Eurycoma longifolia Jack (Simarubaceae); Advances in Its Medicinal Potentials

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ABSTRACT

Eurycoma longifolia Jack is a tall slender shrub-tree which is well-reputed among the natives of South East Asia for its potent aphrodisiac effect. Its root extracts have shown important biological activities such as antitumor, antimalarial, antibacterial, anti-diabetic, anti-hypertensive, Osteoprotective, and ergogenic which are mainly attributed to quassinoids. Commercially it is available in the form of drinks (along with other herbs), capsules, or loose powders. Based on available online databases it was realized that in spite of numerous reports on medicinal properties of *E. longifolia*, a review of recent developments regarding phytopharmacology, safety and toxicology, pharmacokinetics, and clinical applications was lacking. Therefore, this concise yet informative piece of work was prepared by pulling together trustworthy information from all the accessible published and unpublished scientific resources to serve as a reliable source of reference for future investigations.

Keywords: Malaysian Ginseng, Eurycomanone, Tongkat Ali, Quassinoids, Ergogenic, Pasak Bumi.

INTRODUCTION

Traditional/Herbal Medicine is an ancient system of health which has gained great popularity in recent years. In Malaysia alone about USD 500mln is being spent annually on this type of health system.[1,2] To date, over 200 herbal supplements with *Eurycoma longifolia* as their principal ingredient are available in the form of beverages, capsules, and loose powders in the market which are produced mainly from its root extracts.[3] *Eurycoma longifolia* Jack (family; *Simarubaceae*) is a slender evergreen tree which grows in wild at an altitude of about 500 meters above sea level in the forests of Malaysia, Borneo, southern Myanmar, Cambodia, Vietnam, Laos, Thailand, Indonesia, and Philippines. It attains a height of up to 10 meters (Max. 15 meters) with few upright branches capped by umbrella-like rosette of leaves. The flowers are hermaphrodite with very fine pubescent whereas the fruits are hard, oblong in shape, yellowish brown when young and brownish red when ripe. In Malaysia, it is commonly known as "Tongkat Ali" and/or "Malaysian Ginseng" which are assigned mainly due to its aphrodisiac properties.[4–6] The plant is usually cultivated by direct sowing of the germinated seedlings in well-drained soil with sufficient organic matter. An annual rainfall of 2000–3000mm and a temperature range of 25–30°C is said to be ideal for the growth. Generally, it is harvested 2 to 3 years following the cultivation but the exact harvesting age in not known.[7,8] Traditionally, *E. longifolia* is used to treat bleeding gums, fever, malaria, oedema, jaundice, arthritis, cachexia, rheumatism, dysentery, flatulence, indigestion, erectile dysfunction, wounds, syphilis, boils, lumbago, high blood pressure, and to relieve gastric ulcer. Moreover, the bark is useful as blood coagulant after childbirth while the leaves are found to be effective in relieving stomach ache.[3–5] Today, many of its medicinal potentials such as antitumor,[9] antimalarial and antipyretic,[10,11] aphrodisiac,[12] antimicrobial,[13] anti-hyperglycaemic,[14] antiinflammatory,[15] anti-schistosomal,[16] antifungal,[17] anti-toxoplasmic,[18] anti-hypertensive,[19] and Osteoprotective[20] have been uncovered through extensive scientific research.

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CHEMICAL CONSTITUENTS

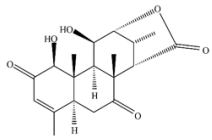
Active constituents

By and large, roots of *E. longifolia* are the powerhouse of this very herb which are home to large amounts of

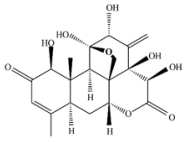
biologically active compounds such as Quassinoids (Scheme 1), Squalene- and Tirucallene-type Triterpenes, Canthin-6-one and *β*-carboline Alkaloids, and Biphenylneolignans, among which quassinoids are well distinguished due to their diverse bioactivity. The term quassinoid itself originates from the word "Quassin" named after a man called "Quassi" who used the barks from Simarubaceae plants to treat fever. Quassinoids are otherwise degraded triterpenes of bitter in nature which are classified based on the number of carbon atoms as C-18, C-19, C-20, C-22, and C-25.[21] Amongst the several quassinoids identified in *E. longifolia* extracts, Eurycomanone (EN) is said to be the major one with strong antiproliferative and steroidogenesis effects.[9–22]

Proteins, minerals and amino acids

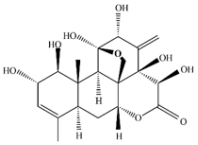
Elemental contents determination of roots of *E. longifolia* by Majid and coworkers[26] revealed the presence of certain elements such as Al, Br, Ca, Ce, Cl, Cs, Cr, Fe, K, La, Mg, Mn, Na, Rb, Sb, Sc, and Zn along with other toxic elements. Subsequent evaluation of aqueous root extracts of *E. longifolia* collected from two climatically and geographically different locations in Malaysia; Perak and Pahang for proteins, minerals, and amino acids showed dissimilarity in the content of these substances. For instance, Tongkat Ali Pahang displayed higher amount of protein matters almost twice as that of Tongkat Ali Perak. Similarly, amino acid



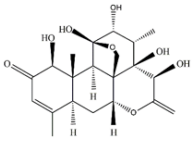
Eurycomalactone[23]



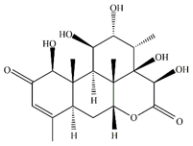
Eurycomanone[24]



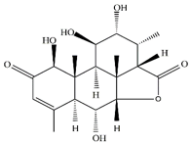
Eurycomanol[24]



13α,21-Dihydroeurycomanone[25]



14,15β-Dihydroxyklaineanone[25]



Longilactone[25]

Scheme 1: Array of some biologically active quassinoids from *Eurycoma longifolia* Jack.

composition determination of the extracts by HPLC unveiled the presence of Lysine and Arginine in Pahang, but not Alanine. Additionally, presence of other nutrient minerals such as Ni, Cu, Co, Ba, Li, In, Sr, Ga, and U detected by Inductively Coupled Plasma Mass Spectrometry were also reported.[27]

BIOLOGICAL ACTIVITIES

Aphrodisiac

E. longifolia has gained tremendous popularity among men in Malaysian peninsula for its potent aphrodisiac action. Based on traditional claims, it has the capacity to augment low testosterone levels in aged men affected with infertility and/or impotence. This was in fact found to be true as the outcomes of recent investigations disclosed its strong steroidogenesis and spermatogenesis in rodents.[28] Elsewhere, its standardized methanol extract reversed the infertility induced by *Andrographis paniculata* through significant boost in testosterone synthesis both in plasma and testes. The resultant testosterone then stimulated spermatogenesis by binding to Androgen Binding Protein (ABP).[29–31] Later, Low and coworkers[12] realized that the action of quassinoids on the generation of testosterone as well as sperm was most likely mediated through hypothalamic-pituitary-gonadal axis.

It was believed that 25mg/kg of F2 fraction obtained from the elution of methanol extract with water, reduced estrogen secretion but rather stimulated the secretion of high amount of LH and FSH. This in turn resulted in enhanced plasma testosterone levels as well as spermatogenesis. Recent advancements suggest the essential role of Eurycomanone (EN) in boosting steroidogenesis process in a dose-dependent manner possibly through inhibiting aromatase and phosphodieasterase enzymes (Figure 1).[22]

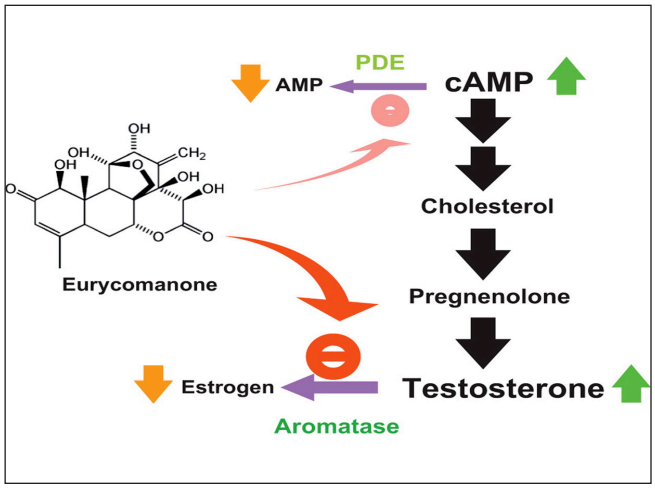


Figure 1: Possible mechanism of action of Eurycomanone (EN) on the stimulation of steroidogenesis.

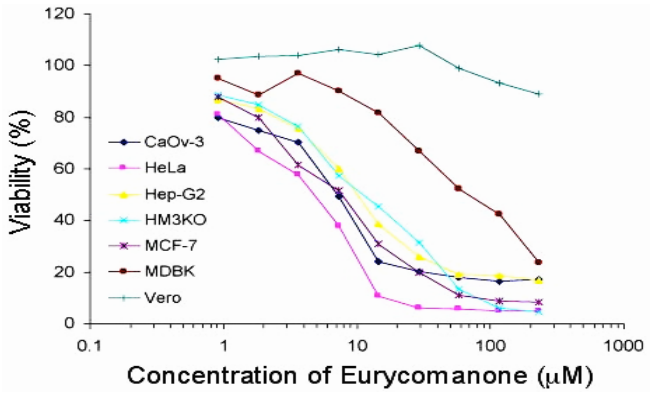


Figure 2: Anti-proliferative effect of Eurycomanone (EN) on normal and cancer cell lines.

Anti-estrogenic activity

Treatment with oestrogen may be associated with deleterious effects on fertility and libido in men.[32] Abdul Wahab *et al.*[33] suggested that the negative impact of estrogen on spermatogenic cells was reversible when treated with *E. longifolia.* Similarly, two quassinoids; 13a, 21-Dihydroeurycomanone (ED) and EN exhibited potent (p<0.001) anti-uterotrophic and antiestrogenic actions in immature and mature female rats by effectively countering the testosterone- and 17a-Ethynyl Estradiol-induced ovarian cystic follicles and irregular oestrous cycles, respectively.[34,35] These actions by *E. longifolia* are probably due to its ability to suppress enzyme aromatase which is responsible for the conversion of testosterone into oestrogen, hence showing anti-oestrogenic effect.

Antitumor

Quassinoids and alkaloids isolated from Simarubaceae plants are known for their robust cytotoxic activity against various cancer cell lines.[36,37] Likewise, antiproliferative activity of *E. longifolia* is attributed to two quassinoids; EN and Longilactone both of which act by inducing apoptosis in tumour cells via diverse pathways. Initially, EN was believed to induce apoptotic cell death via down-regulation of *Bcl-2* protein (anti-apoptotic) and cleavage of caspase-7 and PARP-1.[9] However, later apoptotic cell death induced by EN was found to have been triggered by up-regulation of *p53* and *Bax* (pro-apoptotic) proteins and rather suppression of *Bcl-2* protein in several cancer cell lines (Figure 2).[9] Nevertheless, subsequent evaluations indicated substantial reduction in the expression of certain tumour markers such as Heterogeneous Nuclear Ribonucleoprotein-A2/B1 (hnRNP- A2/B1), p53-tumor suppressor protein and other genes like Endoplasmic Reticulum protein-28 (ERp28), Prohibitin (PHB), and Annexin-1 (ANX-1) in A549 lung cancer cell lines upon administration of EN.[39] Quite the reverse, Longilactone exerted its antitumor action via the activation of caspase-7, caspase-8, and Poly(ADP-ribose) polymerase but not caspase-9, while leaving the levels of both *Bcl-2* and *Bax* proteins unaltered.[40]

Antioxidant & anti-angiogenic

Aging process in human is usually associated with the amount of oxidative stress that is generated by certain endogenous (oxidative burst) and exogenous (cigarette smoking) factors which bring about an imbalance in body's defense mechanism.[41] Human body has developed its own protective mechanism to overcome these stresses. However consumption of natural products with established antioxidant properties could further enhance the process of detoxification in the body. Investigation of standard ethanol root extract of *E. longifolia* (TAF-273) by Purwantiningsih and coworkers[42] for free radical scavenging indicated the presence of 0.253±0.016μg QE/mg d.w. and 17.142±1.102μg GAE/mg d.w. of total flavonoids and of total phenolics, respectively. Moreover, DPPH assay of TAF-273 exhibited an EC_{50} of 754μg/mL (p<0.01).

