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Hilly MO, Adams MN and Nelson SC. 2009. Potential fly-ash utilization in agriculture. *Progress in Natural Sci.*, **19**: 1173-1186.

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Brown WY and White SR.1985. **The Elements of Style**, third ed. MacMillan, New York.

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EDITORIAL PREFACE

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

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

JJBS has been indexed by SCOPUS, CABI's Full-Text Repository, EBSCO, Science Citation Index-Zoological Abstract and is currently under evaluation to be indexed in Thomson Reuters, National Library of Medicine's MEDLINE\ Pub Med system and others. I would like to reaffirm that the success of the journal depends on the quality of reviewing and, equally, the quality of the research papers published.

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

June, 2015

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

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Jordan Journal of Biological Sciences **Guest Editorial**

A New Challenge in the Academic World: Earning Real Money and Eminence by Paper Publishing

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Keywords: Hijacked Journal, Fake Conferences, Social Engineering, Academic World, Bogus Impact Factors.

Nowadays, the academic world is faced with different challenges such as hijacked journals (Jalalian, 2014; Jalalian and Mahboobi, 2014; Dadkhah *et al.*, 2015), bogus impact factors (Jalalian and Mahboobi, 2013), social engineering (Dadkhah and Quliyeva, 2014; Krombholz, 2014), and fake conferences. There are other tricks and non-academic behaviors that we will introduce in this letter to familiarize editors and researchers with them. Although the academic world has many advantages it is an area for jobber people to make money; many people exploit researchers and trick them to earn money. On the other hand, some researchers ignore academic principles and act, in contrast, with these principles. In the following few pages, we will introduce some of the new challenges in the academic world.

1. Plagiarism

Although there are powerful software packages that are being used for detecting plagiarism, still these programs cannot detect conceptual plagiarism. Some people create a n ew article by re-phrasing an entire article, and then publish it under their own names. In this case, the content of the article is not changed, what is actually changed is its wording. To demonstrate the authenticity of the idea above, we performed an easy experiment. First, we analyzed an article published previously by the "Viper Software," plagiarism detecting software (http://www.scanmyessay.com). The software detected the published article and reported the plagiarism rate, which was 85%. Then we used the "Article Rewrite Worker" software (http://www.articlerewriteworker.com) to re-phrase the previous article, and performed the test again and the plagiarism rate was reported as 13%. Therefore, in this state, the detection of conceptual plagiarism is very difficult. Unless the journal is familiar with the original version of this article, such type of plagiarism is not easy to detect. Another type of this conceptual trickery is publishing different versions of the same article about a cer tain subject in different journals; all such versions express the same scientific findings, without presenting any new different ones.

2. Article Sale

Nowadays many jobber people sell articles. They sell these articles to other researchers who seek promotion and enhancement in their academic career. These people obtain new articles through conceptual plagiarism or by tricking researchers, and begin to sell these articles. Nowadays, forgers of valid journals, set up f ree fake journals and, after receiving articles from researchers, upload abstracts and titles of those articles on t heir website, and sell the original articles to people who want them. Therefore, by publishing only the abstract and title of the article on their own website, the plagiarism detection software cannot detect the original article. The concept of "Ghostwriter" - that is, writing speeches, articles and books for other persons, is unfortunately very widespread in the scientific community. The career of graduate students and young scientists depends, in most cases, on the goodwill and the support of the supervisor or the director of the institute, and a showdown is undesirable. Therefore, upon r eceiving a request, the young "indentured Intellect" has to write to the authorities in silence, while remaining invisible to readers. The concept of "Ghostwriter" develops new opportunities on the web; one can sell a scientific publication "for cash" or add a "co-author" (Bagdassarian, 2014).

3. Forced Joint Authorship

The best hidden and almost impossible to be proven, and the most common theft in academia is imposed authorship ("forced joint authorship") or "author-spirit" ("Ghostwriter") (Tzankova, 2008; Zdravkova, 2011; Bagdassarian, 2014). While "co-author," or "joint author," is an author who creates an article with (an) other

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author/authors, the co-intrusive ("forced joint authorship") is a person whose name is added to the list of authors due to his higher administrative position. This person is not usually part of the process of creation, but his name is added with the knowledge of the actual participants. Agreement of the other members of the team is not always compulsory. This act looks completely natural, and "the head," sitting behind his desk, seems as if he actually took part in the creative process. Often, such type of "leaders" squeezes ideas from his subordinates and present them as his own, then "manage" the creative process as "legal." This type of managers often collect the fruit of the labor of other teams whose work is manipulated by this administrative leader, who, in turn, shows "productivity" through the dozens of scientific publications (often in various areas) he publishes per year. These people cannot be "caught up" with their scientific acting career by even the most intense working scientists who present only their own labor or research. Similar to plagiarism, forced joint authorship is in fact "usurping" another person's or group of people's work. The difference is that forced joint authorship is conducted with the knowledge and consent of the whole authorship team. This agreement is usually the result of the abuse of power on the part of the forcedly added "co-author."

4. Conversion of a Journal in to a Print Machine

Some journals, and after receiving a new valid index (specifically the Thomson Reuters indexing), publish a lot of articles with the sole goal of making money in mind. These journals or publishers are known as "predatory publishers." The term "predatory open access" was coined by University of Colorado Denver librarian and researcher Jeffrey Beall (Carl, 2012). These journals also publish articles that are not related to the scope of the journal, and publish articles with different subjects by creating special issues of the journal that do not follow a certain subject. Figure 1 shows the number of published articles in four journals that have published a large number of articles. According to a published article (Bohannon, 2013), most of these journals do not have a Peer Review process.

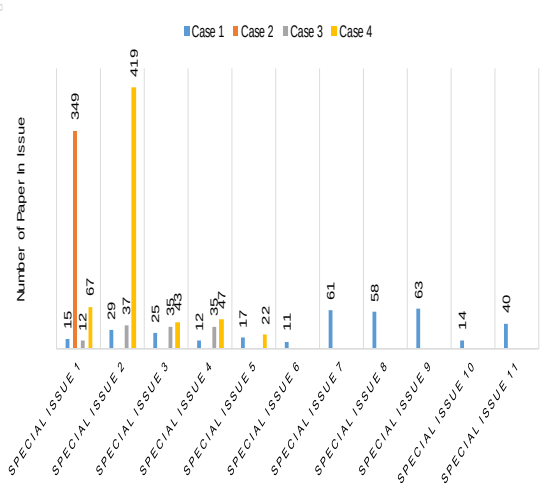

Figure 1. Number of papers in special issues in some journals. Case 1 has 11 special issues, case 2 has 1 special issue, case 3 has 4 special issues and case 4 has 4 special issues.

5. Invalid Conferences

Nowadays, a lot of invalid conferences are designed. These conferences perform the act of validating the articles by machines and, then, the "organizing committee" publishes these articles after receiving the payment (cost of publication). Such conferences claim holding virtual conferences, while in reality such conferences are never held. In addition, some conferences hand in scientific workshop participation certificates to researchers who had never attended any real workshops. In other cases, the "organizing committee," responsible for these fake conferences, publishes articles in hijacked journals; they do that by requesting and receiving payments from the researchers.

6. Concluding Remarks

In this short letter, we presented some of challenges that the academic world is facing nowadays to inform researchers and redactors of journals about such challenges. Although there are a number of jobber people in the academic world, we should not ignore the fact that the basic reasons of such problems do not belong to the jobber people only, the non-academic behaviors made by some of researchers are also involved and can be effective in creating such problems too.

In the World Wide Web, a wide range of scientific journals has been created. Some of them are designated as predatory. There should be lists aimed at revealing the reality and essence of such journals. Still, many journals try to organize critical reviews for the articles submitted to them. The editors of these journals offer editing services; they ask scientists from around the world to get assistance from them to revise and edit their articles. Needless to say, such services are paid. Most manuscript evaluation is voluntary and many scientists abandon it. We, personally, do not ever reject an article, unless it is not within our area of expertise. We consider it our duty, and, in fact, peer reviewing is the duty of all scientists worldwide. It is our belief that this is the only way we can block all the chances of jobber publishers from becoming predators and that through these way only manuscripts with a high scientific value will be published. The quality of scientific publications is at the hands of the scientists themselves. Peer Review is a voluntary act that guarantees good quality. Everyone should be responsible for this process. After peer reviewing (which should become mandatory), the adjustments and modifications made by the reviewers should also be assessed next. Otherwise, the peer review process is pointless and senseless.

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The Effect of Citronella Essential Oil on Controlling the Mango Red-Banded Caterpillar, *Noorda albizonalis* Hampson (Lepidoptera: Pyralidae)

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Abstract

Mango fruit borer (red-banded caterpillar) *Noorda albizonalis* is one of the important pests detrimental to the cultivation of mangoes. A control measure to reduce the level of attacks is necessary in order to prevent a high loss of yield. Plant based pesticides (as citronella essential oil) are potential pest control agents that are environmentally friendly and are safer than the currently used pest-control agents. The present paper was conducted at the Cukurgondang Research Station, Pasuruan-East Java, Indonesia. The experiment was arranged in a randomized block design with 8 replications in which the treatments were concentrations of essential oil 2, 4, 6 cc/l and control (untreated). The parameters observed were the intensity of fruit borer attack and the economic profit obtained from the application of citronella oil. The results showed that the application of citronella essential oil could reduce the rate of fruit borer attack and the production loss on mango, mainly at a concentration of 6 and 4 cc/l. The profit per hectare gained from its application at a concentration of 6 and 4 cc/l was IDR 3,596,000 and IDR 2,864,000, respectively (US$1 = IDR 9,700).

Keywords: Mango, *Noorda albizonalis*, Citronella Oil.

1. Introduction

Mango plays an important role in the life of Indonesian people in terms of health and economy. Mango fruits contain the nutrients required to support human health. These nutrients are protein, fat, calcium, phosphorus, iron, water, vitamins A, B, C, and E. From an economic perspective, mango farming is promising because it can provide high profits due to the benefit/cost ratio of 3.96. In Indonesia, the production of mangoes has steadily risen from year to year. During the years 2007-2011, mango hectarage continuously increased in all the 33 provinces (Agricultural Statistics, 2012). In terms of production and the export value of fruit crops, mango ranks second in production next to banana and second in export value next to mangosteen.

Despite the increase in the total number of production and area, the productivity of mango per area unit has not increased yet. The average productivity per hectare is approximately 5 t ons, much lower compared to other mango growing countries that have reached 12 tons/ha. The main reason behind the low yield is the presence of pests and diseases. One of the important pests affecting mango production is the fruit borer (Red-Banded Caterpillar), Noorda albizonalis Hampson. The yield losses caused by this pest are approximately 10-15% (Anonymous, 2002). This pest should be controlled (Royer, 2009). To date, the control measures used to suppress the pest still mainly depends on the use of pesticides.

The control techniques applied should be safe and environment-friendly to support the program of generating agricultural products that are safe for both the consumers and the environment. One of them is the use of the potential natural pesticides that have short persistence and no residual negative effects but are effective to control pests. Citronella essential oil is one of the natural ingredients, contained in Lemongrass, that have a potential as control agents of pests and diseases. This is based on the results of previous studies revealing that the citronella essential oil has properties as a b actericidal, fungicidal, and insect repellent (Isman, 2000; Kazuhiko *et al.*, 2003; Zaridah *et al*., 2006; Van Tol *et al.,* 2007; Catherine and Hamraoui, 1995). However, to date, there is no information about the use of citronella essential oils for controlling mango fruit borer. Therefore, the current study is intended to evaluate the effectiveness of using citronella essential oils in controlling mango red-banded caterpillar in mango orchard. It is expected that citronella essential oil can reduce mango fruit borer attacks due to its repellent properties.

2. Materials and Methods

The study was carried out at the Cukurgondang Research Station, Pasuruan - East Java from July to December 2011. The problem of red-banded caterpillar almost always occurs in every fruit season in

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Cukurgondang and the surrounding areas. Therefore, the study was carried out in that area. There were four treatments of citronella oil concentration for controlling red-banded caterpillar, i.e., 2 cc/l, 4 cc/l, 6 cc/l, and 0 cc/l (control). An adhesive material, methyl chlor, was added to the water in order to form an emulsion of citronella oil with water. As citronella oil was added with an adhesive material to dissolve into the water, the control treatment was just water with an adhesive material. A concentration of an adhesive material added into water was 3 cc/l. Essential oil was extracted from the leaves and stems of *Cymbopogon nardus* Rendle by a steam distillation process. The main active constituent is citronellal. Mango plants used were Arumanis variety of 20 years old with the height of approximately 8 m. The study was arranged in a randomized block design with 8 r eplications. Spraying application was at 6 days interval. This interval was based on the results of a preliminary test indicating that the persistence of citronella essential oil ranged from 5 to 7 days. Spraying began when the fruits were 3-5 mm (soybean size) up t o 1 week before harvest. A power sprayer that has an extension stick (3 m in length) was used to spray the entire plant canopy (up to 7 m). The maintenance covering fertilization, irrigation, and weeding was steadily conducted to keep the optimum plant growth.

The parameters measured were red-banded caterpillar attack rate, yield loss, and profits of spraying the citronella oil. Since the mango trees were high, the attack rate was determined by counting the fallen fruits caused by red-banded caterpillar per tree sample. Observations were made every week starting from one week after the first application of citronella oil to harvest. The yield loss was calculated using the formula:

$$\text{Percentage of yield loss} = \frac{A}{B} x100\%$$

where **A** = accumulated number of attacked fruits per mango tree since one week after the first application of citronella oil to harvest, **B** = total number of fruits per tree derived from the number of harvested fruits plus the number of fallen fruits since one week after the first application of citronella oil to harvest. All the percentage data collected were subjected to one-way analysis Of variance (ANOVA). The means were separated using the Least Significant Difference (LSD) test at the 5% significance level (Gomez and Gomez, 1984).

The economic profit was assessed by calculating the difference of production, revenue, and additional costs involved in the application of citronella oil compared with no treatment (control). Additional cost of citronella oil applications includes the cost of labor and citronella oil. Labor cost per day was IDR 50,000 (Indonesian Rupiah) and the citronella oil price was IDR 80.00/cc. Revenue was calculated under the assumption of mangoes Arumanis price of IDR 5,000/kg.

3. Results and Discussion

The results of this study indicate that the use of citronella essential oil could reduce the level of the attack intensity of *N. albizonalis* on the mango crop. This could be seen in the data on the number of fruits attacked on all observation dates, especially at a concentration of 6 cc/l citronella (Figure 1).

Figure 1 shows that *N. dorsalis* began attacking mango fruits when fruits are at the young phase until the fruits ripened. Periods of severe attacks took place at the beginning of the young fruit and at the fruit development phase until fruits matured.

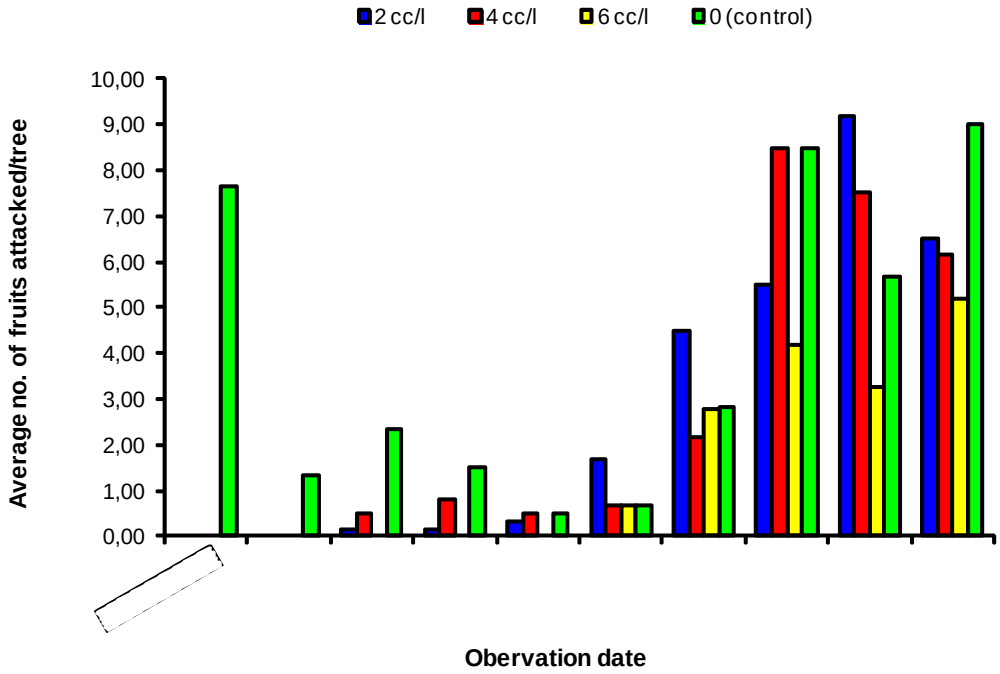


Figure 1. Fruit borer attack rate at each concentration treatment of citronella oil

Therefore, the period when the arumanis fruits are still at a medium size to the period of their maturity is a critical phase for pest attacks. In the young fruit period, from the date of 11/7/2011 to 19/08/2011, citronella

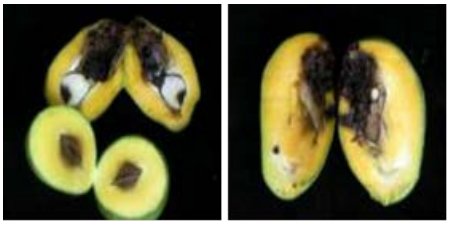
essential oil showed high effectiveness in suppressing the attack of mango fruit borer. This is demonstrated by the fact that, in general, the fruit borer attack in this period was always lower in the treatment of citronella essential oil compared to the control.

The effectiveness of citronella decreased in suppressing the pest attack when fruits were in the middle phase of development up to harvest (from August 25th to October 19th, 2011). This was indicated by the rates of the fruit borer attack that continued to increase until harvest. Nevertheless, the application of citronella essential oil, particularly for the 6 cc/l concentration was still more effective to lessen pest attack rate compared to control.

Different from the treatment of 6 cc/liter citronella, the treatment of 2 and 4 cc/liter had the relatively unstable effectiveness to suppress pest attack that was indicated by a higher attack rate than control treatment only on several observations (August 15th, September 8th and October 10th). Although the effectiveness of citronella essential oils was less stable in suppressing borer infestation, the total percentage of infected fruits, when young to harvest at application of citronella essential oil, was lower than that at the control treatment. Yield losses due to fruit borer attack at application of citronella essential oil at concentration of 2, 4, and 6 c c/l were 20.18%, 18.93%, and 14.77%, respectively, while the control was 26.16% (Table 1). Symptoms of mango fruit borer attacks are shown in Figure 2.

Table 1. Yield losses of Arumanis mango fruits at citronella essential oil treatments

No.	Concentration of citronella (cc/l)	Percentage of yield losses (%)
1	2	20.18 b
2	4	18.93 b
3	6	14.77 c
4	Control (*untreated*)	26.16 a



Means in a column followed by the same letter are not significantly different (P >0.05, LSD)

Figure 2. The attack of the fruit borer on mango fruit cv. Arumanis

The reasons why the essential oils can reduce mango fruit borer attack are that the essential oil of citronella has some negative properties against insects, namely:

1. Repellent Properties**:** citronella essential oil has an insect repellent effect, making the insect unwilling to come to the host. The scent of citronella causes fruit pests less likely to attack the mango fruit. This explanation is based on several previous studies ascertaining that the citronella oil has a repellent effect on some insects, such as fruit piecing and sucking moths *Calpa emarginata* (F.), *Othreis materna* (L.), *O. fullonia* (Cl.), *Cyligramma latona* (Cram.), *Sphingomorpha chlorea* (Cram.) in an apple orchard (Bosch, 1971) and fruit sucking moth *O. materna* on pomegranate and guava fruit (Jayanthi *et al.,* 2010).
2. Insecticidal Properties**:** Even though it is not as strong as some synthetic and botanical pesticides, citronella essential oil can also kill Spodoptera litura, a noctuid moth that attacks tobacco (Hummelbrunner and Isman, 2001), the aphid *Hyadaphis foeniculi* Passerini (Abramson *et al*., 2006), as well as some stored product insects (Issa et al., 2011) like *Callosobruchus maculatus* (Raja *et al.,* 2001; Raja and William, 2008) and *Sitophilus oryzae* (Paranagama *et al.,* 2004). The citronellal compound contained in the essential oil of citronella is responsible for this property and has an insecticidal property against insects (Koul *et al.,* 2008).
3. Inhibitory Properties (Antifeedant): This antifeedant makes the insects unwilling to eat the plant because of its the chemical compound that is not preferred upon initial biting. As a result, the insect keeps away from its host without making any damage. A good example was shown on *S. litura* (Isman, 2000), and *Ostrinia nobilalis*, a p yrallid moth attacking corn (Lee *et al.,* 1999).
4. Ovicidal Properties: These properties lead to reducing the rate of egg hatching. The compound that acts as a cause of this nature is citronellal. Setiawati *et al.* (2011) reported that the egg hatchability of *Helicoverpa armigera* on chili pepper, treated with citronella oil, was reduced up to 95%. Earlier studies also reported that citronella oil signifcantly reduced the hatchability of eggs laid by *C. maculatus* on fresh batches of cowpea seeds (Raja *et al.,* 2001; Raja and William, 2008).

From Figure 1, it appears that the high attacks occuring during the time period of fruit development up to harvest might be attributable to the weak insecticidal and ovicidal effects ag ainst the moth. Hence, of the four properties above, antifeedant or repellent activities seem to be more dominant in suppressing moth attacks.

The data show that the attack rate of mango fruit borer fluctuates following the critical phase of the mango crop. Critical phase of mango fruit borer attack takes place when the fruit is in the development stage until harvest (Figure 1). This can occur when the fruit is in the condition favored most by the pest, such as the strong attractant scent or the presence of eating stimulants (*kairomones* action). The strength of repellent and eating inhibitors (*allomones* action) will also fluctuate depending on the strength of the attractant scent and the availability of materials stimulants. Such interaction causes the fluctuation on the effectiveness of citronella essential oil in suppressing the attack of mango fruit borer. In this study, the use of citronella essential oil as a repellent and/ or antifeedant against the mango fruit borer was more effective at a concentration of 6 cc/l rather than at a concentration of 2 a nd 4 c c/l. This is because the concentration of 6 cc/l can reduce the attractant scent released by mango fruit or make the fruit borer dislike eating the mango fruit.

The results of the profit analysis show that the use of citronella essential oil, particularly at concentration of 6 cc/l and 4 cc/l, is profitable and increased revenues despite the additional costs of the essential oil materials and spraying labors (Table 2).

Table 2. Profit analysis of citronella oil application in controlling mango fruit borer per hectare

Concentration of citronella (cc/l)	Cost of citronella oil (IDR ,000)	Cost of labor (IDR ,000)	Average of production (Kg)	Gross Revenue (IDR ,000)	Net Revenue (IDR ,000)
2	768	1,100	4,700	23,500	21,632
4	1,536	1,100	5,700	28,500	25,864
6	2,304	1,100	6,000	30,000	26,596
Untreated	-	-	4,600	23,000	23,000

As shown in Table 2, the additional benefits (profit) of citronella essential oil application can be determined by calculating the difference between net revenue of citronella oil treatment and control. Based on t hese calculations, the profits gained from the application of citronella oil at a concentration of 4 and 6 cc/l were IDR 2,864,000 and IDR 3,596,000 per hectare. Even though the use of citronella essential oil could only suppress the attack rate up to 11.39%, when converted into IDR, it gave a high enough profit. Further studies on the commercial scale should be carried out in order to confirm these findings.

4. Conclusion

Citronella essential oil could effectively reduce the attack rate of mango fruit borer *N. albizonalis*, especially at a concentration of 4 and 6 cc / liter of water. The yield loss could be avoided as much as 7.23% and 11.39%, as a result from the application of 4 and 6 cc/l, respectively; whilst the profits gained were IDR 2,864,000 and IDR 3,596,000 per hectare.

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Probiotic Properties of *Lactobacillus* Species Isolated from Local Traditional Fermented Products

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Abstract

The present study aims to isolate *Lactobacillus* species from locally fermented vegetables and to characterize selected isolates for their probiotic potential. Seventeen *Lactobacillus* strains (9 *Lactobacillus plantarum* 1, 3 *Lactobacillus pentosus*, 2 *Lactobacillus brevis* 1, 2 *Lactobacillus brevis* 3 and 1 *Lactobacillus salivarius*) were isolated and tested for their probiotic potential. This included survival in gastrointestinal simulated juice, antagonistic activity against *Salmonella typhimurium*, *Escherichia coli*, *Bacillus cereus* and a methicillin-resistant *Staphylococcus aureus* (MRSA) isolate, bile tolerance and antibiotic resistance to 8 antibiotics. Most isolates, especially *Lactobacillus plantarum* 1, were tolerant to the acidity and intestinal conditions after exposure for three and four hours, respectively, with reduction less than one log cycle of the starting CFU/ml. The same trend was observed in respect to bile tolerance with slight variations. All isolates inhibited the growth of the tested pathogens and were the most effective against MRSA isolate. As for antibiotic resistance, it was pronounced against kanamycin, ampicillin, erythromycin and tetracycline. Some isolates (M3, M5, M6, M12, B14) showed a resistance to 6 or more antibiotics of those tested. These results prove that the traditionally fermented vegetables are a good source for probiotic *Lactobacillus*. However, further *in vivo* studies are needed to substantiate the potential of these isolates.

Keywords: *Lactobacillus*, Probiotics, Fermented vegetables, Characteristics..

1. Introduction

The increased demand for complimentary and health foods encourages innovation as well as new and novel product development in the food industries (Guo *et al.,* 2010; Tham *et al.,* 2012; Abbas and Mahasneh, 2014). It is well established that the consumption of probiotic bacteria as formulation or fermented food ingredients stimulates growth of beneficial bacteria and reduces pathogen activity (Chiang and Pan, 2012). To attain the health benefits of probiotic foods, it should contain no less than 10^7 CFU of viable bacteria per gram (Pundir *et al.,* 2013). As a result, probiotic fermented or fortified foods have received a wide interest in an expanding market (Argyri *et al*., 2013). For *Lactobacillus* strains to exert the expected benefits as probiotics, they should fulfill these criteria: the ability to survive in the gastrointestinal tract of high acidity, tolerate bile salts as well as adhesion and persistence to resist pathogens through production of antimicrobial substances (Giraffa *et al.*, 2010) and other means (Lee *et al.,* 2011; Tulini *et al*., 2013).

Lactic acid bacteria mainly *Lactobacillus* and *Bifidobacterium* species are considered very basic in probiotic development; however, lactobacilli are the fundamental group (Rivera-Espinosa and Gallardo-Navaro 2010). They have been used in naturally fermented or fortified dairy products (Granato *et al*., 2010). Recently, a drive towards non-dairy novel probiotics has been observed to span traditionally fermented foods of vegetable origin (Sanchez *et al.,* 2012). No doubt such traditionally fermented foods would be a u nique mining area for new and novel probiotic *Lactobacillus* isolates. It is recognized that wild probiotic strains would compete better in food traditional fermentation settings (Lavilla-Lerma *et al.,* 2013). Accordingly, new and novel specific probiotic candidate bacterial strains are being sought. The efficacy of such strains is mandatory and should be carefully evaluated for safety.

In Jordan, pickled and traditionally fermented vegetables form a reasonable part of the homemade stored foods; for instance, Makdoos, in its different forms, whether it is made of traditionally fermented aubergine stuffed with ground walnuts, garlic, hot dried pepper and left to ferment in vegetable oil, mainly olive oil (Hamady 2003). The other type of Makdoos is the big green pepper Makdoos which is stuffed with cut tomato, parsley, garlic and hot dried pepper and traditionally soaked and

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fermented in vegetable oil preferably olive oil. The fermentation process starts with a w ide variety of indigenous microorganisms present in the vegetables and the stuffing material used. However, the bacteria responsible for fermentation in this case are lactic acid bacteria mainly *Lactobacillus* spp.

Due to the increasing concern of the interrelationship between diet and health, a great attention is given to the functional properties of indigenous lactobacilli involved in traditional fermented foods (Pisano *et al.,* 2014). It is assumed that these foods could provide an alternative source of new novel probiotics with unique properties. This study aims at isolating and identifying selected *Lactobacillus* strains from Makdoos and at studying some of their probiotic characteristics such as acid tolerance, tolerance to gastrointestinal juice and bile, and their antagonistic activity against some pathogens.

2. Materials and Methods

2.1. Collection of Makdoos Samples and Bacterial Growth Enrichment

Homemade and commercial samples of fermented Makdoos were collected and included in this investigation. The enrichment process was carried out by inoculating approximately 1 ml of a mix of the liquor and Makdoos homogenate into 50 ml sterile MRS broth (Oxoid, UK) and incubated anaerobically at 37˚C for (2-5) days (Abbas and Mahasneh, 2014). All samples were collected into sterile glass bottles and were kept in the laboratory at room temperature for further analysis.

2.2. Isolation of Lactobacillus Strains

Enriched Makdoos samples were serially diluted in sterile normal saline. Aliquots of 100 µ l from each dilution were then plated onto de Man, Rogosa and Sharpe agar (MRS, Oxoid, UK) supplemented with 0.01% bromocresol purple as a pH indicator. Plates were incubated anaerobically using anaerogen bags (AnaeroGen, UK) at 37˚C for 24 hours. Presumptive Lactobacillus colonies with yellow halos were randomly picked off the MRS plate and were further subcultured onto fresh plates of the same medium to ensure purity.

2.3. Identification of Bacterial Strains

All isolates were tested for catalase and oxidase activity, Gram reaction, cell morphology as well as spore formation (Guessas and Khal, 2004; Ashmaig *et al.,* 2009). The strains were tested for the production of acids from carbohydrates and related compounds using API 50 CH kits and CHL media (BioMèrieux, France) according to the manufacturer's instructions. Results were scored after incubation at 37˚C for 24 and 48 hours. These results were joined to the apiweb™ identification software with database (V5.1) which uses the phenotypic data to predict a species identity. Interpretations of the fermentations profiles were facilitated by comparing all results obtained for the tested isolates with information from the computer aided database. Isolates were also tested for their hemolytic patterns, gelatinase and DNase activity according to Gupta and Malik (2007).

2.4. Maintenance of Bacteria

Bacterial cultures were maintained in MRS broth with 20% glycerol and kept stored at -80˚C. Working cultures were kept on MRS agar plates at 4˚C and were routinely sub cultured every 2-4 weeks. For comparative purposes, *Lactobacillus reuteri* DSMZ 20056, a probiotic strain, was included in some tests.

2.5. Preparation of Simulated Gastric and Intestinal Juices

Fresh simulated gastric and intestinal juices were prepared daily by suspending pepsin (P7000-25G Sigma-Aldrich, USA) at 0.3% w/v and pancreatin USP (P-1500, Sigma-Aldrich, USA) at 0.1% w/v in sterile 0.5% w/v NaCl. The pH was adjusted to 3.0 for gastric juices using HCl and pH 8.0 for intestinal juices with 0.1M NaOH using pH meter (Eutech 510, Singapore).

2.5.1. Bacterial Tolerance to Simulated Gastric and Intestinal Juices

Overnight bacterial cultures grown in MRS broth of each test isolate were adjusted to 0.5 McFarland and 30 ml aliquot of that suspension was centrifuged (2500 x g, for 20 minutes, at 5˚C), washed twice in 50 mM K2HPO4 (pH 6.5) and resuspended in 3 ml of the same buffer. One milliliter of each isolate suspension was harvested by centrifugation (12,000 x g, for 20 minutes, at 5˚C) and resuspended in 9 m l of gastric solution. Total viable counts on MRS plates were recorded, both before and after incubation period of 3 h ours at 37˚C. Then, one milliliter of gastric juice was taken and added to 9 ml of intestinal juice solution. Total viable counts on MRS plates were also recorded, after an incubation period of 4 hours at 37˚C. The results were expressed as colony counts (log10 orders CFU/ml).

2.5.2. Determination of Total Viable Counts

The total viable counts of *Lactobacillus* species were determined by spread plate method using MRS agar. Serial ten-fold dilutions were prepared using sterile normal saline. Triplicate plates of each suitable dilution were inoculated with 100 µ l each and incubated anaerobically (AnaeroGen, UK) at 37˚C for 48 hours after which numbers of CFU/ml were determined.

2.6. Detection of Antibacterial Activity of the Bacterial Isolates

For the detection of antagonistic activities of the isolates, an agar spot procedure was used. The antibacterial activity of the selected *Lactobacillus* isolates was determined by the test described by Schillinger and Lucke (1989) with some modifications as follows: Five microliters of each overnight culture of *Lactobacillus* isolate grown in MRS broth were spotted onto the surface of MRS agar plates (containing 0.2% glucose) and were then incubated under anaerobic conditions at 37˚C for 48 hours. An overnight culture of four indicator strains (*E. coli* ATCC 25922), (*S. typhimurium* ATCC 14028), (*B. cereus* toxigenic strain TS) and (MRSA clinical isolate) were grown in nutrient broth and were adjusted to 0.5 McFarland solution standard which is equivalent to about 10^8 CFU/ml. Aliquots of 0.25 ml were inoculated into 7 ml of soft/semi-solid nutrient agar (containing 0.2% glucose and 0.7% agar). Inoculated semi-solid agar was

immediately poured in duplicates over the MRS plate on which the tested Lactobacillus isolate was grown. The plates were incubated aerobically at 37˚C for 24 hours. The antibacterial activity was detected by measuring the inhibition zones around the ***Lactobacillus*** bacterial spots. Inhibition was recorded as positive if the diameter of the zone around the colonies of the producer was 2 mm or more (Mami *et. al.* 2008).

2.7. Bile Tolerance Test

The tolerance of the bacterial isolates to bile was tested using MRS broth prepared with 0.3, 0.5, 1 and 2% (w/v) oxgall (Oxoid, UK). Ten milliliter aliquots of bile solutions were transferred into standard glass tubes and sterilized by autoclaving at 121˚C for 15 min. Bacterial cultures were inoculated into sterile MRS broth, incubated overnight and adjusted to 0.5 McFarland at the time of use. Two hundred microliters of the adjusted bacterial cultures were inoculated into different bile concentrations for each isolate. One milliliter aliquots were taken from each inoculated bile tube at zero hours of incubation and after 24 hours, serially diluted with sterile normal saline and inoculated in triplicates onto MRS agar to determine total viable counts.

2.8. Antibiotic Susceptibility Testing

The antibiotic susceptibility test was done according to the agar diffusion method published by the National Committee for Clinical Laboratory Standards (NCCLS, 2000). The determination of minimum inhibitory concentration (MIC) to certain antimicrobial agents recommended by Scientific Committee on Animal Nutrition (SCAN, 2002) included Ampicillin, Ciprofloxacin, Erythromycin, Gentamicin, Kanamycin, Streptomycin, Tetracycline and Trimethoprim. Müller-Hinton agar (Merck, Darmstadt, Germany) plates were used and incubated under anaerobic conditions. Serial dilutions of antibiotics were prepared using sterile distilled water, DMSO and/or ethanol and were sterilized using 0.22 µm syringe filters (Macherey-Nagel, Germany). One ml of each suitable antibiotic concentration was added to 9 ml of molten agar, mixed thoroughly and poured into sterile petri dishes. The agar plates were allowed to set at room temperature. Bacterial inocula were prepared by suspending several bacterial colonies from fresh agar plates in normal saline to a 0.5 McFarland turbidity standard. A spot of 4 µl of the inocula was placed on the agar surface. The inoculated plates were allowed to stand at room temperature for about 30 minutes. The triplicate plates were then transferred into anaerobic jars and were then incubated at 37˚C for 24 hours. The MIC (Minimum Inhibitory Concentration) was recorded as the lowest concentration of the antimicrobial agent that completely inhibited growth.

2.9. Statistical Analysis

The results are presented as means $\pm$ SD. Statistical differences among bacterial isolates were determined by two way ANOVA except for tolerance to simulated gastric and simulated intestinal juices which were determined by three way ANOVA. Differences were considered significant at $P<0.05$.

3. Results and Discussion

3.1. Isolation and Identification of Lactobacillus Potential Probiotic Strains

A total of seventeen isolates from both types of Makdoos were chosen to be used in the present study. All isolates were Gram positive rods, catalase and oxidase negative, non-spore forming, non- hemolytic, and DNase as well as gelatinase negative (Table 1). Absence of hemolytic activity of these isolates is a positive sign in favor of being suitable probiotic isolates irrespect of being *L. plantarum*, *L. pentosus*, *L. brevis* or *L. salivarius*. Similar observations were recorded for *Lactobacillus* isolates from dairy and other sources (Maragkoudakis *et al*., 2006). These isolates were further characterized using API 50 CH strips. Results of the API 50 test confirmed the identity of the 17 *Lactobacillus* isolates (Table 2). Identification of the isolates (Table 2) indicated the dominant presence of *L. plantarum* where 8 out of the 17 isolates belonged to this species, followed by 3 *L. pentosus* isolates and 2 *L. brevis*. Four isolates were not designated to any species.

Probiotic lactobacilli were isolated from foods of plants origin (Husmaini *et al*., 2011) and cereals (Rivera-Espinosa and Gallardo-Navarro 2010). It is recognized that wild type strains that dominate naturally fermented products tend to have higher metabolic capabilities, thus affecting the final quality of the traditionally fermented product (Ayed *et al*., 2002).

