 0.01 % respectively. The essential oils were stored in dark vials at 4 °C. (The trials of the essential oils extraction from both *Urtica urens* and *Zygophyllum coccineum* through hydrodistillation method were failed).

For preparation of different concentrations of the essential oils mixture; equal volumes of the essential oils of both *Myrtus communis* and *Ziziphus spina-christi* were mixed in a dark vial and kept at room temperature for 24 hours, then stored at 4 °C (shake well before it using). 0.1, 0.2, 0.3, 0.4 and 0.5 ml of the mixture then used.

### *2.5. Edible Films Preparation*

Two different edible films were prepared, one of them from soy-starch and the other from gelatin.

#### *2.5.1. Control Films*

Film-forming solution of soy-starch prepared by using a modified method to that described by (Ghorpade *et al*., 1995). While, film-forming solution of gelatin prepared according to the method that described by (de Carvalho and Grosso, 2006).

Each film-forming solution were spread in a rectangular area of (11.5 ×32.5 cm).

#### *2.5.2. Films Enriched with the Essential Oils*

Films-forming solutions incorporated with the essential oils prepared by using a modified method to that described by (Maizura *et al.*, 2008) and by the same steps of preparation of control films.

Seven films sheets prepared for both of soy-starch and gelatin. A sheet containing 0.5 ml *Myrtus communis* essential oil, a sheet containing 0.5 ml *Ziziphus spina-christi* essential oil. And the other five sheets each one contained different concentration of the essential oils mixture (0.1, 0.2, 0.3, 0.4 and 0.5 ml).

### *2.6. Antimicrobial Activity*

Screening of Antibacterial and antifungal activity for plants extractions, the essential oils and the edible films carried out by agar disc diffusion method.

#### *2.6.1. For plant extractions*

0.2 ml of suspension of tested microorganisms spread on the solid media surfaces. Sterilized filter paper discs (5 mm in diameter) saturated with plant extractions, left to dry at room temperature for an hour. Then placed on the cultures surfaces which previously inoculated. Control cultures contained only on sterile filter paper discs.

#### *2.6.2. For Essential Oils*

Sterilized filter paper discs (5 mm in diameter) were saturated with the essential oils, left to dry at room temperature for 30 minutes. Then placed on cultures surfaces which previously seeded with 0.2 ml of the inoculum. Control cultures contained only on sterile filter paper discs.

#### *2.6.3. For Edible Films*

Each film sheet cut into discs (13 mm in diameter), then placed on cultures surfaces which previously seeded with 0.2 ml of the inoculum.

All the plates incubated at 30° C for 48 h for bacteria and 6 days for fungus. Diameters of inhibition zones measured in millimeters. All the tests performed in triplicates, and the results analyzed statistically using ANOVA test (Schott, 1997).

*2.7. Physicochemical Properties of Films*

*Color.* Films Color of soy-starch and gelatin examined by using Kodak camera (dimensions of photo 862 × 962) to show the color of each film.

*Thickness.* The thickness value represented by the mean of five measurements taken along the strips made on each film which used for testing tensile strength and percentage elongation at break. The films thickness measured automatically by a micrometer connected to the Universal Testing Instrument (Zwick \ Z010). The test was carried out at the National Institute for Standards.

*Solubility.* Different pH solutions (1-14 pH) were prepared by using the distilled water and solutions of HCl-KCl Buffer (1-2.2), Citrate-Phosphate Buffer (2.6-7) and Glycine-NaOH Buffer (7.4-14). Small pieces of dried films samples (3 × 4cm) were placed in Petri dishes containing 60 ml of adjusted pH solution. The Test dishes were covered and incubated at room temperature for 48 hrs.

*Water vapor permeability (WVP).* Carried out by using a modified method to that described by (Kunte *et al.*, 1997). Fan was provided for the air circulation inside the desiccator cabinets at the first for 4 hours only after that, the test completed without it. All the tests were performed in triplicates.

*Tensile strength (TS) and percent elongation (%E).* According to (Kunte *et al.*, 1997), tensile testing was performed with the Universal Testing Instrument (Zwick \ Z010) on (50 ×4 cm) dample shape film strips. Initial grip separation set at 25 mm, while cross-head speed set at 50 mm/sec, the used lot cells (100 N). The test was carried out at the National Institute for Standards.

*Scanning electron microscopy (SEM).* Carried out for both films of soy-starch and gelatin by using SEM Model Philips XL 30 attached with EDX unit with accelerating voltage (15 K.V.). Samples were coated with gold and the films surfaces pictures taken by SEM at magnification of (500x).

*Qualitative analyses by (GC-MS)*

Glass jars were tightly sealed with film specimens (8 ×8 cm) of soy-starch film and gelatin film respectively. Film specimens cut from films sheets prepared in rectangular area of (11.5 ×32.5 cm) and enriched with 0.5ml of the essential oils mixture. All the samples were left at room temperature (27°C±2°C) for 7 days.

The air inside the jars examined by Gas Chromatography-Mass Spectrometer (from Agilent Technologies; 6890N, network GC system and 5975 inert XL Mass Selective Detector). It was carried out at the National Institute for Standards. The test performed in duplicate.

*2.8. Applications on Foodstuffs*

*Films Preparation.* The films-forming solutions for soy-starch and gelatin were prepared by the same method which shown before in the preparation of edible films that enriched with the essential oils. For each film-forming solution, 0.5ml from the essential oils mixture was added then spread in rectangular area of (11.5 ×32.5 cm) on foil sheet.

*Preparation of food samples*. Different selected food products were covered with the edible films, each one with film area of (8 ×8 cm). Fruits (orange - apple - lemon), Vegetables (tomato), Bakery products (pizza dough), Meats products (chicken salami- meat salami) and Dairy products (artificial cheese) were directly attached with the films. Where, Ready meal (mayonnaise) and Dairy products (yoghurt-skimmed cheese) were packaged in glass jars and tightly sealed with films.

For control specimens, foil sheets free from films were used. The results were recorded daily during 100 days for odor changes and physical observation.

## 3. Results and Discussion

Four plants have reported in previous studies as medicinal plants were chosen; *Myrtus communis* (Montvale, 2000 and Tsybula and Kazarinova, 1996), *Ziziphus spina-christi* (Glombitza *et al.*, 1994 and Waggas, 2007), *Zygophyllum coccineum* (Batanouny *et al.*, 1999) and *Urtica urens* (Wichtl, 2002 and Randall, 2003) to be safe in using them as food additives. The antibacterial and antifungal activity for all plants extractions gave interesting results. Whereas Myrtus communis and Ziziphus spina-christi were the best of them (Table 1).

**Table 1**. Antimicrobial activities of plants extractions of Myrtus communis, Urtica urens, Ziziphus spina-christi, and Zygophyllum coccineum against the foodborne pathogens, E.coli, S. typhimurium and Aspergillus niger

| plants species | Used part | Extraction solvent | inhibition zones diameter(mm) | | |
|---|---|---|---|---|---|
| | | | *E. coli* | *S. typhimurium* | *A. niger* |
| *M. communis* | Dry Leaves | Distilled Water | 10 | 12 | 9 |
| | | Alcohol | 9 | - | 10 |
| | | Water + Alcohol | - | 11 | 14 |
| | Fresh Leaves | Distilled Water | 9 | 9 | - |
| | | Alcohol | 9 | 10 | - |
| | | Water + Alcohol | 9 | 12 | 12 |
| | Dry Fruit | Distilled Water | 11 | 15 | - |
| *U. urens* | Fresh Leaves | Distilled Water | - | - | - |
| | | Alcohol | 11 | 9 | - |
| | fresh (Leaves+ Flowers+ Stems) | Distilled Water | 6 | - | - |
| | | Alcohol | - | 7 | - |
| | Fresh Root | Distilled Water | 9 | - | - |
| | | Alcohol | 6 | - | 6 |
| | Dry Leaves | Distilled Water | - | - | - |
| | | Alcohol | - | - | 7 |
| | Dry (Leaves+ Flowers+ Stems) | Distilled Water | - | - | 7 |
| | | Alcohol | 8 | - | 7 |
| | Dry Root | Distilled Water | - | - | 8 |
| | | Alcohol | 11 | 6 | 10 |
| | Dry Seeds | Distilled Water | - | - | 6 |
| | | Alcohol | 7 | - | 6 |

| | | | | | |
|---|---|---|---|---|---|
| | Expressed Juice | Fresh Intact Plant | - | - | 10 |
| *Z. spina-christi* | Dry Leaves | Distilled Water | - | 10 | - |
| | | Alcohol | - | - | 6 |
| | | Water + Alcohol | - | 9 | 7 |
| | Fresh Leaves | Distilled Water | - | 7 | 7 |
| | | Alcohol | - | 10 | 7 |
| | | Water + Alcohol | - | 9 | 7 |
| | Dry Fruit | Distilled Water | 9 | - | 7 |
| | Fresh Fruit | Distilled Water | 8 | 10 | - |
| | Seed Embryo | Distilled Water | - | - | - |
| *Z. coccineum* | Fresh Leaves | Distilled Water | - | - | 11 |
| | | Alcohol | 7 | - | - |
| | Fresh Leaves and Stems | Distilled Water | 9 | - | - |
| | | Alcohol | 8 | 8 | - |
| | Expressed Juice | Fresh Intact Plant | 9 | 8 | - |

Each value is the mean of three replicates.
Control Cultures containing only sterile filter paper discs & did not show any inhibition zones. Filter paper discs diameter of 5 mm.
The results are not significant ($p > 0.05$) according to ANOVA test.

The mode of action was attributed to the disturbance of the cytoplasmic membrane, disrupting of the proton motive force, electrolyte flow and active transport and coagulation of bacterial cell contents (Burt, 2004). Whereas, the essential oils components in plants extractions may have an inhibitory effect on the mycelial growth of fungi (Özcan *et al*., 2005).

The extraction of the essential oils from *Urtica urens* and *Zygophyllum coccineum* through hydrodistillation method was failed. Therefore, only the essential oils of *Myrtus communis* and *Ziziphus spina-christi* were used. The method of extraction (Lemberkovics *et al.*, 2003) and the origin of the samples (Tuberoso *et al.*, 2006) have effect on the composition of essential oils in aromatic plants.

According to (Tuberoso *et al*., 2006 and Montvale, 2000), the essential oil yields of *Myrtus communis* were on average 0.5 for the dried leaves and 0.02 for berries. Therefore, the dried leaves were used for the extraction of the essential oil. Although *Myrtus communis* fragrance clearly appeared in its extracts; but the antimicrobial activity for its essential oil against all the tested microorganisms appeared more effective than the extracts (Figure1).

The essential oil of Ziziphus spina-christi showed more potent antimicrobial activity against all the tested microorganisms comparing with all the plant extractions (Figure1).