Dietary supplements containing herbal plants play vital role in the prevention of tumor in human body by means of their antioxidant and anti-angiogenic properties.[43] One good example is *E. longifolia.* Three of its root fractions; TAF-273, F3, and F4 were able to suppress angiogenesis both *in vitro* (rat aortic ring tissues) and *in vivo* (male BALB/c nude mice) 16 days following the treatment (p<0.012).[44]

Antimalarial, toxoplasmicidal, & anti-schistosomal

Quassinoids along with canthine alkaloids isolated from roots of *E. longifolia* are known to possess potent antimalarial activity against various strains of *Plasmodium falciparum* parasites including those resistant to Chloroquine such as Gombak A.[10,11] It is claimed that a combination of standard antimalarial agent and antimalarial herbal extract is beneficial in combating the infection caused by malaria parasite. For instance, co-administration of TAF-164 and Artemisinin brought about substantial suppression of parasitemia by up to 80% well higher than that displayed by a single drug administration ($p<0.05$).[45] It is anticipated that the antimalarial activity presented by quassinoids is probably due to their ability to hinder protein synthesis in *plasmodium falciparum*.[46] In a separate study, Kavitha *et al.*[18, 47] reported toxoplasmicidal action by *E. longifolia* extracts against *Toxoplasma gondii*. Their findings suggested the inhibition of toxoplasmosis 3 hours following the treatment. This was assumed to be mediated through alterations in the cell wall along with formation of invaginations followed by complete distortion of cells. Elsewhere, Longilactone, 11-dehydroklaineanone,and14,15β-dihydroxyklaineanone showed anti-schistosomal effect by successfully impeding the movement and egg laying capacity of *Schistosoma japonicum*.[16]

Antibacterial & anti-mycobacterial

In spite of accumulating large amounts of active constituents, root extracts of *E. longifolia* have failed to demonstrate desirable antibacterial or antifungal activities.[17] In contrast, leaves and stem extracts have shown relatively strong antibacterial action against both gram positive (*Bacillus subtilis, staphylococcus aureus, Enterococcus faecalis, Micrococcus leteus*) and gram negative bacteria (*Proteus vulgaris, Serretia marcescens*).[13] Moreover, various extracts of *E. longifolia* also displayed significant anti-mycobacterial effect by inhibiting *Mycobacterium smegmatis* growth with MIC values ranging from 800 to 3200μg/ml.[48]

Osteoprotective

Supplementation with testosterone is associated with the augmentation of minerals in the bone during the treatment of osteoporosis especially in men with low serum testosterone concentrations.[49] A similar effect was reported by Shuid *et al.*[20] for *E. longifolia* where 6 weeks administration of its aqueous extract in orchidectomized rats restored the depleted bone calcium levels but failed to show a meaningful rise in serum testosterone concentration. However, in their subsequent study treatment with 15mg/kg of aqueous extract (for 6 weeks) by oral gavage showed marked rise in both testosterone and osteoprotegerin levels in androgen-deficient orchidectomized rats. Meanwhile, C-terminal telopeptide of type I collagen (CTX) and Macrophage-Colony Stimulating Factor (M-CSF) genes expressions were down-regulated. Also, Receptor Activator of Nuclear Factor Kappa-B ligand (RANKL) and osteocalcin levels did not experience any noticeable change. Moreover, bone micro-CT assessment of undecalcified femora of the animals revealed ineffectiveness of aqueous extract of *E. longifolia* in restoring the Trabecular Thickness, Trabecular Volume, Trabecular Number, and Trabecular Separation.[51] However, its co-administration with testosterone in orchidectomized rats exhibited a significant development in the strain parameter in femoral bone.[52]

OTHER BIOLOGICAL ACTIVITIES

Effect on nitric oxide, cAMP, & cGMP

For erection to occur presence of certain physiologic mediators is essential which upon release induce vasodilatation followed by penile erection through increase in blood flow to penile tissues. Certain medicinal plants with known aphrodisiac effect like ginseng are reported to stimulate the release of Nitric Oxide (NO) in animals and bring about a state of relaxation.[53] According to a report by Basir[54] effect of *E. longifolia* extract (10mg/kg; i.p.) on NO levels in rats was unsatisfactory as it failed to cause significant elevation of NO levels following acute and chronic treatments. On the contrary, treatment with aqueous extract of *E. longifolia* markedly enhanced the levels of cGMP and cAMP for 30 and 60 minutes, respectively in rabbit corpus cavernosum (Azimahtol, 2001; unpublished data).

Anti-hypertensive & anti-hyperglycemic

As mentioned above, *E. longifolia* is traditionally used by natives in Malaysia to lower high blood pressure for centuries. Upon intravenous administration of 25–50mg/kg of aqueous extract immediate reduction in blood pressure well below the normal level for more than 30 min was noted in normotensive rats which was assumed to be of peripheral origin.[19] Elsewhere, findings of a preliminary investigation by Husen[14] suggested anti-hyperglycemic activity displayed by two aqueous extracts (TA-a & TA-b) of *E. longifolia* upon oral administration (150mg/kg) which was comparable to that of Glibenclamide at 10mg/kg.

Anxiolytic & Cognitive Enhancer

Along with other biological activities mentioned above, *E. longifolia* is said to alleviate anxiety and also enhance cognition in rodents. Anti-stress tests such as open-field, elevated plus-maze and anti-fighting tests conducted in anxious mice produced interesting outcomes. 300 mg/kg of its extracts (Butanol, Chloroform, Methanol, and Water) brought about major improvement in square crossings as well as reduction in immobility and number of fecal pellets in open field test 5 days following the treatment ($p<0.05$). While, the number of entries and time spent in open and closed arms were markedly increased and decreased, respectively, in elevated plus maze-test, the fighting episodes were substantially reduced in all treated groups as compared to control groups ($p<0.05$).[55] On the other hand, ethanol extract from its roots was able to significantly enhance cognition and glutamic acid levels in male Wistar rats which was perhaps mediated via high testosterone production.[56]

PHARMACOKINETICS

Bioavailability

Bioavailability studies are essential in order to define the appropriate dose and route of administration for the drug of choice. Findings of a study by Low and coworkers[57] suggested poor bioavailability of EN upon oral gavage with C_{max} and T_{max} of 0.33±0.03μg/ml and 4.40±0.98h, respectively. High first pass metabolism and/or low membrane permeability were thought to be the cause though. Subsequently, upon intravenous and oral administration of EN, Eurycomanol, 13α, 21-Epoxyeurycomanone (EP), and ED, only EP and EN displayed relatively longer biological life, better membrane permeability, and lower elimination rate.[58]

Drug-drug interactions

Those suffering from erectile dysfunction (ED) due to diabetes or even hypertension might consider consuming *E. longifolia* supplements in conjunction with conventional drugs for improving their sexual life. This may enhance or reduce the metabolism of either of the drugs due to drug-drug interactions. For instance, concurrent administration of *E. longifolia* extract (TAF-273) and Aminopyrine in normal and diabetic rats caused marked ($p<0.05$) enhancement in the metabolism of Aminopyrine in a dose dependent manner via activation of G-protein, Protein Kinases G, and A in the cAMP pathways and Protein Kinase C.[59,60] Likewise, Hussin[61] showed that co-administration of TAF-273 and Rosiglitazone considerably enhanced formaldehyde formation by inducing phase I metabolism of Rosiglitazone in hepatocytes of diabetic and normal rats.

HUMAN TRIALS

Aphrodisiac

Various preliminary human trials have recognized the effectiveness of aqueous extract from *E. longifolia* roots in improving the quality of life and well-being in men particularly those suffering from infertility.[62] More precisely, administration of twice daily of 100mg capsules containing *E. longifolia* in 75 patients suffering from partial infertility showed significant improvement in sperm concentration, motility, normal morphology, and sexual performance.[63] Next, 76 patients identified with hypogonadism and late hypogonadism displayed reduction in Ageing Male Symptoms ($p<0.0001$) and increase in testosterone concentrations by 46.8% following the treatment with 100mg capsule of *E. longifolia* twice daily for one month.[64] This effect by *E. longifolia* was believed to be mediated via the stimulation of Eurypeptides which activated CYP17 enzyme which in turn enhanced metabolism of pregnenolone and 17-hydroxypregnenolone to ultimately produce more testosterone.[65]

Ergogenic

Herbal beverages/drinks containing *E. longifolia* and other herbs such as tea, ginseng, and coffee are normally consumed by men in Malaysia assuming it can augment physical performance. High testosterone production is generally associated with increase in muscular mass and size.[66] It is also known that long term consumption of *E. longifolia* is associated with boost in testosterone level in the body. Hamzah and coworkers[67] claimed that 5 weeks daily administration of aqueous extract (100mg) in men considerably improved muscular size and strength through enhancing mean arm circumference ($p<0.011$) and lean body mass ($p<0.0012$). This was further reconfirmed by a relatively well-structured study by Henkel and coworkers[68] whose findings suggested that the administration of 400mg (twice daily) of aqueous extract for 5 consecutive weeks was associated with substantial elevation of total and free testosterone concentrations as well as augmentation in muscular strength in physically active senior males and females (52–72 years).

Anti-stress

Effect of various extracts of Tongkat Ali on anxiety and stress has been reported in anxious mice linking it to high Testosterone production. Outcomes of latest human trials conducted in 63 moderately stressed subjects (men and women) were indicative of significant anti-stress effect by *E. longifolia* ($p<0.05$). Treatment with 200mg/day of its standardized hot-water root extract for four weeks exhibited meaningful improvements in parameters such as tension, confusion and anger with no effect on vigor, fatigue and depression. Also, stress hormone profile assessment showed increased Testosterone and reduced Cortisol levels which further highlighted the role of Testosterone in stress reduction.[69]

Drug-drug interactions

As per earlier reports, *E. longifolia* was found to strongly interact with other drugs by affecting their metabolism and absorption in animals. The similar effect was observed in human subjects where the co-administration of Propranolol and aqueous extract of *E. longifolia* in fourteen non-smoker males (19–24 years old) caused significant increase in Propranolol concentration in the blood up to 10hr post-treatment which ultimately reduced its absorption. It was assumed *E. longifolia* triggered this action either; (i) by inducing the efflux transport P-glycoprotein which in turn increased the extrusion of Propranolol from the epithelial cells into the intestinal lumen, or (ii) via the formation of insoluble and non-absorbable chemical complexes with Propranolol which hindered its absorption.[70]

SAFETY AND TOXICOLOGY

In general, aqueous extracts of roots of *E. longifolia* are considered to be relatively safe with no reported toxic effects. In contrast, its alcoholic extracts at higher concentrations have been associated with serious toxicities both *in-vitro* and *in-vivo*.

Acute, subacute and subchronic toxicities

Initial toxicity evaluation of *E.longifolia* extracts by Satayavivad[71] recommended a LD_{50} of 1500–2000mg/kg and >3000mg/kg for 34% alcoholic and aqueous extracts, respectively. Subsequent evaluation of EN toxicity in mice and brine shrimp indicated LD_{50} of 0.05g/kg and 3.5µg/ml, respectively. Presence of unsaturated double bound at C-13 and C-21, an α, ß-unsaturated ketone moiety at C-2 in ring A, and oxymethylene bridge linking C-11 to C-8 were said to have contributed to its toxicity.[72] Likewise, F2 fraction showed a LD50 of 2.71g/kg for acute toxicity in rats whereas a dose of up to 51.2mg/kg was found to be safe with no visible damage to vital organs and testes following its sub-chronic (90 days) and chronic (180 days) toxicities. In general, preclinical studies have established the safety of the aqueous extract of *E. longifolia* roots at as high as 5000mg/kg whereas its organic extracts are reported to be relatively toxic[73] Lately, 4 and 13-weeks sub-acute and sub-chronic toxicity evaluation of *E. longifolia* root powder showed no notable toxic effects. Not to mention that, a dose of up to 1.2g/adult/day was recommended to be safe for consumption in human.[74]

Heavy metals contamination

By and large, plants absorb large amounts of heavy metals from earth which mostly get concentrated in the roots. These may pose threat to human life up on consumption by causing various ailments. Over 200 products containing *E. longifolia* are reported to be present in Malaysia most of which lack appropriate toxicological evaluation.[75] Over 200 products containing *E. longifolia* are reported to be present in Malaysia alone most of which lack appropriate toxicological evaluation. For example, heavy metals contamination in about 100 herbal products with *E. longifolia* as the main ingredient showed that about 36 of these products failed to comply with the limits for heavy metals contamination defined by the Drug Control Authority (DCA) of Malaysia. Equally, a similar assessment of 100 more herbal products indicated the presence of 10.3–20.3ppm of lead and 0.53–2.35ppm of mercury in 17 and 26 products, respectively, well above the permitted levels set by DCA.[76–78]

CONCLUSION

E. longifolia possesses numerous medicinal activities some of which have been investigated in detail to explore the underlying mechanism of actions involved particularly with regard to antitumor and aphrodisiac effects. Its traditional reputation as aphrodisiac has attracted scientists to investigate its effect on sexual arousal in both sexes especially in male. Apparently, aphrodisiac activity displayed by *E. longifolia* is linked to its ability to boost testosterone which is the main mediator of sexual function. In this regard, methanol extract has shown relatively stronger steroidogenesis and spermatogenesis effects than that of aqueous extract probably owing to accumulation of large amounts of bioactive quassinoids particularly EN. In a comparative study methanol extract was reported to be

about ten times more potent than the aqueous extract in generating more testosterone in Rodents.[12] Based on the above literature *E. longifolia* seems to have a lot to offer in managing and/or even treating conditions such as erectile dysfunction, malaria, and cancer. Nonetheless, more detailed investigations are necessary to further assess its effectiveness and safety in human subjects.

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Isolation and identification of endophytic fungi in the medicinal plant *Mikania laevigata* (Asteraceae)

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ABSTRACT

The *Mikania laevigata* (Asteraceae) is a Brazilian medicinal plant used to treat respiratory tract diseases. It is produced on a large scale for phytotherapic formulation. No studies on the endophytes of *Mikania laevigata* have been described in the literature. The endophytic fungi isolated from the leaves of *M. laevigata* cultivated in a floral garden located in the south region of the Bahia state, Brazil, were investigated. After surface disinfection, the performing the isolation procedure, a total of forty endophytic fungi were obtained and grouped into different morphospecies. The fungi were characterized by molecular sequencing the ITS rDNA regions, and they were identified by comparing the results published Gen Bank sequences.The phylogenetic analyses revealed four major clades of fungi from the sequenced ITS-rDNA regions: *Nodulisporium* sp. (3 isolates), *Hypoxylon* sp. (3 isolates), *Daldinia* sp. (1 isolate) and *Xylaria luteostromata* (aunique isolate).The Xylariaceae Genus was the dominant group of fungi associated with *Mikania laevigata*.

Keywords: *Mikania laevigata*, Asteraceae, Xylariaceae, Endophytic fungi.

INTRODUCTION

Brazil has a long tradition of using raw vegetable materials in folk medicine. *Mikania laevigata* (Asterceae) is among these material. It is popularly known as "guaco" and possess pharmacological properties for treating the airways[1,2]. *M. laevigata* also possesses antimicrobial,[3] inflammatory, bronchodilator, antispasmodic, antirheumatic and antiulcerant activities[4,5].

Endophytic fungi are a class of microorganisms that live within plant tissues during at least one life-cycles period but do not cause overt symptoms of disease[6,7]. The intensity and diversity of the endophytes in the host plant are related to the age of the plant as well as the geographical and climatic conditions but vary according to the specific type of host and tissue[8]. Studies indicate that many plants depend on fungi for their survival, especially in stressful conditions[9,10]. Plant in a natural setting host one or more endophytes and the recent increase in isolated endophytic fungi and the investigation of the symbiosis between plants and endophytes have created new perspectives on plant-fungal chemical interactions. Endophytic colonization can bio-produce numerous secondary metabolites that provide protection against pathogens, herbivores or abiotic stress, aiding the survival of the plant. Thus, the endophytic fungi have also been recognized as important sources of natural bioactive compounds[11–13].