3.2. Resistance to Simulated Gastrointestinal Juices

In order for probiotic candidates to exert their beneficial activity, they should survive exposure to gastrointestinal environment of low pH and others (Begley *et al*., 2005). The viable counts of all isolates of the different strains of *Lactobacillus* species were less than or equal to 1 log CFU/ml as compared with the zero time count (7-8 log CFU/ml). This was noted at both pH 3 and 8 after 3 h and 4 h exposure, respectively. This high resistance was observed among *L. plantarum*, *L. pentosus* and *L. brevis* isolates (Table 3). Maragkoudakis *et al*. (2006) and Dunne *et al*. (2001) tested the acid resistance of probiotic bacteria isolated from different sources and reported results in agreement with those reported herein. Abbas and Mahasneh (2014) isolated *Lactobacillus* isolates from camel milk and they had a similar trend in tolerance to gastrointestinal juices as those observed in this investigation.

Table 1. Some primary characteristic of bacterial isolates; all were non-sporeforming rods

Isolate	Gram reaction	Catalase	Oxidase	Hemolysis	DNase	Gelatinase
M3	+	-	-	-	-	-
M4	+	-	-	-	-	-
M5	+	-	-	-	-	-
M6	+	-	-	-	-	-
M7	+	-	-	-	-	-
M8	+	-	-	-	-	-
M9	+	-	-	-	-	-
M10	+	-	-	-	-	-
M11	+	-	-	-	-	-
M12	+	-	-	-	-	-
B13	+	-	-	-	-	-
B14	+	-	-	-	-	-
B16	+	-	-	-	-	-
B17	+	-	-	-	-	-
B18	+	-	-	-	-	-
B19	+	-	-	-	-	-
B20	+	-	-	-	-	-

Table 2. Biochemical identification of bacterial isolates according to API CH 50 strips.

Isolate code number	Identity of the bacterial isolate
M3	*Lactobacillus plantarum* 1
M4	*Lactobacillus plantarum* 1
M5	*Lactobacillus pentosus*
M6	*Lactobacillus plantarum* 1
M7	*Lactobacillus plantarum* 1
M8	*Lactobacillus plantarum* 1
M9	*Lactobacillus pentosus*
M10	*Lactobacillus plantarum* 1
M11	*Lactobacillus brevis* 3
M12	*Lactobacillus plantarum* 1
B13	*Lactobacillus brevis* 3
B14	*Lactobacillus plantarum* 1
B16	*Lactobacillus plantarum* 1
B17	*Lactobacillus brevis* 1
B18	*Lactobacillus salivarius*
B19	*Lactobacillus brevis* 1
B20	*Lactobacillus pentosus*

Table 3. Effect of simulated gastric juice and intestinal juice on viability of *Lactobacillus* isolates. Results are presented as mean log CFU/ml ± S.D. after 3 h exposure at pH 3 and 4 h exposure at pH 8.

	Viable count (log CFU/ml ± S.D.)		
Isolate	**Gastric juice (pH 3)**		**Intestinal juice (pH 8)**
	0 h	**3 h**	**4 h**
M3	8.82 ± 0.13	7.68 ± 0.14	6.70 ± 0.20
M4	8.94 ± 0.05	7.78 ± 0.02	6.87 ± 0.38
M5	7.91 ± 0.11	7.35 ± 0.12	6.73 ± 0.15
M6	8.61 ± 0.16	8.04 ± 0.09	6.97 ± 0.14
M7	8.01 ± 0.15	7.83 ± 0.09	7.02 ± 0.06
M8	8.58 ± 0.02	8.20 ± 0.04	7.26 ± 0.07
M9	8.15 ± 0.12	8.15 ± 0.05	6.96 ± 0.12
M10	7.91 ± 0.12	7.95 ± 0.05	6.92 ± 0.08
M11	8.62 ± 0.04	8.19 ± 0.03	7.11 ± 0.16
M12	7.52 ± 0.54	0.00 ± 0.00	0.00 ± 0.00
B13	8.96 ± 0.05	8.30 ± 0.05	7.31 ± 0.08
B14	8.78 ± 0.08	8.07 ± 0.02	6.96 ± 0.08
B16	8.57 ± 0.03	8.26 ± 0.02	7.29 ± 0.09
B17	8.51 ± 0.03	8.59 ± 0.07	7.50 ± 0.08
B18	8.61 ± 0.63	8.43 ± 0.09	7.16 ± 0.06
B19	8.02 ± 0.03	8.05 ± 0.10	7.09 ± 0.14
B20	8.32 ± 0.17	8.12 ± 0.06	7.16 ± 0.27
L. reuteri	7.10 ± 0.17	7.52 ± 0.09	7.06 ± 0.25

3.3. Antagonistic Activity Against Pathogens

All isolates inhibited the pathogenic target bacteria with varying degrees (Table 4). This leads to the assumption that some bacteriocins were being produced by these isolates which need further testing. Antibacterial substance production is a functional property to characterize probiotics (Shah, 2007). The ability to produce such compounds is very basic for competitive exclusion of pathogens and is a critical characteristic for probiotic bacterial candidates (Begley *et al.,* 2005). Of interest is the superior antagonistic activity of *L. plantarum* 1 M6, *L. plantarum* 1 M8 and *L. brevis* 1 B17 against multiresistant *S. aureus*.

Table 4. Antagonistic activity of *Lactobacillus* isolates against pathogenic bacteria. Inhibition zone diameters (mm) are presented as mean ± S.D.; n=2.

Inhibition zone diameters (mm) of indicator strains				
Bacterial isolate	*B. cereus*	*E. coli*	MRSA	*S. typhimurium*
M3	40 ± 0.0	57.5 ± 3.5	30 ± 0.0	40 ± 0.0
M4	54 ± 0.0	30 ± 14.1	19.5 ± 0.7	58 ± 2.8
M5	40 ± 0.0	39.5 ± 2.1	16 ± 0.0	57.5 ± 3.5
M6	40 ± 0.0	38 ± 0.0	63 ± 4.2	50 ± 0.0
M7	44 ± 0.0	32 ± 1.4	20 ± 0.0	40 ± 0.0
M8	40 ± 0.0	45 ± 7.1	60 ± 0.0	45 ± 1.4
M9	44 ± 0.0	36 ± 4.2	40 ± 0.0	50 ± 0.0
M10	22 ± 2.8	41 ± 1.4	22 ± 2.8	52 ± 2.8
M11	49 ± 1.4	55 ± 7.1	34 ± 0.0	55.5 ± 0.7
M12	46 ± 0.0	38 ± 0.0	34 ± 0.0	50 ± 0.0
B13	45 ± 0.0	36.5 ± 0.7	26 ± 8.5	50 ± 0.0
B14	44 ± 0.0	54 ± 0.0	34 ± 0.0	22 ± 0.0
B16	55 ± 7.1	42 ± 0.0	38 ± 2.8	60 ± 0.0
B17	62 ± 2.8	38.5 ± 0.7	53 ± 1.4	50 ± 0.0
B18	50 ± 0.0	38 ± 0.0	49 ± 1.4	60 ± 0.0
B19	46 ± 0.0	39 ± 1.4	36 ± 0.0	52 ± 2.8
B20	40 ± 0.0	26 ± 4.2	24 ± 0.0	50 ± 0.0

3.4. Resistance to Bile Salts

Most isolates were highly resistant to bile salts at the range of 0.3-2% after 24 h of exposure with little viable count reduction to the level of less than 1 log cycle (Table 5). However, *L. pentosus* M9, *L. pentosus* B20 and M12 were drastically affected after exposure to 1% and 2% for 24 h where both M9 and B20 lost viability totally after 24 h at 2% bile concentration. Since bile plays a role in the defenses of the gut, hence, bile tolerance is a paramount marker in choosing probiotic bacterial strains (Charteris *et al.,* 2000). Sanders *et al.* (1996) demonstrated the ability of lactobacilli to grow and metabolize at normal physiological bile concentrations of the gastrointestinal environment. Ganzel *et al.* (1999) reported the effect of food nature and components in the intestine in enhancing probiotics resistance to bile salts.

3.5. Antibiotic Susceptibility

Table 6 presents the Minimum Inhibitory Concentration (MIC) breakpoints for the isolates. Strains were considered resistant if they had higher breakpoints compared with that of the European Food Safety Authority (EFSA, 2008). Most isolates showed resistance to kanamycin, ampicillin, erythromycin and tetracycline. However, resistance varied within the strains of the same species especially isolates of *L. plantarum* 1. The most sensitive isolate *L. plantarum* 1 M8 was resistant only to ampicillin, erythromycin and kamanycin and sensitive towards the 5 other antibiotics used, while B19 was also sensitive to 4 antibiotics. *L. pentosus* M5 and B20 strains were the most resistant isolates exhibiting resistance to 6 antibiotics of the 8 tested. The observed resistance for kanamycin in this study was in agreement with previous reports about *Lactobacillus* in general (Temmerman *et al.,* 2003). Tetracycline resistance among isolates was higher than that reported by others (Choi *et al.,* 2003). Ammor *et al*. (2008) reported high resistance among *Lactobacillus* isolates towards aminoglycosides (gentamicin and kanamycin) which was observed in this study. Resistance to such antibiotics is considered natural and intrinsic in lactobacilli due to it being chromosomally encoded (Charteris *et al.,* 2001; Morrow *et al.,* 2012).

Table 5. Tolerance of *Lactobacillus* isolates to varying concentrations of bile salts after 24 h of anaerobic incubation (results are presented as mean ± S.D. of viable counts at zero and 24 h exposure to bile).

	Bile concentration							
Isolate	0.3		0.5		1.0		2.0	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
M3	6.73 ± 0.05	8.90 ± 0.18	6.52 ± 0.22	8.36 ± 0.10	6.33 ± 0.16	8.03 ± 0.14	5.26 ± 0.24	5.02 ± 0.55
M4	6.37 ± 0.06	6.83 ± 0.16	6.23 ± 0.10	8.70 ± 0.03	6.42 ± 0.07	6.70 ± 0.17	6.10 ± 0.09	7.16 ± 0.02
M5	6.30 ± 0.07	8.97 ± 0.49	6.40 ± 0.16	8.22 ± 0.32	6.27 ± 0.10	9.62 ± 0.20	5.94 ± 0.12	7.61 ± 0.09
M6	6.37 ± 0.15	8.74 ± 0.23	6.14 ± 0.38	9.07 ± 0.20	6.30 ± 0.06	8.95 ± 0.0	6.32 ± 0.09	7.81 ± 0.20
M7	6.55 ± 0.09	8.40 ± 0.17	6.66 ± 0.10	7.97 ± 0.03	6.53 ± 0.08	7.97± 0.03	6.35 ± 0.03	7.66 ± 0.05
M8	6.43 ± 0.06	7.03 ± 0.05	6.56 ± 0.03	5.60 ± 0.23	6.53 ± 0.02	3.49 ± 0.10	6.34 ± 0.09	3.44 ± 0.03
M9	6.05 ± 0.19	8.35 ± 0.69	6.35 ± 0.11	8.25 ± 0.51	4.98 ± 0.03	5.69 ± 0.36	5.00 ± 0.0	0.00 ± 0.0
M10	6.35 ± 0.03	8.58 ± 0.27	6.34 ± 0.05	8.63 ± 0.06	6.39 ± 0.10	8.10 ± 0.17	6.35 ± 0.06	7.74 ± 0.13
M11	6.51 ± 0.08	8.37 ± 0.08	6.59 ± 0.07	8.37 ± 0.09	6.44 ± 0.16	8.42 ± 0.15	6.44 ± 0.02	8.41 ± 0.03
M12	5.49 ± 0.48	0.00 ± 0.0	5.32 ± 0.26	0.00 ± 0.0	5.10 ± 0.17	0.00 ± 0.0	5.07 ± 0.20	0.00 ± 0.0
B13	6.54 ± 0.06	8.57 ± 0.05	6.81 ± 0.14	8.45 ± 0.05	6.73 ± 0.05	9.17 ± 0.08	6.68 ± 0.04	8.40 ± 0.02
B14	6.18 ± 0.07	8.10 ± 0.35	6.06 ± 0.11	7.83 ± 0.30	6.02 ± 0.20	7.97 ± 0.03	6.30 ± 0.25	8.13 ± 0.24
B16	6.62 ± 0.06	8.43 ± 0.12	6.69 ± 0.08	8.35 ± 0.04	6.53 ± 0.04	8.41 ± 0.03	6.62 ± 0.08	8.08 ± 0.0
B17	6.83 ± 0.06	8.64 ± 0.14	6.72 ± 0.11	8.30 ± 0.03	6.69 ± 0.02	8.45 ± 0.09	6.60 ± 0.08	8.08 ± 0.0
B18	6.62 ± 0.07	8.37 ± 0.19	6.73 ± 0.04	8.62 ± 0.13	6.78 ± 0.01	8.54 ± 0.06	6.66 ± 0.01	8.40 ± 0.05
B19	6.51 ± 0.06	9.17 ± 0.10	6.55 ± 0.07	8.92 ± 0.06	6.42 ± 0.09	8.55 ± 0.04	6.55 ± 0.08	8.38 ± 0.05
B20	6.20 ± 0.08	7.08 ± 0.0	5.97 ± 0.18	4.66 ± 0.02	5.74 ± 0.23	3.46 ± 0.19	5.07 ± 0.20	2.27 ± 0.07

Table 6. Antibiotic susceptibility profiles of *Lactobacillus* isolates of probiotic potential.

	Antibiotic breakpoint[a] (µg/ml)							
Isolate	A (2)	C (4)	E (4)	G (1)	K (32)	S (16)	Te (16)	Tr (16)
M3	R	S	R	R	R	R	R	R
M4	R	R	R	S	R	S	R	S
M5	S	R	R	R	R	R	R	S
M6	S	R	R	S	R	R	R	R
M7	R	S	R	R	R	S	R	S
M8	R	S	R	S	R	S	S	S
M9	R	S	R	S	R	R	R	S
M10	R	S	R	R	R	S	R	S
M11	R	R	S	R	R	S	R	R
M12	R	R	R	R	R	S	R	S
B13	R	R	S	S	R	S	R	R
B14	R	R	R	R	R	R	R	R
B16	R	R	S	S	R	R	R	R
B17	R	R	R	S	S	S	R	R
B18	R	R	R	S	S	R	R	R
B19	R	R	S	S	S	S	R	R
B20	R	S	S	R	R	R	R	R
L. reuteri DSMZ 20056	S	R	S	S	S	S	R	S

[a]The breakpoints for *Lactobacillus* sp. by SCAN category. Minimum Inhibitory Concentration (MIC) equal to or higher than the breakpoint is considered as resistant. (S): Susceptible; (R): Resistant; (A): Ampicillin; (C): Ciprofloxacin; (E): Erythromycin; (G): Gentamicin; (K): Kanamycin; (S): Streptomycin; (Te): Tetracycline and (Tr): Trimethoprim.

In conclusion, it is becoming increasingly obvious that the traditional fermented foods offer unlimited reservoir of probiotics. However, *in vitro* results should be substantiated by *in vivo* studies.

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The Inhibitory Effects of Human, Camel and Cow's Milk against Some Pathogenic Fungi in Iraq

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Abstract

The present study investigates the use of the agar dilution method and the inhibitory effects of different concentrations (10%, 20% and 30%) of human, camel and cow's milk on thirteen different species of fungi from the genera *Aspergillus, Trichophyton, Microsporum, Penicillium* and *Fusarium*. The results show that all the tested concentrations of each of the three milks were capable of inhibiting the growth of the thirteen fungal species, but the greatest inhibitory effect was recorded with the concentration of 30%. Human milk gave the highest growth inhibition rate on all fungal species and the complete growth inhibition (100%) was recorded in respect to *Aspergillus fumigatus* with a chi-square x2 value of 9.462. For camel and cow milk, inhibition rates of 96% and 92%, with chi-square-x2 values of 8.684 and 9.140, respectively, were recorded. Overall, the inhibitory effects were observed to be concentration-dependent.

Keywords: Human, Camel and Cow's Milk, Fungi.

1. Introduction

Milk represents a m ajor source of well-known antimicrobial substances, in addition to its recently discovered immunomodulatory effects. These are important in shaping the immune system of new-borns since the neonatal immune system is not fully developed, making it d ifficult for new-borns to protect themselves from infections (Garofola and Goldman, 1999; Mete *et al.*, 2006).WHO (2003) and Oftedal (2012) confirmed that feeding infants with maternal milk for the first six months of life, with continued breast feeding for the first one to two years of life (or longer), is the normative standard, due to the nutritional composition of human milk and the non-nutritive bioactive factors that promote survival and health development.

Some infants may not exclusively breast-feed during the first months of life, replacing human milk with cow milk modified to mimic the composition of human milk (Posati and Orr, 1976). Many nutritional problems have been reported as a result of the use of cow's milk for infant feeding, especially cow's milk allergy (El-Agamy, 2007; El-Agamy *et al*., 2009); therefore, other types of milk have been proposed as a substitute for human milk including buffalo (Shamsia, 2005), goat (Park and Haenlein, 2006) and sheep (Haenlein and Wendorff, 2006).

Camel milk has the ability to inhibit the growth of pathogens not only because it contains more nutrients compared to cow milk, but because it also has therapeutic and antimicrobial agents (El-Ziney and Al-Turkiy, 2007). It has several beneficial characteristics, such as the absence of diabetes in populations that consume it and tolerance by patients who show intolerance to lactose. Even though camel milk does contain lactose, it is however in a lower concentration than the amount in human milk; it is a nutrient for individuals who are allergic to cow milk (Cardoso *et al*., 2010; Ehlayel *et al*., 2011).

Wernerg (2003) and Shamsia (2009) explained that camel milk contained high fat, protein (especially casein), ash, Ca, Mg, P, K, Na, Fe, Cu, whey protein, lactose and Zn, vitamins and niacin. Camel milk proteins contained a satisfactory balance of essential amino acids and many enzymes with antibacterial and antiviral properties, such as lactoferrin, which prevents microbial growth in the gut, lactoperoxidase, peptidoglycan recognition protein (PGRP),which has a broad antimicrobial activity, lysozyme, which inhibits the growth of bacteria and has a highly effective influence on the storage of camel milk, and immunoglobulin, all of which gives camel milk

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tremendous advantages over conventional antibodies. El-Agamy and Nawar (2000) found that camel milk contained 1.64 mg/ml of immunoglobulin G versus 0.67 for cow milk. A comparative study of lysozyme concentration in milk of different species (El-Agamy *et al*.,1997) showed that camel milk contained a significantly higher content of lysozyme than the cow, buffalo, sheep and goat milks, but a very low content as compared to lysozyme content of human, mare and donkey milks. The same study showed that the camel milk also contained a significantly higher level of lactoferrin (0.22 mg/ml) than cow, buffalo, sheep and goat milks, but very low level compared with the human milk.

Many studies have focused on the antimicrobial and antiviral effects of milk or milk constituents, but only few studies have been conducted to investigate its antifungal effects (Anderson *et al*., 2000). The aim of the present study, therefore, is to evaluate the antifungal effects of human, camel and cow milk on clinical fungal isolates.

2. Materials and Methods

2.1. Fungal Isolates

Thirteen different species of fungi from the genera *Aspergillus, Trichophyton, Microsporum, Penicillium* and *Fusarium* were isolated from different sources and characterized, as shown in (Table.1):

Table1. Clinical fungal isolates and their sources

Fungus Species	Sources of Isolation
Aspergillus niger	Lower respiratory tract infection
Aspergillus fumigatus	Lower respiratory tract infection
Aspergillus flavus	Lower respiratory tract infection
Aspergillus terreus	Otomycosis
Trichophyton mentagrophytes	Dermal infection
Trichophyton rubrum	Dermal infection
Trichophyton gypsum	Lower respiratory tract infection
Trichophyton violaceum	Dermal infection
Trichophyton tonsurans	Dermal infection
Microsporum audouinii	Dermal infection
Microsporum canis	Dermal infection
Penicillium spp.	Otomycosis
Fusarium oxysporum	Lower respiratory tract infection

2.2. Milk Samples

Samples of human milk were obtained from lactating women two months after labour (lactation after colostrum, with a breast pump). Fresh camel's and raw cow's milk samples were collected from apparently healthy animals also after two months after labourbred in the living stock station at the College of Veterinary Medicine, Baghdad University, Baghdad.

The milk samples were placed in sterile containers and transported to the laboratory in a cool box. Human, camel and cow milk samples were passed separately through a Millipore filter (0.22mm) (Bio-Rad) before determining their anti-fungal activity.

2.3. Evaluation of Anti-Fungal Activity of Milk

The following technique was used to determine the anti-fungal activity of the studied milk types according to Wang *et al*. (2005):

100 ml of each type of milk was prepared and each of these volumes was mixed separately with sterilized SDA (Sabouroud dextrose agar) in order to prepare the required concentrations (10%, 20% and 30%). These concentrations were shacked well, poured into Petri dishes and left to solidify in sterile conditions. A 5 mm piece of mycelia growth from mould cultured for seven days was deposited in the centre of each plate. The inoculated plates were incubated at 28ºC for 7-10 days. Replicates were prepared for each treatment. The diameters of the fungal colonies were measured and then the anti-fungal activity of each concentration of the studied milk was calculated by measuring the growth inhibition rate using the following formula (1):

Growth inhibition rate (%) = ({Growth in control – Growth in treatment/ Growth in control} x100).

2.4. Statistical Analysis

The statistical evaluation of the results was performed using Analysis System-Microsoft SAS (2012). The chi-square test was used to compare significance between the growth inhibition percentages.

3. Results and Discussion

The inhibitory effects of human, camel and cow milk at concentrations of 10, 20 and 30% for each of the different fungal species were determined as described previously. The results, shown in (Tables 2, 3 and 4), indicate that for all the fungal species the growth inhibition rate (%) was related to the milk concentration, so that a 30% concentration gave the highest growth inhibition rate, followed by the 20% and 10% concentrations.

For the genus *Aspergillus*, a concentration of 30% exhibited the highest growth inhibition rate on *Aspergillus fumigates* (100%, 96% and 92%, respectively, for the three types of milk) with chi-square-x2 values of 9.462, 8.684 and 9.140, respectively. For the genus *Trichophyton*, the greatest inhibition was recorded for *Trichophyton rubrum* (92%, 87% and 88%, respectively) with chi-square-x2 values of 11.053, 9.417 and 10.427. While for the genus *Microsporum, Microsporum canis* was the most inhibited species with the growth inhibition rates of 75%, 79% and 65% and chi-square-x2 values of 10.628, 8.938 and 9.326.Finally, the genus *Penicillium* and *Fusarium* exhibited growth inhibition rates of 73%, 59% and 51%, and 62%, 55% and 51%, respectively, with chi-square-x2 values of 10.819, 9.155 and 8.629 and 10.062, 8.951 and 8.627, respectively. Chi-square-x2values of 10.622, 8.264 and 8.619 were recorded for each type of milk, respectively.

Table2. The inhibition growth rate (%) of human milk on different clinical fungal isolates

Clinical isolates	Inhibition growth rate (%) with different Concentrations of milk (%)			Chi-square–χ^2 value
	10 %	20 %	30 %	
Aspergillus niger	19	38	62	10.316 **
Aspergillus fumigatus	52	80	100	9.462 **
Aspergillus flavus	30	73	92	10.702 **
Aspergillus terreus	12	23	35	7.934 **
Trichophyton mentagrophytes	40	62	88	10.538 **
Trichophyton rubrum	40	73	92	11.053 **
Trichophyton gypseum	18	30	65	10.812 **
Trichophyton violaceum	19	42	73	10.931 **
Trichophyton tonsurans	19	37	72	10.944 **
Microsporum audouinii	15	32	53	8.592 **
Microsporum canis	22	40	75	10.628 **
Penicillium spp.	22	47	73	10.819 **
Fusarium oxysporum	17	30	62	10.062 **
Chi-square–χ^2 value	9.327 **	11.289 **	11.752 **	-----

** (P<0.01).

Table3. The inhibition growth rate (%) of camel milk on different clinical fungal isolates

Clinical isolates	Growth inhibition rate with different milk concentrations (%)			Chi-square–χ^2 value
	10 %	20 %	30 %	
Aspergillus niger	37	51	82	9.745 **
Aspergillus fumigatus	52	75	96	8.684 **
Aspergillus flavus	29	44	65	8.927 **
Aspergillus terreus	14	39	62	8.634 **
Trichophyton mentagrophytes	25	59	77	9.108 **
Trichophyton rubrum	30	52	87	9.417 **
Trichophyton gypseum	19	32	55	8.405 **
Trichophyton violaceum	`15	41	66	10.271 **
Trichophyton tonsurans	22	41	70	9.528 **
Microsporum audouinii	22	40	63	8.623 **
Microsporum canis	37	52	79	8.938 **
Penicillium spp.	19	33	59	9.155 **
Fusarium oxysporum	20	39	55	8.951 **
Chi-square–χ^2 value	8.219 **	9.855 **	9.891 **	-----

** (P<0.01).

Table4. The inhibition growth rate (%) of cow milk on different clinical fungal isolates

Clinical isolates	Growth inhibition rate with different milk concentrations (%)			Chi-square–χ^2 value
	10 %	20 %	30 %	
Aspergillus niger	11	20	37	6.946 **
Aspergillus fumigatus	40	65	92	9.140 **
Aspergillus flavus	22	37	52	7.351 **
Aspergillus terreus	9	22	40	7.150 **
Trichophyton mentagrophytes	20	47	72	8.255 **
Trichophyton rubrum	23	52	88	10.427 **
Trichophyton gypseum	15	29	44	6.922 **
Trichophyton violaceum	`13	32	56	8.437 **
Trichophyton tonsurans	19	37	59	8.922 **
Microsporum audouinii	12	32	45	7.832 **
Microsporum canis	25	44	65	9.326 **
Penicillium spp.	12	29	51	8.629 **
Fusarium oxysporum	12	30	51	8.627 **
Chi-square–χ^2 value	7.849 **	9.535 **	9.702 **	** (P<0.01).

** (P<0.01).

The results of this study show that the human milk represents the most effective type of milk against fungal growth, compared to camel and cow milk which were ranked second and third, respectively, according to their overall inhibitory effect on the studied species of fungi. Our results agree with those of Mete *et al.* (2009) who

demonstrated that human, cow and infant's formula milks have an antifungal activity *against Rhizopus, Penicillium, Alternaria* and *Aspergillus* and determined that the human milk had a more pronounced antifungal effect than that found in cow milk, after comparing the fungal growth in human and cow milk-rubbed agar.

The results obtained by the present study may be indicative of the effect of some of the constituents of the different types of milk as inhibitors of fungal growth. Lönnerdal (2003) referred to some of these constituents, especially of human milk, which contains wide varieties of proteins that contribute to its unique qualities. The positive health effects of milk proteins can be presented as antioxidative, anti-microbial, antihypertensive, immuno-modulatory and anti-thrombotic (FitzGerald and Meisel, 2000).

There are several types of milk proteins with antimicrobial activity, such as immunoglobulin, casein, lysozyme, lactoferrin, haptocorrin, α-lactoalbumin and lactoperoxidase. These proteins are relatively resistant to proteolysis in the gastrointestinal tract and contribute to the defence of breast fed infants against numerous types of microbes (bacteria, fungi and viruses). These enzymes are present in the milk of cows, ewes, goats, buffalos, pigs, camels and humans (Wernerg, 2003; Seifu *et al*., 2005 and Siseciaglu *et al*., 2010), but their concentration fluctuates depending on several factors such as species, health status of the animal and stage of lactation. Thus, the level of immunoglobulin-G in camel milk is 1.64 mg\mL-1 compared to 0.70, 0.67, 0.55, 0.63 and 0.86 mg\mL-1 for goat, cow, sheep, buffalo and human milk, respectively (El-Agamy and Nawar, 2000). While, the content of the glycoprotein lactoferrin, sometimes known as lactotransferrin, in camel milk (0.22 mg\mL-1) is significantly higher than that in goat, sheep, buffalo and cow milks and very low compared with that of human milk (El-Agamy *et al.,* 1997). At the same time, lactoperoxidase, which is purified from different milk sources, exerts bactericidal activity generally on Gram negative bacteria and antifungal activity especially on *Aspergillus niger, Pencillium schrysogeum, Aspergillus flavus, Alternaria* sp., *Trichoderma* sp., *Corynespora cassiicola, Phytopthora meadii, Claviceps* sp. and *Corticiums almonicolor*, and thus, contributes to non-immune host defence systems (Ueda *et al*., 1997; Ozdemir *et al*.,2002; Uguz and Ozdemir, 2005). Camel milk also has a unique property in that it includes the presence of lactic acid bacteria (LAB), especially *Lactobacillus* sp. strains, as shown by Laref and Guessas (2013) who found that these bacteria have the ability to inhibit the germination of candida and completely inhibit the mycelium growth of *Aspergillus* sp., *Trichoderm* sp., *Pencillium* sp. *Fusarium roseum* and *Stemphylium* sp. on a solid medium by using the overlay method and confrontation assay.

Wakabayashi *et al*.(2006), Kruzel *et al*.(2007) and Legraut *et al*. (2008), show that lactoferrin is an essential element of non-specific innate immunity in humans and other mammals (the concentration of lactoferrin in cow's milk is lower than it is in human's milk). At the same time, lactoferrin protects the intestinal epithelium cells and inhibits the growth of *E. coli* and other pathogenic intestinal bacteria, mainly *Enterobacteriaceae*, while stimulating the growth of useful intestinal micro flora like *Bifidobacterium*.

Shamsia (2009) determined the antimicrobial factors of both camel and human milk and concluded that camel milk is richer in Immunoglobulin (1.54 mg/ml) than human milk (1.14mg/ml). However, its contents of lactoferrin and lysozyme were very low, (0.24mg/ml) and (0.06mg/ml), respectively, as compared with human milk, which contains (1.95mg/ml) lactoferrin and (0.65mg/ml) lysozyme. Shamsia (2009) also reported that camel milk contained more fat, protein, especially casein, and ash contents but lower whey protein and lactose contents than the human milk. The lower casein and higher whey protein contents in human milk make it very nutritious for the new born due to the resultant soft coagulum after milk ingestion and the higher digestibility and absorption of soluble proteins (Fox and Mc Sweeney, 1998)

In conclusion, the present study confirms that there is a positive relationship between the concentrations of the milk proteins mentioned above and the inhibitory growth rate of milk against fungi and that human milk has a stronger inhibitory effect than camel or cow milk.

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Oviposition Deterrent and Egg Hatchability Suppression of *Secamone afzelii* (Schult) K. Schum Leaf Extract on *Callosobruchus maculatus* (Fabricius) (Coleoptera: Chrysomelidae)

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Abstract

The efficacy of oil extracted from the leaves of *Secamone afzelii* was evaluated in the laboratory against *Callosobruchus maculatus* infesting stored cowpea. Leaf extracts from *S. afzelii* were obtained through the soxhlet extraction method using methanol and hexane as the solvent. Each of the extracts was tested by exposing five pairs of adult beetles to various levels of 0.5, 1.0, 1.5 and 2.0 ml corresponding to 2.5, 5.0, 7.5 and 10.0% v/w concentrations admixed with 20g cowpea in three replications respectively, in a Completely Randomized Design (CRD). Control treatment was set along. The results showed that oviposition and percentage egg hatched were significantly (P <0.05) suppressed on seeds treated with higher treatment level of extracts. Leaf extract with hexane at 2 ml (10.0% v/w)/20g cowpea seeds was most effective in suppressing oviposition and egg hatched. Therefore, *S. afzelii* exhibit promising degree of oviposition deterrent and ovicidal properties and, thus, have a great potential for use as a plant-based biopesticide as an alternative to synthetic insecticides for controlling *C. maculatus* infestation on stored cowpea grains.

Keywords: Alternative, Egg hatched, Oviposition deterrent, Ovicidal properties, Biopesticide.

1. Introduction

Post-harvest losses of cowpea grains are serious problems in Africa, and as much as 20-50% of grains is lost because of *Callosobruchus maculatus* infestation resulting in weight loss and quality deterioration (Ofuya and Lale, 2001; Lale, 2010; Mailafiya *et al* 2014). Heat, moisture and waste products, produced by the beetles, result into further deterioration and the growth of mould, thus rendering grains unfit for consumption and marketing. Thus, farmers are forced to sell their produce early after the harvest when prices are still low partly because of the anticipated losses in storage (FAO, 1985). The huge post-harvest losses and quality deterioration caused by this insect pest contribute to the inability of achieving food security in developing countries (Rouanet, 1987).

Infestation control of stored grains insect pests has primarily relied upon the use of synthetic chemical insecticides, such as methyl bromide and phosphine. The shortcomings associated with the continuous use of these chemical insecticides, such as high cost of procurement, pest resurgence and resistance, poisonous residue accumulation in foods, risks of user's contamination, effect on both human and environmental health, have necessitated the need for seeking alternative means of insect infestation control that are non-toxic and eco-friendly (Ileke *et al*., 2014).

Secamone afzelii Schult) K. Schum (Family: Apocynaceae) known in major Nigerian languages as arilu, ailu or alu in Yoruba, utunta (Ibo) and Ewuonkwonegie (Bini) (Gill, 1992), is a scandent or creeping woody climber found on fences and trees. It grows to a very long length of about 12m with pinnately compound leaves (Abere and Onwukaeme, 2012; Prota, 2014). *S. afzelii* is used in traditional medicine for stomach problems, diabetes, colic, dysentery, treatment of sexually transmitted infections, purgative for children, sore throat, backache, cough, catarrhal conditions and as galactogogue, purge, also for kidney problems and as a remedy for spinal disease (Abo *et al.,* 2008; Gill, 1992; Watt and Breyer-Brandwijk, 1962; Oliver, 1960).

In literature, there appears to be a dearth of empirical information on the utilization of *S. afzelii* extracts for their insecticidal potential. However, Adesina and Ofuya (2011) and Adesina *et al.* (2012) reported the efficacy of *S. afzelii* leaves and vine powder for the control of *C. maculatus* and *Sitophilus zeamais*. Therefore, the objective of the present study is to evaluate the efficacy of organic solvent extract of *S. afzelii* as protectants for stored cowpea seeds against *C. maculatus* infestation.

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2. Materials and Methods

2.1. Insect Culture and Experimental Conditions

Callosobruchus maculatus stock, used for the present study, was obtained from an established culture from Entomology Laboratory of Crop, Soil and Pest Management of Federal University of Technology, Akure, Ondo state, Nigeria. The insects were sub-cultured on 200g Sokoto white local cowpea cultivar (a well known susceptible cultivar) in Kilner jar under laboratory conditions (32±0.6°C, 68±3% relative humidity and 12L: 12D photo regime) (Idoko and Adesina, 2013; Idoko and Adesina, 2012; Udo, 2005) for oviposition to produce a steady and sufficient supply of beetles of known age for experimental purpose (Adesina, 2012) in the Entomology Laboratory of Crop, Soil and Pest Management Technology Department, Rufus Giwa Polytechnic, Ondo State, Nigeria (Latitude 5° 12' N and Longitude 5° 36' E).

2.2. Collection and Preparation of Plant Materials

Leaves of *S. afzelii* were obtained from Ipesi Akoko, Ondo State, Nigeria and air-dried under a room temperature for about 2 weeks to avoid possible volatilization of the active ingredients (Adesina, 2012). The dried leaves were milled into powder using a hammer mill (Epidi *et al.,* 2009). Thereafter, the milled leaf powder of *S. afzelii* was taken to the laboratory for extraction using ethanol and n-hexane as organic solvent using soxhlet apparatus.

2.3. Soxhlet Extraction

The ordinary method of extraction was not efficient to yield a good amount of active ingredients of the plant material. The plant material was extracted using the Soxhlet extraction method (Anurag Sharma and Raskesh Gupta, 2009). A known amount (500g) of *S. afzelii* was filled into the Soxhlet apparatus. A cotton plug was used at the place of thimble to stop the entry of the crude material into the siphoning tube. The required solvent (ethanol and n-hexane) was filled up five times more than the total amount of the sample material into the flask of the apparatus. The apparatus was then connected with the water supply to the condenser. The temperature of the heating mantle was maintained at 60-65°C (boiling point of ethanol). The process was carried out for 5 to 6 hours for each sample. The extracts were later concentrated using a rotary evaporator to obtain a concentrated extract, which was stored in specimen bottle for future use.

2.4. Sources of Cowpea Seeds

The freshly harvested seeds of Ife brown cowpea variety, which were used in the bioassay experiments, were obtained from the Teaching, Commercial and Research Farms of Rufus Giwa Polytechnic, Owo, Ondo State, Nigeria, were adequately air-dried to 12% moisture content (Adesina, 2013). The seeds were properly sieved and handpicked, thus ensuring that the only whole and uninfested seeds were used (Olotuah *et al.,* 2007; Adesina *et al*., 2012). The clean seeds showed no visible signs of beetle eggs, presence of adults or exit holes. These were nevertheless sterilized in an oven at 50°C for 4 hours to kill any immature stage of insect (if any) and were allowed to cool for 1 hour before use (Idoko and Adesina, 2013). This harvested cowpea had no history of postharvest insecticide treatment.

2.5. Effect of Extracts on Oviposition and Fecundity

S. afzelii, extracted at four dosages, namely 0.5, 1.0, 1.5 and 2.0 ml (corresponding to 2.5, 5.0 7.0 and 10.0% w/v concentration), was measured into 9.0 cm diameter disposable Petri-dishes containing 20g of disinfected cowpea seeds weighed using a digital weigh balance (model TS 400D) in triplicates using a syringe and each is thoroughly mixed using a glass rod to ensure the uniform mixing of the extract with the grains and is left open to dry. Thereafter, five (5) paired sexed adult insects of 1-2 days old *C. maculatus* were introduced into each Petri-dish containing different dosages of plant extract/ food complex of treated and untreated grains (Udo, 2000). The sex of *C. maculatus* was determined by the pattern of Iloba and Ekrakene (2006). There was also a control treatment that does not involve any addition of extract on the seeds. The Petri dishes were then covered to prevent insects from escaping. The number of eggs laid by the female beetles on the seeds was recorded on the 14th day after the introduction of beetles to seeds; this was used to calculate the percentage of egg hatching according to Abdullahi *et al.* (2011) and the percentage reduction of egg laying (Emman and Abass, 2010), respectively, as follows:

Percentage reduction of eggs laid =

$$\frac{\text{no of eggs laid in control} - \text{no of eggs laid on treated grains}}{\text{no of eggs laid in control}} \times \frac{100}{1}$$

egg hatching (%) =

$$\frac{\text{no of eggs hatched}}{\text{no of eggs in each Petri} - \text{dish}} \times \frac{100}{1}$$

2.6. Statistical Analysis

The experiment was laid out in Completely Randomized Design (CRD) and each treatment was replicated there (3) times. Percentage data were transformed to square root of arcsine to normalize the data before analysis. Data from the 3 replicates of the experiment were pooled together and subjected to one-way Analysis of Variances (ANOVA). Treatment means were separated using Least Significant Differences (LSD) at 5% probability level (Gomez and Gomez, 1994).