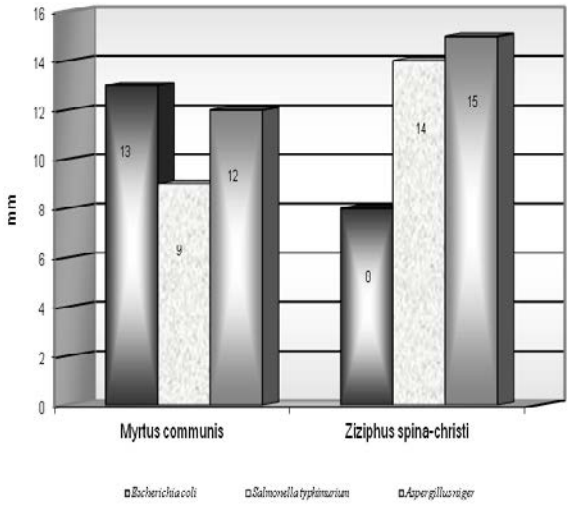



**Figure 1**. The antimicrobial activity of *M. communis* and *Z. spina-christi* essential oils against the foodborne pathogens *E. coli*, *S. typhimurium* and *A. niger* .
Each value represents the mean of three replicates. Filter paper discs diameter of 5 mm.
Control cultures contained only on sterile filter paper discs & did not show any inhibition zone.
The results are not significant ($p > 0.05$) according to ANOVA test.

This can be attributed to the absence of sugars that present in its extractions and include lactose, glucose, galactose, arabinose, xylose and rhamnose (Dweck, 2005). These sugars can be considered as a source of nutrient for the microorganisms.

The results of these experiments emphasize the work of Gill and Holley (2006) which concluded that, "at bactericidal concentrations of the essential oils" the bacterial-cytoplasmic membrane disrupted by increasing its non-specific permeability because the essential oils components may be possess ATPase inhibiting activity. Also there were concluded that, the other secondary effects at sublethal concentrations cannot be discounted and can be expected as a consequence of membrane interactions. By the same way and according to Özcan *et al.* (2005), the essential oils has inhibitory effect on the mycelial growth of fungi. Whereas Curini *et al.* (2003) found that, the essential oil of *Myrtus communis* causing morphological alterations of fungal hyphae.

So it is obvious that, the plants extractions and essential oils have remarkable lethal effects on the tested microorganisms *Escherichia coli*, *Salmonella typhimurium* and *Aspergillus niger* by inhibiting their survival remarkably.

The results of the antimicrobial activity of edible films which incorporated with the essential oils of *Myrtus communis* and *Ziziphus spina-christi* whether individually (Table 4**)** or as a mixture (Table 5) demonstrated wide variation in activities against the tested microorganisms.

**Table 4**. Antimicrobial activity of the films incorporated with *M. communis* and *Z. spina-christi* Essential Oils (separately) on *E. coli*, *S. typhimurium* and *A. niger* by the disc diffusion method.

| Film | Essential Oil | Diameter of inhibition Zones (mm) | | |
|---|---|---|---|---|
| | | *E. coli* | *S. typhimurium* | *A. niger* |
| Soy-Starch | Control | 20 | 20 | 21 |
| | *M. communis* | 21 | 22 | 22 |
| | *Z. spina-christi* | 20 | 21 | 25 |
| Gelatin | Control | 18 | – | 20 |
| | *M. communis* | 21 | 25 | 32 |
| | *Z. spina-christi* | 18 | 22 | 33 |

Each value represents the mean of three replicates. Films discs diameter of 13 mm.
Each prepared film sheet (11.5 ×32.5 cm) containing 0.5 ml of an essential oil.
Control films prepared without adding any of Essential oils. The results are significant ($p > 0.01$) according to ANOVA test.

**Table 5**. Antimicrobial activity of the films incorporated with different concentrations of the essential oils mixture on *E. coli*, *S. typhimurium* and *A. niger* by the disc diffusion method.

| Film | essential oils mixture Conc. (ml) | Diameter of inhibition Zones (mm) | | |
|---|---|---|---|---|
| | | *E. coli* | *S. typhimurium* | *A. niger* |
| Soy-Starch | 0.1 | 28 | 20 | 30 |
| | 0.2 | 28 | 23 | 32 |
| | 0.3 | 30 | 20 | 31 |
| | 0.4 | 25 | 22 | 30 |
| | 0.5 | 26 | 20 | 25 |
| Gelatin | 0.1 | 20 | 20 | 44 |
| | 0.2 | 20 | 18 | 31 |
| | 0.3 | 20 | 20 | 33 |
| | 0.4 | 27 | 25 | 30 |
| | 0.5 | 27 | 21 | 30 |

Each value represents the mean of three replicates.
Films discs diameter of 13 mm.
Each prepared film sheet (11.5 × 32.5 cm) containing different concentration of essential oil mixture.
The results of both soy-starch and gelatin are highly significant ($p < 0.01$) according to ANOVA test.

The films materials come from biological tissues; soy (protein) and starch (carbohydrate) from plants, and gelatin (protein) from animal tissue. Therefore, may be some of the chemicals residues which come from their origin attached to the powders (Pérez-Pérez *et al.*, 2006). This may be explaining the antimicrobial activity of the control films.

There is no single antimicrobial agent can cover all the requirements for food preservation. Therefore, the antimicrobial activity of the essential oils mixture of *Myrtus communis* and *Ziziphus spina-christi* were examined. And according to the presented results, the minimum inhibitory concentration not detectable and it cannot be unified to all the microorganisms (Table 5). This is because of the potential differences of the effect of the essential oils on their cell wall (Gill and Holley, 2006).

Differences between both films were appeared through the physicochemical tests.

Color is an important property because it could affect consumer acceptance of such films for both edible and nonedible packaging applications. Differences observed clearly between both films (Figure 2).


**Figure 2**. Photo of gelatin and soy -starch films.

This is attributed to the used powders color of both soy protein (yellow) and gelatin (white). The Soy protein consists of two major protein fractions referred to as the 7S (conglycinin) and 11S (glycinin). Each fraction has a quaternary (subunit) structure (Kinsella *et al.*, 1985) and make up to 37 and 31%, respectively of the total extractable proteins (Gennadios *et al.*, 1994). Both 7S and 11S contain cysteine residues (Sulfur-containing amino acid) (Kinsella *et al.*, 1985). For that soy powder has the yellow color which became faint with adding of starch. While gelatin contain on high content of the amino acids glycine, proline and hydroxyproline which are free of sulfur.

The films thicknesses were measured automatically by a micrometer connected to the Universal Testing Instrument (Zwick \ Z010). There were 0.1272 mm and 0.1704 mm for soy-starch and gelatin, respectively

Although plasticizers are hydrophilic substances causing an increase in the films solubility (Gennadios *et al.*, 1994). It must be added to the film-forming solutions. Because it decreasing the accumulation of intermolecular forces along polymer chains. Thereby "softening" film structure, decreasing tensile strength and increasing elongation (Mellan, 1961). Whereas, films made without plasticizer were extremely brittle and shattered (Brandenburg *et al.*, 1993).

Polymers which contain groups that can associate through hydrogen or ionic bonding causing the susceptibility of films to absorb moisture (Salame, 1986).

Therefore, both gelatin and soy-starch films are susceptible to moisture absorption. All the film pieces of gelatin were completely dissolved in all the pH solutions. While, not all soy-starch film pieces dissolved. The film pieces that immersed in the pH values 1, 9 and 13 were remained stable; this result confirmed by Gennadios *et al.* (1993). So, the soy-starch film can withstand the highly acidic and highly alkaline solutions.

Also, the edible films that produced from polysaccharides and proteins were showed limited resistance to moisture transmission. This is due to the inherent hydrophilicity of the film-forming substances and to the considerable amount of hydrophilic plasticizers that incorporated into the films to ensure formation of free-standing films (Guilbert *et al.*, 1996 and Callegarin *et al.*, 1997**)**. Therefore, both films were showing limited water vapor permeability.

Tensile strength (TS), elongation (E %) and the elastic modulus (E- Modulus), are measurements helps in the description of the mechanical properties for the films and the relation with their chemical structures (Ninnemann, 1968). Tensile strength is an important mechanical property, which expresses the maximum stress which developed on the film during the tensile testing (Briston, 1988). Whereas the elongation, is the ability of the film to stretch; it determined at the point where the film breaks under tensile testing. And It expresses as, the percentage of change of the original length of the specimen between the grips of the testing machine (Briston, 1988). While the elastic modulus, is the mathematical description of an object or substance's tendency to be deformed elastically (i.e., non-permanently) when a force is applied to it (Hartsuijker and Welleman, 2001). However, soy-starch film showed lower TS, lower E% and lower E- Modulus than gelatin film (Figure 3, Table2 and Figure 4, Table 3 respectively).

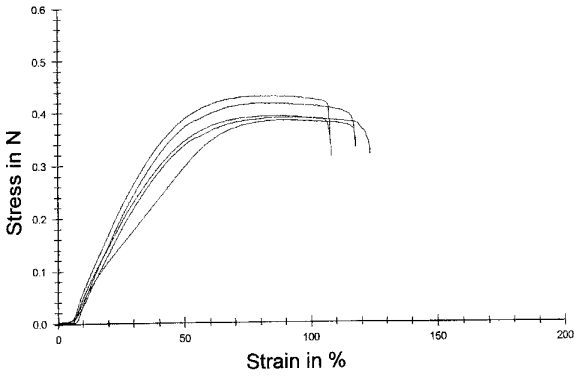



**Figure 3**. Tensile strength (TS), percent elongation (%E) and elastic modulus (E- Modulus) of Soy-starch film
Test standard: ASTM D638-02, Load cell: 100N.
Thickness of protein-carbohydrate film = 0.1272 mm

**Table 2**. Statistics of TS and %E of Soy-starch film.

| n=5 | E-Modulus MPa | RM MPa | ε F max % | RB MPa | ε Break % |
|---|---|---|---|---|---|
| **X** | 1.94 | 0.87 | 81.78 | 0.71 | 110.79 |
| **S** | 0.44 | 0.08 | 6.38 | 0.07 | 6.81 |
| **V** | 22.68 | 9.59 | 7.80 | 9.34 | 6.15 |

**X:** the mean of five measurements. **S:** Standard deviation.
**V:** Poisson's ratio.

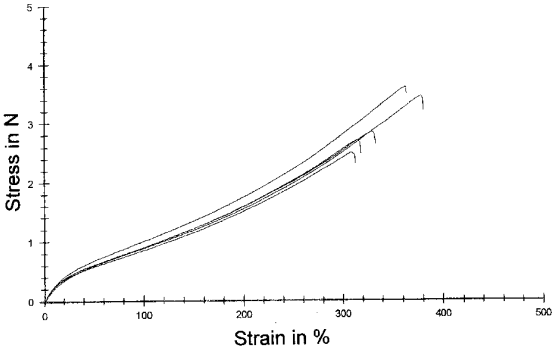



**Figure 4.** Tensile strength (TS), percent elongation (%E) and elastic modulus (E- Modulus) of gelatin film.
Test standard: ASTM D638-02, Load cell: 100N.
Thickness of protein film = 0.1704 mm

**Table 3**. Statistics of TS and %E of gelatin film.

| n=5 | E- Modulus MPa | RM MPa | ε F max % | RB MPa | ε Break % |
|---|---|---|---|---|---|
| **X** | 4.08 | 5.33 | 338.49 | 4.98 | 341.49 |
| **S** | 0.75 | 0.81 | 30.31 | 0.88 | 29.33 |
| **V** | 18.28 | 15.23 | 8.95 | 17.76 | 8.59 |

**X:** the mean of five measurements. **S:** Standard deviation.
**V:** Poisson's ratio.

Those results can be explained as follow; in case of gelatin film. The amino acids chains rearranged with the help of glycerol (plasticizer) during drying the film (Mellan, 1961). This led to the formation of a uniform surface structure (Figure 6). Whereas in case of the soy-starch film, also glycerol helped in the rearrangement of amino acids chains for soy protein. But, although the addition of starch improved the mechanical properties of the film (comparing with a film was prepared free from starch). Addition of starch caused the formation of uneven surface (Figure 5) which affected the film tensile strength, elongation and elastic modulus.