No studies on endophytic microorganisms and *M. laevigata* have been described in the literature. This study shown the isolated and preliminarily identified endophytic fungi from the leaves of *Mikania laevigata* by sequencing of the ITS region as an indicator of phylogenetic relationship.

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MATERIALS AND METHODS

Collection of plant material

The *Mikania laevigata* was collected in a floral Garden located in State University of Santa Cruz (UESC), Ilhéus, Bahia State, Brazil. The gathering was held in September 2009. Three plants were selected for the withdrawal of healthy leaves. From each plant, five leaves were collected, totaling a sample of fifteen leaves which were immediately subjected to endophytic fungi isolation.

Isolation of endophytic fungi

The surfaces of the leaves were disinfected according to the method described by Maitan,[14] with several modifications. The leaves were washed thoroughly under running tap water and were then submerged in 70% ethanol for 1 min and 3% sodium hypochlorite for 5 min, successively. The leaves were then rinsed in distilled water for 1 min (3 times). The apex and base of each leaf were removed and only the central portion of the leaf for analysis. The leaves were cut into 5 mm x 5 mm segments using a sterile scalpel. The leaf blades were macerated with 6.0 ml of sterile 0.5 M sodium phosphate buffer and 100 µL of this extract was spread onto four different culture media: PDA, Sabouraud, ISP-2 (Agar yeast extract-malt extract) and oatmeal agar supplemented with chloramphenicol (100 µg/mL) and Rose Bengal (25 µg / mL). Five replicates were performed for each medium. The plates were incubated at 28ºCfor fifteen days. After the twelfth day of incubation, small pieces of agar containing the newly developed fungi were transferred to plates containing PDA medium to isolate and purify the colonies. The fungi were purified by successive samplings. The fungal isolates were stored according to Castellani (1967)[15].

DNA sequencing

The 40 fungi isolates were grouped as morphospecies based on the culture morphology. The isolates were grown in 1 % malt extract broth and agar for two weeks at 28ºCin the dark. The DNA was extracted using the method described by Doyle & Doyle,[16] with some modifications.ITS5 and ITS4 primers were used to amplify of the ITS regions[17]. Amplification was performed in a solution (25 µL) containing the following components: 1 x PCR reaction buffer (100 mMTris-HCl), 2,5mM $MgCl_2$, 0,2 mMdNTP, 7.5 pmol each oligonucleotide primer, 2.5 unit of Taq polymerase (Phoneutria, Brazil), 1M Betaine,1 µg of BSA, 2 % of DMSO and approximately 1 ng of genomic DNA, and sterilized, deionized water.

The thermocycler program was as follows: an initial cycle of denaturation at 95ºC for 1 min, followed by 35 cycles at 94°C for 1 minute (denaturation), annealing at 60°C for 90 minutes and extension at 72°C for 2 minutes, and a final cycle of extension at 72°C for 10 minutes. The PCR reaction products were examined by electrophoresis on 1% agarose gel, and the concentrations of these products were estimated by comparing the band intensity with the intensity of the bands of the low molecular mass DNA marker (Invitrogen). Analysis and photo documentation of gels was conducted using the Kodak 1D 3.6 program. The purified PCR products were sequenced on an ABI 3130XL automated sequencer (Applied Biosystems).

Identification of isolates

Electropherograms were edited using the Staden package (modules PREGAP4 and GAP4)[18]. Sequences were submitted to BLASTn, NCBI (www.ncbi.nlm.nih.gov/blast) to obtain close matches from Genbank to be included in the matrix, and aligned in Clustal X v. 1.81[19]. The matrix was then manually adjusted by comparing to other sequences obtained from GenBank.Two distinct phylogenetic analysis were performed for a comparison of topologies: Maximum Parsimony (MP) and Bayesin approaches. Maximum Parsimony was carried out using PAUP 4.0[20], with the following criteria: heuristic search with 1000 replications of random taxon addition, TBR algorithm, saving ten trees per replicate. Branch support was evaluated through character bootstrap[21], with 1000 pseudoreplicates, simple addition of taxa and TBR algorithm, saving 10 trees per replicate. Bayesian analysis was conducted using Mrbayes 3.1.2[22]. The evolutionary model and the prior distribution of probabilities were selected using AIC implemented in Mr Model test 2.2[23]. Bayesian analysis consisted of two parallel runs with four chains each sampling trees in each 100 generations for 5 x 10^6 of generations. The remaining trees were used to construct the majority consensus in PAUP 4.0, which allowed estimating a consensus tree and posterior probabilities of clades. The data generated were deposited with the Tree base ID 11520 and the number sequences JN051356-JN051363 in the Genbank. All specimens were deposited in public collection (Culture Collection of Microorganisms from Bahia – CCMB).

RESULTS

We isolated 40 endophytic fungi from the leaves of *Mikania laevigata,* and 13 morphotypes were selected

according to the morphological characters of the fungi. The IT Sr DNA region was sequenced for all morphotypes, and the endophytes were identified by comparing the resulting sequences with the published GenBank sequences. Table 1 shows the endophytic fungi identified from *M. laevigata*.

Table 1: Endophytic fungal isolates obtained from leaves of *Mikania laevigata* and used for DNA sequencing

Code of isolated	Species identity	Family	Order	BLAST match sequence		CCMB no.	GenBank accession no.
				Accession no.	Coverage/ Maxident %		
FEX1	*Nodulisporium sp.*	Xylariaceae	Xylariales	FJ612983	100/99	562	JNO51356
FEX2	*Hypoxylon sp.*	Xylariaceae	Xylariales	FJ612691	100/98	563	JNO51357
FEX5	*Nodulisporium sp.*	Xylariaceae	Xylariales	FJ612983	99/99	564	JNO51358
FEX7	*Daldinia sp.*	Xylariaceae	Xylariales	GQ999505	99/99	565	JNO51359
FEX8	*Hypoxylon sp.*	Xylariaceae	Xylariales	FJ612745	100/99	566	JNO51360
FEX9	*Nodulisporium sp.*	Xylariaceae	Xylariales	FJ612983	100/99	567	JNO51361
FEX11	*Hypoxylon sp.*	Xylariaceae	Xylariales	FJ612745	100/99	568	JNO51362
FEX13	*Xylari auto stroma*	Xylariaceae	Xylariales	GU324739	96/99	569	JNO51363

CCMB: culture collection of microorganisms from Bahia

The amplification products of the ITS region generated a single fragment approximately 600 bp in size. The sequencing of the PCR-products yielded 595–677 bp of informative sequence. The bayesian topology including all of the sequences obtained in this study showed the diversity and the phylogenetic placement of the endophytic fungi associated with *Mikania laevigata* (Figure 1). Species of *Neurospora crassa* M13906[24] were selected for an out-group based on phylogenetic relationships. The ITS alignment consisted of 8 sequences generated from this study and 41 sequences obtained from GenBank. This ITS alignment consisted of 750 characters including alignment gaps, of which 91 were variable.

How was it obtained a high percentage of endophytic fungi in Xylariaceae were selected isolates that had different morphological characteristics (as morphotypes) for sequencing of the ITS-rDNA region. These were made a selection of 8 endophytic fungi in Xylariaceae. According to the topology (Figure 1), the phylogenetic reconstruction showed the formation of four clades were assigned where the endophytes of this study. The Bayesian Analyses showed that these endophytic fungi possess a higher identity to the genus *Nodulisporium, Xylaria, Daldinia* and *Hypoxylon* (Figure 1).

The isolate FEX13 shown to have a high identity with *Xylaria luteo stroma* GU324739 supported bootstrap 100%. The isolate FEX7 showed high similarity to the genus *Daldinia*. FEX11, FEX2 and FEX8 to *Hypoxylon*, FEX 5, FEX1 and FEX9 to *Nodulisporium*. All these clades showed a high statistical support bootstrap value above 90%.

DISCUSSION

The aim of our study was to characterize the endophytic fungi associated with *Mikania laevigata*, a medicinal plant that has important therapeutic properties. The Xylariaceae was the dominant group of fungi associated with this plant, including species in the *Xylaria*, *Hypoxylon*, *Daldinia* and *Nodulisporium* genera[25,26]. The endophytes were identified based on rDNA ITS sequences. Several fungal ITS primers, such as ITS4 and ITS5, were designed to amplify the fungal rDNA from a variety of samples[27]. The phylogenetic analyses revealed four major clades containing fungal sequences. By sequencing of the ITS rDNA region,

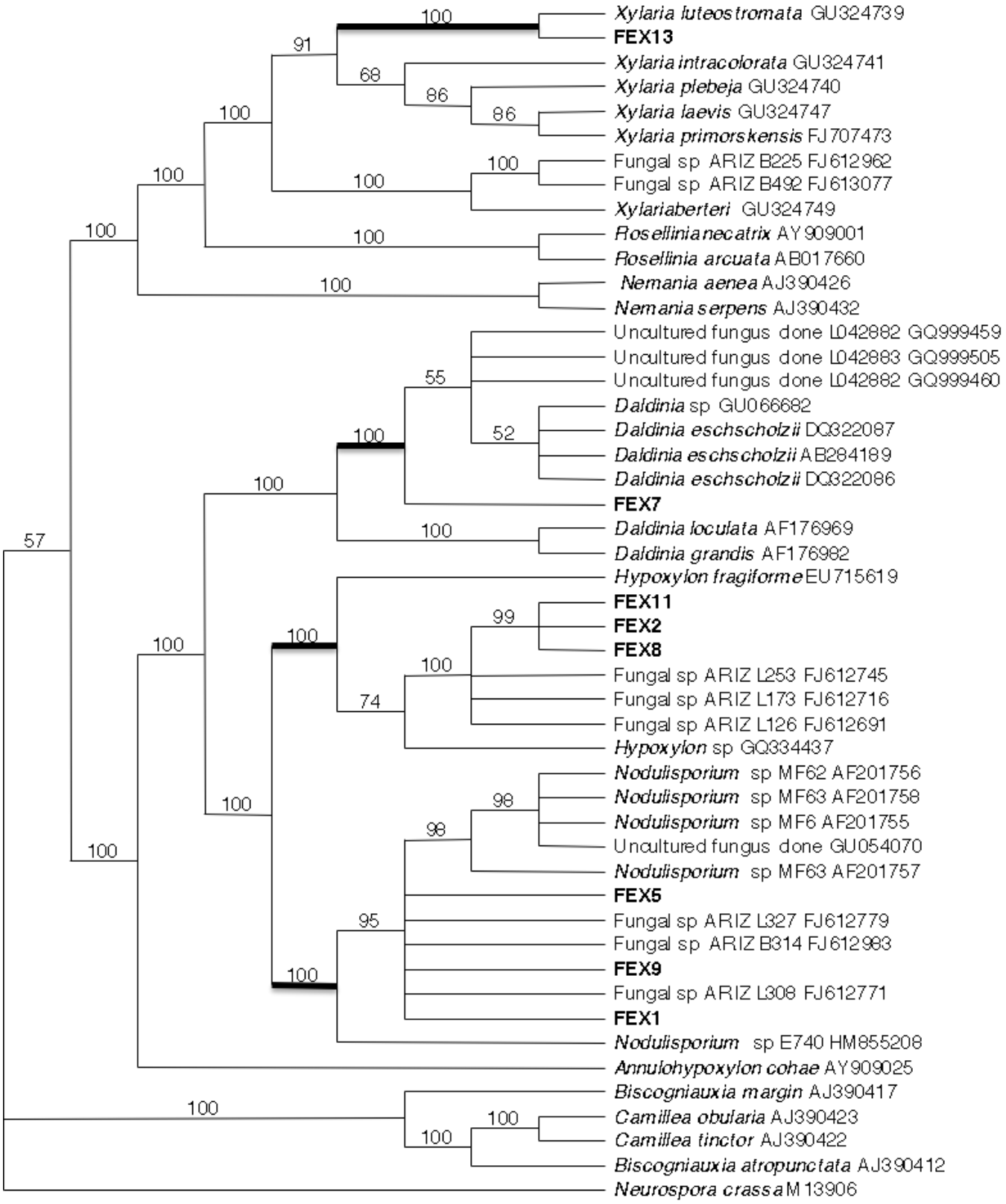

Figure 1: Phylogenetic tree of endophytic fungi isolated from *Mikania laevigata*

we identified 8 Xylariaceae members, which comprised: 3 isolates of *Nodulisporium* sp., 3 isolates of *Hypoxylon* sp., 1 isolates of *Daldinia* sp. and a species identified as *Xylaria luteostromata*.

The endophyticxy lariaceous fungi isolated in this study are widespread and have been found in many different medicinal plants. Fity-two endophytic fungi strains were identified from *Huperziaserrata* based on rDNA ITS analysis. Several isolates belonged to the *Hypoxylon, Daldinia, Xylaria* and *Nodulisporium* genera[28]. In *Nothapodytes foetida, Nodulisporium* species were identified using 28S DNA sequencing[29]. According to Huang,[30] *Xylaria* species are the dominant fungal endophytes in the genus *Artemisia* (Asteraceae). Linnakoski[31] isolated and characterized endophytic fungi of the genera *Xylaria* and *Hypoxylon* from *Khayaanthotheca* cultivated in Ghana.

The Xylariaceae fungi area rich source of secondary metabolites, including the sesquiterpenexy larenal obtained from *Xylaria persicaria*,[32] xylactam, a nitrogen-containing compound obtained from fruiting bodies of *X. euglossa*; and xanthone derivatives obtained from *Xylaria*sp[33]. The xanthones area class of natural products with several pharmacological properties, such as anti-inflammatory,[34] antimicrobial,[35] antioxidants,[36] antifungal[37] and anticancer activities[38]. In addition, crude extracts obtained from the fungi described here were evaluated against four *Salmonella* species, and all of the fungal extracts showed antimicrobial activity[39].

CONCLUSION

The present investigation contributed to the scientific knowledge of endophytic fungi present in a species *Mikania laevigata*. The molecular parameters presented in this paper revealed four major clades of fungi from the sequenced ITS-rDNA regions: *Nodulisporium* sp., *Hypoxylon*sp, *Daldinia* sp. and *Xylaria luteostromata*. The Xylariaceae genus was the dominant group of fungi associated with *Mikania laevigata*.

CONFLICTS OF INTEREST

All authors have none to declare.