3. Results

3.1. Effect of S. afzelii extracts on oviposition and fecundity by C. maculatus

The effect of the extracts on oviposition is summarized in Table 1. The results showed that the extracts at all application levels significantly inhibited the female *C. maculatus* from laying eggs on treated cowpea seeds. In spite of the the early death of *C. maculatus* adults, no concentration of the extracts could completely prevent the females from oviposition. The percentage reduction in the number of the laid eggs was inversely proportional to the extracts concentration tested. The laying capacity

gradually decreased with the increase in the treatment dose of each extract. The maximum reduction in egg laying was noticed with hexane extract 41.0% reduction on grains treated with 2.0ml dosage rate as against 24.21% recorded in control. The same trend was recorded in ethanol extract when at 2ml, 51.6% eggs reduction was observed on treated cowpea against 30.6% in control treatment. Statistically, there was a significant difference between the tested concentrations compared to control.

Table 1. Mean percentage reduction in number of eggs laid by female *C. maculatus*

Treatments/20g cowpea	percentage reduction in eggs laid	
(Conc in %)	Methanol	Hexane
0.0ml (0)	30.62±3.7	24.21±1.8
0.5ml (2.5)	40.84±2.3	28.23±2.2
1.0ml (5.0)	47.55±4.4	20.77±3.4
1.5ml (7.5)	47.89±2.1	41.02±4.2
2.0ml (10.0)	51.61±3.3	52.85±2.3
LSD (5%)	11.01	23.76

3.2. Effect of S. afzelii Extracts on Egg Hatching

In the present study, the effect of *S. afzelii* extracts on the egg hatching capability of *C. maculatus* revealed that there was a significant reduction. The egg hatching capacity gradually decreased with the increase in the treatment dose level of each leaf extract. The maximum reduction in the hatched eggs was noticed at 2ml (10%) level when only 7.94% and 8.79% eggs found to be laid, hatched on the seeds treated with hexane and methanol extracts, respectively, as against 31.93% and 31.37% hatched eggs in control (Table 2). The results revealed that *S. afzelii* extracts, at different dose level, were very effective against *C. maculatus* egg viability on stored cowpea.

Table 2. Mean percentage of egg hatching of *C. maculatus* from treated cowpea seeds

Treatments/20g cowpea	percentage egg hatching	
(Conc in %)	Methanol	Hexane
0.0ml (0)	31.37±4.28	31.93±3.3
0.5ml (2.5)	19.58±2.1	15.78±2.3
1.0ml (5.0)	12.86±1.6	12.76±1.8
1.5ml (7.5)	9.24±0.4	10.87±1.2
2.0ml (10.0)	8.05±1.2	7.94±0.5
LSD (5%)	8.79	4.31

4. Discussion

The results of the study provide empirical evidence that the insecticidal activity of *S. afzelii* extract with ethanol and hexane can be effective to varying degrees in deterring oviposition and egg viability by *C. maculatus*, and ultimately reduced the percentage seed damage and weight loss due to infestation by the foregoing insect pest. However, no concentration of the plant extracts completely prevented the females from laying eggs on the seeds. This study also showed that the plant extracts significantly reduce the number of eggs laid per female compared to that obtained in the control.

The marked decline in egg laying was perhaps a consequence of the mild suppressing effect exerted by these volatiles on the pulse beetles' mating, which is a decisive factor influencing the subsequent number of eggs laid by the beetles (Engelmann, 1970). The present findings corroborate the observation recorded for oil vapors on *C. maculatus* (Paranagama *et al.*, 2003).

These findings are in accord with Elhang (2000), Kim *et al.* (2003), Ghoswal *et al*. (2004) and Abdullahi (2011) who found that the reduction in egg laying of pulse beetle was significantly high when the seeds were treated with various pesticidal plant extracts, and a similar trend was noticed in the case of some vegetable oils too. The reduction in oviposition in the extract treated seeds may also be caused by an extract film on the seeds which becomes unsuitable for oviposition. The results revealed that various pesticidal plants at a different dose level acted as highest ovipositional deterrents. The ability of the extract to reduce the egg laying capability by the female beetles may be attributed to the presence of flavonoids in the plant (Zabri *et al.*, 2008; Zabri *et al.*, 2009). This confirmed the findings of Righi-Assia *et al.* (2010) that flavonoids significantly reduce the egg laying and fertility in *C. Chinensis.* Results from this study equally suggest that the plant extract might interfere with the normal embryonic development by suppressing hormonal and biochemical processes. A similar physiological inference was observed by Ofuya *et al.* (1992), Jayakumar *et al.* (2003) and Raja and William, (2008). The extract could also involve an ovarian change similar to those that caused chemosterilisant by blocking female egg laying. This supports the findings of Saxena and Rohdendorf (1974) and Schmidt *et al.* (1991a and b). Jadhav and Jadhav (1984) reported that *Jatropha curcas* seed oil applied at 0.2% (v/w) concentration significantly reduced the number of eggs laid by adult *C. maculatus* and prevented eggs hatched 33 days after the treatment. Also, Adebowale and Adedire (2006) observed that the cowpea seeds treated with *Jatropha curcas* seed oil reduced the number of eggs laid by *C. maculatus* and prevented the adult emergence at a concentration between 0.5 and 2% (v/w). While, Ofuya (1990) and Tapondjou *et al.* (2002) suggested that the oviposition inhibition property of botanical powders on adult bruchids (in terms of weakening of adults by powder treatments) made them lay fewer eggs and killed the larvae hatching from eggs laid on grains. Oviposition inhibitors have the advantage of attacking a pest at the start of its life cycle. The insect is deterred from laying its eggs on the cereals/grains, thus preventing the pest population from increasing (Pandey *et al.*, 2011).

The leaf extract used in this study was found to be significantly superior in reducing egg hatching; as the concentrations of the extracts increased, their inhibitory effect on egg hatching also increased. The egg mortality and the failure to hatch on seeds, treated with the extract, were probably attributed to the toxic component of the extracts and also to the physical properties, which caused changes in the surface tension and the oxygen tension within the eggs (Singh *et al.,* 1978). The ovicidal effect of

the extracts on the bruchid may also be explained in terms of asphyxiation by blocking the major route of gas exchange between a thin area of the chorion and outside (Credland, 1992), which ultimately reduced the emergence of the insects from the treated seed (Copping and Menn, 2000).

Very few studies on the effect of pesticidal plant on the egg hatching of pulse beetle are available. However, Abdulahi (2011), Abdulahi *et al*. (2011) and Adesina *et al*. (2011) found a significant effect on reduced egg hatchability of *C. maculatus* when laid on seeds treated with plant extracts and powder, respectively, at different doses, which is in agreement with the present study. The current results are in agreement with Ketoh *et al.* (2006) who reported that *Cymbopogon* oil vapor treatment for 24h could be satisfactory for controlling the eggs hatchability of *C. maculatus*. In a related development, Gajmer *et al*. (2002) reported that eggs laid on extract-treated oviposition substrate, exhibited reduced hatching and that marked adverse effects on hatching were noticed when the eggs were dipped in different concentrations of extracts, they also reported that adults, fed on an extract containing sucrose diet, laid significantly fewer eggs with poor hatching.

The extracts coating the seeds may have possible contact effects on the insect during oviposition since eggs are laid on the seed, thus preventing *C. maculatus* eggs to firmly attach to the seed coat (Adebowale and Adedire, 2006) or blocking respiration (Bamayi *et al*., 2007) thus inhibiting or interfering with the normal embryonic development by suppressing hormonal and biochemical processes, which prevent egg hatching. This finding suggests that the *S. afzelii* extracts successfully inhibit egg hatching into the seed and ultimately suppress the F1 progeny emergence.

The present study clearly demonstrates that *S. afzelii* possess anti-oviposition and ovicidal activities that can be employed in the management of *C. maculatus* infestation on stored cowpea. Further studies are needed to explore isolating the bio-active molecule responsible for the insecticidal properties exhibited by the plant.

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The Effect of Dry Extract of *Derris elliptica* Stem on some Enzymatic Changes in the Plasma of African Catfish *Clarias gariepinus* (Burchell, 1822).

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Abstract

The dry extract of different parts of the *Derris elliptica* is used in fish ponds to harvest and control predatory fish. However, the toxicological impact of the extract on fish has not yet been evaluated. The objective of the present study is to investigate the effects of the extract on some enzymes of plasma of the widely consumed African catfish *Clarias gariepinus*. Fish specimen were exposed to sublethal concentrations (0.00 [control], 0.75, 1.50 and 3.00g/L) of dry extract of stems of *D. elliptica* for 24, 72 and 168 h adopting the semi-static renewal bioassay technique and were then subjected to plasma analyses. The level of plasma acid phosphatase and plasma alkaline phosphatase decreased significantly ($P<0.05$) while plasma alanine aminotransferase and plasma aspartate aminotransferase increased significantly ($P<0.05$). It was concluded that the dry extract of the stem of *D. elliptica* produced disorders in liver, kidney and respiratory metabolism of *C. gariepinus*.

Keywords: *Derris elliptica*, African catfish, Enzymatic parameters.

1. Introduction

The pesticidal effect of plant extracts are of unique importance due to their chemical compositions that enhance their properties as medicinal plants, preservatives, insecticides, molluscidices, to mention a few. Thus, they have always been useful to man and aquatic animals (Akinwade *et al.,* 2007). Due to their narcotic, pesticidal and molluscidal properties, many fishermen and fish farmers indiscriminately use the extracts of different parts of these plants to weaken and kill the fishes for easy catch and clean up the aquatic systems of some pests. Some farmers use very high concentrations of these plant extracts which can lead to physiological disturbances in the aquatic organisms and ultimately to a reduction in aquatic productivity (Mondal *et al.,* 2007). Some of these used plants are non-selective in their destruction, thereby interfering with the ecological balance of the immediate environment. The usefulness of plants extracts for pesticidal and medicinal purposes was reported by Akobundu, 1987 and Adewole *et al.,* 2002.

Derris elliptica is a plant that belongs to the family Leguminosae, subfamily Papilionoideae. Over the years, *Derris* has been known as an important source for compounds with broad spectrum of insecticidal properties (Gupta, 2007). It is locally known in Southern East Asian Countries as Derris or Tuba while in Thailand as Lotin or Hang liadaeng. *D. elliptica* is a liana (woody climbing plant) which is up to 16 m long, root is reddish-brown, apical shoots often leafless for several meters and rusty pubescent, leaflets are 7-15cm long, mostly densely rusty hairy on both surfaces when young. Its extract is very poisonous and is used locally by fishermen in Nigeria for catching fish. Recently, it has also been used extensively for controlling insect pests. Different parts of the plant are also used in traditional medicine for the treatment of wounds, calculus, rheumatism and dysmenorrhoea and asthma in man. Extracts and metabolites from this plant have been found to possess significant larvicidal, pesticidal, cytotoxic, anti-fungal, anti-inflammatory, antimicrobial, nitric oxide inhibitory, and cancer chemopreventive activities (Olufayo and Akinpelumi, 2012).

The African catfish *Clarias gariepinus* is the most suitable species for aquaculture in Africa and is with a Pan-African distribution, from Nile to West Africa and from Algeria to South Africa. The African catfish has a high growth rate; the exposure of this catfish to these biocides may cause stress not necessarily leading to death. The stress response is characterized by biochemical and physiological changes which may be manifest in both acute and chronic toxicity tests (Singh and Singh, 2002; Tiwari and Singh, 2004). The disruption of biochemical and physiological integrity is assessable by the changes in

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the enzyme activities in functional organs (de la Torre *et al.*, 2000; Van Der Oost *et al.*, 2003).

Measurement of the enzymatic activities or marker enzymes in tissues plays a significant and well-known role in diagnostic, disease investigation and in the assessment of drug plant extract toxicant for the safety toxicity risk. Enzymes serve a wide variety of functions inside living organisms. They are indispensable for signal transduction and cell regulation often via kinases and phosphatases (Hunter, 1995). Enzymes are biochemical macromolecules that control the metabolic process of organisms, and a slight variation in the enzyme activities would affect the organism (Roy, 2002). The activities of alkaline phosphatase, alanine aminotransferase, aspartate and aminotransferase, are useful marker enzymes of damage to the liver and kidney (Akanji *et al.,* 1993). Changes in the activities of these enzymes may help to forecast the consequence of long-term exposure of fish to chemical pollutants (Adedeji *et al.*, 2010). Moreover, the evaluation of blood biochemistry was considered as a useful tool for the diagnosis of diseases and assessing the physiological status of fish (Stoskopf, 1993). Many studies have investigated the changes in many physiological indices of animals induced by environmental conditions and presence of contaminants (Kori-Siakpere *et al.*, 2006; Maheswaran *et al.*, 2008; Ololade and Oginni, 2010). The biochemical parameters in the fish are valid for physio-pathological evaluation and sensitive for detecting potential adverse effects and relatively early events of pollutant damage (Almeida *et al.*, 2002; Matos *et al.*, 2007; Osman *et al.*, 2010).

Hence, the present study is conducted to evaluate the effects of four concentrations of dry extracts of the stem of *D. elliptica* on the plasma enzymes of the African catfish *C. gariepinus* in order to evaluate its long term effect on fish..

2. Materials and Methods

2.1. Experimental Animals

Live specimens of *C. gariepinus* (mean total length 30.50 ± 0.90 cm; mean weight, 152.78 ± 8.11g) were obtained locally from a commercial fish farm. They were transferred to the Animal and Environmental Biology Research Laboratory, Delta State University, Abraka, Nigeria. The fishes were held in the laboratory in large plastic aquaria of 140L capacity with clean borehole water. They were then acclimatized for 14 days during which they were fed to satiation with commercial fish feed pellets (Coppens 4.0 mm; 35% crude protein diet) twice daily. Uneaten food and faecal matters were removed daily during the acclimation and the experimentation period. Dead fish were also promptly removed to avoid contamination. The percentage of death recorded during acclimatization was less than 2% and as such the fishes were accepted as being adapted to the laboratory conditions.

2.2. Plant Material

Fresh stems of *D. elliptica* were collected from a farm in Oleh, Isoko South Local Government Area, Delta State, Nigeria and transported to the Department of Animal and Environmental Biology Laboratory. The plant was identified as *D. elliptica* by Dr. (Mrs.) N. E. Edema of the Department of Botany, Delta State University, Abraka, Nigeria. They were air-dried for two weeks and later oven-dried for three hours at 60°C to a constant weight. The dried stems were ground into powder with an electric blender (MX - 2071, Nakai Japan), sieved and the fine powder was stored in a dry airtight container.

2.3. Toxicant Preparation

The concentrations of the dry stem extract of *D. elliptica* used for the experiment were 3.00, 1.50, 0.75 and 0.00(control) g/L which were obtained after preliminary investigation. These concentrations were introduced into four sets of tanks, each designed for one of the four mentioned-above concentrations with two replications.

2.4. Experimental Procedure

The containers used in the experiment consisted of plastic containers of 120 L capacity. The upper part of each container was covered with a lid made of fine polyethylene gauze screen of 1mm mesh size. Fifteen acclimatized specimens were stocked in a tank and were exposed to a particular concentration of the aqueous extract of *D. elliptica* for 168 h.

The toxicant and water were renewed (semi-static bioassay) after 24, 72 and 144 h of exposure to maintain the toxicant strength and the level of dissolved oxygen (D.O) as well as to minimize the level of ammonia excretion during the experiment (Kori-Siakpere, 1996). The water quality parameters of the exposure water, used in the tests and control, were temperature 28.30±1.3°C, pH 7.58±0.32, dissolved oxygen 8.32±1.04 mg L^{-1}, free carbon dioxide 4.85±0.08 mg L^{-1}, alkalinity 36.50±1.72 mg L^{-1} and hardness 134.53± 11.75 mg L^{-1}.

2.5. Sampling Procedure

At the end of the exposure periods (24, 72 and 168 hrs), the fish were taken from the control and test tanks, sacrificed and subjected to the analyses.

Five fishes were caught individually in a small hand net from the containers. After the preliminary investigation of the length and weight, the fish were then placed belly upwards and blood samples were obtained from the caudal circulation with the aid of a heparinised 2 cm^3 disposable plastic syringes with a 21 gauge disposable hypodermic needle. Plasma was obtained from blood samples by centrifugation and then drawn into a 1 cm^3 plastic syringe and transferred into a lithium heparin bottle, diluted 1:20 with deionised water. The diluted plasma was then stored in a refrigerator and later used for the analysis of plasma enzymes: acid phosphatase, alkaline phosphatase, alanine aminotransferase, aspartate and aminotransferase. All determinations were carried out in duplicates for each sample.

2.6. Enzyme Analyses

Activities of acid phosphatase, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase were determined spectrophotometrically using Teco Diagnostic, Anahein, USA commercial kit, following the manufacturer's instruction with the aid of spectrophotometer.

2.7. Data Analysis

The results obtained were subjected to two-way analysis of variance (ANOVA). Comparisons of the means were done using Dunnet Multiple Comparison and results were considered significant at the 95% confidence level ($P< 0.05$).

3. Results

3.1. Acid and Alkaline Phosphatase

The level of plasma acid and alkaline phosphatase significantly decreased ($P<0.05$) in the fish exposed to various concentrations of the stem powder of *D. elliptica* when compared with the control (Figures 1 and 2).

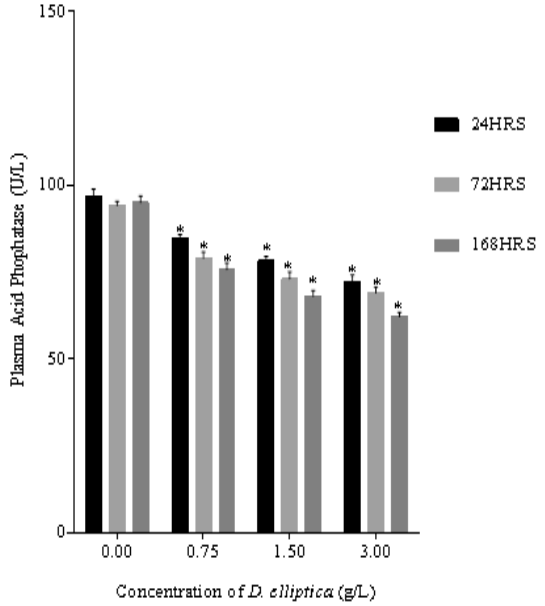

Figure 1. Mean values of acid phosphatase in the plasma of *C. gariepinus.* Each column represents the mean value and vertical bars indicate the standard error of the means. Asterisk represents significant difference between the control and experimental group at (*P<0.05*) level.

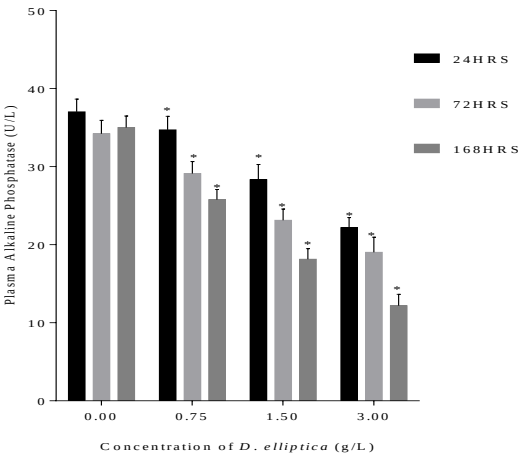

Figure 2. Mean values of alkaline phosphatase in the plasma of *C. gariepinus*

3.2 Alanine Aminotransferase

The level of plasma alanine aminotransferase in *C. gariepinus* is presented in Figure 3. The level of alanine aminotransferase showed a insignificant increase ($P<0.05$) in the fish exposed to various concentrations of the stem powder of *D. elliptica* when compared with the control.

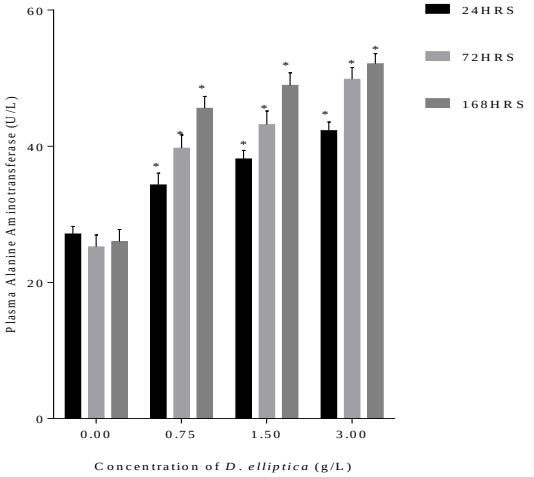

Figure 3. Mean values of alanine aminotransferase in the plasma of *C. gariepinus*.

3.2. Aspartate Aminotransferase

The level of plasma aspartate aminotransferase in *C. gariepinus* is presented in Figure 4. The level of aspartate aminotransferase showed a significant increase ($P<0.05$) in the fish exposed to various concentrations of the stem powder of *D. elliptica* when compared with the control.

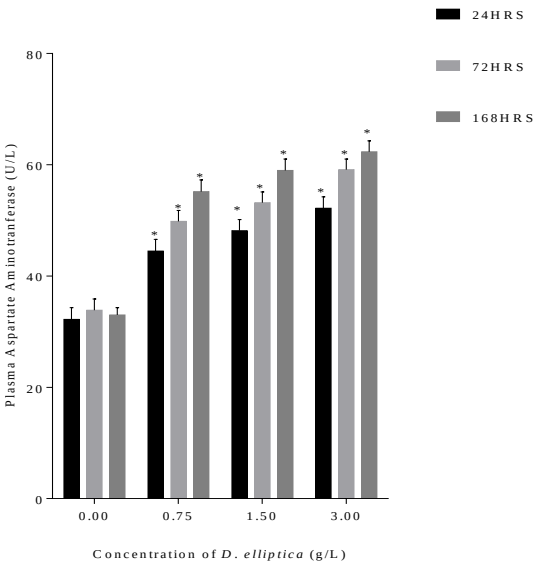

Figure 4. Mean values of aspartate aminotransferase in the plasma of *C. gariepinus*

4. Discussion

The enzymes considered in this study are useful marker enzymes which indicate the cellular damage long before the structural damage which is revealed by some other convectional techniques (Shahjahan *et al*., 2004; Akanji *et al.,* 1993).

Assay of these enzymes are parts of standard laboratory test to detect abnormalities in animals (Ayalogu *et al.*, 2001; Gabriel *et al.*, 2010). Changes in these enzymes activities resulting from toxicant or contaminant effects in various organs of fish were reported by Mgbenka *et al.*, 2005 and Oliverira *et al.*, 2006. Such alterations in fish are aimed at maintaining equilibrium in the presence of these toxicants which are known to disrupt physiological and biochemical processes (Winkler *et al.*, 2007).

Alkaline and acid phosphatase activities decreased with the increase in the concentrations of *D. elliptica* stem extract. Alkaline phosphatase is a marker enzyme for the plasma membrane and endoplasmic reticulum (Wright, 1974). In the present study, there was a significant decrease in the plasma alkaline phosphatase and acid phosphatase activity of fish probably due to the inhibition of the enzymes by the plant extracts (Akanji, 1993). This may result in the alteration of phosphatase metabolism and is an indication of the toxic effect of the stem extract of *D. elliptica*.

The dose and time-dependent inhibition of alkaline and acid phosphatase observed in this investigation is in agreement with the reports of many authors. Adamu (2009) reported a decreased value of plasma alkaline phosphatase in *Heteroclarias* (a Hybrid of *Heterobranchus bidorsalis* and *C. gariepinus*) exposed to sublethal doses of tobacco (*Nicotiana tobaccum*) leaf dust. Ogueji and Auta (2007) reported a reduced value of plasma alkaline phosphatase in African catfish *C. gariepinus*, exposed to lambda-cyhalothrin. Sastry and Sharma (1980) reported an alkaline phosphatase inhibition after 96h exposure to diazinon. Goel *et al*. (1982) reported a plasma alkaline phosphatase inhibition by 15% in *Heteropneutes fossilis*, resulting from the effect of malathion. Similarly, Das and Mukherjee (2003) reported a depletion of alkaline phosphatase due to sublethal exposure of *Labeo rohita* fingerlings to cypermethrin. Rashatwar and Hyas (1983) reported a significant decrease in alkaline phosphatase activity in freshwater fish *Nemachelius denisonii* (day) exposed to sublethal concentrations of Basalin.

The significant (P<0.05) decrease in the acid phosphatase (ACP) concentration with an increase in the concentration in the plant extract in this study is similar to that observed in *C. gariepinus* adults to acute effect of diazinon on blood plasma biochemistry (Adedeji, 2010). Sastry and Sharma (1980) reported a decrease of activities in acid phosphatase in the brain of *Channa punctatus* following the effect of diazinon. Goel *et al.* (1982) reported that plasma acid phosphatase decreased by 15% in *Heteropneutes fossilis*, resulting from the effect of organophosphate malathion. The activities of acid phosphatase in blood plasma of *Cyprinus carpio* were almost identical in the control and test treatment following the exposure to acute effect of diazinon (Luskova *et al.*, 2002).

Aminotransferases are gainfully used in the diagnosis of disease and tissue damage. It functions as a link between carbohydrate and protein metabolism by catalyzing the inter conversion of strategic compounds, respectively (Martin *et al.*, 1983). They are intracellular enzymes which exist in only a small amount of the plasma. Their presence in the plasma may give information on organ dysfunction (Wells *et al.*, 1986; Gabriel and George, 2005). The aminotransferases occupy a central position in amino acid metabolism as they help in retaining amino group (to form a new amino acid) during the degradation of amino acid; they are also involved in the biochemical regulation of intracellular amino acid pool. They also help in providing necessary intermediates for gluconeogenesis. In the present study, the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increased significantly (P<0.05) in the plasma as the concentration of *D. elliptica* increased; this indicated stressed based tissue impairment (Svoboda, 2001). Under stress conditions, fish need more energy resulting from a higher demand for carbohydrate and their precursors to keep the glycolytic pathway and TCA cycles at sustained levels (Tiwari and Singh, 2004). Similarly, in other studies (Ayalogu *et al.*, 2001; Svoboda *et al.*, 2001; Tiwari and Singh, 2004) an alteration in the activities of ALT and AST was recorded, indicating that there was an increased demand for energy due to tissue impairment. Studies carried out by Das *et al.* (2004) also showed that there was an alteration in the activity level of ALT and AST of Indian major carps exposed to nitrite toxicity and suggested that the alteration of the aminotransferases is a result of the diversion of the amino acids in the TCA cycle as keto acids to argument energy production. From the pattern of the results obtained in this plasma aminotransferase, it is conceivable that the plant powder caused an increased energy demand by the exposed fish.

5. Conclusion

It was concluded from this study that the stem powder of *D. elliptica* could affect the liver function of the African catfish by decreasing its plasma ACP, ALP and by increasing the plasma ALT, AST levels. These parameters appeared to be good indicators of the deteriorating health of the fish exposed to the stem powder. Using these parameters, a presumptive prediction can be made on the health status and the possible problem (infection or toxicity) of the fish. Results of the present study provide baseline information. However, the parameter set may be, to some extent, case-dependent and requires information about the history of the fish.

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The Impact of Cassava Effluent on the Microbial and Physicochemical Characteristics on Soil Dynamics and Structure

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Abstract

The effects of cassava effluent on soil microbial and physicochemical properties were studied using culture-dependent and standard analytical methods. Soil samples were collected from sites polluted with cassava effluent and from adjacent sites that were not impacted with the effluent pollution. The isolation and enumeration of microbial population was carried out using standard culture-based methods. Standard analytical methods were used to assay for physicochemical properties. The highest bacterial count of $3.61\times10^8 \pm 0.12$ CFU/g was recorded for polluted soil sampled from Ehor, while the lowest count of $1.3\times10^8 \pm 0.03$ CFU/g was recorded in Isihor. Isihor had the highest fungal count of $2.2 \times10^8 \pm 0.01$ CFU/g from soil contaminated with cassava effluent. The fungal counts of the polluted soil were significantly lower than the bacterial counts generally ($p < 0.05$). The heavy metal contents of the contaminated soils were relatively higher than the uncontaminated soil (control). Unlike in the control soils, pH of the polluted soils ranged from 4.0 - 4.78. The bacteria isolated were *Bacillus subtilis, Bacillus macerans, Pseudomonas aeruginosa*, *Klebsiella aoxytoca* and *Escherichia coli*. Eleven species of fungi belonging to the genera *Aspergillus*, *Penicillium a*nd *Rhizopus* were also isolated. The present study shows that the cassava effluent can have an increasing or limiting effect on the microbial diversity of the polluted soil which could also be attributed to the simultaneous impact on the physicochemical parameters of the soil.

Keyword: Biodiversity; Microbial density; Heavy metal; Pollution; Toxicants.

1. Introduction

The risk to human lives and aquatic organisms constituted by industrial and gaseous effluents cannot be over-stressed (Okafor, 2011). Most industries are responsible of releasing contaminants into the environment. Soil and water bodies are particularly polluted with toxicants from food processing and allied industries and inhabitants of the affected areas are exposed to health related risks as a result of this uncontrollable industrial discharge (Salami and Egwin, 2007). Soil is the uppermost layer of the earth's crust formed as a result of the microbial transformation of weathered rocks (Kolwan *et al.,* 2006). Soil is stratified into several layers and the topsoil is the most prolific. The topsoil consists of soil microorganisms which are involved in the degradation of organic matter and nutrient cycling. This has an effect on global geochemical nutrient (Bunning and Jimenez, 2003). The topsoil gets the ultimate effect from environmental pollutants. Such pollutants include hydrocarbon pollutants, palm oil mill effluent, human and animal wastes, wood waste, waste water from agro-allied industries and refineries, mining effluent as well as cassava mill effluent from cassava processing activities (Wade *et al.,* 2002; Walsh *et al.,* 2002; Ojumu, 2004; Arimoro and Osakwe, 2006).

Cassava (*Manihot esculenta*) belongs to the family Euporbaceae (Nwaugo *et al.,* 2008). It is one of the largest sources of energy-giving foods in the tropics (Fauquet *et al.,* 1990). Cassava is an essential food in Nigeria and other developing countries. Nigeria is the largest producer of cassava while the greatest exporter of this crop is Thailand (FAO, 2004). There has been great upsurge in the production and utilization of cassava in the past few years. This has led to the establishment of cassava milling engines in most environments with the consequence of an extensive ecological pollution associated with the effluent discharge. The unpleasant smell coming from the fermenting effluent calls for the establishment of laws to guide the discharge of cassava waste generated (Nwaugo *et al.,* 2008). In Niger Delta region of Nigeria, cassava tubers are processed for eating either as starch, garri, fufu, dried or wet cassava flour. Garri is widespread among all processed cassava products in Nigeria. Garri production is accompanied with the release of water, hydrocyanic acid, organic matter and sieves from the pulp. Hence, the remnants of the processing of cassava consist of solid and liquid wastes.

Reports have shown that the cassava effluent contains harmful cyanides, copper, mercury and nickel which have

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the capacity to affect native micro-biota (Aiyegoro *et al.,* 2007). Pollution from such effluent could result to a serious imbalance in the living and non-living entities of the ecosystem (Lemke *et al.,* 1997). This could lead to a reduction in the soil fertility. Unlike toxigenic organic matters that are susceptible to degradation, the metals that are discharge into the soil have the tendency to persist indeterminately where they accumulate in living organisms through food chain (Cossic *et al.,* 2002).

Cassava mills, which are mainly on small scale platform, are processed and managed by persons who lack knowledge of environmental safety. While on a small scale platform, there are many of them, when combined together they produce great negative effects on the ecosystem (Nwaugo *et al.,* 2008). Thus, it is essential to estimate the extent of such effects on both biotic and abiotic components of the ecosystem. Although Okafor and Uzuegbu (1987) studied the biochemical transformation associated with the fermentation of cassava effluent there are few reports on the ecological impacts of this effluent in some parts of Edo State, Nigeria. We evaluated the impact of cassava processing mill activities on culturable bacterial and the fungal diversity and the physicochemical status on the soil microbiota to ascertain the degree of pollution on the environment.

2. Materials and Methods

2.1. Samples Collection

Using disinfected soil auger, soil samples were collected in clean sterile bottles from a depth of 0-20 cm from soil polluted with cassava effluent in Oluku, Isihor and Ehor in Edo South region of Edo State, Nigeria. Soil samples, free of cassava effluent, were also collected from control sites outside the processing plant. Samples were collected during the rainy season between August and October, 2014.

2.2. Sample Preparation

One gram (1 g) of the soil samples was measured into a sterile test tube and 9 mL of sterile distilled water was added to make a stock solution; dilution was made from the stock solution. The 10^{-1} suspension was subsequently serially diluted to 10^{-10} dilution. Diluted samples were used for microbial analysis. On the other hand, soil samples were subjected to air-drying for seven days in the laboratory, ground and sieved through a 2-mm stainless-steel sieve and kept in a sealed polyethylene bag at ambient temperature (28±2^{o}C) for 24 h prior to physicochemical analysis.

2.3. Microbial Analysis

The aerobic heterotrophic bacterial and fungal populations were ascertained by a standard pour plate method. Heterotrophic bacteria were isolated using nutrient agar amended with 0.015 % (w/v) nystatin to inhibit fungal growth. The nutrient agar plates were incubated at 28 ± 2^{o}C for 24-48 h. Potato dextrose agar containing 0.05 % (w/v) chloramphenicol was used to isolate fungi upon incubation at 28 ± 2 ^{o}C for 72 h. Purification of representative bacterial and fungal colonies was done by sub-culturing and identifications were made as reported by Staley *et al.* (1989) and Talbot (1978), respectively.

2.4. Physicochemical Properties Analysis

Physicochemical parameters of effluent contaminated and control soil samples were examined. Atomic Absorption Spectrophotometer (AAS) (Perkin Elmer AA Analyst 800 series Graphite Furnace AA) was used to analyze the digested samples for metals contaminant. Soil temperature and pH were determined using a mercury thermometer (Digital Thermometer (Model 6300), Spectrum Technologies, Inc. and pH meter (FieldScout pH 110 Meter), respectively. The Total Organic Carbon (TOC) was ascertained as reported by Nelson and Sommers (1982). Cation Exchange Capacity (CEC) was determined according to the method described by Chapman (1965). Total nitrogen using Kjedahl digestion and steam distribution method and percentage organic matter were investigated as reported by previous studies (Bremner, 1965). The electrical conductivity was determined by the method of Chopra and Kanzar (1988). P, NH^{4+}, NO^{3+} and NO^{3-} were estimated with the use of AAS (Perkin Elmer AA Analyst 800 series Graphite Furnace AA). The particle size analysis of the soil was achieved by hydrometer method as described by Bouyoucos (1962).

2.5. Statistical Analysis

Statistical analyses of data were achieved using Minitab16 software and Microsoft excel. Data were subjected to Anderson-Darling normality test. Comparisons of means were assessed statistically by subjecting data to one-way analysis of variance (ANOVA). Regression analysis was also done. A probability values (*p*-values) of less than 0.05 was considered as significant.

3. Results

The bacteria isolated from the studied areas were *Bacillus subtilis, Bacillus macerans, Pseudomonas aeruginosa, Klebsiella oxytoca* and *Escherichia coli*. On the other hand, eleven (11) species of fungi belonging to the genera *Aspergillus, Penicillium* and *Rhizopus* were isolated. Table 1 represents the occurrence and the distribution pattern of bacterial species or diversity in the studied areas; while Table 2 shows the occurrence and the distribution pattern of fungal diversity in the studied areas. The total heterotrophic bacterial and fungal population ranged from 1.3×10^{8} - 3.61×10^{8} CFU/g and 1.84×10^{4} - 2.2×10^{8} CFU/g, respectively. Table 5 represents the mean values and the standard error of physicochemical parameters of soil samples from cassava effluent polluted and control soil in Oluku, Isihor and Ehor during the period of study.

Table 1. The occurrence and distribution pattern of bacterial population in the in studied areas

Sampling sites	Month	August					September					October				
	Species	B_1	B_2	B_3	B_4	B_5	B_1	B_2	B_3	B_4	B_5	B_1	B_2	B_3	B_4	B_5
O		+	+	-	-	-	+	-	-	-	+	+	+	+	+	+
I		+	+	+	-	+	-	+	+	+	+	+	+	+	-	-
E		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C_O		-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
C_I		+	-	-	-	-	-	+	+	-	-	+	+	+	-	-
C_E		+	+	+	+	+	-	-	-	-	+	-	-	-	+	+

Legend: O- Oluku; I- Isihor; E- Ehor; C_O- Control site at Oluku; C_I- Control site at Isihor; C_E- Control site at Ehor; + -present; - -absent; B_1- *Escherichia coli*; B_2- *Klebsiella oxytoca*; B_3- *Bacillus subtilis*; B_4- *Bacillus macerans*; B_5- *Pseudomonas aeruginosa*

Table 2. The occurrence and distribution pattern of fungal population in the studied areas

Sampling sites	Month	August			September			October		
	Species	F_1	F_2	F_3	F_1	F_2	F_3	F_1	F_2	F_3
O		+	-	+	+	+	-	+	+	+
I		+	+	+	-	+	+	+	+	+
E		+	+	+	+	+	-	+	-	+
C_O		-	-	+	+	-	-	-	-	-
C_I		-	-	-	+	-	-	-	-	+
C_E		+	-	-	-	+	-	-	+	-

Legend: O- Oluku; I-Isihor; E-Ehor; C_O-Control site at Oluku; C_I-Control site at Isihor; C_E-Control site at Ehor; + -present; - -absent; F_1- *Rhizopus* spp; F_2-*Aspergillus* spp;F_3- *Penicillium* spp.

