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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds), Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International. pp 181-190.

Jake OO (2002). Pharmaceutical Interactions between *Striga hermonthica* (Del.) Benth. and fluorescent rhizosphere bacteria Of *Zea mays*, L. and *Sorghum bicolor* L. Moench for *Striga* suicidal germination In *Vigna unguiculata*. PhD dissertation, Tehran University, Iran.

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ARTICLES

Review

**Systematic pharmacognostical, phytochemical and pharmacological review on an ethno medicinal plant, *Basella alba L***

Shankul Kumar, A. K. Prasad, S. V. Iyer, S. K. Vaidya

58

Research Articles

**Pharmacognostic, larvicidal and phytotoxic profile of *Coleus forskohlii* and *Rosmarinus officinalis***

Saeed Ullah, Muhammad Ibrar, Barkatullah, Naveed Muhammad, Roohullah

63

**Antimicrobial efficacy of neutral super-oxidized electrolyzed gel versus chlorhexidine digluconate 0.12% in biofilm formation on orthodontic mini-implants: *An in vitro* study**

Evelyn Torres-Capetillo, Roberto Carrillo-Fuentevilla, Myriam A. De la Garza-Ramos, Roberto Mercado Hernández, Hilda H. H. Torre-Martínez, Juan Carlos Segoviano-Ramirez

71

**Effect of *Hippocratea africana* root bark extract on some biochemical indices of male and female albino Wistar rats**

J. I. Ndem, M. U. Eteng, M. I. Akpanabiatu, A. F. Uwah, O. Otitoju, E. O. Akpanyung

76



Review

# Systematic pharmacognostical, phytochemical and pharmacological review on an ethno medicinal plant, *Basella alba* L.

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The ethno medicinal plant *Basella alba* belongs to the family Basellaceae, and commonly known as malabar spinach, indian spinach, ceylon spinach and vine spinach. Although it has been reported in ancient systems of medicine, it did not gain much importance and has always remained controversial. It has been found to be a good source of calcium, iron, vitamin A and vitamin C. In the Indian system of medicine, the plant has immense potential in androgenic activity, antiulcer activity, antioxidant, cytotoxic and antibacterial activity, anti-inflammatory activity, central nervous system (CNS) depressant activity, nephroprotective and wound healing properties etc. The present is therefore, an effort to give a detailed survey of literature on pharmacognostical, phytochemical, ethnopharmacology as well as the pharmacological activities of the plant.

**Key words:** Nephroprotective, cytotoxic, androgenic, antiulcer activity, antioxidant, spinach.

## INTRODUCTION

To cure human disease, medicinal plants have been a major source of therapeutic agents since ancient times. The revival of interest in natural drugs started in last decade mainly because of the wide spread belief that natural medicine is healthier than synthetic product. Nowadays, there is manifold increase in medicinal plant based industries due to the increase in the interest of use of medicinal plant throughout the world which is growing at a rate of 7 to 15% annually. Despite the major advances in the modern medicine, the development of new drugs from natural products is still considered important. Since 1980, World Health Organization (WHO) has been encouraging countries to identify and exploit traditional medicine and phytotherapy.

The main Indian traditional system of medicine namely Ayurveda and Siddha, are primarily plant based system. The evaluation of new drugs especially phytochemically obtained materials has again opened a vast area for research and development. As per WHO, about 80% of

the population in the world relays on the traditional medicine for the treatment of various diseases. Therefore, the evaluation of rich heritage of traditional medicine is essential. In this regard, one such plant is *Basella alba* which is a fast-growing, soft-stemmed vine, reaching 10 m in length. Its thick, semi-succulent, heart-shaped leaves have a mild flavour and mucilaginous texture. The stem of the cultivar *B. alba* 'Rubra' is reddish-purple. It is popularly known as Malabar spinach, Indian spinach; native of Southern Asia. It is a plant which is used in every household in the eastern and southern parts of India. The leaves and stem part of the plant are used in cooking. The plant is found to be versatile in properties. It has been found to be a good source of calcium, iron, vitamin A and vitamin C.

In the Indian system of medicine, the plant has immense potential in androgenic activity, antiulcer activity, antioxidant, cytotoxic and antibacterial activity, anti-inflammatory activity, central nervous system (CNS)



Figure 1. Plant with green stem.



Figure 2. Plant with purple stem.

depressant activity, nephroprotective and wound healing properties etc. The aim of present review is to highlight the pharmacognostical, phytochemical, ethnopharmacology as well as the pharmacological investigation carried out on the plant so that the more pharmacological studies could be conducted to investigate the unexploited potential.

### Plant profile

*B. alba* (Family: Basellaceae); Synonym: *Basella cordifolia* Linn. *Basella lucida* Linn. is a perennial climber. It is also known as Malabar spinach, Indian spinach, Ceylon spinach and vine spinach. The stem is succulent with tender leaves. Both stem and leaves are used in

Culinary practice in Southern parts of India. It is found to be a good source of calcium, iron, vitamin A and Vitamin C (Palada and Chang, 2003; Sir Ghillean and Mark, 2005). (Figures 1 and 2).

Other names of the plant include: (1). English: Malabar spinach, Ceylon spinach, Indian spinach; (2). Hindi: Poi ki Bhaji; (3). Bengali: Pui shak; (4). Oriya: Poi saga; (5). Konkani: Valchi bhaji (Vauchi bhaji); (6). Kannada: Basale soppu; (7). Telugu: Bachhali; (8). Tamil: Kodip pasaLi; (9). Marathi: Mayalu.

### Taxonomy of plant

- (i) Kingdom: Plantae;
- (ii) Unranked: Angiosperms;
- (iv) Unranked: Eudicots;
- (v) Unranked: Core eudicots;
- (vi) Order: Caryophyllales;
- (vii) Family: Basellaceae;
- (viii) Genus: *Basella*;
- (ix) Species: *Basella alba* Linn.

### Description

It is a fast growing perennial climber, growing up to 9 m in length and belongs to the family Basellaceae. It is a valuable vegetable that can be cultivated from either seed or cutting.

The stem is green or purplish and quadrangular in shape, about 2 to 3 cm thick, with prominent nodes and internodes. The taste is bland and mucilaginous, with no odour.

The leaves are fleshy and ovate or heart shaped, chordate base, dark green in colour, glossy above and glaucous below. The size of the leaves varies from 3 to 9 cm in length and 4 to 8 cm in width. Taste was found to be bland, with no odour.

The flowers are inconspicuous, bisexual white flowers borne on axillary spikes or branching peduncles.

Its fruits are fleshy and stalk less, ovoid or spherical in shape, 5 to 6 mm, and purple when mature (Tropilab Inc. <http://tropilab.com/bas.html>. accessed on 31 July 2012).

The useful parts of the plant include its leaves, young stem, matured fruit, and roots (Plants for a future [http://www.pfaf.org/database/plants\\_phb?Basella+alba](http://www.pfaf.org/database/plants_phb?Basella+alba). Accessed on 31 July 2012).

### Chemical constituents

Dried leaves (per 100 gm) contains, protein 20%, fat 3.5%, carbohydrate 54%, fibre 9%, and ash 19%. The leaves contain a high level of calcium and are rich in vitamins like A, C, thiamine, riboflavin, niacin, and betacyanin, oxalic acid, flavonoid like acacetin, 4,7-dihydroxy kempferol and 4'-methoxyisovitexin and also

phenolic acids like vanillin, syringic and ferulic acid. The fruit contains betacyanins and gomphrenin (Daniel, 2006; Eliana et al., 2007).

### Traditional use

The plant has been known to be a demulcent, a diuretic and an emollient action. The entire plant is used in Chinese medicine where it is claimed to reduce fever and neutralize poison. The pulped or bruised leaves are used as a poultice for ulcers and to hasten the maturation of abscesses. A decoction of the leaves is believed to have laxative properties, and is used to treat constipation in pregnant women and children. The extract mixed with *Hibiscus rosa-sinensis* is given to pregnant women as a safe aperients. The juice of the plant is used as a dye for official seals, as a rouge on the facial skin and food colouring (Daniel, 2006; Herbal Medicine Research Centre, 2002). In southern India, the plant is used for the treatment of Aphthae (Hebbar et al., 2004).

### PHARMACOGNOSTICAL REVIEW

Pharmacognostical studies provide qualitative standard and reveal the type of cell, its arrangement, and cell content. Transverse section of the fresh stem revealed the following features (Vanaliya Saroj et al., 2012).

#### Epidermis

The epidermis is represented by a single layer of compactly arranged, barrel-shaped parenchyma cells. Intercellular spaces are absent. The cells are slightly thick walled. Externally, a thin transparent waxy covering called cuticle is found.

#### Hypodermis

Hypodermis is represented by a few layers of collenchyma cells with angular thickenings. The cells are compactly arranged without any intercellular spaces.

#### Cortex

Cortex comprises of 3 to 5 layers of compactly arranged parenchyma cells without intercellular spaces.

#### Stele

The stele comprises of pericycle, vascular bundles and pith.

#### Pericycle

It is represented by a 3 to 4 layers of compactly arranged sclerenchyma cells.

#### Vascular bundles

Vascular bundles are arranged in the form of a ring. The vascular bundles are conjoint, collateral and closed. Xylem is on the inner surface and phloem on the outer surface. Xylem is described as endarch.

#### Pith

Pith is made of many layers of loosely arranged parenchyma cells with intercellular spaces.

### PHYTOCHEMICAL REVIEW

Phytochemical studies reveal the presence of steroids and phenolic compound (Phadungkit et al., 2012). The chemical composition of the leaf extract include proteins, fat, vitamin A, vitamin C, vitamin E, vitamin K, vitamin B9 (folic acid), riboflavin, niacin, thiamine and minerals such as calcium, magnesium and iron. Kaempferol is the flavonoid present in *B. alba* at a concentration of 1.4 mg/100 g (Yang et al., 2008). *Basella* mucilage is viscous, with low swelling capacity. Its pH is good for skin (5.3 to 5.4). Partial purification of *Basella* mucilage proved to be composed of polysaccharide, with D-galactose as a major compound. The cell toxicity to Chang liver cell shows tendency of mild toxicity. The gel preparation of *Basella* mucilage provide good stability that serve for further development as cosmetic and medicine for skin diseases (Toshiyuki et al., 2001). *B. alba* contains basellasaponins, amino acid such as arginine, leucine, isoleucine, lysine, threonine and tryptophan (Khare, 2007), peptide, and phenolic compounds in various extracts (Maisuthisakul and Ritthiruangdej, 2008). The fruit contains gomphrenin derivative which is a betalain pigment (Glassgen et al., 1993). The mucilage of *B. alba* consists of mixture of polysaccharides (Palanuvej et al., 2009) and starch-type glucan which can be separated by starch iodine complex (Haq et al., 1969). Anthocyanins are a natural pigment which is responsible for the blue, purple, violet and red colours in fruits, flowers, stem and leaves (Glassgen et al., 1993).