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Pharmacognostic evaluation and chrysazin quantitation of *Xyris indica* flowering heads

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ABSTRACT

Objectives: The present study aimed to establish quality specification of *Xyris indica* L. flowering heads. The pharmacognostic parameters were investigated. Chrysazin contents were analyzed by TLC image analysis using ImageJ software compared to TLC-densitometry. **Methods:** *X. indica* flowering heads from 15 different sources in Thailand were collected. Morphological and physicochemical parameters were characterized. Chrysazin was successively extracted and determined by TLC image analysis using ImageJ software and TLC-densitometry. **Results:** Macroscopic study was illustrated as whole plant drawing. The microscopic study showed fragment of corolla, seeds, pollen grain and staminode. The pharmacognostic parameters revealed that the loss on drying, total ash, acid-insoluble ash and water content should be not more than 6.90, 2.50, 0.41, and 11.12 of % dry weight respectively while water and ethanol-soluble extractive values should be not less than 6.59 and 4.03 of % dry weight respectively. TLC fingerprint revealed clearly chrysazin yellow fluorescent band at 365 nm. Chrysazin quantitation by TLC image analysis and TLC densitometry were developed and validated. Chrysazin content was 0.022 ± 0.001 % dry weight by both methods. There was no statistically significantly difference between these methods. **Conclusion:** This study provided pharmacognostic specification and chrysazin content of *X. indica* flowering heads that can be used for basic quality control and standardization of plant material. TLC image analysis using ImageJ software showed reliable and convenient for analysis of chrysazin content in this crude drug.

Keywords: *Xyris indica*, Pharmacognostic specification, Chrysazin, Quantitative analysis, Antimicrobial activities

INTRODUCTION

Herbal medicines have been used for the treatment or prevention of diseases from time immemorial in all cultures. Over the last few decades, many people have been turning back to herbal medicines for remedy. However, adulteration and misidentification of crude drug still exist. Thus, development of standardization and quality control of herbal medicines are needed to be prioritized at the earliest stage.

Xyris indica L. (Xyridaceae), a perennial herb which is grass-like, known locally as Kra thin thung, is widespread species in Thailand and a native plant of east India. *X. indica* flowering heads used as crude drug in traditional Thai medicine Figure 1 (left). It has been used to treat ringworm, constipation and flatulence.

The phytochemical studies of *X. indica* flowering heads showed two isocoumarins; xyridin A and xyridin B, two sterols; stigmasterol and spinasterol and three anthraquinones; chrysazin, 3-methoxychrysazin and 3-hydroxychrysazin[1–3]. The previous antimicrobial studies revealed that 3-hydroxychrysazin showed good antifungal activity against *Trichophyton mentagrophytes* and *T. rubrum*[3] and ± Dihydroxyridin showed strong effect than standard drug against *Aspergillus niger*[4].

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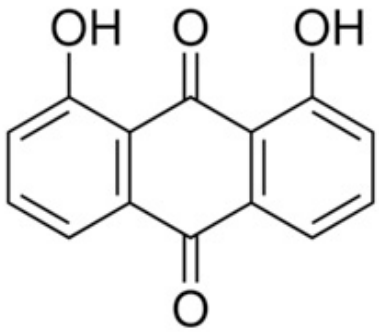
Crude drug of *Xyris indica* L. flowering heads



Chrysazin (1,8-Dihydroxyanthraquinone)

Figure 1: (left) *Xyris indica* L. flowering heads; (right) Chemical structure of chrysazin (1,8-Dihydroxyanthraquinone)

Chrysazin Figure 1 (right), the main component in *X. indica* flowering heads was chosen as quantitative marker in this plant. It had been used as a laxative and as a natural colorant. However, the studies in experimental animals indicated that chrysazin is reasonably anticipated to be a human carcinogen[5]. Thus, chrysazin content in this crude drug should be concerned for safety especially use as oral medicine.

Although *X. indica* has been used for a long time, there have been no reports on standardization of this plant. Therefore, this study aimed to assess the pharmacognostic characters of *X. indica* flowering heads and determine the chrysazin content by TLC image analysis using ImageJ software and TLC-densitometry for standardization of *X. indica* crude drug.

MATERIALS AND METHODS

Plant materials

X. indica flowering heads were collected from 15 different sources in Thailand and authenticated by Ruangrungsi N. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The samples were dried in hot air oven at 45°C and ground to powders.

Plant extraction

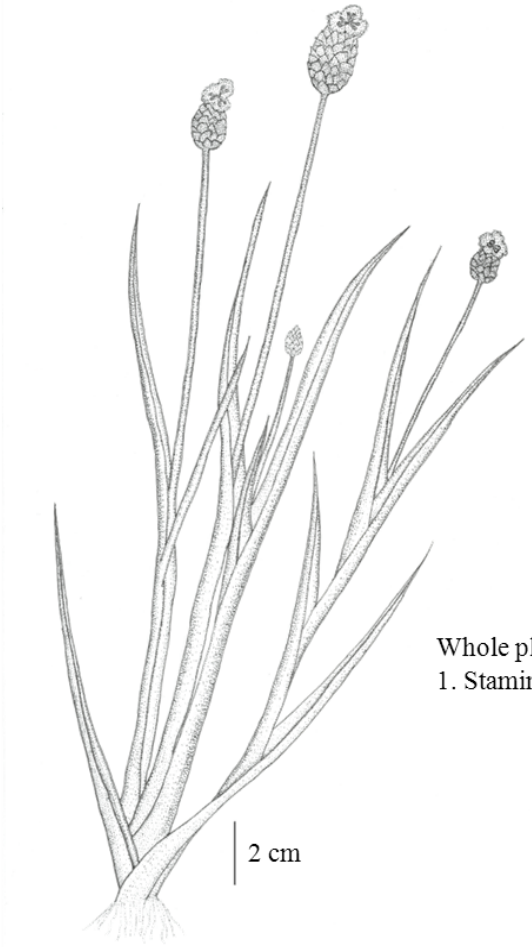
The ground samples (5 g) were exhaustively extracted with benzene by soxhlet apparatus. The extracts were filtered. The filtrate was evaporated to dryness and re-dissolved in ethanol to obtain a concentration of 5.0 mg/ml for analysis of the chrysazin content.

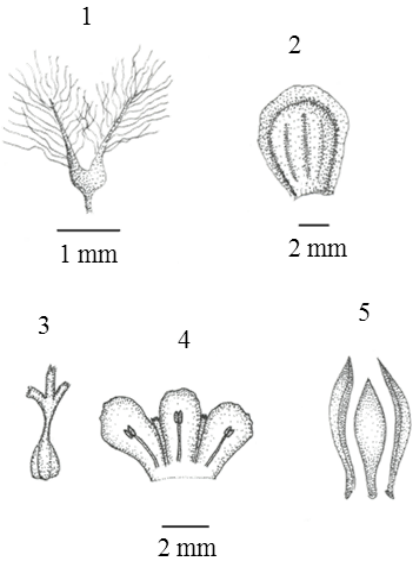
Standard chrysazin

Standard chrysazin (purity 96 %) was purchased from Sigma-Aldrich Co., USA. The stock solution of chrysazin (0.5 mg/ml) was prepared in 95 % ethanol and diluted to obtain the series of standard solutions with concentration of 15, 30, 45, 60 and 75 µg/ml.

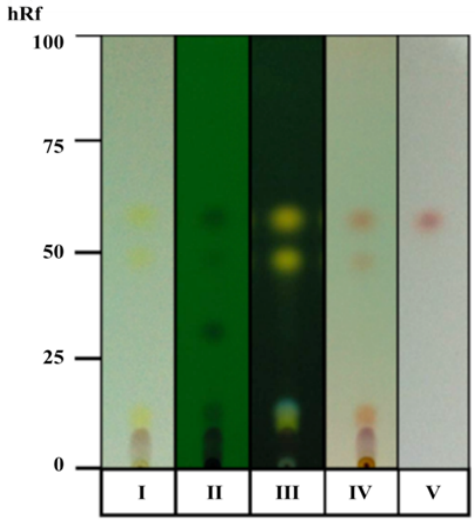
Pharmacognostic evaluations

The pharmacognostic evaluations were performed according to WHO guideline quality control methods for medicinal plant materials[6]. Briefly, macroscopic examination of plant material was illustrated by including shape, size, colour, odour and taste. Microscopic examination of ground sample was observed cell and tissue structures under microscope. Three grams of ground sample were heated at 105˚C until constant weight for determination of loss on drying then ignited in an incinerator at 500˚C until it was white, cooled and weighed to calculate total ash. The remaining ash was boiled gently with 25 ml of hydrochloric acid (70 g/l), filtered and ignited at 500˚C until constant weight, cooled and weighed to calculate acid-insoluble ash. Determination of extractive values was performed with ethanol and water. Water content was determined by azeotropic distillation. TLC fingerprint of ethanolic extract was performed using silica gel 60 GF_{254} as stationary phase and a mixture of petroleum ether and ethyl acetate (8:1) as mobile phase. The plate was visualized under UV light at 254 nm, 365 nm and by staining with 5% potassium hydroxide in methanol.

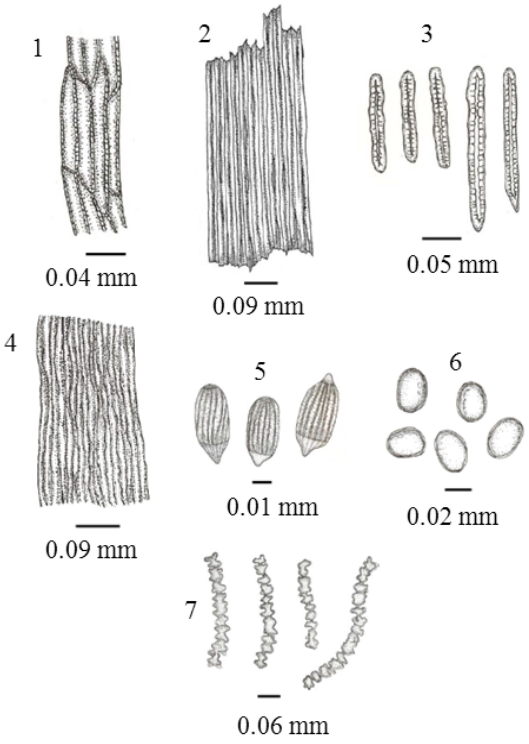




Whole plant of *Xyris indica* L.
1. Staminode 2. bract 3. pistil 4. corolla laid open 5. sepals



TLC fingerprint of ethanolic extract of *Xyris indica* flowering heads (I-IV) and chrysazin standard (V)
DetectionI = under daylight
II = under UV light 254 nm
III = under UV light 365 nm
IV, V = staining with 5% KOH in methanol



Powder of *Xyris indica* L. flowering heads
1. Parenchyma of bract 2. fragment of fibers of bract 3. sclereid of bract
4. fragment of corolla 5. seeds 6. pollen grains 7. staminodes

Figure 2: Macroscopic, microscopic characteristics and TLC fingerprint of *Xyris indica* L.

Chrysazin quantitative analysis

TLC-densitometry of chrysazin

Three microliters of benzene extract and standard chrysazin solutions were applied on the silica gel 60 GF_{254} plate (Merck, Germany; 20 x 10 cm, 0.25 mm thickness) by Linomat 5 applicator (Camag, Switzerland). The plate was developed to a distance of 8.0 cm in a TLC chamber with petroleum ether and ethyl acetate (8:1), then dried and scanned at 430 nm using TLC scanner 3 (Camag, Switzerland) in operation with winCATS software. The quantitative analysis was performed in triplicate.

TLC image analysis of chrysazin by ImageJ software

Developed TLC plate from above was visualized in UV viewing cabinet (Spectroline®, USA) under UV 365 nm and photographed by a charge-coupled device camera (Canon Power shot A650). The images saved as JPEG files were opened with ImageJ software (NIH, USA)[7] and applied with smooth function 5 times. Then a rectangular tool was used to crop the band and create plot profiles. After drawing the line under the plot, the peak areas were measure as square pixels by wand tool.

METHOD VALIDATION

The analytical procedures were validated according to the ICH guideline[8] in terms of specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness.

RESULTS

Pharmacognostic evaluations

X. indica is a perennial herb to 30–60 cm tall. Stems are tufted. Leaves are narrowly linear, acute at apex, 0.4–0.5 × 13–40 cm. The yellow flowers are packed between bracts. Bracts are form a compact head or spike, 0.5–1.4 × 0.5–2.2 cm. The yellowish brown bracts are suborbicular or ovate, 5–8 × 5–7 mm. There are 3 yellow sepals. 2 lateral sepals are boat-shaped, 0.8–1.4 × 5–7mm. Median sepal is cap- shaped, 2–2.5 × 4–6 mm. There are 3 yellow petals, obovate, serrate at apex, 3–4 × 3–4.5 mm. For stamen, there are 3 fertile stamens with anthers 2 lobed and 3 staminodes with hairy. Ovary is obovoid with 3 styles. Seeds are ovoid. The flowering heads powder was slightly characteristic odour and bitter, astringent taste. Dried flowering heads of *X. indica* was used as crude drug. Macroscopic and microscopic characteristics were shown in Figure 2.

The pharmacognostic parameters of *X. indica* flowering heads were shown in (Table 1). The loss on drying, total ash, acid insoluble ash and water content should be not more than 6.899, 2.497, 0.409 and 11.121 % of dry weight respectively while water-soluble extractive and ethanol-soluble extractive values should be not less than 6.592 and 4.030 % of dry weight respectively. TLC fingerprint revealed clearly separated spot of chysazin, appearing yellow fluorescent spot at 365 nm with hRf value of 59. The compound turns into pink spot with 5% potassium hydroxide in methanol (Figure 2).

Table 1: The pharmacognostic parameters of *Xyris indica* flowering heads

Parameter (% by weight)	Mean ± SD*	Range (Mean ± 3SD)
Loss on drying	6.899 ±0.165	6.403 – 7.395
Total ash	2.497 ± 0.033	2.399 – 2.595
Acid-insoluble ash	0.409 ± 0.027	0.329 – 0.490
Water-soluble extractive	6.592 ±0.474	5.170 – 8.014
Ethanol-soluble extractive	4.030 ± 0.486	2.573 – 5.487
Water content	11.121 ± 1.132	7.725 – 14.518

[w]*The parameters were shown as grand mean ± pooled SD. Samples were collected from 15 different sources in Thailand. Each sample was tested in triplicate.