Table 3. Effect of cassava effluent on bacterial load of soil (CFU/g) during the period of study

S	August		September		October	
	Control Soil	Polluted soil	Control Soil	Polluted soil	Control Soil	Polluted soil
O	$1.2\times10^8 \pm 0.02$	$2.14\times10^8 \pm 0.04$	$3.0\times10^8 \pm 0.01$	$3.1\times 10^8 \pm 0.12$	$2.3\times10^8 \pm 0.02$	$3.2\times10^8 \pm 0.02$
I	$1.4\times10^8 \pm 0.04$	$1.72\times10^8 \pm 0.04$	$1.5\times10^8 \pm 0.03$	$1.6\times10^8 \pm 0.01$	$1.0\times10^8 \pm 0.05$	$1.3\times10^8 \pm 0.03$
E	$1.41\times10^8 \pm 0.04$	$3.61\times10^8 \pm 0.12$	$3.3\times10^8 \pm 0.15$	$2.16\times10^8 \pm 0.03$	$1.1\times10^8 \pm 0.01$	$2.0\times10^8 \pm 0.04$

Legend: S-sampling site; O- Oluku; I- Isihor; E- Ehor; Values are means of triplicates; ± - standard deviation

Table 4. Effect of cassava effluent on fungal load of soil (CFU/g) in during the period of study

S	August		September		October	
	Control Soil	Polluted soil	Control Soil	Polluted soil	Control Soil	Polluted soil
O	$1.2\times10^5 \pm 0.01$	$1.84\times10^4 \pm 0.08$	$2.0\times10^6 \pm 0.02$	$2.1\times 10^5 \pm 0.12$	$1.3\times10^6 \pm 0.01$	$2.2\times10^8 \pm 0.01$
I	$1.3\times10^6 \pm 0.03$	$2.45\times10^4 \pm 0.31$	$1.0\times10^4 \pm 0.03$	$0.6\times10^8 \pm 0.01$	$2.0\times10^2 \pm 0.05$	$1.4\times10^7 \pm 0.03$
E	$1.1\times10^4 \pm 0.04$	$8.4\times10^4 \pm 0.49$	$1.3\times10^6 \pm 0.05$	$1.16\times10^7 \pm 0.03$	$2.3\times10^4 \pm 0.01$	$2.0\times10^7 \pm 0.03$

Legend: S-sampling site; O- Oluku; I- Isihor; E- Ehor; Values are means of triplicates; ± - standard deviation

Table 5. Physicochemical parameters of soil samples from cassava effluent polluted and control soil in Oluku, Isihor and Ehor during the period of study

Parameters	Oluku		Isihor		Ehor	
	Polluted soil	Control soil	Polluted soil	Control soil	Polluted soil	Control soil
Temp (°C)	25.00±0.1	25±0.30	20.65±0.1	27.52±0.12	26.52±0.6	25±0.66
pH	4.00±0.2	5.6±0.01	4.78±0.26	5.58±0.44	4.78±0.3	6.6±0.55
EC (µs/Cm)	182±0.17	175±0.14	192±0.00	187±0.00	160±0.00	150±0.00
Cl^- (mg/kg)	47.17±1.0	43±1.00	59.33±0.0	40.67±0.02	50±0.08	49±0.88
SO_4^{2+} (mg/kg)	7.9±0.7	7.4±0.1	9.67±0.36	6.0±0.72	4.00±0.1	3.0±0.99
NO_3^- (mg/kg)	4.0±0.4	3.0±0.02	4.00±0.79	0.67±0.00	7±0.03	8±0.00
PO_4^{3-} (mg/kg)	5.24±0.4	5.24±0.03	6.43±0.34	6.88±0.61	7.22±0.1	5.22±0.01
Fe (mg/kg)	12.0±0.1	12.6±0.1	4.58±0.01	4.38±1.00	5.88±1.1	4.33±0.11
Zn^{2+} (mg/kg	1.87±0.0	1.78±0.02	1.89±0.01	1.44±0.01	1.43±0.1	1.33±0.01
Mn^{2+} (mg/kg)	0.69±0.0	0.61±0.00	0.05±0.00	0.01±0.05	0.06±0.0	0.01±0.01
Cu^{2+} (mg/kg)	2.17±0.0	2.12±0.00	1.69±0.07	1.09±0.06	2.9±0.07	1.9±0.00
O.C (%)	1.21±0.40	0.21±0.20	2.15±0.06	1.26±0.07	2.36±0.5	136±0.03
T.N (%)	0.31±0.0	0.11±0.002	0.37±0.02	0.130±0.01	0.44±0.4	0.33±0.03
Moisture (%)	13±0.1	12±0.01	12±0.02	11±0.02	13±0.05	13±0.03
Sand (%)	89±0.68	87±0.034	86±1.22	87±0.97	89±1.23	76±0.03
Silt (%)	4±0.8	3±0.9	4±0.02	4±0.06	4±0.07	3±0.02
Clay (%)	7±0.51	6±0.50	7±0.32	6±0.38	8±0.38	5±0.01

Legend: EC-electrical conductivity; O.C - organic carbon; T.N - total nitrogen Values are means of triplicates; ± - standard deviation

4. Discussion

The results reveal the following bacterial isolates, which are common micro-biota of the soil: *Bacillus subtilis, Bacillus macerans, Pseudomonas aeruginosa, Klebsiella oxytoca* and *Escherichia coli*; while eleven (11) species of fungi belonging to the genera *Aspergillus, Penicillium,* and *Rhizopus* were isolated. These bacteria and fungi species were isolated by previous authors (Knowles, 1988; Ehiagbonare *et al.,* 2009). These isolates occurred more in cassava effluent polluted soil than in the control soils in all the studied areas (Tables 1 a nd 2). Cassava effluent polluted soil from Ehor had the highest bacterial count of 3.61×10^8 CFU/g in August while the lowest bacterial count of 1.3×10^8 CFU/g was recorded at Isihor in October. These values are similar to those reported by Okoh *et al.* (1999) and Aiyegoro *et al.* (2007).

The fungal counts for the polluted and control soil ranged from 1.84×10^4-2.2×10^8 CFU/g and 2.0×10^2-2.0×10^8 CFU/g, respectively. This suggests that the cassava effluent has effects on the fungal diversity of the polluted soil. The fungal counts were significantly lower than the bacterial counts ($p < 0.05$); and this is in agreement with the report from Aiyegoro *et al.* (2007). The relative increase in the fungal growth in the polluted soil experiments may be due to the acidic pH of the soil which ranged from 4.0-4.78 (Table 5). On the contrary, the relative low fungal diversity, observed in the control soil, could be attributed to the near neutral pH of 5.58-6.6 (Table 5). However, the differences in the soil pH values of the different months for the polluted soil were not observed to be statistically significant ($p < 0.05$). These bacteria may have acquired the genetic traits that enabled them to survive in such an acidic environment. The low pH of the soil could explain the presence of cyanogenic glycosides in the cassava effluent contaminated soil. Factors like low pH, high negative soil charges, and low clay content were reported as soil conditions that increase cyanide mobility (Fuller, 2004). In addition, the high organic carbon contents of the cassava effluent may have contributed to the proliferation of these aerobic microorganisms as reported by (Okwute and Isu, 2007; Nwaugo *et al.,* 2008). Top soil was indicated to harbor the richest microbial diversity because it contains a h igher amount of organic matter and oxygen which decreases with depth (Maloney *et al.,* 1997). Higher bacterial counts of 3.3×10^8 CFU/g were observed in the control soil samples than those in the polluted soil in September from Ehor (Table 3). A similar trend was also observed in control soil samples from Oluku and Isihor in August (Table 4). In the present investigation, there was a drastic increase in the amount of rainfall in August and September, upon which there was a decline; this trend did not correlate generally with the total microbial counts. The soil was made up of sandy, silt and clay particles. The mean percentage of sandy particles of the polluted soil ranged from 86-89 % while that of the control ranged from 76-87 %. Similar results were observed by Uzoije *et al.* (2011). The percentage silt and clay contents were relatively low (Table 5). The organic carbon content ranged from 1.21-2.36 % for the polluted soil. The organic carbon content was higher in all the polluted soil experiments than in the control experiments. This increase could be a result of the high organic matter and organic carbon content of the cassava effluent. There were no significant differences in the amount of organic carbon among the various months ($p > 0.5$). This suggests that

the cassava effluents are perhaps of similar nutrient quality. However, the total organic carbon contents of the polluted soil were significantly higher than those from the control sites in all the studied areas ($p < 0.05$). There was a significant positive relationship between organic carbon and temperature ($p < 0.05$). The total nitrogen ranged from 0.31-0.44%. The total nitrogen recorded was probably due to nitrogen mineralization as a result of the degradation of organic matter. Electrical conductivity is used as a means of appraising soil salinity. The values recorded in the soil samples may be due to the increase in the concentration of soluble salts. With the exception of iron, the heavy metal levels for all sites were significantly higher than the levels observed in the control sites ($p > 0.05$). This implies that the soils receiving the effluent have some levels of heavy metal enrichments. The high concentration of heavy metals like zinc, copper, and manganese in the effluent contaminated soils could also be attributed to the wearing off or abrasion of the cassava milling machine parts and emission of the metals through the exhaust of the machine (Osakwe, 2010).

Intensive research is necessary to examine the impact of cassava effluents on soil microbiomes on non-cultural microorganisms using metagenomics approach to examine the bacterial and fungal strain diversity, the relationships between the microorganism and the ecosystem, phylogenetic relationships among the microorganism.

5. Conclusion

The results of the present study reveal that the cassava effluent has impacts on the microbial diversity of the receiving soil. This is indicated by the significant increase observed in the microbial density of the polluted soil and the simultaneous impact on the physicochemical parameters of the soil.

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Genetic Diversity and Variability in Landraces for Key Agro-economic traits in Vegetable Roselle (*Hibiscus sabdariffa* var. sabdariffa L.)

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Abstract

Landraces of vegetable Roselle (*Hibiscus sabdariffa* var. sabdariffa L.) are under subsistence and commercial cultivation in tribal, rural and peri-urban vegetable farming systems by the tribal folks, small and marginal farmers of south India. Leaf yields of these landraces are low in farmer's conditions either due to poor production potential of landraces or poor agrotechniques. The production potential and the economic value can be enhanced by identifying the promising landraces and their intensive cultivation in the market and truck gardens. In collaboration with National Bureau of Plant Genetic Resources, Regional Station, Rajendranagar, 28 landraces of vegetable Roselle were collected from parts of Andhra Pradesh and Odisha states of India during 2010-2011. These landraces were evaluated in a randomized block design with three replications during summer 2013 at Vegetable Research Station, Rajendranagar to assess the production potential and the genetic variability for various agro-economic traits. The variation recorded within the landrace germplasm for plant height, total biomass, leaf yield, stalk yield, leaf-stalk ratio and harvest index show its potential for use in the genetic improvement. The landraces RNR-16, RNR-20 and RNR-27 were promising as indicated by the high leaf production potential of 14.22, 12.72 and 11.85 g plant^{-1}, respectively. High estimates of heritability coupled with high genetic advance as percent of mean for plant height, total biomass, leaf yield, stalk yield and leaf-stalk ratio indicating the possibility to improve these agro-economic traits through selection programs. Selection is effective for plant height, leaf yield, stalk yield, leaf-stalk ratio and total biomass in vegetable Roselle.

Keyword: Agro-Economic traits, Genetic parameters, Geographic information system, Landrace germplasm, Leaf yield, Shannon diversity index.

1. Introduction

Vegetable Roselle (*Hibiscus sabdariffa* var. sabdariffa L.) belongs to the family Malvaceae. It is known by different synonyms and vernacular names, such as Roselle, Indian sorrel, Jamaica sorrel, Guinean sorrel, red sorrel, Mesta and karkade (Abu-Tarboush *et al*., 1997; Chewonarin *et al.,* 1999; Tsai *et al.,* 2002; Parkouda *et al.,* 2008). Roselle is a tetraploid (2n=4x=72) whose chromosomes are related to the diploid (2n=2x=36) *Hibiscus cannabinus* L. (Wilson and Menzel, 1964; Mclean, 1973). It is probably a native of Asia (India to Malaysia) to tropical Africa (Gomez-Leyva *et al.,* 2008). It is an important annual crop which grows successfully in the tropics and sub-tropics (Cobley, 1968). Being a tropical plant species, Roselle can be found in almost all tropical countries, such as Malaysia, South East Asia, Indonesia, Thailand and Philippines (Rao, 1996; Chewonarin *et al.,* 1999). Cultivation of the plant was reported throughout Indian subcontinent (Cobley, 1968). It is a versatile plant similar to the coconut tree with a number of useful properties (Quezon, 2005). The juvenile or tender leaves are consumed as a green vegetable (Small and Rhoden, 1991; Delgado-Vargas and Parcedes-Lopez, 2003). It is a famous leafy vegetable crop with several uses and benefits (Ottai *et al.,* 2006). It has considerable industrial, pharmaceutical, nutritional and economic values in India and many other countries around the world. Roselle plays an important role in providing nutritional and health security and income generation and subsistence among rural farmers in developing countries (Cisse *et al.,* 2009). Being tolerant to little shade, it can be intercropped and grown in greenhouses. It is resistant to relatively high temperatures throughout the growing season (Tomes, 1990). The crop is grown mainly by traditional farming methods, exclusively under rainfed conditions (El Naim and Ahmed, 2010). It is under subsistence and commercial cultivation in tribal, rural and peri-urban vegetable farming systems by the tribal folks, small and marginal farmers of south India for its juvenile or tender leaves. There have been drastic changes in the

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production systems and marketing units of Roselle over the last few decades in south India. Presently, it has been cultivated as a monthly crop (30-40 days) with bunch of seedlings as unit of marketing in all vegetable farming systems. However, its leaf yields are very low in farmer's conditions in India due to the traditional cultivation and poor potential of cultivated varieties. As this plant has socio-economic importance, there is a constant need for the improvement of Roselle. In order to improve the leaf yield of Roselle, plant breeders should have a b etter understanding of the genetic diversity and variability for yield and it components. The breeding approach may lead to possible improvements in the yield and quality of the cultivars.

The cultivation and diversity of Roselle in India is unique. There are no named varieties available in this crop. A vast range of agro-climatic and socio-cultural settings in the country gave rise to a large number of Roselle landraces adapted to specific niches. A wide range of Roselle diversity on farm is under cultivation as landraces and they have long been adapted to local environments and cultural regimes, being better suited to diverse farming systems, agro-ecological niches, diverse socio-cultural settings and the needs of farmers across all of its altitudinal range. Landraces are the major characteristic of Roselle production systems in India. These landraces constitute a conspicuous source of variation for crop improvement (Zeven, 1998). It is a well-established fact that the progress in improving a crop depends on the degree of the variability in the desired character in the base material *vis-à-vis* germplasm collection. However, the genetic variability for many traits is limited in cultivated germplasm (Sabu *et al.,* 2009). Plant breeding is a process of a genetic change to improve the genetic content to a more superior genotype (variety or hybrid). Landraces or farmer varieties constitute the basic material for developing any variety or hybrid. Screening of local landraces, with the goal of identifying suitable parents for plant breeding, is the first step in the process. Development of any crop improvement program essentially depends on nature, magnitude of genetic variability, genetic advance, characters association, direct and indirect effects on yield and yield attributes (Ibrahim and Hussein, 2006). The genetic improvement of crops, for quantitative traits, requires reliable estimates of genetic variability, heritability and genetic advancement in respect to the breeding material that is presently at hand in order to plan an efficient breeding program (Dudley and Moll, 1969; Chand *et al.,* 2008). H ence, the estimates of the variability of the yield and the yield contributing characters and their heritable components in the materials are more important in any crop improvement program. The overall performance of a genotype may vary due to changes in the environment, and, if the heritability for the traits is higher, the selection process will be simpler and the response to selection will be greater (Larik *et al.,* 1997; Larik *et al.,* 2000; Soomro *et al.,* 2008). Genetic variability provides wider scope for selection. Genetic variation among selected lines is of vital importance to breeding programs that aim to produce improved cultivars for marginal growing environments (Yadav *et al.,* 2001). The knowledge of the existing variability is essential for developing high yielding genotypes in Roselle. Agro-morphological characteristics have long been used to classify and distinguish plant genotypes.

Vegetable Roselle is largely underutilized and underexploited leafy vegetable crop. It has received no attention; not much research has been carried out on its genetic improvement, either. Little is known about its genetic potential, divergence and variability, which are supposed to be large when considering its wide geographical distribution. The level of research on Roselle does not compare to the works done on its closely related species, such as cotton (Kumar *et al.,* 1986). Most breeding of Roselle has been for its fiber yield. Roselle is endowed with a r ich reservoir of genetic variability for various yield components, adaptation and quality traits (El Tahir and El Gabri, 2013). Several studies were done on genetic potential (Ibrahim and Hussein, 2006; Ibrahim *et al.,* 2013; Sabiel *et al.,* 2014) genetic diversity (El Tahir and El Gabri, 2013) and genetic variability of Roselle for calyx production as a seasonal crop (Thirthamallappa and Sherif, 1991; Gasim, 1994; Zayed *et al.,* 1996; Ibrahim and Hussein, 2006; Ahmed *et al.,* 2009; Atta *et al.,* 2011; Sabiel *et al.,* 2014) and no studies have been done on these aspects of leaf production as a monthly crop. In spite of the crop's economic prospects and importance, it has received little attention regarding its genetic improvement.

To understand and assess the value and extent of genetic variability and diversity prevalent in landraces, the present study was made on a collection of landrace accessions from two southern states of India (Andhra Pradesh and Odisha (formerly Orissa) using key agro-economic traits so as to identify the elite landraces for commercial exploitation and to identify the selection indices and breeding program for the improvement of vegetable Roselle.

2. Materials and Methods

2.1. Exploration Surveys

Two exploration surveys, covering different locations of Andhra Pradesh and Odisha states of India, were conducted by National Bureau of Plant Genetic Resources (NBPGR), Regional Station, Rajendranagar in collaboration with Vegetable Research Station, Dr. Y. S. R. Horticultural University, Rajendranagar during two consecutive years 2010 and 2011. Random sampling was adopted and, whenever necessary, the sampling was based on selection; a total of 28 *Hibiscus sabdariffa* var. sabdariffa L. accessions, representing different agro-ecological areas in the above states was collected. Each ecotype was characterized by geographical information (name of the village, latitude, longitude and elevation). Geographical co-ordinates of the collection sites (Table 1) were recorded by Global Positioning System (Garmin 12, USA). Collection sites of the above landraces were mapped (Figure 1) Cotton bags were used for the collection of the samples. The collector number was assigned to all the accessions collected and the passport information collected was subsequently assigned with identification numbers.

Table 1. Passport data of the various landraces of vegetable Roselle

Accession ID	Collector number	Collection site						
		Geo-reference						
		Latitude (°N)	Longitude (°E)	Altitude (m)	Village	Mandal	District	State
RNR-1	SNTV-1	18.32.534	84.01.496	70	Buditi	Buditi	Srikakulam	Andhra Pradesh
RNR-2	SNTV-6	19.17.301	84.24.122	823	Raisinghi	R. Udaygiri	Gajapati	Orissa
RNR-3	SNTV-10	19.09.048	84.15.484	827	Raudiva panchayat	Lakshmipur	Gajapati	Orissa
RNR-4	SNTV-11	18.59.231	84.01.602	375	Kuttum	Gumma	Gajapati	Orissa
RNR-5	SNTV-16	18.59.861	84.02.766	733	Bubani	Gumma	Gajapati	Orissa
RNR-6	SNTV-17	19.00.283	84.04.548	733	Kingdong	Gumma	Gajapati	Orissa
RNR-7	SNTV-23	18.46.148	84.24.517	35	Peddaveedhi	Palasa	Srikakulam	Andhra Pradesh
RNR-8	SNTV-24	18.46.148	84.24.517	35	Peddaveedhi	Palasa	Srikakulam	Andhra Pradesh
RNR-9	SNTV-27	18.39.231	84.18.246	35	Nandigaon	Nandigaon	Srikakulam	Andhra Pradesh
RNR-10	SNTV-35	18.21.573	82.53.408	29	Babajipetha	Srikakulam	Srikakulam	Andhra Pradesh
RNR-11	SNTV-47	18.34.318	83.47.822	61	Palavalasa	Burgi	Srikakulam	Andhra Pradesh
RNR-12	SNTV-53	18.18.797	83.34.237	66	Chipurupalli	Chipurupalli	Vizianagaram	Andhra Pradesh
RNR-13	SNTV-58	18.27.772	83.18.857	131	Arikathota	Rambhadrapuram	Vizianagaram	Andhra Pradesh
RNR-14	SNTV-61	18.27.772	83.18.857	131	Arikathota	Rambhadrapuram	Vizianagaram	Andhra Pradesh
RNR-15	SNTV-64	18.32.465	83.19.619	131	Mettavalasa	Bobbili	Vizianagaram	Andhra Pradesh
RNR-16	SNTV-69	18.35.147	83.21.940	131	Patha Bobbili	Bobbili	Vizianagaram	Andhra Pradesh
RNR-17	SNTV-74	18.29.768	83.16.711	129	Ramabhadrapuram	Rambhadrapuram	Vizianagaram	Andhra Pradesh
RNR-18	SNT-11-7	16.04.20	78.54.22	666	Patha sunnipenta	Srisailam	Kurnool	Andhra Pradesh
RNR-19	SNT-11-9	16.04.20	78.54.22	666	Patha sunnipenta	Srisailam	Kurnool	Andhra Pradesh
RNR-20	SNT-11-10	15.48.46	78.13.20	666	Damagadha	Nandikotkur	Kurnool	Andhra Pradesh
RNR-21	SNT-11-19	15.49.40	78.03.26	280	Banddimetta	Kurnool	Kurnool	Andhra Pradesh
RNR-22	SNT-11-25	15.42.56	78.05.04	340	Narnur	Orvagal	Kurnool	Andhra Pradesh
RNR-23	SNT-11-40	14.41.48	77.39.56	335	Regulakunta	Bukkarayasamudram	Anantapur	Andhra Pradesh
RNR-24	SNT-11-41	14.41.48	77.39.56	335	Regulakunta	Bukkarayasamudram	Anantapur	Andhra Pradesh
RNR-25	SNT-11-43	14.14.32	77.36.53	468	Timbaktu	Chinnakottapalli	Anantapur	Andhra Pradesh
RNR-26	SNT-11-47	14.15.59	77.41.19	442	Venkatrampalli	Chinnakothapalli	Anantapur	Andhra Pradesh
RNR-27	SNT-11-48	14.15.59	77.41.19	442	Venkatrampalli	Chinnakothapalli	Anantapur	Andhra Pradesh
RNR-28	SNT-11-55	13.49.57	77.30.01	634	Hindupur	Hindupur	Anantapur	Andhra Pradesh

RNR= Rajendranagar Roselle

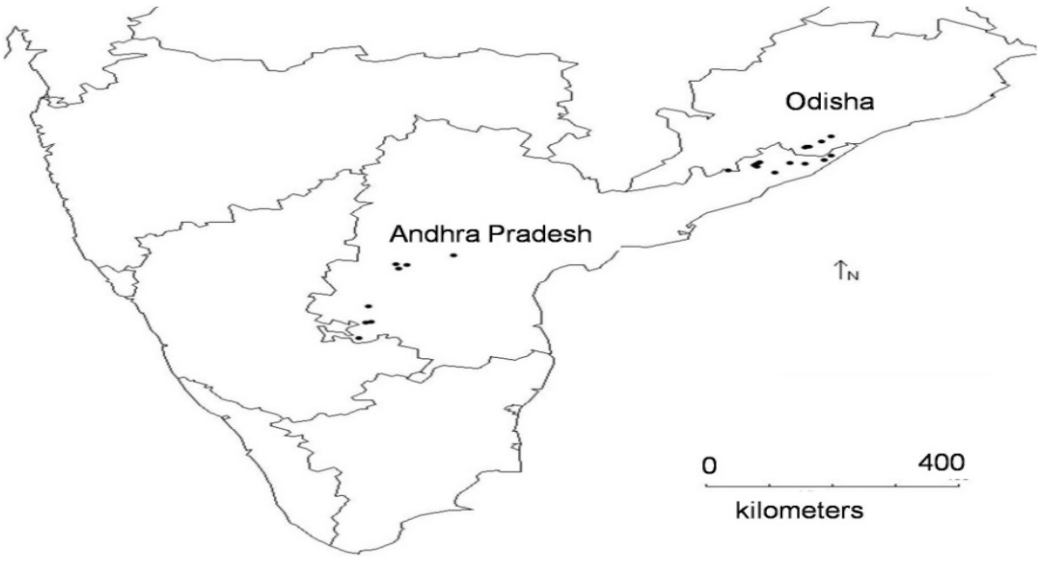

Figure 1. DIVA-GIS mapping of collection sites of landraces of vegetable Roselle from Andhra Pradesh and Odisha

2.2 Pre-breeding

It is short day plant having critical photoperiod of 10-12.5 hours (Hacket and Carolone, 1982). Since natural cross-pollination is reported in Roselle (Sanyal and Dutta, 1954), selfing must be done to maintain the genetic purity of the diverse germplasm accessions, which would be in a close proximity in a breeding nursery. These landraces were pre-bred by selfing during rainy season 2012 a t Vegetable Research Station, Rajendranagar for initial seed increase for further evaluation and conservation. These landraces were christened with accession identification numbers (accession IDs starting with RNR; RNR-1 to RNR-28).

2.3 Experimental Design and Agro-Techniques

Twenty-eight landraces were utilized for the present study. The experiment was conducted at the Experimental Farm of the Vegetable Research Station, Rajendranagar, Hyderabad, Andhra Pradesh, India (latitude 17.19^{o}N and longitude 79.23^{o}E, and altitude 222 m above mean sea level) during summer, 2013. The experimental design was a randomized block design with three replicates. The treatment plot (block) consisted of 28 rows (1 row per genotype). The individual plot was 1.0 m long and 0.20 m wide. In each replication, a plant population of 20 plants per row, plot and genotype was maintained. Three seeds per hill were dibbled with an intra-row spacing of 0.05 m and an inter-row spacing of 0.20 m under high density planting system. Thereafter hills were thinned to one plant at two weeks after sowing. All agronomic practices were maintained for a whole duration of the experiment. Regular plant protection measures were carried out to safeguard the crop from pests and diseases.

2.4. Recording of Biometric Data

All landraces were subjected to harvest at once by pulling out the seedlings along with the root system reasonably intact 40 days after sowing as practiced by farmers under once-over harvest system. Crop plots were judiciously irrigated at the previous night. Four out of six variables were measured on the seedling plants of the 28 accessions of Roselle. Five seedling plants chosen randomly from middle of rows of each plot were tagged for measurements on pl ant height (cm), total biomass (g/plant), leaf yield (g/plant) and stalk yield (g/plant). Height of the plant (cm) from ground level to tip of the main stem was measured in centimeters just before the harvest on the tagged seedling plants. A graduated scale of 100 cm long was used to measure the plant height. These tagged seedling plants were pulled out along with root system reasonably intact. These sampled seedling plants were weighed with a digital analytical balance (±0.001 g) to arrive at the seedling weight or the total biomass (g/plant) consisting of above ground shoot system and below ground root system. These sampled seedling plants were stripped off leaves to facilitate recording of data on separated leaves and stalks to arrive at leaf yield (g/plant) and stalk yield (g/plant), separately. Leaf yield (g/plant) and stalk yield (g/plant) were recorded on a digital analytical balance (±0.001 g). The remaining two variables *viz*., leaf-stalk ratio and harvest index were calculated using replicated mean values of leaf yield, stalk yield and total biomass. Leaf-stalk ratio was calculated as the ratio of leaf yield (g/plant) and stalk yield (g/plant). The harvest index was taken as the ratio of the leaf yield (g/plant) to total biomass (g/plant).

2.5. Statistical Analysis

The replicated means of each individual landrace was employed in the statistical analysis. The data, thus recorded, were subjected to the analysis of variance (Steel and Torrie, 1980). Tests for the significant difference between the means were made using the procedure of analysis of variance (ANOVA) and the Student Newman Keuls test at 0.05 and 0.01 probability levels. A statistical analysis for estimating the mean performance of landraces and major genetic components for six agro-economic traits was performed using SAS Enterprise Guide Version 4.2 (SAS Institute Inc., 2009). From the analysis of variance, the phenotypic, the environmental and genotypic components of variance were estimated as follows, as per the formula suggested by Lush (1940):

Environmental variance = σ^2_e

$$\text{Genotypic variance } (\sigma^2_g) = \frac{Mg - Me}{r}$$

Where,

Mg = mean sum of squares of genotypes (treatments)

Me = mean sum of squares of error

r = number of replications (blocks)

Phenotypic variance (σ^2_p) = Genotypic variance (σ^2_g) + Environmental variance (σ^2_e)

The analysis of variance also permits the estimation of phenotypic and genotypic coefficients of variation (Burton, 1952). The Genotypic Coefficient of Variation (GCV) and Phenotypic Coefficient of Variation (PCV) were computed by adopting the method of Burton (1952) and Burton and de Vane (1953). The PCV and GCV values were classified as low (<10%), moderate (10-20%) and high (>20%) as suggested by Sivasubramanian and Menon (1973):

$$\text{Phenotypic coefficient of variation (PCV)} = \frac{\sqrt{\sigma^2 p}}{\overline{X}} X100$$

where,

PCV = phenotypic co-efficient of variation

$\overline{X}$ = general mean

σ^2p = phenotypic variance

$$\text{Genotypic coefficient of variation (GCV)} = \frac{\sqrt{\sigma^2 g}}{\overline{X}} X100$$

where,

GCV = genotypic co-efficient of variation

$\overline{X}$ = general mean

σ^2g = genotypic variance

The broad sense heritability (h^2_{bs}) was estimated for all characters as the ratio of genotypic variance to total or phenotypic variance (Lush, 1940). The heritability values were classified as low (<30%), moderate (30-60%) and high (>60%) as suggested by Johnson *et al*. (1955):

$$h^2 = \frac{\text{Genotypic variance}}{\text{Phenotypic variance}} \times 100$$

The estimates of genetic advance (GA) at 5% selection intensity (2.06) and genetic advance as per cent of mean were obtained using the procedure given by Allard (1999). The estimates of the genetic advance and the genetic advance as percent of mean were classified as low (<10%), moderate (10-20%) and high (>20%), as suggested by Johnson *et al*. (1955):

$GA = h^2_{bs} \times \sigma_p \times K$

where, h^2_{bs} =Heritability estimate in broad sense

σ_p =Phenotypic standard deviation of the trait

K =Standard selection differential which is 2.06 at 5 per cent selection intensity

$$\text{GA as per cent of mean} = \frac{\text{GA}}{\text{Grand mean}} \times 100$$

A hierarchical clustering was carried out following Ward's minimum variance method (Ward, 1963). DIVA-GIS version 7.5.0, free downloadable software was used for mapping of the collection sites of the above landraces and analysis of diversity of the collected landrace accessions of Roselle and for generating grid maps (Figure 2). Shannon diversity index was calculated for all the agro-economic traits with observed data using DIVA-GIS version 7.5.0 free downloadable software (Hijmans *et al*., 2012).

3. Results

3.1. Analysis of Variance

Analysis of variance (Table 2) revealed significant differences ($P < 0.01$) among 28 genotypes of Roselle for all of the six agro-economic traits *viz*., plant height, leaf yield, stalk yield, leaf stalk ratio, harvest index and total biomass under study. Significant differences were not observed within replications for almost all traits.

Table 2. Analysis of variance for various agro-economic traits of vegetable Roselle

Character	Mean squares		
	Replications (2)	Genotypes (27)	Error (54)
Plant height (cm)	1.1074	240.9060**	6.0603
Total biomass (g/plant)	2.7955	120.9715**	2.0893
Leaf yield (g/plant)	0.6908	37.4995**	0.5284
Stalk yield (g/plant)	0.7482	25.1438**	0.5639
Leaf-stalk ratio	0.0654	1.1004**	0.0289
Harvest index	0.0007	0.0149**	0.0003

**Significant at $P \leq 0.01$ level

Values in parentheses denote degrees of freedom.

3.2. Production Potential of Landraces

The ranges of mean values revealed a s ufficient variation for all the traits under study (Table 3). Large variations between ecotypes were observed for all the traits. A wide range of variations was observed in most of the agro-economic traits. Plant height, total biomass, leaf yield, stalk yield, leaf stalk ratio and harvest index (Table 3) ranged from 16.93-51.23 cm, 3.16-23.66 g/plant, 1.95-14.22 g/plant, 0.8-10.94 g/plant, 1.18-3.25 and 0.54-0.77, respectively. In the landrace germplasm used in the present study, a maximum range of variability (Table 3) was observed for plant height (34.30 cm) followed by total biomass (20.50 g/plant) and leaf yield (12.27 g/shoot). RNR-20 (51.23 cm) had tallest plants followed by RNR-14 (45.87 cm) and RNR-25 (43.07 cm). RNR-20 (23.66 g) had highest total biomass followed by RNR-16 (23.55 g) and RNR-10 (19.91 g). RNR-16 (14.22 g) had highest leaf yield followed by RNR-20 (12.72 g) and RNR-27 (11.85 g). RNR-20 (10.94 g) had highest stalk yield followed by RNR-16 (9.33 g) and RNR-10 (8.74 g). RNR-9 (3.25) had highest leaf-stalk ratio followed by RNR-7 (3.05) and RNR-26 (2.74). RNR-9 (0.77) had highest harvest index followed by RNR-7 (0.76) and RNR-3, RNR-5, RNR-22 and RNR-26 (0.72). On the basis of genetic potential for leaf yield (Table 2), the landraces RNR-16 (14.22 g/plant), RNR-20 (12.72 g/plant), RNR-27 (11.85 g/plant), RNR-10 (11.17 g/plant) and RNR-25 (10.50 g/plant) were promising from consumer point of view.

Table 3. Mean performance of landraces of vegetable Roselle for agro-economic traits

Accession number	Plant height (cm)	Total biomass (g/plant)	Leaf yield (g/plant)	Stalk yield (g/plant)	Leaf-stalk ratio	Harvest index
RNR-1	33.00	7.23	4.73	2.50	1.91	0.65
RNR-2	20.42	3.27	2.30	0.97	2.39	0.71
RNR-3	22.70	4.35	3.12	1.23	2.54	0.72
RNR-4	37.30	8.08	5.31	2.77	1.92	0.66
RNR-5	20.27	3.96	2.82	1.14	2.52	0.72
RNR-6	24.58	8.20	5.57	2.64	2.11	0.67
RNR-7	23.30	7.44	5.61	1.83	3.05	0.76
RNR-8	25.50	10.28	6.85	3.44	1.99	0.66
RNR-9	16.93	3.38	2.57	0.80	3.25	0.77
RNR-10	41.30	19.91	11.17	8.74	1.28	0.56
RNR-11	29.40	6.51	3.65	2.87	1.28	0.56
RNR-12	32.50	8.45	4.63	3.83	1.23	0.55
RNR-13	32.17	7.90	4.37	3.53	1.24	0.56
RNR-14	45.87	16.48	9.12	7.36	1.24	0.55
RNR-15	35.33	13.81	8.51	5.31	1.62	0.61
RNR-16	38.53	23.55	14.22	9.33	1.53	0.61
RNR-17	31.10	13.94	8.28	5.67	1.46	0.59
RNR-18	24.27	6.07	4.23	1.83	2.33	0.69
RNR-19	24.63	7.77	5.48	2.29	2.45	0.71
RNR-20	51.23	23.66	12.72	10.94	1.18	0.55
RNR-21	31.40	3.98	2.43	1.55	1.57	0.61
RNR-22	21.93	4.20	2.99	1.21	2.49	0.72
RNR-23	20.50	3.59	2.50	1.09	2.29	0.70
RNR-24	21.53	3.16	1.95	1.21	1.62	0.61
RNR-25	43.07	18.15	10.50	7.65	1.37	0.58
RNR-26	19.58	3.63	2.65	0.99	2.74	0.72
RNR-27	38.23	18.33	11.85	6.49	1.83	0.64
RNR-28	34.37	9.88	5.34	4.54	1.18	0.54
S. Ed	2.01	1.18	0.59	0.61	0.14	0.01
CV	8.20	15.04	12.31	20.27	8.87	2.62
CD (5%)	4.03	2.36	1.19	1.23	0.28	0.03

RNR= Rajendranagar Roselle

3.3. Genetic Diversity Analysis

The DIVA-GIS grid maps, generated for Shannon diversity index in Roselle (Figure 2), indicated that the Andhra Pradesh is an important pocket for collecting the diversity of Roselle. Shannon diversity index (Table 4) varied from 0.389 to 2.00 for plant height, stalk yield, total biomass and leaf yield, 0.358 to 2.00 for leaf-stalk ratio and 0.322 to 2.00 for harvest index suggests existence of significant variability among the landraces.

