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Preface

Now commencing its seventh year, Jordan Journal of Biological Sciences (JJBS) will continue to provide biologists with first class research articles, review articles and short communications in various disciplines and frontiers of Biological Sciences. Here, I ask active researchers from all over the world to consider JJBS as one of their first choices for submission to publish their data. JJBS is now indexed with and included in DOAJ, EBSCO, CABI, HINARI, Google Scholar, Chemical Abstract Service, Zoological Abstract, Ulrich's, Index Copernicus International, ISC, Directory of Research Journal Indexing (DRJI) and others. Moreover, the journal is under the indexing process with ISI and Scopus. As always submitted research articles will receive fair and constructive comments by peer reviewers and worthwhile articles will get published. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscript or not, is the mentoring nature of our review process. JJBS provides authors with high quality, helpful notes and comments to assist authors in improving their manuscripts.

Moreover, the Editorial Board of JJBS are very much interested in publishing significant review articles that outline and discuss current hot topics in the frontiers of Biological Sciences. P utting such topics in perspective and fitting pertinent data together is of utmost importance in guiding future research and helping new scholars in the field to address important and pertinent issues. I encourage experts in various fields of Biological Sciences who wish to review certain front line topics in their specialties to contact me if they wish to contribute one or more review articles. In this way, the Editorial Board hopes to include at least one major or mini review in each journal issue as of March 2014.

As in prior two years, this seventh volume of JJBS will include four issues with at least twelve articles in each issue. In the coming year, it is my vision to have JJBS publishes more outstanding articles from distinguished scholars in various areas of Biological Sciences. In addition, I will be working on the inclusion of JJBS in Scopus, ISI and other international information retrieval services, which will lead to a good impact number.

Again, I must congratulate and thank all the researchers who contributed to research and review articles published in previous issues of JJBS during the past six years.

Also, I thank my esteemed reviewers of previous articles submitted to the journal. They are assurance of high quality of published research work. To all our former contributors and potential new ones, I welcome further manuscripts for submission. Your manuscripts will receive careful consideration to maintain a high quality publication in JJBS.

I would like to thank the JJBS International Advisory board members for their continuous support. Furthermore, I would like to thank the JJBS Editorial board members for their exceptional work and continuous support to JJBS. Finally, I very much appreciate the support of The Hashemite University and Jordanian Scientific Research Support Fund for their continuous support to JJBS.

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Jordan Journal of Biological Sciences **Review**

Phytochemistry, Pharmacological Properties and Industrial Applications of *Rhus coriaria* L. (Sumac)

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Abstract

Rhus coriaria L. (Sumac), belonging to the Anacardiaceae family, is an important species and is the most used species of the genus *Rhus* in the Mediterranean region since antiquity. Sumac has long been used as a flavoring spice, drink, appetizer, and as acidulant in food recipes, in addition to its use in traditional medicine. The role of the plant in leather and textile industry is also significant. *R. coriaria* is very rich in phenolics mainly and tannins as well as flavonoids; let alone its abundance with organic acids. The leaves and fruits of *R. coriaria* are recognized to have defensive and beneficial effects on a wide set of diseases, including, but not limited to, diabetes mellitus, cancer, stroke, oral-diseases, inflammation, diarrhea, and dysentery. On the other hand, Sumac extracts were found to possess a potential antiviral, antimicrobial, antifungal, antioxidant and hypolipidemic activities. This review updates the current phytochemical, biological and therapeutic knowledge that so far exists on *R. coriaria.* It also aims at highlighting the importance of Sumac extracts as a promising and potential source of functional ingredients and nutraceuticals with desirable bioactivities, prompting the further use of Sumac in food preservation, pharmacology and functional food industries.

Keywords: *Rhus Coriaria* L. (Sumac); Anacardiaceae; Pharmacology; Phytochemistry; Antioxidant, Antimicrobial, Tannins; Organic Acids.

1. Introduction

Rhus coriaria L. (Tanner's Sumac or Sicilian Sumac) (Figure 1) grows wild mainly in the Mediterranean bordering countries, South Europe, North Africa, Iran and Afghanistan (Nasar-Abbas and Halkman, 2004). The plant is also originated in temperate and tropical regions worldwide, often growing in areas of marginal agricultural capacity. Sumac is the common name of the *Rhus* genus, which comprises 91 of accepted species names in the Anacardiaceae (The plant list 2010). The name "Sumac" comes from "summāq" which means "dark red" in Arabic and Syriac (Quattrocchi, 1999). *Rhus coriaria* has been used in spice blends and in traditional medicines for hundreds of years (Ali-Shtayeh *et al.*, 2008). The word "sumac" will be henceforth used to indicate the spice product of *R. coriaria*.

Sumac has long been used as a seasoning spice, either in pure form or in combination with other spices, as a drink, appetizer, sauce, and also as a natural acidulant in food recipes (Abu-Reidah *et al.*, 2014). It is worth noting that *R. coriaria* has an attractive economic importance due to its increasing use in cosmetic and pharmaceutical industries, coloring or preservation of foods, veterinary practices and animal skins processing technology (Bahar and Altug, 2009; Kizil and Turk, 2010). In the past, the leaves, bark, roots and branches of *R. coriaria* were used in dyeing as mordant natural dyes. In addition, *R. coriaria* possesses high fixation, retention and fungal resistance properties, and is useful against wood decay (Sen *et al.*, 2009). So far, a big deal of nutritionally and medicinally considerable metabolites (such as phenolic acids, tannins, anthocyanins, organic acids, proteins, essential oils, fatty acids, fiber, and minerals) have previously been identified from various parts of *R. coriaria* (Shabir, 2012).

Figure 1. *Rhus coriaria* L. plant and fruits; a. Sumac plant (leaves, fruits, and flowers) b. Sumac fruits c. Sumac fruit powder.

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Rhus coriaria has been reported to possess antibacterial (Aliakbarlu *et al.*, 2014; Kossah *et al.*, 2013; Ali-Shtayeh *et al.,* 2013; Iauk *et al.,* 1998), antifungal (Onkar *et al.,* 2011), antioxidant (Aliakbarlu *et al.*, 2014), anti-inflammatory (Panico *et al.*, 2009), DNA protective (Chakraborty *et al.*, 2009), vascular smooth muscle cell migration inhibition (Zargham and Zargham, 2008), hypoglycemic (Anwer *et al.*, 2013; Golzadeh *et al.*, 2012), and hypolipidemic activities (Madihi *et al.*, 2013). Moreover, this plant has traditionally and widely been used in the treatment of diabetes (Mohammadi *et al.*, 2010), stroke and cancer (Zargaran *et al.*, 2013), in the digestive tract maladies such as ulcer, diarrhea, stomach tonic, stomachache, and hemorrhoids pain (Ahmad *et al.*, 2013), diuresis, anorexia, measles, smallpox, hyperglycemia, gum ailments (Abu-Reidah *et al.*, 2014), hypertension (Polat *et al.*, 2013), atherosclerosis (Setorki *et al.*, 2012), dysentery, conjunctivitis, hematemesis, hemoptysis, and leucorrhea, dermatitis, ophthalmia, and liver disease, besides it was used also for throat treatment and in addition as abortifacient. Other medicinal uses have also been reviewed including weight loss, treatment of skin, hair, burns, digestive system, headache and temperature reducing (Ali-Shtayeh *et al.*, 2013). *R. coriaria* leaves have been reported to be useful in the treatment of chronic diseases as osteoarthritis and may form a potential application in joint disease therapy (Panico *et al.*, 2009). It was reported that the acute consumption of sumac might have a protective effect on some of the risk factors of atherosclerosis, oxidative stress and liver enzymes, due to high fat food stress (Setorki *et al.*, 2012).

The present review suggests increasing the size of research on and development efforts for obtaining bioactive whole extracts or individual functional components from *R. coriaria*, which makes the plant an appealing species of *Rhus,* as well as a source of functional food and nutraceutical ingredients. Additionally, it may help to further establish mechanisms of action of *R. coriaria* components, leading to a better understanding of the plant extracts and components' bioactivity. Moreover, this review attempts to focus on the traditional use of *R. coriaria* based on actual research data for its multivalent actions as health promoting dietary additives as well as putative therapeutic agents. In the current work, we critically review the so far known biological activities of *R. coriaria* extracts in an attempt to update the current knowledge on the plant.

2. History of Sumac use

Sumac has been used as a natural and traditional source of medication in different dietary cultures all over the world; the use of the plant in seasonings and flavoring agents has been the mainstay of indigenous remedies across the world.

Sumac is used as a spice, and has been used in cooking for millennia. About 2,000 years ago, the Greek physician Pedanius Dioscorides (40-90 A.D.) wrote in his voluminous "De Materia Medica" ("Of Medical Matters") about the healthful properties of Sumac, principally as a diuretic and anti-flatulent (Norton, 2006).

One practice of ancient Rome continues today in certain cuisines in which *R. coriaria* berries are pressed to extract their essential oils. The oil is then mixed with either olive oil or vinegar, depending on the type of condiment sauce being made. Nevertheless, the medicinal properties of *R. coriaria* had been noted since antiquity. For instance, sumac was used in folk medicine for the treatment of stroke chronic symptoms, as was described by Avicenna (Ibn-Sina) in his well-known book, *Canon of Medicine* (Zargaran *et al.*, 2013).

Interestingly, in Iran and Palestine, sumac represents pure ground fruit epicarps of the plant, while in Turkey the whole fruit is ground with salt crystals (Mirhadi *et al.*, 2011). It is commonly used as a seasoning spice in the Mediterranean region, especially in meat and fish dishes (Nasar-Abbas *et al.*, 2004). The ground sumac seeds, mixed with olive oil, are also used in food industry in salads and other meals (Kizil and Turk, 2010).

Today, a large mass of literature indicates that adding sumac into food stuff or water can have beneficial effects on human and animals (Chakraborty *et al.*, 2009).

3. Morphological Characterization of Different Parts of the Plant

Rhus coriaria L. is a shrub 3-4m high, the leaves pinnate with 6-8 pairs of small oval leaflets of different sizes, and white flowers in terminal inflorescences. The fruits are globose, villose and reddish drupe when ripe; with one seed, they contain tannins, essential oils, various organic acids, anthocyanins and fixed oil. The leaves contain gallic acid, (bi)flavonoid, sugar, wax and essential oils (Ünver and Özcan, 2010). Generally, investigations have focused on the tannin and flavonoid contents of *R. coriaria* leaves. Physical properties, such as length (4.70 mm), weight (0.20 g), volume (19.50 mm^3), geometric diameter (3.64 mm), sphericity (0.77), and thickness (2.64 mm) of *R. coriaria* fruits have been estimated at 4.79 % moisture content levels. At an identical moisture content level porosity (68.50%), static friction (0.48-0.68), bulk projected area (0.16 cm^3), terminal velocity (3.50 m/s), and density (304.25 kg/m^3) of the fruits were also determined (Özcan and Haciseferogullari, 2004).

4. Phytochemical Significance

In the light of the significance of sumac uses in food seasoning, folklore medicine and industry, *Rhus coriaria* has long been investigated to expose its chemical composition. *R. coriaria* plant is known as an abundant source of tannins (condensed and hydrolysable), phenolic acids, anthocyanins, gallic acid derivatives, flavonoid glycosides, organic acids (Abu-Reidah *et al*., 2014).

Parts like leaves, fruits, and seeds of *R. coriaria* were reported to contain a number of phyto-constituents as shown in Figure 2. The presence of gallotannins (mainly hydrolysable tannins) is a characteristic property of the *Rhus* genus, mostly *R. coriaria* species, which is an abundant source of tannins with different isomers and conjugations; besides, it contains other metabolites or phytochemicals, which have been described in various parts of the plant.

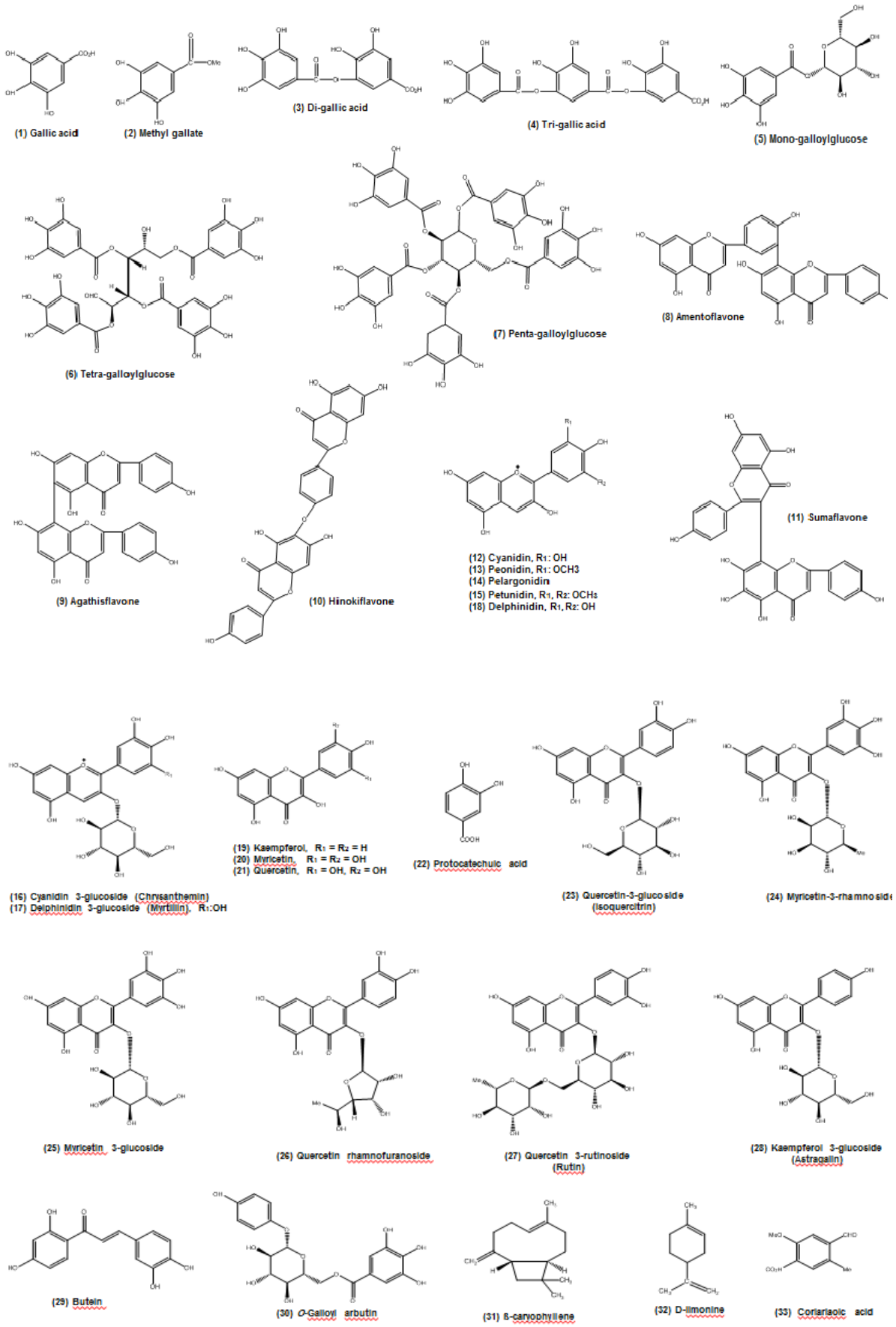

Figure 2. Structure of some selected phytochemicals from *Rhus coriaria*

Tannins are polyphenolic secondary metabolites of plants (MW's: 500 to 3,000), containing sufficient hydroxyls and carboxyls' groups (Haslam, 1989) which form hydrogen bonds in solutions. Tannins are astringent and bitter compounds, which can form strong complexes with various macromolecules that bind to and can precipitate proteins and other organic compounds including amino acids. They play a vital role in protecting plants from predation; and perhaps also as pesticides, as well as in plant growth regulation (Thorington and Ferrell, 2006). Lately, these substances have gained attention as they may trim down the risk of chronic diseases, by reinforcing the defenses against reactive oxygen species (Panico *et al.*, 2009).

The tannin compounds are widely distributed in many plant species, where they play a role in protection from predation, and plant growth regulation (Katie *et al*., 2006).

Structurally, tannins are divided into two classes: hydrolysable and condensed ones. *Rhus coriaria* has been reported as one of the major commercial hydrolysable tannin sources (Sarioezlue and Kivanc, 2009).

The methanol extracts from *R. coriaria* fruits were reported as a rich source of natural antioxidants phenolics, mainly tannins, which has an inhibitory function in the migration of vascular smooth muscle cells, suggesting an atheroprotective role for this chemical. *In vitro* and *in vivo* studies have shown that tannins have anticarcinogenic effects (Ram *et al.*, 1997).

The aqueous and aqua-methanol extracts of *R. coriaria* leaves and fruits were investigated using HPLC to reveal the presence of gallotannins derivatives, namely gallic acid (1), methyl gallate (2), digallic acid (3), tri-gallic acid (4), and ellagic acid, together with mono- (5), di- , tri- , tetra- (6), penta- (7), …, deca-, undeca- and dodeca-gallolyl glycoside derivatives as representative tannins present in *R. coriaria* (Regazzoni *et al*., 2013). Some of the above-mentioned galloylglucose derivatives were reported to have the ability to reduce blood urea nitrogen and blood pressure (Djakpo and Yao, 2010).

In fact, the galloylated-glucose derivatives were previously studied in *R. coriaria* leaves using UV, paper chromatography, and IR measurements, in addition to the column chromatography technique which was used to uncover the existence of flavonoid glycosides (El Sissi *et al.*, 1972). Flavonoid dimers (with antiviral activity) like amenthoflavone (8), agathisflavone (9), hinokiflavone (10), and sumaflavone (11) have also been identified in the leaves and fruits via LC and LC-MS (Van Loo *et al.*, 1988; Abu-Reidah *et al.*, 2014). Other anthocyanins were also established: cyanidin (12), peonidin (13), pelargonidin (14), and petunidin (15) structures and coumarates, anthocyanins were peonidin-3-glucoside, petunidin-3-glucoside (coumarate), delphinidin (coumaroyl) glucosides, and cyanidin coumaroyl glucoside. However, the presence of cyanidin-3-glucoside (16), delphinidin-3-glucoside (17) and delphinidin (18) has already been reported from the fruits of *R. coriaria* (Mavlyanov *et al.*, 1997).

Furthermore, some other phenolics have been isolated from *R. coriara*, including gallic acid, methyl gallate, kaempferol (19), myricetrin (20), quercetin (21), *p*-benzoic acid, vanillic acid isoquercitrin, protocatechuic acid (22), kaempferol 3-galactoside, quercetin 3-glucoside (isoquercitrin) (23), quercetin 3-rhamnoside, myricetin 3-rhamnoside (24), myricetin 3-glucoside (25), myricetin 3-glucuronide, myricetin 3-rhamnoglucoside, have also been already identified in the *R. coriaria* leaves and fruits (Shabana *et al.*, 2011; Abu-Reidah *et al.*, 2014). The separation of gallotannins and flavonoids was carried out by HPLC-ESI-MS, which allowed the structure resolution of the isobaric flavonoid glycosides.

Lately, a detailed profiling of phytochemical compounds has been carried out by analyzing the hydromethanolic extract of the fruits using HPLC-DAD-ESI-MS/MS technique, where more than 200 phytochemical components have been tentatively identified. Curiously, the occurrence of the conjugated form of aglycone with hexose-malic moieties (24 compounds) has been very recently identified for the first time in the Palestinian *R. coriaria* (Abu-Reidah *et al.*, 2014). In the same work, five cyanidin derivatives have been newly detected anthocyanins in the fruit epicarps. Moreover, the following flavonoid glycosides have been also identified: quercetin-rhamnofuranoside (26), rutin (27), and kaempferol 3-glucoside (Astragalin) (28).

Butein (29) is a recently identified chalconoid derivative from *R. coriaria*. Notably, this compound exhibited a significant anti-breast cancer activity (Li *et al.*, 2014). Another galloyl derivative compound was also characterized in the fruits: *O*-galloyl arbutin (30).

Minerals are essential chemical elements for supporting the human health, indispensably obtained from the diet. Once minerals intake is inadequate, deficiency symptoms may take place (McDowell, 2003). However, minerals like potassium, calcium and magnesium were found to be predominant in sumac. Other minerals have also been explored, namely sulfur, cadmium, phosphor, lead, titanium, vanadium, copper, silicon, barium, chromium, lithium, brome, aluminum, chloride, manganese, iron, sodium, zinc, strontium, and nitrogen (Kizil and Turk, 2010).

On the other hand, β-caryophyllene (31) a bicyclic sesquiterpene has been recently described to be a major essential oil component isolated from *R. coriaria* (Gharaei *et al.*, 2013). An anti-inflammatory effect for this terpenoid has been described elsewhere (Gertsch *et al.*, 2008).

Interestingly, *R. coriaria* fruits were found to possess various fatty acids, including azelaic, tetradecanoic, elaidic, stearic, eicosadienoic, arachidic, and tetracosanoic acids, with oleic (ω 9), palmitic, and linoleic (ω 6) acids being as major fatty acids in sumac. The polyunsaturated fatty acid (ω 6+ ω 3) contents of the total fatty acids were found to be between 34.84 and 37.36% (Dogan and Akgul, 2005). The main fatty acids of sumac were found to be: oleic (33.78-52.57%), palmitic (17.00-29.80%), linoleic (11.60-21.90%), linolenic (0.33-1.33%) and stearic (17 %) acids. On the other hand, linoleic (49.35-60.60%), oleic (24.60-32.05%), palmitic (8.30-13.60%), stearic (1.60-3.00%) and linolenic (0.46-0.74%) acids were described to be major fatty acids of *R. coriaria* seeds (Ünver and Özcan, 2010).

It is worth noting that the major volatiles determined from *R. coriaria* were aliphatic, farnesyl acetone, aldehydes, hexahydrofarnesylacetone, and oxygenated terpenes, among others. Terpene hydrocarbons were reported to be the main constituents. Polyisoprenoids from the leaves were investigated via GC–MS (Mamatkulova *et al.*, 2012). A-tocopherol was found as the predominant existing substance; besides, other minor components, such as tocopherol mannoside, farnesylacetate, pentadecanal, and hexadecanal, have also been determined. D-limonene (32), a monoterpene derivative isolated from the plant, was found to have hypocholesterolemic effects (Golzadeh *et al.*, 2012). A recent study reported that cembrene (21.40 %) and β-caryophillene (30.70 %), as main terpinoid derivatives, are found in *R. coriaria* (Gharaei *et al.*, 2013).

However, the sourness of sumac is mainly due to the presence of organic acids, such as malic, citric and tartaric acids (Kossah *et al.*, 2013), while the astringent taste is ascribed to its tannins composition. It is interesting to know that *R. coriaria* fruits and seeds are incredibly rich in antioxidants, Vitamins A and C.

5. Biological Properties

Many literature reports indicate that the addition of sumac to the food/feed or water can impart a beneficial effect on both human beings and animals (Capcarova *et al.*, 2012). Some of the recently published information about the biological activities in literature is illustrated in Table 1.

Table 1. Digest of reported biological activities of different used parts of *Rhus coriaria* L.

Pharmacological Properties	Plant part used	Used extract/plant part (form)	Result/Activity	Reference
Antibacterial activity	**Fruits**	Hydrodistilled extract	Demonstrated a desirable antibacterial activity	Sağdıç, and Özcan, 2003
	Fruits	Ethanol and methanol extracts	Sumac extracts were effective against Gram positive and Gram negative bacteria	Nasar-Abbas and Halkman, 2004
	Fruits	Ethanol 95% extract	Significant antibacterial activities against all tested species have been shown	Nimri,*et. al*., 1999
	Fruits	Methanol extract	A strong *in vitro* antioxidant activity indication of the methanolic extract of sumac fruits	Candan, and Sökmen, 2004
	Fruits	Water extract solution extract 0.8:10 (wt/vol)	Bacteriostatic/bactericidal effects by bacteria cycle reduction exerted by sumac extract have demonstrated	Gulmez, *et al.,* 2006
	Plant	Water, Methanol 80 %, Ethanol 80 % extracts	Antibacterial activity can be exerted individually or conjointly with other spice	Adwan, *et. al*., 2006
	Fruits	Ethanol 80% extract	Effective antibacterial agents on both Gram-positive and Gram-negative bacteria	Fazeli *et al*., 2007
	Plant	Ethanol extract	*R. coriaria* extract can have an antimicrobial effect on total microbial and *Salmonella* count in minced meat for one week	Radmehr and Abdolrahimzade, 2009
	Leaves	Ethanol 95% extract	Showed a high antibacterial activity in comparison with other plants	Ertürk, 2010
	Plant	Ethanol 80% extract	Sumac extracts exhibited a moderate activity on *Brucella* strains	Zandi, *et al.,* 2012
	Fruits	Ground and fermented sumac	*R. coriaria* could decrease the formation of biofilm, a major virulence factor in staphylococcal infections	Kırmusaoğlu, *et al.,* 2012
	Plant	Ethanol extract	Results indicated that among other plant extracts, the sumac one, was found to have the most potent against: *Propionibacterium acnes*, *S. aureus*, *E. coli*, *P. aeruginosa*	Ali-Shtayeh, *et al.,* 2013
	Fruits	Ethanol 20% extract	A remarkable inhibitory activity was shown by sumac extract against *B. cereus*. Also it strongly inhibited the growth of *H. pylori*. The fruit extract exhibited a good antioxidative capacity, justifying its use as a natural antibacterial preservative	Kossah, *et al.,* 2013
	Plant	Water extract	Sumac water extracts showed the strongest antibacterial activity among other 10 extracts studied.	Aliakbarlu, *et al.,* 2014; Aliakbarlu, *et al.,* 2014
Antioxidant activity	**Fruit epicarps**	Methanol extract	From results it can be noted a desirable antioxidant activity of sumac which in turn could delay the oxidation of palm oil	Ozcan, 2003
	Plant	Ethyl acetate and 80% methanol fractions after initial defatting by petroleum ether	The ethyl acetate fraction of plant materials exhibited a noticeable antiradical activity on DPPH	Bozan, *et al.,* 2003
	Fruits	Methanol extract	Results indicate a strong *in vitro* antioxidant activity of the methanolic extract of *Rhus coriaria* fruit based on hydroxyl radical scavenging	Candan , 2003

Pharmacological Properties	Plant part used	Used extract/plant part (form)	Result/Activity	Reference
	Fruits	Water extract	Sumac extract was more effective than BHT, and could be added to meat products (e.g. sausage)to enhance quality	Bozkurt , 2006
	Fruits	Methanol 70% extract	Sumac extracts and fractions showed remarkable antioxidant activity against inhibition of lipid peroxidation and scavenging activity based on DPPH radical assay.	Kosar, *et al.,* 2007
	Aerial parts	Methanol 50% extract	A desirable antioxidant activity was shown	Serteser *et al.,* 2009
	Fruits	Ethanol and water extracts	Water extracts of sumac with effective antioxidant and radical scavenging activities as compared to ethanol extracts.	Bursal and Köksal, 2011
	Fruits	Ethanol 20% extract	Sumac fruit extract exhibited a good antioxidative capacity also it showed a remarkable inhibitory activity against *B. cereu,* besides it strongly inhibited the growth of *H. pylori.*	Kossah, *et al.,* 2013
	Plant	Water extract	Sumac water extracts showed the strongest antioxidant activity among other 10 extracts	Aliakbarlu, *et al.,* 2014
Antidiabetic activity	**Fruits**	Methanol extract after fractionation with ethyl acetate and hexane	Ethyl acetate fraction of sumac fruits showed appreciable biological activity through α-amylase inhibition indicating significant hypoglycemic activity.	Giancarlo, *et al.,* 2006
	Fruits	Ethanol 96%	The Sumac extract raised markedly HDL and also reduced LDL, increasing superoxide dismutase and catalase activities. Also, it inhibited maltase and sucrase activities.	Mohammadi, *et al.,* 2010
	Fruits	Ethanolic extract	Antidiabetic activity in vivo: Alloxan-induced diabetic wistar rats	Sharma and Arya, 2011
	Seeds	Methanol extract	Antidiabetic effect of Sumac on blood glucose and glycosylated hemoglobin levels in NIDDM rats.	Anwer *et al.,* 2013
DNA-Protect activity	**Fruits**	Sumac extract in water solution	Sumac showed to be a potent antioxidant which may protect humans against oxidative DNA-damage suggesting gallic acid as main contributor for Sumac effects	Chakraborty *et al.,* 2009
Lipid-lowering and hypocholesterolic activity	**Fruits**	Methanol 80% and 100% extracts	Sumac fruit extract was of use in decrease the high serum lipid levels, and moderate the elevated cardiac lipid concentrations.	Shafiei, *et al.,* 2011
	Fruits	Dietary supplement Sumac fruits, and methanol 80% extracts	Decrease in cholesterol in the blood of rabbits resulted after the oral administration of sumac during 90 days, showing thus a positive effect on cholesterol and VLDL levels in adult male rabbits.	Capcarova *et al.* 2012
	Fruits Powder	Dietary Sumac powder oral administration	Dietary supplementation of sumac, reduces the blood VLDL-c, TC, and FBS concentrations in broiler chicken	Golzadeh, *et al.,* 2012
	Fruits Powder	Fat diet with 2% of Sumac powder	A protective effect of consuming sumac with food on some risk factors of atherosclerosis and oxidative stress (LDL-C, total cholesterol) has been demonstrated	Madihi *et al.,* 2013
	Fruits	Dietary Sumac powder oral administration	Sumac can be useful to decrease the negative effects of mild heat stress on broiler chickens due to its richness in tannins.	Alishah, *et al.,* 2013
Antimigratory activity	**Fruits**	Acetone 70% extract	Tannin extract from Sumac has an inhibitory role on the migration of VSMC and suggesting an atheroprotective role.	Zargham and Zargham, 2008
	Fruits	Dietary Sumac powder oral administration	Acute consumption of Sumac might be having protective effects on some risk factors of atherosclerosis, and liver enzymes, due to high fat food stress.	Setorki, 2012
	Leaves	Ethanol extract	Moderate antifungal activity was found for Sumac	Ertürk, 2006

5.1. Antibacterial and Antifungal Properties

It is worth mentioning that a large number of antibacterial activity studies have specifically focused on sumac because of its wide-range use in the Mediterranean area as a seasoning spice.