Chrysazin quantitative analysis

TLC was selected for quantitative analysis of chrysazin using the mixture of petroleum ether and ethyl acetate (8:1) as mobile phase. This mobile phase showed good separation for chrysazin on developed TLC plate. The yellow fluorescence spot of chrysazin was clearly detected under UV 365 nm (Figure 3). The percent yield of benzene extracts were 2.26 - 6.91% w/w and the average was 3.55±1.34 % w/w. The chrysazin contents in 15 samples of *X. indica* flowering heads analyzed by TLC-densitometry were between 0.0126–0.0390 % w/w (0.0223 ± 0.0011% w/w). The contents by TLC image analysis were between 0.0124–0.0377 % w/w (0.0219 ± 0.0007 % w/w). The chrysazin contents of both methods were not statistically significantly different ($P > 0.05$) as determined using paired t-test.

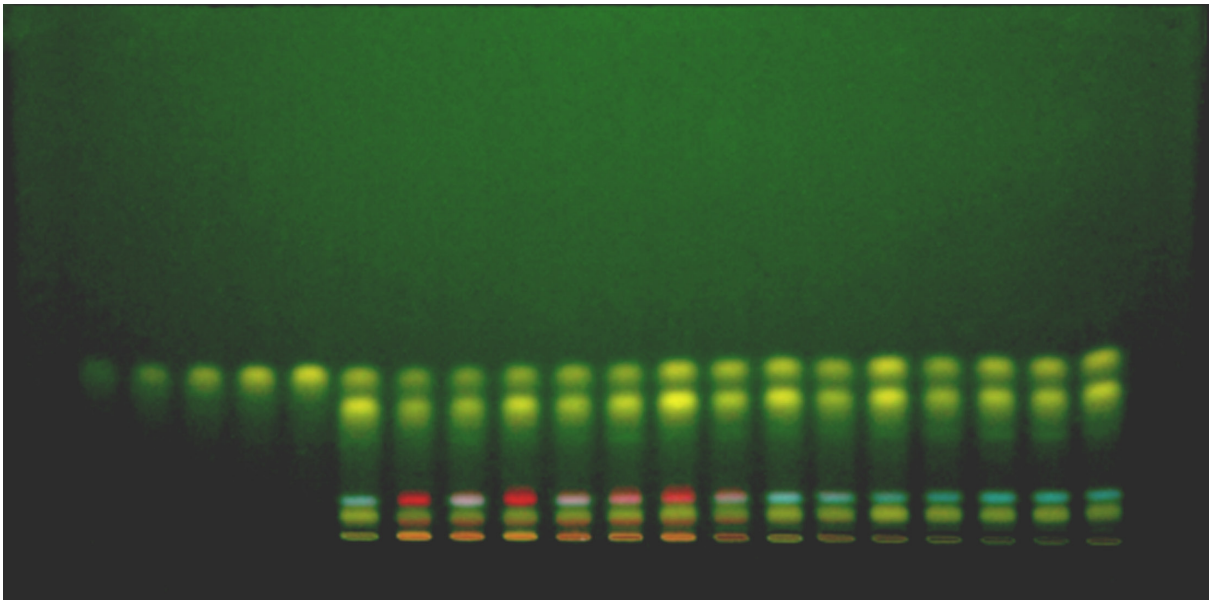


Figure 3: The TLC plate developed with petroleum ether: ethyl acetate (8:1) visual under 365 nm original image; lane 1 to 5 – standard chrysazin, lane 6 to 20 – sample no. 1–15

METHOD VALIDATION

The specificity of the TLC method was confirmed by comparing UV/VIS spectrum of the chrysazin peak in the sample with standard chrysazin peak. The result showed the identical absorption spectra with maximum absorbance at 430 nm. The calibration curves between peak area and concentration of standard chrysazin were linear regression over the range of 15.0–75.0 µg/ml. For TLC-densitometry, the equation was y = 98.67x - 147.7 (R^2 = 0.9997) whereas the equation of TLC image analysis was y = 480.2x + 586.7 (R^2 = 0.9986) where y is peak area and x is concentration. The accuracy was examined from percent recovery by spiking known amount of chrysazin (10, 25 and 45 µg/ml) in a sample. The recovery values of both methods were between 90.67–99.16 % (Table 2). The precision of these methods was studied in aforementioned three concentrations of spiked samples. The % RSD for repeatability and intermediate precision of TLC densitometry were 0.76 - 2.77% and 1.47 – 4.48 % respectively. The %RSD for repeatability and intermediate precision of TLC image analysis were 0.78 - 3.10 % and 2.46 - 3.95% respectively. LOD and LOQ were evaluated based on the standard deviation of y-intercepts and the slope of the calibration curve. The LOD and LOQ of TLC-densitometry and TLC image analysis were 0.79 and 2.39 µg/ml and 1.69 and 5.13 µg/ml, respectively. The robustness studied by changing composition of mobile phase (petroleum ether: ethyl acetate 8:1, 8.1:0.9, 8.2:0.8, 7.9:1.1, 7.8:1.2 v/v) was 4.44 % RSD of peak area from TLC-densitometry and 3.60 %RSD from TLC image analysis. Table 2 demonstrated the method validity of both methods.

Discussion and conclusion

The quality control methods are important tool in traditional medicines which serve as useful information for identification, authentication and standardization of herbal medicine[9]. The safety and efficacy of herbal

Table 2: Validity of chrysazin quantitative analysis in *Xyris indica* flowering heads by TLC-densitometry and TLC image analysis

Parameter	TLC-densitometry	TLC image analysis
Linearity	y = 98.67x - 147.7	y = 480.2x + 586.7
R^2	0.9997	0.9986
Range	15.0–75.0 µg/ml	15.0–75.0 µg/ml
Accuracy: % Recovery	90.67–99.16 %	91.87–96.00 %
Precision		
Repeatability	0.76–2.77 %RSD	0.78–3.10 %RSD
Intermediate precision	1.47 – 4.48 %RSD	2.46–3.95 %RSD
Limit of detection (LOD)	0.79 µg/ml	1.69 µg/ml
Limit of quantitation (LOQ)	2.39 µg/ml	5.13 µg/ml
Robustness	4.44 %RSD	3.60 % RSD

medicine are dependent on the standardization and quality of plant materials[10]. Macroscopic and microscopic methods can help to identify and authenticate plant materials. The microscopic characteristics of powdered *X. indica* flowering heads were remarkable diagnostic characteristics of this plant part. The constant numbers from pharmacognostic evaluation could be used for quality and purity of plant materials. TLC fingerprint showed the pattern of phytochemical characteristic components.

To develop alternative TLC method for analyzing chrysazin content, TLC-densitometry and TLC image analysis using ImageJ software were performed and validated to confirm that the analytical procedure employed reliable and accurate results. Chrysazin content in dried flowering heads was 0.022 ± 0.001 % w/w by both methods. The previous phytochemical study of anthraquinones in *X. indica* flowering heads showed that chrysazin was major component[3]. In addition, chrysazin was able to be isolated from dried leaves and stems of *X. semifuscata*[11]. Chrysazin also occurs naturally in *Cassia*, *Aloe*, *Rheum* and *Rhamnus* species[5].

The specificity of the TLC method indicated that 430 nm is maximum absorption of chrysazin used as peak identity. This wavelength is optimal wavelength for scanning densitometer in this study that quantified chrysazin accurately. The calibration curves of both methods showed good linearity relationships ($R^2 > 0.99$). Moreover, the percent recovery exhibited an acceptable accuracy of both methods[12]. The precision showed that repeatability and intermediate precision of the methods were satisfactory as RSD at each concentration level less than 15%[13]. The LOD and LOQ values from both methods displayed sufficient sensitivity of the methods. However, TLC-densitometry showed better sensitivity than TLC image analysis. The result of robustness indicated that changing mobile phase composition was not affected in both methods.

Thus, the result from method validation and paired t-test indicated that TLC image analysis is efficient, reliable and suitable technique for using in quantitative analysis of chrysazin in *X. indica*. Moreover, TLC image analysis can be use as alternative method for any laboratory due to its advantages which is easy to perform, fast, inexpensive instruments. Charge-coupled device camera becomes more widely used because it is much faster and efficient than scanning densitometer[14]. Image analysis using ImageJ software was not required sophisticated instrument and easily applicable[15]. Besides, ImageJ software, other image analysis softwares can also be used in TLC quantitative analysis. For example, Photoshop 7.0 and Scion image software were chosen to determine the amount of three curcuminoid in *Curcuma longa*. The techniques were validated and showed to be accurate and reliable method[16–17].

In conclusion, this research provides pharmacognostic specification and chrysazin content of *X. indica* flowering heads that can be used for basic quality control and standardization of plant material. TLC image analysis method could be use as alternative method for the simultaneous analysis of chrysazin content in plant material.

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The *In-Vitro* Toxic Effect of The Glycoalkaloids for Some *Solanum* Species Against The *LIM-1863* Cell Line

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ABSTRACT

Background The LIM-1863 Cell Line is one of the colon cancer types considered to be responsible for a high rate of deaths, and the glycoalkaloids being natural substances existing in the *Solanum* species have anticancer effects. **Objective** This research aims at studying the effect of the glycoalkaloids on viability of the LIM-1863 cancerous cells in-vitro. **Materials and Methods** The glycoalkaloids in this study are extracted by the ultrasonic waves technique and detecting them by the Thin Layer Chromatography (TLC) in addition to incubating of the LIM-1863 cells with different concentrations of the glycoalkaloids for 48 hours and then assessing of the cell viability using the MTT assay.**Results** The findings showed that the glycoalkaloids have a toxic effect on the LIM-1863 cells and that half of the inhibiting concentration (IC_{50}) of the *Solanum* fruits extract: (*Solanum nigrum* L.), (*Solanum villosum* Mill.) and (*Solanum elaeagnifolium* Cav.) on the LIM-1863 cells have the (164.7, 35.91 and 12.14 μg/ml) values successively. **Conclusion** The observations indicated that the glycoalkaloids are able to inhibit the colon cancer cell proliferation.

Keywords: *Solanum*, Glycoalkaloids, TLC, LIM-1863, Viability, MTT.

INTRODUCTION

Historically, plants have a useful effect on many diseases that infecting the human life, as the World Health Organization estimates that about 80% of the health problems of the globe's population should be treated by the medical plants drugs[1,2]. The medical plants have a long history for cancer treatment because the active constituents of *Angelica gigas*, *Campototheca acuminate*, *Catharanthus roseus*, *Ocrosia elliptica*, *Podophyllum emodii*, *Podophyllum peltatum* and *Taxus brevifolia* have been used to treat advanced stages of several malignant tumors[3]. The colon cancer is responsible for a high rate of deaths being directly connected to age and diet that spreads more over time[4,5].

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The glycoalkaloids are considered among the most important compounds with an anticancer effect which are found in the butanol's extracts of fruits (BEF) of the Solanum species spreading in the Syrian wilderness. The (*S. nigrum*), (*S. villosum*) and (*S. elaeagnifolim*) (Figure 1) categorized under the Solanaceae family according to Syria's Poisonous Plants Information System (SPPIS)[6].

The *Solanum* genus is regarded one of the largest genus of the Solanaceae family since it contains more than 1500 species many of which are economically important throughout their cosmopolitan distribution such as annual and perennial plants, forbs, vines, sub-shrubs, shrubs and small trees, and they often have attractive fruits and flowers, The *Solanum* constituents moreover have medicinal and toxic values together[7,8].

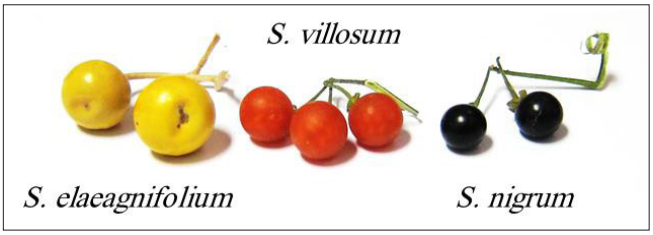

Figure 1: The profile of fruits of *Solanum Species*

The *Solanum* contains the glycoalkaloids that showed biological effects like the antifungal[9,10] and the antiviral ones[11]. but its most important feature in fact lies that it also shows considerable anticancer effects. For example, The Solamargine causes the human hepatoma cells death (Hep3B) by apoptosis;[12] α-solasonine (Figure 2) from *S. crinitum* and *S. jabrense* has a cytotoxic effect on the leukemia cells;[13] chaconine, solanine, tomatine, and their derivatives inhibit the human colon (HT29) and liver (HepG2) cancer cells to grow;[14] β-2-solamargine from (*Solanum nigrum* L.) has a toxic effect on the cell lines: HT-29 (colon), HCT-15 (colon), LNCaP (prostate), PC-3 (prostate), T47D (breast), and MDA-MB-231 (breast)[15].

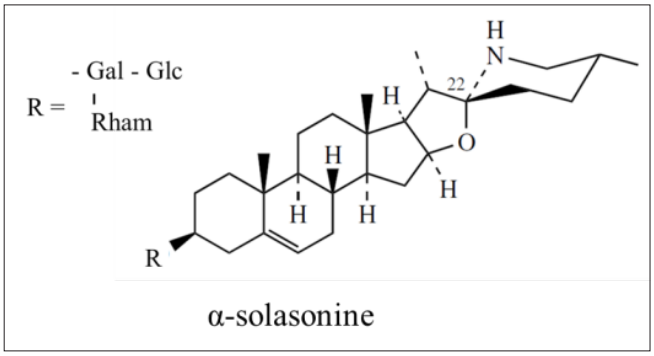

Figure 2: The structure of α-solasonine

This research has the aim to find the toxic effect of the glycoalkaloids existing in the BEF of (*S. nigrum*, *S. villosum* and *S. elaeagnifolium*) on a type of the human colon cancer being the LIM-1863 cell line,[16,17] to extract the glycoalkaloids from the *Solanum* plant fruits and then to separate and detect them using the Thin Layer Chromatography (TLC) and the chemical reagents.