#### Ethnopharmacology

*B. alba* has been used for many of its useful products in ancient times. Nowadays, its properties have been utilized

for the extraction of some useful material so that it can be used for the benefit of human activities. Some of the uses of this plant parts in the cure of certain problems which occurred in humans has been explained here:

1. Daily consumption of *B. alba* has a positive effect on total-body vitamin A storage in men (Haskell et al., 2004).
2. The paste of root of red *B. alba* along with washed rice water is taken in the morning on empty stomach for one month to cure irregular periods by the rural people of Orissa, India. Leaves of *B. alba* is used for the treatment of hypertension by Nigerians in Lagos, and malaria in Cameroonian folk medicine. The plant has been reported for its antifungal, anticonvulsant, analgesic, anti-inflammatory and androgenic activities and for the treatment of anemia. The leaves of *B. alba* are traditionally used in Ayurveda system of medicine to bring sound refreshing sleep when it is applied on head about half an hour before bathing (Anandarajagopal et al., 2011).
3. A paste of the root is applied to swellings and is also used as a rubefacient. The sap is applied to acne eruptions to reduce inflammation. Decoction of leaves is used for its mild laxative effect. Pulped leaves are applied to boils and ulcers to hasten suppuration. Sugared juice of leaves is useful for catarrhal affections. Leaf-juice mixed with butter is soothing and cooling when applied to burns and scalds. In Ayurveda, it is used for hemorrhages, skin diseases, sexual weakness, ulcers and as laxative in children and pregnant women. The plant is febrifuge, its juice is a safe aperient for pregnant women and a decoction has been used to alleviate labour. It is also an astringent, and the cooked roots are used in the treatment of diarrhea. The leaf juice is a demulcent, used in cases of dysentery (Kumar, 2010).
4. This plant serves as a Thai traditional vegetable. The fruit provides dark violet colour for food colorant. *Basella* mucilage has been used in Thai traditional medicine as topical application for irritant, bruise, ringworm and labour. Stem and leaves are used as mild laxative, diuretic and antipyretic (Chou, 1997).
5. In India, it has been used for antipruritis and burns (Saikia et al., 2006), and has been used in Bangladesh for acne and freckle treatment (Akhter et al., 2008).
6. The Ayurvedic treatment in India used *B. alba* leaves and stem as anticancer such as melanoma, leukemia and oral cancer (Premalatha and Rajgopal, 2005). Roots and leaves has been used for the removal of after birth, stomach pains and increase milk production (Pascaline et al., 2010).
7. *B. alba* is administered orally for the treatment of anal prolapsed or hernia. Ground leaves of *B. alba* are rubbed on the human hand the whole preparation introduce into animal's vagina every morning for the treatment of sterility (Chifundera, 1998).
8. The leaf juice is used in Nepal to treat dysentery, catarrh and applied externally to treat boils. The

mucilaginous qualities of the plant make it an excellent thickening agent in soups, stews, etc. The purplish sap from fruits is used as a colouring agent in pasteries and sweets (Ramu et al., 2011).

9. *B. alba* has been used for the treatment of Anemia in women, coughs, cold (leaf with stem), and cold related infections (Rahmatullah et al., 2010).

10. Maceration is taken orally for infertility, pelvic inflammatory disease, orchitis, epididymitis, threatened abortion and spurious labour (Focho et al., 2009).

11. Leaves are used in constipation, poultice for sores, urticaria and gonorrhea. It is also used in poultice local swellings, intestinal complaints etc (Yasmin et al., 2009).

12. The mucilaginous liquid obtained from the leaves and tender stalks of plants is a popular remedy for headaches (Jadhav et al., 2011).

## PHARMACOLOGICAL REVIEW

Pharmacological review reveals the pharmacological activity and its therapeutic value of plant. The popularity of the plant was highly enhanced by ideological belief in the herb as a cure for multiple diseases. The detailed pharmacological activities of plant are thus given:

### Androgenic activity

In this study, Leydig cells were purified from 70 day-old Sprague Dawley male rats and incubated with 10 and 100 µg/ml of methanolic extract of *B. alba* (MEBa) for 4 h, followed by the evaluation of cell viability, steroid (testosterone and estradiol) production, and the level of aromatase mRNA. The results showed that MEBa did not only affect Leydig cell viability but also significantly stimulated testosterone and estradiol production ( $p < 0.01$  and  $p < 0.03$ , respectively), and enhanced aromatase mRNA level ( $p < 0.04$ ). These observations suggest that MEBa directly stimulated testosterone, estradiol and aromatase mRNA levels in isolated Leydig cells (Edouard et al., 2011; Moun dipa et al., 2006).

### Anti-inflammatory activity

The methanolic extract of *B. alba* (MEBa) and aqueous extract of *B. alba* (AEBa) were studied for its *in vitro* anti-inflammatory activities. The potency of the extracts was compared with standard Diclofenac sodium (50 and 100 µg/ml). The aqueous extract showed the most significant membrane stabilizing action on human red blood cell membrane (Vijender et al., 2011). In cotton pellet induced granuloma, the test drugs 50% (BLE 250 mg/kg) and 60% (BLE 500 mg/kg) were found to be less potent than phenyl butazone as a standard (Krishna, 2012).

### Haematological and biochemical parameters studies

The effects of the aqueous leaf extract of *B. alba* on haematological and biochemical parameters were studied in Wistar strain albino rats and the results showed that *B. alba* significantly increased red blood cell count, white blood cell count, packed cell volume, haemoglobin concentration and platelet count. However, the extract significantly reduced the activity of the liver enzymes such as alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In conclusion, adding *B. alba* leaves as part of daily diet may reduce anemia and maintain good health (Bamidele et al., 2010).

### Anti ulcer activity

In the anti ulcer studies, parameters such as ulcer index, percentage of ulcer inhibition, gastric pH, pepsin content, thiobarbituric acid reactive substances, lipid hydro peroxides, superoxide dismutase (SOD), glutathione peroxidases (GPx), CAT, Glutathione (GSH), vitamin C, vitamin E have been analysed, and the results suggest that aspirin altered the parameters to considerable extent, which were restored to near normal with *B. alba* leaf extract (Venkatalakshmi and Senthamaraiselvi, 2012).

### CNS depressant activity

Petroleum ether, methanol and aqueous extracts of dried aerial parts of *B. alba* were studied. CNS depressant activity of all the extracts of *B. alba* was evaluated by pentobarbitone induced sleeping time test, open field test and hole cross test in mice. Methanol extract (100 and 200 mg/kg, *p.o.*) showed highly significant ( $p < 0.001$ ) CNS depressant activity than other extracts tested. All the results were compared with reference drug, diazepam (Anandarajagopal et al., 2011).

### Cytotoxic and antibacterial activity

The methanolic extract shows the significant growth inhibition on human cancer cell lines and momentous zone of inhibition for microorganisms studied. The overall result of this study indicates that the methanolic extract from *B. alba* have interesting anticancer and antibacterial properties, and the traditional use of this plant may also derive from its antibacterial and anticancer properties (Rathee et al., 2010).

### Antioxidant activity

*B. alba* fruit with dark blue skin and deep red violet flesh

is a potential source of natural colorant. This study were aimed to evaluate the total betacyanin content, total phenol and to analyse the antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide anions, hydroxyl radical, metal chelating, hydrogenperoxide, fluorescence recovery after photobleaching (FRAP), 2,2'-azino-bis (ABTS) and deoxyribose degradation in a dose dependent manner. Betacyanin extracted from *B. alba* fruit exhibited excellent antioxidant activity (Reshmi et al., 2012; Sivasankar et al., 2011; Chanda and Dave, 2009).

### Wound healing activity

The aqueous extract of leaves of *B. alba* were formulated as a gel and investigated for its physicochemical as well as for its burn wound healing activity. Different gel formulations of aqueous extract of *B. alba* (2% w/v) were prepared using polymers carbopol 934 and carbopol 940 by varying their concentration. Wound healing studies of aqueous extract revealed that *B. alba* treated animals were found to epithelise in 23 days while the solvent control and untreated rats epithelised within 35 and 39 days, respectively. The formulation 1.5% w/w carbopol 934 were found to be more promising as it shows better physicochemical characteristics, higher pharmacological activity and better stability compared to other formulations (Mohammed et al., 2012).

### Nephroprotective effect

The nephroprotective effect of an ethanolic extract of *B. alba* L. on gentamycin (GM)-induced nephrotoxicity in Wistar albino rats were studied by administering GM only (100 mg/kg, *i.p.*) for 8 days. In drug treated groups, rats were pretreated with *B. alba* (250 and 500 mg/kg per day orally) for 14 days and co-treated with GM for 8 days. After 24 h of the last dose, blood, urine, and tissue samples were collected from the animals. GM when administered induced a marked renal failure characterized by a significant increase in serum and urine creatinine, urea, uric acid, gamma-glutamyl transferase (GGT), and protein levels. Besides, there were elevation of malondialdehyde (MDA) level and decrease in the concentration of total proteins (TPs) and sulphhydryl group (SH) free in kidney tissue, which are indicators of oxidative stress of kidney. The extract also significantly reduced the GM-induced elevated serum and urine levels of sodium, potassium, calcium, protein, creatinine, urea, uric acid, and GGT. The tissue MDA level was also significantly diminished; the decreased free-SH and TP levels were significantly replenished by an ethanolic extract of *B. alba* treatment (Saleh, 2011).



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Full Length Research Paper

## Pharmacognostic, larvicidal and phytotoxic profile of *Coleus forskohlii* and *Rosmarinus officinalis*

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This study was performed to conduct the pharmacognostic study of *Coleus forskohlii* Linn. and *Rosmarinus officinalis* and find larvicidal and phytotoxic effects of these plants. The pharmacognostic parameters for both plants were set out using macroscopic and microscopic methods and the larvicidal and phytotoxic potential was determined using recommended methods. Various pharmacognostic parameters such as stomatal index, palisade ratio, vein islet number and vein termination number were determined for both plants. In the powder drug of both plants, various fragments were detected such as epidermal cells, non-glandular trichomes, and glandular sessile and stalked trichomes, epidermal cells with stomata. Biological activity of ethanolic extract of *C. forskohlii* and *R. officinalis* showed that *R. officinalis* extract is more effective than *C. forskohlii* against *Culex quinquefasciatus* larvae. In case of phytotoxic effect, the effect of *R. officinalis* was better than *C. forskohlii*. Both tested plants were found to be phytotoxic and larvicidal.