Table 4. Shannon diversity index for various key agro-economic traits of vegetable Roselle

Agro-economic trait	Shannon diversity index range
Plant height (cm)	0.389 - 2.00
Total biomass (g/plant)	0.389 - 2.00
Leaf yield (g/plant)	0.389 - 2.00
Stalk yield (g/plant)	0.389 - 2.00
Leaf stalk ratio	0.358 - 2.00
Harvest index	0.322 - 2.00

A dendrogram (Figure 3), illustrating the genetic divergence of landraces, was generated following Ward's minimum variance method using semi-partial R^2 values. A cluster analysis, based on s emi-partial R^2 values, is shown in Figure 3. The dendrogram constructed revealed two major clusters (cluster-I and cluster-II). The first major bifurcation in the dendrogram (Figure 3) separated the 28 accessions into two major clusters (cluster-I and cluster-II). Clusters II could be further divided into sub-clusters (cluster-IIA and cluster-IIB). Cluster I c onsisted of six accessions, while cluster IIA and cluster IIB consisted of 13 a nd 9 landraces, respectively. The genotypes of the sub-cluster-IIA showed high genetic distance than the sub-cluster-IIB. The multiple accessions collected from single collection sites like Peddaveedhi (RNR-7 and RNR-8), Arikathota (RNR-13 and RNR-14), Patha sunnipenta (RNR-18 and RNR-19), Regulakunta (RNR-23 and RNR-24) and Venkatrampalli (RNR-26 and RNR-27) formed separate groups. Further, certain Roselle accessions, from different collection sites, were clustered together; for example RNR-11 from Palavalasa and RNR-21 from Bandimetta in one group and RNR-10 from Babajipetha and RNR-25 from Timbaktu in another group. There are several duplications out of the 29 accessions collected.

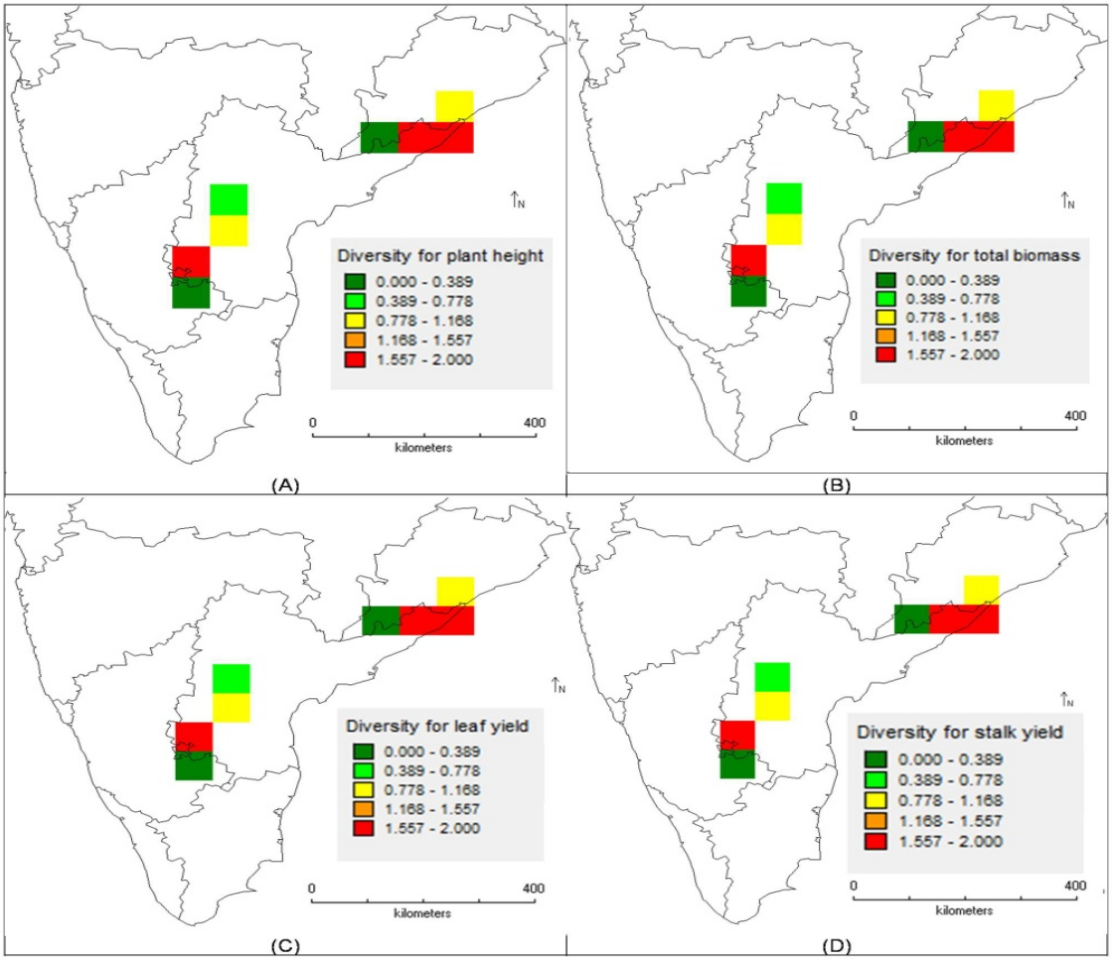

Figure 2. Shannon diversity index for (A) plant height; (B) total biomass; (C) leaf yield; (D) stalk yield of vegetable Roselle landraces

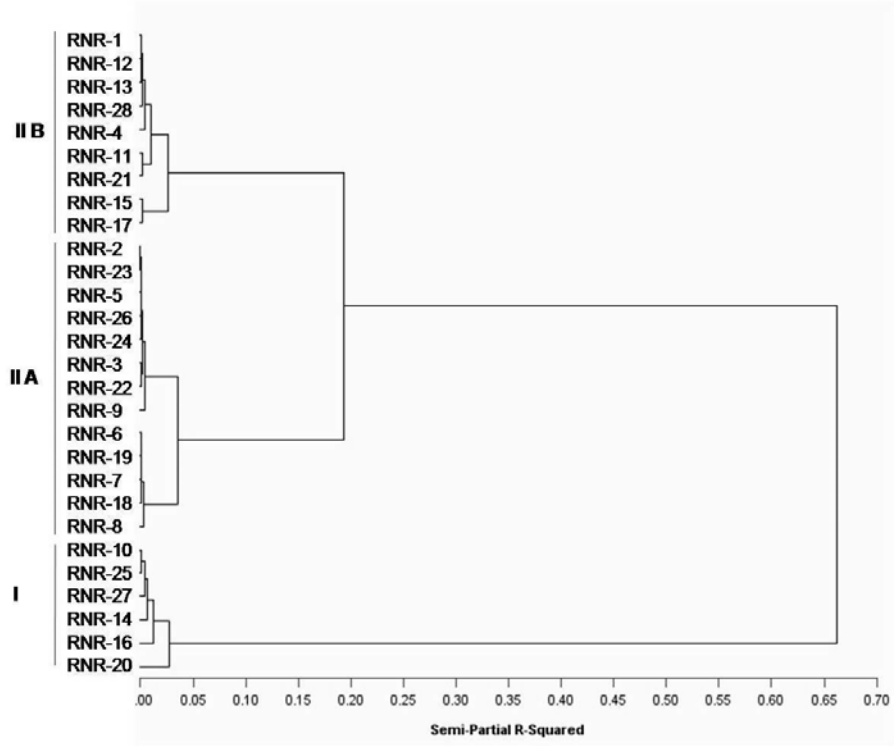

Figure 3. Ward's minimum variance dendrogram showing clustering of landraces of vegetable Roselle

3.4. Genetic Variability Analysis

In general, phenotypic variances were higher than the corresponding genotypic variances for all the characters under study, except for the harvest index (Table 5). The phenotypic variance was the highest for plant height (84.34) followed by total biomass (41.72) and leaf yield (12.85). Similarly, the genotypic variance was the highest for plant height (39.63), followed by total biomass (41.72) and leaf yield (12.32). The degree of variability, shown by different parameters, can be judged by the magnitude of GCV and PCV. PCV showed that the extent of genetic variability in the population ranged from 11.18 (harvest index) to 79.87 (stalk yield). GCV showed that the extent of genetic variability in the population ranged from 10.84 (harvest index) to 77.25 (stalk yield). The estimates of PCV (Table 5) were the highest for stalk yield (79.87%) followed by the total biomass (67.18%) and leaf yield (60.67%), while the lowest for the harvest index (11.18%) followed by plant height (30.58%) and leaf-stalk ratio (32.44%). The estimates of GCV (Table 5) were the highest for stalk yield (77.25%), followed by the total biomass (65.48%) and leaf yield (59.41%), while the lowest for the harvest index (10.84%), followed by plant height (29.46%) and leaf-stalk ratio (31.20%). The estimates of PCV (Table 4) were of a high magnitude (>20%) for almost all the traits except for the harvest index with a moderate magnitude (10-20%). The estimates of GCV (Table 5) were of a high magnitude (>20%) for almost all the traits except for the harvest index with moderate magnitude (10-20%). In general, the magnitude of PCV was higher than the corresponding GCV for all the six characters under study (Table 5). The magnitudinal differences between the estimates of GCV and PCV were the highest for stalk yield (2.62), followed by leaf yield (1.26) and leaf-stalk ratio (1.24). The estimates of heritability (Table 5) were of a high magnitude (>60%) for almost all the traits except for the harvest index with a moderate magnitude (30-60%). The estimates of genetic advance as percent of mean (Table 5) were of a high magnitude (>20%) for all the traits under study. High estimates of heritability (>60%), coupled with a high genetic advance as percent of mean (>20%), were observed for almost all the traits except for the harvest index.

Table 5. Genetic parameters for various agro-economic traits in vegetable Roselle

Character	Variance		Coefficient of variation (%)		Heritability (%)	Genetic advance (%)	Genetic advance as per cent of mean
	Phenotypic	Genotypic	Phenotypic	Genotypic			
Plant height (cm)	84.34	78.28	30.58	29.46	0.93	17.56	58.47
Total biomass (g/plant)	41.72	39.63	67.18	65.48	0.95	12.64	131.46
Leaf yield (g/plant)	12.85	12.32	60.67	59.41	0.96	7.08	119.84
Stalk yield (g/plant)	8.76	8.19	79.87	77.25	0.94	5.70	153.93
Leaf-stalk ratio	0.39	0.36	32.44	31.20	0.93	1.18	61.82
Harvest index	0.01	0.01	11.18	10.84	0.41	0.14	21.67

4. Discussion

In general, species and cultivars are chosen according to their fitness for local conditions. *Hibiscus sabdariffa* has an extensive intraspecific variation, a differentiation into two botanical varieties (sabdariffa and altissima) and a further differentiation into different cultivar groups as landraces. Landraces are most often heterogeneous with a blend of different individual plants maintained by farmers in a local environment and constitute a significant portion of the cultivated crop gene pool. Landraces are the heterogeneous crop populations (Harlan, 1975) which have not originated due to modern plant breeding (Louette, 2000) and constitute a significant source of diversity in the crop gene pools. The selection imposed by farmers makes landraces significant with their social, cultural and religious dimensions in farming. Landraces are crop populations, selected and maintained by farmers within the natural system of evolution (Allard, 1999). They are passed from one generation of farmers to another generation and are exposed to natural and human selections in a local environment. Landraces are known to be resistant to several biotic/ abiotic stresses, nutritionally diverse and considered as valuable genetic resources in crop improvement. These landraces are the only resource available in a resource-poor environment and this genetic variation could be exploited in plant breeding where access to new technology is difficult (Witcombe, 1999). A range of diversity in the form of vegetable Roselle (*Hibiscus sabdariffa* var. sabdariffa L.) landraces is under cultivation by the farmers in open fields in traditional leafy vegetable production and management systems of India. Roselle, planted by traditional farmers in India, is usually of different types and cultivars. These landraces are grown for diverse uses such as for home consumption and sale and are adapted to a range of agro-ecological niches. Such diverse genetic resources of Roselle in India are threatened by different factors, including environmental and production factors. Their utilization in the crop improvement programs depends largely on their availability, genetic diversity and variability. The collection and the characterization of local landrace germplasm have become necessary. Though, the use of the changing land pattern and the habitat destruction have led to the genetic erosion of landraces of Roselle, the collected and reserved gene serves as a natural genetic resource for crop improvement programs of Roselle.

4.1. Production Potential of Landraces

In general, the leafy vegetable crop production and the marketing systems are interdependent. Various kinds of leafy vegetable crop production and marketing systems have evolved over the years in south India. Being an annual crop (Berhaut, 1979), it is traditionally cultivated as a seasonal crop (150-180 days) with periodical leaf pickings or periodical stem cuttings under low density planting (40-30 × 40-30 cm) and medium density planting (30-20 × 30-20 cm) systems, respectively. The low density planting system, with periodical leaf pickings in which 'heap of tender leaves' as a unit of marketing, was practiced upto 1980s. The plants may be stripped off leaves 6 weeks after sowing leaving the stalks in the field with subsequent leaf pickings at 4 weeks interval. The medium density planting system, with periodical stem cuttings in which 'bunch of tender stems' as a unit of marketing, was practiced in 1990s. The plants may be cut off 6 weeks after sowing leaving only 7.5-10 cm of stem in the field with subsequent cuttings at 4 weeks interval. The high density planting with once over harvest, in which 'bunch of seedlings' as a unit of marketing, became more common in 21st century. Currently, it is cultivated as a monthly crop (30-40 days) under a high density planting and once over harvest system in all vegetable farming systems. Under this high density planting and once over harvest system, vegetable Roselle is grown either by broadcasting or drilling at a spacing of 10-20 × 5-10 cm so as to harvest 500000-2000000 plants per hectare just 30-40 days after sowing depending on variety and plant type. In the present study, the production potential of 28 landraces of vegetable Roselle under a high density planting and once over harvest system was discussed.

The analysis of variance revealed significant differences ($P < 0.01$) among 28 genotypes of Roselle for all of the six agro-economic traits, indicating the presence of a significant amount of variability for effective selection. Similar results were reported by many workers (Ibrahim and Hussein, 2006; Ibrahim *et al.,* 2013) indicating that the diversity can be attributed to genetic as well as their interaction with the environment (Koorsa, 1987; Thirthamallappa and Sherif, 1991; Zayed *et al*., 1996). In the germplasm, used in this study, a maximum variability (Table 3) was observed for plant height (34.30 cm), followed by total biomass (20.50 g/plant) and leaf yield (12.27 g/shoot). The ranges of mean values revealed a sufficient variation for all the traits under study. Large variations among landraces were observed for plant height, total biomass, and leaf yield. Such large variation indicated the scope for improving the population for these characters. A great variability among landraces was observed in leaf yield, indicating the possibility to increase leaf production through selection.

In conventional breeding, the choice of parents is based on a high mean performance; out of the six quantitative traits under study, high mean values are desirable for plant height, total biomass, leaf yield, stalk yield, leaf-stalk ratio and harvest index. Hence, the breeders are in absolute need of a desirably high or low mean value, depending upon the character, which is considered a main criterion for effective selection forever. In Roselle, of the six quantitative traits under study, high mean values are desirable for plant height, total biomass, leaf yield, stalk yield, leaf-stalk ratio and harvest index. In the present study, significant differences in plant height were observed among the landraces. Ibrahim and Hussein (2006) also observed significant differences among genotypes of Roselle for plant height. Short plants are often preferred in breeding programs, because they can reduce the lodging problems and responds in a better way to fertilizers. However, plants with longer main stems are stronger and do not fall easily in production levels compared to short plants in Roselle (Chang *et al.,* 2006). Shorter plants with due reduction in internodal length adds the more number of leaf production points in plant. The leaf biomass percentage decreased as the Roselle

plant increased in height. There was advantage of harvesting maximum leaf yields from tall Roselle plants.

Stalk and leaf yields have been currently referred to in terms of yield per plant. Both of these references are usually made on a fresh weight basis. Typically, the leaf and stalk yields will increase throughout the growing season, with the rate of growth reduced by drought, decreasing heat units, and flowering. Leaf production continues throughout the growing season, and is reduced by the same factors, but unlike stalk yields, leaf yields will often increase only during the first half of the growing season and level off or even decrease in the second half of the season. The leaf production continues, but, as a result of leaf abscission, the older lower leaves will drop off, as the plant grows taller. The loss of the lower older leaves limits leaf yields, and results in a decreasing leaf percentage on the whole-plant basis. The leaf yields and total biomass (plant weight) are also important considerations when selecting cultivars for leaf production, because the leaves are the primary source of protein (Webber, 1993a). Researchers reported the differences among cultivars for stalk and leaf biomass percentages (Webber, 1993a and 1993b). These stalk and total biomass can also be greatly affected by plant maturity at harvest. The high leaf-stalk ratio and the harvest index are the key aspects. The harvest index is not directly a yield contributing trait but is considered an important parameter for the genetic improvement of genotypes. It is normally accepted within the Roselle industry to report plant yields on total biomass, leaf yield, leaf-stalk ratio and harvest index. It is much more important to consider the leaf yield when discussing plant yields.

On the basis of genetic potential for leaf yield (Table 3), accessions RNR-16 (14.22 g/plant), RNR-20 (12.72 g/plant) and RNR-27 (11.85 g/plant) were found promising owing to their high yielding potential under high density planting and single harvest system. These genotypes can be evaluated for their stability in various ecological zones across the state of Andhra Pradesh. Upon assessing yield stability through multi-location trials, these genotypes may be used for large-scale cultivation if found suitable. These landraces, with a high leaf yield potential, could be used as donor parents for improvement of indigenous varieties for higher leaf productivity in Roselle improvement programs. The landraces were ranked by the breeders; the adoption rate should be maximal if these local varieties keep the same performance in the farmer's fields. These promising landraces are to be disseminated using on-farm conservation in some sites in the state and at Research Stations. These local varieties of Roselle could be tapped and used in the breeding program, which necessitates the on-farm maintenance of landraces. In order to increase the adoption rate of the promising landraces of Roselle, a participatory selection, on the promising landraces based on users' criteria preferences, should be carried out at the Research Stations, with heavy involvement of the users (producers, consumers and traders). The identification of the promising Roselle landraces will enable the Roselle industry to further move forward in terms of providing choices for varietal selection. By promoting the raising of these promising landraces of this underutilized species, extension workers can help diversify farming systems throughout the tropics, thereby increasing food and economic security on marginal lands.

4.2. Genetic Diversity of Landraces

A better understanding of the genetic diversity distribution is essential for its conservation and use. It will help us in determining what to conserve and where to conserve, and will improve our understanding of the taxonomy and the origin and evolution of the plant species of interest. The distribution of Roselle diversity varied across locations and states (Figure 2). Shannon diversity index (Table 4) varied from 0.389 to 2.00 for plant height, stalk yield, total biomass and leaf yield, 0.358 to 2.00 for leaf-stalk ratio and 0.322 to 2.00 for the harvest index suggesting the existence of a significant variability among the landraces. DIVA-GIS grid maps, generated for Shannon diversity index for Roselle (Figure 2), indicated that Andhra Pradesh and Odisha states had important pockets of diversity in Roselle. The study showed a greater diversity in Andhra Pradesh followed by Odisha, indicating that earlier is important habitat for an on-farm conservation. DIVA-GIS has been used successfully in assessing biodiversity and in identifying areas of high diversity for Phaseolus bean (Jones *et al.,* 1997), wild potatoes (Hijmans *et al.,* 2000), Piper (Parthasarathy *et al.,* 2006), horse gram (Sunil *et al.,* 2008), and black gram (Abraham *et al.,* 2010). However, there is a need for measuring the impact on the change of genetic diversity over time both at village and landscape levels.

The genetic divergence analysis estimates the extent of diversity among the selected genotypes. A dendrogram (Figure 3), illustrating genetic divergence of landraces, was generated following Ward's minimum variance method using semi-partial R^2 values. A multivariate analysis, following Ward's minimum variance method, revealed that the landrace germplasm of Roselle under study seems to be quite diverse agronomically, with a high level of divergence among the accessions under study. The diversity in Roselle germplasm could be attributed to the fact that the studied germplasm represented local farmers' cultivars that are used for commercial production in India, rather than improved advanced varieties. The multivariate analysis, following Ward's minimum variance method, revealed distinct clustering pattern (Figure 3). The present evaluation shows the existence of an intra-specific diversity of the vegetable Roselle studied. The dendrogram constructed by the UPGMA clustering method also revealed the genetic relationship and demonstrated a considerable divergence among 16 accessions of Roselle and kenaf (Omalsaad *et al.,* 2014). The diversity assessment of a collection of 124 roselle accessions and 16 accessions of its close relatives *Hibiscus cannabinus* and *Abelmoschus esculentus* based on ten agro-morphological traits identified two major distinct groups in *H. sabdariffa* using a Bayesian method wherein these two genetic groups were associated with statistical differences for three phenological characteristics: number of days to flowering, 100-seed weight and calyx size (Bakasso *et al.,* 2014). The genotypes of the sub-cluster-IIA showed a higher

genetic distance than those of the sub-cluster-IIB, which clearly indicates the genetic closeness of the groups of Roselle landraces. In the breeding program, the distantly related genotypes should be of a great interest to get the desirable segregates with wide genetic traits. Roselle is a self-pollinating crop species. Flowers being cleistogamous, pollination occurs naturally in the bud stage before the flower blooms. This phenomenon has become a barrier to natural or artificial hybridization to produce genetic variation, and, hence the reason why the breeding program is rarely carried out in a conventional manner. Roselle is a tetraploid (2n = 72) species and, therefore, their segregating populations need a longer time for purification compared to the diploid species. Furthermore, Roselle has cleistogamous flowers. Thus, crop improvement through conventional hybridization is very difficult to be carried out (Jain, 1979; Vaidya, 2000). To avoid these limitations, mutation breeding is recommended to generate a new source of genetic variability. The scoring matrix should be used. The determination of the nature and degree of the genetic diversity of accessions is extremely important to the plant breeder in choosing the diverse parents for a purposeful hybridization in the breeding of crop plants. Hence, it is indispensable that the natural genetic diversity of Roselle for crop improvement be scrutinized and exploited.

The clustering pattern revealed that the landraces, collected from different collection sites, are grouped into different clusters with certain exceptions. For example, the multiple accessions collected from single collection sites like Peddaveedhi (RNR-7 and RNR-8), Arikathota (RNR-13 and RNR-14), Patha sunnipenta (RNR-18 and RNR-19), Regulakunta (RNR-23 and RNR-24) and Venkatrampalli (RNR-26 and RNR-27) formed separate groups. These studies showed that accessions from the same geographical region may differ genetically and phenotypically as well as in their adaptability. Further, certain Roselle accessions, from different collection sites, were clustered together; for example RNR-11 from Palavalasa and RNR-21 from Bandimetta in one group and RNR-10 from Babajipetha and RNR-25 from Timbaktu in another group. The pattern of clustering did not show any distinct relationship with the collection site of landraces. This indicates that the geographic diversity is not the only factor in determining the genetic diversity. Differences of genetic differentiation was probably associated with the differences in the sampling methods and the accession handling. The genotypes with the same geographic origin could have undergone a change in different characters under the selection during the process of evolution. The free clustering of the landraces suggested the influence of the direction of the selection pressure for realizing a maximum yield in different ecosystems; the nicely evolved homeostatic devices would favor the constancy of the associated characters and, thus, show indiscriminate clustering.

There are several duplications out of the 28 accessions collected. For example, RNR-10 from Babajipetha and RNR-25 from Timbaktu were the duplicates. Similarly, RNR-1 from Buditi and RNR-12 from Chipurupalli were also the duplicates. For landraces, the genetic variability is maintained not only between but also within the accessions. The molecular markers, along with the morphological traits, made it possible to evaluate the genetic diversity contained within and between the cultivars and also helped in identifying the duplicate accessions in the gene banks (Virk *et al.,* 1996; Zhu, 1996). However, there is relatively little information available on the intra-accession (cultivar) variation in landraces compared with the variation between them. It is important that the agro-morphological characterization be further explored by more sophisticated studies, both biochemically and molecularly to test the validity of our results. The identification of duplication will lead to a good ex-situ conservation strategy of the accessions. The high duplication rate shows the importance of the exchange of the seeds between the producers within a village or at the state level. The occurrence of duplicates, evident in the dendrogram, indicated the existence of a movement of the seeds within and between localities. Markets remain the main exchange place for the seeds. The enhancement of the number of accessions of the same species and the use of different modes of characterizations can contribute to the implementation of a core collection, which is the best strategy of conserving this landrace germplasm.

4.3. Genetic Variability of Landraces

The objective of the present study is to estimate the amount of the genetic variability available for selection in the accessions and to estimate the amount of heritability and genetic advance among agro-economic traits. The amount of genetic variability is a major determiner of the genetic gain from selection. The estimates of the phenotypic variances were higher than their respective genotypic ones for almost all the traits under study except for the harvest index. In general, the agronomic characters had larger phenotypic variances than their respective genotypic ones. However, the influence of the environmental factors on the expression of the agronomic characters, as indicated by the magnitude of the phenotypic variance, was quite evident. This indicates that a large proportion of phenotypic variance was due to environmental causes; thus, such characters do not possess a promising genetic variation. For them, therefore, selection will not be effective; solely, it would prove to be very low.

The degree of variability, shown by different parameters, can be judged by the magnitude of GCV and PCV. In general, there were differences in the magnitude of genotypic and phenotypic coefficients of variation for all the traits under study. The PCV showed that the extent of the genetic variability in the population ranged from 11.18 (harvest index) to 79.87 (stalk yield). Presence of a high variability for stalk yield, total biomass and leaf yield, as evident from their GCV and PCV values for the above parameters, can form the basis for the effective selection of superior lines in Roselle. The PCV was slightly higher than the corresponding GCV for all the characters under study, which indicates the environmental influence on the character expression. For these characters, the differences between PCV and GCV were narrow, and the PCV and GCV values were close to one another, indicating a low environmental influence in the expression of these characters, implying that the genotype

contributed more in the expression of these characters than the environment, suggesting greater possibilities of improvement through selection. With the help of GCV alone, it is not possible to determine the extent of heritable variation. Thus, the estimates for the heritability indicate the effectiveness with which the selection may be expected to exploit the existing genetic variability.

The high magnitude (>60%) of the estimates of the heritability for plant height, stalk yield, leaf yield, total biomass per plant and leaf stalk ratio indicates that these characters possessed a wide range of genetic variability and their improvement could be achieved by mass selection alone. These high estimates of heritability could be attributed to the difficulty of the separation of all the genotype and environment interactions from genotypic variance since the study was carried out in one location and during one season, and, thus, the heritability estimates were biased upward. The moderate heritability for the harvest index was due to the fact that it depends on many components which are greatly influenced by the environment. Falconer (1980) opined that more variable conditions reduce the heritability, whereas uniform conditions increase it. The high heritability for plant height was also reported by Ibrahim and Hussein (2006) in Roselle. In a selection program where the primary objective is character improvement, a study of genetic gain is more advantageous than the heritability studies.

The high estimates of the heritability (>60%), coupled with a high genetic advance as percent of mean (>20%), were observed for almost all the traits except for the harvest index (Table 5). The high heritability, coupled with a high genetic gain and coefficient of variability, was observed for fresh sepal's weight, number of capsules and plant height (Sanoussi *et al.,* 2011; Falusi *et al.,* 2014). High estimates of heritability (>60%), coupled with a high genetic advance as percent of mean (>20%) for plant height, total biomass, leaf yield, stalk yield and leaf-stalk ratio, indicate the possibility to improve these agro-economic traits through selection programs to develop new varieties. However, the association of the genetic advance and heritability does not follow the same pattern as that between the genetic advance and the genotypic coefficient of variation. The increase in the heritability value was not always accompanied with an increase in the genetic advance. The nature of association between the heritability and the genetic advance was explained by Panse (1957) who reported that the association of high heritability with a high genetic advance is an indication of additive gene effects and, consequently, a high genetic gain from the selection could be expected. On the other hand, the association of low heritability with a low genetic advance is an indication of non-additive gene effects and, consequently, a low genetic gain would be expected from the selection. However, heritability does not provide an actual measurement of the amount of the genetic variation, as the magnitude of the heritability depends on the degree of the association between the genotypic and the phenotypic variances regardless of being high or low; while, the genetic gain depends on the amount of the genetic variability (Johnson *et al.,* 1955). A similar line of reasoning was expressed by Allard (1999).

5. Conclusion

In conclusion, the analysis of variance revealed that the mean squares, due to genotypes, were significantly different, indicating significant differences in the landraces for all the six agro-economic traits under study. The landraces RNR-16 (14.22 g/plant), RNR-20 (12.72 g/plant), RNR-27 (11.85 g/plant) were of a high leaf yielding potential and, thus, are highly suitable to grow as monthly crops. The multivariate analysis, following Ward's minimum variance method, revealed a distinct clustering pattern of landrace germplasm. The distinct clustering pattern, as evident from the dendrogram, presumably reflects the divergence of landraces. Using more landrace accessions and other evaluation methods could help set up a core collection, which is the best way for germplasm conservation. This Roselle landrace germplasm was endowed with a rich genetic variability for all agro-economic traits, as evident from the genotypic and phenotypic coefficients of variation. The broad sense heritability and the genetic advance indicated that the selection for plant height, leaf yield, stalk yield, leaf-stalk ratio and total biomass would be more effective in boosting the leaf yield performance of the vegetable Roselle genotypes. Selection is effective for plant height, leaf yield, stalk yield, leaf-stalk ratio and the total biomass in Roselle.

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Antimicrobial Activity of Secondary Metabolites from a Soil *Bacillus* sp. 7B1 Isolated from South Al-Karak, Jordan

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Abstract

Soil is considered as a promising environment for discovering and isolating bacterial strains that are able to produce novel natural products. Therefore, a Gram positive *Bacillus* sp. 7B1 was isolated from a soil sample collected from south Al-Karak, Jordan. The bacterial isolate was cultivated, identified and its culture medium was extracted. The crude extract was purified using silica gel column chromatography. The antibacterial activity of the crude extract and the purified fractions was evaluated by agar diffusion test as well as by measuring the minimum inhibitory concentration in microbroth dilution assay. The produced crude extract was active only against the tested Gram positive bacteria, namely *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus*. Bioactivity-guided fractionations of the resulted crude extract led to the purification of compound ($\mathbf{C_2}$). In the present work, *Micrococcus luteus* was the most susceptible bacterial test strain to compound ($\mathbf{C_2}$) with a minimum inhibitory concentration of 25 µg/ml. Our findings highlighted the importance of soil bacterial isolates for production of compounds with interesting bioactivities that may contribute to the drug research field.

.**Keyword:** *Bacillus*, Antimicrobial, Partial purification, Al-Karak, Jordan.

1. Introduction

Microorganisms are able to synthesize secondary metabolites of various structures and, hence, bioactivities. Their production is regulated by nutrients, growth rate, enzyme inactivation and induction (Demain, 1998). They are produced to help the organism in competing successfully with other organisms in their natural habitat and to adapt with the changed environmental conditions (Teasdale *et al.,* 2008). These metabolites have played a key role in the discovery and development of many antibiotics (Motta *et al.,* 2004). Consequently, a large number of drugs approved for marketing nowadays are of microbial origin (Harvey, 2008; Butler *et al.,* 2014).

Despite the large number and diversity of these metabolites and due to the emergence of new infectious diseases and resistant pathogens that represent a serious problem for human life (Cragg *et al.,* 1997; El-Banna *et al.,* 2007; Disriac *et al.,* 2013), the need for new therapeutic agents from nature is still urgent. As less than 1% of soil bacterial species are currently known (Torsvik and Ovreas, 2002), soil represents a promising habitat for discovering and isolating new natural products (MacNeil, 2001).

Various reports confirmed the ability of *Bacillus* species to produce antimicrobial agents and compounds with potential biotechnological and pharmaceutical applications (Awais *et al.,* 2007; Hassan, 2014). In addition, numerous studies were conducted on the bioactivity of crude extracts produced by bacterial strains isolated from different habitats in Jordan. Soil *Streptomyces* species were able to inhibit the growth of multi-drug resistant *Pseudomonas aeruginosa* (Saadoun *et al.,* 2008); actinomycin C2 and actinomycin C3 were isolated from red soil *Actinomyces* strain and were reported to inhibit the growth of both *Micrococcus luteus* and *Staphylococcus aureus* (Falkinham *et al.,* 2009); El-Banna *et al.* (2007) showed that the substances produced by different *Bacillus* species isolated from various Jordanian sources have antibacterial activity against methicillin-resistant *S. aureus*.

In an ongoing work on natural products from microorganisms and due to our interest in isolating soil bacterial strains with the ability to produce biologically active metabolites, herein, we report the isolation, identification, cultivation and extraction of bioactive crude extract from a soil *Bacillus* sp. 7B1. Moreover, partial purification of the resulted crude extract using chromatographic methods is described.

2. Materials and Methods

2.1. Isolation and Identification of Bioactive Crude Extract Producing Strain

Bacillus sp. 7B1 was isolated from a soil sample collected from Al–Mazar (south of Al-Karak, Jordan). One gram of the sample was suspended in 10 ml sterile normal saline (0.85% NaCl) and serially diluted up to 10^{-6}. 50 µl from each dilution was spread on Nutrient Agar Media (Oxoid, UK) supplemented with 50 µ g/ml of cyclohexamide and nystatin to inhibit the growth of fungi

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and yeasts. The agar plates were incubated at 27 °C and the isolate was streaked several times till a pure culture is obtained. 2 ml of *Bacillus* sp. 7B1 culture medium was stored in 80% glycerol (1:1) at – 20 °C for further use.

Bacillus sp. 7B1 was characterized morphologically (colony form and appearance, cell shape and size, etc.) and biochemically (Gram stain, oxidase, catalase, nitrate reduction, etc.) for 48-72 hours grown colonies on Nutrient Agar plates. The biochemical characteristics were determined using procedures described by Collins *et al.* (2004) and York *et al.* (2007). The production of acid from different carbohydrates was done as described by Helmke and Weyland (1984). Moreover, the identification of the isolate was confirmed using the commercial MICROGEN® BACILLUS-ID kit (Microgen bioproducts, UK) provided with Microgen Identification System Software (MID-60).

2.2. Bacterial Cultivation, Extraction and Purification of Produced Bioactive Crude Extract With Partial Characterization of The Active Compound

Bacillus sp. 7B1 was inoculated in 20 L of Luria-Bertani (LB) broth (0.5% tryptone, 0.5% yeast extract, 1% NaCl) (8 X 5 L Erlenmeyer flasks containing 2.5 L broth) with continuous shaking (130 rpm) at 27 ° C. During the cultivation process, 50 ml daily samples were collected and used to measure the changes in the bacterial growth parameters and the increases in bioactivity. The growth was followed by measuring the increase in turbidity reading (OD_{600nm}) (UV. Spectrometer, Lambda 16, Perkin-Elmer, LANGEN) and changes in pH values while the bioactivity was measured by the agar diffusion test against *B. subtilis*. The cultivation was ended directly after the OD_{600nm} value decreased or the bioactivity reached its maximum. The cells were harvested by centrifugation (8000 rpm, 15 min, Sorvall ® RC-5B Refrigerated super speed, Dupont company/USA). The pH of the supernatant was adjusted to 8 and extracted with an equal volume of ethyl acetate. The organic phase was dried over sodium sulfate (anhydrous) and concentrated *in vacuo* at 45 °C using vacuum evaporator (Büchi Rotavapor R-215, Switzerland). The resulting crude extract was dissolved in methanol to 50 mg/ml and stored at 4 °C.

The resulting crude extract was applied onto a column of silica gel (Merck 60, 0.063-0.2 µm, Ø 3 cm x 14 cm). An elution was performed with organic solvents of increasing polarity starting from 100% cyclohexane through cyclohexane-ethyl acetate mixture, ethyl acetate-methanol mixture till 100% methanol as mobile phase and under atmospheric pressure. Each eluted fraction was collected and monitored by Thin Layer Chromatography (TLC) and used to measure its bioactivity by agar diffusion test. The fractions that exhibited bioactivity were further purified using size exclusion chromatography (Sephadex LH-20, Ø 3 cm x 33 cm) with 0.1% formic acid : methanol as eluent under atmospheric pressure. 10 ml fractions were collected and monitored by TLC. The fractions with bands of similar migration pattern on TLC were combined together and concentrated *in vacuo* using vacuum evaporator at 45 °C.

The resulting combined fractions were further purified by preparative TLC (PTLC) (20 cm x 20 cm glass plates coated with Merck 60 silica gel) using the Toluene : acetone : formic acid as mobile phase (30 : 70: 1). Each resulting band was scratched from the plate and its constituents were dissolved in acetone. The dissolved constituents were used to follow the bioactivity by agar diffusion test and the purity was tested by re-spotting them on TLC plates. The spotted compounds were detected using UV light at 254 nm and 366 nm. Moreover, developed spots were visualized with ninhydrin (0.1% spray-reagent of 2,2-dihydroxyin-1,3-dandion) with heating at 120 °C for detection of amino acids; bromocresol green (0.1 g bromocresol green in 500 ml ethanol and 5 ml 0.1 M NaOH) for detection of organic acid; and bromothymol blue (0.1% bromothymol blue in 10% aqueous ethanol) for detection of lipid and phospholipids (Touchstone, 1992).

2.3. In Vitro Antibacterial Activity Measurement

Antibacterial activities of the bacterial crude extract, partially purified fractions and the resulting pure compound, were determined by agar diffusion test; the minimum inhibitory concentration (MIC) was also measured by microbroth dilution assay using the standard methods mentioned in Clinical and Laboratory Standards Institute (CLSI, 2012) and as described by Al-Zereini (2014). Five bacterial test strains were included in the present study; three Gram positive bacteria [*Staphylococcus aureus* (ATCC 43300), *Bacillus subtilis* (ATCC 6633) and *Micrococcus luteus* (ATCC 10240)] and two Gram negative bacteria [*Proteus vulgaris* (ATCC 13048) and *Escherichia coli* (ATCC 25922)]. They were seeded on Muller Hinton Agar plates (Oxoid, UK).