Most of the antibacterial assays carried out on *R. coriaria* used either ethanol or water extracts. In this context, the water and hydro-methanol extracts from the fruits were found to have a great activity against more than 10 different bacteria species, among which are Gram positive and Gram negative bacteria strains, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Yersinia enterocolitica, Shigella dysentariae*, and *Salmonella enteritidis* (Nasar-Abbas and Halkman, 2004). Among several plants tested, the aqueous extracts of sumac had the strongest antibacterial activity against the tested bacteria (Aliakbarlu *et al.*, 2014). According to Gulmez *et al*. (2006), the water extract of *R. coriaria* fruits had an antimicrobial activity against coliform applied on the stored poultry meat. It is worth noting that the mature fruits of *R. coriaria* possess higher antimicrobial activity in comparison to the immature ones.

The ethanol extract of sumac was reported to be effective in count-decreasing of the total microbial count and salmonella in the minced meat, in which a significant antimicrobial potential was shown for the ethanol extract compared to controls (Radmehr and Abdolrahimzade, 2009). Furthermore, the methanol extract of sumac fruit demonstrated an important antibacterial activity against *Bacillus pumilus, Bordetella bronchiseptica, Staphylococcus epidermidis*, and *Klebsiella pneumonia*, using the agar well-diffusion method (Shabir, 2012).

The antimicrobial activity of *R. coriaria* extracts were tested against six strains, including three Gram-positive and three Gram-negative. *Bacillus subtilis* was found to be the most sensitive Gram-positive with MIC of 0.5 mg/ml, while Gram-negative bacteria were affected by higher concentrations of sumac extracts ranging 10-20 mg/ml. Among bacteria, the inhibitory effects increased with the increase of *R. coriaria* fruit extracts concentration from 0.1 to 20 mg/ml (Raodah *et al*., 2014). The antibacterial activity of the plant extract against *Brucella* has been also assessed, in which the mean zone of growth inhibition for Sumac was 22.55 mm for disks that contained 40 mg/mL, and (MIC: 3.26 mg/mL), whilst the minimum bactericidal concentration (MBC) was 9.03 mg/mL (Motaharinia *et al.*, 2012). The antibacterial effect of *R. coriaria* on the biofilm formation of *S. aureus* has been also evaluated. Significant differences between varying concentrations of the plant extract were observed in several strains of methicillin resistant/sensitive *S. aureus*, indicating dose-related diminishes in the slime formation noted in bacteria. Briefly, the plant extract could reduce the formation of biofilm, a major play factor in staphylococcal infections (Kırmusaoğlu *et al.*, 2012). Among the fifty Palestinian medicinal plants that were examined to investigate their antimicrobial activities against acne *vulgaris*, the ethanolic extract of *R. coriaria* exhibited a strong inhibitory effect and was found to be among the most active plant extracts against all bacterial strains tested including, *P. acnes*, and Gram-negative strains of aerobic bacteria (Ali-Shtayeh *et al.*, 2013). It is worth mentioning that the observed *in vitro* antimicrobial potential of *R. coriaria* has been mainly referred to the presence of tannins.

On the other hand, the antifungal activity results, reported by Onkar and coworkers (2011), have indicated that the sumac methanol extract, including other three individual compounds (coriorianaphthyl ether, coriariaoic acid, and coriarianthracenyl ester) thereof, were found to be able to reduce the growth of several fungus strains. Thus, coriariaoic acid (33) was effective against both *A. flavus* and *C. albicans* at the lowest tested concentration of 25 mg/ml, analogous to the standard (Fluconazole) at higher tested concentrations against *A. flavus,* unlike the case of *C. albicans*. Moreover, coriarianaphthyl ether has exhibited a comparable activity at higher concentrations of the reference drug; it was also found to be active against all the fungal strains tested at all concentrations used (Onkar *et al.*, 2011). In one more study, it was shown that the alcohol extract of *R. coriaria* to possess a high antifungal activity against *C. albicans* and *A. niger* (Ertürk, 2010).

From the results given about the antimicrobial and antifungal activities, it can be concluded that the aqueous and alcoholic extracts of *R. coriaria* possess compounds with valuable antibacterial and antifungal activities that can be potentially used as antimicrobial agents and in the treatment of infectious diseases including acnes and those caused by resistant microorganisms.

5.2. Antiviral Activity

The antiviral activity of twenty five species of various medicinal plants in Iran was investigated, of which the aqueous extract of *R. coriaria* exhibited a significant antiviral activity against HSV-1 and adenovirus type 5 at non-toxic concentration (Monavari *et al*., 2007).

Interstingly, four biflavones, viz. amentoflavone (8), agathisflavone (9), hinokiflavone (10), and sumaflavone (11), were isolated from the leaves and fruits of different *Rhus* species. Amentoflavone (8) and agathisflavone (9) have shown an activity against influenza A and B viruses. Amentoflavone exhibited moderate anti-HSV-1 and anti-HSV-2 activities with EC50 =18 and 48 μg/mL, respectively (Lin *et al.*, 1999). On the other hand, hinokiflavone, amentoflavone, and agathisflavone demonstrated significant activities against HIV-1 reverse transcriptase, with IC_{50} values ranged from 65-100 μM (Lin *et al.*, 1997). In a previous work, hinokiflavone isolated from *Podocarpus macrophyela* has shown an antiviral activity demonstrated by its inhibitory action noted on the Epstein-Barr virus genome expression in Raji cells, which suggested an important antiviral potency of this biflavone (Kozuka *et al.*, 1989).

5.3. Antioxidant Activity

Antioxidant activity of *R. coriaria* fruit methanol extract against lipid peroxidation and free radicals has been previously reported indicating that the plant extract may prevent the development of chronic diseases such as atherosclerosis (Shafiei *et al.*, 2011). On the other hand, Aliakbarlu *et al.* (2013) studied the antioxidant activity of water extract of sumac among other spices and found that the water extracts of the plant have one of the highest antioxidant potential among the extracts studied.

The results of antioxidant activities indicated that the antioxidant effects are due to phenolic components, especially, gallic acid and its derivatives (Chakraborty *et*

al., 2009). Ferk and coworkers (2007) estimated the antioxidant effect of sumac to be 50 fold more than vitamin C and E. Besides, they reported that the daily consumption of 0.2 mg per kg body weight gallic acid for three days, in male rats, showed protective effects on lymphocytes, brain, liver, colon and lung.

In a very recent study, Gabr and coworkers (2014) extracted the active constituents of sumac like, alkaloids, glycosides, phenol and terpenoids using GC-MS. The antioxidant activity of *R. coriaria* extract and its constituents were determined using DPPH and β-carotene-linoleic acid scavenging activity assays. Antioxidant activity showed a range of (72.70-87.9%) for the plant extract compared to a lower antioxidant activity of its active constituents. However, phenols showed a higher range of antioxidant activity (70.1-75.8%) compared to glycosides (65.7-67.6%), alkaloids (53.4-58.4%) and terpenoids (50.7-51.3%), respectively (Gabr *et al*., 2014).

Antiradical activities of water and ethanol extracts of *R. coriaria* were studied comparatively. The study indicated that antioxidant capacity and radical scavenging of water extract was significantly higher than that of ethanol extract. Also, amounts of both total phenolic and total flavonoid contents of water extract were higher than those of ethanol extract (Bursal and Köksal, 2011).

5.4. Antidiabetic Activity

Diabetes mellitus is a metabolic disorder of the endocrine system which is emerging as a severe problem. It continues to increase both in numbers and in the impact upon the quality of life, as changing lifestyles leads to reducing physical activity and to increasing obesity. In 2010, 285 million adults worldwide were estimated to have DM (approaching 7% of the adult population). It is anticipated that by 2030, the number of DM patients will exceed 438 million people, almost 8% of the adult population (Ali-Shtayeh *et al*., 2012). The fruits could improve the life of type 2 diabetic patients by exerting mild antihyperglycemic and potent antioxidant properties. Moreover, *R. coriaria* is highly recommended for the blood lipids adjustment in diabetic patients.

The hypoglycemic efficacy of the plant extracts has been previously investigated via hindering the α-amylase enzyme. Ethyl acetate extract of sumac was suggested as beneficial in the treatment and prevention of hyperglycaemias and diabetes (IC_{50}: 28.7 mg/mL), suggesting a considerable blood sugar decreasing activity of sumac extracts, whereas, the methanol extract of fruits showed 87% inhibition activity at 50 µg/mL (Mohammadi *et al.*, 2010).

Another study carried out by Anwer *et al.* (2013) suggested that the methanol extract of *R. coriaria* can notably delay the onset of hyperinsulinemia and glucose intolerance, and it can also improve insulin sensitivity in rats. Above all, the gallotannin; penta-galloylglucose (7) which was repeatedly reported in sumac plant was found to have an antidiabetic effect, exhibited by acting as an inhibitor of PTP1B enzyme (Baumgartner *et al.*, 2010). In contrast to this, it was found, by other researchers, that the plant extracts increase the levels of blood sugar in rats (Mirhadi *et al*., 2011; Pashazadeh *et al*., 2013). These findings demonstrate that sumac can positively affect the blood sugar level in diabetic patient.

5.5. Hypolipidemic Activity

Positive effects of sumac consumption on antioxidant status and cholesterol level in rabbits have been demonstrated in a recent study, suggesting that the plant may have a lowering effect on blood cholesterol level in animals and human beings (Capcarova *et al.*, 2012; Golzadeh *et al.*, 2012). On the other hand, Shafiei and coworkers (2011) showed that the sumac extract was able to decrease high serum lipid concentrations and could adjust the elevated cardiac lipid levels in the hypercholesterolemic conditions.

Additionally, Valiollahi and others (2014) have shown that the triglyceride and cholesterol level decreased significantly in broiler chicks that consumed sumac; also, the LDL level decreased significantly and HDL levels increased in the same group (Valiollahi *et al*., 2014). Similarly, Santiago *et al.*, (2010) reported reduced serum cholesterol concentrations in rats consuming d-limonene. It was recently monitored that the acute consumption of sumac might have a protective effect on some of the risk factors caused by high fat food stress, such as atherosclerosis, oxidative stress and liver enzymes (Setorki *et al.*, 2012). Again, a significant decrease in the blood levels of total cholesterol, LDL-C, and fibrinogen compared to the high-cholesterol diet group have been described elsewhere (Madihi *et al.*, 2013), a protective effect demonstrated on some risk factors including atherosclerosis and oxidative stress, followed consuming the Sumac with food.

5.6. Scolicidal Activity

In nature, only few anthelmintics are available for healing hydatid disease caused by the parasite *Echinococcus granulosus*. Lately, Moazeni and Mohseni (2012) have studied the scolicidal effect of the methanol extract of sumac as anthelmintic. Thus, three concentrations of the plant extract (10, 30 and 50 mg/mL) were used for 10, 20 and 30 min. Whereas 16.93% rate in the control group was for the dead protoscolices, the rate increased to 94.13%, 97.67% and 100% after 10, 20 and 30 minutes, respectively, obtained when the protoscolices were exposed to sumac extract at the concentration of 10 mg/mL. However, at the concentration of 50 mg/mL, one hundred percent mortality rate was observed after 10 min of exposure, suggesting the methanol extract to be an effective natural scolicidal agent.

5.7. Anti-Mutagenic Activity

Chakraborty *et al.* (2009) have suggested that *R. coriaria* can protect against genotoxic carcinogens, which are degraded by specific enzymes, namely glutathione S-transferase GST–π, and GST-α were clearly enhanced by 40%, 26%, and 52%, respectively. Actually, gallic acid, a major constituent of the plant, is known to possess multiple biological activities, including anticancer function (Liu *et al.*, 2011). However, this work indicated an inhibitory role of invasion and migration of PC-3 cell dose-dependently of gallic acid. Consequently, it was postulated that the gallic acid might modulate in the course of blocking the several signaling pathways and

dropping the NF-κB protein level, resulting in human prostate cancer cells inhibition.

Hinokiflavone (10) has been previously characterized as the cytotoxic principle from *R. succedanea* and *R. coriaria* berries; however, its significant cytotoxicity was referred to the ether linkage between the apigenin aglycones (Lin *et al.*, 1989).

6. Applications of Sumac in Food Safety and Technology

There is an increasing interest in using plant extracts by the food industry as natural preservatives. Lipid oxidation and microbial growth in food can be controlled by the use of plant extracts. Water extracts of *R. coriaria* possess a strong antioxidant and antibacterial activity against food-born pathogenic bacteria, suggesting the use of water extracts of the plant as effective and natural preservatives in food manufacturing (Aliakbarlu *et al.*, 2014). Industrially, the seeds are by-products in the production of the spice; however, they are rich in linoleic and oleic acids that qualify the plant seeds to be considered as a valuable raw material for the oil industry. In this context, the mixing of *R. coriaria* seeds oil with olive oil for the use in salads and cooking has already been proposed (Ünver and Özcan, 2010).

The plant extract has shown to be more effective than BHT (butylated hydroxytoluene) in enhancing the quality parameters of the fermented sausage, suggesting the use of the plant in sausage industry to enhance its total quality (Bozkurt, 2006).

Nasar-Abbas and Halkman (2004) established that the level of inhibitory action exerted by the *R. coriaria* extract on the bacteria tested was analogous to that commonly used in food products, the concentration at which the plant extract exerts a desirable antibacterial effect may be higher in foods than that studied in vitro, but joined with other agents, it may help to control bacterial growth in foods. The antimicrobial properties of the plant on pathogenic bacteria in meat have also been evaluated (Radmehr and Abdolrahimzade, 2009). From the results, sumac exhibited significant antimicrobial effects on the total microbial and *Salmonella* count in minced meat for one week. The plant extract was found to be effective in stabilizing peanut oil compared with BHA, in which the antioxidant efficiency lasted for about 4 weeks. Curiously, the inhibition of autoxidation was proportional to the concentration, suggesting that high concentrations of sumac extract contribute mainly to the demonstrated antioxidant activity. Also, the data can support the application of *R. coriaria as* a natural antioxidant in oily foods (Özcan, 2003).

Waste extracts of *R. coriaria* are considered a potential source of natural, safe, plentiful, and also a cheap antimicrobial resource for food, acting as a surface decontaminant replacement by the use of the synthetic and chemical antimicrobials in the poultry industry, since it showed to be superior to lactic acid (antimicrobial preservative) in terms of the quality of poultry meats (Vatansever *et al.*, 2008). The activity of the plant extract might be due to the synergistic activity of slowing down the growth rate of contaminants, originated from both water soluble tannins and organic acids (Gulmez *et al.*, 2006).

However, the production of pure sumac extract powder by using a carrier (maltodextrin) through spray drying has been recently developed (Caliskan and Nur Dirim, 2013). This sumac powder mix can be effectively used in poultry and meat food production chains.

7. Conclusions

Since ancient times, *R. coriaria* has been used as an important seasoning spice, in medicine, as well as in the industry of leather. Different earlier studies suggest that this plant possesses varied therapeutic uses, including antioxidant, anti-inflammatory, antibacterial, antifungal, and hypoglycemic properties. These observed biological properties may be attributed to the presence of individual phytochemicals, mainly phenolic compounds. *R. coriaria* is thought to be very rich in these compounds.

Leaves and fruits of *R. coriaria* are recognized to have defensive and beneficial effects on numerous diseases, such as diabetes mellitus, some cancers, inflammation, dysentery, and digestive tract system ailments. Moreover, it possesses potential antiviral, antibacterial, antifungal, antioxidant and hypolipidemic activities.

In this review, we have explored the recent phytochemical and biological research available on this well-known plant. Therefore, a comprehensive account of its healing activity, both from a traditional and pharmacological point of view, is presented along with phytochemical components which are nutritionally and medicinally significant. From the present review, it can be concluded that the plant extracts possess compounds with antibacterial and antifungal potential that can be used to treat microbial infectious diseases, as well as in the food industry. The plant extracts can be used to search for bioactive natural products that help in the development of new drugs and food preservatives; it is also worthy to point out the important role of the plant in industry in view of many recent findings and its potential for future research.

The review also aimed at updating the current phytochemical, biological and medicinal knowledge available so far on *R. coriaria;* it also highlights the importance of *R. coriaria* extracts as a promising and potential source of functional ingredients and nutraceuticals with desirable bioactivities, urging further uses of sumac as a food preservative in pharmacology and functional food industries.

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Extracellular Synthesis of Silver Nanoparticles Using *Pseudomonas aeruginosa* KUPSB12 and Its Antibacterial Activity

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Abstract

The use of microorganisms like bacteria in the synthesis of nanoparticles emerges as an eco-friendly approach and an alternative to the chemical method. In the present investigation, we report the biosynthesis of silver nanoparticles (AgNPs) using the phosphate solubilizing bacterium *Pseudomonas aeruginosa* KUPSB12. Silver nanoparticles were synthesized through the reduction of aqueous Ag^+ ion using the bacterial culture supernatants at room temperature. Synthesis of AgNPs was initially observed by color change from greenish yellow to brown which was confirmed by UV-visible spectroscopy. The silver nanoparticles were further characterized using Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopic (SEM) analyses. The synthesized nanoparticles were found to be spherical in shape with a size in the range of 50-85 nm. The synthesized AgNPs were found to have antibacterial activity against six tested pathogenic bacteria (*Escherichia coli* MTCC 443, *Vibrio cholera*e MTCC 3904, *Shigella flexneri* MTCC 1457, *Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 3160 and *Micrococcus luteus* MTCC 1538). Thus, the biosynthesis of silver nanoparticles using *Pseudomonas aeruginosa* culture supernatant deserves to be a good candidate as an antibacterial agent.

Keywords: *Pseudomonas aeruginosa,* Silver nanoparticles, Antibacterial activity, Phosphate solubilizing bacterium (PSB).

1. Introduction

Nanotechnology involving synthesis and applications of nanoscale materials is an emerging field of nanoscience with significant applications in biology, medicine and electronics owing to their unique particle size and shape dependent physical, chemical and biological properties (Albrecht *et al*., 2006; Mahasneh, 2013). To date, nanoparticles are mostly prepared from metals, i.e. silver (Sinha and Paul, 2014), gold (Arunachalam *et al*., 2014), copper (Lee *et al*., 2013), zinc (Darroudi *et al*., 2013), iron (Nadagouda *et al*., 2010), palladium (Khazaei *et al*., 2013) and titanium (Rajakumar *et al*., 2012). Among the metal nanoparticles, silver nanoparticles (AgNPs) have received much attention in various fields, such as antimicrobial activity (Agarwal *et al*., 2014), therapeutics (Mukherjee *et al*., 2014), water treatment (Con and Loan, 2011), bio-molecular detection (Tomšič *et al*., 2009), silver nanocoated medical devices (Furno *et al*., 2004) and optical receptor (McFarland and Van Duyne, 2003).

The nanoparticles have been synthesized by using toxic chemicals and high energy physical procedures. To overcome this problem, biological materials have been used for the synthesis of various metal and oxide nanoparticles. Hence, the biogenic approach, the usage of natural organisms or materials in particular, has offered a reliable, simple, nontoxic and eco-friendly method (Gopinath *et al*., 2013). The microbial synthesis of nanoparticles has significant advantages over other processes since it takes place at relatively ambient temperature and pressure (Gade *et al*., 2008; Mukherjee *et al*., 2008; Wei *et al*., 2012). In such a situation, screening of unexplored microorganisms for AgNPs synthesizing property is very important, as the size and shape of nanoparticles can also be controlled in microbial synthesis (Narayanan and Sakthivel, 2010).

Microbial synthesis of metal nanoparticles can take place either intracellularly or extracellularly (Kowshik *et al*., 2003, Korbekandi *et al*., 2013). Extracellular biosynthesis is cheap and it requires a simpler downstream processing than the intracellular biosynthesis which requires additional steps such as ultrasound treatment or reactions with suitable detergents to release the synthesized nanoparticles (Kalimuthu *et al*., 2008). This favors large-scale production of silver nanoparticles to explore its potential applications. Because of this, many studies focused on extracellular methods for the synthesis of metal nanoparticles (Duran *et al*., 2005). *Escherichia coli* (Gurunathan *et al*., 2009), *Staphylococcus aureus* (Nanda and Saravanan, 2009), *Bacillus megaterium* (Saravanan *et al*., 2011), *Bacillus cereus* (Sunkar and

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Nachiyar, 2012), *Salmonella typhimurium* (Ghorbani, 2013), *Serratia nematodiphila* (Malarkodi *et al.*, 2013), *Pseudomonas fluorescens* (Silambarasan and Jayanthi, 2013) etc., proved its property to form extracellular nanoparticles very effectively. Biofabrication of silver nanoparticles (AgNPs) has offered a consistent, nontoxic and eco-friendly approach for the management of plant diseases owing to their strong antimicrobial properties (Navrotsky, 2000; Hu *et al.*, 2006; Moonjung *et al.*, 2010). Phosphate solubilizing bacteria are found to be agriculturally important. As a result, the development and application of biosynthesized nanoparticles has opened new avenues in agricultural research oriented to developing eco-friendly and effective means of controlling plant diseases. Though several works regarding the synthesis of nanoparticles of a large number of bacteria have been made, no comprehensive work is their relating to the nanoparticles synthesis using phosphate solubilizing bacteria. Furthermore, considering the significance of agriculturally important microbes, their utilization to synthesize AgNPs with potent antimicrobial properties can certainly provide an alternate means for plant protection.

Therefore, the present investigation deals with the phosphate solubilizing bacteria *Pseudomonas aeruginosa* KUPSB12 mediated extracellular synthesis and characterization of silver nanoparticles and their biomedical application.

2. Materials and methods

2.1. Chemicals and Tested Bacteria

All the chemicals were purchased from Merck, India. All the chemicals used were of an analytical grade. The tested bacterial strains for the antibacterial activity were obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

Figure 1. Map of the sampling site.

2.2. Strain used for Silver Nanoparticles Synthesis

Pseudomonas aeruginosa KUPSB12, a phosphate solubilizing bacterial strain isolated from a jute mill effluent exposed area of river Ganga at Bansberia (22°58'17"N and 88°24'03"E), West Bengal, India has been used for the synthesis of silver nanoparticles (Figure 1). Previously, the bacterium was isolated and screened on Pikovskaya's agar medium by pour plate technique (Pikovskaya, 1948). After 48 h of incubation, discreet colonies showing halo zones were picked up with an inoculating needle and reinoculated in Pikovskaya's broth for further plating and isolation by streaking on Pikovskaya's agar. The methods were followed three times to procure a pure colony of phosphate solubilizing bacteria. Physiological, morphological and biochemical tests of the selected bacterial strain were carried out for their identification as per the procedures outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) (Figure 2). The bacterium was also characterized based on 16S rRNA technique and the sequence has been submitted to the Genbank with the accession number KJ131180 (Thompson *et al*., 1997).

Figure 2. Pure culture of *Pseudomonas aeruginosa* KUPSB12 used for synthesis of AgNPs

2.3. Extracellular Synthesis of Bacterial Silver Nanoparticles

The *P. aeruginosa* KUPSB12 strain was freshly inoculated in an Erlenmeyer flask containing 100 ml nutrient broth. The flasks were incubated in orbital shaker at 37°C and agitated at 200 rpm for 24 h. After incubation, the cell filtrates were obtained by centrifugation at 10,000 rpm for 10 min and followed by decantation. The final concentration of 1 mM $AgNO_3$ was added in to 100 ml of cell filtrate in 250 ml Erlenmeyer flask. The flasks were incubated in a dark room condition up to 48 h. The control was maintained without addition of $AgNO_3$ with the experimental flask containing cell filtrate. The brown colored solution of silver nanoparticles was stored under ambient condition for further characterization and applications.

2.4. Characterization of Silver Nanoparticles

The bioreduction of the Ag^+ ions in the solution was monitored by changes in color. The absorption spectrum of this solution was recorded using a UV–visible spectrometer (Shimadzu UV-2450) from 300 nm to 800 nm at regular intervals. Further characterization of AgNPs

involved Fourier Transform Infrared Spectroscopy (FTIR) by scanning the spectrum in the range 400–4000 cm^{-1} at resolution of 4 cm^{-1}. To reveal the shape and the size, AgNPs Scanning Electron Microscopic (SEM) analysis was applied using Hitachi S-4500 SEM machine.

2.5. Antibacterial Activity

Antibacterial activity was performed with synthesized silver nanoparticles by Well diffusion method against three Gram negative (*Escherichia coli* MTCC 443, *Vibrio cholera*e MTCC 3904 and *Shigella flexneri* MTCC 1457) and three Gram positive (*Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 3160 and *Micrococcus luteus* MTCC 1538) bacteria. The bacterial cultures were brought into broth culture for antibacterial assay. Approximately 7 mm diameter of well was made on Mueller Hinton agar plate with the help of sterilized cork borer. The cultures were uniformly spread on solid culture media with the help of sterilized glass spreader. 25 µl of synthesized AgNPs were poured into the well, and then the plates were incubated for 37^{O} C for 24 h and the zones of inhibition were measured.

2.6. Statistical Analyses

All experiments were carried out in triplicate, and the results were expressed as the mean. Means and standard deviations (SD) were analyzed by using the SPSS 13.0 software package.

3. Results and Discussion

A study on extra-cellular biosynthesis of AgNPs by the culture supernatant of *Pseudomonas aeruginosa* KUPSB12 was carried out in this work. Physiological, morphological and biochemical characteristics of isolate KUPSB12 were outlined in Table 1. On the basis of above characteristics as well as 16S rRNA study, the isolate was identified as *Pseudomonas aeruginosa.*

Visual observation of the culture supernatant incubated with $AgNO_3$ showed a color change from greenish yellow to brown (Figure 3). The appearance of a brown color in $AgNO_3$-treated culture supernatant due to reduction of silver ions suggested the formation of AgNPs (Priyadarshini *et al*., 2013; Ranjitham *et al*., 2013). This supports the fact that change in color as observed in the experiment can be considered as an indication of AgNPs formation.

The confirmation of the particle synthesis and stability of the AgNPs in colloidal solution was monitored by UV–vis spectral analysis for which aliquots of the reaction mixture (after completion of the reaction) were withdrawn and used for UV–vis spectroscopy measurements. In the UV–vis absorption spectrum, a strong, broad peak, located at about 442 nm, was observed for nanoparticles synthesized using the culture supernatant (Figure 4). This peak indicated a surface plasmon resonance (SPR), which has already been well documented for various metal nanoparticles with sizes ranging from 2 nm to 100 nm (Henglein, 1993; Ravindra and Rajasab, 2014). As evident from previous reports, the presence of single SPR peak indicates spherical shape of AgNPs which was further confirmed by scanning electron microscopy (Kanchana *et al.*, 2011).

Table 1. Morphological, physiological and biochemical characteristics of *Pseudomonas aeruginosa* KUPSB12

Characters/tests	*Pseudomonas aeruginosa* KUPSB12
Cell shape	Rod
Gram reaction	-
Motility	+
Growth at 5% NaCl	+
Catalase	+
Oxidase	+
IMViC test	
Indole production	-
Methyl red	-
Voges-Proskauer	-
Citrate	+
Urease	-
H_2S production	-
NO_3^- reduction	-
Gelatine liquefaction	+
Starch hydrolysis	-
Hugh-Leiffson (O/F) reaction	O/F
Utilization of carbon source	
Glucose	+
Fructose	+
Sucrose	+
Raffinose	-
Cellobiose	-
Xylose	+
Mannitol	-
Sorbitol	-
Dulcitol	-

+ indicates presence or positive; - indicates absence or negative; O= Oxidation; F=Fermentation

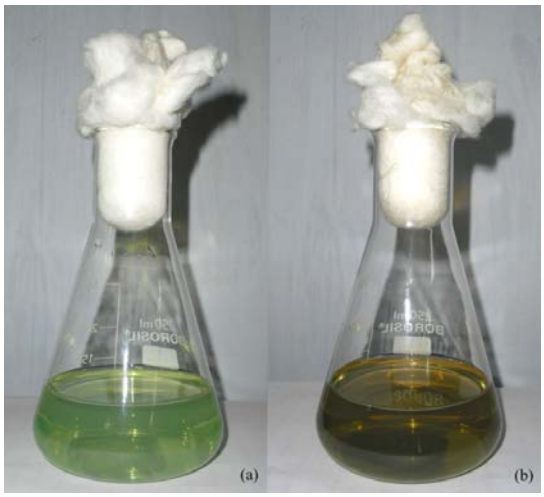

Figure 3. (a) Cell filtrate of *Pseudomonas aeruginosa* KUPSB12 without silver nitrate (control), (b) cell free extract with $AgNO_3$ after 24 h incubation**.**

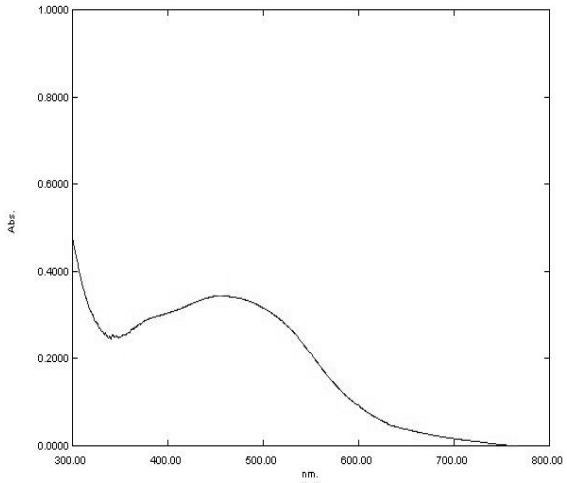

Figure 4. UV-visible spectra of synthesized silver nanoparticles

To explore the reduction process of $AgNO_3$ by the culture supernatant of *P. aeruginosa*, FTIR measurements were carried out to identify possible interactions between silver salts and protein molecules, which could account for the reduction of Ag^+ ions and stabilization of AgNPs (Figure 5). The amide linkages between amino acids residues in proteins give rise to the well known signatures in the infrared region of the electromagnetic spectrum. The bands seen at 3449.08 cm^{-1} and 2633.72 cm^{-1} were assigned to the stretching vibrations of primary and secondary amines respectively. The band observed at 1863.63 cm^{-1} is characteristic of –C=O carbonyl groups and –C=C stretching. The band seen at 1487.23 cm^{-1} is due to amine group. The overall FTIR pattern confirms the presence of proteins in synthesized nanoparticles. The free amine and carbonyl groups present in the bacterial protein could possibly perform the function for the formation and stabilization of silver nanoparticles (Babu and Gunasekaran, 2009; Balaji *et al*., 2009). Thus, the higher stability of the synthesized AgNPs could be attributed to the complex nature of the *Pseudomonas aeruginosa* KUPSB12 strain culture supernatant (Malhotra *et al.*, 2013; Mishra *et al.*, 2014).