**Key words:** Pharmacognostic, *Coleus forskohlii* Linn., *Rosmarinus officinalis* Benth.

### INTRODUCTION

The pharmacognostic study of crude drug is very essential for the identification of medicinal plants and prevention of adulteration (Muhammad et al., 2012; Ismail et al., 2011). Medicinal plants have acquired increasing significance in development of cooperation among various organizations in the recent years. The medicinal use of the plants is well established even in the modern world, which is indicated by the fact that more than 30% of the allopathic drugs are of plant origin and about 80% of the world population relies chiefly on traditional medicines (Saeed et al., 2010; Shinwari, 2010). *Rosmarinus officinalis* (Lamiaceae), locally known as rosemary is a long lived semi woody perennial shrub, with evergreen leaves, stem of 1 to 2 m long, calyx densely white tomentose, corolla bilabiate, two prominent stamens with simple filament and carpel consisting of long

style. *Coleus forskohlii* (Lamiaceae) is a perennial shrub, with fleshy leaves, stem of 18 to 24 inch long, calyx and corolla bilabiate, stamens didynamous, and carpel consist of bifid style (Qaiser and Omer, 1985).

In the traditional system of medicines, *R. officinalis* is used as anti-asthmatic, bronchodilator, antiplasmodial, antioxidant (Inatani et al., 1983), anti-inflammatory, anticancer and as hepatoprotective agent (CA R, 1999). Antimicrobial activities of this plant have also been reported by Oluwatuyi et al. (2004). *C. forskohlii* (local names are Coleus, mainmul, and karpuravali) is also a well known medicinal plant and has been reported with various pharmacological activities like anti-obesity and anti-asthmatic. Its use in glaucoma and heart disease has been reported by Kavitha et al. (2010).

This study was conducted with aim to document the

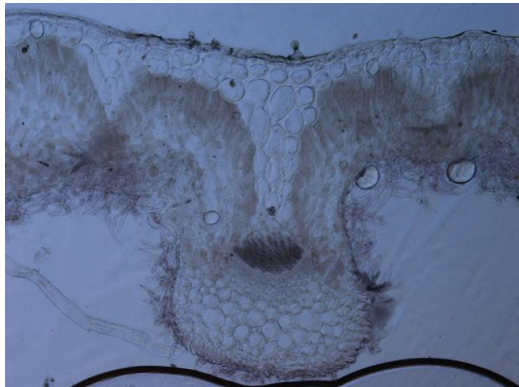


Figure 1. Transverse section of *Rosmarinus* leaf.



Figure 2. Lower epidermis of *Rosmarinus* with gland and trichomes.

pharmacognostic profile of both plants and the ethanolic crude extract of both plants was screened for their larvicidal activity.

## MATERIALS AND METHODS

### Collection of plants and larvae

*R. officinalis* and *C. forskohlii* were collected from plant growing at Department of Botany University of Peshawar, Peshawar. Each of the collected plant samples was cleaned washed and dried at room temperature.

*L. minor* plants were collected from the fresh running water in University of Peshawar and were transferred to the Pharmacognosy Laboratory, Department of Botany, University of Peshawar for phytotoxic assay.

Mature larvae of *Culex quinquesfasciatus* were collected and identified by Prof. Dr. Khesroon, Department of Zoology, University of Peshawar, Peshawar, Pakistan.

### Preparation of extract

The leaves of both plants were soaked in pure ethanol of commercial grade and ethanolic extract was prepared following our previous published methods (Muhammad et al., 2010a; Muhammad

et al., 2010b; Barkatullah et al., 2011). The powder of *R. officinalis* and *C. forskohlii* were soaked in ethanol for one week. There after each plant extract was passed through filter papers. The obtained extracts were evaporated in a rotatory evaporator at 45°C obtained concentrated extract of each plant. These concentrates of each extract were stored at 4°C prior to use. The final collected crude ethanolic extract was scrutinized for larvicidal activity.

### Pharmacognostic study

The dried leaves were powdered with the help of an electric grinder and were stored in airtight bottles. Some fresh specimens of the plants were used to study morphological and anatomical characters, and for the palisade ratio, vein-islets number, vein-termination number and stomatal studies. The hands cut sections were made of fresh specimens. The material was mounted in the center of potato pith, by making a whole, cylindrical or longitudinal according to the type of material. The thin sections were selected for staining, which was carried out on a microscopic glass slide (Evans et al., 2002). The powdered drug was studied on electric microscope at different power of eyepieces. The powder material was macerated with chloral hydrate suspension. One drop of solution was taken on a slide and then it was heated on spirit lamp and then examined under microscope. Different tissues were observed under the microscope and were photographed (Muhammad et al., 2012).

### Larvicidal activity

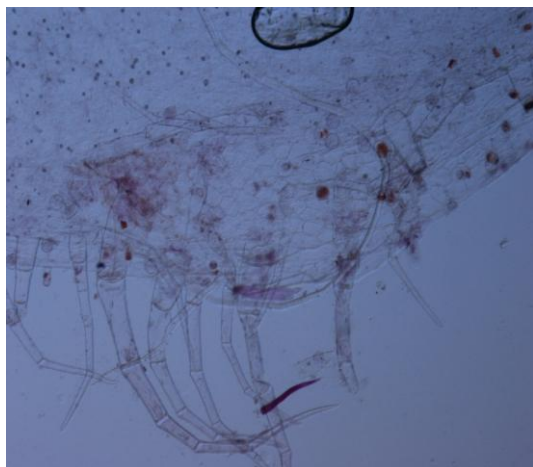
One gram of each crude extract was first dissolved in 10 ml of ethanol to prepare a stock solution. From the stock solution, different concentration (50, 100 and 200 ppm) were prepared with tap water. Experiments were conducted for 24 and 48 h, respectively at room temperature. The larvicidal activity was assessed following the well recommended procedure (Rahuman et al., 2000). For bioassay, 10 test larvae were taken in 3 replicates in 50, 100 and 200 ppm concentration of the desired plant extract. And the numbers of dead larvae were counted after 24 and 48 h exposure, respectively and the percentage mortality was reported from the average of 3 replicates.

### Phytotoxic activity

The crude ethanolic extract of both plants concentrations of 50, 100 and 200 ppm were tested for their phytotoxic effect using our previous published method (Muhammad and Saeed, 2011).

## RESULTS AND DISCUSSION

Microscopic characteristics of the leaf of *R. officinalis* show that the upper epidermis which is unicerate cuticle is thick, and palisade cell present only below the upper epidermis (Figure 1). The trichomes are of two types, glandular and non-glandular or covering trichomes. The stomatal index of *R. officinalis* is non-measurable due to the peltate hair. The stomata were present only on the lower surface. Microscopic characteristics of the leaves of *C. forskohlii* showed that the cuticle is thin, and stomata were present on upper as well as on the lower surface but more on the lower surface (Figures 2 and 3). Non-glandular as well as glandular trichomes were present on

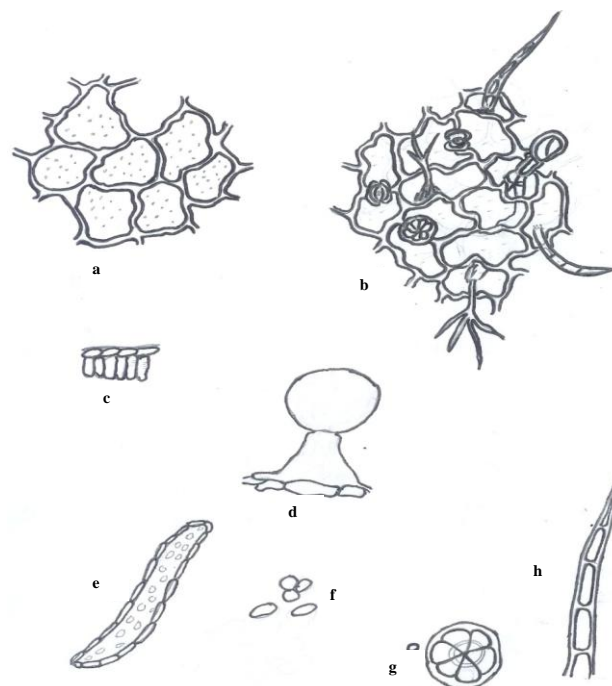


**Figure 3.** Transverse section of *Coleus* leaf.

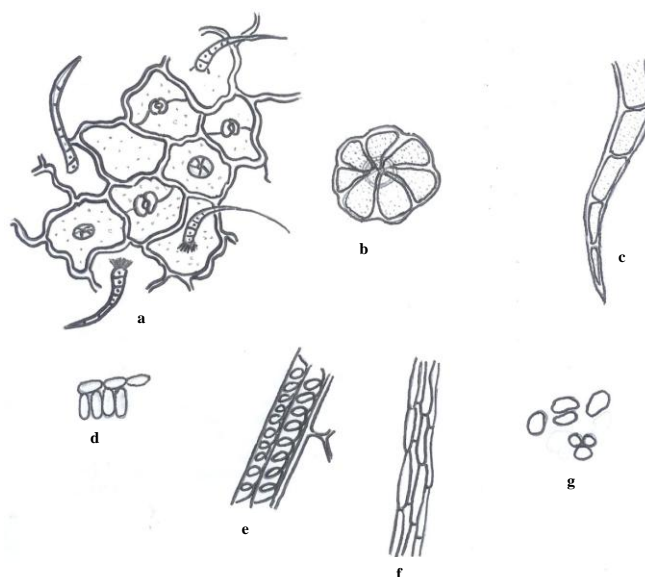
both surfaces. Palisade cells were present on both lower as well as on upper sides.

Glandular and non-glandular trichomes were present on the lower surface, which is the characteristic feature of family Lamiaceae, but a few stalked glandular trichomes were also present on the upper epidermis. The glandular trichomes were mostly sessile and with multicellular head but few stalked glandular hairs were also present in *R. officinalis* (Figure 4). The stomatal index of *R. officinalis* was non-measurable, because the stomata present only on the lower epidermis which is covered with dense peltate trichomes which have hidden the elliptical stomata (Annalisa et al., 2003). In *C. forskohlii*, non-glandular as well as glandular trichomes were present on both surfaces. Rejdali (2008), who reported different types of trichomes which were not uniformly distributed on the leaf surface stomata of diacytic type were present on both lower and upper epidermis in *Sideritis* (Lamiaceae). In *Coleus*, the covering trichomes are more numerous, unicerate and unbranched. Palisade cells were present on both lower as well as on upper sides (Figure 5) between palisade cell, and spongy mesophylls were present. The average vein islets number of *R. officinalis* and *C. forskohlii* as observed under microscope during this work were 11 to 14 and 9 to 12, respectively and the vein termination number of the *R. officinalis* and *C. forskohlii* were worked out and tabulated as shown in Table 1. The powder drug characters especially the microscopic character have got prominent role in the identification and standardization of drugs. The organoleptic study of the powder drug of *R. officinalis* and *C. forskohlii* were also carried out.

The powder drug of *R. officinalis* was dull green in color, slightly bitter in taste with cooling effect and pungent odor. Two types of trichomes covering (trichomes and glandular trichomes), epidermal cell with elliptical stomata, tracheid, parenchyma's cell and starch



**Figure 4.** (a) Upper epidermis, (b) lower epidermis with stomata, glandular, non-glandular and branch trichomes, (c) palisade cells, (d) glandular trichome, (e) tracheid, (f) starch grains, (g) multicellular head of sessile gland, (h) covering trichome.