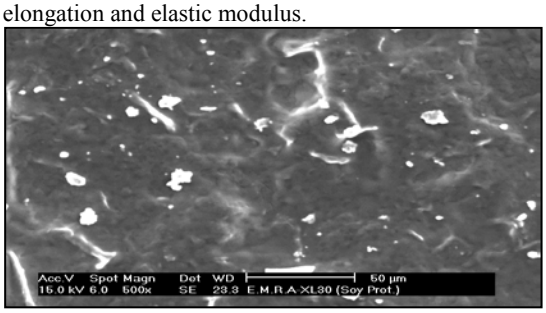



**Figure 5**. SEM of soy-starch film surface.

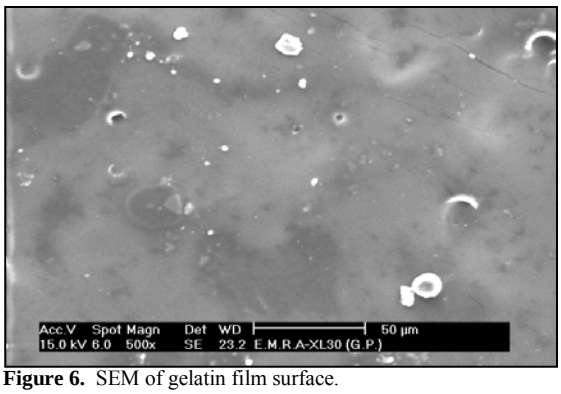



**Figure 6.** SEM of gelatin film surface.

The results that detected quantitatively by GC-MS showed the presence of α-pinene in soy-starch film, while α-pinene and limonene in gelatin (Figure7 and Figure 8, respectively**)**.

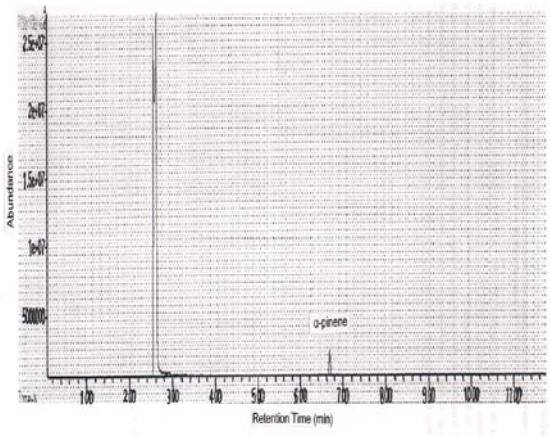



**Figure 7**. Chromatogram of GC-MS for soy-starch film.
**Temp. :** 22± 2°C **RH:** 43±5%
**Area for α-pinene =** -2.76 (Representing the amount in the area of film specimen of (8 ×8 cm)).

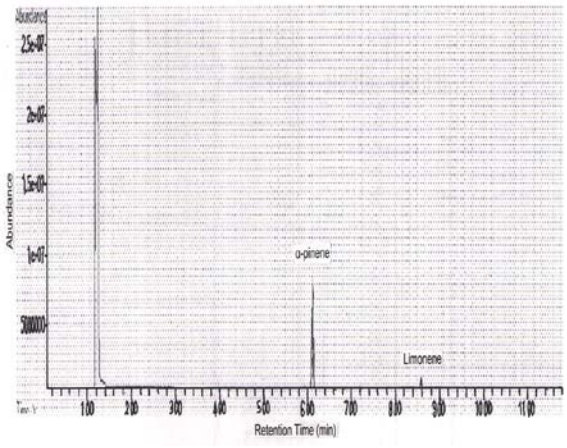



**Figure 8**. Chromatogram of GC-MS for gelatin film.

**Temp. :** 22± 2°C **RH:** 43±5%
**Area for α-pinene =** - 11.4 & **for limonene =** - 0.83
(Representing the amount for both of them in the area of film specimen of (8 ×8 cm))

This test proved that, the antimicrobial activity of the films which directly attached to the food products was due to the existence of the essential oils components in the films. Also, the antimicrobial activity of the films which covered the jars was due to the liberalized essential oils components from the films to the space above the surface of the packaged food (not packaged under vacuum condition).

α-pinene is an organic compound of the terpene class, one of two isomers of pinene (Figure 9).

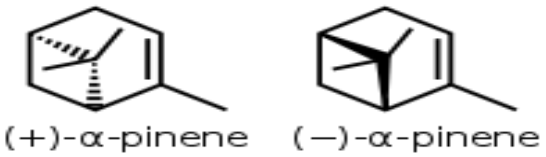



**Figure 9**. α-pinene structure

**(1S, 5S)-2, 6, 6-Trimethyl bicycle [3.1.1] hept-2-ene ((−)-α-Pinene).**

It is an alkane, contains a reactive four- membered ring and of melting point 64° C. It is found in the oils of many plant species (Simonsen, 1957). The four-membered ring makes it a reactive hydrocarbon (Richter, 1945); therefore, α-pinene easily attached to both soy-starch and gelatin amino acids.

Limonene is a hydrocarbon, classified as a cyclic terpene and is a chiral molecule in which biological sources produce one enantiomer (Simonsen, 1947) (Figure 10).

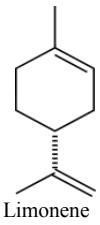


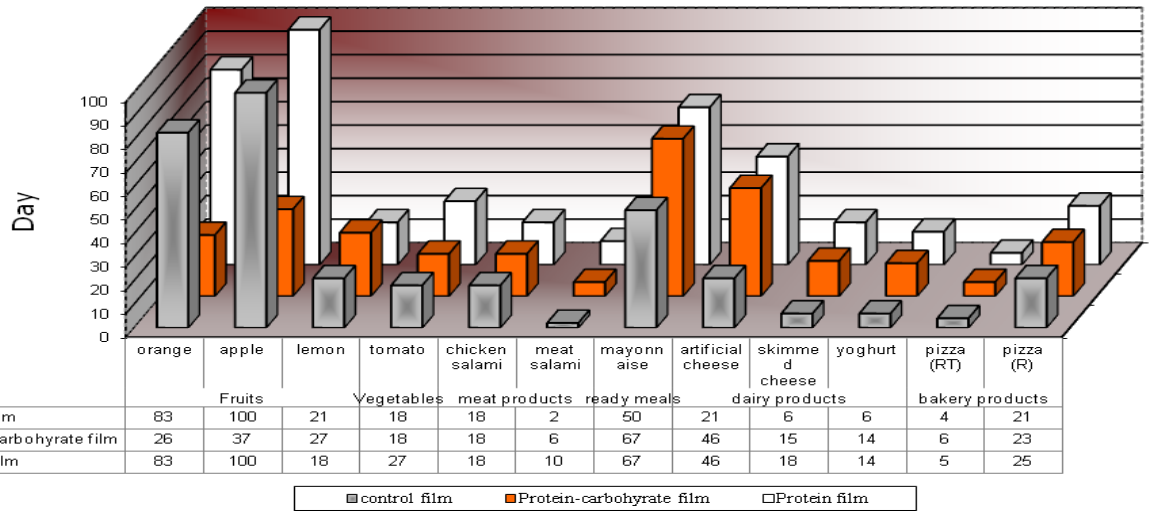

**Figure 10**. Limonene structure

**1-methyl-4-prop-1-en-2-yl-cyclohexene (Racemic: DL-limonene).**

It is a relatively stable terpene, which can be distilled without decomposition, although at elevated temperatures it cracks to form isoprene (Pakdela *et al.*, 2001). It is considered by some researchers to be a significant chemopreventive agent (Crowell, 1999).

Gelatin structure contains on high content of amino acids glycine, proline and hydroxyproline (Bourtoom, 2008). While, it contains on many of glycine (almost 1 in 3 residues arranged every third residue), proline and 4-hydroxyproline residues (Chaplin, 2009). So, the availability of free hydrogen bond in glycine amino acid in gelatin skeleton permit the binding of limonene through the second C=C double bond with gelatin. This explains the presence of limonene only in gelatin film.

The used food products showed different susceptibility towards both films of control and those enriched with the essential oils mixture (Figure 11).




| | orange | apple | lemon | tomato | chicken salami | meat salami | mayonnaise | artificial cheese | skimmed cheese | yoghurt | pizza (RT) | pizza (R) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fruits | | | Vegetables | meat products | | ready meals | | dairy products | | bakery products | |
| control film | 83 | 100 | 21 | 18 | 18 | 2 | 50 | 21 | 6 | 6 | 4 | 21 |
| Protein-carbohyrate film | 26 | 37 | 27 | 18 | 18 | 6 | 67 | 46 | 15 | 14 | 6 | 23 |
| Protein film | 83 | 100 | 18 | 27 | 18 | 10 | 67 | 46 | 18 | 14 | 5 | 25 |

**Figure 11**. The effect of films enriched with essential oils mixture on the shelf-life of different food products.

Pizza (RT): Pizza dough kept at room temperature. Pizza (R): Pizza dough kept in the refrigerator.

Although, the direct attachment of the films which enriched with the essential oils mixture have the limited benefits because the active substances either neutralized on the food surface or diffused rapidly from the surface into the food mass (Quintavalla and Vicini, 2002). Good results obtained by this way with Meat Salami, Artificial Cheese, and the refrigerated Pizza dough. Whereas, the essential oils components can be "in some cases" insufficient to inhibit the microbial growth or adsorbed rapidly on the food stuff surface. This supposition can be explaining the results obtained with Chicken Salami, and Pizza dough which kept at room temperature.

Theoretically, food products packaged in containers and sealed with the films enriched with the essential oils mixture can be the best process. That is because; the air which filled the space over the food stuff be saturated with essential oils components (evidenced by GC-MS test), adsorbed and diffused slower than that directly attached with the food surface.

However, the use of such packaging materials is not meant to be a substitute for good preservation practices, but it should enhance the safety of food as an additional hurdle for the growth of pathogenic microorganisms.