MATERIALS AND METHODS

Plant materials

The botanical materials that are studied in this research have been collected in September 2010 where the (*S nigrum*) and the (*S. villosum*) fruits were collected from Ebla University's Garden (GPRS-data: 35° 54 ' 34" N and 36° 51' 17" E) while the (*S. elaeagnifolium*) fruits were collected from Al-Raqqa city (GPRS-data: 35° 57 ' 1" N and 39° 9' 40" E) (Figure 3) and all documented by Dr. Amin Salkiny (ICARDA, Aleppo, Syria). The fruits are placed on stainless clean trays and stored at the room temperature for 7 days. The dry fruits are weighed, ground to become a fine powder and kept within dry containers in a fridge.

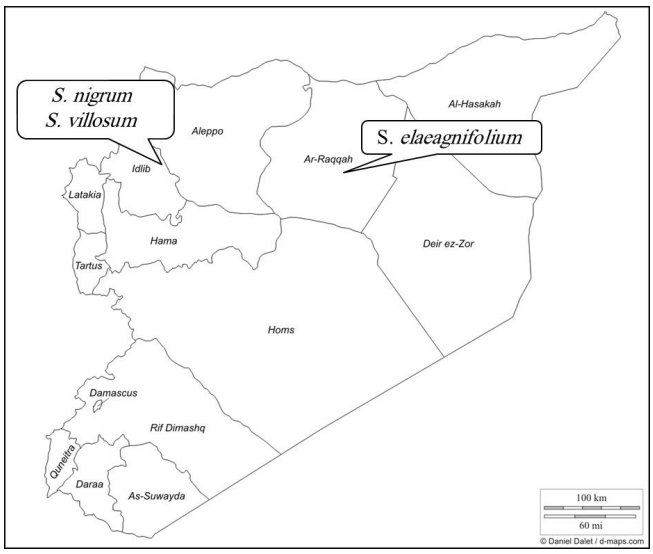

Figure 3: Collection sites of (*Solanum spp.*) fruits Syria

Extraction of glycoalkaloids

The glycoalkaloids were extracted from the *Solanum* species fruits by the ultrasound technique[18] using the butanol as a solvent[19]. A design of such extracting is found in (Figure 4). The obtained butanol's extract is filtered through filtering papers and concentrated using the rotary evaporator (B465, Buchi, Switzerland) to give (4.5, 2.5 and 2.5% w/w) for BEF of (*S. nigrum*), (*S. villosum*) and (*S. elaeagnifolium*), respectively. The BEF of *Solanum* species were stored in -20 ˚C until their use. The concentration used in the experiment based on the extract's dry weight (μg/ml).

Analysis of glycoalkaloids by TLC

The preliminary examination of the previously extracted glycoalkaloids using TLC (Silica Gel 60 GF254, Merck, Darmstadt, Germany). The solvent system used was chloroform: methanol: water (14:6:1), the chromatogram is sprayed in Dragendorff's reagent,[20] phosphomolybdic acid reagent,[21] ninhydrin reagent 2% (then ultra-violet UV 365nm),[22] antimony trichloride reagent 2%[23] and blood hemolysis reagent[24].

Cell culture and glycoalkaloids treatment

LIM-1863 cell line was obtained from Prof. Nizar Mhaidat, Jordan University of Science and Technology. The LIM-1863 cells were cultured in DMEM medium added to which 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin and 1% streptomycin (All from Gibco, Scotland) at 37 ˚C within an incubator contains Co2 5%. The cells which were harvested by

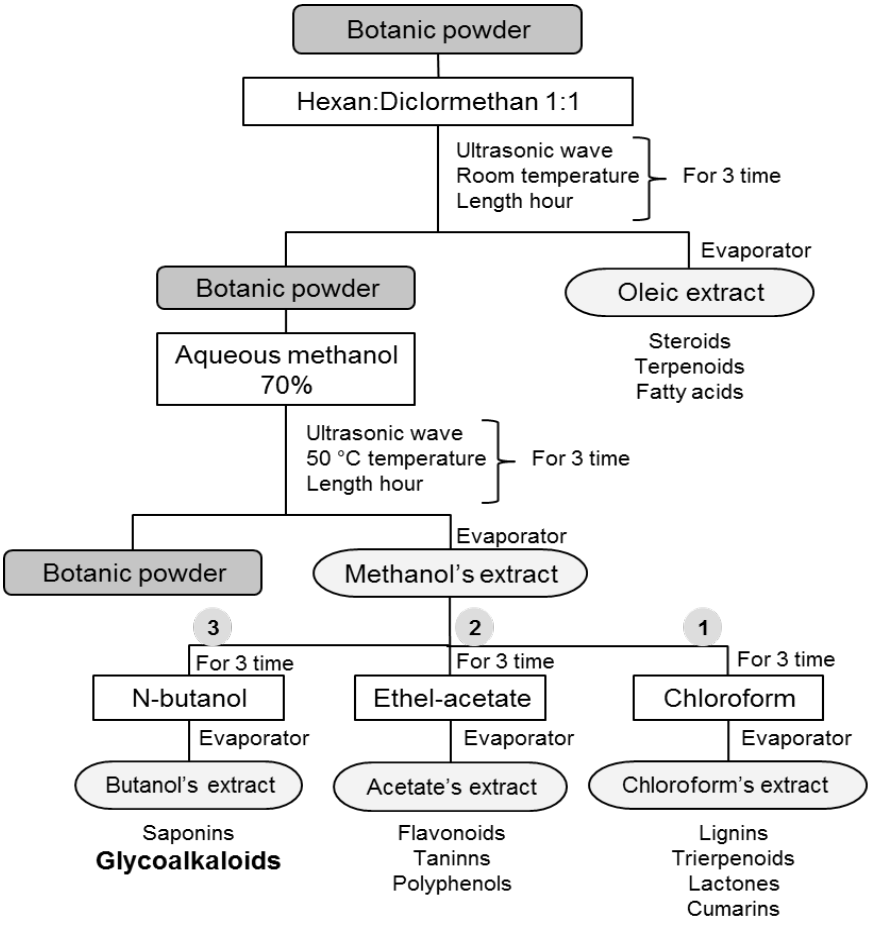

Figure 4: A design of extraction of the glycoalkaloids

trypsin 10% (Sigma, USA) are counted using neubauer slide and trypan blue, and they were then cultured in 96-well plate (1 × 10^4cells/well). The BEF was dissolved in ethanol 50%. The cells were incubated with different concentrations of BEF (5, 20, 80 and 320 µg/ml) for 48 hours. Each concentration was tested on four wells of the 96-well plates which contain 1 × 10^4 LIM-1863 cells. In every experiment, four LIM-cells cultured wells without a sample are used as a negative control and four culture-medium wells without cells are used as a blank[25].

Determination of cell viability

The cell viability is determined using the methyl thiazolyl tetrazolium bromide (MTT) (Sigma, USA) assay[25]. The MTT was dissolved in PBS (phosphate buffer saline) at a concentration of 5 mg/ml. After the cells have been incubated for 48 hours, 20 µl of MTT solution is added to each well containing 150 ml of culture medium. The yellowish MTT solution is changed to insoluble purple formazan crystals within 24 hours of incubation (Figure 5). Presence of active mitochondrial enzyme in the living cells is the reason for this change while such enzyme is not available in the dead cells (Figure 6).

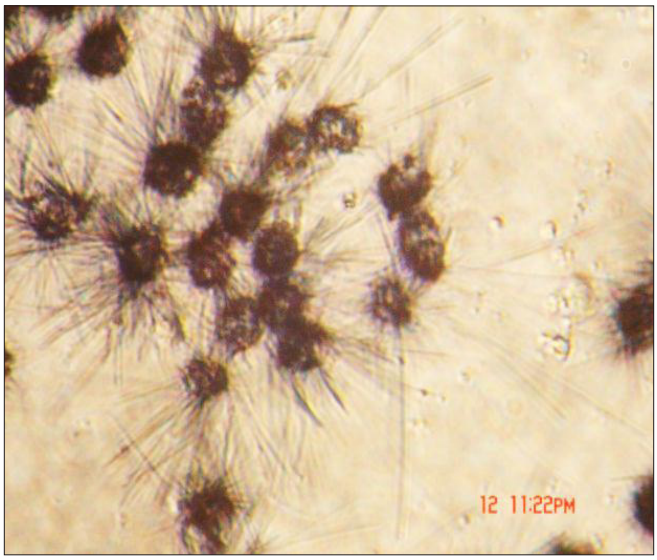

Figure 5: The insoluble purple formazan crystal

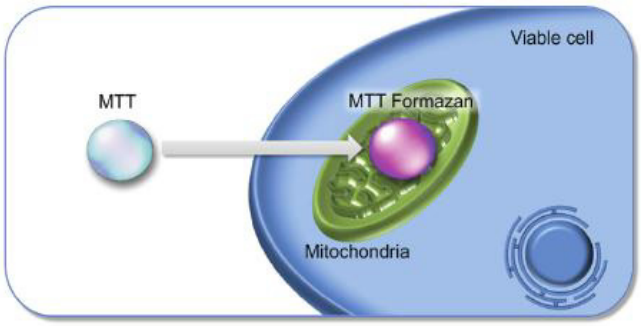

Figure 6: The mechanism of action of the MTT

The purple formazan crystals are dissolved in 150 ml of DMSO (Sigma, USA) (Figure 7). Absorbency of the solution is read using a multi-well spectrophotometer (ELISA reader, Organon Teknika, Netherlands) at a wavelength of 570 nm. The percentage of viability was calculated according to following formulas:

$$\% \text{ cell viability} = (At\text{-}Ab) / (Ac\text{-}Ab) \times 100$$

Where, At= Absorbance value of test compound, Ab= mean absorbance value of blank, Ac= mean absorbance value of negative control.

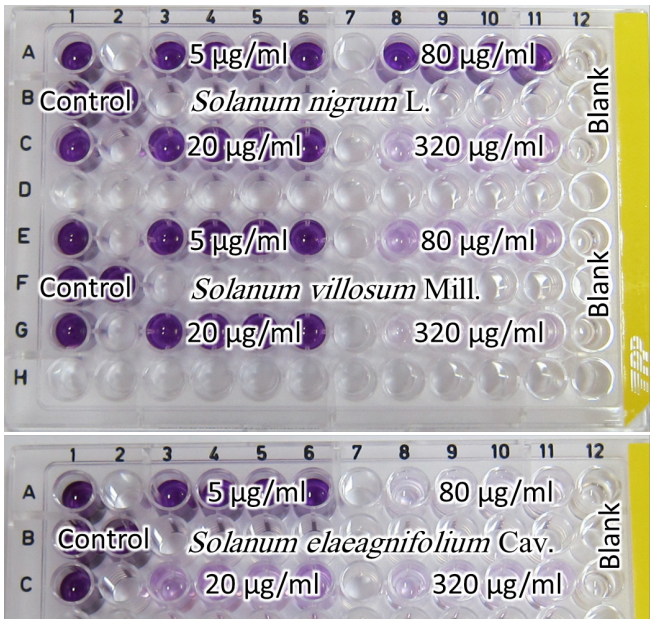

Figure 7: The profile of 96 well for measurement of cell viability, wherefrom the cells were incubated with different concentrations of (BEF) of *Solanum* Species (5, 20, 80 and 320 µg/ml) at 48 h.

Figure 8: The profile of 96 well for measurement of cell proliferation, wherefrom the cells were incubated with the concentrations of glycoalkaloids (0.5, 6, 12,18,25,37 and 50 µg/ml) from (*S. villosum*) and (0.25, 3, 6, 9, 12,18 and 25 µg/ml) from (*S. elaeagnifolium*) at 48 h.

Repeat the above steps, but the concentrations of (BEF) are (0.5, 6, 12,18,25,37 and 50 µg/ml) from (*S. villosum*) and (0.25, 3, 6, 9, 12, 18 and 25 µg/ml) from (*S. elaeagnifolium*) (Figure 8).

Statistical analysis

Results were expressed as mean ± SD by Excel MS Office[26].

RESULTS

Detection of glycoalkaloids by TLC

It has been indicated through the TLC chromatogram the availability of several compounds in the butanol's extracts of the *Solanum* species fruits (Figure 9), some of these compounds are alkaloids with R_f values the following: (0.07, 0.13, 0.17, 0.20, 0.27, 0.39, 0.52, 0.59, 0.63) and among such compounds there are ones with saponin properties called alkaloid steroid saponins having R_f values the following: (0.27, 0.52). It is noted here that the Dragendorff's reagent and the blood reagent are less sensitive to the little-amount concentrations than the rest of the used reagents (Table 1).

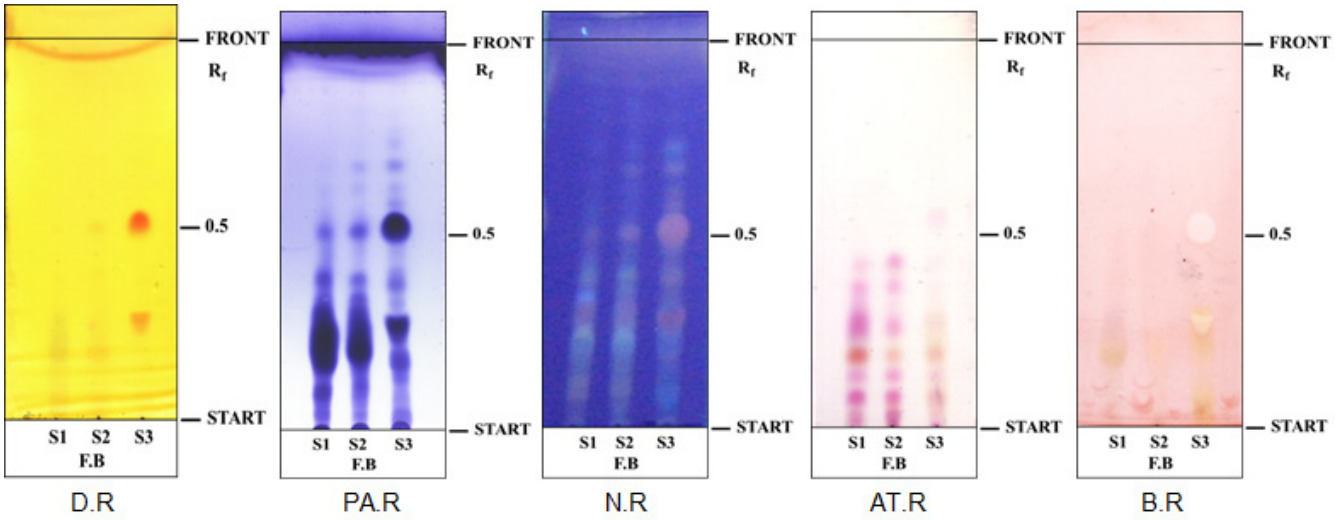

Figure 9: The TLC chromatogram of (BEF) of *Solanum* species. Where S1: *S. nigrum*, S2: *S. villosum*, S3: *S. elaeagnifolium*; F.B: butanol's extract of *Solanum Species* fruits; Merck 60F Silica gel plate; mobile phase: chloroform: methanol: water (14:6:1); developed with PA.R: phosphomolybdic acid reagent, D.R: Dragendorff's reagent, N.R :ninhydrin reagent 2% (then UV 365nm), AT.R: antimony trichloride reagent 2%, B.R: blood reagent.