500 µg of crude extract or 200 µg of the partially purified fractions was applied on a 6-mm blank filter disk and used in the agar diffusion test, while concentrations starting from 100 µg/ml of purified compound or positive control (Penicillin G) were used in microbroth dilution assay to measure the MIC. The biostatic or biocidal effect of the tested compounds was determined by re-streaking 10 µl from the culture in the first well where there is no obvious growth on a new Nutrient Agar plate. All assays were performed in triplicate and the average values are presented.

3. Results and Discussion

Throughout the present study, one bacterial isolate (*Bacillus* sp. 7B1) exhibited a promising bioactivity against the tested bacterial strains. *Bacillus* sp. 7B1 formed circular creamy yellow colonies on Nutrient Agar Medium. It is a Gram positive, 2-2.5 µm long and 1 µm wide bacilli (Figure 1), oxidase negative, catalase positive, hydrolyze starch and is able to reduce nitrate to nitrite. The biochemical characteristics are summarized in Table 1. It was identified with a 99% similarity to species belonging to the genus *Bacillus* by the Microgen Identification System Software (MID-60) supplied with the MICROGEN® BACILLUS-ID kit

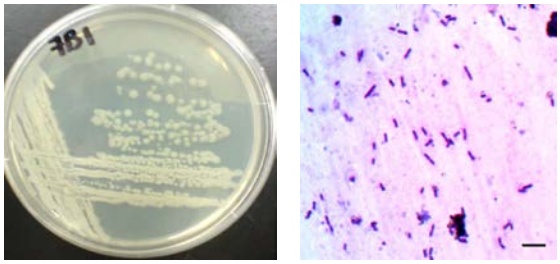

Figure 1. Microphotography of *Bacillus* sp.7B1. A) Colonies gown on Nutrient Agar Medium. B) Cell shape and Gram staining. Scale bar is 10 µm.

Table 1. Biochemical characteristic of bacterial isolate *Bacillus* sp.7B1

Test tests	Experimental result
Oxid/Ferm:[a]	
D-Glucose	+/+
Sucrose	+/+
Sorbitol	+/+
Maltose	+/+
D-Xylose	+/-
Organic compounds as N[b] and C[c] - source:	
L- Leucine	+
L- Phenylalanine	+
L- Histidine	+
L- Asparagine	+
L- Tyrosine	+
Glycine	+
Organic compounds as C-source:	
Sodium acetate	+
Sodium pyruvate	+
Hydrolysis of :	
Starch	+
Gelatine	-
DNA	-
Catalase	+
Oxidase	-
Nitrate reduction	+
H_2S – production	-

[a)] Oxidation/Fermentation [b)] Nitrogen source [c)] Carbon source

The cultivation of *Bacillus* sp. 7B1 was carried out for 196 hours, during which the pH value increased from 7.1 up to 8.5 at the end of cultivation period; the cells entered the stationary phase after 120-144 hours and the cultivation ended after the OD_{600nm} value started to decrease and the bioactivity of the crude extracts from daily samples reached their maximum. In contrary to typical idiolites (secondary metabolites that are produced during idiophase or stationary phase), the antibacterial activity of 500 µg/disc crude extract against *B. subtilis* could be detected early in the logarithmic growth phase (exponential phase), after 48 hours. The maximum activity was obtained at the beginning of the stationary growth phase, with no significant decrease during prolonged incubation (Figure 2).

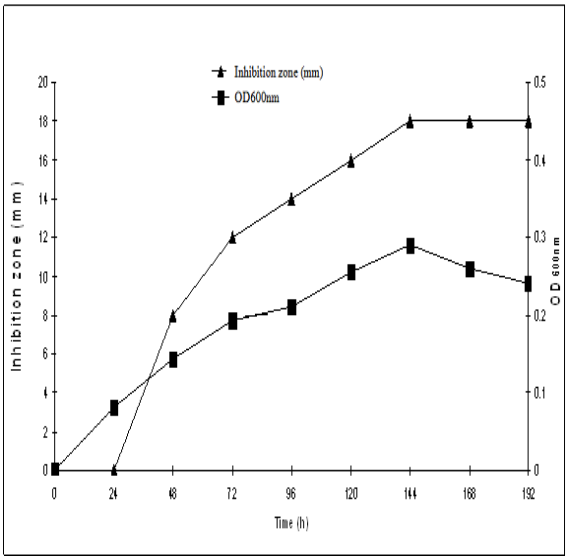

Figure 2. Cultivation parameters during growth of *Bacillus* sp.7B1 in LB medium. Changing in cell density (OD_{600nm}) and increase in inhibition zone diameter caused by 500 µg/disc of applied crude extract against *B. subtilis* are shown.

The crude extract, at 500 µg/disc, of strain 7B1 exhibited a promising anti-bacterial activity against the Gram positive test strains. It caused inhibition zones of 16, 18 and 26 mm against *S. aureus*, *B. subtilis* and *M. luteus*, respectively. Gram negative bacteria were resistant to the tested crude extract. This resistance may be attributed to the low permeability of the Gram negative bacteria outer membrane and the lipopolysaccharide barrier for the hydrophobic compounds (Delcoue, 2009; Wiener and Horanyi, 2011).

Several studies reported that *Bacillus* strains were able to produce a large number of antimicrobial peptides with different chemical structures, such as bacteriocins, iturin A and surfactin (Belhara *et al*., 2011). The production of these metabolites was reported to be a g rowth phase-dependent and produced, as typical secondary metabolites, during the stationary growth phase (Benitez *et al*., 2012). However, the early noticed production of the bioactive crude extract from *Bacillus* sp. 7B1 coincided with reported production kinetics observed for other antimicrobial peptides, namely subtilin, subtilosin and bacteriocin-like substances by different *Bacillus* species (Barboza-Corona *et al*., 2007; Stein, 2005). Nevertheless, the proteinaceuos nature of spotted compounds from *Bacillus* sp. 7B1 was not confirmed after spraying the TLC plates with ninhydrin reagent.

The extraction of culture fluid (19 L) with ethyl acetate yielded 859 mg of crude extract. Bioactivity-guided fractionations of the crude extract using silica gel column chromatography resulted in elution of eight fractions, three of which are polar antibacterial fractions (E-G) (Figure 3). Further purification of the combined bioactive fractions on sephadex LH-20 yielded eight sub-fractions. Only sub-fraction S_3 (100 mg) conferred antibacterial activity and caused an inhibition zone of 18 mm to *B. subtilis* at 200 µg/disc.

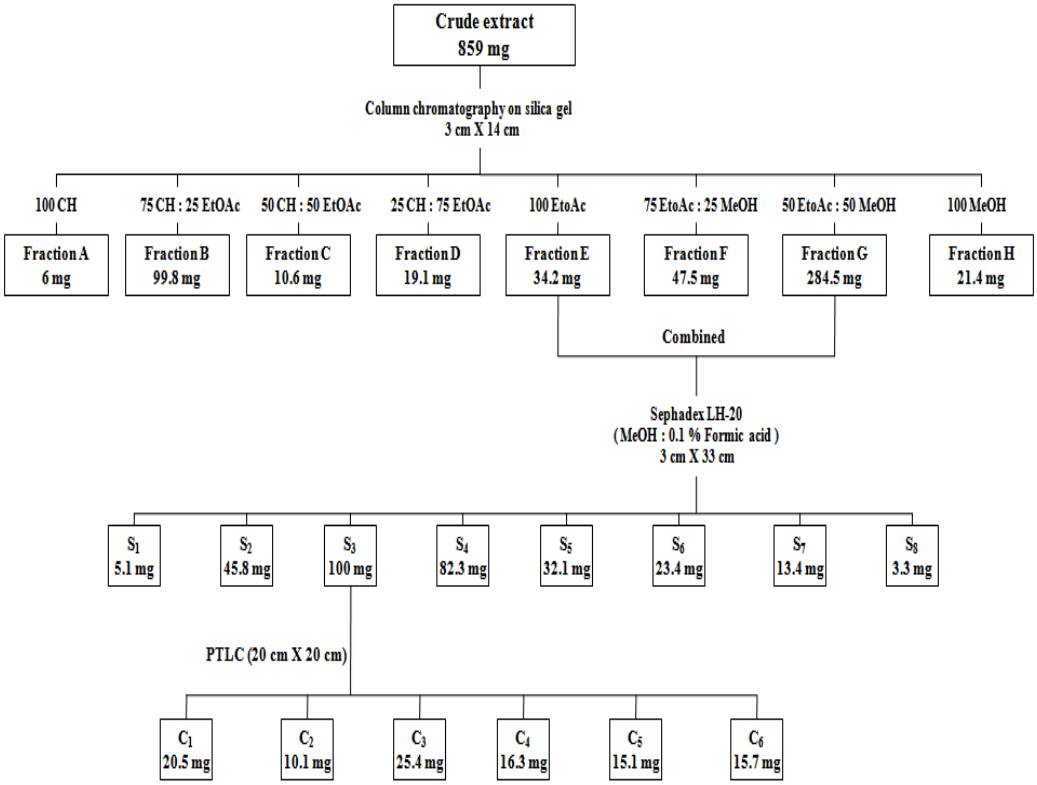

Figure 3. Purification scheme of crude extract resulted from *Bacillus* sp.7B1 cultivated in LB medium.

Generally, the percentage of the bioactive compound in the resulting fractions after each step of purification is increased and, thus, it e xhibits an activity at a concentration less than that at the beginning of screening. Therefore, in the agar diffusion test, we started our screening with a h igher concentration of crude extract (500 µg/disc) and after each step of purification, the fractions were examined at a concentration of 200 µg/disc.

The separation of sub-fraction S_3 constituents by preparative TLC resulted in the production of six bands (Figure 4), of which band number (2) (rate of flow, R_f: 0.25) was the only band that revealed an antibacterial activity in the agar diffusion test. Scratching of band number (2) from TLC plate, dissolving it in acetone followed by drying *in vacuo* using vacuum evaporator and re-spotting on a new TLC plate to examine its purity resulted in 10 mg of pure white colored compound ($\mathbf{C_2}$). 50 µg/disc of compound ($\mathbf{C_2}$) caused inhibition zones of 13, 15 and 17 mm against *S. aureus*, *B. subtilis* and *M. luteus* respectively. In microbroth dilution assay, the MIC value of compound ($\mathbf{C_2}$) was 25–100 µg/ml (Table 2). Compound ($\mathbf{C_2}$) had a bacteriostatic effect as it inhibited growth but did not kill the tested microorganisms. This may enable the human immune system to take its role and overwhelm the bacterial infections (Pankey *et al.,* 2004), especially those caused by Gram positive bacteria.

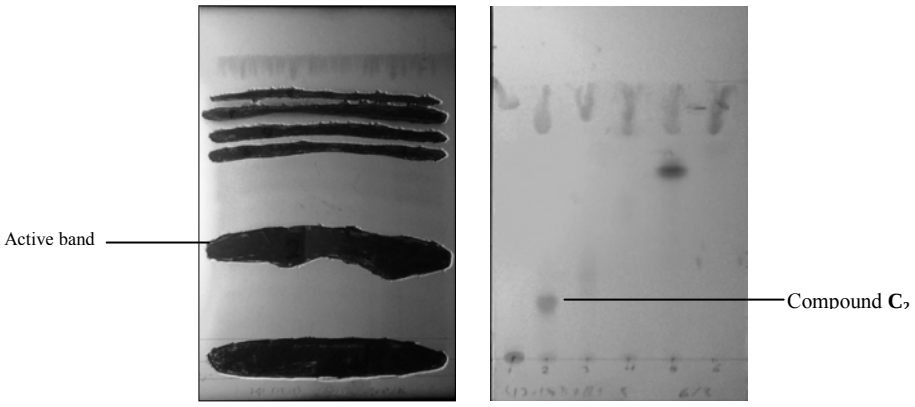

Figure 4. Thin layer chromatography of the partially purified and purified active compound obtained from *Bacillus* sp. 7B1. A) Migration profile of the partially purified S_3. B) The purified compound ($\mathbf{C_2}$). Bands on TLC plates were visualized by UV_{366nm}.

Table 2. Minimal inhibitory concentration (MIC) of the purified compound ($\mathbf{C_2}$) from *Bacillus*.sp.7B1 against susceptible bacterial strains

Tested microorganisms	MIC (µg/ml) *Bacillus* sp.7B1 (C_2)	Penicillin
M. luteus	25s	<8
B. subtilis	>100s	<8
S. aureus	100s	62.5s

s: bacteriostatic

In contrary to bacteriostatic antibiotics that slow or stop the bacterial growth, usually by inhibiting the protein synthesis without inducing the production of toxic radicals, bactericidal antibiotics promote the generation of hydroxyl radicals and, thus, leading to bacterial cell death (Bernatová *et al.*, 2013). Consequently, bactericidal agents induce ROS overproduction in mammalian cells causing an oxidative damage to DNA, proteins, and membrane lipids and mitochondrial dysfunction (Kalghatgi *et al.*, 2013). Furthermore, bactericidal agents may cause a sudden increase in the bacterial products and toxins in the human body which may potentially cause a harmful inflammation due to the cytokine production (Finberg *et al.*, 2004).

Herein, the bioactivity reported for the crude extract and the purified compound ($\mathbf{C_2}$) from *Bacillus* sp. 7B1 against the methicillin-resistant *S. aureus* is more than that obtained from similar strains isolated from Jordan. El-Banna *et al.* (2007) reported that the air flora *Bacillus megaterium* NB-3 and *Bacillus subtilis* NB-6 were inactive against some methicillin-resistant *Staphylococcus aureus* isolates and weakly active to other *S. aureus* isolates from meat samples collected from retail shops and slaughter houses located in Amman area, Jordan. Unfortunately, due to the low amounts of compound ($\mathbf{C_2}$) obtained from *Bacillus* sp. 7B1, it was not possible to perform additional physico-chemical characterizations nor structural elucidation. Therefore, a large scale of 100 L cultivation process is intended to be done in the future in order to gain adequate amount of $\mathbf{C_2}$ compound and elucidate its chemical structure.

Finally, several antibacterial agents were isolated and identified from several *Bacillus* species, among which were the *B. subtilis* group of bacteria including *B. amyloliquefaciens* (Wulff *et al.*, 2002; Al-Tarawneh, 2011; Cao *et al.*, 2011). Moreover, they are known as a source of antifungal compounds (Athukorala *et al.*, 2009) and the most promising candidates for microbial biocontrol agents (Arguelles-Arias *et al.*, 2009). Therefore, *Bacillus* species may be considered a promising source for isolation secondary metabolites with potential application in pharmaceutical and agricultural industry.

4. Conclusion

The present study pointed out the importance of soil bacterial isolates as a source of biologically active and potentially novel secondary metabolites. These metabolites are more effective against Gram positive bacteria than Gram negative strains. Moreover, members of the genus *Bacillus* are still a proliferative group of bacteria with species that are able to produce compounds that may contribute to pharmaceutical industry.

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Tramadol-Induced Liver and Kidney Toxicity among Abusers in Gaza Strip, Palestine

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Abstract

Tramadol, a centrally acting analgesic opioid, is considered to have a low abuse potential and is devoid of side effects like drug dependence, liver and kidney toxicity.. The present study was conducted to assess the tramadol addiction effects on both liver and kidney functions among tramadol abusers. A total of 50 male individuals with a tramadol abuse problem were referred to psychiatric clinics in Gaza Strip, along with an age matched normal control. An informed consent was obtained, a questionnaire was filled, blood samples were collected, serum was separated, and tested serologically to detect antibodies to hepatitis A virus, the presence of hepatitis B surface antigen, and IgM antibodies to hepatitis A virus. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities, bilirubin, creatinine, uric acid and blood urea nitrogen (BUN) levels were measured in the serum. Serum ALT, AST and LDH levels were significantly higher in abuser group compared to the control group. Serum ALP, direct bilirubin, total bilirubin, BUN, uric acid and creatinine levels significantly increased in the abusers that were addicted to tramadol for more than 5 years. Our results point out the risk of increased hepatic and renal damage due to a long-term use of tramadol. Although opioids are reported to be effective in pain management, their toxic effects should be kept in mind during the chronic usage. In addition, measures to control the spread of tramadol abuse should be implemented.

Keywords: Tramadol addiction, Liver toxicity, Kidney toxicity, Gaza Strip, Palestine.

1. Introduction

Tramadol (Tramal TM) is a centrally acting synthetic opioid analgesic agent, used parenterally and orally for the treatment of moderate to severe pain. The mechanism of its analgesic action is complex. Most reports suggest that the analgesic activity and other clinical effects of tramadol are a result of opioid and non-opioid mechanisms. Tramadol binds to the μ-opioid receptor, although much more weakly than morphine. It also inhibits the neuronal reuptake of norepinephrine and serotonin as do the antidepressant drugs such as amitriptyline and desimpramine (Raffa *et al.,* 1992; Raffa, 1996; Dayer *et al.,* 1997; More *et al.,* 1999; Grond and Sablotzki, 2004; Gillman, 2005).

Tramadol has a high oral bioavailability in the range of 70- 80%. Peak blood levels are reached in about 2 hours after an oral dose. It is metabolized in the liver by the cytochrome p450 and by-products are excreted through the kidney. Its biotransformation occurs in the liver and results in O-desmethyl-tramadol, which itself is an active substance and 2 to 4 times more potent than tramadol (Subrahmanyam *et al.,* 2001; Wu *et al.,* 2001; Tao *et al.,* 2002; Wu *et al.,* 2002; Halling *et al.,* 2008). Further, biotransformation results in inactive metabolites, which are excreted by kidneys (Lee *et al.,* 1993; Matthiessen *et al.,* 1998).

Tramadol has a dose-dependent analgesic efficacy that lies between that of codeine and morphine, with a parenteral potency comparable to that of pethidine, i.e., about 10- 20 % of the standard morphine (Wilder-Smith *et al.,* 1999; Pang *et al.,* 2003). A long-term of tramadol administration for management of pain, as well as its use as an acceptable alternative for persons with drug-seeking behavior is controversial. However, the tramadol's effects at a cellular level are not clearly understood (Brena and Sander, 1991; McCarberg and Barkin, 2001; Atici, 2005).

The adverse effects associated with the medicine used in clinics constitute a serious problem for patients and health care providers (Asis *et al.,* 2009). It has been estimated that about 10% of drugs are associated with severe, undesirable side effects (Hussaini and Farrington, 2007; Shi *et al.,* 2010). However, this number is probably underestimated, given that drug-induced adverse effects are difficult to detect due to pre-existing medical conditions, multiple drug usage, and lack of diagnostic standards (Asis *et al.,* 2009).

The central role of liver and kidney in detoxification and drug metabolism increases the risks of toxic injury. Almost certainly, every drug has been associated with hepatotoxicity due to the essential role of the liver in drug

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metabolism (Poppers, 1980; Tolman, 1998). Metabolites of the drugs that are excreted from kidneys may also cause a cellular damage leading to a kidney dysfunction and may have a higher activity and/or a greater toxicity than the original drug (Singhal *et al.,* 1998).

Tramadol is considered to have a low abuse potential and is devoid of side effects, like drug-dependence. A tramadol overdose may lead to a Central Nervous System (CNS) depression, nausea and vomiting, tachycardia, seizures, coma, respiratory depression and cardiovascular collapse (Jones, 2005; Chandrasekaran, 2007). Very few fatalities have been reported so far (Pothiawala *et al.,* 2011). Therefore, a tramadol dosage should be adjusted according to the pain severity. The total daily dose should not exceed 400mg in adult therapeutic blood levels of 0.1–0.8 mg/L (Clarot *et al*., 2003).

Unfortunately, the unregulated access of tramadol to Gaza Strip leads to the misuse of the drug and this is considered a serious problem that may have a negative impact on liver and kidneys among users. A cross-sectional prospective study was conducted to assess the liver and kidney functions among chronic users of tramadol in Gaza Strip. In addition, an investigation of the possible causes of tramadol abuse was attempted.

2. Materials and Methods

2.1. . Study Population

The present study is analytical and descriptive. A total of 50 male individuals with tramadol abuse problems, ranging from 15 to 35 years old, were referred between September 2013 to June 2014 to psychiatric clinics in Gaza Strip. A normal control group with matching age and sex was also included, with the condition that they did not take any medication, and did not suffer from any chronic diseases. All the selected abusers were free of chronic diseases, such as diabetes mellitus, hypertension, heart diseases and kidney or liver failure; all had a history of addiction.

The purpose and the procedures of the present study were explained to all participants. An informed consent was obtained, a questionnaire filled, and a blood sample was collected. Helsinki ethical committee in Gaza Strip approved of this study.

2.2. Sample Collection

Blood samples were collected into plain tubes, and then the specimens were transported within one hour to the Clinical Chemistry Laboratory of the Faculty of Health Sciences, Islamic University - Gaza. Blood samples were centrifuged at 4000 rpm for 10 minutes, serum was separated immediately. All blood specimens were tested serologically to detect antibodies to hepatitis C virus (HCV), the presence of hepatitis B surface antigen (HbsAg), and IgM antibodies to hepatitis A virus (HAV) by enzyme linked immunosorbent assay (ELISA) (CTK Biotech Inc, San Diego). Then, biochemical tests were performed within two hours.

2.3. Biochemical Analysis

Levels of Serum enzymes Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities, bilirubin, creatinine, uric acid and blood urea nitrogen (BUN) were determined in the serum using (BioSystem, Barcelona) protocols. Data entry and analysis were done using Statistical Package for Social Sciences (SPSS version 20) software. Comparisons were made using Chi-square test and t-test. A P-value of < 0.05 was considered indicative of a statistically significant difference.

3. Results

3.1. Description of Study Subjects

A total of 100 specimens (50 tramadol abusers who are admitted to psychiatric clinics in Gaza Strip and 50, of matching age and sex, as control) were included in the study. 28 (56%) of the abusers were from the age group of 21-25 years old, followed by the age group of 26-30 (24%), the age group of 31-35 (12%) and the age group of 15-20 (8%) (Figure 1). All abusers were male.

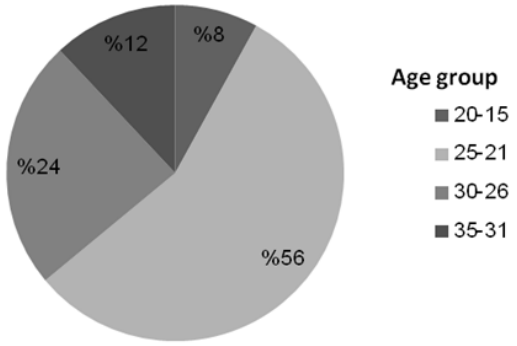

Figure 1. Abusers distribution by age

Twenty-five (50%) of the patients were at the secondary educational level, 27 (54%) of the patients were single. The addiction profile for the patients showed that the average addiction period was 4 years (ranging from 0.5 to 15 years). Most of the patients, 33, (66%) were addicted to a tramadol dose of more than 500 mg and 17 (34%) were addicted to a tramadol dose of less than 500 mg (Table 1) with an average intake of 5 times per day. 48 (96%) of the patients were smokers. Patients selected were free of any abnormalities that may introduce abnormal results in liver and kidney function tests; any patient with positive HAV, HBV, or HCV tests was excluded. None of the patients had a chronic disease such as diabetes, hypertension, heart, and kidney or liver diseases.

Table 1. Tramadol dose intake among patients (N=50)

Daily dose	Frequency	Percent
Less than 500 mg	17	34.0
500-1000 mg	13	26.0
1001-1500 mg	7	14.0
1501-2000 mg	5	10.0
More than 2000 mg	8	16.0

3.2. Liver Function Tests

Liver toxicity does not solely occur by drug metabolites that accumulate due to over dosage (Singhal *et al.*, 1998); it can also occur through viral infection. In the present study, all patients were tested for hepatitis viruses (HAV, HBV, and HCV) and only those with negative results were included in the study.

With regard to liver function tests, ALT results were significantly higher at *p*-value of less than 0.05 in tramadol abusers (72%) against the control group (0%) (Figure 2A).

About 50% of the tramadol abusers showed abnormal AST and LDH results in comparison with the control group that scored only 16% (Figures 2B and 2D). ALP, direct billirubin, and total bilirubin results showed no significant difference between the two groups. When the addiction period was considered in comparing the results, it was found that 4% of the "more than 5 years tramadol abusers" showed an abnormal result against a 100% normal result among the control group (Figure 2C).

3.3. Kidney Function Tests

During the vital role of kidney in blood clearance from toxic compounds, some of these compounds, such as drug metabolites, may cause a c ellular damage, leading to a kidney dysfunction (Singhal *et al.*, 1998).

Kidney function was evaluated among the tramadol addicts and the results showed that a low percentage of the abusers (ranging from 6% -10%) had abnormal BUN and Uric acid value. Those patients were categorized as chronic addicts for more than five years (Figure 3 A and B); this indicates that the long-term tramadol addiction may cause kidney injury. The creatinine levels showed no significance differences among abusers and controls.

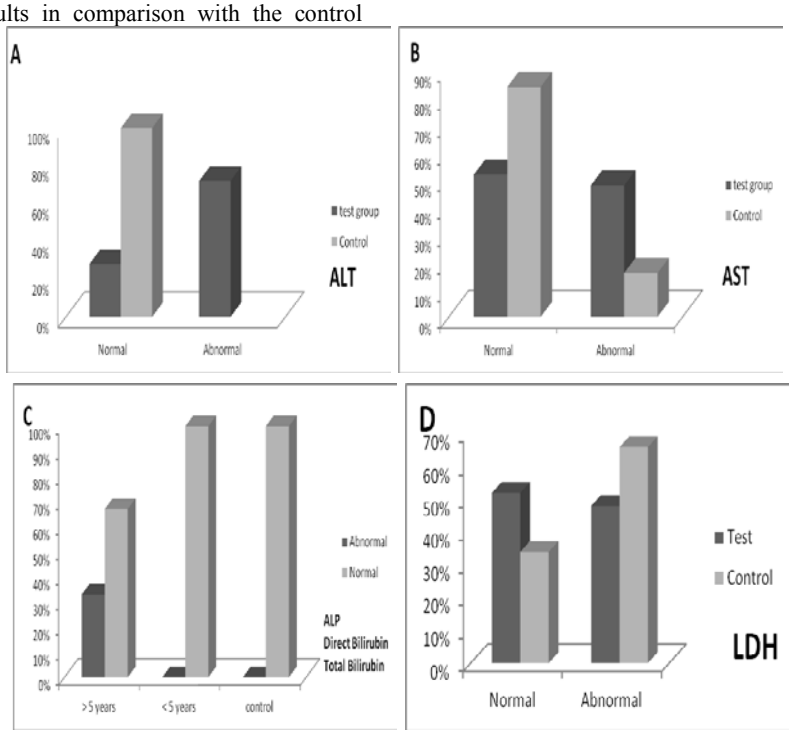

Figure 2. Liver enzymes results in patients and control serum. At $P < 0.05$ patients showed significant higher ALT results (A) , AST results (B). ALP, Direct bilirubin and Total bilirubin and bilirubin was only elevated in patients addicted to tramadol more than 5 years (C). Data significantly showed high levels of LDH among patients at $P < 0.05$ (D).

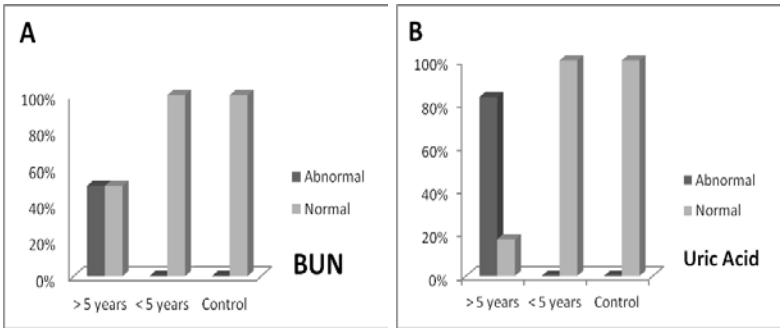

Figure 3. Kidney function tests results in patients and control serum. At $P < 0.05$ patients with long term of addiction had abnormal BUN results (A), uric acid results (B).

4. Discussion

Tramadol overdosing has been one of the most frequent causes of drug poisoning in the recent years, especially in young adult males with a history of substance abuse and mental disorders (Shadina *et al*., 2008). When overdosed, it is, as believed, associated with significant morbidity and mortality (Jones, 2005; and Chandrasekaran, 2007). The present is the first study that attempts to assess the effects of tramadol addiction on liver and kidney functions among the chronic users of tramadol in young adults of Gaza Strip.

The hepatic function in drug metabolism involves converting drugs and other compounds into products that are more easily excreted (Coughtrie *et al.,* 1989; Milne *et al.*, 1997; Tolman, 1998). Metabolites may have a higher activity and/ or a greater toxicity than the original drug. These metabolites, excreted via kidneys, may also cause a cellular damage and, thus, a kidney dysfunction (Singhal *et al.,* 1998). The liver and kidney are responsible for the tramadol metabolism and excretion and the high risk of hepatotoxicity and nephrotoxicity (Wu *et al.,* 2001; Jansen, 2005).

In the present study, tramadol abusers' personal data showed that about 56% of the abusers were 21 to 25 years old, and 50% did not go to college. This age is particularly critical, especially with the difficult socioeconomic status along with a life of hardship and unemployment. Another key factor that could contribute to the use of tramadol and other drugs by young adults is the absence of awareness of the hazardous effects of such drugs and also to the relatively easy accessibility. This is in agreement with a study performed in Iran, showing that the majority of people seeking tramadol from pharmacies are adolescents/ young adults taking tramadol orally, with the criteria for drug addiction (Zabihi *et al.,* 2011). Most of patients, 33 (66%), were addicted to a tramadol dose of more than 500mg by means of opioid overdose according to previous published data that considered the total daily dose should not exceed 400mg for adult therapeutic blood levels of 0.1–0.8mg/L (Clarot *et al*., 2003)

The liver function was evaluated through measuring Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities and serum bilirubin. The results showed a significantly higher ALT result among tramadol abusers compared with the control group. About 50% of the tramadol abusers (> 5 years abuse) showed abnormal AST and LDH results; and this is in agreement with the findings of a study carried out by Panchenko *et al.* (1999). A significant increase in the level of ALT and AST was reported among rats that received 40 and 80 mg/kg of tramadol with a pronounced effect caused by the large dose and the duration of drug administration (El-Gaafarawi, 2006). Those findings were similar to previous studies conducted on long-term tramadol treated mice (Borzelleca *et al.,* 1994; Aitic *et al*., 2005; Elyazji *et al.,* 2013; Rukhshanda *et al.,* 2014).

ALP and billirubin results showed no significant difference between tramadol abuser and control groups except for the chronic abusers (more than 5 years) who showed abnormal levels. This indicates that the long-term use of tramadol intake is more harmful to liver and causes a serious cellular toxicity and a liver failure. Previous histopathological studies indicated the necrosis, vacuolization, central vein dilation, hemorrhage, cytolysis and complete cell membrane degeneration in hepatocytes in tramadol long-term treated mice (Aitic *et al.,* 2005; Rukhshanda *et al.,* 2014).

Moreover, the results of the kidney function tests showed an increase in BUN, uric acid and creatinine levels in samples obtained from the-more-than-5-years tramadol abuser. This suggests that the long-term use of tramadol has negative impacts on kidney functionality. This result is in accordance with Aitic *et al.* (2005) who reported an increase in BUN and creatinine levels in rats with long-term tramadol receiving; others studies reached similar results (Borzelleca *et al.,* 1994; Aitic *et al.,* 2005; Elyazji *et al.,* 2013; Rukhshanda *et al.,* 2014).

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The Chemical Composition and the Antibacterial Properties of *Ruta graveolens* L. Essential Oil Grown in Northern Jordan

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Abstract

Very little has been published on the essential oil composition of *Ruta graveolens* worldwide. Herein, we report on the essential oil composition of *Ruta graveolens* growing in Jordan. The essential oil was isolated using hydrodistillation from the aerial parts of *Ruta graveolens* L and a chemical composition analysis was conducted using Gas Chromatography/Mass Spectrometry (GC-MS). The antibacterial activity was evaluated using a disc diffusion method. Thirty components, accounting for 98.1% of the oil, were identified. Ketones (43.02%), aldehydes (37.12%), esters (9.33%) and sesquiterpene hydrocarbons (5.22%), were the major constituents. The major compounds identified were 2-nonanone (37.13%), undecanal (34.69%), 2-acetoxydodecane (5.0%), and 2-decanone (3.31%). The essential oil of *Ruta graveolens* showed a good antibacterial activity against all the tested bacterial isolates. The oil proved to be more effective on Gram-positive than Gram-negative bacterial species.

Keywords: *Ruta graveolens*, Gas Chromatography-Mass Spectrometry, Disc diffusion, Essential oil.

1. Introduction

For thousands of years, nature has been serving as a rich source of medicines, to which a large number of modern drugs owe their origin. A t present, natural products and their derivatives represent over 50% of all drugs in clinical use, with plant-derived natural products representing about 25% of the total (Ellof, 1998; Thomas and Devi, 2013).

Plant-derived traditional medicines continue to play an essential role in health care, with about 80% of the world's population using traditional medicine at some time or other (Owolabi *et al.,* 2007). Needless to say, such plants should be investigated to better understand their properties, their safety, and efficacy.

The emergence of bacterial resistance to the currently available antimicrobial agents is an increasing concern since the treatment options available for infected patients are severely limited (Carlet *et al.,* 2014). To make the matter even worse, many pharmaceutical companies seem to have lost interest in developing new antimicrobial agents due to their reduced profitability. It may be noted that the development of new antimicrobial agents costs between $0.8 and $1.7 billion (Stanton, 2013; Spellberg, 2012). Thus, there is a need for other strategies, such as the use of plant-derived essential oils, to fight against resistant bacteria.

Ruta graveolens L. belongs to the Rutaceae family, originally native to the Mediterranean region (Asgarpanah and Khoshkam, 2012). *Ruta graveolens* is an evergreen shrub with bluish-green leaves that emit a powerful odor and have a bitter taste. *Ruta graveolens* is an ornamental, aromatic, and medicinal plant. In Jordan, it is used as a flavoring agent in foods and beverages. In traditional medicine, it is used for its antispasmodic, diuretic, sedative, and analgesic effects, and externally for its anti-rheumatic effect (Khouri and El-Akawi, 2005). In addition, *Ruta graveolens* promotes menstruation, and is used as a contraceptive (Browner, 1985; Steenkamp, 2003).

The chemical composition of the oil of *Ruta graveolens*, growing in Jordan, has never been established. The aim of the present study is to determine the chemical composition of the essential oil from the aerial parts of *Ruta graveolens* grown in northern Jordan and its antibacterial activity.

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2. Materials and Methods

2.1. Collection and Authentication of Plants

Fresh *Ruta graveolens* plants were collected before flowering from Qumeim town, Irbid, northern Jordan. The plants were taxonomically identified and authenticated by the Botanical Survey Division of Yarmouk University, Jordan.

2.2. Isolation of Essential Oil

Fresh aerial parts of *Ruta graveolens* were finely chopped and subjected to hydrodistillation for 4 h using a Clevenger-type apparatus, yielding 0.27% (v/w) pale yellowish oil. Subsequently, oil was dried over anhydrous sodium sulfate and immediately stored in GC-grade hexane at 4ºC until analysed by Gas Chromatography/ Mass Spectrometry (GC-MS).

2.3. Essential Oil Composition

2.3.1. Gas Chromatograph-Flame Ionization Detector (GC–FID) analysis

The oil was analyzed in an Agilent (Palo Alto, USA) 6890N gas chromatograph fitted with a 5% phenyl–95% methylsilicone (HP5, 30 m × 0.25 mm × 0.25 µm) fused silica capillary column. The oven was programmed to run from 60°C to 240°C at 3°C/min with hydrogen being used as the carrier gas (1.4 mL/min). One microliter of a 1% solution of the oil in hexane was injected in split mode (1:50). The injector was kept at 250°C and the flame ionization detector (FID) was kept at 280°C. Concentrations (% content) of oil constituents of *Ruta graveolens* were determined using their relative area percentages obtained from the GC chromatogram, assuming a unity response by all components.

2.3.2. Gas Chromatography-Mass Spectrometry (GC–MS) analysis

The chemical analysis of the essential oils was carried out using GC-MS (Agilent 6890N gas chromatograph, Palo Alto, USA). The chromatographic conditions were as follows: column oven program; 60ºC (1 min, isothermal) to 246ºC (3 min, isothermal) at 3ºC/min, the injector and detector temperatures were 250ºC and 300ºC, respectively. Helium was the carrier gas (flow rate 0.90 mL/min), and the ionization voltage was maintained at 70 eV. An HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thicknesses) was used. A hydrocarbon mixture of *n*-alkanes (C_8-C_{20}) was analyzed separately by GC-MS under the same chromatographic conditions using the same HP-5 column. Kovats Retention Indexes (KRIs) were calculated by injection of a series of *n*-alkanes (C_8–C_{20}) in the same column and conditions as above for gas chromatography analyses.

The identification of the oil constituents was based on a computer search using the library of mass spectral data (http://www.massbank.jp) and a comparison of the calculated KRIs with those of the available authentic standards and literature data was drawn.

2.4. Maintenance and Preparation of Cultures

Six clinical isolates of bacteria were used in the present study; three strains of Gram-positive bacteria (Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, and *Bacillus subtilis*) and three strains of Gram-negative bacteria (*Escherichia coli*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*). Pure isolates were obtained by subculture on nutrient agar plates and characterized by standard microbiological and biochemical methods, including Gram-stain, catalase test, coagulase test, and the API system (bioMerieux, France).