If the types of food products can be divided in general into solid, semi-solid and soft food stuffs; this work succeeded in designing a protection process to the solid and semi-solid one by using the simplest techniques and materials. According to the results "and after the purification and chlorophyll removal process", extractions of *Myrtus communis, Urtica urens, Ziziphus spina-christi* and *Zygophyllum coccineum* can be used as antibacterial food additives.

## 4. Conclusion

The microbicidial activities for the films of soy-starch and gelatin that enriched with 0.5ml of the essential oils mixture were attributed to the presence of α-pinene in soy-starch film, and α-pinene and limonene in gelatin film. From the physicochemical properties of films, those edible films were found to be suitable for packaging solid and semi-solid food products.

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# Effects of Three Medicinal Plant Products on Survival, Oviposition and Progeny Development of Cowpea Bruchid, *Callosobruchus maculatus* (Fab.) [Coleoptera: Chrysomelidae] Infesting Cowpea Seeds in Storage

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**Abstract**

Powders from three medicinal plants were evaluated for their efficacy as contact and fumigant insecticides on cowpea bruchid, *Callosobruchus maculatus* (Fab.) in the laboratory at ambient tropical conditions of temperature and relative humidity. The plant powders tested included *Capsicum frutescens* L. (fruit and seed), *C. annum* Miller (fruit and seed) and *Citrus sinensis* Osbeck (peel). The powders were applied at rates 0.0 (control), 2g and 3.0g/20g of cowpea seeds either directly for contact with the insect pest or in plastic containers to assess fumigant toxicity of their volatiles. Results of contact toxicity assay showed that powders of *C. frutescens* and *C. annum* seeds were more effective against the adult *C. maculatus* evoking 100% mortality within 2 days of application at 3g/20g of cowpea seeds. There was no progeny development of the bruchid in samples treated with *Capsicum* species. The survival of the bruchid from eggs to adults when treated with the plant powders showed that there was significantly ($P<0.05$) more % progeny development in the control (69.32%) compared to others. However, the results of fumigant assays showed that *C. sinensis* had the highest insecticidal activity causing ~~2~~81.25% mortality of *C. maculatus* within 4 days of application at rate 3g/20g of cowpea seeds. This study showed that all the tested plant products were toxic to cowpea bruchid and the powders can be mixed with cowpea seeds to prevent hatching of the eggs thereby helping in their management.

**Key Words:** Insecticidal activity, progeny development, *Callosobruchus maculatus*, *Capsicum frutescens*, *Capsicum annum*, *Citrus sinensis*, cowpea seed.

## 1. Introduction

Cowpea, *Vigna unguiculata* (L.) Walp, belongs to the family Leguminosae, subfamily Papilionaceae and Tribe Phaseolae (Gbaye and Holloway, 2011). It is a legume widely cultivated in tropical and subtropical countries and largely produced in West Africa, Brazil and India. Cowpea is a staple component of the diet in several developing nations and a major source of protein to combat malnutrition in young children in lieu of expensive animal protein in such countries.

One of the major problems encountered in agriculture in developing countries is post harvest losses which usually occur during storage (Adedire *et al*., 2011). The cowpea bruchid, *Callosobruchus maculatus* has been recognized for decades as the major post harvest insect pest of cowpea seeds. It is a cosmopolitan species (Ofuya, 2001; Ileke and Bulus, 2012a). Initial infestation of cowpea starts in the field just before harvest and the insects are carried into the store where the population builds up rapidly (Appert, 1987; Ofuya, 2001; Ileke *et al*., 2012). The huge post-harvest losses and quality deterioration caused by this insect is a major obstacle to achieving food security in developing countries such as Nigeria. Annual production loss of 5% to *C. maculatus* in Nigeria alone would amount to 40,000 tonnes of cowpea seeds cost about $100 million (Singh and Ntare, 1985). The larvae are the major destructive stage because adult cowpea bruchid do not feed (Ofuya, 2001; Gbaye and Holloway, 2011).

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Efficient control of stored products insect pests has long been the aim of entomologists throughout the world. Synthetic insecticides have been used for many years to control stored product insect pests (Salem *et al*., 2007; Ashouri and Shayesteh, 2010). Development of resistance of insect pests to these conventional storage insecticides, increased concern by consumers over insecticide residues, ecological consequences and increasing cost of application call for new approaches to control stored products insect pests that are readily available, affordable and less detrimental to the environment (Adedire and Lajide, 2003; Udo, 2005; Ileke and Oni, 2011; Ashouri and Shayesteh, 2010; Ileke and Bulus, 2012b). A number of plants used locally for medicinal purposes, have also demonstrated potential as insect control agents (Arannilewa *et al.,* 2006; Oni, 2011). For example, cowpea seeds mixed with *Alstonia boone*i and *Eugenia aromatic* have been found to reduce infestation by cowpea beetle (Ofuya *et al*., 2007; Ileke *et al*., 2012). Plants such as *Capsicum frutescens, C. annum* fruit and *Citrus sinensis* peel have previously been shown to cause mortality of adult *Dasyses rugosella* in yam tuber (Ashamo, 2010). Oni (2011) examined contact toxicity of *Capsicum* species to adult *Sitophilus zeamais* and *C. maculatus*. In the present investigation, survival, oviposition and progeny development of cowpea bruchid, *C. maculatus* exposed to *Citrus sinensis* peel, *Capsicum frutescens* and *C. annum* fruit and seeds powders were evaluated.

## 2. Materials and Methods

This study was conducted in the Environmental Biology and Fisheries Research Laboratory, Faculty of Science, Adekunle Ajasin University, Akungba Akoko, Ondo State, Nigeria.

### *2.1. Insect culture*

The insects used to establish a laboratory colony of *C. maculatus* came from a batch of infested cowpea seeds, *Vigna unguiculata* (L.) Walp variety Ife brown collected from Agricultural Development Project, Akure, Ondo State, Nigeria. Beetles were reared subsequently by replacement of devoured and infested cowpea seeds with fresh un-infested cowpea seeds in 2-L kilner jars covered with muslin cloth to allow air circulation. Insect rearing and the experiments were carried out at ambient temperature of 28±2°C and 75±5% relative humidity.

### 2.2. *Plant collection*

The plants evaluated in this work were *Capsicum frutescens* (fruit and seed), *Capsicum annum* (fruit and seed) and *Citrus sinensis* (peel). They were obtained in fresh form, free of insecticides from Oja-Oba market, Akure, Ondo State, Nigeria and authenticated by the Plant Science and Technology Department of Adekunle Ajasin University, Akungba Akoko, Ondo State. These plant materials were rinsed in clean water to remove sand and other impurities, cut into smaller pieces before air dried in a well ventilated laboratory and ground into very fine powder using an electric blender. The powders were further sieved to pass through $1mm^2$ perforations. The powders were packed in plastic containers with tight lids and stored in a refrigerator at 4°C prior to use.

### *2.3. Collection of Cowpea Seeds*

Cowpea seeds used for this study were obtained from a newly stocked seeds free of insecticides at Agricultural Development Program (ADP), Akure, Ondo State, Nigeria. Firstly, the seeds were cleaned and disinfested by keeping at -5°C for 7 days to kill all hidden infestations. This is because all the life stages, particularly the eggs are very sensitive to cold (Koehler, 2003). The disinfested cowpea seeds were then placed inside a Gallenkamp oven (model 250) at 40°C for 4 hours (Jambere *et al*., 1995) and later air dried in the laboratory to prevent mouldiness (Adedire *et al*., 2011) before they were stored in plastic containers with tight lids.

### *2.4. Effect of contact toxicity of plants powders on adult mortality, oviposition and progeny development of Callosobruchus maculatus*

Fine powders of *Capsicum frutescens*, *Capsicum annum*, *Citrus sinensis* were admixed with cowpea seeds at the rates of 2 and 3g /20g of cowpea seeds in 250ml plastic containers. Ten pairs of adult *C. maculatus* (2 to 3 days old) sexed according to the methods described by Halstead (1963); Appert (1987); Odeyemi and Daramola (2000) were introduced into the treated. Male *C. maculatus* have comparative shorter abdomen and the dorsal side of the terminal segment is sharply curved downward and inward. In contrast the females have comparatively longer abdomen and the dorsal side of the terminal segment is only slightly bent downward. The female also has two dark visible spots on their elytra (Odeyemi and Daramola, 2000). Untreated cowpea seeds were similarly infested. Four replicates of the treated and untreated controls were laid out in Complete Randomized Block Design in insect cage. Insect mortality was assessed every 24 hours for four days. Adults were assumed dead when probed with sharp objects and made no responses. At the end of day 4, all insects, both dead and alive were removed from each container. The experiment was kept inside the insect cage for another 30 days to allow for the emergence of the first filial ($F_1$) generation. The number of adults that emerged from each replicate was counted with an aspirator and recorded. The percentage adult emergence was then calculated using the method described by Odeyemi and Daramola (2000).

$$\% \text{ Progeny development} = \frac{\text{No of adult emerged}}{\text{No of eggs laid}} \times \frac{100}{1}$$

### *2.5. Fumigant effect of plants powders on adult mortality, oviposition and progeny development of C. maculatus*

Ten grams of the cowpea seeds were weighed into 50ml transparent plastic tubes that had been cut opened at the bottom and sealed with muslin cloth. Fine powders of *Capsicum frutescens*, *Capsicum annum*, *Citrus sinensis* weighing 2g and 3g concentrations were put into another half-cut 25ml plastic tubes. The 50ml tube and 25ml tube were then joined together with the aid of gum (Ileke and Bulus, 2012a). Ten pairs of adult *C. maculatus* (2 to 3 days old) sexed according to the methods described above were introduced to the tube containing 10g of cowpea seeds and tightly sealed (Ileke and Bulus, 2012a). Untreated cowpea seeds were similarly infested. Four replicates of the treated and untreated controls were laid out in Complete Randomized Block Design in insect cage.

Bruchid mortality was assessed every 24 hours for four days. Adults were assumed dead when probed with sharp objects and made no responses. At the end of day 4, all insects, both dead and alive were removed from each container. The experiment was kept inside the insect cage for another 30 days to allow for the emergence of the first filial ($F_1$) generation. The number of adults that emerged from each replicate was counted with an aspirator and recorded. Percentage adult emergence was calculated as described above.

### *2.6. Statistical analysis*

Data were subjected to analysis of variance and where significant differences existed, treatment means were separated using the Tukey's test.