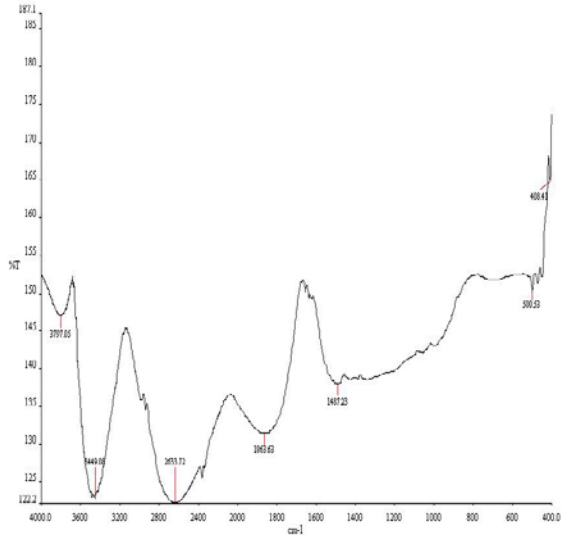

Figure 5. FTIR spectra of synthesized silver nanoparticles

Scanning electron microscopy (SEM) was used to determine the size and shape of the synthesized nanoparticles. SEM images revealed the average size of particles as 50-85 nm. SEM images show that they are relatively uniform in diameter and have a spherical shape (Figure 6). The size ranges of silver nanoparticles produced by the *P. aeruginosa* KUPSB12 fall closer to the size of silver nanoparticles produced by other bacteria (Shahverdi *et al*., 2007; Das *et al.*, 2014).

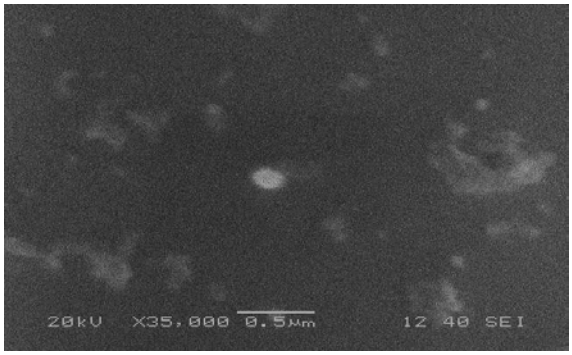

Figure 6. SEM image of synthesized silver nanoparticles

The antibacterial activities of synthesized silver nanoparticles were tested against six pathogenic bacteria as shown in Table 2. The silver nanoparticles exhibited antibacterial activity against both Gram positive and Gram-negative bacteria. The highest inhibition zone of 19.0 mm diameter was formed against *Escherichia coli* and the lowest of 13.6 mm was produced against *Staphylococcus aureus* by the synthesized nanoparticles. In general, Ag ions from nanoparticles are believed to become attached to the negatively charged bacterial cell wall and lyse it, leading to protein denaturation and finally cell death (Lin *et al.*, 1998). Priyadarshini *et al.* (2013) reported that the Gram negative bacterium *E. coli* showed a greater antibacterial activity compared to that of the Gram positive bacteria *Bacillus cereus* and *Streptococcus pyogenes* which was probably due to their thick cell walls.

Table 2. Antibacterial activity of synthesized silver nanoparticles against tested pathogenic bacteria (mean ± SD)

Tested bacteria	Zone of inhibition (mm in diameter)
Escherichia coli	19.0 ± 0.24
Vibrio cholerae	15.3 ± 0.28
Shigella flexneri	16.0 ± 0.34
Bacillus subtilis	17.6 ± 0.21
Staphylococcus aureus	13.6 ± 0.36
Micrococcus luteus	18.6 ± 0.18

The exact mechanism behind the extracellular synthesis of nanoparticles using microbes is not clearly established. But it is believed that enzymes like nitrate reductase secreted by microbes help in the bioreduction of metal ions to metal nanoparticles (Duran *et al*., 2005). Such a mechanism was found to be operative in *Bacillus licheniformis* where nitrate reductase secreted by the bacteria was found to be responsible for the reduction of Ag^+ to nanoparticles (Kalimuthu *et al*., 2008). Nangia *et al.* (2009) also suggested that the biosynthesis of

nanoparticles and their stabilization via charge capping in *Stenotrophomonas maltophilia* involved NADPH-dependent reductase enzyme through electron shuttle enzymatic metal reduction process.

4. Conclusions

In conclusion, we have reported the simple biological way for synthesizing the silver nanoparticles using the culture supernatant of *P. aeruginosa* KUPSB12. The present investigation indicates the extracellular synthesis of highly stable silver nanoparticles. The results of FTIR suggested that the protein might have played an important role in the stabilization of silver nanoparticles. Synthesized silver nanoparticles showed a potent antibacterial activity against six pathogenic bacterial strains. These study results demonstrated that the phosphate solubilizing bacteria *P. aeruginosa* KUPSB12 is a cheap and environment-friendly bio-resource for the synthesis of silver nanoparticles with antibacterial activity. Considering the significance of phosphate solubilizing bacteria an agriculturally important microbes, their utilization to synthesize AgNPs with potent antibacterial properties can certainly provide an alternate means for plant protection. Further studies are required on fundamental understanding of the mechanism of nanoparticles synthesis at cellular and molecular levels.

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Antioxidant and Hepatoprotective Activity of Fruit Extracts of *Tetrapleura tetraptera* (Schum & Thonn) Taubert

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Abstract

The antioxidant and hepatoprotective activity of *Tetrapleura tetraptera* extracts in carbon tetrachloride (CCl_4)-induced liver injury in rats was investigated. *T. tetraptera* extracts showed varying levels of protective action against CCl_4-induced liver damage as evidenced through the significant reduction in the activities of serum marker enzymes for liver damage (alanine transaminase, aspartate transaminase, and alkaline phosphatase), and bilirubin levels when compared with CCl_4-intoxicated control rats. The extracts decreased the elevation in the activities of the enzymes in the liver. They also protected against CCl_4 induced lipid peroxidation. The extracts reduced CCl_4-liver induced necrosis in dose dependent manner. These results indicated that fruit extracts of *T. tetraptera* possess a hepatoprotective property against CCl_4-induced liver damage which was mediated through its antioxidative defenses.

Keywords: *Tetrapleura tetraptera*, Antioxidant, Hepatoprotective, Serum marker enzymes, Vitamins.

1. Introduction

Oxidative Stress is a term used to describe the oxidative damage of cells, tissues, or organs, caused by the Reactive Oxygen Species (ROS) (Noda and Wakasugi, 2001). It is caused by an imbalance between the production of reactive oxygen species and the detoxifiers (antioxidants). The most common source of ROS is the leakage of activated oxygen from mitochondria during normal oxidative respiration. Enzymes capable of producing superoxide are xanthine oxidase, reduced nicotinamide adenine dinucleotide phosphate oxidases and cytochrome P450. ROS play an important role in cell signaling and are constantly cleared by the auto antioxidants such superoxide dismutase, catalase, glutathione peroxidase and glutathione-s-transferases. Oxidative stress has been linked to disease conditions like inflammation (Cai *et al.*, 2004), cancer (Banerjee *et al*., 2005), diabetes, cataracts and aging (Halliwell and Gutteridge, 1999). The liver is one of the largest organs in human body and the chief site for intense metabolism and excretion. Liver helps in maintaining and regulating the homeostasis of the body.

Tetrapleura tetraptera (Schum & Thonn), Taubert (*Fabeceae*) is a tree that grows in the tropical deciduous forest of West Africa, extending from Senegal to West Cameroon; it is also found in Sudan, Uganda and Zaire (Burkill, 1995). The aqueous or alcoholic decoctions of the plant are used in herbal medicine for the management of convulsion, leprosy, inflammation and or rheumatoid pains (Dalziel, 1948). Extracts of *T. tetraptera* have been reported to have antischistosomiasis (Adetunji, 2007), antiulcer (Kota *et al*., 2012), antimicrobial (Ekwenye and Okorie, 2010), contraceptive (El-Izzi *et al*., 1990) and anticonvulsant (Akah and Nwambie, 1993) activities. The fruits and seeds of *T. tetraptera* are used in the Niger Delta region of Nigeria for various ailments and have a significant antiplasmodial effect (Lekana-Douki *et al*., 2011; Igwe *et al*., 2012). The anti-inflammatory and hypoglycaemic effects of *T. tetraptera* (Taub) fruit aqueous extract in rats has been investigated using egg albumin-induced pedal oedema and streptozotocin (STZ)-induced diabetes mellitus (Ojewole and Adewunmi, 2004). The pods notably have an appealing culinary use for mothers from the first day of delivery to post parturition and as a lactation aid (Ekwenye and Okorie, 2010). Here we investigated the antioxidant and hepatoprotective activities of the fruit extracts of *T. tetraptera*.

2. Materials and methods

2.1. Material

The fruits of *Tetrapleura tetraptera* were purchased in Ogige Market Nsukka, Enugu State. The chemicals used for this work were purchased from Sigma-Aldrich, Germany. Serum alanine aminotransferase (ALT) and Serum aspartate aminotransferase (AST) kits

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(Randox,U.K), a lkaline phosphate (ALP) kit (Quimica Clinica Aplicada, Spain), serum bilirubin kit (Boehringer Mannheim GmbH, Diagnostica, Germany) and UV/Visible Spectrophotometer (Perkin Elmer, Lambda 25) were used in the study.

2.2. Extraction Procedure

Five hundred grams (500 g) of the pulverized fruit of *T. tetraptera* were extracted with *n*–hexane (1.0 L). The marc was dried and re-extracted with methanol (1.0 L) using Soxhlet method. The extracts were concentrated *in vacuo* at 40ºC and freeze-dried to produce crispy extracts.

2.3. Animals

Wistar rats and mice of both sexes were obtained from the Department of Veterinary Medicine, University of Nigeria Nsukka. The animals were maintained on standard pellet diet and tap water *ad libitum* and acclimatized for 14 days before use. The use and care of laboratory animals were conducted in accordance with the best internationally accepted practices that are contained in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and approved by the local Ethics Committee of our institution.

2.4. Design for Hepatoprotective Assay

The rats were divided randomly into nine groups of six rats each. The hepatoprotective activity of the plant extracts was tested using CCl_4 model. Group I (normal control) received neither the plant extract nor CCl_4 for 5 days, i.e., they received only food and water. The rats were treated with the extracts as a fine suspension in 3% Tween 80 for 5 days. The test samples were administered to rats for 2 days before the injection of CCl_4 on the third and fourth days.

Group I: Served as normal control and received only food and water.

Group II: Served as induction control and received 0.75 mg/kg b.wt of CCl_4

Group IIIA: Received 100 mg/kg b.wt of *n*-hexane extract

Group IIIB: Received 300 mg/kg b.wt of *n*-hexane extract

Group IIIC: Received 500 mg/kg b.wt of *n*-hexane extract

Group IVA: Received 100 mg/kg b.wt of methanol extract

Group IVB: Received 300 mg/kg b.wt of methanol extract

Group IVC: Received 500 mg/kg b.wt of methanol extract

Group V: Served as positive control and received Vitamin E (1000 UI)

2.5. Assay for Hepatoprotective activity of the Extracts

The hepatoprotective activity of the extracts were assessed biochemically and histopathologically

2.5.1. Biochemical evaluation of ALT and AST

The animals were treated for 5 days and sacrificed under chloroform anesthesia. Blood samples from each rat were withdrawn directly by heart puncture with syringes into properly labeled EDTA bottles and allowed to clot for 1 h a t room temperature. Serum was separated by centrifugation at 3000 r pm and 40°C for 20 min. The separated serum was used for the estimation of the biochemical parameters like Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and bilirubin. Serum ALT and AST were determined colorimetrically according to the method of Reitman and Frankel (1957) with slight modification. Briefly, ALT and AST determination was done by monitoring the concentrations of pyruvate hydrazone formed with 2, 4 dinitrophenyl hydrazine. 0.5 ml of Glutamic-Pyruvic Transaminase (GPT) substrate phosphate buffer solution was added into test tubes labeled blank, sample, control blank and control, respectively, for ALT and AST, respectively. 0.1 ml serum sample was added to the test tubes and the tubes were incubated at 37ºC for 30 min. A volume of 0.5 ml each of 2, 4-Dinitrophenylhydrazine was added into all the test tubes. 0.1ml of sample and control was added into their respective blank test tube. The contents of each test tube was mixed and allowed to stand for 20 min at 20-25ºC. 5.0 ml of 0.4N sodium hydroxide was added to each tube, mixed and their absorbencies read at 546 nm against the respective blank prepared.

2.5.2. Biochemical evaluation of Alkaline Phosphatase

Alkaline Phosphatase activity was done by Phenolphthalein monophosphate method (Braide *et al*., 2011). Briefly, test tubes were respectively labeled sample, standard and control. A volume of 0.1 ml of distilled water was pipette into the test tubes followed by a drop of the substrate (Phenolphthalein monophosphate) into each of the test tubes. The contents in the tubes were mixed and incubated at 37ºC for 5min in a water bath. A volume of 0.1 ml of sample, standard and control were added into the respective test tubes and the tubes incubated at 37ºC for 20 min. A volume of 5.0 ml of color developer was added to each test tubes, mixed, and their absorbance read at 550 nm using water as blank. The activity of sample was calculated using the absorbance of sample against absorbance of standard multiplied by concentration of standard (Babson *et al*., 1966).

2.5.3. Biochemical evaluation of Bilirubin

The total and direct bilirubin activity was assessed by colorimetric method as outlined in the diagnostic kit described by Sunday *et al*. (2014).

2.5.4. Histopathological study

For the histopathological study, liver from each rat was removed, sliced and preserved in 10% neutral formalin solution for 7 da ys. Thin sections of the liver were washed and dehydrated in ethanol and subsequently immersed in xylene solution for 15 mins. Then representative blocks of the liver tissues were taken and possessed for paraffin embedding using the standard microtechnique (Ahsan *et al*., 2009). Thin sections (5 µm) of the livers were stained with haematoxylin and eosin (H & E) and observed microscopically for histopathological studies.

2.6. Assay for Antioxidant activity

The lipid peroxidation was determined as ThioBarbituric Acid-Reactive Substance (TBARS) as described by Atawodi *et al*. (2014) based on the principle that peroxide intermediates generated release malondialdehyde (MDA) upon cleavage (Torres *et al*., 2004). The thiobarbituric acid-reactive substance and malondialdehyde react with thiobarbituric acid to give a red or pink adduct which absorbs maximally at 532 nm. One ml of serum was added 0.45 ml of normal saline and mixed thoroughly before adding 0.5 ml of 25% trichloroacetic acid and 0.5 ml of 1% thiobarbituric acid. A blank was prepared with distilled water. The mixture was incubated at 95^{o}C for 40 min in a water bath followed by centrifugation at 3000 r pm for 10 min. MDA was measured colorimetrically at 532 nm and the level of thiobarbituric acid reactive substances were quantified as lipid peroxidation products by referring to a standard curve of malondialdehyde (MDA) concentration (Asuku *et al*., 2012).

2.7. Statistical Analyses

A statistical analysis of the results was performed using one-way analysis of variance (ANOVA), using GenStat 7.22 Discovery Edition 3 (VSN International Limited). All values were expressed as mean ± S.D., and a value of $P < 0.05$ was considered significant as compared to the respective control group.

3. Results

3.1. Biochemical and antioxidant parameters

Three doses of the *n*-hexane and methanol extracts of *Tetrapleura tetraptera* were evaluated for their antioxidative and hepatoprotective activities in carbon tetrachloride (CCl_4)-induced liver injury in rats. It was observed that the extracts reduced the level of liver function biomarker (ALT, AST, ALP, and bilirubin) and antioxidant parameter (MDA) compared with the CCl_4 group. The levels of the biochemical parameters (ALP, AST, ALT) in the sera were elevated in CCl_4 treated animals, indicating liver damage. Also, the levels of these biochemical parameters in the rats treated with *Tetrapleura tetraptera* fruit extracts showed a remarkable reduction (Table 1 and 2). The LPO levels by thiobarbituric acid reaction showed an increase in LPO in the CCl_4 treated rats. Treatment with *Tetrapleura tetraptera* fruit extracts at 100-500 mg/kg significantly ($P < 0.005$) reduced the LPO level comparable to the normal control (Table 3).

Table 1. Effect of extracts of *Tetrapleura tetraptera*, on various biochemical parameters in rats with carbon tetrachloride induced hepatotoxicity.

Treatment	Doses (mg/kg)	ALP (IU/L)	AST (IU/L)	SGPT (IU/L)
Normal control	-	50.67±14.36	10.33±2.98	8.6±1.75
CCl_4	0.75	107.0±7.73	20.0±3.53	17.59±5.24
CCl_4 + *n*-hexane extract	100	54.50±7.79*	15.50±3.64	16.67±2.63*
	300	54.03±6.39	10.90±2.23	14.0±3.81
	500	50.50±16.46	7.10±0.64	12.20±1.73
CCl_4 + Methanol extract	100	66.00±19.05*	13.40±1.30	16.96±5.01*
	300	57.53±14.06	11.40±4.16	14.23±5.11
	500	56.23±9.73	10.50±4.85	11.85±5.08
Vitamin E	1000 IU	66.47±6.67*	11.17±3.22	12.93±3.74

All values are mean ±SD, n=6 rats in each group. *P<0.005 as compared with the normal control.

Table 2. Total and direct bilirubin levels in rats treated with extracts of *T. tetraptera* fruits for 5 d.

Treatment	Dose (mg/kg)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
Normal control	-	1.03±0.26	0.93±0.66
CCl_4	0.75	1.85±0.26	0.37±0.05
CCl_4 + *n*-hexane extract	100	1.34±1.65	0.27±0.33*
	300	1.10±0.00	0.22±0.00
	500	0.93±1.58	0.19±0.31*
CCl_4 + Methanol -extract	100	1.35±0.43	0.27±0.09
	300	1.18±0.57	0.24±0.12*
	500	1.13±0.74	0.23±0.07*
Vitamin E	1000 IU	1.05±0.31	0.21±0.07

All values are mean ±SD, n= 6 rats in each group. *P<0.005 as compared with the normal control.

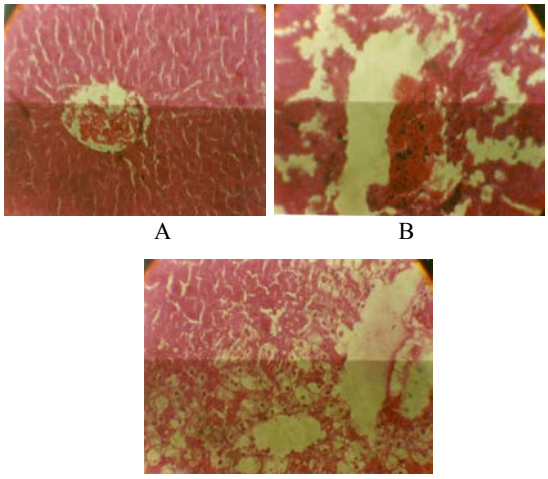
Table 3 Malondialdehyde (MDA) levels in rats treated with extracts of *T. tetraptera* fruits for 5 d.

Treatment	Dose (mg/kg)	TBARS (µg/ml)
Normal control	-	6.25±0.00
CCl_4	0.75	14.17±11.80
CCl_4 + *n*-hexane extract	100	9.50±2.96*
	300	7.00±4.76
CCl_4 + methanol extract	100	12.36±2.82
	300	9.75±2.49*
	500	8.86±4.12
Vitamin E	1000 IU	9.67±1.84

All values are mean ±SD, n= 6 rats in each group. *P<0.005 as compared with the normal control.

3.2. Histological Observations

The histopathological study of the liver treated with CCl_4 and the extracts are shown in Figure 1. The CCl_4 treated rat liver shows a damage of hepatocytes with hepatocellular vacuolization, focal necrosis and congestion of hepatic sinusoids while the liver from the extract treated rats showed a mild vacuolization.



A B C

Figure 1. Histopathology of (a): normal liver having histological structures of normal hepatic lobules; (b): Toxicant treated liver (CCl_4, 0.75 mg/kg body weight) showing damage to hepatocytes with hepatocellular vacuolization, focal necrosis and congestion of hepatic sinusoids;(c): *Tetrapleura tetraptera* (methanol extract) treated liver (200 mg/kg body weight) showing mild vacuolization. H & E x400.

4. Discussion

The liver is very susceptible to damage by xenobiotics and non-xenobiotics induced oxidative stress. Liver damage is manifested in increased serum levels of aspartate transaminase (AST) or serum glutamate oxaloacetate transaminase (GOT), alanine transaminase (ALT) or serum glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP) and serum bilirubin, as well as pronounced necrosis of hepatic cells. Carbon tetrachloride (CCl_4) induced hepatotoxicity model is widely used for the study of hepatoprotective effects of drugs and plant extracts (Ahsan *et al*., 2009). Treatment of animals with *T. tetraptera* extracts and Vitamin E significantly reduced the level of liver function biomarker (ALT, AST, ALP, and bilirubin) and antioxidant parameter (MDA) compared with the CCl_4 group. The test extracts mediated reduction in levels of AST, ALT as well as repair of hepatic tissue damage caused by CCl_4. These effects are in agreement with the commonly accepted view that serum levels of transaminase return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew *et al*., 1987). Liver damage is manifested in increased serum levels of ALP, AST, ALT and bilirubin; as well as, pronounced necrosis of hepatic cells. The rats that received the extracts had a significant decrease in serum ALP, AST, ALT and bilirubin when compared with those of the normal control rats (Tables 1-2). These indicate that the extracts have a hepatoprotective effect that was found to be more pronounced at higher doses. The *n*-hexane extract showed a higher hepatoprotective activity than the methanol extract. Studies have shown that phenolic compounds and flavonoids present in plant extracts enhance their hepatoprotective activities (Maheswari *et al*., 2008). Thus, the observed hepatoprotective activity of the fruit extract could be attributed to the presence of flavonoids in the fruit extract.

Lipid peroxidation induced by CCl_4 is a commonly used experimental animal model for studying oxidative injury in biological systems (Srilakshmi *et al*., 2010). Cytochrome P-450 enzymes are believed to metabolize CCl_4 to trichloromethyl radicals that can initiate peroxidation of unsaturated fatty acid and initiate chain reactions of lipid peroxidation. The lipid solubility of CCl_4 allows it to cross the cell membrane and to be distributed to the organs. However, the liver is the major target organ of CCl_4–induced toxicity owing to its high content of cytochrome P-450 enzyme. Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a n umber of degradation products. During lipid peroxidation, malonaldehyde (MDA), one of the thiobarbituric acid reactive substances (TBARS) is produced and has been studied widely as an index of lipid peroxidation and as a marker of oxidative stress (Njayou *et al*., 2013). In the detoxification process of CCl_4 from the body, free radicals were generated which can initiate lipid peroxidation and reactions leading to the generation of thiobarbituric acid reactive substance (TBARS). The extracts lowered TBARS in the sera of treated rats compared with those of the normal control rats. The *n*-hexane extract lowered TBARS more than the methanol extract (Table 3). This is an indication that the extracts have antioxidative potential. The fruit extracts of *T. tetraptera* lowered the formation of TBARS in a concentration-dependent manner. This suggests that the fruit extracts prevent the oxidation of cell membranes lipids. Secondary metabolites like flavonoids have been isolated from the stem bark of *Tetrapleura tetraptera* (El-Izzi *et al*., 1990). Flavonoids have been reported to have antioxidant activity (Kostić *et al*., 2013). The antioxidative activity reported in our study could be attributed to these flavonoids present in the fruit.

The result of the histopathological studies provided supportive evidence for biochemical analysis. Histology of liver section of normal control rats exhibited normal hepatic cells each with well defined cytoplasm, prominent nucleus and nucleolus and well brought out central vein whereas that of CCl_4 intoxicated group animal showed total loss of hepatic architecture with hepatocellular necrosis and wide area of hyperaemia. The liver section of the rats treated with 300 mg/kg b.wt methanol extract showed patches of hepatic cells undergoing mild degeneration and produced mild necrosis of hepatocytes in the portal areas (Figure 1).

5. Conclusion

The *n*-hexane and methanol extracts of *Tetrapleura tetraptera* have both antioxidative and hepatoprotective properties, which have been attributed to presence of secondary metabolites in the extracts. Hence further studies are required for the standardization of these fruit extracts.

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Effect of Fenugreek (*Trigonella foenum-graecum*) on Ethylene Glycol Induced Kidney Stone in Rats

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Abstract

Fenugreek is one of several herbal medicines whose seeds and leaves are used either as food or as an ingredient in folk medicine. In the existing literature, there is evidence regarding the effects of fenugreek on ethylene glycol induced kidney stone formation of rats. Regarding the other drugs, such as Cystone, the seeds of *Trigonella foenum-graecum* (fenugreek) are reported to have been used as anti-urolithiatic in traditional medicine. Thus, the present study was undertaken to investigate the effect of fenugreek on the prevention of kidney stone formation. Twenty male albino rats were divided into 4 groups: Normal, Ethylene Glycol (EG), Cystone and Fenugreek. The duration of the experiment was 28 days. Ethylene glycol group led to increases in kidney weight, malondialdehyde (MDA) and platelet count, while Cystone and Fenugreek combat the effect of EG. Haematological examination showed that the hemoglobin and red blood cell count in rats treated EG were significantly lower than those in the controls while Fenugreek and Cystone decreased the EG effect. Our studies demonstrate the anti-urolithiatic and anti-oxidative potential effects of *T. foenum-graecum*, which could exert beneficial effects against the kidney stone formation and the associated free radicals complications in kidney tissues. Further clinical trials are needed for evaluating its benefits and the possible side effects.

Keywords: Fenugreek, kidney stone, Ethylene glycol, Cystone, rats.

1. Introduction

Trigonella foenum-graecum (Linn.) (Tfg) is an annual dicotyledon herb belonging to the family Fabaceae commonly known as fenugreek; it is 30 to 60 cm tall, cultivated throughout Asian countries (Shishtoppo *et al*., 2009). 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). Its seeds are considered to be of commercial interest as a source of a steroid diosgenin, which is important in the pharmaceutical industry. In Iranian traditional medicine, the seeds are used as tonic and blood sugar lowering (Nasroallah and Kolsum, 2013). Fenugreek was used to ease childbirth and to increase milk flow. It is still taken by Egyptian women for menstrual pain and as hilba tea to ease stomach problems of tourists (Rashmi *et al*., 2011). Renal stone is one of the most painful urologic disorders. Urolithiasis is the medical term used to describe stones occurring in the urinary tract. Calcium containing stones, especially calcium oxalate monohydrate (whewellite), calcium oxalate dihydrate (weddellite) and basic calcium phosphate (Javed *et al*., 2011). Kidney stone formation is a complex process that results from a succession of several physico-chemical events including supersaturation, nucleation, growth, aggregation and retention within renal tubules (Vijaya, *et al*., 2013).

Rashmi and his worker showed that the fenugreek seeds reduces the amounts of calcium oxalate in the kidneys, which often contributes to the production of kidney stones (Rashmi *et al*., 2011). Laroubi and his worker showed that the amount of calciifcation in the kidneys and the total calcium amount of the renal tissue in rats treated with Tfg were signiifcantly reduced compare with untreated group (Laroubi *et al*., 2009). Consequently, Tfg may be a useful agent in the treatment of patients with calcicurolithiasis (Laroubi *et al*., 2007).

The present study was designed to investigate the anti-urolithiatic activity of fenugreek and its effect on some physiological parameters.

2. Materials and Methods

2.1 Plant Materials

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

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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 normal group (N) which was supplied with normal water and diet.