The bacteria were inoculated into Tryptone Soy Broth (TSB) (Oxoid, Hampshire, England) and incubated at 37°C for 24 h. One milliliter of the broth, containing 1 X 10^8 CFU/mL, was used as inoculums and spread on each Mueller Hinton agar plate (Oxoid, Hampshire, England).

2.5. Disc Diffusion Assay

The antibacterial activity of the *Ruta graveolens* essential oil was determined by the disc diffusion method according to the Clinical Laboratory Standards Institute guidelines (2012). Sterile paper discs (Oxoid, Hampshire, England) of 6 mm diameter were impregnated with 10 µL of essential oil and deposited on the agar surface. Petri dishes were placed at 4°C for 2 h to facilitate the dissemination of extract on the culture medium, followed by incubation at 37°C for 24 h. For each sample, a negative water control and a positive antimicrobial agents disc control (Oxoid, Hampshire, England) were used. At the end of the period, inhibition zones (if any), formed on the medium, were evaluated in mm. Studies were performed in triplicate in three independent experiments.

3. Results

3.1. Chemical Composition of the Essential Oil

Hydrodistillation of the aerial parts of the *Ruta graveolens* sample gave pale yellowish oil with a yield of 0.27%. The chemical composition of the oil was investigated using the GC-MS techniques.

The identified component groups are shown in Table 1 with ketones (43.02%), aldehydes (37.12%), esters (9.33%), and sesquiterpene hydrocarbons (5.22%), being the major constituents.

Table 1. Constituents groups of the essential oil of *Ruta graveolens*

Compounds	Peak area %
Monoterpene hydrocarbons	0.61
Oxygenated monoterpenes	1.49
Sesquiterpene hydrocarbons	5.22
Oxygenated sesquiterpenes	0.2
Nitrogen-containing compounds	0.21
Aldehydes	37.12
Ketones	43.02
Alcohols	0.9
Esters	9.33

The identified components of the essential oil, their percentages and retention indices are given in Table 2. Thirty components, accounting for 98.1% of the oil, were identified. The major identified compounds were 2-nonanone (37.13%), undecanal (34.69%), 2-acetoxydodecane (5.0%), and 2-decanone (3.31%).

Table 2. Constituents compounds of the essential oil of *Ruta graveolens*

No.	Kovats Index	Compound	Peak area %
1	844	2E-Hexenal	0.28
2	847	3Z-Hexenol	0.28
3	860	Tetrahydro-3,6-dimethyl - 2H-pyran-2-one	0.1
4	970	Sabinene	0.10
5	987	2-Octanone	0.73
6	1026	d-Limonene	0.45
7	1080	3-Ethyl-2,2-dimethyloxazoldine	0.11
8	1107	2-Nonanone	37.13
9	1109	2-Nonanol	0.62
10	1110	Nonanal	0.35
11	1135	Geijerene	0.11
12	1139	2-Octanol acetate	0.36
13	1142	E-Myroxide	0.77
14	1160	2E-Nonen-1-al	0.1
15	1193	2-Decanone	3.31
16	1210	Octanol acetate	0.06
17	1239	2-Acetoxydodecane	5.0
18	1256	Cis- piperitone	0.37
19	1288	Pereijerene	5.22
20	1307	Undecanal	34.69
21	1314	2-Nonanyl acetate	0.63
22	1364	2-Methylundecanal	1.7
23	1393	3-Dodecanone	0.95
24	1433	Methylundecanoate	1.27
25	1457	2-Pentadecanone	0.05
26	1494	2-Tridecanone	0.8
27	1532	Citronellyl butanoate	0.1
28	1545	Elemol	0.35
29	1555	Alpha-agarofuran	0.2
30	1805	2-Ethyl hexyl salicylate	1.87
		Total	98.1

3.2. Antimicrobial Activity

The disc diffusion results, presented in Table 3, provide data on the activity of *Ruta graveolens* essential oil against several bacterial clinical isolates. The results show that the essential oil has a very potent activity against all the tested species. The *Ruta graveolens* essential oil was more active than the tested antimicrobial agents on M RSA, *S. epidermidis, B. subtilis*, and *E. aerogenes*. The essential oil was more active than neomycin, but less active than nitrofurantoin, against *E. coli*. It was more active than ceftazidime, and was similarly active to cefotaxime, against *P. aeruginosa*. Overall, the essential oil of *Ruta graveolens* was more active against Gram-positive than Gram-negative bacteria.

Table 3. Antibacterial activity of *Ruta graveolens* essential oil.

Bacterial species	*Ruta graveolens* essential oil zone of inhibition (mm); Mean± SD	Antimicrobial agent	Antimicrobial agent zone of inhibition (mm); Mean± SD
MRSA	20±0.20	Vancomycin	5±0.15
		Rifampicin	16±0.19
S. epidermidis	22±0.23	Cefuroxime	5±0.11
		Cefotaxime	11±0. 17
B. subtilis	28±0.65	Vancomycin	20±0.45
		Chloramphenicol	26±0.21
E. coli	14±0.2	Neomycin	12±0.29
		Nitrofurantoin	16±0.15
E. aerogenes	26±0.26	Neomycin	20±0.40
		Nitrofurantoin	22±0.25
P. aeruginosa	10±0.11	Ceftazidime	6±0.10
		Cefotaxime	10±0.14

4. Discussion

Hydrodistillation of the aerial parts of *Ruta graveolens* yielded pale yellowish oil. The chemical composition of the oil was investigated using GC-MS techniques, leading to the identification of thirty components, accounting for 98.1% of the oil content. The major constituent groups, given in Table 1, were ketones (43.02%), aldehydes (37.12%), esters (9.33%) and sesquiterpene hydrocarbons (5.22%). The major identified compounds, as shown in Table 2, were 2-nonanone (37.13%), undecanal (34.69%), 2-acetoxydodecane (5.0%), and 2-decanone (3.31%). A point to note is that the essential oil content of plants of a given species can vary depending on a number of factors, including the harvest time and the stage of growth at the time of picking (Adzet *et al.,* 1992). Table 4 summarise the essential oil content of *Ruta graveolens* according to previously published research.

Table 4. Reported major components of *Ruta graveolens* essential oil

Compound	Zhu *et al*. (1993)	Formacek and Kueczka (1982)	Aboutab *et al.* (1988)	El-Sherbeny *et al*. (2007)	Fredj *et al* (2007)	Soleimani *et al.* (2009)
2-Undecanone	36.5%	90.42%	49.2%	51%	27.34%	33.9%
2- Nonanone	23.17%	4.27%	24.7%	10.15%	38.66%	8.8%
2-Nonyl acetate	22.03%	-	6.2%	-	-	-
Limonene	-	-	6.06%	-	-	-
2-Nonanol	-	-	-	-	12.25%	-
2-Octyl acetate	-	-	-	-	7.71%	-
2-Heptanol acetate	-	-	-	-	-	17.5%
1-Dodecanoln	-	-	-	-	-	11%
Geyrene 10.4%	-	-	-	-	-	10.4%

The increase of the bacterial resistance to commonly used antimicrobial agents' presents itself as a major challenge for therapy. The essential oil of *Ruta graveolens* showed a very potent activity against all the tested bacteria being more effective against Gram-positive than Gram-negative bacteria. The results indicate that the essential oil of *Ruta graveolens* may have a potential as an effective and affordable means of combating bacterial infection. The complex mixtures of different compounds of the essential oil may provide multiple mechanisms for an antimicrobial activity. This could explain why this essential oil, and indeed other essential oils, has such a potent activity. The modes of action of only a limited number of the essential oil components were studied. The major components of an essential oil are thought to play a more significant role in the antimicrobial activity, while the minor constituents are thought to result in synergistic outcomes (Li *et al.,* 2014). Published data show that the most active essential oil components are phenols, followed by cinnamic aldehyde, alcohols, aldehydes and ketones, and ethers, while the least active being hydrocarbons (Kalemba and Kunicka, 2003).

The major components of *Ruta graveolens* essential oil were ketones (43.02%) and aldehydes (37.12%). These two components normally show a moderate antimicrobial activity (Fredj *et al.,* 2007). Li *et al.* (2014) reported that 70% of the *Litsea cubeba* essential oil, which has a very good antibacterial activity, was aldehydes. The antibacterial activity of *Ruta graveolens* essential oil may be similarly attributed to its aldehydes and ketones content, while the minor constituents could be responsible for other synergistic effects.

The main target of the essential oil compounds is the cell membrane. They lead to a cell membrane damage causing increased membrane permeability, ions leakage, and inhibition of different enzymes and proteins (Saad *et al.*, 2013; Hyldgaard *et al.,* 2012; Cox *et al.,* 2000). As to why the essential oil of *Ruta graveolens* was more effective on Gram-positive than on Gram-negative bacteria, it may be noted that the cell wall structures of the Gram-positive and the Gram-negative bacteria are different, being more complex in the latter. The cell wall of Gram-negative bacteria has an outer membrane that lies outside and is linked to the peptidoglycan layer below it (Nazzaro *et al.,* 2013; Saad *et al.,* 2013; Hyldgaard *et al.*, 2012). This outer membrane possesses pores that allow only small hydrophilic molecules to pass through and is almost impermeable to hydrophobic molecules. This outer membrane is absent in Gram-positive bacteria. This may explain why the hydrophobic essential oil is more active against Gram-positive than Gram-negative bacteria (Nazzaro *et al.,* 2013; Saad *et al.,* 2013; Hyldgaard *et al.,* 2012; Kavanaugh and Ribbeck, 2012; Gao *et al.,* 1999). However, the susceptibility of Gram-negative bacteria may vary according to genus and species. For example, the essential oil of cinnamon inhibits the growth of *E. aerogenes* through the interaction with different amino acid decarboxylases. Thus, the activity of essential oils may vary depending on the presence or absence of some targets (Boire *et al.,* 2013). This may explain why *Ruta graveolens* essential oil yielded a high antimicrobial activity toward *E. aerogenes*.

5. Conclusions

This study is the first to report on the essential oil composition of *Ruta graveolens* from Jordan. The essential oil of *Ruta graveolens* had an antimicrobial activity to several bacterial clinical isolates including MRSA and *P. aeruginosa*.

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Comparative Studies on the Phytochemical Composition, Phenolic Content and Antioxidant Activities of Methanol Leaf Extracts of *Spondias mombin* and *Polyathia longifolia*

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Abstract

Spondias mombin and *Polyathia longifolia* leaves are popular choices in traditional herbal medicine practice. *P. longifolia* leaf extracts have not enjoyed profound investigation as more attention is paid to its stem bark and roots. The present study, therefore, evaluates the phytochemical constituents, the phenolic content and the antioxidant activity of the methanol leaf extract of *P. longifolia* and draws comparisons with *Spondias mombin* leaf extracts. Results from the phytochemical screening test on *S. mombin* extract revealed the presence of alkaloids, reducing sugars, cardiac glycosides, flavonoids, tannins, saponins, steroids and terpenoids. *P. longifolia* extract contained alkaloids, flavonoids, tannins and reducing sugars. The *S. mombin* extract had significantly higher total phenol content, while the total flavoniod and proanthocyanidin content were significantly higher in the *P. longifolia* extract. The *S. mombin* extract had better antioxidant activities as evidenced by the lower IC_{50} values for DPPH radical scavenging, a higher FRAP value and a higher inhibition of lipid peroxidation *in vitro*. The results suggest that the extracts of *S. mombin* and *P. longifolia* leaves possess moderate antioxidant activities, which may be of nutraceutical and pharmaceutical importance.

Keywords: *Polyathia longifolia, Spondias mombin,* Antioxidant, Phenol, Flavonoid

1. Introduction

Spondias mombin Linn is a fructiferous tree that belongs to the family Anacardiaceae with about 14 species worldwide. In Nigeria, it is called with several local names, such as *okhikghan* (Bini), *akika* (Yoruba), *tsadermasar* (Hausa), *ijikara*. (Igbo) (Igwe *et al.*, 2011).A decoction of the plant leaves is used by the Senegalese and Edo people in Nigeria to treat dysentery and other intestinal disorders. The leaves have also been used as a remedy for malignant tumors in Nigeria. It is also documented to possess antimicrobial, anti-malaria, and anti-epileptic properties as well as wound healing properties (Asuquo *et al*., 2013). *Spondias mombin* is also employed to manage several conditions arising within the female reproductive system. For instance, it is used to induce labor, reduce pain and bleeding during and after childbirth, to prevent miscarriage and treat uterine/ vaginal infections (Asuquo *et al.*, 2013; Igwe *et al.*, 2013). Igwe *et al*. (2013) also reported that the extract has no detrimental toxicological effects on the organ/tissue function of the animals studied.

The genus *Polyalthia* includes about 120 species occurring mainly in Africa, South and South-Eastern Asia, Australia, and New Zealand. *Polyalthia longifolia* is a small medium-sized tree, native to India and Sri Lanka that has now been introduced in gardens of many tropical countries across the world. It is an evergreen tree and can grow up to a height of 15-20 meters. The leaves are long, narrow, dark green and glossy (Jothy *et al*., 2013). In the last few decades, a lot of phytochemical studies have been carried out on *P. longifolia*. However, most of these studies focused on the stem with very few reports on the leaves (Jothy *et al*., 2013).

The search for new natural compounds with potent biologic activities has been increasing with an estimated eighty percent (80%) of the human population in developing countries relying, to some extent, on medicinal plant materials for their primary healthcare. Medicinal plant based drugs have the added advantage of being readily available, effective, and offering a broad spectrum of activity with a greater emphasis on preventive action. These medicinal plants are usually known to exert their diverse health benefits through the numerous phytochemicals they contain (Jeyachandran *et al.*, 2010; Jothy *et al.*, 2013). The present study, therefore, evaluates the phytochemical composition, the phenolic content and the antioxidant activities of methanol leaf extracts of *S. mombin* and *P. longifolia*. Hopefully, the extracts will be used in future in the pharmacological industry for drug discovery.

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2. Materials and Methods

2.1. Collection of Plant Leaves

Spondias mombin and *Polyalthia longifolia* leaves were collected during the period between January and March, 2013 from private farms at different locations in Benin City, Nigeria. The leaves were identified and authenticated by a Botanist in the Department of Plant Biology and Biotechnology, University of Benin. The leaves were then washed, dried and macerated.

2.2. Preparation of Extracts

100g of the macerated *S. mombin* and *P. longifolia* leaves were soaked in 1000mL of absolute methanol for 72 hours with occasional stirring. The extracts were then filtered using a double layered muslin cloth and the filtrate concentrated to dryness by using a rotary evaporator at reduced pressure. The dried extracts were stored at 4°C until use.

2.3. Phytochemical Screening

Phytochemical screening was carried out on the plant samples using established protocols as described by Harbone (1998), Sofowora (1993) and Trease and Evans (1989).

A stock solution of each extract, with a concentration of 10 mg extract/mL distilled water, was prepared and used for the phytochemical screening.

2.3.1. Determination of Total Phenolic Content

The total phenolic content was determined using the Folin - Ciocalteau method as described by Cicco *et al.* (2009). Concentrations, ranging from 0.2 - 1 mg/mL of gallic acid or extracts, were prepared in methanol. Then, 4.5mL of distilled water was added to 0.5 mL of the extract and mixed with 0.5 mL of a ten-fold diluted Folin-Ciocalteau reagent. Five milliliters of 7% sodium carbonate was then added to the tubes and another 2mL of distilled water was added. The mixture was allowed to stand for 90 min at room temperature; absorbance was then read at 760 nm. All determinations were performed in triplicates with gallic acid utilized as the positive control. The total phenolic content was expressed as Gallic Acid Equivalent (GAE).

2.3.2. Determination of Total Flavonoid Content

The total flavonoid content was determined using the method of Miliauskas *et al.* (2004). Two milliliters of 2% $AlCl_3$ in ethanol was mixed with 2mL of varying concentrations of the extracts (0.1-1.0mg/mL), in methanol. The absorbance was measured at 420 nm after one hour incubation at room temperature. Similar concentrations of quercetin, the positive control were used. The total flavonoid content was calculated as mg quercetin equivalent /g of extract.

2.3.3. Determination of Proanthocyanidin Content

The determination of proanthocyanidin was carried out according to the method of Sun *et al.* (1998). To 0.5mL of 1.0mg/mL of each extract was added 1mL of 4 % methanol solution and 0.75mL of concentrated hydrochloric acid. The mixture was left undisturbed for 15 minutes and the absorbance was read at 500nm. Ascorbic acid was used as standard.

2.4. Estimation of Diphenyl-2-Picryl-Hydrazyl (DPPH) Radical Scavenging Activity

The free radical scavenging capacity of the leaf extracts against 1,1–diphenyl–2–picrylhydrazyl (DPPH) radical was determined by a slightly modified method of Brand-Williams *et al*. (1995). Briefly, 0.5 mL of 0.3 mM DPPH solution in methanol was added to 2mL of various concentrations (0.2 - 1.0 mg/mL) of the extracts. The reaction tubes were shaken and incubated for 15 min at room temperature in the dark; absorbance read at 517 nm. All tests were performed in triplicate. Ascorbic acid was used as standard control, with similar concentrations as the test samples prepared. A blank containing 0.5mL of 0.3 mM DPPH and 2mL methanol was prepared and treated as the test samples.

The radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = $[(A_0-A_1)/(A_0)] \times 100$,

where A_0 was the absorbance of DPPH radical + methanol; A_1 was the absorbance of DPPH radical + sample extract or standard. The 50% inhibitory concentration value (IC_{50}) was calculated as the effective concentration of the extract that is required to scavenge 50% of the DPPH free radicals.

2.5. Ferric Reducing Antioxidant Power (FRAP) Assay

The Ferric Reducing Antioxidant Power (FRAP) assay was carried out using a modified method of Benzie and Strain (1996). To 1.5 mL of freshly prepared FRAP solution (25 mL of 300 mM acetate buffer pH 3.6, 2.5 mL of 10mM 2,4,6-tripyridyls- triazine (TPTZ) in 40mM HCl, and 2.5 mL of 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$)solution) was added to 1mL of the extracts at concentrations of 0.1 - 1.0 mg/mL. The reaction mixtures were incubated at 37°C for 30 min and the increase in absorbance at 593nm was measured. $FeSO_4$ was used for the calibration curve and ascorbic acid served as the positive control. FRAP values (expressed as mg Fe (II)/g of the extract) for the extracts were then extrapolated from the standard curve.

2.6. Estimation of Thiobarbituric Acid Reactive Substances (TBARS)

TBARS was estimated according to the method of Ohkowa *et al*. (1979). Egg yolk homogenate (0.5 mL of 10% v/v) and 0.1 mL of extract were added to a test tube and made up to 1mL with distilled water. 0.05mL of $FeSO_4$ (0.07 M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 mL of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 mL of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulphate and 0.05 mL 20% TCA were added and the resulting mixture was vortex; it was then heated at 95ºC for 60 min. The generated color was measured at 532 nm.

Inhibition of lipid peroxidation (%) was calculated with the formula:

(C-E)/C x 100%;

where C is the absorbance value of the fully oxidized control and E is (Abs_{532}+TBA -$Abs_{532\text{-}TBA}$).

2.7. Statistical Analysis

All analyses were carried out in triplicate and the results were expressed as mean ± SEM. The data were subjected to one-way analysis of variance (ANOVA) where applicable. P values of < 0 .05 were regarded as significant.

3. Results

The results of phytochemical screening are shown in Table 1. More phytochemicals were detected in the *S. mombin* leaf extract than in the *P. longifolia* leaf extract. Alkaloids, flavonoids, tannins and reducing sugars were detected in both extracts while saponins, steroids, terpenoids and cardiac glycosides were detected in *S. mombin* extract only.

Figure 1 de picts the total phenol, flavonoid and proanthacyanidin content of extracts of *S. mombin and P. longifolia* leaf. *S. mombin* extract had a higher total phenol content (213.50 ± 1.25 mg gallic acid equivalent / g extract) than the *P. longifolia* extract(110.00 ± 10.00 mg gallic acid equivalent / g extract). However, the *P. longifolia* extract had higher total flavonoid and proanthocyanidin contents (98.58 ± 1.75 mg quercertin equivalent/ g extract and 47.25 ± 0.15 mg ascorbic acid equivalent / g extract, respectively) than the *S. mombin* counterpart (78.75 ± 0.42 mg quercertin equivalent/ g extract for total flavonoid and 20.00 ± 0.00 mg ascorbic acid equivalent / g extract for proanthocyanidin content).

The results of the DPPH radical scavenging activities of *S. mombin and P. longifolia* leaf extracts are shown in Figure 2 with the IC_{50} values in Table 2. The results show that at lower concentrations, (2 – 10 µg/mL) the *P. longifolia* extract was a better inhibitor of the DPPH radical than the *S. mombin* extract. Whereas, at higher concentrations (25 – 100 µg/mL), the *S. mombin* extract had a better DPPH radical scavenging activity than the *P. longifolia* extract. The IC_{50} values (Table 2) were144.89 µg/mL for the *S. mombin* extract and 139.72µg/mL for the *P. longifolia* extract in contrast to that of ascorbic acid (118.55µg/mL).

The Ferric Reducing Antioxidant Potential (FRAP) results are presented in Figure 3. The results revealed that the *S. mombin* extract had a significantly higher ($p < 0.05$) FRAP value (68.13 ± 2.13mg Fe(II)/0.05g extract) than the *P. Longifolia* extract(23.63 ± 1.88mg Fe(II)/0.05g extract).

Figure 4 s hows the thiobarbaturic acid reactive substances (TBARS) inhibitory activities of the *P. longifolia* and *S. mombin* extracts. The percentage inhibition of lipid peroxidation was significantly higher ($p < 0.05$) in the *S. mombin* extract (11.7 ± 0.00%) than in the *P. longifolia* extract (1.40 ± 0.01%).

Table 1. Phytochemical composition of methanol extracts of *P. longifolia* and *S. mombin* leaves.

Phytochemicals	*Polyalthia longifolia*	*Spondias mombin*
Cardiac glycosides	_	++
Terpenoids	_	+
Akaloids	++	+++
Reducing sugars	++	+++
Flavonoids	+	+
Tannins	++	+
Saponins	_	+
Steroids	_	++

KEY:+++ = Very highly detected; ++ = Highly detected; + = Less detected; – = Not detected

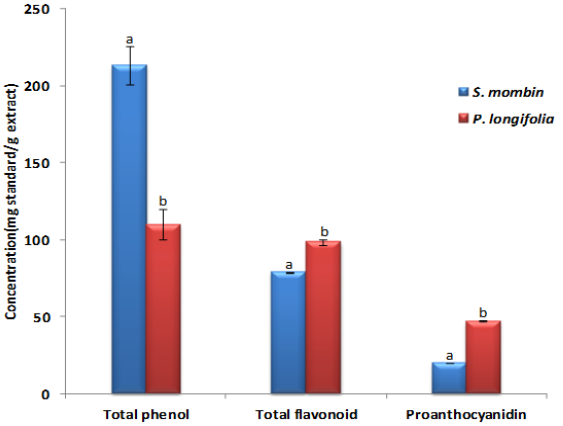

Figure 1. Total phenol, flavonoid and proanthocyanidin content of extracts of *S. mombin and P. longifolia* leaves. Total phenol is expressed as mg Gallic Acid Equivalent / g extract, Total flavonoid is expressed as mg Quercertin Equivalent / g extract and Proanthocyanidin content is expressed as mg Ascorbic acid Equivalent / g extract. Values are expressed as mean± SEM, *n* = 3/group. Different lowercase letters represent significant difference between means at $P < 0.05$.

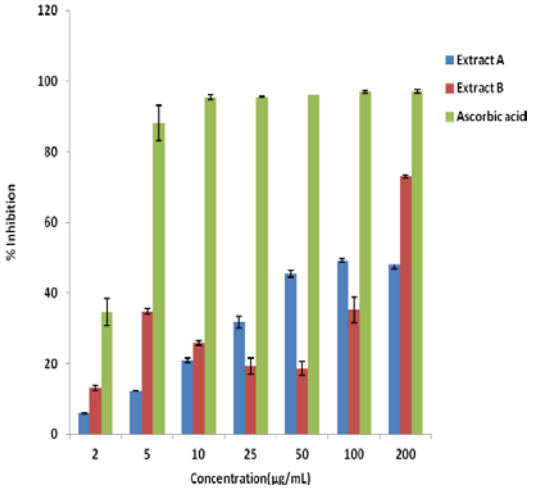

Figure 2. DPPH's radical scavenging activity of methanol extracts of *S. mombin* and *P. longifolia* leaves. Extract A= *S. mombin*; Extract B= *P. longifolia,* Values are expressed as mean± SEM, *n* = 3/group. Different lowercase letters represent significant difference between means at $P < 0.05$.

Table 2. IC_{50} values of extracts of *S. mombin and P. longifolia* leaves.

Plant	IC_{50} (µg/mL)
Ascorbic acid	118.55[a]
Spondias mombin	144.89[b]
Polyalthia longifolia	139.72[b]

Data represent mean ± SEM of triplicate analysis. Different lowercase letters within column indicate significant difference at $P \leq 0.05$.

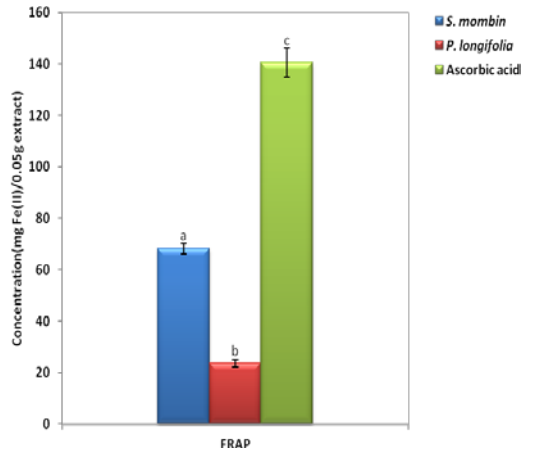

Figure 3. Ferric acid reducing antioxidant potential (FRAP) assessment of methanol extracts of *P. longifolia* and *S. mombin* leaves. Values are expressed as mean± SEM, n = 3/group. Different lowercase letters represent significant difference between means at $P < 0.05$.

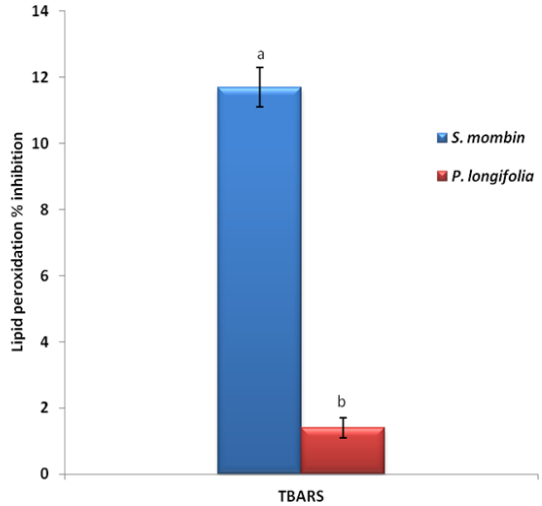

Figure 4. Thiobarbaturic Acid Reactive Substances (TBARS) of extracts of *S. mombin and P. longifolia* Values are expressed as mean± SEM, n = 3/group. Different lowercase letters represent significant difference between means at $P < 0.05$.

4. Discussion

The WHO describes a medicinal plant as any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or that are precursors for chemo-pharmaceutical semi synthesis. These non-nutrient plant chemical compounds or bioactive components often referred to as phytochemicals or phyto-constituents are responsible for protecting the plant against microbial infections or infestations by pests (Doughari, 2012). Screening plant materials for their phytochemicals is usually the first step in research protocol aimed at the isolation and the purification of natural compounds with bioactivities. It allows the researcher to see at a glance the phytochemicals present in a plant material and, hence, gives an idea of the potential use of the plant material (Oikeh *et al.,* 2013).

The result of the phytochemical screening of *S. mombin* leaf extract revealed the presence of several phytochemicals, viz saponins, flavonoids, terpenoids, cardiac glycosides, reducing sugars, alkaloids, tannins and steroids. These findings are consistent with those of Njoku and Akumefula (2007) who also detected the presence of some of these phytochemicals. Phytochemical screening of *P. longifolia* leaf extract revealed the presence of flavonoids, tannins, alkaloids and reducing sugars. Cardiac glycosides, steroids, terpenoids and saponins were not detected. These results corroborate, in part, with the findings of Saha *et al.* (2008). The differences between both research findings may be due to the different extracting solvents used.

Reactive Oxygen Species (ROS) are continuously produced *in vivo*. At low concentrations, they exert beneficial effects on cellular response and immune function, but at high levels, these radicals become toxic and disrupt the antioxidant defense system of the body, which may lead to oxidative stress (Jothy *et al*., 2012). Antioxidants scavenge free radicals in biological systems and hence, help in disease prevention. The antioxidant activity of plants is believed to be due to their phenolic compounds. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching reactive oxygen species (Jothy *et al.,* 2012). Phenolics are chemical components that occur ubiquitously in plants and play important roles in plant defense against pathogens and herbivore predators, hence their use in the control of human pathogenic infections. Flavonoid phenolics are a major group of plant phenolics with proanthocyanidins, being a sub group of flavonoids (Doughari, 2012). The proanthocyanidins (condensed tannis) are believed to possess potent antioxidant activities and exert several protect effects on humans (Gu *et al.,* 2003).

The *S. mombin* extract contained a h igher total phenolic content but a lower total flavonoid and proanthocyanidin content than the *P. longifolia* extract. The lower flavonoid content of the S. mombin extract may suggest that its total phenolic content is majorly of non-flavonoidal origin. Proanthocyanidins are condensed tannins formed as a result of coupling reactions between flavanyl units (Prakash and Gupta, 2009). The higher quantification of proanthocyanidins in the *P. longifolia* extract than in the *S. mombin* extract is consistent with the result of the phytochemical screening wherein tannins gave more intense coloration in the *P. longifolia* extract when compared with the *S. mombin* counterpart.

Antioxidants can deactivate radicals by three major mechanisms, namely via Hydrogen Atom Transfer (HAT), Electron Transfer (ET), and combination of both

HAT and ET (Jothy *et al*., 2012). HAT measures the ability of an antioxidant to quench free radicals by hydrogen donation, while ET detects the ability of antioxidant to transfer one electron to reduce radicals, metals and carbonyls. Ferric Reducing Antioxidant Power (FRAP) is an ET assay while DPPH assay combines both HAT and ET mechanisms (Jothy *et al*., 2012). As a rapid and simple measure of antioxidant activity, the DPPH radical scavenging capacity is based on the reduction of the stable radical DPPH to yellow colored diphenylpicrylhydrazine in the presence of a hydrogen donor (Jothy *et al.,* 2012). The IC_{50} values obtained from the extracts were relatively low and compared favorably with that of the ascorbic acid, the standard antioxidant. These plant extracts may thus have potent radical scavenging properties. The ferric reducing antioxidant potential test is based on the ability of antioxidants present in the test extracts to reduce Fe^{3+} to Fe^{2+}. The *S. mombin* extract was observed to have a s ignificantly higher (P<0.05) FRAP value than the *P. longifolia* extract. Both extracts however have significantly lower (P<0.05) FRAP values than the standard antioxidant.

Lipid peroxidation has been implicated in many diseases. The results on t he inhibition of lipid peroxidation revealed that the *S. mombin* extract had a significantly greater inhibitory effect on lipid peroxidation than the *P. longifolia* extract. Moreso, polyphenols are strong natural antioxidants that possess free radical scavenging activity (Prakash and Gupta, 2009). This may explain why the *S. mombin* extract, which was observed to contain a higher total phenolic content, is a better inhibitor of lipid peroxidation and a better ferric reducing antioxidant potential than the *P. longifolia* extract.

5. Conclusions

This study reveals that *S. mombin* contains higher amounts of the tested phyto-constituents than the *P. longifolia* extract. The *S. mombin* extract also contains a higher total phenolic content than the *P. longifolia* extract. These may be responsible for the better antioxidant properties observed, especially in the *S. mombin* extract which may be of importance in the drug discovery process.

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Phytosociological Analysis and Species Diversity of Herbaceous Layer in Rashad and Alabassia Localities, South Kordofan State, Sudan

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Abstract

The objective of the present study is to analyze the phytosociological characteristics and the diversity patterns of herbaceous plants in Rashad and Alabassia localities. The study was conducted in selected 14 vegetation sites. Important Value Index (IVI) was used to estimate the phytosociological characteristics, the Shannon index to measure the plant diversity and the Pielou index for species evenness. During the study period, a total of 48 species, representing 42 genera from 20 families, were recorded. The phytosociological characteristics revealed that *Tetrapogon cenchriformis* dominated herbaceous species in sites 1, 7, 10, 11 with IVI values 139.3, 113, 70.3 and 95.8, respectively, followed by *Spermacoce pusilla* dominating sites 3, 4, 5 and 6 with IVI values 65.1, 50.4, 104.2 and 133.5, respectively. The distribution pattern revealed that 87.5% species showed aggregated distribution, while 12.5% were randomly distributed. The highest density was 110 plants/m^2 recorded in site 4. Species richness varied through different sites; the highest number of species was 19, recorded in site 2. The highest values of Shannon diversity index and evenness index were in site 12. The highest similarity was recorded between site 9 and site 10 (73.39%) and the lowest (41.83%) between site 1 and site 3. The herbaceous flora of the area indicated its importance as one of the productive range region.

Keywords: Importance Value Index, Aggregation, Species Richness, Species Evenness.

1. Introduction

Phytosociology deals with plant communities, their composition and development, and the relationship between the species within them. A phytosociological system is a system for classifying these communities. The aim of phytosociology is to achieve a coefficient empirical model of vegetation using plant taxa combination that characterizes vegetation units. Phytosociology is useful to describe the population dynamics of each plant species occurring in a particular community and to understand how they relate to the other species in the same community (Mishra *et al.,* 2012). The herbaceous layer composition is changing continuously in space and time due to a multitude of factors, such as grazing, fire, and rainfall which differs in intensity and duration (Shameem, *et al.,* 2010). Maintaining or increasing the plant species diversity is an important goal of habitat managers in semi-arid environments (West, 1993; Fulbright, 1996).

Species diversity is an important property of communities because it is often related to their functioning and potential for change (Stachowicz *et al.,* 2007; Gamfeldt and Hillebrand, 2008). Diversity is a measure of how likely two randomly selected individuals in a community belong to different species. Thus, diversity is affected by two other properties of communities: richness, and evenness (Magurran, 1988; Krebs, 1999). Species richness is a biologically appropriate measure of alpha (α) diversity and is usually expressed as the number of species per sample unit (Whittaker, 1972). Evenness is the degree of similarity in abundance among the species (Krebs, 1999).

The study is carried out in Rashad and Alabassia localities, South Kordofan State, Sudan, which is part of the regions involved in the civil war in the country (The Nuba Mountains).The study area is characterized by a high diversification in vegetation cover components. According to Harrison and Jackson (1958), the vegetation of the area was classified as a low rainfall woodland savanna on clay and as special areas of the low rainfall wood land savanna under Hill catena's are divided into five zones, namely the rocky summit, the rocky steep slopes, the hard surfaced soils at the food of the steep slopes, a dark cracking clay plain surrounding the hill and the seasonal watercourses. Topography plays an important role in the formation of the soils of the study area. Soils

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suitable for cultivation of the basic food staples of the area are limited. They are divided broadly by local people into the HADABA which are fertile cracking clay soils of the plains (vertsol), GARDUD the sandy/clay pediment or transitional soils found at the foot of the mountains, KARKAR the rocky soils found in the mountains (Entisol or nonsol) which are shallow and confined to the mountainous areas, and Loamy alluvial soil deposits which are limited to seasonal streams and valleys (Harragin, 2003).

The Nuba Mountains are inhabited by more than 50 tribes composed of Nuba as well as a minority of cattle-raising Arabs (WFP, 2001). Agriculture is the main activity and is practiced by all the population. Nevertheless, its contribution to household food needs is declining (UNDP, 2003). The main crops cultivated are sorghum, maize, sesame, groundnuts, cowpeas and fruit trees (WFP, 2001). Nomadic pastoralism, ranking as the second major activity after agriculture in the area, is practiced by two nomad groups: the Baggara (cattle raisers) and the Abbala (camel raisers) (Bashir and El Tahir, 2006).

The aim of the present investigation is to analyze the phytosociological characteristics and the diversity pattern of the herbaceous plants of the area. The present study sheds light on the importance of the study area as one of the main pastoral resource in Sudan.

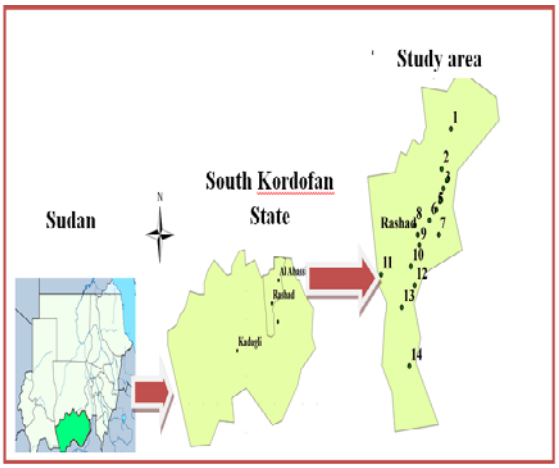
2. Materials and Methods

A field study was conducted in Rashad and Alabassia localities in the northern part of eastern Nuba Mountains of South Kordofan State extending from latitude 11° 33` to 12° 33` N and from longitude 31°08` to 31° 18` E (Map 1). The rainy season extends from mid-May to mid-October, with an annual rainfall ranging from 400 to 800 mm, allowing grazing and seasonal rain-fed agriculture (Starbase, 2003).