**Figure 5.** (a) Epidermis with stomata, sessile glandular and covering trichomes, (b) multicellular head of sessile gland, (c) covering trichome, (d) palisade cells, (e) spiral ducts, (f) vessels, (g) starch grains.

grain were observed. The powder of *C. forskohlii* was yellowish brown with pleasant smell and bitter taste powder shows numerous ovoid simple circular elliptical



**Table 1.** Leaf histological parameter of *R. officinalis* and *C. forskohlii*.

Parameter	Average ratio	
	<i>R. officinalis</i>	<i>C. forskohlii</i>
Palisade ratio	4.6 to 5.20	4.6 to 6.10
Stomatal index	Non measurable	27.60 (lowered) 24.40 (upper)
Vein islet number	11-14	9-12
Vein termination number	12-15	9-11

**Table 2.** Larvicidal activity of *Rosmarinus officinalis* and *Coleus forskohlii*.

Plant	Dose (ppm)	Mortality (% after 24 h)	Mortality (% after 48 h)
Control	-	0	0
	50	13	16
	100	20	20
<i>C. forskohlii</i>	200	33	40
	50	20	43
	100	46	66
<i>R. officinalis</i>	200	56	83

**Table 3.** Organoleptic evaluation of *R. officinalis* and *C. forskohlii*.

Parameter	Observation	Observation
Color	Dull green	Light brown
Odor	Pungent	Aromatic
Taste	Slight bitter with cooling effect	Slight bitter
Fracture	Hard	Soft
Touch	Hard	Soft

starch grain, trachied, vessels and paranchymatus cells. Two types of trichomes (Glandular and Non-glandular), epidermal fragment with diacytic stomata and palisade cells were also observed. Larvicidal activities of *R. officinalis* and *C. forskohlii* were carried out. The present results (Table 2) showed that the leaf ethanolic extract of *R. officinalis* was more effective than *C. forskohlii* against *Culex* larvae. The activity was observed in different concentration of the crud extracts, that is, 50, 100 and 200 ppm at 24 and 48 h exposure.

In this study, both plants (*R. officinalis* and *C. forskohlii*) at 50 ppm concentration showed low mortality rate after 24 and 48 h, respectively. But at higher concentration (100 and 200 ppm) and after 48 h, the mortality rate for *R. officinalis* was 66.67 and 83.33, respectively, whereas for *C. forskohlii* the mortality is 20.00 and 40.00, respectively as shown in Table 3. This shows that *R. officinalis* can be used effectively as a larvicide against *Culex*. Whenever there is an effective crude alternative to synthetic drugs or Larvicides, the present trend is to go for the former one not only because it is cheaper, but due

to its lesser toxicities to human being and due to their biodegradability. The member of Lamnaceae family is a suitable organism to investigate physiological processes. *Lamna* (*L. minor*) plant consists of a central oral frond or mother frond with two daughter frond and filamentous root. The phytotoxic effect was tested according the well recommended methods (Atta-ur Rehman et al., 2001).

The crude ethanolic extract of the leaves of both plants were tested at concentration of 10, 100 and 1000 ppm. The percent phytotoxic effect of *R. officinalis* was 13, 20 and 67% at the tested concentrations of 10, 100 and 1000 ppm, respectively, while the percent phytotoxic effect of *C. forskohlii* was 10, 15 and 35% at the same tested concentrations, respectively.

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Full Length Research Paper

## Antimicrobial efficacy of neutral super-oxidized electrolyzed gel versus chlorhexidine digluconate 0.12% in biofilm formation on orthodontic mini-implants: An *in vitro* study

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The objective of this study was to compare the antimicrobial efficacy and cytotoxicity in fibroblasts of neutral super-oxidized electrolyzed gel and chlorhexidine digluconate. Cultures were prepared with *Streptococcus intermedius*, *Porphyromonas gingivalis* and a mixture of both. Thirty sterile orthodontic mini-implants were impregnated with neutral super-oxidized electrolyzed gel and chlorhexidine digluconate for 10 min, then immersed in culture media and incubated at 37°C for 24 h. Samples were taken for colony forming units (CFU), turbidity tests to determine bacterial absorbance and concentration, and for cytotoxicity testing in fibroblasts. Neutral super-oxidized electrolyzed gel had a lower inhibitory effect on *S. intermedius* with a statistically significant bacterial absorbance and CFU ( $P = 0.012$  and  $P = 0.000$ , respectively). This was also observed for *P. gingivalis* with a statistically significant CFU ( $P = 0.000$ ). A similar behavior was found with the mixture of *S. intermedius* and *P. gingivalis* with a significant bacterial absorbance and CFU ( $P = 0.003$  and  $P = 0.000$ , respectively). Chlorhexidine digluconate showed no bacterial growth and a greater cell cytotoxicity (22.08% cell viability), compared with super-oxidized gel (97.16%). Super-oxidized gel inhibited bacterial growth around the mini-implant. Chlorhexidine digluconate acted as a bactericide. Chlorhexidine digluconate had a greater cytotoxic effect when compared with neutral super-oxidized electrolyzed gel.

**Key words:** Mini-implants, chlorhexidine, neutral super-oxidized electrolyzed gel, bactericide.

### INTRODUCTION

Current orthodontics has systematized the placement of mini-implants as part of a treatment plan, primarily to obtain and improve skeletal anchorage and to reduce the need for patient co-operation. Peri-implantitis is an inflammation of the mucosa that surrounds the implant

and has clinical and radiographic evidence of bone loss, bleeding upon probing, suppuration, epithelium infiltration and progressive mobility, which affects the soft tissue and supporting bone around a functioning implant (Kravitz et al., 2007). Chlorhexidine is a positively ionized bisbiguanide

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that absorbs different negative charges from the site where it is used, such as the mucus membrane, the Salivary film on teeth, and titanium surfaces, as well as the different components of the biofilm on the tooth surface (bacteria, extracellular polysaccharides and glycoproteins) (Zanatta et al., 2007). Chlorhexidine is used after mini-implant placement as an antibacterial and to minimize tissue inflammation. It promotes a slow epithelialization and may reduce the possibility of increased soft tissue around the mini-implant (Kravitz et al., 2007).

*In vitro* studies (Zanatta et al., 2007; Longworth, 1964; Xie, 2000; Kozlovsky, 2006) have shown that at low concentrations, chlorhexidine causes cell membrane damage and leakage of low molecular weight molecules of microorganisms. In contrast, at high concentrations, it causes protein precipitation and coagulation in the cytoplasm of the exposed microorganisms. These properties interfere with biofilm formation and prevent its growth.

Gianelli et al. (2008) found that chlorhexidine affects cell viability depending on the time of exposure, particularly in osteoblasts. Its toxic effect is the induction of apoptosis and autophagocytosis of dead necrotic cells involving damage of mitochondrial function, an increase in intracellular  $Ca^{+2}$ , and cell oxidation. This suggests that chlorhexidine is highly cytotoxic *in vitro* and that precaution is necessary when used as an antiseptic in surgical procedures of the oral cavity.

Studies report that the mechanism of action of super-oxidized gel is the oxidation of sulfhydryl and amino groups of the bacterial wall, which affects the respiration and nutrition process of microorganisms, resulting in oxidation of respiratory components, inhibition of protein synthesis, and altered cell metabolism with decreased production of high energy phosphates (adenosine phosphate), regardless of the breakage of chains and repression of RNA synthesis (Esteripharma Mexico, 2012). Super-oxidized solutions have been found effective in wound care and *in vitro* studies have demonstrated activity against different bacteria, viruses, and spores (Gutierrez, 2006; Tanaka et al., 1996). The potential toxicity of superoxide solution has also been studied in fibroblast cultures comparing hydrogen peroxide versus pH-neutral superoxide solution. It was found that superoxide solution is significantly less cytotoxic than antiseptic hydrogen peroxide (Gonzalez-Espinoza et al., 2007).

It was considered important to conduct a prospective experimental, longitudinal and comparative *in vitro* study of the efficacy of neutral super-oxidized electrolyzed antimicrobial gel (Esteripharma Mexico, SA de CV, Mexico City, Mexico) and chlorhexidine digluconate 0.12% (Farmacia Morlan, Toledo, Spain) in biofilm formation and their cytotoxic effect on fibroblasts, because there are no previous reports in the literature comparing the antimicrobial and cytotoxic properties of super-oxidized gel and chlorhexidine digluconate 0.12%.

## MATERIALS AND METHODS

### Bacterial inoculation of samples

A prospective, experimental, longitudinal and comparative study was conducted of 30 orthodontic mini-implants in three different bacterial culture media (*Streptococcus intermedius*, *Porphyromonas gingivalis* and a bacterial mixture of *S. intermedius* and *P. gingivalis*). To determine the sample size and the experimental methodology with regard to obtaining bacterial cultures, we used the previous experience with bacterial cultures of Ferraz et al. (2007) and Chin et al. (2007).

Sterile titanium orthodontic mini-implants (Ancoragem Ortodôncica®, Neodent, Curitiba, Brasil), trypticasein culture medium, and sterile chemicals products (neutral super-oxidized electrolyzed gel and chlorhexidine digluconate 0.12%) were used. Titanium orthodontic mini-implants that were contaminated or damaged or that had contaminated culture medium, or contaminated chlorhexidine digluconate and super-oxidized gel solutions were excluded.

The variables studied were bacterial absorbance, bacterial cell concentration, colony forming units (CFU), and cytotoxicity. Bacterial cultures of *S. intermedius*, *P. gingivalis*, and a mixture of *S. intermedius* and *P. gingivalis* were prepared. Once bacterial cultures were active, we proceeded to conduct the experiment in 50 sterile Eppendorf tubes. Samples were divided into 6 groups and controls were included.

### Bacterial count analysis

The bacterial suspension was pipetted onto a microscope slide, through the edge of the cover slip, filling the counting chamber by capillarity. Within minutes, the cells precipitated to the bottom and counting began. Bacterial cells from each of the 25 larger squares drawn on the slide were counted. The bacteria from several large squares were counted and means were obtained. The number present in the largest square multiplied by 25 is the number present in  $0.02 \text{ mm}^3$ . The number multiplied by 50 is the number in  $1 \text{ mm}^3$ . This number times 1000 is the number in 1 ml. If the number of cells in a small square is counted, this number is multiplied by a factor of 16. Example:  $16 \times 25 \times 50 \times 1000 = 20,000,000$ .