Table 1: TLC of glycoalkaloids of three of Solanum Species

R_f ±0.01	Fruits			Color				
	S1	S2	S3	PA.R	D.R	N.R	AT.R	B.R
0.07	+	+	+	Blue	-	Blue	purple	-
0.13	+	+	+	Blue	-	Blue	purple	-
0.17	+	+	+	Blue	-	Blue	purple	-
0.20	+	+	+	Blue	-	Blue	brown	-
0.27	+	+	+	Blue	Orange	brown	purple	White
0.39	+	+	+	Blue	-	brown	purple	-
0.52	+	+	+	Blue	Orange	brown	purple	White
0.59	+	+	+	Blue	-	brown	-	-
0.63	+	+	+	Blue	-	brown	-	-
0.69	+	+	+	Blue	-	brown	-	-
0.74	+	+	+	Blue	-	Blue	-	-

-: Absent, +: Present

Glycoalkaloids cytotoxicity on LIM-1863 cells

It appeared by the preliminary experiment in comparison to the control that the Glycoalkaloids have a toxic effect on the LIM-1863 cell line (Figure 10), and the IC_{50} values were like this: (164.7, 35.91 and 12.14 μg/ml) for the butanol's extracts of (*S. nigrum*), (*S. villosum*) and (*S elaeagnifolium*) fruits, respectively (Figure 11). While appeared by the second experiment in comparison to the control that the IC_{50} values were like this: (14.6 and 57.6 μg/ml) for the butanol's extracts of the (*S. villosum*) and (*S. elaeagnifolium*) fruits, respectively (Figure 12), being approximate to the IC_{50} values in the first experiment.

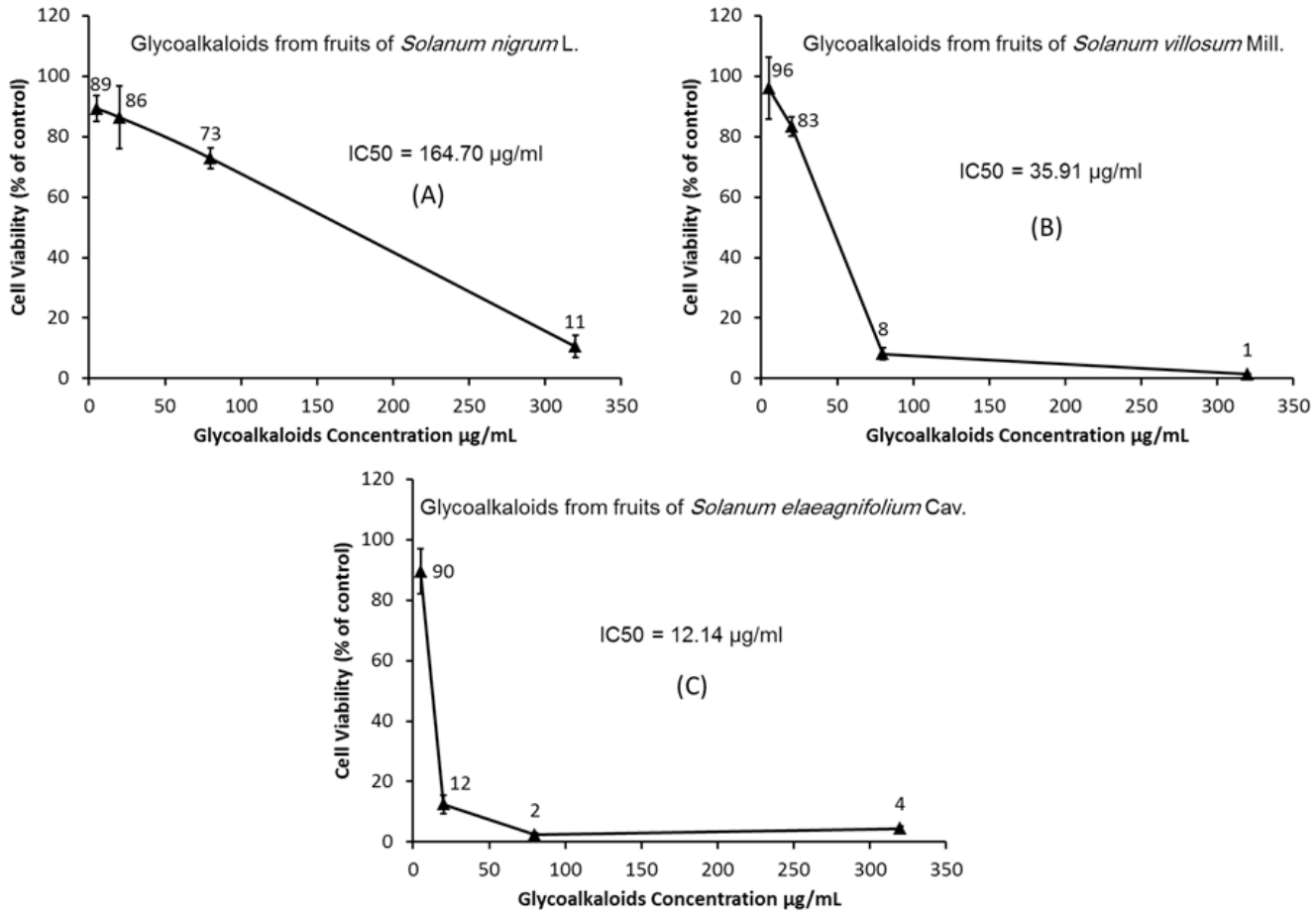

Figure 10: The first test: the viability of LIM-1863 colon carcinoma cells after 48 hours of exposure to different concentrations of each extract, which was assessed using MTT assay. Results are expressed as a percentage of viability compared to control and are presented as mean ± SD of three independent experiments.

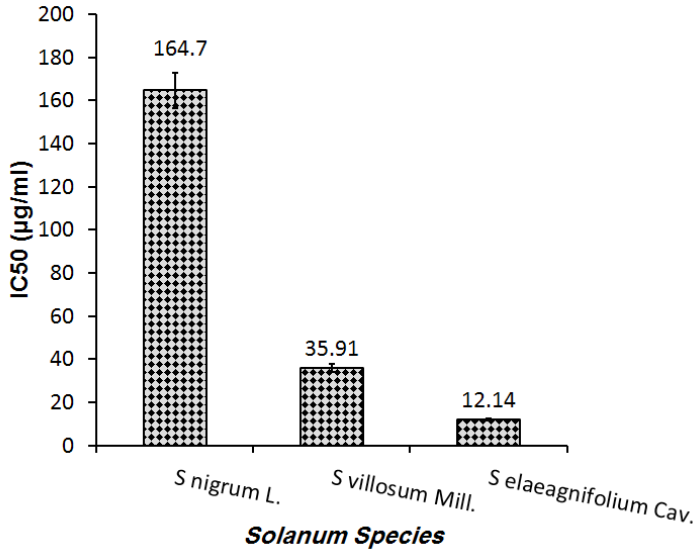

Figure 11: The IC50 values for Glycoalkaloids affecting on LIM-1863 cells during 48 h incubation. That were assessed by MTT assays. Results were expressed as mean ± SD.

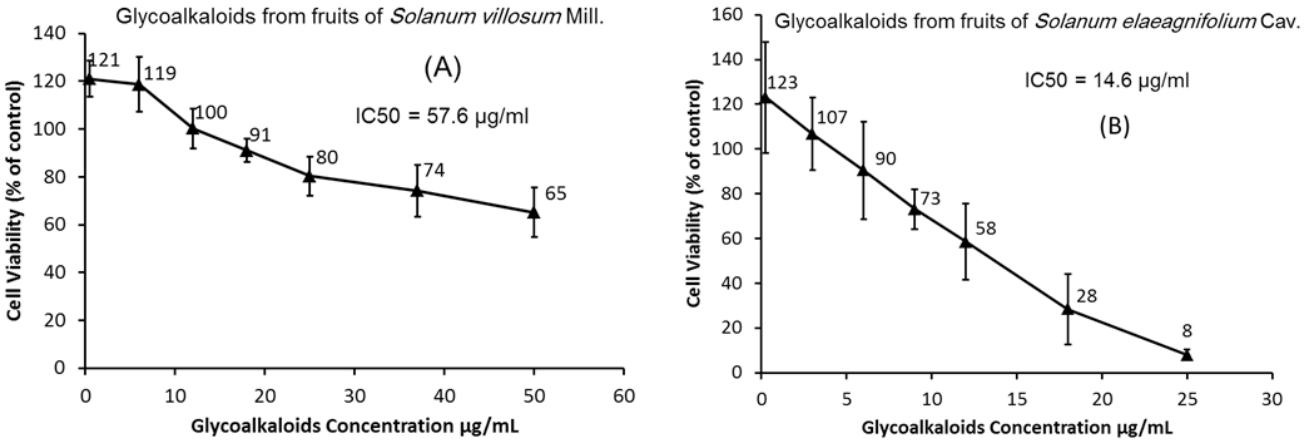

Figure 12: The second test: the viability of LIM-1863 colon carcinoma cells after 48 hours of exposure to different concentrations of each extract, which was assessed using MTT assay. Results are expressed as a percentage of viability compared to control and are presented as mean ± SD of three independent experiments.

DISCUSSION

It has been observed that the natural products are good and important sources to develop new drugs including anticancer drugs. But in the present study, the butanol's extract of (*S. elaeagnifolium*) fruits has the greatest toxic effect on cell viability and then follows the butanol's extract of (*S. villosum*) fruits. Then, the one with the least effect is the butanol's extract of (*S. nigrum*) fruits.

In comparison with the reference studies exhibiting the *Solanum* species is a rich source of glycoalkaloids and plants of this species are in the first place contain glycoalkaloids such as solamargine, solasonine and solanine, and it appeared that solanine shows an anticancer effectiveness at (14.47 µg/ml) IC_{50} value, solamargine shows an anticancer effectiveness at about (~ 4.6 µg/ml) IC_{50} value and solasonine shows an anticancer effectiveness at an average value of (2.3 µg/ml) IC_{50}, our results have been approximate to the glycoalkaloids' role in toxicity generating and because both butanol's extracts of (*S. villosum*) and (*S. elaeagnifolium*) fruits have reduced the LIM-1863 cell viability at (4,12 µg/ml) IC_{50} values respectively, which means they both contain a larger percentage of glycoalkaloids, while IC_{50} value of (*S. nigrum*) fruits' butanol's extract (164.7 µg/ml) is high indicating they contain a less concentration of the glycoalkaloids (Table 2). This corresponds with the reference study

Table 2: Comparison of IC_{50} values between results of reference studies and results of this study

Material and Species	Dry part used	Solvent used in extraction	Cell line	Origin	IC_{50} µg/ml	Ref.
S. nigrum	fruits	butanol	LIM-1863	colon	164.7	This study
S. villosum	fruits	butanol	LIM-1863	colon	35.91	This study
S. elaeagnifolium	fruits	butanol	LIM-1863	colon	12.14	This study
S. nigrum	fruits	methanol	HELA	cervical	265	[27]
S. nigrum	herb	ethanol 70%	HELA	cervical	227	[28]
solanine	-	-	HepG2	liver	14.47	[29]
solamargine	-	-	H441	lung	3.5	[30]
solamargine	-	-	H520	lung	7.7	[30]
solamargine	-	-	H661	lung	8.3	[30]
solamargine	-	-	H69	lung	6.7	[30]
solamargine	-	-	HBL-100	breast	2.4	[31]
solamargine	-	-	SK-BR-3	breast	3.5	[31]
solamargine	-	-	ZR-75-1	breast	2.5	[31]
solamargine	-	-	HCT116	colon	4.6	[32]
solasonine	-	-	HCT116	colon	2.3	[32]

which shows the IC_{50} value of (*S. nigrum*) extract on average equals (246 μg/ml).

CONCLUSION

The present study shows that glycoalkaloids from *Solanum Species* clearly have the capacity to cancer cell death and these natural products represent interesting lead compounds for the development of potential cancer therapeutics. This is the first report which was tested effect (BEF) of *Solanum Species* on the viability of LIM-1863 human colon carcinoma cell line. Our data suggest that the presence of glycoalkaloids in *Solanum Species* is associated with cancer cell death.

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Study on the antioxidant activities of root extracts of *Zizyphus lotus* from the western region of Algeria

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ABSTRACT

Objective: To find a new natural source of antioxidant, the phenolic contents and antioxidant activities of some secondary metabolites extracted from roots of *Ziziphus lotus* were evaluated. **Methods:** The total polyphenol contents were determined spectrophotometrically. Pyrrocatechol, catechin and cyanidin equivalents were used for these parameters. The antioxidant activities of the extracts were determined by several in vitro systems of assays, namely DPPH radical scavenging activity method, β-Carotene Bleaching assay (BCB), Ferric Reducing Antioxidant Power assay (FRAP) and Total Antioxidant capacity test (TAC). **Results:** The quantitative estimation showed that the roots of *Z. lotus* were rich in polyphenols (20.09 mg PE/g DW) and proanthocyannidins (1.56%) and they contained a small amount of flavonoids (0.02 mg CE/g DW). The most fractions exhibited high antioxidant activities, and some even showed higher potency than the standard synthetic antioxidants in some instances. In DPPH assay, all extracts had shown significant inhibition (58.535 – 94.730% at 1 mg/mL). In addition, the IC_{50} values ranged from 0.211 to 0.816 mg/mL, compared to 0.110, 0.214, 0.214 and 0.413 mg/mL for gallic acid, tannic acid, butylated-hydroxyanisol and ascorbic acid respectively. In (BCB) assay, the extracts showed strong inhibition (55.55 – 100.00% at 1 mg/mL) and the IC_{50} values ranging from 0.123 to 0.850 mg/mL compared to 0.433 mg/mL for gallic acid. A dose dependant curve was obtained for all extracts in the FRAP assay. However, the antioxidant potencies of ascorbic acid and extracts were comparable at low concentrations. The majority of extracts showed the highest value of antioxidant activity, based on TAC test (0.073 – 0.398 mg ascorbic acid / mg extract). **Conclusion:** The results indicate that *Z. lotus* roots could be an important sources of natural antioxidants.