Depending on Cystone leaflet it was comprised of the following substances: shilapuspha (Didymocarpus pedicellata) 130mg, Pasanabheda (Saxifaga lgulata Syn. Bergenia ligulata/ciliata) 98 mg, Manjishtha (Rubia cordifolia) 32 mg, Nagarmusta (Cyperus scariosus) 32 mg, Apamarga (Achyranthes aspera) 32 mg, Gohija (Onosma bracteatum) 32 mg, Sahadevi (Vernonia cinerea) 32 mg, Shilajeet (Purified) 26 mg, and Hajrul yahood bhasma 32 mg. Group (E): drinking water was supplemented with EG (1%) for 28 days.

Group (C) was given 2.5 tablets of Cystone (750 mg\ kg body weight) in 100 ml of water and 2.5 tablets in 100 g of standard diet.

Group (F) was given 10 gm of fenugreek in 100 ml of water and 10 gm in 100 gm of standard diet.

At the end of the experiment, the blood (hematological parameter and serum for electrolyte determination) and urine samples were collected for determining the presence of any CaOx in kidney.

2.3 Statistical Analysis

The results were presented as means ± S.E.M and the comparison between the experimental groups were made using Newman-keuls test and ANOVA. *: $P < 0.05$ was considered as indicative of degree of significance by using GrphaPad prism 6.0.

3. Results and Discussion

From the results of the present study, it was observed that ethylene glycol caused an increase in kidney weight and kidney/body weight ratio when compared with normal; our results are comparable with results of Schladt *et al*. (1998) who concluded that a significant increase in kidney weights in rats treated with ethylene glycol which induced necrosis, fibrosis and crystal deposition in renal tubules (Figure 1).

Cystone decreases kidney weight and kidney/body weight ratio when compared with EG group (Figure 2). This might mean that Cystone prevents the accumulation, deposition and super saturation of calciurologenic chemicals in the kidneys. Likewise, oxalic acid and calcium hydroxyproline were reduced in urine. This action inhibits the formation of kidney stones (Rafiq *et al*., 2012). The fenugreek highly significant decrease of kidney weight is clear when compared with EG group; this indicates that fenugreek may be protective against kidney stones.

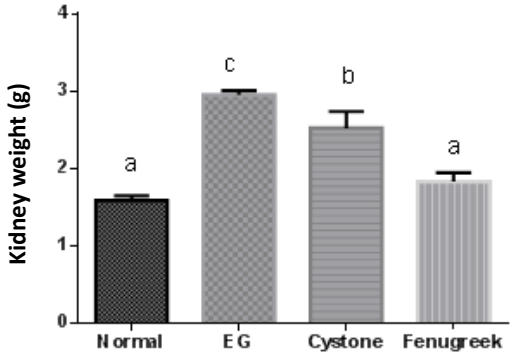

Figure 1. The effect of Fenugreek seeds on kidney weight of rats. Each column and vertical bar represents mean ± SEM of 5 animals. Normal, EG, Cyctone and Fenugreek.

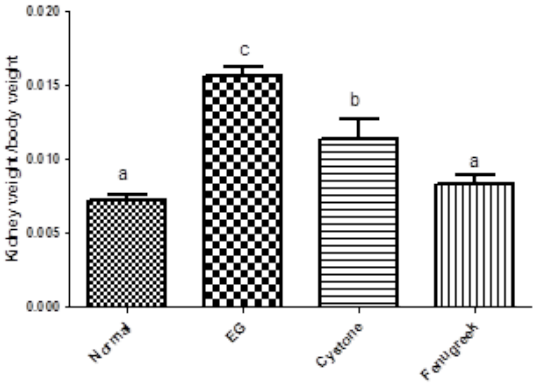

Figure 2. The effect of Fenugreek seeds on kidney/body weight ratio of rats. Each column and vertical bar represents mean ± SEM of 5 animals. Normal, EG, Cyctone and Fenugreek

Body weight gain was significantly reduced in rats of the ethylene glycol group during the days of the experiment (Figure 3); these results are in agreement with the result of Gaunt *et al*. (1974). While treated groups of Cystone and fenugreek, when compared with the untreated group, decreased body weight because fenugreek decreases body fats and is effective on obesity but the differences between them is not statistically significant (Nasroallah and Kolsum, 2013) (Figure 3).

Supplementation of fenugreek in group 4 signiifcantly countered EG induced renal hypertrophy when compared to the control group 1 since its seeds contain alkaloids, including trigonelline, gentianine and carpine compounds, unique amino acid, 4-hydroxy isoleucine (Ajaya *et al*., 2009).

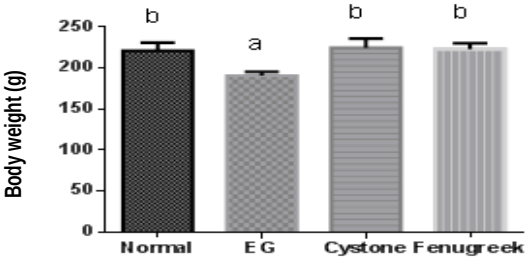

Figure 3. The effect of Fenugreek seeds on body weight of rats. Each column and vertical bar represents mean ± SEM of 5 animals. Normal, EG, Cyctone and Fenugreek.

Animals treated with EG for 28 days revealed a significant increase in the MDA level with a mean value as compared with control group which was 36.67 ± 1.188 nmol/ml (Figure 4). On the other hand, animals treated with Cystone showed a decrease in MDA level in sera compared with EG group; after 28 days, the mean value was 8.484 ± 1.023 while the mean value for fenugreek was 8.18 ± 1.125 which showed high significant differences ($P < 0.001$) when compared with EG group. It counteracts its effect to the normal level 7.804 ± 0.3053.

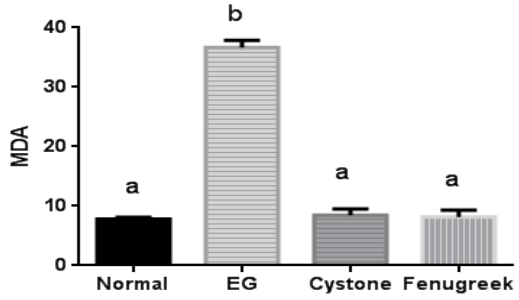

Figure 4. The effect of Fenugreek seeds on MDA of rats. Each column and vertical bar represents mean ± SEM of 5 animals. Normal, EG, Cyctone and Fenugreek

Devi *et al*. (2012) showed that fenugreek led to decrement in MDA level in fenugreek treated rats, which could be due to the consumption of the extracellular antioxidant, ceruloplasmin, by the oxidants, to combat oxidative stress.

The ethylene glycol administration resulted in non-significant increases in the level of serum values of sodium, potassium, chloride and calcium (Figures 5, 6 and 7) compared with normal rats; these results are in agreement with the results of Sunitha *et al*. (2012). The present results are comparable with the study of Al-Atwi (2010), who investigated the serum values of sodium, potassium, chloride and calcium and did not reveal any significant change in Cystone and fenugreek treated rats when compared with the control values.

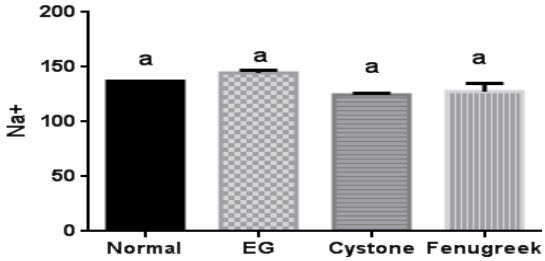

Figure 5. The effect of Fenugreek seeds on Na+ of Rats. Each column and vertical bar represents mean ± SEM of 5 animals. Normal, EG, Cyctone and Fenugreek

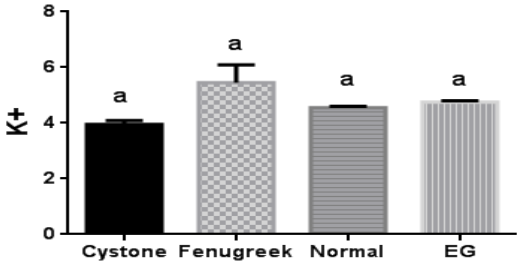

Figure 6. The effect of Fenugreek seeds on K+ of rats. Each column and vertical bar represents mean ± SEM of 5 animals. Normal, EG, Cyctone and Fenugreek.

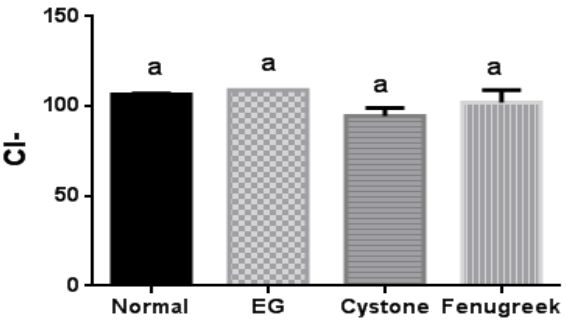

Figure 7. The effect of Fenugreek seeds onCl-+ of Rats. Each column and vertical bar represents mean ±SEM of 5 animals. Normal, EG, Cyctone and Fenugreek

The results of the present study showed a significant decrement in RBC count and hemoglobin concentration (Figures 8, 9), which leads to anaemia in the EG group and our results are comparable with the study of Shih *et al*. (2000) who proposed that anemia is caused by a bone marrow toxin rather than haemolysis or peripheral toxicity.

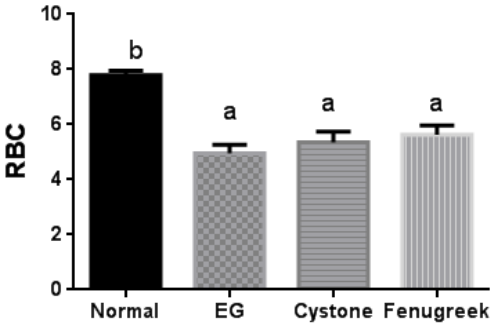

Figure 8. The effect of Fenugreek seeds on RBC of Rats. Each column and vertical bar represents mean ± SEM of 5 animals. Normal , EG, Cyctone and Fenugreek

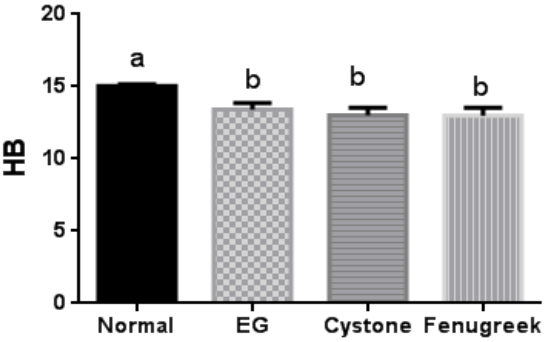

Figure 9. The effect of Fenugreek seeds on Hb of Rats. Each column and vertical bar represents mean ± SEM of 5 animals. Normal, EG, Cyctone and Fenugreek

These ifndings are consistent with previous studies which showed that the high exposure to EG will induce haematological defects. Haematological disorders induced by exposure to EGME have also been conifrmed in anima studies. The haematological toxicity in animals included decreased concentrations of haemoglobin, packed cell volume, white and red blood cell counts, bone marrow cellularity, and erythropoiesis and enhanced haemolysis (NIOSH, 1992).

The reduction in RBC count and hemoglobin was observed in the 15 days treated animal with EG and was also found to be significantly restored in fenugreek and

Cystone treated rats (Sindhu *et al*., 2012) (Figures 8 and 9). Platelet count, obtained in the present study, demonstrated that non-significant change in EG, Cystone and fenugreek when compared with normal rats (Figure 10).

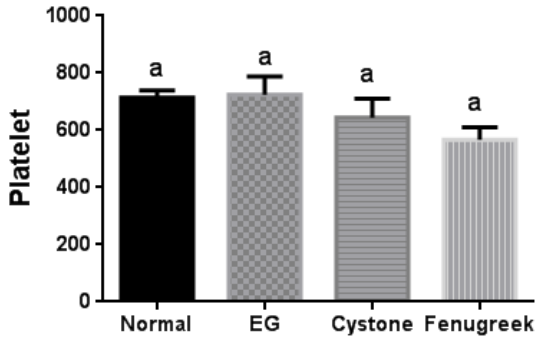

Figure 10. The effect of Fenugreek seeds on platelet of rats. Each column and vertical bar represents mean ± SEM of 5 animals. Normal, EG, Cyctone and Fenugreek

The microscopic examination of urine revealed the presence of CaOx in EG rats, but in Cystone and fenugreek groups, no CaOx appeared, it was similar more or less to that normal group as shown in Figure 11.

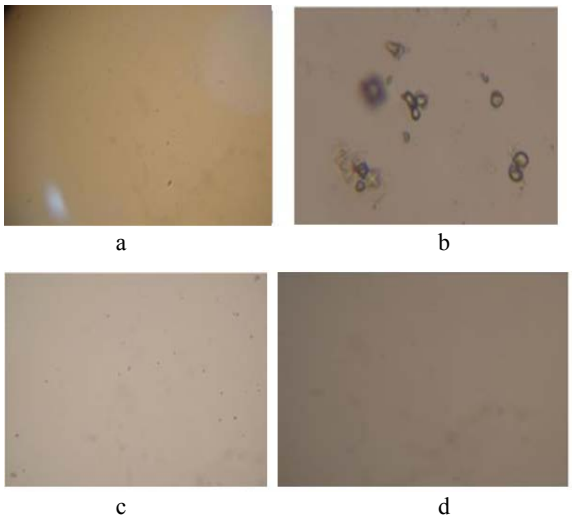

Figure 11. (a) Presence of CaOx in urine smear in Normal rats; (b) Urine smear in EG rats; (c) Urine smear in Cystone rats, (d) Urine smear in Fenugreek rats

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Effect of Probiotic Hummus on Blood Lipids of Rats

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Abstract

The present study investigates the synbiotic effect of probiotic bacteria and hummus as a prebiotic on blood lipids in Sprague-Dawley rats. A developed probiotic hummus that contained *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bb-12 was added to a previously heated hummus at 75ºC/5 min., followed by an anaerobic incubation at 37ºC/8 h. The experimental diets included cholesterol diet, probiotic + cholesterol diet, hummus + cholesterol diet and probiotic hummus + cholesterol diet. Animals were divided randomly, according to their weights, into four groups (8 rats/group). Each group of the rats was fed one of the four diets for 8 weeks. Inclusion of probiotics to the cholesterol diet showed hypocholesterolemic effect, since it significantly (p<0.05) decreased TC and LDL-C by 14.5% (from 73.38 to 62.75) and 28.5% (from 19.70 to 14.08), respectively, as compared with the control group. No significant effects (p> 0.05) in HDL-C and TG were shown due to this inclusion. The hummus addition to the cholesterol diet caused a significant (p<0.05) reduction of 9.0% (from 73.38 to 66.75), 22.1% (from 19.70 to 15.35) and 14.0% (from 93.88 to 80.75) in TC, LDL-C and TG, respectively. The combined effects of probiotics and prebiotics in probiotic hummus + cholesterol diet caused significant (p<0.05) reductions of 14.1% (from 73.38 to 63.00), 27.5% (from 19.70 to 14.29) and 24.4% (from 93.88 to 71.00) in TC, LDL-C and TG, respectively. It could be concluded that the addition of probiotic hummus to the cholesterol-rich diet caused significant reductions in TC, LDL-C and TG. However, these reductions were not significantly different from those reductions caused by the addition of probiotic or hummus alone except for TG.

Key words: Probiotics, Hummus, Blood lipids, Rats, *Lactobacillus*, *Bifidobacteria.*

1. Introduction

Widespread interest in the possibility that selected foods might promote health has resulted in the coining of the term "functional foods." Prebiotics and probiotics may positively affect various physiologic functions of the body that will permit them to be classified as functional foods (Douglas and Sanders, 2008). Probiotic bacteria have been the focus of much scientific and commercial interest. This interest is due to a range of possible health effects of these bacteria (O'Bryan *et al*., 2013).

Cardiovascular diseases (CVD) are an important public health concern. In Western countries, they are considered as major causes of mortality and morbidity (Jones *et al*., 2013). In Jordan, the analysis of the mortality data of 2008 showed that the number one killer is CVD with a 36.1 % (Ministry of Health (MOH), 2011). For more than 20 years, the primary focus of public health strategies has been aiming at reducing risk of the coronary heart disease (CHD) and atherosclerosis, by reducing cholesterol concentration in circulating blood (Lye *et al*., 2010).

It was reported that there is a relationship between the consumption of probiotics and the reduction of serum cholesterol levels in human beings (Xiao *et al*., 2003), rats (Liong and Shah, 2006), and hens (Alkhalf *et al*., 2010). Certain strains of probiotic bacteria act directly on bile acids in the gastrointestinal tract (GIT) and are beneficial in reducing serum cholesterol levels (Lye *et al*., 2010).

Legumes, including chickpeas, are one of the most important crops in the world because of their good nutritional quality (Wang *et al*., 2010). Legumes have shown numerous health benefits, e.g., lowering of glycemic index for people with diabetes, increased satiation, cancer prevention and protection against cardiovascular diseases due to their dietary fiber content (Tosh and Yada, 2010). The resistant starch and the raffinose family of oligosaccharides (the α-galacto-oligosaccharides raffinose, stachyose and verbascose), which are found in appreciable concentrations in legumes, are potential prebiotics. The total contents of these a-galacto-oligosaccharides in dry beans, peas, lentils and chickpeas range from 2 to 10 g/100 g dry weight (Tosh and Yada, 2010). The resistant starch content in chickpea is 2.3 g/100 g dry weight (Queiroz-Monici *et al*., 2005).

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Many traditional foods in Jordan and most Arab countries are based on legumes as a raw material. Hummus, chickpeas paste, is one of the most popularly consumed traditional foods in our region (Fares and Takruri, 2002). With the globalization of the food market, the consumption of hummus has increased dramatically**.** The paste is made of ground re-hydrated cooked seeds of the chickpea legume, to which salt, spices and in many cases sesame seeds paste or 'Tahini' (*Sesamum indium*) are also added (Yamani and Al-Dababseh, 1994). Hummus is considered a relatively cheap protein source and has a nutritive and cultural value (Fares and Takruri, 2002).

Nowadays, there is an increasing trend toward using probiotics in different food systems and in the global market for such functional foods are on the increase (Sudha *et al*., 2009). Probiotics have been proved to be beneficial for health since their use reduced blood cholesterol both in experimental animals and humans; Xiao *et al*. (2003) reported a 22%, 41.2% and 13.2% significant reduction in serum TC, LDL-C and TG concentrations, respectively of rats fed bifidobacterium milk as compared with the control group. Fukushima and Nakano (1996) also found that the rat group receiving the mixture of probiotic bacteria showed a significantly greater decrease in serum TC concentrations (33.2% reduction) than the group receiving *L. acidophilus* bacteria only (21.3% reduction).

Although most of the current probiotic foods are mainly dairy based, there is a growing interest in the development of non-dairy probiotic products due to problems such as lactose intolerance in many people and the unfavorable cholesterol content of fermented dairy products. Additionally, there is an increasing demand for vegetarian probiotic products (Ranadheera *et al*., 2010). This has led to the development of probiotic products from various food metrics including fruits, vegetables, legumes and cereal products (Ranadheera *et al*., 2010). Probiotic fermented barley-based food (Sindhu and Khetarpoul, 2003), probiotic soy cheese, probiotic sausages and other probiotic food products have been developed (Ranadheera *et al*., 2010).

Since there are no studies on developing a "probiotic hummus," the proposed study aims to investigate the synbiotic effect of both prebiotics in hummus and the added probiotics in reducing blood lipids in rats.

2. Materials and methods

This research was approved by the Department of Nutrition and Food Technology Committee for Animal Experimentation and the Faculty of Graduate Studies at the University of Jordan.

Lactobacillus acidophilus NCFM (Danisco, Copenhagen, Denmark) and *Bifidobacterium lactis* Bb-12 (Chr. Hansen, Hørsholm, Denmark) were used after subculturing (for activation) from their freeze dried form. These bacteria are probiotic starter culture used commercially in the production of probiotic dairy products.They were selected after determining their suitability for use as probiotics by performing tests for acid tolerance (Pereria and Gibson, 2002), bile tolerance (Haddadin *et al*., 1997), adhesion to the intestine (Brink *et al*., 2006), antibacterial activity against *Escherichia coli*, *Salmonella typhmarium* and *Staphylococcus aureus* (Mishra and Prasad, 2005), cholesterol assimilation (Gilliand and Walker, 1990) and after the viability in the feed was tested (Al-Awwad *et al*., 2009).

2.1. Propagation and Maintenance of Probiotic Bacteria

The two selected strains were maintained by subculturing in MRS broth, containing 0.05% L-cyseine-HCL (L-cys), using 1% inoculum and 18-20 h of incubation at 37 °C in an anaerobic jar (Oxoid, UK). The cultures were kept in the refrigerator at 4°C between preparation of subcultures. Each isolate was subcultured two to three times prior to every test (Al-Awwad *et al*., 2004).

2.2. Development of Hummus Broth as A Culture Medium for Probiotic Bacteria

Hummus Broth (HB) was developed as a culture medium for probiotic bacteria before their incorporation in hummus to develop probiotic hummus. This broth contained 0.05% L-cys, 0.5% glucose 1.2% yeast extract and 3 % skim milk (Al-Awwad *et al*., 2014).

2.3. Development of Probiotic Hummus

Hummus was prepared under hygienic conditions according to the procedure followed by Yamani and Dababseh (1994). Hummus was heated at 75 ºC/ 5 min, then cooled to 35-40 ºC and inoculated with 10% of hummus broth that contained *B. lactis* and *L. acidophilus*. Probiotic hummus was incubated anaerobically at 37 °C/ 8 h in an anaerobic jar (Oxoid, UK) using anaerobic kit (Anaerbic Gen Gas-Pack 2.5 L, Oxoid, UK), then it was refrigerated.

2.4. Diet Preparation and Incorporation Of Probiotic Hummus

2.4.1. Chemical Analysis of Hummus and Probiotic Hummus

Proximate analyses of hummus and probiotic hummus used in the experiment were determined according to the Official Methods of the Association of Official Analytical Chemists (AOAC, 1995).

2.4.2. Counts of the Probiotic Isolates in the Feed

100 g sample of each experimental diet was weighed in separate beakers. 10% (w/w) of probiotic culture in HB, control hummus and probiotic hummus were added and mixed well with each sample. The beakers were kept at room temperature and the total viable count of the bacteria was determined at 24, 48, 72 and 96 h. Consequently, the proper time for changing the rat diets that contained probiotic bacteria was detected in order to keep the probiotic bacterial counts > 10^6 CFU/g in the diet (Tharmaraj and Shah, 2003).

2.5. Preparation of the Experimental Diets

A 1% cholesterol diet was prepared according to AIN – 93 recommendations (Reeves, 1997). Probiotic, hummus and probiotic hummus were added as10% (w/w) to 1% cholesterol diet and blended to obtain three homogenous mixtures. The 1% cholesterol diet was used

as a control. The four experimental diets were kept at 4 °C until used for feeding.

2.6. Animal Experimentation

Animal experimentation was conducted making use of the conditions described by Liong and Shah (2006) and Al-Awwad *et al.* (2004). Initially, 32 male Sprague-Dawley rats were used. Animals were distributed randomly according to their weights (with an average of 219.8 g/ group) into 4 groups (8 rats/ group). They were individually housed in plastic cages with wire mesh bottoms (B. Holden & Crew 2001, North Kent Plastic Cages Ltd) at a temperature of 25 ± 2 ºC with 12 hrs light-dark cycle in the animal room.

Both diet and water were provided *ad libitum.* Total food intake and animal weight were measured once a week throughout the experimental period. At the end of the experiment, after 8 weeks, rats were fasted for 12 hrs and were anaesthetised using chloroform. Blood samples were collected from the right ventricle of each rat heart and were centrifuged at 3200 rpm for 15 min (Hermle, Z 200 A Centrifuge, Germany) to obtain serum. Sera were stored in conical plastic tubes, duplicate for each rat, at – 18 °C for later analyses of serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and, low-density lipoprotein cholesterol (LDL-C).

2.7. Biochemical Tests

The analyses of serum lipids were done in the Medical Laboratories of the Khalidi Medical Center (Amman, Jordan). An automated clinical chemistry analyser, COBAS INTEGRA 400/700/800 system, was used for the analysis. Before performing the analysis tests, calibration of the analyser for TG, TC, LDL and HDL was done according to the manufacturer's instructions.

2.8. Statistical Analysis

The statistical analyses were performed using the Statistical Analysis System, (SAS, 2008) version 9. Analysis of variance (ANOVA) with t -test was used to determine any significant differences between the means (Steel and Torrie, 1980). Values in tables are expressed as mean ± standard error of the mean (SEM). Levels of significance were at (P<0.05).

3. Results

The *L. acidophilus* and *B. lactis* strains used in this study have the characteristics of the probiotics, since they had the acid resistance activity, bile resistance property, cholesterol assimilation ability, adhesion ability, antibacterial activity and they could survive in the feed. These characteristics are related to their intestinal origin.

3.1. Counts of the Probiotic Isolates in the Feed

It was found that the counts of *B. lactis* and *L. acidophilus* in rat's diets were acceptable and more than 10^6 CFU/g until 72 h of keeping the inculated diets under aerobic conditions at room temperature. Accordingly, the probiotic diet and probiotic hummus diet were changed every 72 h.

3.2. Composition of the Experimental Diets

The composition of the diets that were fed to the rats is shown in Table 1 and the proximate analysis of these diets is shown in Table 2.

Table 1. Composition of the four diets (g/kg) which were fed to the rats for eight weeks *

Compositio	Diet Group			
	Cholesterol Group	ProbioticCholesterol Group	Hummus-Cholesterol Group	Probiotic Hummus-Cholesterol Group
Minerals	35	35	35	35
Vitamins	10	10	10	10
Casein **	140	138.93	133.76	133.54
Corn Starch	620.7	521.77	543.84	545
Soybean Oil	30	30	19.2	18.8
Cholesterol 1% †	10	10	10	10
Sucrose	100	100	100	100
Fiber ö	50	50	43.9	43.36
L-Cystine	1.8	1.8	1.8	1.8
Choline €	2.5	2.5	2.5	2.5
TBHQ £	8 mg	8 mg	8 mg	8 mg
Hummus	0	0	100	0
Probiotic Hummus	0	0	0	100
HBSM ¥	0	100	0	0

* Reference: Reeves *et al*. (1997).

** Casein (> 85 % protein), International Ingredient corporation, USA.

† 1 % (w/w) cholesterol (Purity >99%, Bioworld, USA) were added to each cholesterol-enriched diets.

ö Solka-Floc 100 FCC. International Fiber Corporation, USA.

€ Choline bitartarate (41.1 % choline)

£ TBHQ: Tert – Butylhydroquinone (mg)

¥ Every 100 g powder skim milk contains 35.5 g protein. A 100 ml HBSM containing 3 g SM≈1.065 g protein.

Table 2. Proximate analysis of the diets used in the experiment (g/kg (%) wet matter basis) *

* Mean of triplicate with coefficient of variation CV<5

Diets	Moisture	Ash	Crude protein	Ether extract	Crude fiber	Nitrogen-free extract**	Energy ö Kcal (MJ)
Cholesterol	54.0 (5.40)	62.0 (6.20)	119.2 (11.92)	44.8 (4.48)	50.0 (5.00)	669.9 (66.99)	3559.6 (149.05)
Probiotic-Cholesterol	130.9 (13.09)	40.3 (4.03)	123.9 (12.39)	45.7 (4.57)	47.5 (4.75)	611.7 (61.17)	3699.0 (154.84)
Hummus-Cholesterol	104.3 (10.43)	53.1 (5.31)	119.5 (11.95)	42.9 (4.29)	69.1 (6.91)	611.1 (61.11)	3808.5 (138.49)
Probiotic Hummus-Cholesterol	101.7 (10.17)	52.9 (5.29)	110.6 (11.06)	39.3 (3.93)	75.3 (7.53)	620.2 (62.02)	3276 (137.13)

** The values of nitrogen-free extract are calculated by difference

ö Calculated by multiplying grams of crude protein and nitrogen-free extract by 4 kcal and ether extract by 9 kcal; 1 Kcal=4.186 KJ and 1 MJ is equivalent to 1000 KJ.

3.3. Serum Triglycerides and Lipoprotein Cholesterol

Table 3 shows serum lipids and lipoprotein cholesterol values in mg dl^{-1} of rats fed the four experimental diets for 8 weeks. TC and LDL-C values were significantly decreased ($P< 0.05$) in the probiotic + cholesterol diet (62.75 ± 2.02 and 14.08 ± 0.71), hummus + cholesterol diet (66.75 ± 2.20 and15.35 ± 0.76) and probiotic hummus + cholesterol diet (63.00 ± 2.15 and14.29 ± 0.90) groups as compared to cholesterol diet group(73.38 ± 2.19 and 19.70 ± 1.00).

No significant differences were found in the HDL-C values ($P> 0.05$) among the different groups. TG value was greater in cholesterol diet group (93.88 ± 2.19) and significantly higher ($P< 0.05$) than hummus + cholesterol diet (80.75 ± 2.45) and probiotic hummus + cholesterol diet (71.00 ± 2.54) groups but not significantly higher than probiotic + cholesterol diet group.