## 3. Results

### *3.1. Effectiveness of plants powders as contact insecticides*

The effectiveness of the various plant powders on the survival of cowpea bruchid, *C. maculatus* at different periods after treatment is presented in Tables 1 and 2. The results revealed that in each treatment, the mortality of *C. maculatus* increased gradually with time of exposure. Chilly pepper, *C. frutescens* seed powder caused 100% mortality of *C. maculatus* at rate 2g/20g of cowpea seeds within 2 days of exposure (Table 1). The corresponding value for *C. sinensis, C. frutescens* fruit and *C. annum* fruit and seed powders were 34.5%, 87.5%, 51.25% and 71.25% mortality of adult cowpea bruchid respectively. The contact toxicities of these plant powders increased with increase in dosage as well as increase in the period of exposure to plant powders (Table 2). At rate 3g/20g of cowpea seeds, 100% mortality was obtained in sample treated with *C. frutescens* seed at 1 day after application of powder. The results indicated that various plant powders tested as contact insecticides significantly ($P<0.05$) reduced number of tested insect. In general, *Capsicum* species seeds powders were more toxic than other tested plant powders.

**Table 1**. Percentage mortality of adult *Callosobruchus maculatus* treated with various powders at rate 2g/20g of cowpea seeds for contact toxicity.

| Powder | Mortality % ± SE mean after | | | |
|---|---|---|---|---|
| | 1 day | 2 day | 3 day | 4 day |
| *Citrus sinensis* (p) | 17.25± 1.44b | 34.50± 1.44b | 68.25± 2.39b | 80.00± 1.25b |
| *Capsicum frutescens* (f) | 45.00± 2.04c | 87.50± 5.20e | 100.00± 0.00c | 100.00± 0.00c |
| *Capsicum frutescens* (s) | 70.00± 1.25d | 100.00± 0.00f | 100.00± 0.00c | 100.00± 0.00c |
| *Capsicum annum* (f) | 20.00± 4.05b | 51.25± 2.39c | 100.00± 0.00c | 100.00± 0.00c |
| *Capsicum annum* (s) | 41.25± 2.39c | 71.25± 2.39d | 100.00± 0.00c | 100.00± 0.00c |
| Control | 0.00± 0.00a | 0.00± 0.00a | 0.00± 0.00a | 0.00± 0.00a |

Each value is a mean ± standard error of four replicates. Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.
Keys: f- fruit, s - seed, p – peel.

**Table 2**. Percentage mortality of adult *Callosobruchus maculatus* treated with various powders at rate 3g/20g of cowpea seeds for contact toxicity.

| Powder | Mortality % ± SE mean after | | | |
|---|---|---|---|---|
| | 1 day | 2 day | 3 day | 4 day |
| *Citrus sinensis* (p) | 28.75± 1.25b | 47.50± 1.44b | 78.75± 1.25b | 88.75± 1.25b |
| *Capsicum frutescens* (f) | 50.00± 1.25c | 71.25± 2.39c | 100.00± 0.00c | 100.00± 0.00c |
| *Capsicum frutescens* (s) | 100.00± 0.00e | 100.00± 0.00d | 100.00± 0.00c | 100.00± 0.00c |
| *Capsicum annum* (f) | 32.75± 1.25b | 60.00± 1.25c | 100.00± 0.00c | 100.00± 0.00c |
| *Capsicum annum* (s) | 71.75± 1.25d | 100.00± 0.00d | 100.00± 0.00c | 100.00± 0.00c |
| Control | 0.00± 0.00a | 0.00± 0.00a | 0.00± 0.00a | 0.00± 0.00a |

Each value is a mean ± standard error of four replicates. Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.
Keys: f- fruit, s-seed, p - peel

### *3.2. Fumigant effect of various plant powders*

Table 3 and 4 showed the fumigant effect of various plant powder against *C. maculatus*. At day 4 after post treatment, *C. sinensis* was able to cause 66.75% mortality of adult *C. maculatus* while the corresponding values for *C. frutescens* and *C. annum* seeds powders were 55% and 40% mortality of bruchid respectively at 2g/20g of cowpea seeds. (Table 3). The toxicities of these plant powders to cowpea bruchid increased with an increase in concentration and period of exposure to plant powders. *C. sinensis* powder caused 81.25% mortality of *C. maculatus* while the corresponding value for *C. frutescens* seed powder was 72.5% mortality of *C. maculatus* at rate 3g/20g of cowpea seeds (Table 4).

**Table 3**. Percentage mortality of adult *Callosobruchus maculatus* treated with various powders at rate 2g/20g of cowpea seeds for fumigant toxicity.

| Powder | Mortality % ± SE mean after | | | |
|---|---|---|---|---|
| | 1 day | 2 day | 3 day | 4 day |
| *Citrus sinensis* (p) | 20.00± 1.25b | 238.75± 1.25c | 45.00± 2.04d | 66.75± 1.25d |
| *Capsicum frutescens* (f) | 0.00± 0.00a | 0.00± 0.00a | 10.00± 1.25b | 20.00± 1.25b |
| *Capsicum frutescens* (s) | 10.00± 1.25b | 22.50± 1.44b | 31.25± 1.25c | 55.00± 2.04d |
| *Capsicum annum* (f) | 0.00± 0.00a | 0.00± 0.00a | 0.00± 0.00a | 12.50± 1.44b |
| *Capsicum annum* (s) | 0.00± 0.00a | 10.00± 1.25b | 20.00± 1.25bc | 40.00± 1.25c |
| Control | 0.00± 0.00a | 0.00± 0.00a | 0.00± 0.00a | 0.00± 0.00a |

Each value is a mean ± standard error of four replicates. Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.
Keys: f- fruit, s-seed, p – peel.

**Table 4**. Percentage mortality of adult *Callosobruchus maculatus* treated with various powders at rate 3g/20g of cowpea seeds for fumigant toxicity.

| Powder | Mortality % ± SE mean after | | | |
|---|---|---|---|---|
| | 1 day | 2 day | 3 day | 4 day |
| *Citrus sinensis* (p) | 45.00± 2.04c | 67.50± 2.04d | 78.75± 1.25e | 81.25± 2.39d |
| *Capsicum frutescens* (f) | 0.00± 0.00a | 12.50± 1.44b | 25.00± 1.25c | 31.25± 2.39b |
| *Capsicum frutescens* (s) | 20.00± 1.25b | 38.75± 1.25c | 51.25± 2.39d | 72.50± 3.15cd |
| *Capsicum annum* (f) | 0.00± 0.00a | 0.00± 0.00a | 8.75± 1.25b | 20.00± 1.25b |
| *Capsicum annum* (s) | 8.75± 1.25b | 20.00± 1.25b | 38.75± 1.2d | 62.00± 1.25c |
| Control | 0.00± 0.00a | 0.00± 0.00a | 0.00± 0.00a | 0.00± 0.00a |

Each value is a mean ± standard error of four replicates. Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.
Keys: f- fruit, s-seed, p - peel

### *3.3. Effect of various pant powders applied as contact and fumigant insecticides on oviposition and progeny development of C. maculatus*

Table 5 shows the oviposition and percentage progeny development of *C. maculatus* after being exposed to various plant powders as contact insecticide at two concentrations after 4 days. Progeny development was significantly suppressed by various plant powders with *Capsicum* species and completely inhibited the emergence of *C. maculatus* (100% efficiency).

**Table 5**. Fecundity of *Callosobruchus maculatus* treated with various plant powders as contact insecticides

| Plant Powder | 2g/20g of cowpea seeds No of egg laid | % no of Progeny development | 3g/20g of cowpea seeds No of egg laid | % no of Progeny development |
|---|---|---|---|---|
| *Citrus sinensis* (p) | 36.25± 1.70c | 15.84 | 31.25± 2.39c | 9.60 |
| *Capsicum frutescens* (f) | 10.50± 1.32b | 0.00 | 2.75± 1.25ab | 0.00 |
| *Capsicum f rutescen* (s) | 0.00± 0.00a | 0.00 | 0.00± 0.00a | 0.00 |
| *Capsicum annum* (f) | 12.75± 2.02b | 0.00 | 7.25± 2.39b | 0.00 |
| *Capsicum annum* (s) | 0.00± 0.00a | 0.00 | 0.00± 0.00a | 0.00 |
| *Control* | 88.75± 1.25d | 69.32 | 88.75± 1.25d | 69.32 |

Each value is a mean ± standard error of four replicates. Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.
Keys: f- fruit, s-seed, p - peel

In Table 6, fecundity of *C. maculatus* showed that more eggs were laid on samples treated with plants powders applied as fumigant. Oviposition and % progeny development were high in cowpea seeds treated with *Capsicum* species fruit powders.

**Table 6**. Fecundity of *Callosobruchus maculatus* treated with various plant powders as fumigant insecticides

| Plant Powder | 2g/20g of cowpea seeds No of egg laid | % no of Progeny development | 3g/20g of cowpea seeds No of egg laid | % no of Progeny development |
|---|---|---|---|---|
| *Citrus sinensis* (p) | 11.25± 2.39a | 11.33 | 9.50± 3.15a | 10.32 |
| *Capsicum frutescens* (f) | 38.75± 1.25bc | 32.47 | 30.00± 2.04bc | 28.76 |
| *Capsicum frutescens* (s) | 25.00± 2.04ab | 21.67 | 12.00± 0.00a | 19.49 |
| *Capsicum annum* (f) | 41.25± 2.39c | 35.86 | 37.50± 3.15c | 31.11 |
| *Capsicum annum* (s) | 32.50± 3.15bc | 27.17 | 21.25± 2.39ab | 24.58 |
| *Control* | 77.50± 1.44d | 80.00 | 77.50± 1.44d | 80.00 |

Each value is a mean ± standard error of four replicates. Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.
Keys: f- fruit, s-seed, p - peel

## 4. Discussion

Results reported in this study show that *Capsicum* species and *C. sinensis* powders have insecticidal effects on cowpea bruchid, *C. maculatus* at all levels of treatment but varied with the method of application, exposure period and plant powder concentrations. The two *Capsicum* species seed and fruit powders applied as contact insecticides were very effective against *C. maculatus* causing 100% mortality of adult *C. maculatus* at rate 3g/20g of cowpea seeds within 4 days of application. They also reduced oviposition and completely inhibited progeny development. This shows that *Capsicum* species probably have oviposition deterrent, ovicidal and lavicidal properties. The observed activity may be due to the "pepperich" nature and pungency of the *Capsicum* species (Ashamo, 2010). The pungency of *Capsicum* species was attributed to capsacin (Miyakado *et al*., 1979; Ashamo, 2010). This result is in agreement with the results of Ivbijaro and Agbaje (1986), and Asawalam *et al*. (2007). They both found that *C. frutescens* considerably reduced all stages of *C. maculatus*. The result of this investigation are also similar to the observation of Ashamo (2010) who obtained 100% mortality of adult *Dasyses rugosella* in yam tuber treated with powders and oils of *Capsicum* species. Oni (2011) reported that *Capsicum* species seeds and fruits powders significantly toxic to *Sitophilus zeamais* and *C. maculatus* in stored maize and cowpea seeds, respectively.

The high mortality and low progeny development caused by the powder of *C. sinensis* can be attributed to strong choky odour disrupting respiratory activity of the beetles. Sweet orange peel powders may probably have the same insecticidal properties when applied as contact and fumigant. The results obtained from this study agreed with those reported by Don Pedro (1996a; b) in studies with six *Citrus* species peel oils against *C. maculatus, S. zeamais* and *Dermestes maculatus*. He reported on fumigant action of toxic vapour of *Citrus* species peel oils against *C. maculatus, S. zeamais* and *Dermestes maculatus*.