#### Group 1

The study started with 5 Eppendorf tubes to which 50  $\mu\text{l}$  of bacterial broth of *S. intermedius* (Si) was added. Subsequently, 1 sterile mini-implant was deposited in each of the tubes for 1 min to impregnate them with bacteria. Following this, we impregnated the mini-implants with neutral super-oxidized antiseptic gel for 1 min and then carefully removed the gel irrigating with sterile double distilled water. Finally, each mini-implant was placed in an Eppendorf tube containing 1000  $\mu\text{l}$  of sterile broth culture and samples were incubated at  $37^\circ\text{C}$  for 16 h.

#### Group 2

The study started with 5 Eppendorf tubes to which 50  $\mu\text{l}$  of bacterial broth culture of *P. gingivalis* (Pg) were added; afterwards, the same procedure as for group 1 was conducted.

#### Group 3

We started with 5 Eppendorf tubes to which 50  $\mu\text{l}$  of bacterial broth

culture of *S. intermedius* and *P. gingivalis* were added, carrying out the same procedure as for groups 1 and 2.

#### Group 4

The same procedure was performed as for group 1 using chlorhexidine digluconate 0.12%.

#### Group 5

The same procedure was performed as for group 2 using chlorhexidine digluconate 0.12%.

#### Group 6

We carried out the same procedure as for group 3 using chlorhexidine digluconate 0.12%.

#### Negative controls

Negative controls were carried out to confirm the sterility of the culture medium, of the mini-orthodontic implant, and of the study chemicals. Samples were incubated at 37°C for 24 h.

#### Positive controls

These controls were performed to verify that there was bacterial growth in the different bacteria broth cultures used in the study. Samples were incubated at 37°C for 24 h.

After 24 h, real-time polymerase chain reaction (PCR) was performed for DNA extraction to identify bacterial colonies. This was done to confirm that the bacteria had been inoculated by first intention, and were present at the end of the experiment. Likewise, spectrophotometry was carried out to identify turbidity of the culture medium in order to quantify absorbance and concentration of each of the samples.

#### Turbidity test by absorbance

A Smart Spec™ Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA) was used, in which a cuvette with a concentration of 50 µl of sterile double distilled water for 45 µl of bacterial sample was placed. Absorbance and concentration were then measured to confirm inhibition of bacterial growth around the mini-implant when neutral super-oxidized electrolyzed gel and chlorhexidine digluconate were used.

#### Cytotoxicity measurement in fibroblasts

To assess the cytotoxic effects of neutral super-oxidized electrolyzed gel and chlorhexidine digluconate, normal human cell lines (ATCC Hs68) were used. We placed 50,000 cells per well in a 16 chambered slide containing different concentrations of chlorhexidine and super-oxidized electrolyzed gel in media culture. These were grown for 24 h. After incubation new media was added to wash the cells and eliminate the drugs. Phosphate buffered saline (PBS) was used to replace media culture and perform morphologic analysis. Buffered formalin (4%) was used as a fixative, and an autofluorescence inductor were mixed in the chamber with PBS. Ten minutes later, cells were washed with PBS again and the remaining autofluorescence inductor was eliminated. Slides were

mounted with fluoroshield and analyzed with a Zeiss LSM 710 confocal microscope using a 360 nm line laser to excite 4',6-diamidino-2-phenylindole (DAPI) and a 514 one to excite autofluorescence of cytoplasm induced by the fixative. Morphologic analysis to evaluate the effect of drugs on the cytoplasm was implemented. Images of negative controls and cell cultures exposed to different drug concentrations were acquired in the same conditions.

#### Absorbance, concentration and CFU

To determine differences in absorbance, concentration, and CFU relative to the bacterial dilution between treatments we applied simple linear regression and then the slopes obtained were compared for the following groups: (1) Si + super-oxidized gel, (2) Si + chlorhexidine digluconate, (3) Pg + super-oxidized gel, (4) Pg + chlorhexidine digluconate, (5) Si + Pg + super-oxidized gel, (6) Si + Pg + chlorhexidine digluconate.

#### Statistical analysis

Simple linear regression was used to correlate absorbance to the logarithm of the dilution and concentration, and also to the logarithm of CFU with the logarithm of dilution.

## RESULTS

### Cytotoxicity

A clear difference in the toxicity of chlorhexidine was observed. The test was carried out for only 2 h, because if cells were left longer, there would be no living cells to count and make a comparison.

Chlorhexidine digluconate 0.12% showed a high degree of cytotoxicity causing a considerable decrease in cell viability in comparison to neutral super-oxidized electrolyzed gel, which had a lower percentage (Figures 1 to 6).

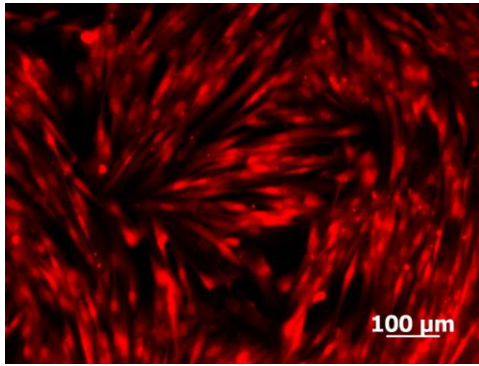
### Results for absorbance, concentration and CFU explained by treatment

Simple linear regression was used to correlate absorbance to the logarithm of dilution, and concentration and also, the logarithm of CFU with the logarithm of dilution (Table 1).

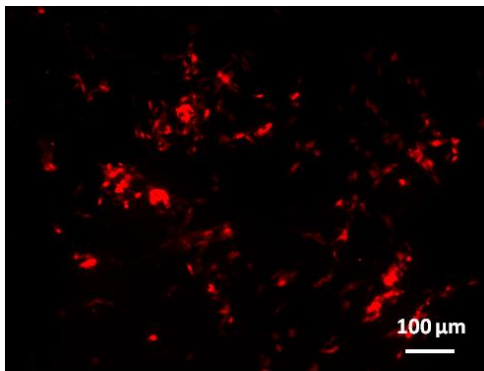
#### Group 1

For Group 1 (Si + super-oxidized gel), taking into consideration zero dilution, it was found out that although there was a certain bacterial growth, absorbance was not statistically significant ( $P = 0.156$ ).

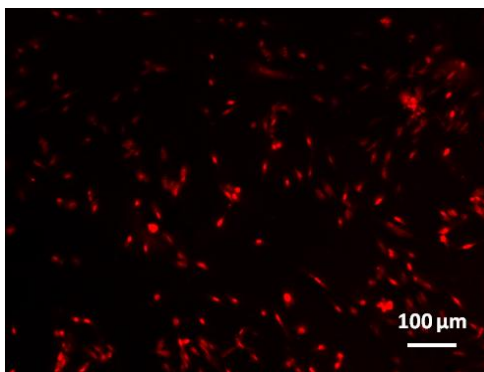
When compared with the same treatment eliminating the zero dilution (that is, the experiment), it was found that there was bacterial growth, which reflected in a statistically significant absorbance level ( $P = 0.012$ ). The



**Figure 1.** Mesenchymal cells of human dental pulp without treatment in culture medium, control group (scale bar 100  $\mu\text{m}$ ).

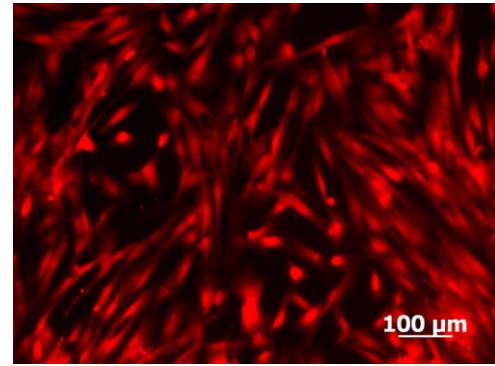


**Figure 2.** Mesenchymal cells of human dental pulp after exposure to chlorhexidine 0.12% for 30 s (scale bar 100  $\mu\text{m}$ ).

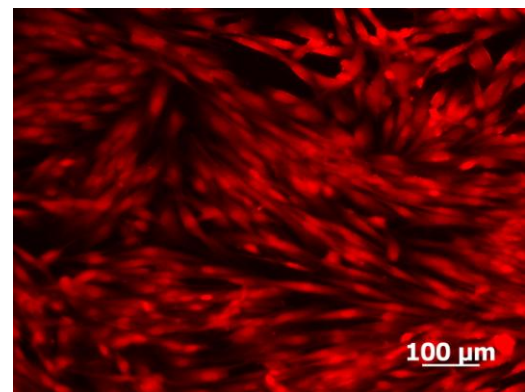


**Figure 3.** Mesenchymal cells of human dental pulp after exposure to chlorhexidine 0.12% for 1 min (scale bar 100  $\mu\text{m}$ ).

concentration for this treatment, although it showed some bacterial growth, was not statistically significant ( $P = 0.318$ ). Instead, the CFU bacterial count was highly significant ( $P = 0.000$ ), demonstrating that super-oxidized



**Figure 4.** Mesenchymal cells of human dental pulp after exposure to neutral super-oxidized electrolyzed gel 15 ppm for 30 s (scale bar 100  $\mu\text{m}$ ).



**Figure 5.** Mesenchymal cells of human dental pulp after exposure to neutral super-oxidized electrolyzed gel 15 ppm for 1 min (scale bar 100  $\mu\text{m}$ ).

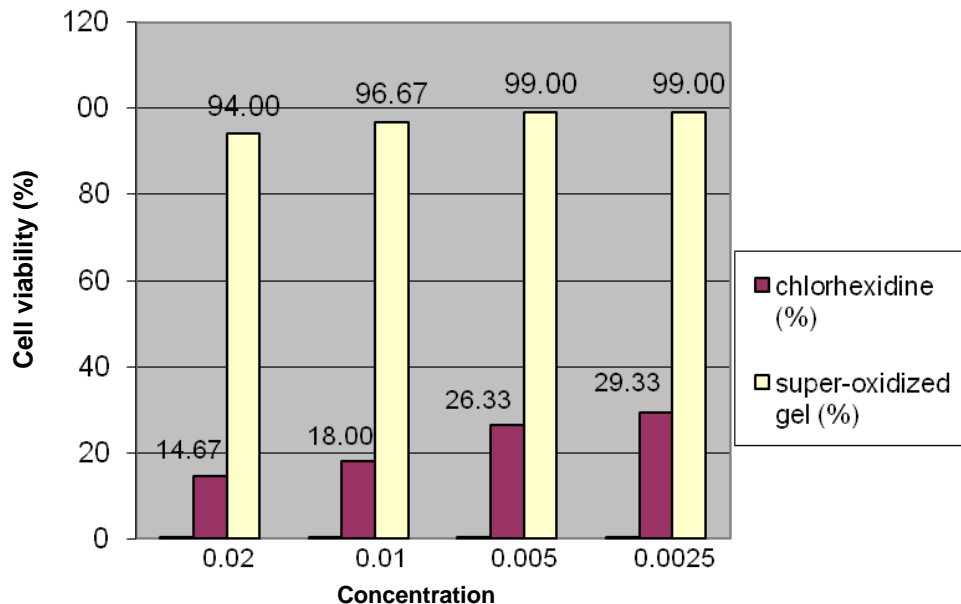
gel did not inhibit *S. intermedius* cell growth in the same measure as chlorhexidine, therefore, it was concluded that there was some bacterial growth with super-oxidized gel.