Table 3. Levels of blood lipids (mg/dl) for rats fed the four experimental diets for eight weeks *, **

Rat groups	Blood Lipids ϕ			
	TC	HDL-C	LDL-C	TG
Cholesterol	73.38 ^{a} ± 2.19	40.78 ^{a} ± 1.13	19.70^{a} ± 1.00	93.88^{a} ± 2.19
Probiotic + cholesterol	62.75 ^{b} ± 2.02	42.76 ^{a} ± 1.17	14.08 ^{b} ± 0.71	88.13 $^{a\,b}$ ± 2.66
Hummus + cholesterol	66.75 ^{b} ± 2.20	40.78 ^{a} ± 1.41	15.35 ^{b} ± 0.76	80.75 ^{c} ± 2.45
Probiotic hummus + cholesterol	63.00 ^{b} ± 2.15	41.99 ^{a} ± 1.42	14.29 ^{b} ± 0.90	71.00 ^{d} ± 2.54

* Each value is represented as mean of eight readings ± SEM.

** Means with different superscripts within the same column are significantly different ($p< 0.05$).

ϕ TC: Total Cholesterol, HDL-C: High density lipoprotein- cholesterol, LDL-C: Low density lipoprotein-cholesterol, TG: Triglyceride

4. Discussion

This study aimed mainly at investigating whether there is a synergistic hypocholesterolemic effect of probioitics (*L. acidophilus* and *B. lactis*) when combined with the proposed prebiotic effect of hummus in rat experimental diets containing 1% cholesterol.

The results showed that the incorporation of probiotics (*L. acidophilus* and *B. lactis*) to the cholesterol diet decreased significantly ($p<0.05$) the TC and LDL-C by 14.5% and 28.5%, respectively. An insignificant ($p>0.05$) decrease in the TG of 6.1% was observed. There is an increase in HDL-C value of 4.9% in probiotics + cholesterol group but this increase is not significant as compared with the cholesterol group (Table 3). These effects of probiotic addition on serum TC, LDL-C, HDL-C and TG are in consistence with the results reported by many researchers using rats (Xaio *et al*., 2003; Al-Awwad *et al*., 2004; Huang *et al*., 2013).

The two strains used in the present study have shown a hypocholesterolaemic effect both *in vitro* and *in vivo*. These findings are in agreement with those of other researchers (Taranto *et al*., 2000; Alkhalaf *et al*., 2010; Huang *et al*., 2013) who supported the finding that cultures, actively assimilated cholesterol in the presence of bile salts from a laboratory medium, would function *in vivo* to exert a h ypocholesterolaemic effect in the experimental animals.

The hypocholesterolaemic effect can be attributed to the inhibition of exogenous cholesterol absorption from the small intestine by binding of cholesterol and bile acids with the bacterial cells, assimilation of cholesterol, as well as deconjugation of bile in the small intestine due to bacterial bile salt hydrolase activity (Lye *et al*., 2010; O'Bryan *et al*., 2013).

The deconjugated bile acids are not reabsorbed in the large intestine and are excreted through the feces. Excretion of bile acids results in the decrease of the extrahepatic recycling of bile acids, and, thus, cholesterol serum level is decreased due to its uses in *de novo* bile synthesis (Sudha *et al*., 2009; Ooi and Liong, 2010).

Another proposed mechanism of the cholesterol-lowering effect of probiotics is the production of Short Chain Fatty Acids (SCFAs). It is reported that acetate, a SCFA, increases the total cholesterol and decreases the fatty acids, while propionate lowers the hypercholesterolaemic response caused by acetate, which is a precursor of cholesterol (St-Onge *et al*., 2000).

Additionally, the cholesterol lowering effect of probiotic bacteria is due to the inhibition of 3-hydroxy 3-

methyl glutamyl CoA reductase, which is a rate-limiting enzyme and responsible for the endogenous cholesterol biosynthesis in the body (Sudha *et al*., 2009). Hydroxy-methyl glutarate (a 3 HMG-COA reductase inhibitor) was suggested to be an active factor that is produced or enhanced by probiotic bacteria (Sindhu and Khetarpaul, 2003). Lye *et al*. (2010) proposed the conversion of cholesterol to coprostanol by cholesterol reductase produced by Lactobacilli probiotic bacteria.

Taranato *et al*. (2000) proposed that the hypotriglyceridaemic effect of *L. reuteri* might be due to a lowering of intestinal absorption of lipid or to an increase in lipid triacylglcerol synthesis from [^{14}C] acetate with a similar concentration dependency in rat hepatocytes. This inhibition was claimed by Lin *et al*. (1995) to be due to the lowering activity of acytel-CoA synthetase by propionate, or to lowering triglycerides by the production of lipase by probiotic organisms (Sudha *et al*., 2009).

From Table 3, it can be observed that the addition of hummus to the cholesterol diet caused a significant reduction ($p<0.05$) of 9.0%, 22.1% and14.0% in TC, LDL-C and TG values, respectively, as compared with the cholesterol group value. The main constituent of hummus is chickpea to which the obtained results of the hummus + cholesterol group can be attributed. Mathur *et al*. (1984) found that the incorporation of whole chickpea flour (75%) to the rat diet containing 1 % cholesterol caused a significant reduction in TC. Similarly, Sihag and Kawatra (2003) found that rats fed Bengal gram seed coats in 1% cholesterol diet resulted in 35% lower serum cholesterol, as compared to the control group. The researchers attribute this to the high hemicelluloses content of the Bengal gram seed coats.

In a study on human beings, Pittaway *et al*. (2007) found that there were reductions in TC of 0.25 mmol/L and in LDL-C of 0.20 mmol/L following the consumption of the chickpea diet for five weeks, as compared to wheat diet. In a meta-analysis of eleven clinical trials (Anderson and Major, 2002), it was concluded that the sum of the whole rather than individual components (soluble dietary fiber, oligosaccharides, isoflavones, phospholipids and PUFAs, phytosterols, saponins and vitamins and minerals) were responsible for the hypocholesterolaemic effect of legumes.

Prebiotics contribute to hypocholesterolaemia via two mechanisms: decreasing cholesterol and bile acids absorption accompanied by enhancing their excretion via feces (Lin *et al*., 1995), and the production of SCFAs upon selective fermentation by intestinal bacterial microflora. Prebiotics are fermented in the colon by large bowel bacteria, yielding SCFAs such as butyrate, acetate and propionate (Ooi and Liong, 2010).

The hypotriglyceridaemic effect of prebiotics is mostly due to the decrease in the *de novo* lipogenesis in the liver (Ooi and Liong, 2010) and to the increase in TG-rich lipoprotein catabolism (Delzenne and Kok, 2001). Furthermore, propionate was also reported to inhibit fatty acid synthesis, thereby lowering the rates of triacylglycerol secretion (Ooi and Liong, 2010).

In the present investigation, total cholesterol of the probiotic hummus + cholesterol group was significantly ($p<0.05$) lowered by 14.1% as compared with TC of the cholesterol group. However, the reduction of TC in this group was not significantly ($p>0.05$) different from that of the probiotic + cholesterol and hummus + cholesterol groups. The LDL-C of the probiotic hummus + cholesterol group was significantly ($p<0.05$) lowered by 27.5% when compared with the cholesterol group, but it was not significantly ($p>0.05$) different from LDL-C of the cholesterol + probiotic and hummus + cholesterol groups.

The HDL-C value of the probiotic hummus + cholesterol group shows a non-significant ($p>0.05$) difference as compared with the cholesterol, probiotics + cholesterol and hummus + cholesterol groups. The TG of the probiotic hummus + cholesterol group was significantly ($p<0.05$) lowered by 24.4%, 19.4% and 12.1% when compared with the cholesterol, probiotics + cholesterol and hummus + cholesterol groups, respectively.

Little information is available on the effective synbiotic dosage of probiotics and prebiotics needed to exert their hypocholesterolaemic effects) (Ooi and Liong, 2010). Kikuchi-Hayakawa *et al*. (1998) found that the addition of Bifidobacterium-fermented soya milk to 0.5% cholesterol-enriched diet decreased the levels of plasma TC, TG and VLDL + LDL-C in hamsters. Liong and Shah (2006) concluded that the synbiotic effect of *L. casei*, fructooligosaccharide, and maltodextrin that were incorporated into rat diet, that contained 1% cholesterol, significantly lowered serum TC and TG levels. However, The HDL-C and LDL-C levels were not affected by this synbiotic effect.

To the best of our knowledge, this is the first time in Jordan a probiotic product based on the traditional food "hummus" is produced; it is also the first study in Jordan, to the best of our knowledge, that studies the synbiotic effect of this product in reducing blood lipids. However, further studies are needed for optimizing probiotic hummus production, studying its shelf-life and using the "Tetra Pak" technique to maintain the anaerobic conditions for extending it. Also, there is a need for finding the suitable methods or techniques for administering probiotic bacteria in hummus, such as the microencapsulation technique.

5. Conclusion

According to the results of this study, it could be concluded that the addition of probiotic hummus to the cholesterol-rich diet caused significant reductions in TC, LDL-C and TG. However, these reductions were not significantly different from those reductions caused by the addition of probiotic or hummus alone except for TG.

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Conflict of Interest

The authors have equally contributed to the work and agree to submit it for consideration to the ***Jordan Journal of Biological Sciences,*** and they also declare that they do not have any conflict of interest.

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Effect of Various Levels of Raw *Citrullus lanatus* Seed Meal Diets on Growth Performance of *Cyprinus carpio* Fingerlings

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Abstract

The nutritional value of feeding *Cyprinus carpio* various levels of raw watermelon seed meal was evaluated in this study. Five diets of 35% crude protein were formulated with different levels of raw seed meal at 0%, 5%, 10%, 15% and 20% inclusion. Twenty fingerlings were randomly allocated in triplicate for each treatment in 70 liters plastic bowls, aeration was provided to culture bowls throughout the 12-week trial period. The results revealed that the inclusion level of 10% raw watermelon seed meal in the diet gave the best mean weight gain, feed conversion ratio, feed conversion efficiency, protein efficiency ratio, apparent net protein utilization, specific growth rate and survival rate. The growth performance decreased with increasing the inclusion level of raw seed meal beyond 10% (P<0.05). The inclusion level of 10% raw watermelon seed meal in the diet of common carp is found to be ideal for enhancing growth and better nutrient utilization.

Key words: Watermelon Seed, Unconventional Feed Stuff, Common Carp.

1. Introduction

Common carp is the most extensively cultivated freshwater fish species in the world (Komen, 1990; Zhou *et al.*, 2004). This fish has several advantages that make it popular for commercial culture: very fast growth rate, high environment tolerance, ease of handling, ability to be raised in high density, ability to utilize artificial diet with a relatively low protein content, and occurrence of highly productive strains and breeds produced from long-term domestication and selective breeding (Kirpitchnikov, 1999). Common carp is a largely benthic species that prefers shallow water habitats covered with aquatic weeds and grasses. It is an omnivorous fish that mostly feeds on the bottom but can exploit all levels in the water column. The natural diet of carp is dominated by chironomids, snails, young clams, shrimps and other benthic animals. This species also consumes aquatic plants, filamentous algae, seeds of plants and organic detritus. Under pond culture conditions, common carp will take soybean and peanut cakes, rice and wheat bran (Christopher *et al.*, 2007).

The importance of artificial feeding in aquaculture cannot be over-emphasized. It promotes faster growth, allows higher stocking density and shorter cultivation periods. However, the unavailability and affordability of adequate fish feed has significantly affected the development of aquaculture in Nigeria. A well prepared and carefully formulated fish feed plays a significant role in fish culture (Ukagha, 2003). The higher cost and competition imposed on some feed ingredients, such as soybean, groundnut cake, maize and sorghum used by human population as food, have necessitated the use of unconventional material for fish feed formulation. The price of finished feed in our part of the world continues to be on t he rise, thereby removing the margin of profit accruing to the fish producers (Amubode and Ogogo, 1994). Thus, overcoming the burden of feed ingredients and reducing the cost of fish feed and fish products have been the burden of numerous researchers (Ani and Okeke, 2003). In fish farming, nutrition is critical because feed represents 40-50% of the production cost (Steven, 2001).

Watermelon belongs to the genus *Citrullus* and family *Cucurbitaceae* (Huxley, 1992). The *Cucurbitaceae* is a medium sized plant family, primarily found in warmer regions of the world. Watermelon seeds are a s ource of protein, B vitamins, minerals (such as magnesium, potassium, phosphorus, sodium, iron, zinc, magnesium and copper) and fat, among others (Collins *et al.*, 2007; Vandermark, 2011). Thus, the consumption of a variety of plant foods, including watermelon seeds, may provide additional health benefits (Cutter, 2000). The quality of the protein was moderate with methionine and cysteine as limiting amino acids, with supplementation of the deficit amino acids watermelon seeds can form a good source of protein. The chemical composition suggests its suitability as a matrix for mineral fortification and its functionality suggests that the watermelon seed is suitable for food formulations (Lakshmi and Kaul, 2011). This study

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attempts to evaluate the watermelon seed's nutritional value as an unconventional feed source, a byproduct protein and a lipid source in the diet of *Cyprinus carpio*.

2. Materials and Methods

2.1. Experimental Site

The feeding trial was carried out at the fish farm of Bauchi State Agricultural Development Program (BSADP) Bauchi, Nigeria. A circular plastic bowls system of 70 liters capacity was used. The experiment lasted for twelve weeks. Aeration was provided using air pumps, and water in bowls was siphoned every two days to avoid fouling.

2.2. Experimental Fish

The *Cyprinus carpio* (common carp) fingerlings were obtained from the Fish Farm of Bauchi State Agricultural Development Program (BSADP) along Dass Road Bauchi. Twenty fingerlings of mean weight 1.77±0.25g were acclimatized for two weeks. After the period of acclimatization, the fishes were randomly distributed into the plastic bowls.

2.3. Diet Formulation

Raw watermelon seeds were cleaned, sundried and milled to produce raw meal of watermelon seeds; they were then stored for diets formulation. The soybeans used were toasted for 30 minutes in an electric oven set at 100˚C and milled after cooling to get a soybean meal (SBM). The yellow maize used was milled also to get a yellow maize meal (YMM); the selected feed ingredients were stored in an air-tight and moisture-free container. Analysis was carried out to determine the nutrient composition of the raw watermelon seed meal diets, as well as the experimental fish before and after the experiments.

The experimental diets were formulated using Pearson square method to contain the same level of crude protein at 35% to meet the protein requirement of *Cyprinus carpio* fingerlings as stated by Hossain *et al*. (1997).

As a result, five experimental diets were formulated with raw watermelon seeds meal at different inclusion levels: Diet 1 (DT1; 0%), Diet 2 (DT_2; 5%), Diet 3 (DT3; 10%), Diet 4 (DT4; 15%) and Diet 5 (DT_5; 20%) while Coppens® fish feed Diet 6 (DT_6) was included as reference diet among the experimental diets.

2.4. Experimental Design and Management

The dietary treatments were in triplicates using Completely Randomized Design (CRD). After the acclimation period, fish were weighed and randomly distributed in bowls, each replicate contained 20 fingerlings of *C. carpio*. The experimental fish were fed twice daily (9.00 AM and 4.00 PM) at 5% body weight for twelve weeks.

The fingerlings were weighed every week to determine weight gain, and feed quantity given was adjusted accordingly. The growth parameters determined include mean weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio, apparent net protein utilization and survival rate.

2.5. Computation of the Growth Parameters

Calculation of the growth parameters were done according to the formulae described by Balfour (1998).

2.5.1. *Mean Initial Weight:* Twenty **(**20) of *C. carpio* fingerlings were counted and weighed, the total weight obtained was divided by 20, to obtain the mean initial weight of the fingerling.

MIW= N_W/N Where MIW =Mean Initial Weight N =Number of fingerlings W = Weight of fingerling

2.5.2. *Mean Final Weight:* The surviving fingerlings were counted and weighed; the weight obtained was divided by the number of the surviving fingerlings to obtain the mean final weight:

$$MFW = N_{sf}W/N_{sf}$$

Where MFW =Mean Final Weight (g)
N_{sf} =No. of surviving fish
W = Weight of the fish

Mean Weight Gained = Mean Final Weight – Mean Initial Weight

2.5.3. Survival Rate:

$$\text{Survival (\%)} = \frac{No - Ne}{No} \times 100$$

No = Initial total number of fingerlings
Ne = Total number of fish mortality at the end of feeding trial (12 weeks)

2.5.4. *Specific Growth Rate (SGR):* This parameter was determined according to the formula stated below:

$$SGR = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} \times 100$$

Where ln = natural logarithm
W_2 = final weight
W_1 = initial weight
$T_2 - t_1$ = time duration (in days)

2.5.5. *Feed Conversion Ratio (FCR):* According to the formula stated below:

$$\textbf{FCR} = \frac{Feed\ Intake}{Weight\ Gain}$$

2.5.6. *Protein Efficiency Ratio (PER):* It was determined according to the formula stated below:

$$\textbf{PER} = \frac{Weight\ Gain}{Protein\ IntCake}$$

Where protein Intake = $\frac{\%\ Protein\ in\ Diet \times Total\ Feed\ Consumed}{100}$

2.5.7. *Apparent Net Protein Utilization (ANPU):* According to the formula stated below:

$$\textbf{ANPU} = \frac{Protein\ Gained}{Protein\ Consumed} \times 100$$

Where protein gain = $Final\ Carcass\ Protein - Initial\ Carcass\ Protein$

2.6. Proximate Analysis of the Experimental Diets

The proximate composition of differently processed watermelon seed meal diets were carried out at Grand Cereals Jos. Moisture content, protein, ether extract, crude fiber, ash and nitrogen free extract of the diets were determined before the experiment, and those of the carcass of the experimental fish were determined before and after the feeding trial according AOAC (2000).

2.7. Data Analysis

Data obtained from the feeding trials were subjected to analysis of variance (ANOVA) where significant differences were observed between treatments; the means were compared using Fishers Least Significant Difference of the means (LSD). Genstat Discovery Edition 4 a nd Minitab 14 software were used for statistical analysis.

3. Results

The proximate composition of the experimental diets containing varying levels of raw watermelon seed meal diets is presented in Table 1. The diets were isonitrogenous at approximately 35% crude protein. DT_5 (20% inclusion) had the highest value for ether extract of 12.31±0.10% among the formulated diet, whereas DT_1 (0% inclusion) contained the least value of 8.53± 0.11%; however, the reference diet had a higher value compared to all formulated diets. The highest crude fiber was observed in diet 20% inclusion (DT_5, 11.81±0.12%); whereas the reference diet (DT_6) had the least value (2.01±0.01%). DT_3 (10% inclusion) had the lowest ash content of 10.26±0.02%, while DT_6 (reference diet) had the highest values of 14.80±0.10%. The moisture content ranged between 10.76±0.02% to 11.89±0.11%, the highest value was recorded in DT_2 (5% inclusion) whereas the nitrogen free extract (NFE) was highest in DT_1 (28.89±0.33%) and lowest in DT_6 (3.43±0.08%).

The growth parameters, nutrient utilization and survival rate of common carp fingerlings fed varying levels of raw watermelon seed meal diets are presented in Table 2. The Mean Final Weight (MFW), Mean Weight Gain (MWG), Feed Conversion Ratio (FCR), Feed Conversion Efficiency (FCE), Protein Efficiency Ratio (PER), Apparent Net Protein Utilization (ANPU), Specific Growth Rate (SGR) and Survival Rate (SR) of fish fed all the experimental diets differed significantly (P<0.05).

The mean final weight of the fish fed DT_3 (10% inclusion level of watermelon seed meal) was significantly high (15.33±0.04g), while DT_5 had the lowest value of 11.58±0.20g. The Mean Initial Weight (MIW) of the experimental fish was 1.775± 0.00, showing no significant difference (P>0.05). The mean weight gain (MWG), feed conversion ratio (FCR) and feed conversion efficiency (FCE) differed significantly (P<0.05) and were best with fish fed DT_3 with values of 13.55±0.04g, 1.75±0.00 and 57.15±0.02, respectively. DT_5 had the least MWG (9.81±0.02g) and the least FCE (48.26+0.12) while DT_3 and DT_6 showed the best FCR of 1.75. The protein efficiency ratio (PER), apparent net protein utilization (ANPU) and specific growth rate (SGR) also differed significantly (P<0.05) with the highest values of 1.65±0.00, 23.73±0.11 and 2.57±0.00, respectively, recorded from the fish fed DT_3, while the lowest values 1.38±0.01, 18.03±0.09 and 2.23±0.00, respectively, were obtained from the group of fish fed DT_5. The percentage survival ranged from 93.30± 3.33% (DT_5) to 100±0.00% in DT_2, DT_3 and DT_4, respectively.

Table 1. Proximate Composition of Diets containing different inclusion levels of raw watermelon seed meal

Treatment	Moisture %	Crude Protein %	Ether Extract %	Crude Fiber %	Ash %	NFE %
Diet 1 (DT_1)	10.76±0.02^{c}	35.23±0.06^{b}	8.53±0.11^{d}	5.70±0.08^{e}	10.89±0.02^{b}	28.89±0.33^{a}
Diet 2 (DT_2)	11.89±0.11^{a}	35.26±0.08^{b}	11.61±0.07^{c}	7.26±0.08^{d}	11.23±0.15^{b}	22.75±0.14^{c}
Diet 3 (DT_3)	10.77±0.01^{c}	34.70±0.05^{c}	12.26±0.10^{b}	8.54±0.11^{c}	10.26±0.02^{c}	23.47±0.07^{b}
Diet 4 (DT_4)	11.50±0.12^{b}	34.83±0.14^{c}	12.28±0.11^{b}	10.15±0.10^{b}	11.12±0.07^{b}	20.12±0.09^{d}
Diet 5 (DT_5)	11.00±0.12^{c}	34.99±0.11^{bc}	12.31±0.10^{b}	11.81±0.12^{a}	11.16±0.14^{b}	18.73±0.09^{e}
Diet 6 (DT_6)	10.97±0.08^{c}	52.24±0.15^{a}	16.55±0.08^{a}	2.01±0.01^{f}	14.80±0.10^{a}	3.43±0.08^{f}

Means in the same column followed by different superscripts letters differed significantly (P<0.05)

Table 2. Growth Parameters of the *Cyprinus carpio* Fingerlings Fed different inclusion levels of raw watermelon seed meal Diets

Treatment	MIW	MFW	MWG	FCR	FCE	PER	ANPU	SGR	% Survival
Diet 1	1.775±0.00	12.45±0.03^{e}	10.68±0.03^{e}	1.98±0.01^{b}	50.45±0.16^{d}	1.43±0.01^{d}	19.46±0.18^{c}	2.32±0.00^{e}	96.67±1.67^{ab}
Diet 2	1.775±0.00	14.99±0.05^{c}	13.21±0.05^{c}	1.81±0.01^{d}	55.29±0.25^{b}	1.57±0.01^{b}	21.56±0.15^{b}	2.54±0.00^{c}	100.00±0.00^{a}
Diet 3	1.775±0.00	15.33±0.04^{b}	13.55±0.04^{b}	1.75±0.00^{e}	57.15±0.02^{a}	1.65±0.00^{a}	23.73±0.11^{a}	2.57±0.00^{b}	100.00±0.00^{a}
Diet 4	1.775±0.00	13.47±0.05^{d}	11.69±0.05^{d}	1.90±0.01^{c}	52.60±0.23^{c}	1.51±0.01^{c}	21.45±0.20^{b}	2.41±0.00^{d}	100.00±0.00^{a}
Diet 5	1.775±0.00	11.58±0.02^{f}	9.81±0.02^{f}	2.07±0.01^{a}	48.26±0.12^{e}	1.38±0.01^{e}	18.03±0.09^{d}	2.23±0.00^{f}	93.30±3.33^{b}
Diet 6	1.775±0.00	16.28±0.04^{a}	14.50±0.04^{a}	1.75±0.01^{e}	57.05±0.27^{a}	1.09±0.01^{f}	16.31±0.09^{e}	2.64±0.00^{a}	100.00±0.00^{a}

Means in the same column followed by different superscripts letters differed significantly (P<0.05)
MIW: Mean Initial Weight; MFW: Mean Final Weight; MWG: Mean Weight Gain; FCR: Feed Conversion Ratio; FCE: Feed Conversion Efficiency; PER: Protein Efficiency Ratio; ANPU: Apparent Net Protein Utilization; SGR: Specific Growth Rate

Figure 1 shows the trend of weekly weight gain of common carp (*Cyprinus carpio*) fingerlings fed different inclusion levels of raw watermelon seed meal diets for the 12 weeks experimental period. The results showed significant different (P<0.05) among the treatments. The increase in weight gains was recorded for all the fishes fed the experimental diets. The highest weight gain was observed for fish fed diet DT6 (16.28g) followed by DT_3 (15.33g) while the lowest weight gain was recorded by fish fed DT_5 (11.58g) at the end of the feeding trial.

The initial and final proximate composition of the carcasses of the fish fed the experimental diets containing various levels of raw watermelon seed meals are presented in Table 3. The proximate analysis revealed significant differences (P<0.05) for moisture content, crude protein, ether extract, crude fiber, ash and Nitrogen Free Extract (NFE) of the experimental fish carcass before and after the feeding trial.

The result of proximate analysis of fish carcass fed the experimental diets indicated an increase in crude protein, ether extract and ash but a decrease in moisture content and crude fiber when compared with the initial carcass. The fish fed DT_5 had the highest carcass moisture content (79.58±0.03%), while the lowest value was observed in fish fed DT_6 (78.60±0.00%). The fish fed DT_6 had the highest carcass crude protein (14.59±0.01%) followed by DT_3 (14.10±0.06%), while DT_5 fish carcass had the lowest value (12.87±0.02%). Ether extract and crude fiber were highest in carcass of the fish fed DT_6 and DT_3 (4.02±0.01% and 0.10±0.01%, respectively), while DT_2 (2.75±0.06%) had the least ether extract and DT_6 fish carcass had the lowest crude fiber (0.04±0.01%). DT_5 had the highest ash content 3.32±0.03% and the lowest was recorded in the carcass of DT_6 (2.56±0.01%).

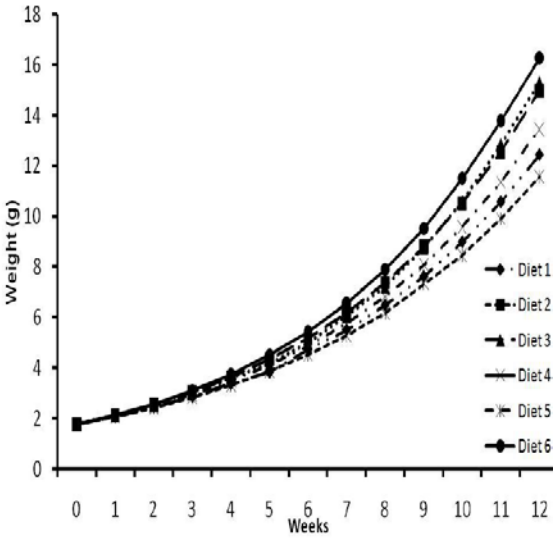

Figure 1. Weekly Weight Gain of *C. carpio* Fed different inclusion levels of raw watermelon seed meal Diets

Table 3. Proximate composition of carcass of fish fed different inclusion levels of raw watermelon seed meal diets

Treatment	Moisture %	Crude Protein %	Ether Extract %	Crude Fiber %	Ash %	NFE %
Initial	80.20±0.06^{a}	11.75±0.06^{g}	1.87±0.06^{d}	0.10±0.00^{a}	2.15±0.01^{e}	3.93±0.02^{a}
Diet 1	78.70±0.01^{de}	13.34±0.06^{e}	2.82±0.01^{c}	0.09±0.01^{ab}	3.11±0.06^{b}	1.94±0.03^{b}
Diet 2	78.68±0.08^{de}	13.51±0.06^{d}	2.75±0.06^{c}	0.09±0.02^{ab}	3.15±0.03^{b}	1.84±0.06^{c}
Diet 3	79.30±0.12^{c}	14.10±0.06^{b}	3.04±0.03^{b}	0.10±0.01^{a}	2.72±0.06^{c}	0.74±0.01^{f}
Diet 4	78.84±0.06^{d}	13.88±0.01^{c}	2.81±0.01^{c}	0.06±0.01^{cd}	3.27±0.04^{a}	1.14±0.03^{e}
Diet 5	79.58±0.03^{b}	12.87±0.02^{f}	2.83±0.02^{c}	0.07±0.00^{bc}	3.32±0.03^{a}	1.33±0.02^{d}
Diet 6	78.60±0.00^{e}	14.59±0.01^{a}	4.02±0.01^{a}	0.04±0.01^{d}	2.56±0.01^{d}	0.23±0.01^{g}

Means in the same column followed by different superscripts letters differed significantly (P<0.05)

4. Discussion

Davies and Gouveia (2010) reported that the nutrient requirement for growth, reproduction and normal physiological function of fish must be met, like other animals; but fish have much higher requirement for protein, so feed mixture with 25-45% of raw protein are mainly used. Furthermore, Mazid *et al.*, (1997) reported that the protein requirement for carp should be between 35-45% until the size of the fish reaches approximately 50-60g. However, Wilson and Halver (2005) suggested that herbivorous and omnivorous fish require a diet with 25-35% crude protein. According to the report of Akiyama (1999), usually fish growth will be directly proportional to the level, if the level within the range of approximately 20 to 40% crude protein. Similarly Noor *et al.* (2011) reported that the protein requirement of carp is 35%, which is in line with the diet formulated for this study.