The study was conducted during the period from Oct. 2010 – Nov. 2011, covering both dry and wet seasons. 14 vegetation sites were selected (Map 1) on the basis of physiognomy, exposure and altitude representing vegetation variation (Table 1). The enumeration was carried out in 280 quadrate 50×50cm dimensions

In order to assess the dominance of species in the vegetation communities, density, frequency and abundance were converted to relative values and summed to obtain importance value index (IVI) following Dangoli and Shivakoti (2001) and Chaudhry *et al.* (2006). The species distribution profile was measured using Bio-Diversity Pro software (McAleece *et al.*, 1997). Species richness was determined as the total number of species present in the studied site; species diversity was measured using Shannon diversity index (H) after Shannon and Weaver (1963). Pielou index was used for the estimation of species evenness (E) after Pielou (1966).

Bray-Curtis (CN, quantitative version of Sorensen index) index was used to determine the degree of similarity in the species composition between the different sites (Magurran, 2004). The similarity dendrograms obtained from the results of cluster analysis were plotted. Data were analyzed using the program BioDiversity Pro (Version 2) (McAleece *et al*., 1997).



Map (1). study area

Table 1. Characteristic feature of the studied sites

Site	Location	Latitude N	Longitude E	Altitude M	Soil Type
1	Um Fakareen	12° 33` N	31° 18` E	500 m	Cracking clay
2	J. Damra	12° 10` N	31° 15` E	739 m	Rocky soil
3	Elmigreh	12° 02` N	31° 14` E	826 m	Rocky soil
4	J. Elmigreh	12° 00` N	31° 13` E	885 m	Rocky soil
5	Sug-Eljabal	11° 57` N	31° 12` E	914 m	Rocky soil
6	Elawai North Rashad	11° 52` N	31° 08` E	849 m	Rocky soil
7	Tabaldia	11° 50` N	31° 09` E	860 m	Rocky soil
8	Rashad Dam	11° 52` N	31° 02` E	894 m	Rocky soil
9	J. Rashad	11° 49` N	31° 03` E	852 m	Rocky soil
10	South Rashad	11° 45` N	31° 02` E	781 m	Rocky soil
11	Um Abdalla	11° 45` N	30° 52` E	664 m	*GARDUD* soil
12	Awai South Rashad	11° 43` N	31°03` E	723 m	Rocky soil
13	Tandek	11° 42` N	31°02` E	695 m	Cracking clay
14	Dibekkir	11° 33` N	31°08` E	618 m	Cracking clay

3. Results

A total of 48 species, representing 42 genera from 19 families, were recorded from the studied quadrates. Poaceae was the dominant family with 13 species, followed by Leguminosae (6), Malvaceae (4), Convolvulaceae and Euphorbiaceae (3 species each), Acanthaceae, Amaranthaceae, Lamiaceae, Solanaceae and Cyperaceae (2 species each), while the other 9 families were represented by only one species (Table 2). The herbaceous plants in the study area included 45 annuals and 3 perennials; most of them are economically important: 28 are fodder plants, 19 species are known to be used for medicinal purposes, 5 species are edible as

human food, while *Striga hermonthica* (Del.) Benth. parasitised the cultivated *Sorghum* spp.

Natural vegetation in a certain area reflects the interaction among plants, animals, soil types and the different climatic factors. The dominance of species was assigned based on the calculated IVI values. *Tetrapogon cenchriformis* was the dominant species in the studied sites with IVI 139.3 in site 1. It also dominated sites 7, 10 and 11 in different soil types with IVI values 113, 70.3 and 95.8, respectively. Its dominance at the specific sites indicated the availability of optimum conditions for its growth and ecological success. *Spermacoce pusilla* dominated sites 3, 4, 5 and 6 in rocky soil with IVI values 65.1, 50.4, 104.2 and 133.5, respectively. *Brachiaria xantholeuca* dominated sites 8 and 13 with IVI values 57.15 and 58.5, respectively. *Pennisetum pedicellatum* dominated site 2 and site 12 with IVI values 49.1 and 38.5, respectively, while *Setaria pumila* dominated site 14 with IVI 65.3 and *Zornia glochidiata* dominated site 9 with IVI 84.9. The aggregation (the distribution pattern) of plant species, in different habitats, was assessed; results revealed that 12.5% were randomly distributed and 85.5% were aggregated. (Table 2).

Figure 1 shows the different phytosociological parameters. The highest density of herbaceous plants was recorded in site 4 at 885 m a.s.l. (110 plants /m^2), followed by site 5, while site 13 at 695 m a.s.l. showed the lowest density (36 plant /m^2). The composition among the different sites in terms of species richness showed that the highest species diversity was observed in sites 1, 2, 4 and 12, sharing the same number of species (18); site 8 contained 16 species; sites 3, 6, and 11 contained 13 species; sites 7, 10 and 14 contained 12 species; sites 5 and 13 each contained 11 species, while the lowest species richness was 9 species recorded in site 9. The highest Shannon diversity index was 2.45 in site 12, followed by 2.19 in site 8, whereas the least Shannon diversity index was 1.21 in site 6. The highest species evenness index was recorded in site 12 (1.94), whereas the least evenness index was 1.09 in site 6.

The highest similarity (73.39%) was recorded between Jebel Rashad (site 9) at 852 m a.s.l and South Rashad (site 10) at 781 m a.s.l. On the other hand, Um Fakareen (site 1) at 500m. a.s.l. in clay plain m a.s.l and Elmigreh (site 3) 826 m. a.s.l. in rocky soil showed the least similarity (41.83%) (Figure 2).

Table 2. Importance value index (IVI) and distribution pattern of plant species

plant species			Values of Importance value index (IVI)														Aggregation
	Life cycle	Economic importance	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10	Site 11	Site 12	Site 13	Site 14	(distribution pattern)
Poaceae																	
Aristida hordeacea Kunth.	Ann.	Fd	85.1	-	-	-	41.1	20.74	-	-	-	-	-	-	6.22	-	Aggregated
Aristida mutabilis Trin. & Rupr.	Ann.	Fd	-	-	-	22.8	13.6	20.74	-	13.92	6.05	7.39	12.49	-	-	-	Aggregated
Brachiaria xantholeuca (Schinz) Stapf.	Ann.	Fd	59.01	42.0	-	42.9	10.7	20.67	-	57.15	10.5	6.36	24.09	-	58.5	-	Aggregated
Chloris pilosa Schumach. & Thonn.	Ann.	Fd	-	-	-	8.39	6.61	-	-	-	-	-	-	-	33.5	-	Aggregated
Cymbopogon nervatus (Hochst.) Chiov.	Ann.	Fd	-	-	-	-	16.7	-	-	-	-	-	-	-	-	-	Aggregated
Dactyloctenium aegyptium (L.) Willd.	Ann.	Fd	22.08	13.7	-	11.42	7.74	-	15.4	19.48	38.8	45.1	22	32.7	19.02	-	Aggregated
Echinochloa rotundiflora Clayton.	Ann.	Fd	-	-	-	4.66	-	-	-	-	-	-	-	-	-	9.18	Aggregated
Hyparrhenia confinis var. *nudiglumis* (Hack.) Clayton	Ann.	Fd	-	-	-	20.4	-	-	-	30.1	-	-	-	-	-	-	Random
Microchloa indica (L.f.) P.Beauv.,	Ann.	Fd	-	-	-	27.6	-	22.37	33.1	38.8	39.1	-	-	16.6	-	-	Aggregated
Pennisetum pedicellatum Trin.	Ann.	Fd	43.5	49.1	19.2	20.1	6.87	-	-	36.96	15.97	17	5.73	38.5	-	8.69	Aggregated
Setaria pumila (Poir.) Roem. & Schult.	Ann.	Fd	24.2	-	-	4.27	-	-	-	8.5	-	-	5.84	6.8	9.09	65.3	Aggregated
Tetrapogon cenchriformis (A. Rich.)W. D. Clyton.	Ann.	Fd	139.3	30.02	24.5	42.7	57.7	19.22	113	19.5	60.1	70.3	95.8	28.9	55.2	34.3	Aggregated
Tetrapogon tenellus (Roxb.) Chiov.	Ann.	Fd	8.87	-	-	-	-	-	-	-	-	-	-	-	-	-	Aggregated

Leguminosae																	
Alysicarpus glumaceus (Vahl) DC.	Ann.	Fd	-	-	-	-	-	-	-	19.14	25.1	28	18.24	-	-	-	Random
Indigofera hochstetteri Baker	Ann.	Fd	-	26.3	12.1	4.66	13.6	10.62	15.4	8.9	-	8.25	53.27	15.5	-	7.2	Aggregated
Indigofera nummulariifolia (L.) Livera	Ann.	Fd	-	-	-	-	-	13.14	-	-	-	-	-	-	-	-	Aggregated
Indigofera spicata Forssk.	Per.	Fd	-	3.52	-	-	-	-	-	5.91	-	-	27.64	-	-	-	Random
Senna obtusifolia (L.) H.S.Irwin & Barneby	Ann.	E	14.3	8.69	57.03	-	-	-	4.84	3.14	-	4.47	3.77	13.4	6.22	5.16	Aggregated
Zornia glochidiata DC.	Ann.	Fd	-	16.7	25.5	7.08	16.1	45.99	28.3	14.5	84.9	53.4	21.57	12.4	-	-	Aggregated
Malvaceae																	
Corchorus tridens L.	Ann.	E	25.6	-	-	-	-	20.74	4.84	-	-	-	-	-	-	-	Aggregated
Triumfetta pentandra J.M. Garg	Ann.	Fd ,E, M	-	6.96	-	-	-	-	-	-	-	-	-	30.3	-	44.8	Aggregated
Hibiscus diversifolius Jacq.		Fb, M	-	3.52	-	-	-	-	-	-	-	-	-	-	-	-	Random
Sida alba L.	Per.	M	7.67	-	7.61	4.66	-	-	-	3.14	-	-	-	31.9	6.22	39.96	Aggregated
Convolvulaceae																	
Astripomoea lachnosperma (Choisy) A. Meeuse	Ann.	M	-	22.18	-	-	-	-	-	-	-	-	-	-	-	-	Aggregated
Ipomoea cordofana Choisy.	Ann.	Fd	-	-	-	-	-	-	-	-	-	-	-	-	-	11.2	Aggregated
Ipomoea sinensis (Desr.) Choisy.	Ann.	Fd	-	-	-	-	-	-	-	-	-	-	5.84	-	-	-	Aggregated
Euphorbiaceae																	
Acalypha indica L.	Ann.	M	22.6	-	-	-	-	-	-	-	-	-	-	-	-	-	Aggregated
Dalechampia scandens L. var. *cordofana* (Webb) Müll	Ann.	Fd	-	5.24	-	-	-	-	-	-	-	-	-	-	-	-	Random
Euphorbia hirta L.	Ann.	M	8.87	-	10.6	2.9	-	-	8.01	8.99	-	-	-	5.44	-	-	Aggregated
Acanthaceae																	
Blepharis linariifolia Pers.	Ann.	Fd	7.67	-	-	-	-	-	-	-	-	-	-	-	-	-	Aggregated
Peristrophe paniculata (Forssk.) Brum.	Ann.	M, Fd	-	-	8.65	-	-	20.74	-	-	-	-	-	-	-	-	Aggregated
Amaranthaceae																	
Amaranthus hybridus L.	Ann.	Fd	33.7	-	49.8	-	-	-	-	-	-	-	-	-	-	8.69	Random
Achyranthes aspera L.	Ann.	M	-	3.52	-	-	-	-	-	-	-	-	-	-	-	-	Aggregated
Lamiaceae																	
Leucas martinicensis R. Br.	Ann.	M	-	-	5.53	5.65	-	-	-	-	-	-	-	5.4	-	-	Aggregated
Ocimum americanum L.	Ann.	M	12.7	-	-	-	-	-	-	-	-		-	8.3	-	-	Aggregated
Solanaceae																	
Physalis peruviana L.	Ann.	M	-	-	9.01	-	-	-	-	-	-	-	-	-	-	-	Aggregated
Solanum incanum L.	Per.	M	25.6	-	5.43	8.29	-	-	-	-	-	-	-	5.99	-	-	Aggregated
Cyperaceae																	
Cyperus amabilis L.	Ann.	M	-	-	-	-	-	-	-	11.91	-	-	-	-	-	-	Aggregated
Cyperus rotundus L.	Ann.	M	-	-	-	-	-	-	2.97	-	-	-	-	-	-	-	Aggregated
Rubiaceae																	
Spermacoce pusilla Wall.	Ann.	M	21.7	11.5	65.1	50.4	104.2	133.5	76.9	-	19.16	44.0	-	14.9	43.7	60.4	Aggregated
Asteraceae																	
Acanthospermum hispidum DC.	Ann.	M		47.6	-	-	-	20.74	12.9	-	-	-	3.77	23.3	-	-	Aggregated
Nyctaginaceae																	
Boerhavia erecta L.	Ann.	Fd	-	-	-	-	-	12.53	-	-	-	11.3	-	-	-	-	Aggregated
Cleomaceae																	
Cleome gynandra L.	Ann.	E	-	5.68	-	-	-	-	-	-	-	-	-	5.99	-	-	Aggregated

Aristolochiaceae																		
Aristolochia bracteolata Lam.	Ann.	M	7.76	-	-	-	-	-	-	-	-	-	-	-	-	-	Aggregated
Commelinaceae																		
Commelina imberbis Ehrenb. ex Hassk.	Ann.	Fd	-	12.1	-	-	-	-	-	-	-	-	-	3.68	-	-	Aggregated
Cucurbitaceae																		
Cucumis prophetarum L.	Ann.	M	-	3.52		-	-	-	-	-	-	-	-	-	-	5.16	Aggregated
Araceae																		
Stylochaeton hypogaeus Lepr	Ann.	M,E	-	-	-	11.1	-	-	14.3	-	-	4.5	-	-	6.22	-	Aggregated
Scrophulariaceae																		
Striga hermonthica (Del.) Benth.	Ann.	(Parasitic)	-	-	-	-	-	-	-	-	-	-	-	-	10.1	-	Aggregated

Ann.= annual, per.=perennial, M= medicinal, Fd= fodder, E= edible

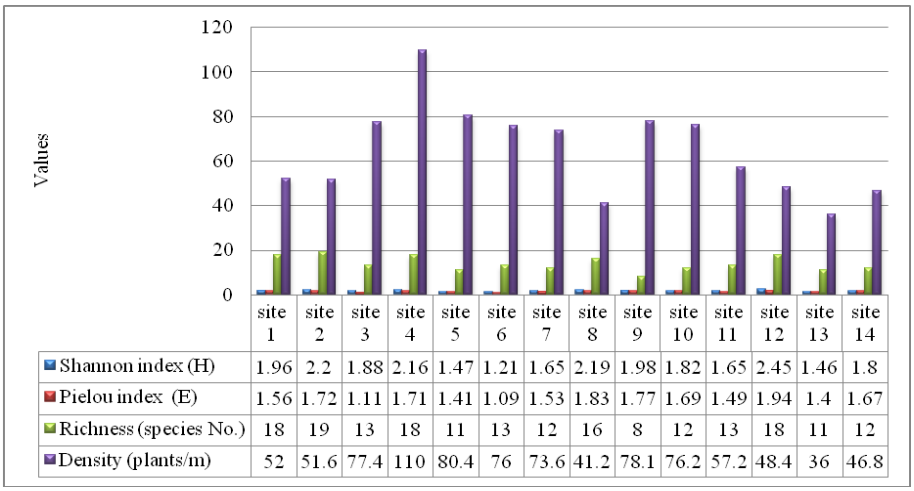


Figure 1. Species richness, diversity indices and density of herbaceous layer.

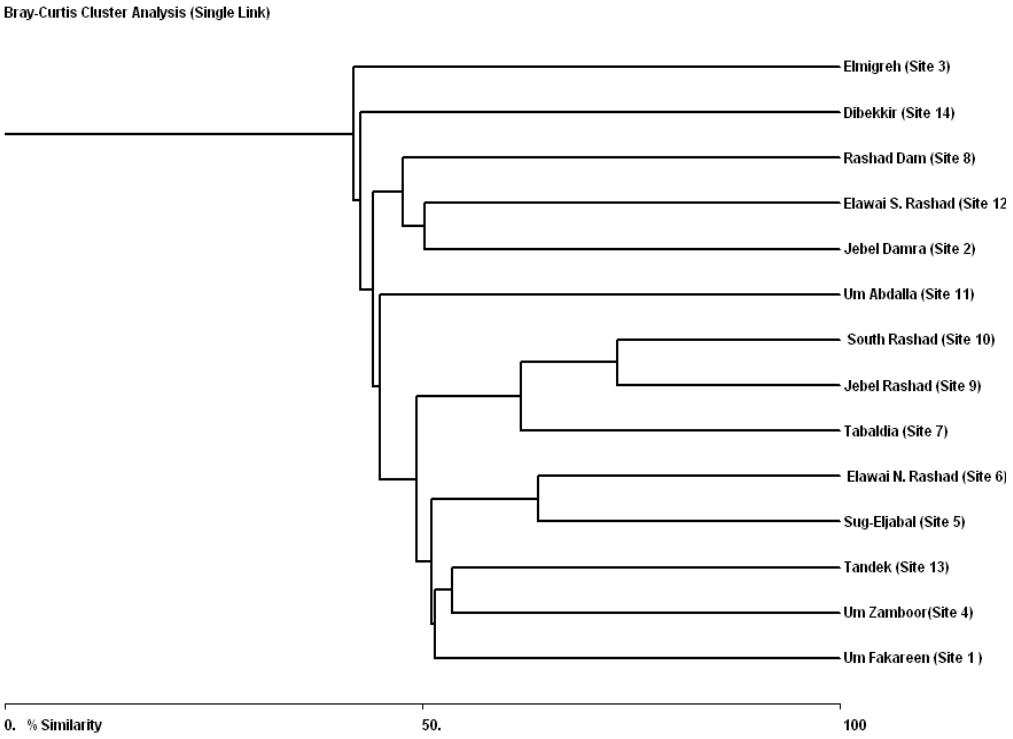


Figure 2. Similarity between different sites of study area, using Bray-Curtis (CN, quantitative version of Sorensen index) index

4. Discussion

According to Bhandari *et al.* (1999), any species in a community plays a specific role and there is a definite quantitative relationship between abundant and rare species. The differences in IVI may be due to the changes in the surrounding conditions and the anthropogenic activities around the sites. The dominance of *Pennisetum pedicellatum* in site 2 is in agreement with Harrison and Jackson (1958) who reported its presence in the rocky steep slopes and the seasonal watercourses. Most of the dominant species in the studied sites are members of the Poaceae family which is known for producing a large number of seeds for sexual reproduction and possessing different means for vegetative reproduction. The dominance of *Tetrapogon cenchriformis* and *Spermacoce pusilla* may be attributed to the fact that these two species are important fodder plants in the study area located in one of the important pastoralist routes in Sudan, which facilitates the dispersal of their seeds; grazing is known to activate the vegetative buds and increase the growth of some range plants, especially grasses.

Aggregation of plant species results (12.5% randomly distributed and 85.5% aggregated) indicate the suitability of these habitats for the aggregated species. The results are in line with Das *et al.* (2012) who stated that the aggregated distribution indicated the habitat preference, while the random distribution indicates that the environment in which these plant species grow is homogeneous and has many factors acting on the population (Ewusie, 1980).

According to Wilsey and Stirling (2007), richness and evenness can be negatively related across the plant communities, and evenness can account for more variation in Shannon's diversity index (H) than richness, which suggests that relationships among the diversity components can be complex. Generally, a strong correlation between species richness, evenness and productivity was not evident in the present study. The differences in the phytosociological parameters may be attributed to different biotic and/or abiotic factors other than soil and elevation.

The highest similarity (73.39%), recorded between site 9 and site 10, may be attributed to the fact that the two sites were characterized by similar soils and relatively close elevations. Site 1 in clay plain and site 3 in rocky soil showed the least similarity (41.83%), this may be due to the differences between the two sites in terms of elevations and soil types.

5. Conclusions

The herbaceous cover diversity of the studied sites was represented by 48 plant species belonging to 41 genera under 19 families. While the dominant family Poaceae is represented with 13 species, 9 families are monotypic. *Tetrapogon cenchriformis* showed the maximum IVI values at 4 sites and also *Spermacoce pusilla* dominated 4 sites. The number of species in the studied sites was in the range of 8-19 and most of them can be considered as fodder plants, which indicated that the area is productive.

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Anti-Urolithiatic and Anti-Oxidant Effects of Fenugreek on Ethylene Glycol-Induced Kidney Calculi in Rats

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Abstract

Renal calculi formation is one of the most common urological disorders. Urinary stone disease is a common disease which affects 10-12% of the population in industrialized countries. The objective of the present study is to investigate the antiurolithiatic and antioxidant activity of fenugreek on ethylene glycol-induced urolithiasis in rats. Twenty male rats weighing 203-263 were used for this study. Group A animals received distilled water for 28 days. Group B to group D animals received 1% v/v ethylene glycol in distilled water for 28 days. Groups C and D received cystone and fenugreek, respectively, from day 15 to day 28. On day 28, blood was collected for serum malondialdehyde (MDA) and calcium level monitoring. Kidneys of rats from all the groups were removed, and histopathologically examined using Paraffin method. Samples of kidney were viewed under a scanning electron microscope. Histologically, the kidney of fenugreek treated group appeared mostly to be calculi-free, even better than the cystone treated group. Similarly, the calcium oxalate deposits, the serum (MDA), the renal tissue calcium content were all significantly lower than those in the other groups. The results of the present study suggest that fenugreek has a strong antiurolithiatic and antioxidant activity.

Keywords: Antilithiatic, Fenugreek, Nephrolithiasis, Urolithiasis, Renal Calculi, Ethylene Glycol, Cystone.

1. Introduction

Kidney stone disease is a common disorder that has been on the rise in Western societies for the last five decades. Kidney stone formation is a complex process and it results as a cascade of events, including crystal nucleation, growth and aggregation, crystal retention within the renal tubules (Coe and Parks, 1983). Usually kidney stones are yellow or brown color with a smooth or gagged structure. Some common type of kidney stones are calcium oxalate, calcium phosphate, struvite, uric acid and cysteine, among of which calcium stones are the most common form of kidney stones in both humans and rats (Sunitha *et al.,* 2012). Urolithiasis, also called calculi or uroliths, is a condition which involves the process of stone formation in the kidney. Renal stones are a universal cause of blood in the urine and pain in the abdomen, with a reported incidence about 12% in the general population (Araújo Viel *et al*., 1999; Kumar *et al.,* 2005).

Fenugreek is one of several herbal medicines whose seeds and leaves are used either as food or as an ingredient in folk medicine (Bellakhdar, 1997). Fenugreek leaves and seeds are consumed in different countries around the world for various purposes, such as medicinal uses, e.g., as an anti-diabetic, for lowering blood sugar and cholesterol levels (Lafta, 2010), as an anti-cancer and anti-microbial; it is also used in food and drinks in many Eastern and Western countries. Fenugreek can be a very useful legume crop for the incorporation into a short-term rotation and for hay and silage for the livestock feed, for the fixation of nitrogen in soil and its fertility (Sadeghzadeh-Ahari, 2009). The fenugreek seeds have been proven to have both hypoglycaemic and anticholesterolemic properties (Sharma, 1986 a; b). It can attenuate oxidative stress indirectly by reducing the lipid peroxidation (Shekha *et al.,* 2014). Calcium oxalate urolithiasis model has commonly been used to investigate the influence of urolithiasis on experimental model in rats. This model is induced by ethylene glycol (EG), a precursor to oxalate formation (Bayir *et al.,* 2011). EG poisoning can lead to acute renal failure which is characterized by a proximal tubular necrosis and an accumulation of calcium oxalate monohydrate crystals in the urine and kidney tissue. The prices mechanism is probably due to the calcium oxalate monohydrate's adherence to tubular cells primary hyperoxaluria and kidney stone formation (McMartin, 2009). Many studies indicated that the Cystone® has a potent anti-lithiatic (prevents the formation of kidney stones) and lithotriptic (dissolves kidney stones) properties by decreasing urinary supersaturation or micropulverizes and diuretic that flushes out small kidney stones (Karamakar and Patki, 2010; Kumaran and Patki, 2011). On the other hand, it was also reported that malondialdehyde production

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increased in the presence of oxalate and stone forming since oxalate in the kidney induces cell death mediated by a cellular necrosis because it induces changes in the membrane integrity, cellular enzyme release and membrane lipid peroxidation (Park *et al.,* 2008). In fact, several of the published studies reported that serum calcium did not change in ethylene glycol induced kidney stone in rats (Laroubi *et al*., 2007; Pareta *et al.,* 2011), while other studies reported an increased in serum calcium (Jafar *et al.,* 2011); sometimes decreased in serum calcium (Soundararajan *et al.,* 2006). Some of the therapeutic uses of fenugreek include its use as an anti-urolithiasis (Laroubi *et al.,* 2007). Fenugreek seeds have been used by traditional medicine for problems of kidney (Snehlata and Payal, 2012). The mechanism underlying the anti-urolithiasis effect is still unknown, but apparently it is related to increased diuresis, antioxidant activity and lowered urinary concentrations of stone forming constituents (Laroubi *et al*., 2007).

The present study is designed to investigate the anti-urolithiatic activity of fenugreek and its effect on kidney abnormalities induced by ethylene glycol in rats.

2. Materials and Methods

2.1. Plant Material

Trigonella foenum graecum L. seeds were purchased from a local market, Erbil city, Kurdistan, Iraq. A voucher specimen was deposited at the Herbarium of Department of Biology, College of Science.

2.2. Animal and Treatment

Male Wistar albino rats (203 to 263 g) were obtained from the Animal House, College of Science, University of Salahaddin, Kurdistan region of Iraq. Twenty Wistar rats, maintained for ten days under experimental conditions, were divided equally into four groups, each of five animals.

All animals had a free access to drinking water ad libitum and regular food, and they were kept under controlled conditions.

Hyperoxalurea and CaOx deposition in the kidney was induced by adding Ethylene Glycol (EG) to the drinking water to a final concentration of 1% for all groups except for the control group (A) which was supplied with normal water and diet. Group (B) received drinking water supplemented with EG (1%) for 28 days. Group (C) was given 2.5 tablets of Cystone in 100 ml of water and 2.5 tablets in 100 g of standard diet+ 1% EG, while group (D) was given 10 gm of fenugreek in 100 ml of water and 10 gm in 100 gm of standard diet+ 1% EG. At the end of the experiment, the serum (MDA) and calcium were determined.

2.3. Determination of Serum Malondialdehyde (MDA)

The level of serum (MDA) was determined spectrophotometrically with a thiobarbituric acid (TBA) solution. In brief, to a 150µl serum sample following were added: 1ml of trichloroacetic acid (TCA) 17.5 % with 1ml of 0.66% TBA were mixed well by vortex, incubated in boiling water for 15 minutes, then allowed to cool. One ml of 70 % TCA was added and the mixture was left to stand at room temperature for 20 minutes, centrifuged at 2000 rpm for 15minutes, then the supernatant was taken out for scanning spectrophotometrically. The concentration of (MDA) was calculated as follows:

MDA= (AxD)/(LxE)

(MDA) (µmol/L) = Absorbance at 532 nm X D/ L x Eo,

where L: light bath (1cm)

Eo: Extinction coefficient 1.56 x 105 M-1.Cm-1

D: Dilution factor = 1 ml Vol. used in ref. / 0.15 =6.7(Burtis and Ashwood, 2005)

2.4. Paraffin Method

Kidney pieces were removed and fixed in Bouin's fluid, dehydrated, cleared, embedded in paraffin and cut into 4-5µm thick section, then stained by hematoxylin and eosin (Kierrnan, 1981).

2.5. Scanning Electron Microscopy

Kidneys were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2-7.4 for 24 hours. After being washed by cacodylate buffer 0.1M, they were postfixed in 1% Osmium tetroxide for two hours, and dehydrated in ethanol (50%, 70%, 85%, 100% and 100%). Then the samples were put in desiccator for air drying, after mounting they coated with gold by coating system (E5200 AUTO SPUTTER COATER) and then examined by SEM in Malaysia (ZEISS, super A, 55VP) (Rasul and Aziz, 2012).

3. Results and Discussion

. The histological structure of the ethylene glycol treated rat kidney showed a lot of alterations compared with the normal structure of control kidney (Figures 1-3). As seen in Figure 2, highly inflammated regions were seen in the kidney of the EG treated rat kidney in which large numbers of leukocytes forming large foci occupied the cortical region. Furthermore, a kidney tubule lumen widening was obvious. The high number of kidney tubules filled with stone crystals is shown in Figure 2b. The crystals caused a further widening of the tubules. The crystals appeared colorless. Further occurrence of crystals within the tubulesis is shown in Figures 3a-d. In Figure 3c, the dilatation of kidney tubules and the deposition of crystals caused a compression on the glomerulus. The scanning electron images showed the crystals more clearly (Figures 3e and f).

With respect to the rats exposed to EG plus cystone, still dilatation of kidney tubules and the occurrence of tubules containing crystals were detected but with a lower density compared with EG treated group. Infiltration of inflammatory leukocytes also appeared in this group (Figure 4).

Treating the rats with fenugreek in addition to EG showed a disappearance of the crystals, and the normal kidney tubules structure was approximately similar to the control group (Figure 5).

As seen in Figure 6, a significant elevation in MDA (µmol/L) levels resulted after 28 days of EG administration (36.67±1.188) compared to control group (4.804±0.3053). Fenugreek and cystone significantly diminished (P<0.0001) the levels of serum (MDA)

(8.180±1.125, 8.484 ±1.023, respectively). Malon-dialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes an overproduction of the MDA. Malondialdehyde level is commonly known as a marker of oxidative stress (Gawel *et al.,* 2004). It seems that fenugreek plays an antioxidant role against oxidative stress that is induced by the ethylene glycol, since the MDA level was elevated in this group. However, serum calcium did not show a significant change (Figure7).

Several experiments suggested the primary contribution of the increased production of ROS in ethylene glycol group (Green *et al.,* 2005), cystone treatment may lead to an increase in the citrate concentration which might have reduced the crystallization of calcium oxalate (Ruckmani *et al.,* 1998).

The present investigation showed a quite disappearance of stone crystals in the EG treated rats after being exposed to fenugreek extract compared to the EG treated group in such a way that exceeds the anti-urolithial effect of cystone. In addition to the disappearance of kidney stone in the fenugreek plus EG treated group, no inflammation was noticed and much less cell degeneration was detected; this may be due to the antioxidant effect of this plant (Shekha *et al.,* 2014), since the oxidative stress is an important cause of cell death, especially the necrotic mode of cell death (Choi *et al.,* 2009; Hanus *et al*., 2013) which is accompanied by infiltration of inflammatory leukocytes(Zitvogel *et al.,* 2010).

The extract of some plant leaves treatment suppresses the increase of the intracellular calcium. The exact reason of this effect is not clear; however it might be due to the increased bioavailability of NO (nitric oxide) which, in turn, activates cGMP (3,5 cyclic guanosine monophosphate) that controls the increase of the intracellular calcium levels (Makasana *et al.,* 2014). Nitric oxide donors have the capacity to control the intracellular rise in the calcium levels. Thus, the plant extract could effectively control the levels of both the salts by the mechanism, such as inhibiting the oxalate or increasing the bioavailability of NO to sequester calcium through the cGMP pathway (Pragasam *et al.,* 2005).

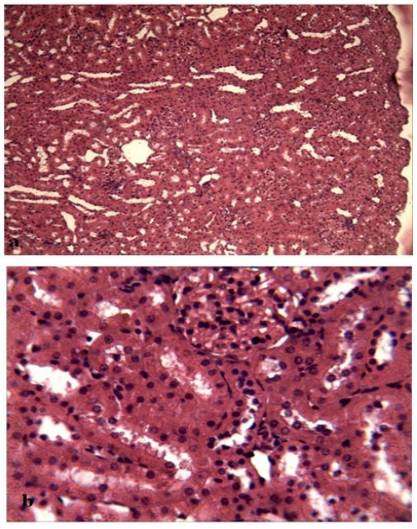

Figure 1. Section through rat kidney in control group, a) lower power, 100X, b) high power, 400X.Both images show the normal structure of the kidney.

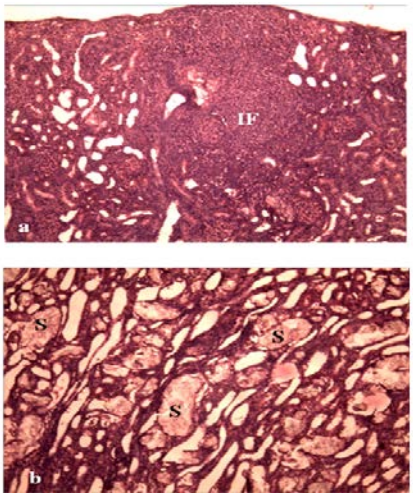

Figure 2 . Sections through the kidney of rat exposed to ethylene glycol showinga) accumulation of high number of inflammatory blood cell infiltration (IF), 100X, b) the high number of the kidney tubules filled with stone crystals (S), 100X. Both images show kidney tubule dilation.

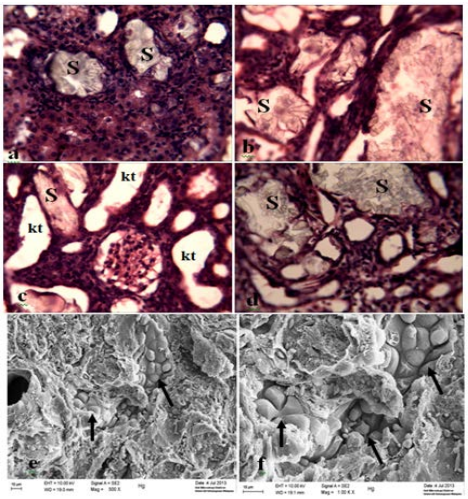

Figure 3. Ethylene glycol treated rat kidney showing the appearance of the crystals within the ratkidney tubules (S), inflammatory leucocytes (IF) and tubule dilation (kt), a-d) paraffin section, 400X, e, and f) scanning electron microscopy showing the crystal in the tubules (arrows).

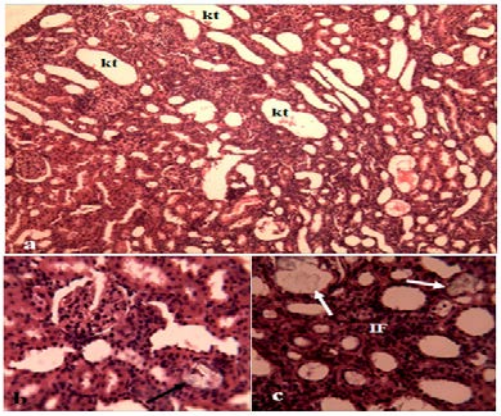

Figure 4 . Sections through the kidney of rat exposed to cystone plus ethylene glycol, still few crystals deposition (arrows) are seen in addition to inflammatory cells(IF) and the kidney tubule dilatation(kt) , a) 100X and c)400X.

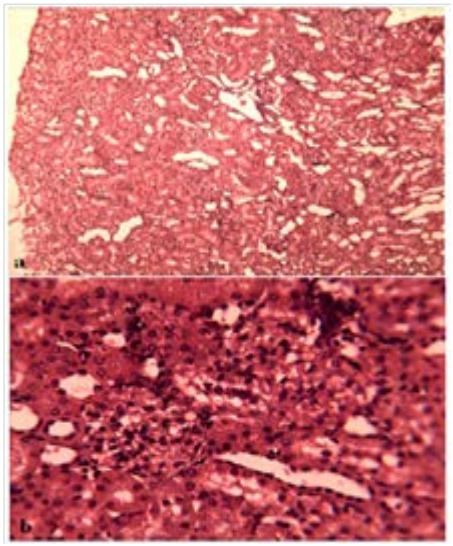

Figure 5 . Sections through ethylene glycol plus fenugreek treated rat kidney showing normal histological structure, a) 100X and c)400X.

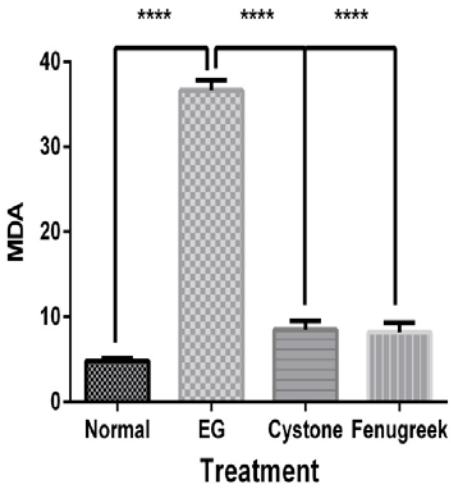

Figure 6. The effect of Fenugreek seeds on (MDA) of rats. Each column and vertical bar represents mean ± SEM of 5 animals.

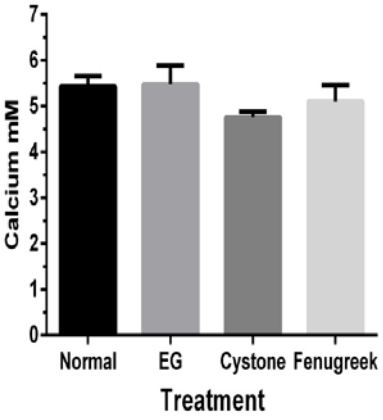

Figure 7. The effect of Fenugreek seeds on serum calcium of rats. Each column and vertical bar represents mean ± SEM of 5 animals.

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المجلة الأردنية للعلوم الحياتية

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تصدر بدعم من صندوق دعم البحث العلمي

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