### Group 2

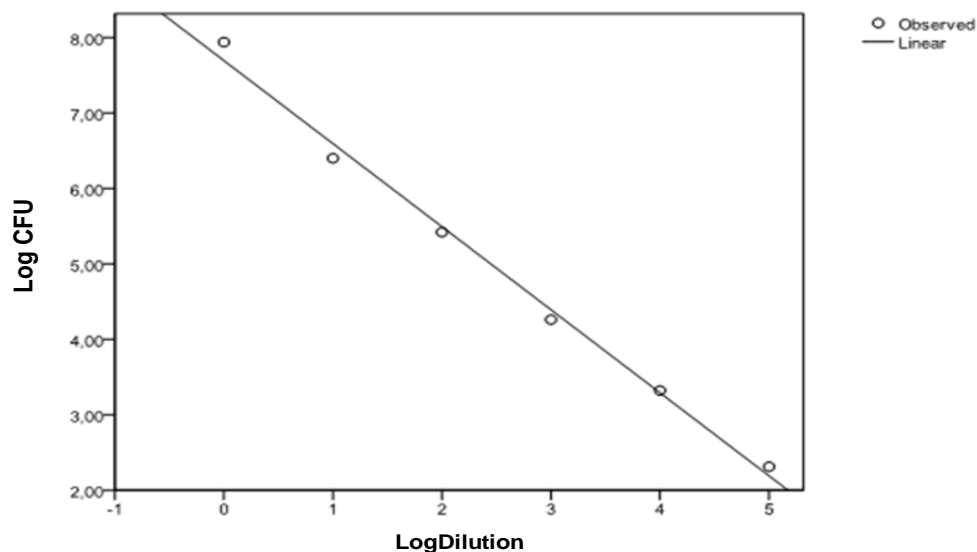
In Group 2 (Pg + super-oxidized gel), taking into account the zero dilution, it was found that despite the observed bacterial growth, absorbance ( $P = 0.119$ ) was not statistically significant.

When compared with the same treatment eliminating the zero dilution, a statistically significant absorbance was not found ( $P = 0.095$ ). This means that there was growth of *Pg* with super-oxidized gel. However, the bacterial count of CFU was highly significant ( $P = 0.000$ ), demonstrating that with super-oxidized gel, growth of *P. gingivalis* was not inhibited (Figure 7).





**Figure 6.** Comparison of cell viability. Chlorhexidine has greater toxicity than neutral super-oxidized electrolyzed gel since there is a greater percentage reduction in cell viability with chlorhexidine with a range of 79.33 with regard to the greater concentration and of 69.67 with regard to the lesser concentration of both products.

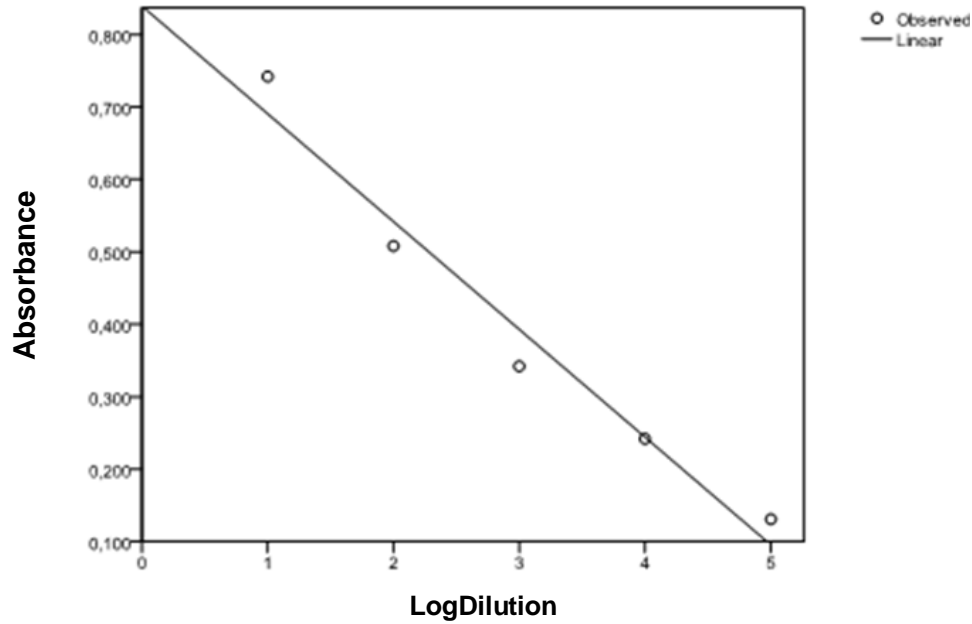


**Figure 7.** Number of colony forming units for Group 2.  
Equation:  $\text{Log} = 7810\ 1082 (\text{log dilution}), F = 4854,898, P = 0.000.$

### Group 3

In Group 3 (Si + Pg + super-oxidized gel), taking into account the zero dilution, it was found that, notwithstanding bacterial growth, absorbance was not statistically significant ( $P = 0.083$ ) (Figure 8). When compared with the same treatment eliminating the zero

dilution, it was found out that growth of the bacterial mixture was such that a highly significant absorbance ( $P = 0.003$ ) was found. Despite the observed bacterial growth, the concentration for this treatment was not statistically significant ( $P = 0.639$ ). However, the CFU bacterial count was highly significant ( $P = 0.000$ ), which represents a lack of effectiveness of super-oxidized gel



**Figure 8.** Results of absorbance for Group 3 eliminating the zero dilution. Equation: Absorbance = 0042 - 0004 (LogDilution), F = 29 547, P = 0.012.

**Table 1.** Correlation of absorbance with the logarithm of dilution and concentration, and correlation of the logarithm of CFU with the logarithm of dilution.

Group	Function or regression correlation	Condition	Significance
1	Absorbance = F (log of dilution)	With dilution = 0	NS
		Without dilution = 0	*
	Concentration = F (log of dilution)	-	NS
	Log of CFU = F (log of dilution)	-	**
2	Absorbance = F (log of dilution)	With dilution = 0	NS
		Without dilution = 0	NS
	Concentration = F (log of dilution)	-	NS
	Log of CFU = F (log of dilution)	-	**
3	Absorbance = F (log of dilution)	With dilution = 0	NS
		Without dilution = 0	**
	Concentration = F (log of dilution)	-	-
	Log of CFU = F (log of dilution)	-	-

NS = not significant; \* = significant ( $P < 0.05$ ); \*\* = highly significant ( $P < 0.01$ )

on bacterial mixtures.

**Group 4**

In Group 4 (Si + chlorhexidine), it was found that all the variables measured were zero, that is, there was no bacterial growth, which means that chlorhexidine digluconate

had a bactericidal effect against *S. intermedius*.

**Group 5**

In Group 5 (Pg + chlorhexidine), it was found that all the variables measured were zero, that is, there was no bacterial growth, which means that chlorhexidine digluconate

is effective in inhibiting proliferation of *P. gingivalis*.

### Group 6

For Group 6 (Si + Pg + chlorhexidine), it was found that all the variables measured were zero, that is, there was no bacterial growth.

## DISCUSSION

We compared the antimicrobial and cytotoxic properties of neutral super-oxidized electrolyzed gel and chlorhexidine digluconate 0.12% and found that neutral super-oxidized electrolyzed gel inhibits biofilm formation and acts as an antibacterial agent on the surface of orthodontic mini-implants.

Kravitz and Kusnoto (2007) determined that to prevent ulceration of tissue around the mini-implant and improve patient comfort, the use of chlorhexidine 0.12% (10 ml) is recommended. This is because ulceration can cause severe inflammation of tissue. This is why chlorhexidine is used after mini-implant placement. It is also used to minimize tissue inflammation, promote slow epithelization and reduce the possibility of increased soft tissue around the mini-implant. In this study, chlorhexidine digluconate 0.12% was employed around the surface of mini-implants and it was found out that its antibacterial properties were significantly greater (CFU = 0) when compared with neutral super-oxidized gel; however, super-oxidized gel was less cytotoxic. Our results showed that there was no bacterial growth in any of the mini-implants used. In contrast, Dennison et al. (1994) found that chlorhexidine had poor efficacy in removing bacteria from the implant surface treated with hydroxyapatite.

Järvinen et al. (1993) conducted a study that demonstrated the susceptibility of *Streptococcus mutans* to chlorhexidine and six other commonly used antibacterial agents such as amoxicillin, cefuroxime, penicillin, sulfamethoxazole, trimethoprim, tetracycline and erythromycin. It was found out that the bacteria exposed to various antimicrobial agents remained susceptible to all, and more importantly to chlorhexidine. The results obtained in the studies mentioned earlier agree with those obtained in this study with regard to chlorhexidine.

In an *in vitro* study conducted by De Baun (2008), in order to prove the antimicrobial properties of chlorhexidine digluconate against seven different bacterial samples, it was found out that this compound reduced bacterial content after 3 min of exposure and its efficacy continued. In this study, the antimicrobial effectiveness of chlorhexidine digluconate was compared only against neutral super-oxidized gel. The results showed that chlorhexidine had a better response by significantly reducing bacterial counts around the mini-implant surface. Other *in vivo* studies conducted by

Persson et al. (2007) and by Paolantonio et al. (2008) also found that chlorhexidine has important antimicrobial properties.

Noiri et al. (2003) conducted an *in vitro* study that examined the effects of chlorhexidine on *P. gingivalis* biofilms, finding that the extracellular matrix of the latter was altered in the presence of this substance. This coincides with the results found in this research although when applying neutral super-oxidized gel, this bacterium had greater resistance.

The susceptibility to chlorhexidine of various types of bacterial samples was demonstrated in a study by McBain et al. (2003). The most susceptible was *Actinomyces naeslundii* followed by Gram-negative anaerobic bacteria such as *Prevotella nigrescens*, *P. gingivalis*, *S. mutans* and *Streptococcus sanguinis*. The results of this study are similar to those in the present study in which chlorhexidine showed a bactericidal effect on the microorganisms used.

Melsen (1986) conducted research that measured the reaction of periodontal and gingival tissues to intrusion forces applied to teeth, as well as the influence of oral hygiene in *Macaca fascicularis* monkeys, using chlorhexidine. The results showed that on the side of hygiene, there were clear signs that bone deposition remained present after the applied eruption forces, something that did not occur with hygiene. This suggests that chlorhexidine inhibits bacterial growth, as was demonstrated in our *in vitro* study.