In this study, the lethal effect of tested plant powders on cowpea bruchid could be as a result of contact toxicity. Insects breathe by means of trachea which usually opens at the surface of the body through spiracles (Adedire *et al*., 2011). These spiracles might have been blocked by the powders thereby leading to suffocation. The powders also prevented oviposition and progeny development when applied as contact insecticides. The choky effect of these powders also disrupt mating activities, sexual communication and inhibit locomotion an effect that have been reported by many researchers (Ofuya, 1992; Adedire 2002; Maina and Lale, 2004; Akinkurolere *et al*., 2006; 2009; Adedire *et al*., 2011; Ileke *et al*., 2012).

Adult bruchids do not feed on stored cowpea seeds but only deposit their eggs. Thus, the use of oviposition inhibitors would be advantageous for the management of cowpea bruchids. The powders of these plants could be mixed with stored cowpea seeds before storage.

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# Reserve Mobilization, Total Sugars and Proteins in Germinating Seeds of Durum Wheat (*Triticum durum* Desf.) under Water Deficit after Short Period of Imbibition

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**Abstract**

Imbibition during the first few hours is crucial for the success of seed germination process. The effect of water deficit after 2 and 8 hours (h) of imbibition of seeds of durum wheat was studied in terms of reserve mobilization, total sugars and proteins at 24, 48 and 72 h post treatment. Imbibition for 2 and 8 h was not enough to support normal mobilization of seed reserves and solubilization of sugars. But, a significant increase in total proteins was shown after 24 and 48 h of water deficit for the two imbibition periods (2 and 8 h). Our results indicate that most of the biochemical and molecular changes are intensified during the first 2-8 h of imbibition. Therefore, biochemical and molecular dissection of this phase of seed germination is of great value in the improvement of our understanding of plant's response to water deficit.

**Keywords**: Durum wheat, imbibition, reserve mobilization, water deficit.

## 1. Introduction

One of the most detrimental factors that plants face is water deficit. Water deficit is one of the major abiotic stresses that threaten food security worldwide (Chaves *et al.*, 2003; Passioura, 2006); hence, there is an urgent need for multilevel investigations and dissection for better and comprehensive understanding of how plants respond to water deficit. In addition, this will help find effective ways to increase crop yield without consuming too much water.

Plants are exposed to drought at all stages of their growth and development. Plant's response to water deficit is dependent on the developmental stage (Blum *et al*., 1980). No correlation was found between the response of seeds and photosynthesizing seedlings to osmotic stress induced by polyethylene glycol (PEG) in ten cultivars of bread wheat, *Triticum aestivum* L. and one cultivar of durum wheat, *Triticum durum* Desf. (Blum *et al*., 1980). Therefore, one cannot extrapolate results about stress response from one developmental stage to another stage, which necessitates independent studies of the response for each developmental stage.

The first stage in plant development is seed germination, which starts by water uptake (imbibition), followed by reserve mobilization and protein synthesis and ends with the emergence of the radicle from seed tissues (Bewley, 1997). Water uptake by seeds was shown to occur in three phases: initial rapid water uptake, then a plateau phase, which is followed by rapid water uptake during which the radicle elongates and emerges (Bewley, 1997). In germinating wheat seeds, it was shown that protein synthesis starts to increase 30 min after imbibition, and it keeps increasing in the next 6 hours of imbibition (Marcus *et al*., 1966). This coincides with the first phase of imbibition, which is characterized by the rapid water uptake. In addition, this suggests a crucial role for this phase in the success of the germination process.

Seeds of bread wheat (*Triticun aestivum* L.) were imbibed for 24 hours, and then they were dehydrated for the subsequent days. The results showed a decrease in reserve mobilization started at day 5 of germination (Miazek *et al*., 2001). Indeed, germinating seeds of bread wheat showed tolerance to dehydration up to the 4$^{th}$ day post-imbibition (Miazek *et al*., 2001). Whereas, in other studies, the effect of osmotic stress on the germination process of seeds revealed a highly significant decrease in the germination percentage, germination rate and reserve mobilization (Almansouri *et al*., 2001; Sayar *et al*., 2010; Soltani *et al*., 2006). This indicates the differential response of germinating seeds to the different water deficit treatments. Indeed, exposing *Arabidopsis thaliana* plants

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to two different water deficit regimes resulted in significant differences in the transcriptome profiles of the two treatments (Harb *et al*., 2010). Therefore, it is of high importance to study the response of germinating seeds to water deficit that simulates to some extent the real stress in the field. This will lead to a better understanding of how water deficit affect the germination process of seeds, and help in the development of strategies to protect plants from the negative effect of water deficit as early as possible.

In this study, the effect of water deficit after the first hours of imbibition was tested in terms of reserve mobilization, total sugars and proteins during the first 3 days of germination after the onset of water deficit.

## 2. Materials and Method

### *2.1. Plant Material*

Seeds of durum wheat (*Triticum durum* Desf. cv. Hourani 27) were provided by the National Center for Agriculture Research and Extension (NCARE), Jordan.

### 2.2. *Water Uptake*

Six groups of seeds of durum wheat (*Triticum durum* Desf. cv. Hourani 27) each group with 20 seeds was prepared. Then, their initial weight (W1) was taken before imbibition. After that, the seeds were put on wet filter paper in 9 cm plates and the plates were kept in an incubator set at 25ºC under dark conditions for different time intervals: 1, 2, 3, 4, 6, 7, and 8 h. Final weight (W2) was taken every one hour after imbibition, and water uptake percentage was calculated:

Water uptake% = (W2 – W1)/ W1 * 100

### *2.3. Reserve Mobilization*

Dry seeds without imbibition were oven dried at 104ºC overnight. After that, their weight was taken and considered the original seed dry weight. The effect of water deficit after few hours of imbibition was tested; wheat seeds were kept on wet filter paper in 9 cm plates at 25ºC under dark conditions for 2 and 8 h. Then, wheat seeds were transferred to dry filter paper in 9 cm plates after blotting the excess water. Samples were taken from treated and control (well-watered) plates at: 24, 48 and 72 h. Samples were oven dried at 104ºC overnight, and their dry weights were taken (dry weight of seed remnants).

Reserve mobilization was calculated as follows:

Weight of mobilized reserve (mg seed$^{-1}$) = (Original seed dry weight – Dry weight of seed remnants)/Number of seeds

Reserve depletion %= (Weight of mobilized reserve / Original seed dry weight) *100

### *2.4. Germination Under Water Deficit*

Seeds were considered germinated when the radicle is 1 mm long. The germination percentage of wheat seeds exposed to imbibition for 2 and 8 h, followed by water deficit was calculated at 48 and 72 h of treatment.

### *2.5. Quantification of Sugars*

Sugars were quantified by anthrone method (Yemm and Willis, 1954). Briefly, seeds were ground in 80% ethanol. Then, a volume of the supernatant was reacted with anthrone reagent under boiling for 5-10 min. Glucose was the standard, and absorbance was read at 630 nm.

### *2.6. Quantification of Total Proteins*

Total proteins in wheat seeds were quantified by Bradford's method (1976). Briefly, seeds were ground in distilled water, and then the mixture was centrifuged. Four (4) µL of the supernatant was mixed with 200 µL of Bradford reagent. The absorbance of the resultant product was read at 575nm. Standard curve of bovine serum albumin was run with the samples.

### *2.7. Statistical Analysis*

All data were analyzed by Student's - T test using Excel software. Differences with p-value less than 0.05 were considered significant.

## 3. Results

### *3.1. Water Uptake*

To determine at what time point water uptake by seeds will reach 25 and 50%, the water uptake of wheat seeds was monitored for the first 8 hours of imbibition. During this time period, an increase in the accumulated water content in seeds was shown (Fig. 1). After 2 h of imbibition, the accumulative water uptake was about 25%, and then it increases to reach 50% after 8 h of imbibition (Fig. 1).

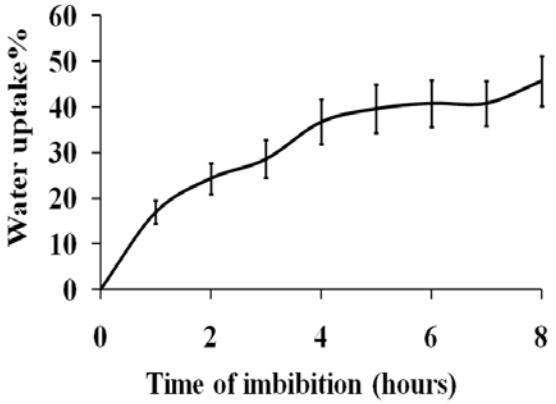


**Figure 1.** Water uptake during the first hours of imbibition in seeds of durum wheat. Error bars represent the standard errors of the means (n=8). The experiment was repeated with the same results.

### *3.2. Reserve Mobilization in Response to Water Deficit*

To test if water absorbed during the first 8 h is enough to support all the phases of the germination process; wheat seeds imbibed for 2 and 8 h were dehydrated for 24, 48, and 72 h. After that, reserve mobilization was determined for both treatments at the three time points of water deficit. The results showed no change in reserve mobilization between the treated and the control seeds after 24 h of treatment. Whereas, the amount of water absorbed after 2 and 8 h was not enough for normal reserve mobilization after 48 and 72 h of water deficit. After 48 h of water deficit, reserve mobilization was 54% and 61% of the well-watered control at 2 and 8 h of imbibition, respectively

(Fig. 2A). Whereas, after 72 h of water deficit reserve mobilization decreased to 44% and 25% at 2 and 8 hours of imbibition, respectively (Fig. 2A).

The results of seed reserve depletion showed the same trend as that shown for reserve mobilization. No change in seed reserve depletion was shown after 24 h (Fig. 2B). Seed reserve depletion was reduced by about 5 and 4 % after 48 h of water deficit for the two imbibition periods (2 and 8 h), respectively (Fig. 2B). After 72 h of water deficit, the reductions were 13 and 11 % after 2 and 8 h of imbibition, respectively (Fig. 2B).

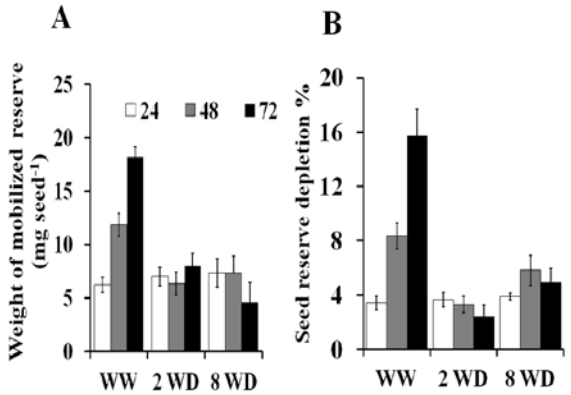


**Figure 2.** The effect of water deficit on reserve mobilization of seeds of durum wheat. A) Weight of mobilized reserve (mg.seed$^{-1}$) in wheat seeds exposed to water deficit at 2 and 8 hours of imbibition (2 WD and 8 WD, respectively) compared to the well-watered (WW) control. B) Seed reserve depletion % in wheat seeds exposed to water deficit at 2 and 8 hours of imbibition (2 WD and 8 WD, respectively) compared to the well-watered (WW) control. Error bars represent the standard errors of the means (n = 6). The experiment was repeated with the same results.