The present study revealed that the lipid content of the experimental diet ranged from 8.53±0.11 to 12.31±0.10. The result conforms to the report of Andras *et al*. (2011) who reported that the lipid content of larvae and fingerling feed should be around 8 to 15%, the oil content would be increased until it reaches 12-15% according to the species and size of the fish. It was also observed, from the results, that the crude fiber contents in this study were

higher than the values reported by Gatlin (2011) who indicated that cellulose and other fibrous carbohydrate were found in the structural component of plant and are indigestible to monogastric (simple-stomach) animals, including fish. In fact, the amount of crude fiber in fish feed is usually suggested to be less than 7% of the diet to limit the amount of undigested materials entering the culture system.

The growth parameter in this study showed that the mean final weight (MFW) and mean weight gain (MWG) of fish fed experimental diets differed significantly (P<0.05). Both the MFW and MWG of DT_2, DT_3 and DT4 were better than DT_1 and DT_5. However, fish fed (DT_3) 10% level of watermelon meal was significantly (P<0.05) higher compared to the fish fed control DT_1 (0% inclusion) and the remaining diets. This is similar to the result of Shazali *et al.* (2013) who reported that the inclusion of watermelon seed meal for broiler diets up to 10% significantly induced better growth and feed utilization efficiency.

This may be attributed to differences in the level of ether extract, fiber content and carbohydrate (NFE) of the experimental diets. This result conforms to the report of Craig and Helfrich (2002) that lipid fats are high-energy nutrients that can be utilized to partially spare protein in aquaculture feed and typically comprise about 10 to 25% of the fish diet. Moreover, the result collaborates with the findings of Oladunjoye *et al.* (2005) that the high fiber content could be responsible for growth depression. Similarly, Lovel and Leary (1990) pointed out that increasing fiber content beyond the basal level could reduce the growth of fish due to poor digestion of cellulose. This is likely to be responsible for the poor growth performance of fish fed DT_5 (20% inclusion) containing a high crude fiber. This also agrees with the findings of Sawaya *et al*. (1986) who recommended that the watermelon seed should not be included at levels higher than 20%, because these levels bring up the fiber content of the ration to be over 10%, which reduces the feed intake.

The results of this study also reveal that the feed conversion ratio (FCR), feed conversion efficiency (FCE), protein efficiency ratio (PER), apparent net protein utilization (ANPU) and specific growth (SGR) followed the same trend with mean weight gain (MWG) which revealed that DT_3 and DT_2 had the best growth performance. However, all the experimental fish exhibited good SGR values (>2% body weight) using the standards reported by FAO (2004) that daily growth rate of carp can be between 2 to 4% body weight. This might be influenced by the variation in crude fiber, fat content and NFE in the diets. This result agrees with the report of Craig and Helfrich (2002) who pointed out that protein is used for growth if adequate levels of fat and carbohydrates are present in the diet; if not, protein may be used for energy and life support rather than growth. Increasing the dietary lipid with high quality fats improves growth, feed conversion and protein utilization, thus reducing nitrogen excretion (Steffens, 1993).

The study also shows a higher percentage survival rate (93.30±3.33-100±0.00%) of the common carp fingerlings fed the experimental diets during the 12 weeks experimental period. This shows that all diets formulated were not harmful to the fish and agrees with the findings of Basavarajah and Anthony (1997) who reported a survival rate of 98% for common carp fry fed conventional feed and 100% for fry fed supplementary feed for a 35-day feeding trial. Similarly, Singh and Dhawan (1996) pointed out that 100% survival rate of carp can be achieved under a very minimal stress and a well-fed condition.

The proximate composition of the experimental fish carcass revealed that the crude protein and lipid content in the fish fed DT_3 is higher than the initial carcass and the fish fed remaining diets. Fish fed DT_2 and DT_4 had a moderate crude protein content in their carcass; however, the results exhibited that all the fingerlings utilized the diets well. The difference may be a result of high lipids and low crude fiber content in DT_3 compared with the rest of the diets containing watermelon seed meal. This is in agreement with the findings of Abbas (2007) and Manjappa *et al.* (2011) who pointed out that a better utilization of nutrients in the carcass of fish fed high lipids diets and protein utilization for growth are related to both the dietary protein level and the availability of non-protein energy sources with lower inclusion of dietary fiber.

5. Conclusion

The feeding trial on varying inclusion levels of raw watermelon seed meal revealed that DT_3 which had 10% inclusion level emerged as the best in both growth performance and nutrient utilization, while DT_5 (20% inclusion level) was the least and poorest growth performance among the treatments. Inclusion level of 10% raw watermelon seed meal in the diet of common carp is suggested to enhance growth performance and nutrient utilization.

As evidenced by the findings of this study, raw watermelon seeds can be successfully used in common carp feed at 10% without adverse effects on the growth responses and nutrient utilization of the fish; further studies should be performed on processing raw seeds so as to improve their growth potential.

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Antioxidant Profile of Saliva among Young Men Using Mobile Phones

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Abstract

Oxidative stress has been implicated as a mechanism of potential health effects that may result from exposure to Radio Frequency Electromagnetic radiation (RF-EMR). A cross sectional study was designed to investigate and analyze the biochemical effects of RF-EMR emitted from mobile phones at 835 MHz and 1900 MHz bands on some biochemical markers: Superoxide dismutase (SOD), albumin, amylase, uric acid and cytochrome C in the saliva of young men (18-37 years; average age 27.74 ± 8 .08). EMF caused a significant increase in the activity of SOD but a significant decrease in that of amylase in the saliva of people after using mobile phones. The increases in the activity of cytochrome C and the concentrations of albumin and uric acid were not significant. A true correlation between the salivary antioxidant biomarkers and the number of calling min., rather than the number of calls, was found. These oxidative changes may result in metabolic changes in the living cells up to oncogenic transformation. Thus, based on these findings, it is recommended that a long-term/or excessive use of mobile phones, especially by young individuals, should be avoided. This goal can be accomplished by telehealth technology promotion activities targeting the more sensitive ages, children and adolescents, since their developing brains absorb more EMR from a mobile phone. Such activities include: group discussions, public presentations and mass communication through available electronic and print media sources.

Keywords: Antioxidants; Human saliva; Mobile phone; Oxidative stress.

1. Introduction

Currently, of the world's 7 billion people, 6 billion have mobile phones and the initial age of youngest users of the cell phone is estimated as three years old. Over time, the number of mobile phone calls per day, the length of each call, and the amount of time people use cell phones have increased (Khurana *et al*., 2009; Awadalla, 2013). Radiofrequency (RF) radiation is an important part of electromagnetic human exposure. This is due to the fact that in this frequency range, the electromagnetic energy penetrates skin depth in a way that the entire body is affected, not just the surface layers. Secondly, it is the frequency range where the outer membranes of mammalian cells are no longer barriers to electric fields, allowing access of the RF to subcellular structures (Goldsworthy, 2012; Awadalla, 2013). If a risk exists, it is likely to be greatest for regions with greatest energy absorption in close proximity to the head (Cardis *et al*., 2008; Awadalla, 2013; Bhargavi *et al*., 2013). Epidemiological studies of the association between exposure to routine mobile phone radio frequency-electromagnetic radiation (RF-EMR) and adverse health effects, including brain tumors, have been inconsistent (some, but not all, studies showed increased risk); the issue remains unresolved (Kundi, 2005; Ahlbom *et al*., 2009; Dubey *et al*., 2010; Yakymenko *et al*., 2011; Hardell *et al*., 2013). The parotid gland is one potential target of interest, since mobile phones are typically pressed up against the side of the face in front of the ear where the gland is located. In fact, an association between the mobile phone use and parotid gland tumors has been reported (Sadetzki *et al*., 2008; Czerninski *et al*., 2011; Duan *et al*., 2011; Bello *et al*., 2012; de Vocht *et al*., 2013). For example, the increase in the annual incidence

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*Abbreviations: 8-Oxo-dG:8-Oxo-7, 8-dihydro-2'-deoxyguanosine; EMR: Electromagnetic Radiation;GSM: Global System for Mobile Communications; GSH-Px: Glutathione Peroxidase; RF: Radio Frequency; RF-EMR: Radio Frequency Electromagnetic Radiation; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase.

of head tumors was correlated with increased rate of mobile phone subscription (de Vocht *et al*., 2013).

One of the priorities in the RF-EMR research is to elucidate the underlying mechanisms of the biological effects of RF-EMR exposure. Despite the increasing number of reports concerning these effects in various biological systems (Sivani and Sudarsanam, 2012), no satisfactory mechanism has been proposed to explain these effects. One of such proposed mechanisms is the stimulation of oxidative stress. However, a comprehensive picture regarding the relationships between oxidative stress and the exposure to RF is still lacking. An oxygen damage of DNA in human spermatozoa (De Iuliis *et al*., 2009), and saliva (Khalil *et al*., 2014), as well as in rat urine (Khalil *et al.*, 2012) through formation of 8-Oxo-7, 8-dihydro-2'-deoxyguanosine (8-Oxo-dG) under non-thermal microwaves radiation has been demonstrated. The antioxidant capacity, measured by Oxygen Radical Absorption Capacity (ORAC), and Hydroxyl Radical Averting Capacity (HORAC) of human saliva did not significantly increase following a short-time mobile phone talk (Khalil *et al*., 2014). Mobile phone radiation induced a significant increase in the activity of superoxide dismutase (SOD) enzyme (Abu Khadra *et al*., 2014). In the latter study, the concentration of other salivary proteins, albumin and uric acid as well as the activity of the enzymes catalase and cytochrom C were not significantly altered.

The present study examines whether the human body responds to exposure to RF-EMR by investigating variations in the salivary profile among young mobile phone male users. This study will not only demonstrate whether the human body recognizes mobile phone radiation as an external stressor but it also provides information on whether molecules, proteins and genes respond (either activated or inactivated) to mobile phone radiation.

2. Materials and Methods

2.1. Design, Setting, and Participants

A randomized cross-sectional study was conducted between June 1 and October 31, 2012. To avoid gender effect and interlaboratory variations, the experiment was performed in one laboratory only on 109 healthy males recruited from North Jordan community. The age of participants ranged between 18 and 37 years (average 27.74 ± 8.08 years). The study was approved by the local institutional review board (Committee on Research Involving Human Subjects, Yarmouk University). All subjects were screened in a short personal interview in order to assure that they corresponded to our criteria of selection, including being nonsmokers, nondiabetic, and not suffering from significant dental, gingival, or chronic systemic inflammatory diseases. All were regular users of the mobile phone, making at least one phone call per week for a period of at least 6 months (less than 8 hours a month).

The mobile phones of all participants used a Global System for Mobile Communications (GSM), which operates in the 900 MHz or 1800 MHz bands, where the maximum power level is 1 watt or 2 watts at 1800 MHz and 900 MHz, respectively (Bhargavi *et al*., 2013). The goal of the study was explained to all, and each individual filled in a questionnaire that incorporates information on the intensity of mobile phone use, the total number of calls per day, the total number min. utilized per day when using mobile phone, and the calendar period of use. A written informed consent was obtained from all participants. Volunteers were not compensated for participation in the study. Volunteers were asked not to eat, drink, or brush their teeth 1h before the collection of saliva. Twenty ml of whole unstimulated saliva samples were collected for 10 min. in sterilized tubes and kept on ice during and after the collection. The sample collection procedure was done in the morning to avoid any diurnal variation in the assessed variables. Thereafter, the samples were centrifuged at 14000 g for 20 min. at 4 ºC. The supernatant fraction was aliquotted into storage vials and kept at -80 °C until required for analysis.

2.2. Biochemical Analyses

After thawing, the saliva samples were centrifuged at 250 g for 5 min. before chemical analysis. Protein concentration was determined according to the procedure described in Bradford (1976). The SOD activity was assessed using SOD assay kit-WST (Fluka Analytical, St. Louis, MO, USA), which utilizes Dojindo's highly water-soluble tetrazolium salt that produces water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase activity and the reaction is inhibited by SOD, which can be determined colorimetrically (Durak *et al*., 1996). The IC_{50} (50% inhibition activity of SOD) was quantified by measuring the decrease in the color development at 440 nm using ELISA-Reader (Awarness Technology Inc., Palm City, FL, USA). Cytochrome C assay was used to detect the extracellular release of the superoxide radical anion, extracellular superoxide radicals reduce ferricytochrome to cytochrome^{+2} that can be measured at 550 nm. 100µM cytochrome C (Sigma-Aldrich, St. Louis MO, USA) in PBS/EDTA and 100 µl of saliva from each sample were added to each well of a 96-well plate. The plates were incubated at 37 ºC for 1 h and the absorption was measured at 550 nm using ELISA-Reader (Awarness Technology Inc.)

Salivary amylase activity was measured by a quantitative colorimetric enzyme kinetic method using BioAssay Systems' EnzyChromTM alpha-amylase assay method (BioAssay System, Hayward CA, USA) as described previously in Gullbault and Rietz (1976) and as modified later in Mashige *et al.* (1982). Alpha-amylase in the saliva hydrolyzes starch and the product is rapidly converted to glucose by alpha-glucosidase and hydrogen peroxide by glucose oxidase. The hydrogen peroxide concentration was determined with a colorimetric reagent. Saliva was diluted with double-distilled water. Then the diluted saliva and standard were transferred into transparent 96-well microplates in duplicates. The standard for the assay was prepared ranging from 5 to 326 U/L Amylase (Roche Diagnostics, Mannheim, Germany). The optical density was measured at 585 nm using ELISA plate-Reader (Awarness Technology Inc.). One unit of enzyme catalyzes the production of 1 mole of glucose per

min. under the assay conditions. Uric acid concentration was determined using uric acid assay kit (Biosystem S.A. Barcelona, Spain). Uric acid is oxidized by uricase to allantoin with the formation of hydrogen peroxide that in turn is oxidized by peroxidase enzyme to form a quinoneimine dye. Quinoneimine was quantified by measuring the absorption spectrophotometry at 505 nm which is proportional to the concentration of uric acid in the sample. The absorption was measured using spectrophotometer (Spectro UV-Vis Auto UV-2602, LaboMed, Inc. Culver City, CA, U S A). The salivary albumin concentration was determined using albumin assay kit (Biosystem S.A). Albumin reacts with bromocresol green at slightly acidic pH. The color was quantified by measuring the absorption spectrophotometry at 630 nm. The intensity of the color formed is proportional to the albumin concentration in the sample.

2.3. Statistical analysis

The correlation between the participants' age, number of calls performed per day and the total number of calling min. per day with the various salivary oxidative stress biomarkers was evaluated using SPSS 17 (Statistical Package for social sciences) software. Initially, the results of the five oxidative stress biomarkers were examined for normality of distribution. The Kolmogorov-Smirnov test revealed that only the amylase activity data demonstrated a normal distribution. In consequence to violation of this parametric assumption, the Spearman rho correlation coefficient as a n on-parametric statistical test was calculated and used to evaluate the effect of mobile phone usage on the levels of the various salivary antioxidants of the participants. The correlation was considered significant if $p \leq 0.05$.

3. . Results

Table 1 provides the baseline characteristics of the subjects volunteered in the mobile phone usage study whose data were used in the analysis. The Spearman rho correlation coefficient between number of calls per day and calling min. per day is 0.477, which is significant at the 0.01 level (2-tailed). However, as shown in Table 2, the levels of the salivary antioxidant biomarkers are related and correlated to the total usage of mobile phone and that is the total min. of calling time. In other words, it is not about the number of calls per day. A significant correlation was obtained between the number of calls per day and the SOD as well as the amylase levels. The correlation was much less than that between the total calling time and the level of these two enzymes: SOD and amylase. The true correlation is with the number of calling min. rather than the number of calls. It is just that the total calling min. per day (which is the true indicator) is related to the number of calls per day (R = 0.477). While increasing the calling time causes a progressive and a significant enhancement of SOD activity, it induces reductions in the activity of amylase (Figure1). The increases in the cytochrome C activity as well as the elevations in the concentration of albumin and uric acid were not significant.

Table1. Baseline Characteristics of Study Participants (N= 109). All Participants Were Males.

Characteristic	Mean ± S.D.
Age (years)	27.74 ± 8.08
Number of calls per day	15.55 ± 8.19
Calling min. per day	67.47 ± 47.81
SOD (U/ml)	61.60 ± 24.39*
Albumin (µg/ml)	155.55 ± 86.04*
Amylase (U/ml)	84.07 ± 25.29*
Uric Acid (mg/dl)	5.75 ± 2.47*
Cytochrome C (Abs.)	0.05 ± 0.02*

Table2. The Spearman's Rho Correlation Coefficient between the Investigated Factors and the Various Salivary Antioxidant Biomarkers.

	SOD (U/ml)	Albumin (µg/ml)	Amylase (U/ml)	Uric Acid (mg/dl)	Cytochrome C (Abs.)
Age (years	0.01	0.02	0.09	0.03	-0.14
Number of calls per day	0.36[a]	0.08	-0.37[a]	-0.10	0.13
Calling min. day	0.74[a]	0.08	-0.81[a]	0.32[a]	0.19 [b]

[a] Correlation is significant at the 0.01 level (2-tailed);

[b] Correlation is significant at the 0.05 level (2-tailed).

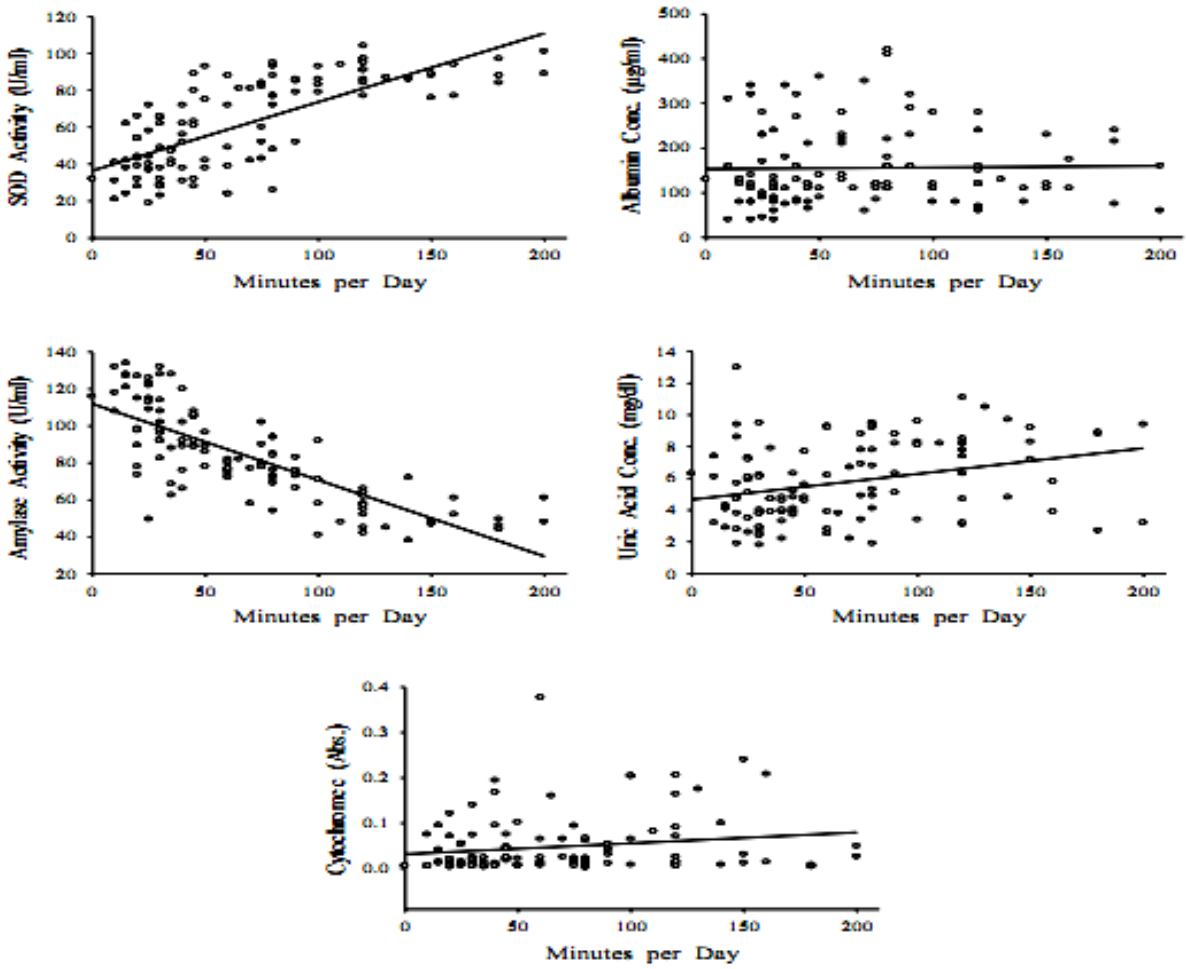

Figure1. Effect of total calling usage of mobile phone on the salivary levels of various antioxidants investigated. Each data point represents mean of three separate readings. The solid line represents a linear regression fit of the data as a function of the cumulative calling minutes per day.

4. Discussion

Saliva is an important biological fluid that plays an important role in maintaining oral homeostasis and constitutes a first line of defense against free radical-mediated oxidative stress. Furthermore, many salivary proteins offer a great potential in clinical and epidemiological research, in oral as well as in general health studies (Fleissig *et al*., 2009; Goldwein and Aframian,2010; Sathishkumar *et al*., 2010). The findings of the present study demonstrate that the exposure of human male subjects to RF-EMR emitted by mobile phone increases SOD activity in their saliva. The linear association between mobile phone-related increases in SOD activity may suggest that mobile phone induced free radical formation in human saliva. The mechanisms by which RF-EMR from mobile phones could affect the activity of salivary SOD are unclear. Some studies suggested that the exposure of certain cell types to RF-EMR could change gene and/or protein expression (Li *et al*., 2005; Gerner *et al.*, 2010). In contrast, other studies did not show significant changes in gene expression following an exposure of cultured cells to RF-EMR (Gurisik *et al*., 2006; Remondini *et al*., 2006; Zeng *et al*., 2006). Further, the overproduction of Reactive Oxygen Species (ROS) in living cells has been implicated as the first step in tissue injury and the response of the cells under RF-EMR exposure (Burlaka *et al*., 2013). The main ROS that have to be considered are superoxide anion which is predominantly generated by the mitochondria, hydrogen peroxide produced from O_2 by the action of SOD and peroxynitrite, generated by the reaction of O_2 with nitric oxide. ROS are scavenged by SOD, glutathione peroxidase (GSH-Px) and catalase *in vivo* (De Iuliis *et al*., 2009; Khalil *et al*., 2012, 2014; Ozgur *et al*., 2010) and *in vitro* (Zmyślony *et al*., 2004; Luukkonen *et al*., 2009). ROS are scavenged by SOD, glutathione peroxidase (GSH-Px) and catalase (Oktem *et al.*, 2005). Disturbance of redox balance, uncontrolled activation of free radical processes, overproduction of ROS and/or suppression of antioxidant defense in cell are often the important signals of some hazardous changes in cell metabolism (Burlaka *et al*., 2013).

In the present study, no significant increases were observed, neither in the concentrations of albumin and uric acid nor in the activity of cytochrome C. In contrast, exposure of rats to EMR caused significant reductions in

serum albumin levels in rats exposed for 3 and 6 months to RF-EMR from mobile phones at 900MHz (El-Bediwi *et al*., 2011). This discrepancy is likely to reflect differences in the species, the type of biological fluid and/or the exposure conditions, such as dose, time pattern, and frequencies of exposure used by different laboratories. In this regard, it has been indicated that the effect of EMR on living organisms depends on the frequency, intensity and duration of the exposure to phone radiation (Andersen *et al*., 2000; Sivani and Sudarsanam, 2012). Since amylase is the abundant protein in saliva, the decrease in amylase activity is intriguing in this study, a lower total parotid saliva protein concentration in dominant, compared with the non-dominant, mobile phone side has been found (Kelsh *et al*., 2011). Furthermore, a h igher saliva secretion rate from the parotid gland in the dominant mobile phone side usage has been reported (Goldwein and Aframian, 2010); it was suggested that a t hermal effect or modified cutaneous blood flow may contribute to this result. Integrating exposure over time is further complicated by the fact that sources vary markedly over very brief time periods relative to the time periods of interest (Kelsh *et al*., 2011). Also, the type of location (urban, suburban, rural) where the phone is predominantly used also appears to influence power levels across the different technologies; the power level may be as low as 1 mW depending on the location of the mobile phone with respect to the base station. Therefore, such location data would capture additional exposure information that could improve the precision of exposure assessment for epidemiological research. Ideally, geographic differences in RF power output levels, the dose, time pattern, and frequencies (wavelengths) of exposure from all key sources should be estimated for each individual in the study (Awadalla, 2013).

In conclusion, the easy access and noninvasive collection make saliva a suitable fluid type to investigate surrogate biomarkers to detect the exposure to genotoxic agents or in intervention studies. Exposure to electromagnetic radiation from a mobile telephone can cause an increase in the activity of SOD and a decrease in the activity of amylase enzyme of the exposed people. This result, along with a previous one (Gerner *et al*., 2010), may suggest that human cells recognize mobile phone radiation as an external stressor and react to them through the activation of proteins and/or the synthesis of new protein molecules.

We have included a relatively large sample size (N = 109) to improve our ability to detect small effects that may have been missed in prior studies with smaller sample sizes (Abu Khadra *et al*., 2014). However, one restriction of this study is the lack of a properly matched control. Even from rural residential areas, it w as not possible to get those so-called mobile phone non-users, or RF-EMR-"unexposed." Continuation of the research on mobile phone radiation effects is needed to assess if these effects could have potential long-term harmful consequences and to improve the basis and the reliability of the safety standards. Based on this study, therefore, it is recommended that a l ong-term and/or excessive use of mobile phones should be avoided. This can be accomplished by a t elehealth technology education program targeting the more sensitive ages: children and adolescents, since their developing brains absorb more EMR from a mobile phone (Gandhi *et al*., 1996). Such activities include: group discussions, public presentations and mass communication through the available electronic and print media sources.

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Serum Enzymes as a Result of *Azadirachta indica* Extract Injection to African Catfish *Clarias gariepinus* (Burchell, 1822)

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Abstract

The aim of this study is to evaluate the effect of the concentrations (0.0, 0.5, 1.0, 2.0 and 4.0g/L) of the leaves of *Azadirachta indica* for seven (7) days on the serum enzymes acid phosphatase (ACP), alkaline phosphate (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of African catfish *Clarias gariepinus* which is widely cultured in Nigeria because of its remarkably fast growth rate and high market value. The level of serum ACP , ALP, ALT and AST revealed a significant decrease ($P<0.05$). In this study, the general inhibition of ALT, AST, ALP implies that there were hepatic disorders (liver disease) or renal injury, a disruption of the activity of the TCA cycle, respiratory process and glycolytic pathways. The present study, thus, tries to provide baseline information on the involvement of these biochemical parameters in toxicity assessment of aquatic system and also on the alteration of the enzymatic parameters maybe used in assessment of these plant extract.

Keywords: *Azadirachta indica*, African catfish, Serum, Enzymatic Parameters.

1. Introduction

The piscicidal plants are of a unique importance in the sense that their chemical compositions enhance their properties as medicinal plants, preservatives, insecticides, molluscidices, to mention a few, hence their usefulness to man and aquatic animals (Akinwade *et al*., 2007). Due to their narcotic, pesticidal and molluscidal properties, many fishermen and fish farmers indiscriminately use various parts of these plant extracts to weaken and kill the fishes for easy catch and clean up the aquatic systems of some pests. Invariably stronger concentrations than necessary are used and this could lead to a physiological disturbance of the aquatic organism and ultimately to a reduction in the aquatic productivity (Mondal *et al*., 2007). Some of the plants used are non-selective in their destruction, thereby interfering with the ecological balance of the immediate environment. The usefulness of these plants for piscicidal and medicinal purposes has been reported (Akobundu, 1987; Adewole *et al*., 2002).

The neem plant, *Azadirachta indica* (L) of the Family Meliaceae and native of eastern Asia is a known medicinal plant that contains margosine, eriterperenoid, azatin, rotinine and quinine among other active ingredients as reported by Ade-Serrano (1982) and Adewole *et al*. (2002). The leaves, barks, fruits and roots of the plant have been highly appraised for their medicinal purposes. As a natural insecticide, the plant contains tetranitroterpenoid compounds, known as meliatoxins that are highly toxic to insects and mammals (Ascher *et al*., 1993). Omoregie and Okpanachi (1992) and Oti (2003) reported on the sublethal and actute effects of water extract of the bark of *A. indica* on *Tilapia zillii*, mudcatfish hybrid and African pike. Neem is the vernacular name used in this part of the world; in Nigeria, it is 'dongoyaro' (Brahmachari, 2004). Martinez (2002) stated that aqueous extract of neem leaves and other neem-based products have been extensively used in fish-farms as an alternative for the control of fish parasites and fish fry predators like dragon-fly larvae.

The African Catfish *Clarias gariepinus* is the most suitable species for aquaculture in Africa. *C. gariepinus*, which is widely considered one of the most important tropical catfish species for aquaculture, has a Pan-African distribution, from Nile to West Africa and from Algeria to South Africa. The African catfish has a high growth rate; exposure of this catfish to these biocides may cause stress without necessarily leading to death. Stress response is characterized by biochemical and physiological changes which may be manifested in both acute and chronic toxicity tests (Singh and Singh, 2002; Tiwari and Singh, 2004). The disruption of the biochemical and physiological integrity is assessable by the changes in the enzyme activities in functional organs (de la Torre *et al.*, 2000; Van Der Oost *et al.*, 2003).

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Enzymes are biochemical macromolecules that control the metabolic process of organisms, thus a slight variation in enzyme activities would affect the organism (Roy, 2002). They are indispensable for signal transduction and cell regulation, often via kinases and phosphatases. 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).