The findings of this study with regard to cytotoxicity coincide with studies performed by Zanatta et al. (2007), and Kozlovsky et al. (2006) in which it was found that low concentrations of chlorhexidine cause cell membrane damage and the release of low molecular weight molecules of microorganisms. With regard to cytotoxicity, there is contradiction in the literature. Campos et al. (2010) showed that in all concentrations, chlorhexidine had a high direct cytotoxic effect on cell cultures. This is consistent with the results in this study, since chlorhexidine was shown to be cytotoxic with increasing concentrations. In contrast, Bonacorsi et al. (2004) reported that chlorhexidine did not have immunostimulatory activity and subtoxic concentrations did not affect macrophage response, a condition that we did not analyze in this study.

Definitely, one of the studies whose results are similar to those obtained in this investigation is that of Gianelli et al. (2008) who found that chlorhexidine affects cell viability depending on the time of exposure particularly osteoblasts; in our study, this condition was analyzed in fibroblasts. These authors explained the induction of apoptosis and autophagocytosis of necrotic cells as the cause of the cytotoxic effect and also implicated mitochondrial function, increased intracellular  $Ca^{+2}$ , and cellular oxidation. In our study, as in that of Gianelli, it was concluded that chlorhexidine is highly cytotoxic *in vitro* and dentists are urged to use caution in oral cavity

procedures.

The potential toxicity of super-oxidized solutions was studied by Gonzalez-Espinosa et al. (2007). These researchers compared the solution with hydrogen peroxide in dermal fibroblasts. They found out that hydrogen peroxide was more toxic. The super-oxidized solution had less effect on cell viability and genotoxicity. Cell viability in our study was >90% after 30 s and 1 min. This is in contrast with Gonzalez-Espinosa et al (2007) who found a viability of 75 and 70% after 5 and 30 min of exposure, respectively. This discrepancy is probably due to the difference in exposure times.

## Conclusions

Neutral super-oxidized electrolyzed gel inhibits biofilm formation on the surface of orthodontic mini-implants and acts as an antibacterial agent. In summary, super-oxidized gel offers a nonirritating inhibitory benefit in cells allowing a regenerative therapeutic effect, which is healthier since this promotes internal recovery.

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## Abbreviations

**CFU**, Colony forming units; **Si**, *Streptococcus intermedius*; **Pg**, *Porphyromonas gingivalis*.

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Full Length Research Paper

## Effect of *Hippocratea africana* root bark extract on some biochemical indices of male and female albino Wistar rats

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The benefits of medicinal plants may not be without some biochemical impediments. Anti-plasmodial activity of *Hippocratea africana* has been studied; its effects on some biochemical parameters were examined in rats in this study. It was observed that in the female test groups, there was no significant ( $p > 0.05$ ) changes in urea concentration but there was significant ( $p < 0.05$ ) increase in glucose concentration. There was also a slight increase in total protein and albumin concentrations which was not significant ( $p > 0.05$ ). The male treatment groups however recorded significant ( $p < 0.05$ ) increases in glucose, total protein, urea and albumin except for serum bilirubin which showed slight but non-significant ( $p > 0.05$ ) decrease. The result generally does not suggest any biochemical imbalance and therefore the herb may be safe for use for its anti-plasmodial property.

**Key words:** *Hippocratea africana*, biochemical imbalance, glucose, total protein, urea, albumin, bilirubin, anti-plasmodial property, health benefit.

### INTRODUCTION

There has been wide acceptability of phyto-remedies because of their availability, efficacy and affordability for the treatment of ailments, especially among the peasants and low income earners. Available evidence shows that herbal remedies have been used in the treatment of ailments for time immemorial and have continued to play significant roles in the general provision of good health to people all over world (Farombi, 2003). All parts of plants, leaves, barks, roots and even latex are used medicinally by Traditional Medicine Practitioners (TMPs) for the treatment of various body ailments such as hypertension, diabetes mellitus, ulcer, malaria, typhoid fever, gonorrhoea, syphilis, cancer etc. (Farombi, 2003). Many botanicals are now sold in United States as dietary supplements (Borchers et al., 2000). Research interest is now focused

hypolipidaemic, anti-platelet, antihypertensive, anti-diabetic, anti-tumour, immune stimulating or anti-malarial properties etc. That may be useful in complementary properties or alternative medicine (Borchers et al., 1997). One of such plants is *Hippocratea africana* (Willd.) Loes. (Hippocrateaceae). The plant inhabits green forests and is a perennial climber with hairs (glabrous), and reproduced from seeds (Dalziel, 1956). The plant is widely distributed in tropical Africa. It is called "godyi" in Hausa, "ponju owiwi" in Yoruba, and "ipungwa" in Tiv tribes of Nigeria. The Ibibio tribe of the Niger Delta region of Nigeria calls it "Mba engang engang". In Ghana, the Akan-Asante call it "nnoto" and Fula-Pulaa in Senegal calls it "Rdelbi" while the Loko in Sierra Leone call it "njabo" (Burkill, 1985). The roots are used traditionally in

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**Table 1.** Distribution of experimental groups.

Group	Number of animals		Treatment (mg/kg body weight)
	Female	Male	
I	6	6	Control
II	6	6	<i>H. africana</i> (100)
III	6	6	<i>H. africana</i> (200)
IV	6	6	<i>H. africana</i> (300)

Control = distilled water

the treatment of various ailments such as fever, malaria, body pains, diabetes and diarrhea (Okokon et al., 2006). It has been reported to possess *in vivo* anti-plasmodial activity with lethal dosage (LD<sub>50</sub>) of 2.45 mg/kg body weight in mice. Okokon et al. (2006) also report that *H. africana* possesses anti-inflammatory, analgesic and anti-pyretic properties which are probably mediated via inhibition of various autocoid formation and release. Other species of *Hippocratea* which possess anti-inflammatory activity are *H. excelsa* (Perez et al., 1995) and *H. indica* (Ogbole et al., 2007). In the phytochemical and anatomical studies of *H. africana* Willd. (*Celastraceae*), Essiet et al. (2006) reported that the plant contains significant quantities of phytochemicals such as alkaloids, cardiac glycosides and flavonoids.

Okokon et al. (2006) have reported the anti-malarial activity of *H. africana* in mice. Studies carried out showed that ethanolic root extract of *H. africana* possess promising blood schizontocidal activity, both in early and established infection at oral doses of 200 to 600 mg/kg/day in mice. The chemo-suppressive effect of *H. africana* at 400 and 600 mg/kg were 81.8 and 90.9%, respectively while that of chloroquine at 5 mg/kg was 92.5%. In this study, the effect of graded doses of root bark extract of *H. africana* on biochemical indices of both female and male albino Wistar rats were assayed.

## MATERIALS AND METHODS

### Experimental animals and animal housing for biochemical studies

Forty-eight mature albino Wistar rats consisting of twenty four males and twenty four females weighing 163 to 227 g each were obtained from the animal house of the College of Health Sciences, University of Uyo, Uyo and used in this study. The animals were randomly divided into four groups of six rats to a group on sex basis. They were caged in plastic cages made of stainless steel bottom. Stainless steel mesh were placed at the bottom of the cages for collection of faeces and feed droppings. The males were caged separately from the females to prevent mating during the treatment period.

### Experimental design and administration of extract for biochemical studies

Group I animals served as the control and were administered distilled water while Groups II, III and IV animals were administered

graded doses of 100, 200 and 300 mg/kg of the crude root bark extract of *H. africana* calculated on the basis of the body weight of the animals. The experimental design is as shown in Table 1. The extract was administered orally once daily for fourteen (14) days by the use of a canular attached to syringe. All the experimental animals were given normal rat chow and water *ad libitum* throughout the treatment period.

### Collection of blood samples

At the end of the fourteen days treatment, the animals were denied their feeds but still had water *ad libitum* for sixteen hours before they were chloroform anaesthetized and dissected. Blood sample was obtained by cardiac puncture using sterile syringes and needles into plain sample bottles for serum separation. The serum was obtained by centrifugation of clotted blood in a MSE table top centrifuge at 4,000 rpm for 10 min and used for analyses.

## Biochemical assays

### Serum concentration of glucose

The serum glucose concentration was determined using enzymatic colorimetric test. Randox laboratory reagent kit method was used in this test based on method by Barham and Trinder (1972). Glucose is determined after enzymatic oxidation in the presence of glucose oxidase (GOD). The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoneimine dye which was measured spectrophotometrically at 546 nm.

### Serum total protein

The serum total protein concentration was estimated by biuret method using Randox Laboratory reagent kit method (Tietz, 1995). Cupric ions in an alkaline medium interacted with protein peptide bonds resulting in the formation of a coloured complex which was measured spectrophotometrically at 540 nm. This was used to quantify the protein in the sample.

### Serum urea concentration

Randox laboratory reagent kit method (Fawcett et al., 1960; Weatherburn, 1967; Chaney and Marbach, 1962; Mackay and Mackay, 1927) was used to determine the serum urea concentration. Urea in serum was hydrolysed to ammonia in the presence of urease. The ammonia was then measured photometrically at 546 nm by Berthelot's reaction.

### Serum albumin concentration

Serum albumin concentration was determined by the use of Randox laboratory reagent kit method (Grant, 1987). The measurement of serum albumin is based on its quantitative binding to the indicator 3, 3<sup>1</sup>, 5, 5<sup>1</sup> - tetrabromo-m cresol sulphonephthalein (bromocresol green, BCG). The albumin - BCG - complex was measured spectrophotometrically at 630 nm, the absorbance being directly proportional to the concentration of albumin in the sample.

### Serum total bilirubin concentration

Serum total bilirubin was determined using Randox Laboratory

**Table 2.** Biochemical indices of female and male albino Wistar rats administered graded doses of ethanol extract of *Hippocratea africana* root bark.

Grouping/dosage	Glucose (mmol/l)		Total protein (g/l)		Urea (mmol/l)		Albumin (g/l)		Total bilirubin ( $\mu$ mol/l)	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
I	4.14 $\pm$ 0.20	3.27 $\pm$ 0.32	68.53 $\pm$ 3.47	63.37 $\pm$ 1.24	10.22 $\pm$ 0.72	7.34 $\pm$ 0.61	43.19 $\pm$ 1.87	38.31 $\pm$ 2.41	29.14 $\pm$ 2.88	26.64 $\pm$ 3.08
II	4.63 $\pm$ 0.70*	3.32 $\pm$ 0.35*	73.56 $\pm$ 2.14	73.36 $\pm$ 1.83**	9.44 $\pm$ 0.26	8.85 $\pm$ 0.97**	46.03 $\pm$ 3.53	44.57 $\pm$ 2.83**	22.36 $\pm$ 2.06**	24.18 $\pm$ 1.49
III	5.75 $\pm$ 0.71**	3.68 $\pm$ 0.77*	71.97 $\pm$ 2.91	66.66 $\pm$ 2.01*	10.68 $\pm$ 1.59*	7.74 $\pm$ 0.76*	44.76 $\pm$ 2.69	41.73 $\pm$ 1.75*	26.89 $\pm$ 1.15*	26.46 $\pm$ 3.08
IV	6.01 $\pm$ 0.53**	5.66 $\pm$ 0.93**	69.75 $\pm$ 2.53	71.24 $\pm$ 1.05**	10.04 $\pm$ 0.88	8.84 $\pm$ 1.37*	45.28 $\pm$ 3.08	41.66 $\pm$ 1.74*	24.45 $\pm$ 1.67	23.19 $\pm$ 2.38

Results are presented as mean  $\pm$  SD; n = 6, \* = significantly different from control at p < 0.05, \*\* = significantly different from control at p < 0.01, HA = *Hippocratea africana* root bark extract; Group I = control, Group II = *H. africana* root bark extract (100 mg/kg body weight), Group III = *H. africana* root bark extract (200 mg/kg body weight), Group IV = *H. africana* root bark extract (300 mg/kg body weight).

reagent kit method (Jendrassik and Grof, 1938; Sherlock, 1951). Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotised sulphanilic acid. This was measured spectrophotometrically at 578 nm.