Reserve mobilization was determined after 7 days of water deficit at 2 and 8 h of imbibition. Both 2 and 8 h of imbibition showed a reduction in reserve mobilization and depletion of 84% compared to the well-watered control (Fig. 3A and B).

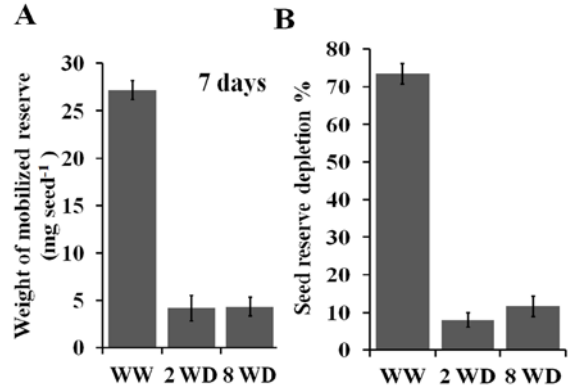


**Figure 3.** Reserve mobilization after 7 days of germination of seeds of durum wheat under water deficit (WD) compared to well-watered control (WW). A) Weight of mobilized reserve (mg.seed$^{-1}$) in seeds exposed to water deficit at 2 and 8 hours of imbibition (2 WD and 8 WD, respectively) compared to the well-watered control (WW). B) Reserve depletion percentage in seeds exposed to water deficit at 2 and 8 hours of imbibition (2 WD and 8 WD, respectively) compared to the well-watered control (WW). Error bars represent the standard errors of the means (n= 6 for WD and 10 for WW). The experiment was repeated with the same results.

### *3.3. Total Sugars in Response to Water Deficit*

Soluble sugars were quantified in dehydrated seeds imbibed for 2 and 8 h of imbibition after 24, 48 and 72 h of water deficit. No change in soluble sugars was shown after 24 h of water deficit for the two imbibition periods (2 and 8 h) (Fig. 4). After 48 h of water deficit, soluble sugars decreased to 76 and 78 % compared to the control at 2 and 8 h of imbibition, respectively (Fig. 4). The reductions were 73 and 47 % at 2, and 8 h of imbibition after 72 h of water deficit, respectively (Fig. 4).

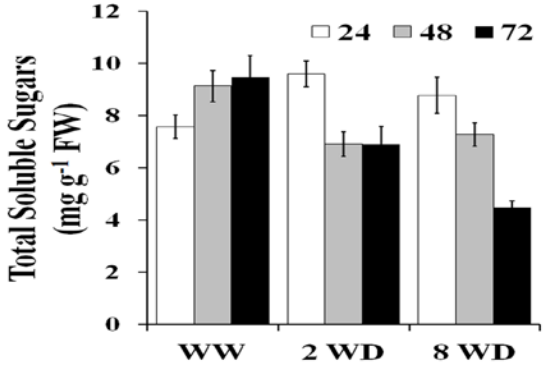


**Figure 4.** The effect of water deficit on the total soluble sugars in durum wheat seeds exposed to water deficit at 2 and 8 hours of imbibition (2 WD and 8 WD, respectively) compared to the well-watered (WW) control. Error bars represent the standard errors of the means (n = 6). The experiment was repeated with the same results.

### *3.4. Total Proteins in Response to Water Deficit*

After 24 and 48 h a highly significant increase in the concentration of total proteins was shown (Fig. 5). At 2 h of imbibition, the increases were 24 and 22 mg.g$^{-1}$ fresh weight (FW) after 24 and 48 h of water deficit, respectively (Fig. 5). At 8 h of imbibition, the increases were 20 and 32 mg.g$^{-1}$ FW after 24 and 48 h of water deficit, respectively (Fig. 5).

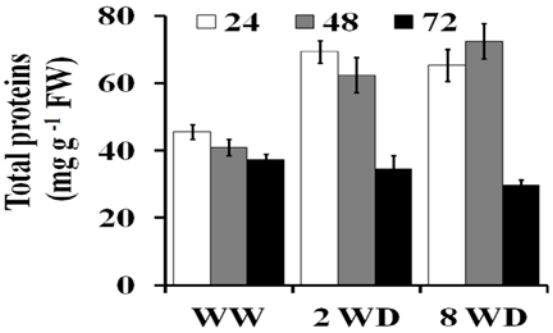


**Figure 5.** The effect of water deficit on the total proteins in durum wheat seeds exposed to water deficit at 2 and 8 hours of imbibition (2 WD and 8 WD, respectively) compared to the well-watered (WW) control. Error bars represent the standard errors of the means (n = 5). The experiment was repeated with the same results.

### *3.5. Seed germination in response to water deficit*

Water deficit at 2 and 8 h of imbibition resulted in a complete inhibition of germination after 48 and 72 h of water deficit.

## 4. Discussion

The results of water uptake during the first 8 h of imbibition showed an increase in the accumulative water with time. This is consistent with the study of water uptake in barley (*Hordeum vulgare*) and bread wheat (*Triticum aestivum* L.) (Davidson *et al*., 1976; Clarke, 1980, respectively). Factors related to the sowing medium and to the seed were found to affect water uptake by seeds (Davidson *et al*., 1976; Clarke, 1980). Water potential and hydraulic conductivity of the soil impose limiting factors on the water uptake process (Ward and Shaykewich, 1972). Moreover, the hydraulic conductivity of the seeds is also playing a major role (Ward and Shaykewich, 1972). To exclude the effect of the initial seed size and other factors on the imbibition process, wheat seeds of the same size and from the same batch were used for the determination of water uptake in this study.

A reduction in reserve mobilization was revealed after 48, 72 h and after day 7 of water deficit at 2 and 8 h of imbibition. These results suggest that water deficit during the first hours of imbibition is detrimental to the biochemical and molecular changes needed for seed germination. In agreement, a study on durum wheat showed a highly significant decrease in reserve mobilization in response to osmotic stress after 7 days of imbibition (Soltani *et al*., 2006).

Soluble sugars are one biochemical indicator of the efficiency of seed reserve mobilization. Water deficit at 2 and 8 h of imbibition inhibited the solubilization of sugars after 48 and 72 h of the treatment. This is in agreement with the results of the effect of osmotic stress on the degradation of sugars in durum wheat, which revealed a drastic decrease of soluble sugars after 48 h of treatment (Almansouri *et al*., 2001). In contrast with our results, studies of germinating seeds of bread wheat showed an increase in soluble sugars started at day 2 of water deficit up to day 5 after 24 h of imbibition (Miazek *et al*., 2001).

Proteins are major components of cereals' grain (Shewry and Halford, 2002; Šramková *et al*, 2009; Triboï *et al*., 2003). Seed proteins can be classified into: storage, structural, metabolic, and protective proteins (Shewry and Halford, 2002). In this study, the concentration of total proteins was increased after 24 and 48 h of water deficit following a short period of imbibition of 2 and 8 h. This increase in protein content might be explained as an acclimation strategy to water deficit (Nakashima *et al*., 2009). It is known that structural and functional proteins are induced by environmental stresses such as water deficit (Bartels and Sunkar, 2005; Ramanjulu and Bartels, 2002). Indeed, in a study on two wheat cultivars with different tolerance to salinity, protein synthesis under salt stress was found as an adaptation strategy adopted by the salinity-tolerant cultivar (Delláquila and Spada, 1993). In germinating wheat seeds, albumins and globulins started to increase at day 2 of water deficit under light conditions (Miazek *et al*., 2001). In addition, in germinating wheat seeds, RNA synthesis started as early as 3 h of germination (Rejman and Buchowicz, 1973). This coincides with the first phase of imbibition, which is characterized by the rapid water uptake. This may suggest a crucial role for this phase in the success of the germination process.

In this study, germination was inhibited by water deficit after 2 and 8 h of imbibition. In bread wheat, dehydrated seeds after 24 h of imbibition were tolerant to water deficit up to the fifth day of imbibition (Miazek *et al*., 2001). The effect of osmotic stress using polyethylene glycol (PEG) and/or mannitol on seed germination of wheat, showed a drastic inhibition of the percentage and rate of germination staring at day 2 of imbibition (Almansouri *et al*., 2001; Blum *et al*., 1980; Sayar *et al*., 2010). The complete inhibition of germination as a result of water deficit can be explained by the complete absence of moisture, whereas, under osmotic stress, some moisture is available to allow low germination.

Intensive seedling growth occurs during the first 4 days of imbibition due to the intensive mobilization of seed reserves (Miazek *et al*., 2001). Imbibition during the first hours of the germination process is crucial to provide the germinating seed with enough moisture for the biochemical and molecular changes during normal germination process. Hence, understanding the biochemical and molecular basis of water-seed relationship during the first hours of imbibition is invaluable for the improvement of plant's resistance to water deficit.

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Short Communication

# A Novel Report on the Prevalence of Enterohaemorrhagic *Escherichia coli* non-O157 Isolated from Cattle in Kaduna State, Nigeria

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**Abstract**

This study was carried out to investigate the presence of enterohaemorrhagic *Escherichia coli* (EHEC) of cattle that may pose a risk to human beings. Two hundred and forty (240) faecal samples were obtained from 8 randomly selected commercial cattle farms in Kaduna state, Nigeria. *E. coli* colonies from 76 (31.2 %) faecal samples were confirmed by Gram staining reactions and biochemically using indole, methyl red, Voges Proskauer and citrate (IMViC), triple sugar iron and motility tests respectively. Characterization of the isolates revealed three heterogeneous serogroups (O111, O118 and O126) from apparently healthy cattle, while no *E. coli* serogroup was isolated from diarrhoeic cattle. The prevalence of non-O157 isolates was 4.5 %. Association between the serogroups and source of samples (farms) was significant ($P<0.05$). The O126 serogroup isolated from apparently healthy cattle occurred more frequently, followed by O118 and O111 respectively. Although it is not known whether the presence of EHEC subgroups in apparently healthy cattle in the study areas may pose a health threat, it is safe to assume that the human population in these areas, including cattle rearers and veterinarians, is at risk of exposure to the EHEC subgroups reported in the study. Data from the study possibly suggest cattle as important source of enterohaemorrhagic *E. coli* in Kaduna State, Nigeria.