The complex of unspecified biochemical indicators of blood and organs reveals the general effect of pollutants and toxin on fish makes it possible to forecast the consequences of the long-term exposure to chemical pollutants (Adedeji *et al*., 2009). Moreover, 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 changes in many physiological and biochemical, blood and organ indices induced by environmental conditions and the presence of contaminants (Kori-Siakpere *et al*., 2006; Maheswaran *et al*., 2008; Ololade and Oginni, 2010). The biochemical parameters in 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). Little attention has been given to the enzymatic effect of *A. indica* on the African catfish *C. gariepinus*.

Hence, the present study was conducted to evaluate the effect of the concentrations of *A. indica* on the serum enzymes of African catfish *C. gariepinus* which is widely cultured in Nigeria because of its remarkably fast growth rate and high market value.

2. Materials and Methods

2.1. Experimental Animals

Tank-raised *C. gariepinus* (mean total length 31.75 ± 0.47 cm; mean weight, 183.26 ± 13.85g) were obtained locally from a co mmercial 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 da ys during which they were fed to satiation with commercial fish feed pellets (Coppens 4.0mm; 35% crude protein diet) twice on a daily basis. Uneaten food and faecal matters were removed daily during the acclimation and experimentation period. Dead fish were also promptly removed to avoid contamination. The percentage of death recorded during acclimatization was less than 2% as such the fishes were accepted as being adapted to the laboratory conditions.

2.2. Plant Material

Fresh leaves of *A. indica* were collected from within the campus of the Delta State University, Abraka and transported to the Department of Animal and Environmental Biology Laboratory. The plant was identified as *A. indica* 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 leaves were ground into powder with an electric blender (MX – 2071, Nakai Japan), sieved and the fine powder was stored in a dry airtight container. An aqueous extract was prepared by weighing out 200g of the milled powder leaves of *A. indica*, adding in 200ml of distilled water in a 500ml beaker and stirring vigorously with a glass rod. The combination was then allowed to settle for 3 hours using the infusion method. The extract was then filtered using Whatman No. 1filter paper. Soxhlet extraction was found to give a higher yield of the extract. The extract was then concentrated by evaporation to dryness using a rotary vacuum evaporator (RE52-2, Benjing China) at a temperature of 40^{o}C. A dark-grey colored mass was obtained and stored in airtight bottles at 4^{o}C in a refrigerator until ready for use.

2.3. Toxicant Preparation

The stored extract was reconstituted using distilled water to obtain extracts of stock solution of 10g/l of the aqueous solution of *A. indica.* From this stock, four test concentrations (0.5, 1.0, 2.0, and 4.0g/L) were prepared by serial dilution for injection of the fish.

2.4. Experimental Procedure

After acclimatization, the experimental fish were divided into six (6) groups (10 specimens per aquaria with replicates) to assess the sub-lethal effect of *A. indica* on serum enzymatic parameters. A dose of two ml of the extract for each concentration (0.0, 0.5, 1.0, 2.0 and 4.0g/L, respectively) was injected intramuscularly above the lateral line of the fish and then introduced into their respective aquaria. Fish in the control were injected with the same dose of distilled water. The aquaria used for the experiments were made of plastic having 140 L capacity. The injected fish and control fish were kept separately throughout the experimental period.

Borehole water was used throughout the acclimation and experimental periods. The water quality parameters of the exposure water used in the tests and control were determined by standard methods (APHA, 1998), as presented in Table 1.

Table 1. Water quality parameters

Parameter	Values
pH	7.58±0.32
Temperature (^{o}C)	28.30±1.3
Dissolved oxygen (mg L^{-}1)	8.32±1.04
Free carbon dioxide (mg L^{-1})	4.85±0.08
Alkalinity (mg L^{-1})	36.50±1.72
Hardness (mg L^{-1})	134.53±11.75

2.5. Sampling Procedure

At the end of the exposure period of seven (7) days, the fish were taken from the control and test tanks, sacrificed and subjected to the analysis.

Six fishes were caught individually in a small hand net from the containers. A fter the preliminary investigation of the length and weight, the fish were then placed belly upwards and blood samples obtained from the caudal

circulation with the aid of a heparinised 2cm^3 disposable plastic syringes and a 21 g auge disposable hypodermic needle. Serum was obtained from blood samples by centrifugation after coagulation and then drawn into a 1 cm^3 plastic syringe and transferred into a universal bottle, diluted 1:20 with deionised water. The diluted serum was then stored in a refrigerator and used later for analysis of serum enzymes: alkaline phosphatase, alanine aminotransferase, aspartate and aminotransferase. All determinations were carried out in duplicates for each sample.

2.6. Enzyme Analyses

The various serum enzymes: acid phosphatase, alkaline phosphate, alanine aminotransferase and aspartate aminotransferase were all determined spectrophotometrically, using Teco Diagnostic, Anahein, SA commercial kit, following the manufacturer's instruction with the aid of a spectrophotometer.

2.7. Data Analysis

The results obtained were subjected to analysis for mean and standard error. The mean values of the treatment were subjected to statistical analysis using one-way analysis of variance (ANOVA) to test the level of significance between the various concentrations of *A. indica.* Multiple comparisons of the means were analyzed with the Dunnet's Test. All statistical analysis was performed using the software programme (GraphPad Prism® Software version 5.0, San Diego, CA). Results were considered significant at the 95% confidence level $P < 0.05$.

3. Results

3.1. Acid Phosphatase

The level of serum acid phosphatase in *C. gariepinus* is presented in Figure 1. The level of acid phosphatase showed a significant decrease (P<0.05) in fish injected with 0.5g/L, 1.0g/L, 2.0g/L and 4g/L of the crude extract of the leaves when compared with the control using Dunnet's Multiple Comparism Test.

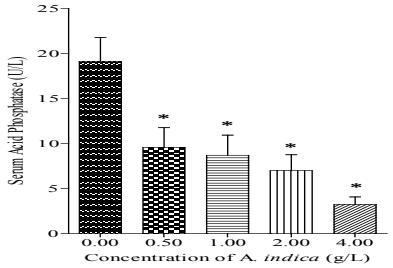

Figure 1. Mean values of acid phosphatase in the serum of *C.* Each column represents the mean value, and vertical bars indicate the standard error of the means. Asterisk represents the significant difference between the control and experimental groups at (*P<0.05*) level.

3.2. Alkaline Phosphatase

The level of serum alkaline phosphatase in *C. gariepinus* is presented in Fig. 2. The level of alkaline phosphatase showed an insignificant decrease (*P>0.05*) in fish injected with 0.5g/L, 1.0g/L, 2.0g/L and a significant decrease (*P<0.05*) in 4g/L of the crude extract of the leaves when compared with the control using Dunnet's Multiple Comparism Test.

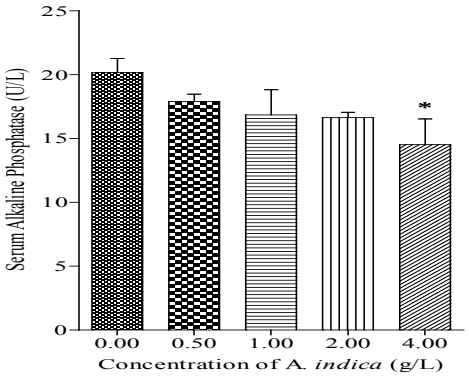

Figure 2. Mean values of alkaline phosphatase in the serum of *C. gariepinus*

3.3. Alanine Aminotransferase

The level of serum alanine aminotransferase in *C. gariepinus* is presented in Fig. 3. The level of alanine aminotransferase showed an insignificant decrease (P>0.05) in fish injected with 0.5g/L, 1.0g/L and a significant decrease (P<0.05) in 2.0g/L and 4.0g/L of the crude extract of the leaves when compared with the control using Dunnet's Multiple Comparism Test.

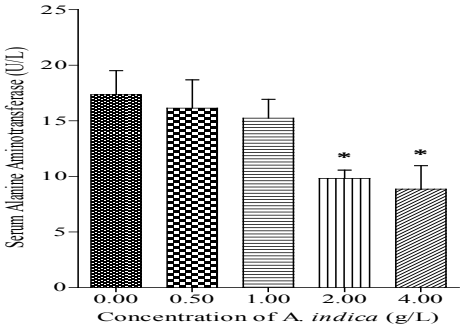

Figure 3. Mean values of alanine aminotransferase in the serum of *C. gariepinus*

3.4. Aspartate Aminotransferase

The level of serum aspartate aminotransferase in *C. gariepinus* is presented in Fig. 4. The level of aspartate aminotransferase showed a significant decrease (P<0.05) in fish injected with 0.5g/L, 1.0g/L, 2.0g/L and 4g/L of the crude extract of the leaves when compared with the control using Dunnet's Multiple Comparism Test.

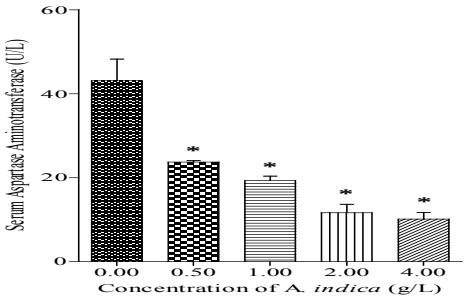

Figure 4: Mean values of aspartate aminotransferase in the serum of *C. gariepinus.*

4. Discussion

The enzymes considered in this study are useful marker enzymes that indicate the cellular damage long before revealing the structural damage by some other convectional techniques (Shahjahan *et al.*, 2004). The activities of the enzymes (alkaline phosphatase, alanine aminotransferase, aspartate and aminotransferase), considered in this study, are useful marker enzymes of damage in the liver and kidney (Akanji *et al.*, 1993).

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 plant extract toxicant for 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).

Enzyme (such as alkaline phosphate (ALP), aspartate transaminase (AST), alanine transaminase (ALT) and acid phosphatase (ACP)) assays 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, have been reported (Mgbenka *et al*., 2005; 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 as the concentrations of *A. indica*. Alkaline phosphate is a marker enzyme for the plasma membrane and endoplasmic reticulum (Wright, 1974). In this study, there was a significant decrease in the serum alkaline phosphatase and acid phosphatase activity of fish; this may be due to the inhibition of the enzyme by some components of the plant extracts (Akanji, 1993). This may result in a decrease in the phosphatase metabolism, an indication of the toxic effect of *A. indica*.

The dose-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 *Clarias gariepinus*) exposed to sublethal Effects of Tobacco (*Nicotiana Tobaccum*) Leaf Dust. Ogueji and Auta (2007) reported a reduced value of plasma alkaline phosphatase in African catfish *Clarias gariepinus* exposed to lambda-cyhalothrin. Sastry and Sharma (1980) reported alkaline phosphatase inhibition after 96 h of exposure to diazinon. Goel *et al*. (1982) reported 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 of 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) and this may support the assumption that the tissue of the experimental fish was markedly affected. 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 treatments following exposure to acute effect of diazinon (Luskova *et al.*, 2002).

Aminotransferases are gainfully used in the diagnosis of disease and tissue damage. They function 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 only in 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 this study, the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) decreased significantly ($P<0.05$) in serum as the concentration of *A. Indica* increased; this indicates a stressed based tissue impairment (Svoboda, 2001). Under stress conditions, fish need more energy resulting in 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 nitritetoxicity, suggesting that the alteration of the aminotransferases is as 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 serum aminotransferase, it is conceivable that the plant extract caused an increased energy demand by the exposed fish.

In summary, extracts of neem affected the liver function by decreasing the serum ACP, ALP and ALT, AST levels in African catfish and can be good indicators of deteriorating health in African catfish. Using some selected parameters, a presumptive prediction can be made on the health status and the possible problem (infection or toxicity). However, the parameter set may be, to some extent, case-dependent and requires information on the history of the fish.

Hence, this study was able to provide baseline information on t he involvement of these biochemical parameters in toxicity assessment of aquatic system as well as in reporting organ dysfunction and the disease conditions of fish when exposed to *A. indica* extracts.

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Bile Synthesis Peculiarities Following Changes in the Functional State of the Endothelin Receptors

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Abstract

Endothelin-1(ET-1) regulates a variety of biochemical processes in liver. However, there is no clear view concerning endothelin receptors participation in the regulation of qualitative and quantitative characteristics of liver secretory function. The purpose of this study is to evaluate the choleretic effect of ET-1 and to determine the role of ET_A receptors functional state in mediating the effect of ET-1 on bile and its organic components secretion. Endothelin-1 and BQ-123 (cyclo-Asp-pro-Val-Leu-Trp) were intraportally injected. Bile flow, bile acid concentration and content, hydroxylation and conjugation coefficients were estimated. The results of this study showed that the secreted bile volume was decreased under the effect of endothelin-1and BQ-123, although this decrease was more prolonged and profound in BQ-123 treated animals. Concentration of taurocholates, glycocholic acid and free bile acids was increased in the endothelin treated rats. When BQ-123 was administered, an increase in glycochenodeoxycholic acid+glycodeoxycholic acid (GCDCA+GDCA) and the taurin-conjugated bile acids concentration was found, whereas the free bile acids concentration altered reversely. Coefficient of hydroxylation was diminished when entholelin receptors were blocked. Activation of endothelin receptors by exogenous endothelin-1 intensified bile acids biosynthesis via "neutral pathway,"involving microsomal oxidation enzymes. The present study concludes that the endothelin receptors blockade eliminated the regulatory function of endogenous endothelin and caused a shift in bile acids synthesis to mitochondrial enzymes through "acidic pathway".

Key words: Endothelin-1, BQ-123, Bile Acid, Coefficient.

1. Introduction

Regulatory peptide endothelin-1(ET-1), the predominant isoform of endothelin, is a potent vasoconstrictor agent that was originally isolated from bovine aortic and pulmonary endothelium. The translation of preproendothelin-1 mRNA results in the formation of a big ET-1 precursor, processing into ET-1(Yanagisawa *et al.*, 1998), activates G_i-protein–coupled 7-transmembrane domain receptors. There are different types of ET receptors that, with various levels of expression, are distributed in divers tissues in human and animal organs (Kawanabe and Nauli, 2011;Yanagisawa *et al*., 1998). ET_A receptor is selective for ET-1, whereas ET_B receptor reveals similar affinities with all isopeptides (Kawanabe and Nauli, 2011; Watts, 2010). Endothelin-1 has been recognized not only as a vasoconstrictor but also as a multifunctional agent. This peptide, through activation of ET_A receptors on intrahepatic vascular smooth muscle cells (Chan *et al*.,2004), the common bile duct (Huang, 2003), Kupffer cells (Yang *et al*., 2003) and hepatocytes (Hartman *et al*., 2010), elicits different pathophysiological effects. Moreover, a wide variety of biochemical processes in liver, including glycogenolysis, gluconeogenesis and hemodynamic action, are all regulated by endothelin (Monti *et al*.,2000).There are some investigations confirming an inhibitory effect of ET-1on bile secretion. According to Rodriguez *et al.* (2013), ET-1induces a dose-dependent decrease in bile flow in isolated perfused rat liver, while Tanaka *et al.* (1994), applying a similar experimental model, demonstrated that a low dose ET-1 increased bile acid-dependent bile secretion. However, these results do not give a clear view concerning endothelin receptors participation in regulation of qualitative and quantitative characteristics of liver secretory function.

The purpose of this study is to evaluate the choleretic effect of ET-1 and to determine the role of ET_A receptors functional state in mediating the influence of ET-1 on bile formation and biliary organic components secretion *in vivo* in rats.

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2. Materials and Methods

The present study has been conducted in acute experiments on 35 l inear (male, 180-250g) Wistar rats, obtained from the Institute of Gerontology, Academy of Medical Sciences of Ukraine (Kyiv, Ukraine) after 18 hours of food deprivation. Rats were anaesthetized with ethyl urethane (100mg/100g rat body weight) which is sufficient for 3-4 hours of acute experiment. Common bile duct was then cannulated with polyethylene catheters to collect bile samples and register choleresis changes following laparotomy, respectively. Endothelin-1 (Sigma, USA, 0.1 μg/100g rat body weight) and ET_A-receptors antagonist, BQ-123 (cyclo-Asp-pro-Val-Leu-Trp; Sigma, USA, 6μg/100g rat body weight) were separately dissolved in 100 μl 0.9% sodium chloride and injected into the portal vein. According to previous studies, endothelin-1 in this concentration causes clear liver vascular movement that is an evidence for the active interaction between peptide and endothelin-1 receptors.

The animals of the control group were intraportally injected with 0.9% sodium chloride (100 μl/100g rat body weight). The secreted bile was collected every 30 minutes (consisted of three ten-minute fractions) during a 3-hour trial. The bile flow was measured by μl per g rat body weight.

Free and conjugated bile acids were divided and determined in each sample by a t hin-layer chromatography method that was initially patented by Veselsky and colleagues and was completely described by Parchami Ghazaee *et al*. (2010). We divided the mixture of the bile acids into the following fractions: taurocholic acid (TCA), taurochenodeoxycholic acid+ taurodeoxycholic acid (TCDCA+TDCA), glycocholic acid (GCA), glycochenodeoxycholic acid+ glycodeoxycholic acid (GCDCA+GDCA), cholic acid (CA), chenodeoxycholic acid (CDCA) + deoxycholic acid (DCA). Bile acids concentration and content were estimated by mg% and μg/g rat body weight. Bile acid hydroxylation and conjugation coefficients were determined by calculating the ratios of 3-hydroxycholates to dihydroxycholates and conjugated to free bile acids contents.

All experiments were performed after entherahepatic circulation abortion, following stabilization of bile flow for 30 m inutes at the beginning of experiment. The stability of rat body temperature was controlled by intrarectal thermometer. The statistical analysis was performed using the statistical package "Statistica."Data were expressed as means±SEM. Student's t-test for normally distributed values (Shapiro-Wilks W test) was used to compare variables between groups (p<0,05 was considered significant).

The study protocol was approved by the Institutional Review Board and Ethics Committee of Faculty of Biology, National Taras Shevchenko University of Kyiv.

3. Results

In the experiment on control animals, we observed a gradual reduction in secreted bile volume by 13.4% during the 3 hours of the experiment (from 0.298±0.09 μl/g in the first 10 minute interval to 0.258±0.11 μl/g in the last one). As illustrated in Figure 1, under the effect of endothelin, there was a considerable retardation in the level of bile secretion. A maximum choleresis reduction was registered in the 40th minute following the endothelin-1 injection by 15.6% (p<0.05) compared to control. Afterwards, a gradual bile flow restoration was observed and its level reached control values towards the end of the experiment. However, its level was lower than that in the first 10 minute interval by 8.6% (Figure 1).

Biochemical analysis of the half-hour bile samples evidenced that endothelin-1 exhibited a diverse effect on the concentration of different bile acids in rats. Although concentration of TCA was gradually reduced during the 3 hours of the experiment, both in control and ET-1 treated animals, its level in the sixth half-hour sample was higher by 9.3% (p<0.05) in the second group (141.5±2.3 mg% in control versus 154.7±2.8 mg% in ET-1 treated animals). However, in ET-1 treated total amount of TCDCA+TDCA was insignificantly higher in the majority of bile samples compared to control rats.

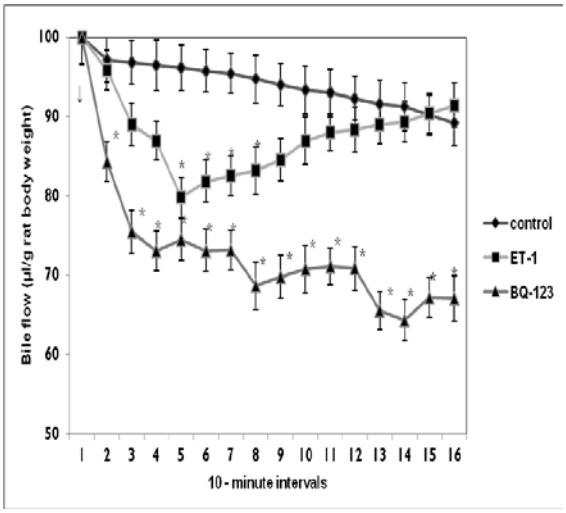

Figure 1. Effects of endothelin-1 and BQ-123 on choleresis, Mean±SEM; n=21, $^*p$<0.05 as compared to control rats.

The most drastic changes were observed in GCA concentration following ET-1 administration. Although the concentration of GCA in control animals was gradually reduced during the whole time of the experiment, it exceeded in the second half-hour, toward the end of the experiment when ET-1 was applied. Particularly, the GCA concentration increased by 12.3% (from 131.3±3.4 mg% to148.3±4.1 mg%; p<0.01), 19.7% (127.1±3.7 mg% to 152.1±4.3 mg%; p<0.01) and 16.3% (125.3±3.5 mg% to 145±7 mg%; p<0.01) in the fourth, fifth and sixth half-hour samples. At the same time, under the effect of ET-1,only a tendency for reduction in GCDCA+GDCA concentration was observed. Endothelin-1 caused an increase in CA concentration. Maximum changes were observed in the third and sixth half-hour bile samples by 20.6% and 19.8% (p>0.05), whereas, the CDCA+DCA concentration in ET-1 treated animals was next to control values (Table 1).

Table 1. Changes of the bile acids concentration (mg%) under the effect of endothelin-1 and BQ-123

Bile acids	Interval (min)	Bile acids concentration (mg%)		
		Control	Endothelin1	BQ-123
	30	168.6±5.5	173.1±4.7	167.8±6.2
	60	171.8±5.1	169.5± 3.9	182.5±5.3
TCA	90	163.1±5.2	155.1± 4.9	172.7±5.2
	120	159.0± 4.7	157.9± 4.2	177.6±5
	150	148.7±3.9	158.7± 4.4	174.3±4.9
	180	141.5±3.6	154.1±3.8*	177.3±4.7
	30	106.8±5.6	95.9± 8.9	103.7±5.5
	60	99.1± 6.1	104.6± 7.4	120.3±4.3
TCDCA+	90	95.7± 5.4	101.7± 5.2	112.3±4.7
TDCA	120	91.6± 5.2	96.8± 4.8	108.7±4.4
	150	86.7± 4.7	91.6± 4.3	109.4±4.2
	180	83.0± 4.3	92.1± 4.5	113.3±4.3
	30	135.8±5.4	132.7± 4.9	124.8±3.9
	60	138.0±5.8	136.5± 4.7	132.6±4.3
GCA	90	134.6±3.6	145.8± 3.8	132.9±4.2
	120	131.3±3.4	148.3±4.1*	134±3.7
	150	127.1±3.7	152.1±4.3**	129.9±4.1
	180	125.3±3.5	145.7± 3.9*	129.3±3.2
	30	36.8± 2.7	32.6± 2.9	31.7±2.1
	60	37.4± 2.5	28.1± 3.2	41.2±2.7
GCDCA+	90	35.3± 2.8	27.9± 3.0	43.3±2.6
GDCA	120	32.9± 2.6	26.8± 2.9	47.6±2.3
	150	29.0± 2.7	25.4± 2.7	46.7±2.7
	180	26.9± 2.5	24.3± 2.6	44.3±2.6
	30	23.2± 2.2	24.5± 2.3	22.7±2.4
	60	22.5± 2.1	25.4± 3.2	20.4±2.3
CA	90	21.8± 1.9	26.3± 2.9	21±2.4
	120	20.7± 1.8	23.2± 2.2	19.9±2.8
	150	19.1± 1.7	20.5± 1.9	17.8±2.5
	180	18.2± 1.9	21.8± 2.1	17.4±2.9
	30	7.4± 1.5	8.3± 1.7	6.9±1.3
	60	7.9± 1.8	8.1± 1.9	7±1.7
CDCA+	90	6.8± 1.2	7.2± 1.4	6.1±1.2
DCA	120	6.5± 0.9	7.4± 1.2	6.7±1.3
	150	6.3± 0.7	6.8± 1.1	5.7±1.1
	180	5.9± 0.8	6.3± 0.9	6.2±1.5

Mean±SEM; n=21, as compared with the changes in control rats. TCA, taurocholic acid; TCDCA+TDCA, mixture of tauro-chenodeoxycholicacid+ taurodeoxycholic acid; GCA, glycol-cholic acid; GCDCA+GDCA, mixture of glycochenodeoxycholic acid+glycodeoxycholic acid; CA, cholic acid, CDCA + DCA mixture of chenodeoxycholic acid +deoxycholic acid;* - $p<0.05$; ** - $p<0.01$

Peculiar changes in bile secretion were revealed when ET_A-receptors were blocked with ET-1 antagonist, BQ-123. It suppressed the action of both endogenous and exogenous endothelin-1 via this subtype of receptors. These functional changes in the ET_A-receptors state led to a more profound and prolonged decrease in choleresis. The bile secretion was decreased by 15.7% ($p<0.05$) immediately 10 minutes following the BQ-123 injection. Further, a choleresis reduction was observed in the next two ten-minute periods toward the end of the third period. It was decreased by 26.9% ($p<0.05$) compared to the initial state and 24.2% ($p<0.05$) compared to the control level. A maximum bile flow reduction was observed in minute 140 by 35.6% ($p<0.05$), compared to the initial state (Figure 1).

The chromatography analysis depicted considerable changes in the qualitative composition and the quantitative content of the bile acids in BQ-123 injected animals. The most marked increase in GCDCA+GDCA concentration was found in the fifth and sixth half-hour bile samples by 56% ($p<0.05$) and 60.6% ($p<0.05$) compared to the control values. At the same time, the concentration of GCA remained almost the same. The concentration of taurin-conjugated cholates was characterized by fluctuated changes during BQ-123 injection. Significant changes were only observed when it was enhanced; the TCA concentration in the fifth and the sixth half-hour samples was higher than in the control by 17.5% ($p<0.05$) and 25.3% ($p<0.05$). Also, the level of TCDCA+TDCA concentration was increased by 26.9% ($p<0.05$) and 35.5% ($p<0.05$). It is important to note that free bile acids concentration, simultaneously, conversely altered (Table 1).Figure 2 reveals the total contents of the different bile acids in the whole time of the experiment following ET-1 and BQ-123 injection.

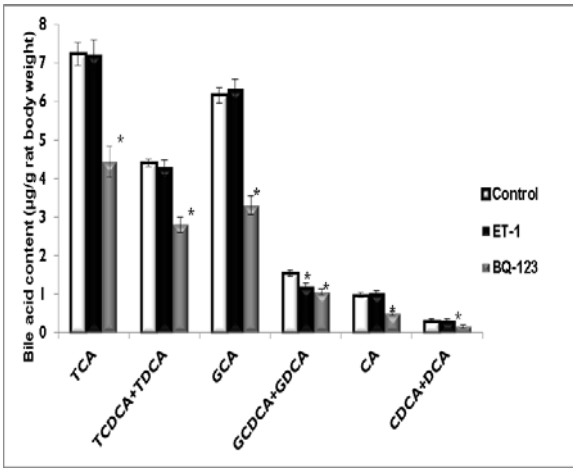

Figure 2. Bile acidscontent in endothelin-1 and BQ-123 treated rats; Mean±SEM; n=21 ;*$p<0.05$ compared to control

The dihydroxycholic acids concentration, in most bile samples, was significantly higher in BQ-123 treated animals compared to controls. This effect is clearly revealed in hydroxylation coefficient when the endothelin receptors were blocked (Table 2).

Table 2. Effect of endothelin-1 and BQ-123 on hydroxylation and conjugation coefficients of bile acids in rats

interval (min)		3-hydroxycholates mg%	Dihydroxy Cholates mg%	Hydroxylation coefficient	Conjugated bile acids mg%	Free Bile acids mg%	Conjugation ccoefficient
30	Control	327.6±3.8	151±2.7	2.2	443.1± 5.6	30.6± 2.7	14.5
	ET-1	330.5± 4.7	136.9±2.9	2.4	434.3± 6.5	32.8 ±2.9	13.2
	BQ-123	315.3± 4.8	142.3 ±3	2.2	428± 5.3	30.1± 2.8	14.2
60	Control	330.1±4.1	144.2±2.4	2.3	447.2± 4.7	30.4± 2.5	14.7
	ET-1	331.4 ±5.2	140.7±3.2	2.4	433.8± 6.3	33.5± 3.1	12.9
	BQ-123	335.7± 5.1	168.8±3.3	1.9	476.9± 4.7	27.6± 3.1	17.3
90	Control	319.5±3.6	137.8±2.2	2.3	428.7± 5.2	29.6± 2.1	14.5
	ET-1	327.2± 4.8	136.9±2.7	2.3	430.5± 5.9	33.3± 2.7	12.4
	BQ-123	326.8 ±4.4	161.8±2.7	2	461.3± 5.5	27.2± 2.4	17
120	Control	311.6±3.4	131.2±2.3	2.4	415.6± 4.8	27.2 ±2.2	15.3
	ET-1	329.4± 3.6	131.3±2.5	2.5	429.8± 5.4	30.6± 2.5	14.1
	BQ-123	331.8± 3.8	163.3±2.6	2	468.3± 5.2	26.8± 2.7	17.5
150	Control	294.9±3.2	122±1.8	2.4	391.5± 4.5	25.4± 1.9	15.4
	ET-1	331.3± 3.4	123.3±2.2	2.6	427.8± 4.7	27.2± 2.3	15.7
	BQ-123	332± 3.7*	161.8±2.5	2	460.3± 4.2	23.5± 2.5	19.6
180	Control	285±2.9	115.3±1.9	2.5	375.9± 3.8	24.1± 2.1	15.6
	ET-1	321.6± 3.5	122.4±2.3	2.6	416.5± 4.3	28.1± 1.8	14.8
	BQ-123	324.2± 3.7*	163.9±2.4	1.9	464.3± 4.4	23.8 ±1.9	19.5

ET-1: endothelin-1. Means±SEM; n=21, * $p<0.05$

4. Discussion

It is of great importance to clarify the bile synthesis peculiarities via evaluating the particular poly enzymatic systems efficiency in liver, which provides bile acid hydroxylation and amino acid conjugation processes, altering the bile colloidal system properties. Determination of hydroxylation and conjugation coefficients, as well as separate bile acid content under the effect of endothelin-1 and BQ-123, indicated significant disturbances in bile formation process.