#### Statistical analysis

The results of all determinations were expressed as mean  $\pm$  standard deviation. Data between treatment groups were analyzed using one way analysis of variance (ANOVA). Pairwise comparison was done using the student's t-test. Values of p < 0.05 were regarded as being significant.

#### RESULTS

The impact of graded doses of extract on glucose (mmol/l), total protein (g/l), urea (mmol/l), albumin (g/l) and total bilirubin ( $\mu$ mol/l) of female and male biochemical imbalance following the administration of *H. africana* root bark extract. The results are as presented in Table 2. The female rats recorded significant (p < 0.05) increase in glucose concentrations of 4.63  $\pm$  0.70, 5.75  $\pm$  0.71 and 6.01  $\pm$  0.53 mmol/l for test groups II, III and IV administered 100, 200 and 300 mg/kg body weight of *H. africana* root bark extract compared with the control group I rats concentration of 4.14  $\pm$  0.20 mmol/l, administered distilled water. Test

groups III and IV also showed significant increases (p < 0.01) compared with the control group I rats. The result showed no significant (p > 0.05) increase in total protein concentration of 73.56  $\pm$  2.14, 71.97  $\pm$  2.91 and 69.75  $\pm$  2.53 g/l for test groups II, III and IV compared with 68.53  $\pm$  3.17 g concentration for the control group I rats.

The urea concentrations were 10.22  $\pm$  0.72, 9.44  $\pm$  0.26, 10.68  $\pm$  1.59 and 10.54  $\pm$  0.88 mmol/l for control group I rats, test groups II, III and IV, respectively. The test groups II, III and IV concentrations showed no significant (p > 0.05) difference compared with control. There was no significant (p > 0.05) increase in albumin concentrations of 46.03  $\pm$  3.53, 44.76  $\pm$  2.69 and 45.28  $\pm$  3.08 g/l for test groups II, III and IV rats, respectively compared with the control group I concentration of 43.19  $\pm$  1.87 g/l. The result showed significant (p > 0.05) reductions of 22.36  $\pm$  2.06, 26.89  $\pm$  1.15 and 24.45  $\pm$  1.69  $\mu$ mol/l in total bilirubin concentrations for test groups II, III and IV rats, respectively compared with the control group I concentration of 29.14  $\pm$  2.88  $\mu$ mol/l. Test group II rats concentration also showed significant reduction at p < 0.01. Generally, there were no significant changes in urea concentration but significant increase in glucose concentration was observed. However, there was slight increase in total protein and albumin and decrease in

serum bilirubin concentrations in the female. The male Wistar rats recorded significant (p < 0.05) increase in glucose concentrations of 3.32  $\pm$  0.35, 3.68  $\pm$  0.77 and 5.66  $\pm$  0.93 mmol/l for test groups II, III and IV rats administered 100, 200 and 300 mg/kg body weight of *H. africana* ethanol root bark extract compared with the control group I rats concentration of 3.27  $\pm$  0.32 mmol/l. Test groups IV concentration further showed significant increase at p < 0.01 compared with the control.

Significant (p < 0.05) increase in total protein concentrations of 73.36  $\pm$  1.83, 66.66  $\pm$  2.01 and 71.24  $\pm$  1.05 g/l were recorded for test groups II, III and IV, respectively compared with the control group I albino Wistar rats were used to assess for the concentration of 63.37  $\pm$  1.24 g/l. The increase in test groups II and IV further showed significant difference at (p < 0.01) compared with the control group. The urea concentration depict significantly (p < 0.05) increased values of 8.85  $\pm$  0.97, 7.74  $\pm$  0.76 and 8.84  $\pm$  1.37 mmol/l for test groups II, III and IV, respectively compared with the control group I concentration of 7.34  $\pm$  0.61 mmol/l. Test group II further showed significant increase at p < 0.01 compared with the control. The albumin concentrations were 38.31  $\pm$  2.41, 44.57  $\pm$  2.83, 41.73  $\pm$  1.75 and 41.66  $\pm$  1.74 g/l for the control group I, test groups II, III and IV, respectively. The test groups II, III and IV increasing concentrations

were all significant ( $p < 0.05$ ) compared with the control. Test group II value further showed significant different at ( $p < 0.01$ ). The test groups II, III and IV rats recorded non dose-dependent and non significant ( $p > 0.05$ ) reductions in total bilirubin concentrations of  $24.18 \pm 1.49$ ,  $26.46 \pm 3.08$  and  $23.19 \pm 2.38$   $\mu\text{mol/l}$  compared with the control concentration of  $29.14 \pm 2.88$   $\mu\text{mol/l}$ . For the males, there was significant increase in all biochemical parameters except for total serum bilirubin which showed slight decrease.

## DISCUSSION

Administration of graded doses of *H. africana* root bark extract to both female and male rats of the test groups showed hyperglycemic effect compared with the control. The glucose level is regulated in a special way in the liver and other extra hepatic tissues and the role of hormonal systems cannot be ignored (Crook, 2006). Plant extracts such as *Eleophobia drupifera* (Eno and Itam, 1996), *Gongronema latifolium* (Ugochukwa, 2003), and *Vernonia amygdalena* (Gidado et al., 2005) have been reported in related studies to have hypoglycemic effects in experimental animals. Anti-malarial such as quinine and xenobiotics such as bidisomide have also been reported to have hypoglycemic effect (Vandenberghe et al., 1995). The hyperglycemic effects observed in this study suggest that there may be phytochemical constituent(s) present in the herb that has glucagon-like activity. Various liver diseases result in decrease blood glucose (Bolarin, 1997). The observed increase in glucose concentration is favorable to the organisms owing to the metabolic roles of glucose in several tissues.

There was a non significant ( $p > 0.05$ ) increase in protein concentration in all the female test groups' rats and significant increase for the rats in the male test groups compared with the control. In a study, Iwu et al. (1986) showed that *A. indica* has a hyper-proteinaemic effect on chicks. Acetaminophin and an anti-malarial, sodium artesunate, are known to bind plasma proteins (Miller and Panosium, 1997). The reported hypo-proteinaemic effects of *A. indica* and acetaminophen were attributed to glutathione (GSH) decrease resulting in increase glutathione disulfide (GSSG) concentration known to inhibit the incorporation of essential amino acids into protein. The reverse was observed for *H. africana* root bark extract. Why there was increase in the serum protein in this study is not known but may imply that the extract did not affect protein absorption and that there was no possible upset in liver function.

The same pattern was observed in the serum urea concentration except that the female 100 mg/kg body weight extract treatment group recorded non significant reduced urea concentration compared with the control. Excess amino acids cannot be stored, thus the liver break them into nitrogen containing amino parts which it

then converts to urea for excretion by the kidney; and carbon compounds (Keto acids) which it converts into glucose, glycogen or fats in the process known as deamination (Crook, 2006). Serum urea levels and blood urea nitrogen (BUN), alongside other parameters like serum creatinine level, are used to assess renal functions (Crook, 2006). The non significant decrease/increase observed in female test rats implied that glomerular functions of the renal tubules of the female experimental animals were not distorted by the extract. The significant increase observed in the male test rats compared with control may suggest extract induced increased protein absorption and catabolism, resulting in the increase urea concentration and not toxicity. Histopathology of the kidney showed normal kidney.

The study revealed a non significant ( $p > 0.05$ ) increase in albumin concentrations that was not dose-dependent for the female test groups rats compared with the control, and significant ( $p < 0.05$ ) increase for all the male test groups compared with the control. Albumin is the major protein present in the blood and is synthesized primarily by the hepatic parenchymal cells, except in early foetal life when it is synthesized largely by the yolk sac (Gitlin and Perricelli, 1970). It is a major synthetic protein and is a major market for the ability of the liver to synthesize protein (Quinn and Johnston, 1997). The synthetic rate is controlled primarily by colloidal osmotic pressure (COP) and secondarily by protein intake (Peter (Jr), 1996; Rothschild, 1972). The observed increase in albumin concentration may be attributed to extract induced protein intake, a secondary reason for its synthetic rate. Markedly increased levels of albumin are seen only in acute dehydration. As a result, the finding of elevated values has no clinical utility. This is because the synthetic rate and intravascular-extravascular shifts usually occur fairly rapidly to stabilize relative osmotic pressures (Burtis and Ashwood, 1999).

Physical examination and observed weight increase in all the test groups animals (both female and male) does not indicate dehydration and therefore the increase may not imply toxicity.

Bilirubin, the end product of haem degradation by the mononuclear phagocytes system, when released into the blood, is transported to the liver bound to serum albumin (conjugated bilirubin) or free (unconjugated bilirubin). The unconjugated bilirubin is a small fraction which may increase in severe haemolytic disease or when protein binding chemicals displace bilirubin from albumin. Both conjugated and unconjugated bilirubin in the serum (total serum bilirubin) accumulate systematically and deposit in tissues, resulting in the yellow discolouration of jaundice (Crook, 2006; Burtis and Ashwood, 1999) that is, bilirubin production exceeds the hepatic capacity to excrete it or conjugate it (or if there is obstruction in the biliary flow) (Crook, 2006).

Generally, both conjugated and unconjugated, bilirubins are increased in the serum in hepatitis (Crook,

2006). In this study, the level of total serum bilirubin was observed to be significantly ( $p < 0.05$ ) decreased in all the female test groups rats compared with the control. There was observed non significant ( $p > 0.05$ ), non dose-dependent decrease for all the male test groups rats compared with the control. The observed decrease indicate that the extract did not cause haemoglobin lyses and that the hepatic capacity to excrete or conjugate bilirubin was not compensated, it may also indicate that there was no impaired intrahepatic and extrahepatic bile flow induced by the herb in both female and male test groups rats.

## Conclusion

Although there was hyperglycemia and hyperproteinaemia, the other biochemical indices taken together do not suggest kidney and liver malfunctioning or derangement in critical tissues or organ function. Hence the herb may be safe for use for its anti-plasmodial property.

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