**Key Words**: Cattle, enterohaemorrhagic *Escherichia coli*, serogroups, Nigeria.

## 1. Introduction

The term 'enterohaemorrhagic *Escherichia coli* ' (EHEC) was originally used to describe strains that cause haemorrhagic colitis (HC) and haemolytic-uraemic syndrome (HUS) (Nataro and Kaper, 1998), express shiga toxins (stx), cause attaching and effacing (A/E) lesions on epithelial cells and possess large plasmid. In accordance with the latest nomenclature, these strains are called shiga toxin-producing *E. coli* (formerly shiga-like toxin-producing *E. coli*) (WHO, 1998). Cattle appear to be the main reservoir of EHEC from which the organisms have been isolated (Clarke, 2001; Djordjevic *et al*., 2001). *E. coli* O111 is the most frequently implicated non-O157 strain causing gastroenteritis with HUS, particularly in the United States of America and Europe (Bettelheim, 2000; Pearce *et al*., 2006). Most studies indicated that majority of O111 serogroups were recovered from individuals with HC and HUS than from cattle (Bettelheim, 2003). Cattle and human O118 serogroups represent the same clones and are similar in virulence attributes. Evidence for zoonotic transmission of *E. coli* O118 serogroups have been documented (Buchanan and Doyle, 1997).

*E. coli* O126 has been reportedly isolated from the faecal samples of cattle and human beings. The serogroup O126 has not been implicated in cases of haemolytic uraemic syndrome (Buchanan and Doyle, 1997; Bettelheim, 2000). Some other non-O157 serogroups of EHEC have been implicated in diarrhoea, HC and HUS in humans (Eklund *et al*., 2001; Bettelheim, 2003). In the present study, we report the prevalence of enterohaemorrhagic *E. coli* from the faeces of cattle in Kaduna State, Nigeria for the first time.

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## 2. Materials and Methods

### 2.1. Study Area

The study area was Kaduna State, which is located between latitude 10 $^0$ and 11 $^0$N and longitude 7 $^0$ and 8 $^0$E, North-Western Nigeria. Eight commercial cattle farms were randomly selected from five different local government areas of Kaduna State, Nigeria.

### 2.2. Sample Collection

A total of two hundred and forty (240) faecal samples from apparently healthy (233) and diarrhoeic (7) cattle were collected from 8 randomly selected commercial farms using stratified sampling technique (Field and Graham, 2003). Faecal material (1-2 g) was aseptically collected from the rectum of each animal using clean disposable hand gloves. The samples were placed in separate sterile bottles containing 9-10 mL of tryptone soya broth (TSB) and kept in a cold box at 4 $^o$C, and transported to the Bacteriology Diagnostic Laboratory, Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria, Nigeria and processed immediately.

### 2.3. Isolation and Identification of Suspected Colonies

Bacterial isolation, identification and biochemical tests were carried out using standard procedures described elsewhere (Barrow and Feltham, 1993; Cheesbrough, 2000). Briefly, samples were streaked on sorbitol macConkey agar and suspected positive colonies were confirmed using biochemical tests.

### 2.4. Biochemical Characterization

Colonies growing on sorbitol macConkey agar (SMAC) suspected to be *E. coli* were subjected to biochemical tests (indole, methyl red, Voges-Proskauer, citrate (IMViC), triple sugar iron, TSI and motility) (Cheesbrough, 2000).

### 2.5. Serogrouping of Somatic 'O' Isolates

All confirmed *E. coli* isolates were sub-cultured onto nutrient agar slants and stored at 4 $^o$C for serogrouping (Blanco *et al.,* 2006). Somatic 'O' isolates of enterohaemorrhagic *Escherichia coli* O111, O118 and O126 were identified using monospecific *E. coli* antisera (SIFIN Berlin, Germany) (Blanco, 2006).

### 2.6. Statistical Analysis

Data obtained from the apparently healthy and diarrhoeic cattle were analyzed using Student's t-test and values of $P<0.05$ were significant.

## 3. Results

### 3.1. Spatial Distribution of Enterohaemorrhagic E. coli

Out of the 240 faecal samples collected from 8 randomly selected commercial cattle farms, the specific prevalence rate for each farm ranged between 0.0 % (Farm A, FA; Farm B, FB; Farm G, FG) and 17.4 % (Farm E, FE) respectively. A total of 11 (4.5 %) *E. coli* serogroups from apparently healthy cattle were found, of which 2 (8.7 %) isolated from Farm E (FE) and 1 (3.0 %) from FH were O111, 2 (8.7 %) from FE and 1 (4.4 %) from Farm F (FF) were O118, 1 (3.0 %) each from Farms C (FC), D (FD) and H (FH), and 2 (8.7 %) from farm F (FF) were O126 serogroups respectively. *E. coli* serogroup O126 occurred more frequently, followed by O111 and O118 respectively. All the farms had one or more serogroups, except FA, FB and FG where no *E. coli* serogroup was isolated. A prevalence rate of 2.1 % was recorded for *E. coli* O126 and 1.2 % each for O111 and O118 respectively. The prevalence rate of non-O157 which was 4.5 % was statistically significant ($P<0.05$) (Table 1).

**Table 1**. Distribution of *E. coli* serogroups among commercial cattle farms in Kaduna State, Nigeria

| Farm | Positive *E. coli* serogroups | | | |
|---|---|---|---|---|
| | Specific Prevalence (%) | O111 | O118 | O126 |
| Farm A | 0.0 | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Farm B | 0.0 | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Farm C | 3.0 | 0 (0.0) | 0 (0.0) | 1 (3.0) |
| Farm D | 3.1 | 0 (0.0) | 0 (0.0) | 1 (3.0) |
| Farm E | 17.4 | 2 (8.7) | 2 (8.7) | 0 (0.0) |
| Farm F | 13.0 | 0 (0.0) | 1 (4.4) | 2 (8.7) |
| Farm G | 0.0 | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Farm H | 6.1 | 1 (3.0) | 0 (0.0) | 1 (3.0) |
| Total | 4.5 | 3 (1.2) | 3 (1.2) | 5 (2.1) |
| $\alpha^2 = 0.04$, $P< 0.05$ | | | | |

### 3.2. Distribution of Enterohaemorrhagic E. coli in Relation to Age

The age distribution of serogroups isolated from commercial cattle farms identified 1 (3.2 %) *E. coli* serogroup as O111, isolated from the young (0-1 year). *E. coli* O111, O118 and O126 were isolated from adults where O126 serogroup had the highest prevalence (7.4 %) rate. However, the specific prevalence varied among the adult species of cattle ranging between 4.5 % (in cattle older than 3 years) and 7.4 % (for those older than 1-2 years). The relationship observed between age and *E. coli* serogroups was not statistically significant ($P>0.05$) in this study.

### 3.3. Distribution of Enterohaemorrhagic E. coli in Relation to Breed

The specific prevalence rate ranged between 0.0 % (Holstein and Simmentals) and 8.1 % (Friesian) in the exotic breeds of cattle and 6.9% in locals (Rahaji). One (2.7 %) O111 and 2 (5.4 %) O126 serogroups were isolated from Friesian breed of cattle. At least one non-O157 serogroup was isolated from different types of local breeds. The relationship between breed and serogroups was not statistically significant ($P>0.05$).

### *3.4. Distribution of Enterohaemorrhagic E. coli in Relation to Sex*

The relationship between sex and *E. coli* serogroups showed that *E. coli* serogroups were distributed according to the sex of cattle. A total of 1 (2.1 %) each for O111 and O118 serogroups and 2 (4.2 %) for O126 were isolated from males, while 2 (1 %) each for O111, O118 and 3 (1.5 %) for O126 were isolated in females. Overall, 11 (4.5 %) with one or more serogroups were identified. The relationship between sex and *E. coli* serogroups was not statistically significant (P>0.05).

### *3.5. Distribution of Enterohaemorrhagic E. coli in Relation to Health Status*

The relationship between health status and *E. coli* serogroups plummeted (0 %) in diarrhoeic and increased (4.5 %) in apparently healthy cattle respectively. Thus, the relationship between health status and *E. coli* serogroups from commercial cattle farms was not significant (P>0.05) (Fig. 1).

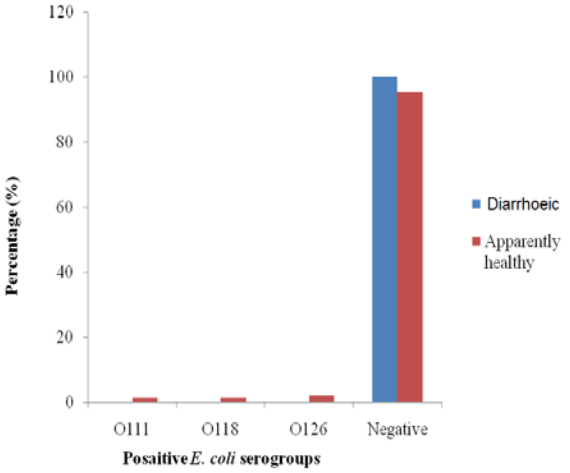


**Figure 1**. Relationship between health status (%) and *E. coli* serogroups isolated from commercial cattle farms in Kaduna State, Nigeria

## 4. Discussion

The prevalence of *E. coli* non-O157 isolated from commercial cattle farms in Kaduna State, Nigeria, was 4.5 %. The authors found individual rates of 2.1 % in males and 1 % in females each for O111 and O118 serogroups respectively. This result agreed with the findings of Bettelheim (2003) and Pearce *et al*. (2006) who reported prevalence rate of 1-2 % for *E. coli* O111 and O118. Serogroup O126 is not frequently associated with disease in humans. Thus, the most common serogroups associated with disease in humans, which were also isolated from apparently healthy cattle in this study were *E. coli* O111 and O118. The prevalence of 1.7 % for O111 serogroup recorded in young animals agreed with the work of Blanco *et al*. (2000), who reported that calves are important reservoirs of *E. coli* non-O157. No *E. coli* serogroup was isolated from diarrhoeic cattle in this study, further supporting our suspicion that cattle may be reservoirs of colibacillosis in the area investigated.

The relationship between sex and the *E. coli* serogroups revealed that males (8.4 %) recorded higher number of *E. coli* non-O157 serogroups as compared to females (3.5 %). Montenegro *et al*. (1990) reported 11.6 % and 3.0 % prevalence rates in cows and bulls using DNA hybridization technique, but the serogroups, except *E. coli* O126 were different. In addition, differences in the areas of study and the changing dynamics of disease may have contributed to this disparity. It is not known whether the presence of EHEC subgroups in apparently healthy cattle, reported in the current study may pose a significant health hazard to human beings residing in the areas investigated. However, it is safe to assume that the human population in these areas, including cattle rearers and veterinarians, is at risk of exposure to the EHEC subgroups reported in the study. Thus, there is the need for veterinary and human public health officials to educate the communities on the public health hazards of colibacillosis. In conclusion, this study revealed that cattle are important reservoir of EHEC in Kaduna state, Nigeria and research should be carried out to establish the extent to which human beings are at risk of being exposed, especially in tropical Nigeria, where cattle owners maintain close contact with animals in the residential areas.

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