The results revealed that endothelin-1 intensified biosynthesis of both free and glycine conjugated 3-hydroxycholates, synthesis of which is closely associated with the microsomal oxidation enzymes activity (neutral pathway), and so depends on tissue supply with oxygen. This agrees with the thesis that the oxygen consumption by the liver tissue is reduced after endothelin-1 administration (Baveja *et al*., 2002, Vlahcevic *et al*.,1997, Yanchuk *et al*.,2008).

Following BQ-123 injection, the coefficient of bile acids hydroxylation reduced from 2.2 in the first sample to 1.9in the last one, whereas in the control group, it increased from 2.2 to 2.5. This is evidences for the intensification of dihydroxycholates biosynthesis in ET_A-receptors blockade that is confirmed by a s ignificant improvement in their glycine and taurine conjugated concentration. It is known that the biosynthesis of the initial chenodeoxycholic acid in liver is realized by an active participation of the mitochondrial enzymes that in our investigation is supported by a significant increase in its conjugates under the effect of BQ-123. Therefore, ET_A-receptors blockade points out an important role of endogenous endothelin-1 in neurohumoral regulation of bile organic components biosynthesis.

The study on rats has demonstrated that endothelin-1 and BQ-123 actively influence bile acids biosynthesis efficiency according to these metabolites content in bile. Endothelin-1 in applied dose promoted both biosynthesis of glycocholic acid and content of majority of bile acids in the whole time of the experiment, whereas endothelin receptors antagonist caused a reversed effect.

It is important to note that the significant and long-lasting choleresis retardation under the influence of BQ-123 is due to the partial removal of the bile acids from osmotic and diffusion processes in bile formation following a considerable decrease in their biosynthesis in hepatocytes.

We conclude that endothelin receptors activation by exogenous endothelin-1 provokes the short-term choleresis retardation which is accompanied by an intensification of bile acids biosynthesis via “neutral pathway, ”involving microsomal oxidation enzymes, the latter is proved by the alteration of 3-hydroxycholates to dihydroxycholates ratio and the increase of glycocholic acid content. Endothelin receptors blockade with BQ-123, which eliminates the regulatory function of endogenous

endothelin, causes a sharp and long-lasting decrease in bile flow, simultaneously, shifting process of bile acids synthesis, mainly to "acidic pathway," involving mitochondrial enzymes that is confirmed by enhancing the concentration of both free and conjugated dihydroxycholates.

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Jordan Journal of Biological Sciences **Short Communication**

A New Record of *Cephalaria paphlagonica* Bobrov (Dipsacaceae) for the Iraqi Flora

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Abstract

Cephalaria paphlagonica Bobrov is a new record to the Dipsacaceae family in Iraq, from Sakran mountain (north-east of Erbil) within Rowanduz district (MRO). Description, photographs, differential morphological characters and map of distribution are conducted.

Keywords: *Cephalaria paphlagonica*, Dipsacaceae, Rowandus district, Iraq.

1. Introduction

The Dipsacaceae is one of the Iraqi flora families. This family involves 350 species throughout the world; these are distributed on 11 genera (Heukles, 2000). Iraq involves 24 species distributed on 4 genera (Al-Rawi, 1964). In Europe, the family is called Teasel (Heukles, 2000), and the genus *Dipsacus* L., from the same family, is also called Teasel (Knopf, 2000). Komarov (1957) mentioned 23 species of the genus *Cephalaria* in the Flora of U.S.S.R. In Turkey, Matthews (1972) recognized 29 species of the genus involving *C. paphlagonica*, while Gokturk and Sumbul (2014) mentioned 39 species. In Europe, Ferguson (1986) listed 14 species of the genus *Cephalaria*. In Saudi Arabia, Migahid and Hammouda (1978) mentioned 1 species of the genus. In Iran, Ghahreman and Attar (1999) mentioned 8 species of the genus. Rechinger (1964), in the Flora of low land Iraq, mentioned 2 species. While Al-Rawi (1964) and Ridda and Daood (1982) found 5 species in Iraq. Khalaf (1980) mentioned 2 species present in Sinjar mountain. Faris (1983) mentioned 3 species in Piramagrun mountain, while Fatah (2003) mentioned only 1 species of the genus in Haybat Sultan mountain.

The present study aims to revise the data concerning the presence of the plant *C. paphlagonica* in Iraq and to study the morphological characters and the geographical distribution of the species, as a contribution to the development of the Flora of Iraq.

2. Materials and Methods

For plant collecting, about 30 excursions were carried out to different regions of northern districts: MAM (Amadiya District), MRO (Rowanduz District), MSU (Sulaimaniya District), FKI (Kirkuk District), FAR (Arbil District) and FNI (Nineveh District) during Spring and Summer seasons of year 2014. Some Iraqi herbarial specimens were used; these specimens were identified through the help of some keys, especially the Flora of Turkey. The specimens were made herbarially to become formal specimens, and putted in herbarium of the Education College (ESUH). The geographical distribution of the species was cleared with fixation of some ecological notes, and a map (plate 4) was putted.

3. Results

Cephalaria paphlagonica Bobrov in Bot. Zhurn. 17: 486 (1932), Fl. Turkey, Matthews, 4: 585 (1972).

Perennial, herbs (70-115 cm), rootstock brown, (5-7)x(1.5-2.0) cm, stem numerous, erect, glabrous, green, (30-40)x(0.4-0.6) cm. Leaves opposite-decussate, become smaller upwardly, basal leaves narrowly oblanceolate, narrowly oblanceolate-very narrowly elliptic, margin dentate, apex acute, base attenuate, glabrous, green, (45-75)x(10-15) mm, lower cauline leaves narrowly oblanceolate, very narrowly elliptic, margin dentate, entire or pinnatifid at base with 1-2 pairs of small segments, apex acuminate, acute, base attenuate, glabrous, green, (75-160)x(15-30) mm., upper cauline leaves very narrowly elliptic, linear, linear-very narrowly elliptic, cultrate-linear, margin entire or pinnatifid at base with 1-2 pairs of small segments, apex acuminate, base attenuate, truncate, glabrous, green, (12-130)x(1.5-20) mm. Inflorescence a cyme head, obconical, globular-semi globular, not radiant, (9-12)x(8-18) mm, peduncle multi-costate, green, glabrous, (16-230)x(0.4-1.2) mm involucral bracts membranous, numerous rows (4-6), each

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row with 4-5 bract, narrowly obovate, orbicular, deltate, margin ciliated, apex acute, apiculate, rounded, obtuse, base acute, rounded, obtuse, truncate, pubescent and pilose, yellow, (2.5-6.5)x(2.3-6.5) mm receptacular bracts membranous, oblanceolate, lanceolate, margin ciliated, apex acute, apiculate, obtuse, base acute, obtuse, truncate, yellow, pubescent and pilose, (6.0-9.0)x(1.8-2.7) mm flowers numerous, peripheral flowers sterile. Outer calyx or involucel of tube and limb, the tube of central flowers cup-shaped, with 8 farrows and ridges, pilose, yellow, (1.7-2.0)x(1.3-1.5) mm, of peripheral flowers (0.6-0.8)x(0.5-0.7) mm, the limb of central flowers with 8 minute teeth, (0.8-1.2)x(1.6-1.9) mm, of peripheral flowers (0.4-0.6)x(0.9-1.2) mm. Inner calyx of tube and limb, the tube of central flowers tubular-ellipsoid, with 8 farrows and ridges, yellow, (1.6-2.0)x(0.9-1.2) mm, of peripheral flowers (0.4-0.6)x(0.4-0.7) mm, the limb of central flowers with 20-24 minute teeth, (1.4-1.8)x(1.7-2.2) mm, of peripheral flowers (0.7-1.0)x(1.0-1.3) mm. Corolla of tube and limb, the tube of central flowers pubescent and pilose, white-light yellow, (5.0-8.0)x(1.4-3.5) mm, of peripheral flowers (6.5-7.2)x(1.6-2.0) mm, the limb of central flowers with 4 equal lobes, oblanceolate, apex acute-obtuse, (3.0-4.2)x(5.0-6.4) mm, of peripheral flowers (4.0-4.5)x(4.2-4.6) mm. Stamens 4, exerted, epipetalous, inserted at the base of the corolla limb lobes and alternate with them, filaments filiform, yellow, (7.0-7.5) x (0.15-0.30) mm, anthers cultrate, narrowly oblong-cultrate, brown-yellow, versatile attechment with the filaments, (2.0-2.7)x(0.5-0.8) mm. Pistil single, ovary inferior, uni-locular, single pendulous ovule, narrowly lanceolate, lanceolate-narrowly ovoid, light yellow, (2.0-2.6) x (0.8-1.2) mm, (the ovary does not grow in the sterile peripheral flowers), (0.4-0.9)x(0.2-0.4) mm, style filiform, terminal attachment with the ovary,

yellow, (5.0-7.0)x(0.30-0.45) mm, [in the peripheral flowers, the style lengths are short (0.9-1.1) mm, very short (0.4-0.6) mm, extremely short (0.15-0.30) mm], stigma oblong, reach to the middle of the corolla limb lobes, dark yellow, (0.25-0.35)x(0.15-0.20) mm. Fruiting heads globular, semi-globular, (9-13)x(9-12) mm, fruiting calyces similar the flowering ones but differ in their dimensions, outer fruiting calyx or involucel tube (6.5-7.2)x(2.2-3.0) mm, the limb (1.5-2.0)x(0.1-0.2) mm, the inner fruiting calyx tube (5.5-6.7)x(1.2-2.6) mm, the limb (2.4-3.0)x(2.0-2.5) mm, fruit simple, dry, indehiscent, achenial, cypsela, very narrowly ellipsoid, very narrowly ellipsoid-narrowly ellipsoid, yellow-brown, (5.0-6.2)x(1.0-2.2) mm. Seed single, similar to the fruit, green-yellow, (4.0-5.2)x(0.8-1.8) mm (plates 1-3).

Type: [Turkey A5] Amasia: in montis Ak-dagh regione alpine, alt. 16-1900 m.s.m., 13 viii 1889, Bornmuller 1074 (holo. LE!).

Material examined

MRO: ESUH/ Sakran mountain (north-east of Erbil), 2400 m, 19.6.2014, A. Sardar & S. Al-Dabagh, 7141.

Environment & Geographical Distribution

Found as population within the area, usually in wet exposed places, in clay and rocky clay soils; altitude: 2200-2400 m; flowering: June-July. Found in Sakran mountain within Rowanduz district (MRO) (plate 4).

Plate 1: Field photograph of *C. paphlagonica*

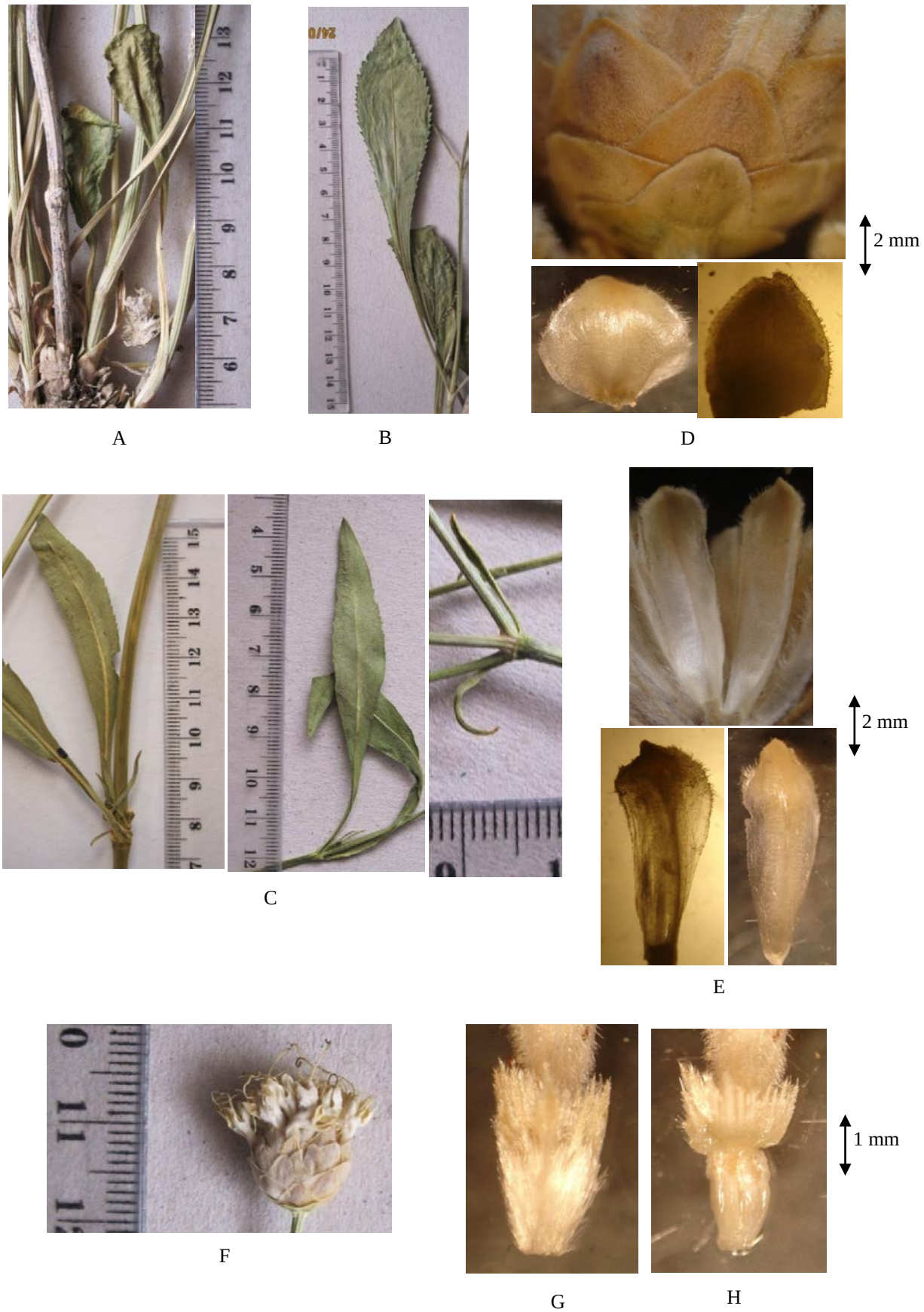

Plate 2: Vegetative and reproductive parts of *C. paphlagonica*: A. Basal leaves, B. Lower cauline leaves, C. Upper cauline leaves, D. Involucral bracts, E. Receptacular bracts, F. Flowering head, G. Outer calyx, H. Inner calyx

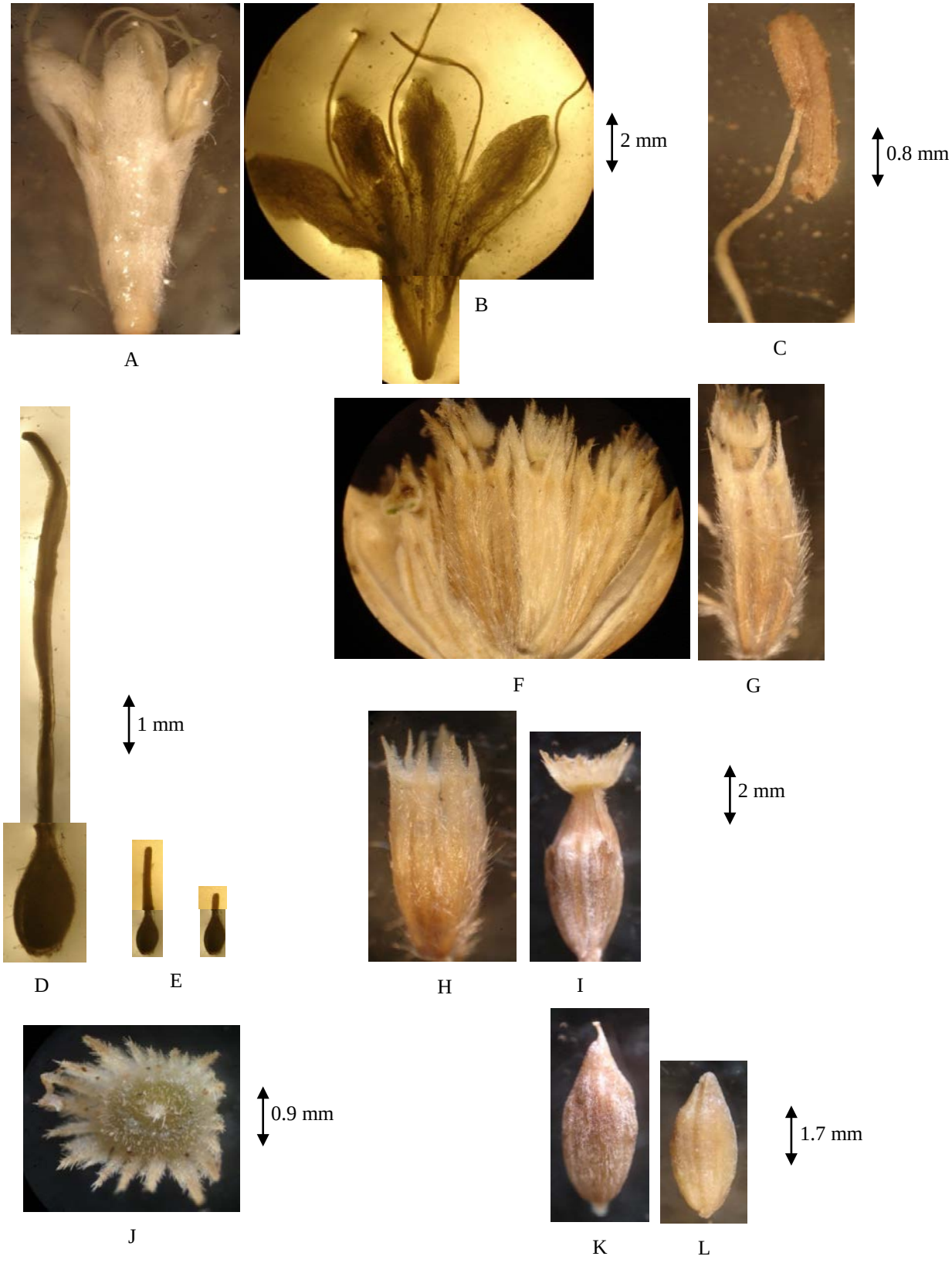

Plate 3: Reproductive parts of *C. paphlagonica*: A. Corolla, B. Opened corolla, C. Stamen, D. Fertile pistil, E. Sterile pistils, F. Section of fruiting head, G. Outer with inner fruting calyx, H. Outer calyx, I. Inner calyx, J. Inner fruiting calyx limb: upper view, K. Fruit, L. Seed

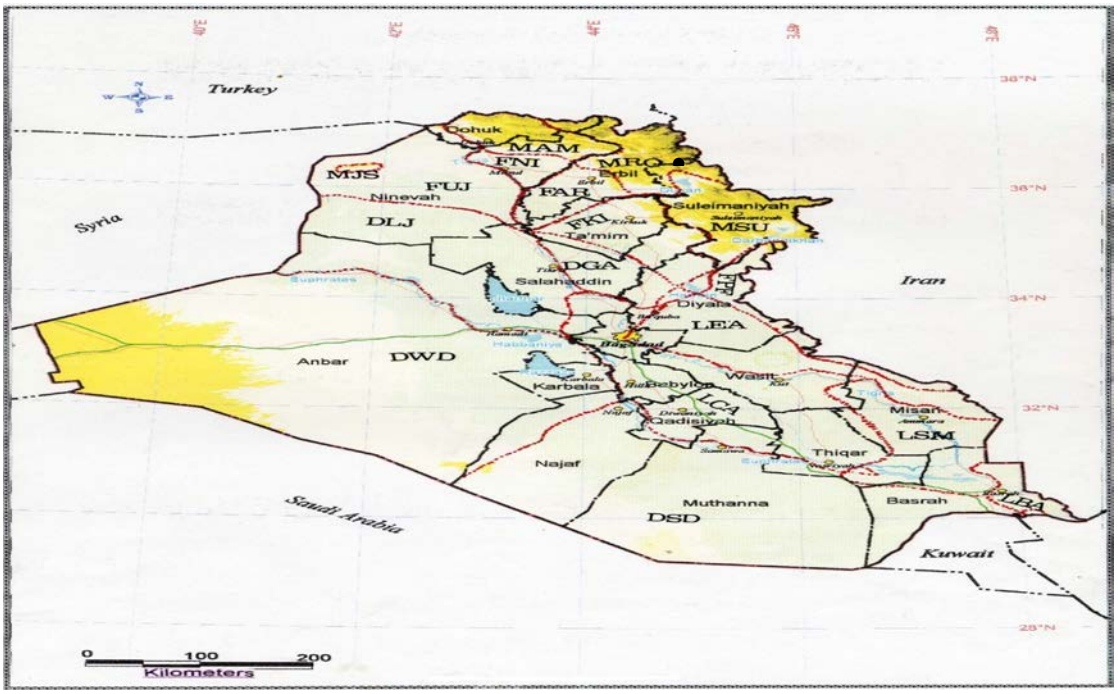

Plate 4: A map of Iraq shows the location of: ● *Cephalaria paphlagonica*

4. Discussion

The present study deals with a new record of *Cephalaria paphlagonica* from the Dipsacaceae family. The study includes specific aspects, such as the morphological characters and the environment with the distribution in the studied area. Within the literature related to the genus *Cephalaria*, including the specimens of National Herbarium of Iraq and University Herbaruim (in Baghdad University), the researcher did not find any species belonging to *C. paphlagonica*. Therefore, this contribution can be regarded a new record in Iraq from Sakran mountain.

C. paphlagonica has some characteristics different from the nearest species which is *C. microcephala* that found in Iraq, and these characteristics include the glabrous stem and leaves, entire or pinnatifid upper cauline leaves, not radiant flowering heads, narrowly obovate, orbicular, deltate involucral bracts with their yellow colors, 8 minute teeth of the involucel.

It is worth mentioning that the flowers are hermaphrodite, entomophilous, and the pistil is single and 2-syncarpous (Watson and Dallwits, 1992)

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Jordan Journal of Biological Sciences **Short Communication**

Ipomoea Muelleri Benth. (Convolvulaceae) – a new record for Asian Continent

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Abstract

Ipomea muelleri Benth (Convolvulaceae) is an endemic Australian plant species. It is reported here for the first time from the Southern Western Ghats of Coimbatore region of India in the Asian continent. A detailed description, illustration and relevant notes are provided for its collection and identification..

Keywords: Climber, Maruthamalai, Tamil Nadu, Western Ghats, India.

1. Introduction

The Convolvulaceae family, consisting of 58 genera and approximately 2,000 species (Staples and Yang, 1998), is cosmopolitan in distribution (Fang and Staples, 1995). *Ipomoea* L. comprises the largest genus of the Convolvulaceae family, represented by c. 650 species and mainly distributed in tropical and warm temperate regions of the world (Mabberly, 2008). In India, the genus is represented by c. 60 species (Santapau and Henry, 1973; Bhellum, 2012) and, in Tamil Nadu state, by c. 33 species (Henry *et al.,* 1987).

During the floristic studies on the climbers of the Southern Western Ghats, India, the authors collected an interesting invasive species from the Maruthamalai foot hills of Coimbatore district, Tamil Nadu. On critical examination and perusal of literature (Johnson, 2011), it was identified as *Ipomoea muelleri* Benth. So far it is known in Australia only. The present collection of *Ipomoea muelleri* Benth, therefore, forms a new distributional record for the Asian continent, particularly in India.

2. Plant Description

***Ipomoea muelleri* Benth** Fl. Austral. 4: 423, 1868; Jessop, J.P. and Toelken, H.R., eds. (1986). Flora of South Australia, 4(4). 330. 1986; Jhonson in Kellerman. Flora of South Australia (ed. 5), pp.20, 2011.

Prostrate climbers, sparsely hirsute, with trailing or twining stems; leaves broadly ovate to triangular, 1.5-8 x 1.5-7 cm, base cordate, apex obtuse, emarginated; inflorescence cymose; peduncles longer than the petioles, bearing 1-3 flowers; bracts very small, pedicel thicker than the peduncle, 1-40 mm long. Sepals thick, outer sepals longer than the inner sepals, ovate to ovate-lanceolate, acuminate, mucronate at apex, 6-8 x 2-3 mm; corolla funnel-shaped, pale rose-pink with a slightly darker throat, glabrous, 1.5-2cm long; filaments unequal, adnate at the base; ovary 4-celled; stigma 2 lobed, globular. Capsule globular to ovoid-globular, depressed, 8-11 mm diam., splitting often tardily into 4 longitudinal valves. Seeds 4, villous, c. 2 mm long, (Figure 1 and 2).

Flowering & Fruiting: January-April

Habitat: Waste land and along roadside as a weed between 430-440 msl, Coimbatore District, Tamil Nadu, India.

Distribution: An invasive species occur rarely along the road side of the Maruthamalai foot Hills of the Coimbatore district, Tamil Nadu, India, (Map 1).

Specimen examined: India: Tamil Nadu, Coimbatore District, Maruthamalai Road. 12 Feb-2013, Sarvalingam, Sivalingam & Rajendran, 006165 (BH).

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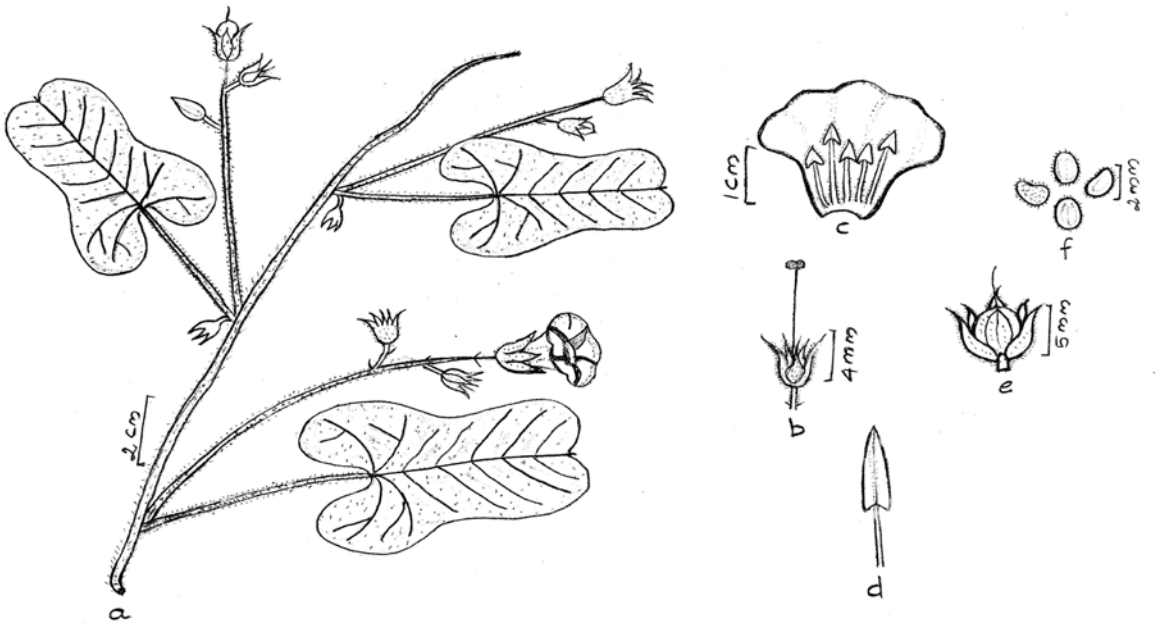
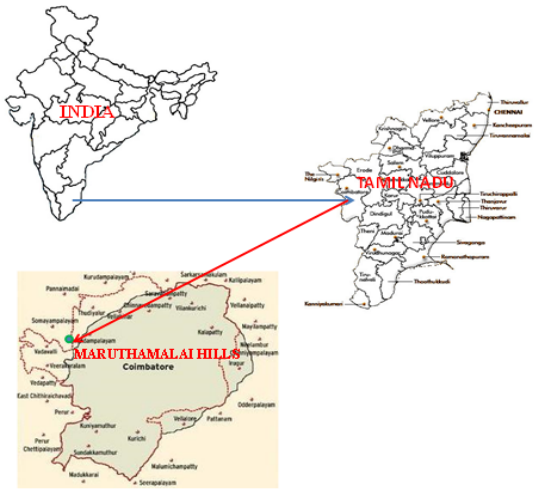


Figure 1. *Ipomoea muelleri* Benth., a. Habit; b. Sepals with pistil; c. Corolla with stamens; d. Anther; e. Fruit; f. Seed

Figure 2. Habit of *Ipomoea muelleri* Benth.



Map 1. Study area

3. Conclusions

Ipomoea muelleri Benth, growing in disturbed areas such as roadside, now intermingled with naturalized plants. The novel report of the species *Ipomoea muelleri* Benth revealed that though a large amount of environmental data are available about their role in biodiversity conservation, field surveys are still vital to enhance knowledge about the Asian biodiversity heritage, particularly in India.

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Appendix A
Reviewers 2014
We would like to acknowledge especially the contributions of the following reviewers during the year 2014

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