Keywords: *Ziziphus lotus*, Polyphenol contents, DPPH radical scavenging activity assay, β-Carotene Bleaching assay, Ferric Reducing Antioxidant Power assay, Total Antioxidant Capacity test.

INTRODUCTION

The medicinal plants are largely used either for the prevention, or for the curative treatment of several diseases. Among the properties behind these virtues, the antioxidant activity holds a place of first order[1–2]. Many of the medicinal plants contain a broad spectrum of phytochemical substances which are sources of natural antioxidants such as α-tochopherol, phenolic acids, flavonoids and tannins. In addition to their antioxidant activities, these compounds have other biological properties like anti – inflammatory, antimicrobial and anti – cancer activities[3]. The use of these natural substances is not limited to the therapeutic field but also with the industrial field because they are more preferred instead of using of synthetic antioxidants such as Butylated – hydroxytoluene (BHT), Butylated - hydroxyanisol (BHA) and propyl gallate (PG)[4]. Today, the identification of new sources of natural substances is one of the most active fields of research in the world. *Ziziphus* (family *Rhamnaceae*) is widespread in tropical and sub-tropical regions: Asia, Africa, North America, South America, Oceania

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and Europe with the center of diversity in Asia[5]. The North African species of the genus *Ziziphus* are known with the vernacular names 'sedra', 'addhal', 'roubaidh', 'dhou achaouk', 'sder alberri' and 'çder nabga' and are used in the traditional therapeutic[6,7]. *Ziziphus lotus* (L.) Desf. is known in Algeria as 'sedra', the fruits and the leaves are used as emollient[8] and in the treatment of diarrhea and intestinal diseases[7]. Cyclopeptides alkaloids[9–11], dammarane saponins[12,13], and a new flavonol glycoside[14] have been isolated from this plant. In addition, the anti – inflammatory, the analgesic, anti – ulcerogenic and the anti – spasmodic activities of this plant were demonstrated in rodents[15–18]. In the view of the traditional use and the chemical composition of *Z. lotus*, the present study was carried out to examine the possible antioxidant activity *in – vitro* of some secondary metabolites**:** polyphenols, flavonoids and tannins of *Z. lotus* roots. The antioxidative properties were evaluated by the 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), the β-Carotene Bleaching method (BCB), the Ferric Reducing Antioxidant Power assay (FRAP) and the determination of Total Antioxidant Capacity test (TAC). The results were compared with antioxidant standards. This work lies within the scope of continuation of the published works describing some of biologically active extracts from Algerian *Z. lotus*[19,20]. To the best of our knowledge, the antioxidant properties of the extracts from *Z. lotus* using the β-Carotene Bleaching method (BCB) and Total Antioxidant Capacity test (TAC) have not been studied.

MATERIALS AND METHODS

Plant material

The roots of *Z. lotus* were collected from Zarifet, a region near Tlemcen city in the West Northern of Algeria (1100 m, 34°50'49"N, 01°21'45"W), in October 2011.

Botanical identification of plant was conducted by Prof. Noury BENABADJI, Laboratory of Ecology and Ecosystem Management, Department of Biology, University of Tlemcen, Algeria. A voucher specimen has been deposited in the same Department.

Before extraction, the fresh roots were extended by ground, in one layer, in an open room protected from the sun. During drying time, plants were turned over to allow homogeneous drying. After drying, the roots were cut to obtain the fine smithereens, which were used for extractions.

Preparation of plant extracts

Polyphenols extraction

The dried plant materials (10 g) were ground and defatted with n-Hexane (Soxhlet extraction). The defatted powdered plant materials (roots) were extracted with a mixture of acetone – water (100 mL, 70/30, v/v) by maceration at room temperature for 24 hours[21]. Then the extract was filtered through whatman filter paper under vacuum and concentrated to dryness under reduced pressure at 45°C using an evaporator. The extract (total polyphenol extracts of roots : PolyR) was stored at 4°C and afterward, used for further investigation (antioxidant activity).

Flavonoids extraction

The dried plant materials (10 g of roots) were extracted with 400 mL of mixture MeOH – Water (70/30, v/v) by maceration at room temperature for 24 hours[22]. After filtration through whatman filter paper, the MeOH of resultant hydroalcoholic extract was evaporated at 40°C under reduced pressure and affording the aqueous extract. Subsequently, the aqueous extract was then fractionated by solvent – solvent extraction, first with ethyl acetate and then with n-butanol, using a separating funnel (Pyrex). Two fractions of flavonoids, namely ethyl acetate rich fraction (EAR) and n-butanol rich fraction (ButR), were obtained. All the fractions were concentrated and dried to a constant weight in a vacuum oven at 45°C. These fractions were then subjected to the antioxidant activity evaluation. The fractions were kept at 4°C when not in use.

Tannins extraction

Powdered materials (10 g of roots) were extracted in refrigerator at 4 °C using 200 mL of a mixture of acetone – water (35/15, v/v) for 3 days [23]. The extract was filtered under vacuum through filter paper and acetone was evaporated at 40°C under reduced pressure. Subsequently, the dichloromethane (2 x 60 mL) was used for the extraction of lipids and pigments from the aqueous extract using a separating funnel. Afterward, the aqueous phase was extracted with ethyl acetate, this process was repeated 4 times. After filtration, the organic phase (ethyl acetate) containing tannins was recovered and concentrated to dryness under vacuum at 40 °C using a rotary evaporator. The residue obtained (TR) after rotary evaporation was kept in glass vials and stored in a refrigerator. Portions were taken from the refrigerator to be used for each of the experiments below.

Total phenolic content determination

The quantitative determination of total phenolic content using Folin-Ciocalteu (F-C) reagent involves oxidation in alkaline solution of phenols by the yellow molybdotungstophosphoric heteropolyanion reagent and colorimetric measurement of the resultant molybdotungstophosphate blue according to the method of Singleton and Rossi and modified by Dogÿan et al[24,25]. The polyphenol fraction (extract corresponding to 1 g of dry plant material) was dissolved in 5 mL of double distilled water. An aliquot of 100 µL of this solution was diluted with double distilled water to 3 mL. Afterward, the obtained solution was added to 300 µL of double distilled water and 500 µL of the F-C reagent. After shaking, the mixture was incubated for 3 min at room temperature. Then 2000 µL of 20 % Na_2CO_3 solution was added. The volume obtained was mixed vigorously, and held for 60 min in the dark at ambiant temperature. The absorbance of the solution was then measured at 650 nm against a blank in a spectrophotometer (UV/VIS Spectrophotometer OPTIZEN POP). The sample was analysed in triplicate and the average content was noted for each measure. The total phenolic content, expressed as mg of pyrrocatechol (PE) equivalents per g of dry weight (DW) of plant material (mg PE/g DW), was calculated through the calibration curve obtained using the equation given bellow :

Absorbance = 0.0828 x C, R^2 = 0.999
where C was the concentration (mg/L).

Total flavonoid content determination

The total flavonoid was measured by the colorimetric assay developped by Zhishen et al.[26] 1 mL aliquot of appropriately diluted sample (polyphenol extract corresponding to 1 g of dry plant material which was dissolved in 5 mL of methanol) or standard methanolic solution of catechin (at different concentrations) was added to 10 mL volumetric flask containing 4 mL double distilled H_2O. At zero time, 0.3 mL of $NaNO_2$ (5 %) was added to the flask. After 5 min, 0.3 mL of $AlCl_3$ (10 %) was added. At 6 min, 2 mL of NaOH (1M) was added to the mixture. Immediately, the reaction flask was diluted to volume (10 mL) with the addition of double distilled H_2O and thoroughly mixed. Absorbance of the mixture – pink color – was determined at 510 nm compared to control water. The sample was analysed in triplicate and the average content was noted for each measure. The total flavonoid extracts were expressed as mg of catechin (CE) equivalents per g of dry weight of plant material (mg CE / g DW). For catechin, the curve absorbance versus concentration C (mg/L) was described by the equation :

Absorbance = 1.340 x C + 0.117, R^2 = 0.9950

Total condensed tannin content determination

The proanthocyannidins were determined by spectrophotometer method[27,28]. This method relies on the reaction with vanillin under acidic conditions. A 2 mL aliquot of a freshly prepared solution of vanillin in methanol (1 %) in 70 % sulfuric acid was added to 1 mL of suitably diluted sample (polyphenol extract corresponding to 1 g of dry plant material). The mixture was incubated in 20°C – water bath and after exactly 15 min, the absorbance at 500 nm was measured against a proper blank in a spectrophotometer. The assay was performed in triplicate and the average content was noted for each measure. The concentration of proanthocyannidins (%) was expressed as cyanidin (CA) equivalents by the following formula:

Proanthocyannidins (%) = ($5.2 \times 10^{-2} \times A_{500} \times V \times DF$)/P

Where A_{500} was the absorbance at 500 nm ; DF = the dilution factor ; V = the volume of extract (mL) ; P = the dry weight of plant material (g).

Antioxidant activity evaluation

DPPH (2,2-Diphenyl-1-Picrylhydrazyl) radical scavenging assay

The hydrogen atoms or electron donating ability of the corresponding extracts was determined from the bleaching of purple colored ethanol solution of DPPH. This spectrophotometric assay uses the stable radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) as a reagent[29,30]. Radical scavenging activity of extracts was measured by slightly modified method of Mighri et al. and Braca et al. as described below[31,32]. Different concentrations of each extract were prepared in ethanol: 0.1 – 1 mg/mL. A solution of DPPH in ethanol (25 µg/mL) was prepared and 2 mL of this solution was added to 50 µL of extract solution in ethanol at different concentrations (0.1 – 1 mg/mL). The solution of DPPH was prepared daily before measurements. The sample solutions were shaken vigorously and left standing at room temperature for 60 min in the dark. Then the absorbance was measured at 517 nm against ethanol. The blank sample was used as 2 mL of DPPH solution (25 µg/mL in ethanol) with 50 µL of ethanol. Decreasing of the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity (% of inhibition). This activity is given as percent

DPPH radical scavenging, which is calculated with the following equation:

% DPPH radical scavenging = $[(A_0 – A_t)/A_0]$ x 100

A_0: Absorbance of blank;

A_t: Absorbance of tested sample solution at the time t.

The experiment was performed in triplicate and the average absorbance was noted for each measure. The same procedure was followed for the positive control: ascorbic acid (AA), gallic acid (GA), tannic acid (TA) and BHA. Ethanol was used for baseline correction. The free radical scavenging activity is usually expressed as percentage of DPPH inhibition but also by the antioxidant concentration required for 50% DPPH reduction (IC_{50}). Basically, a higher DPPH radical scavenging activity is associated with a lower IC_{50} value.

IC_{50} value was determined from plotted graph of scavenging activity against the different concentrations of *Z. lotus* roots extracts, AA, GA, TA and BHA. The scavenging activity was expressed by the percentage of DPPH reduction after 60 min of reaction.

β-Carotene Bleaching assay (BCB)

The β-carotene bleaching method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants[33]. A modified method described by Koleva et al. was used[34]. β-Carotene (2 mg) was dissolved in 20 mL of chloroform and to 4 mL of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40°C and 100 mL of oxygenated ultra-pure water was added, then the emulsion was vigorously shaken. Reference compounds and sample extracts were prepared in methanol. The emulsion (3 mL) was added to a tube containing 0.2 mL of extracts. The absorbance was immediately measured at 470 nm and the test emulsion was incubated in a water bath at 50 °C for 120 min, when the absorbance was measured again. GA and BHA were used as positive control. In the negative control, the extract was substituted with an equal volume of methanol. The antioxidant activity (%) of the extracts was evaluated in terms of the bleaching of the β-carotene using the following formula:

% Inhibition = $[(A_t – C_t)/ (C_0-C_t)]$ x 100

where A_t and C_t are the absorbance values measured for the test sample and control, respectively, after incubation for 120 min, and C_0 is the absorbance values for the control measured at zero time during the incubation. The results are expressed as IC_{50} values (mg/mL), the concentration required to 50% β-carotene bleaching inhibition. Tests were carried out in triplicate.

Ferric Reducing Antioxidant Power assay (FRAP)

The reducing power assay was conducted as previously described by Wang et al. and Oyaizu with AA and BHA being used as the positive controls [35,36]. In brief, 2.5 mL of individual deionized water diluted *Z. lotus* extract (ranged from 0.1 to 1 mg/mL) was sequentially mixed with equal volume of phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1% w/v). After incubation at 50°C for 20 min, 2.5 mL of trichloroacetic acid (10 % w/v) was then added to the mixture followed by centrifuging at 3000 rpm for 10 min. Consequently, 5 mL of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride (0.1 % w/v). After 30 min of incubation at room temperature in the dark, absorbance of the resulting solution was measured at 700 nm. The ferric reducing power capacities of the plant extracts and standard antioxidants were expressed graphically by plotting absorbance against concentration. Samples for the assay were prepared in triplicate.

Determination of Total Antioxidant Capacity (TAC)

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH and determined by the method described by Prieto et al.[37] and Dasgupta and De[38]. Aqueous extract (0.3 mL) was added to 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm against a blank. The antioxidant activity was expressed as the number of equivalents of ascorbic acid.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was carried out using STATISTICA, version 4.1 (Statsoft, Paris, France). Multiple comparisons were performed using ANOVA followed by the LSD (least significant difference) test. $P<0.05$ was considered to represent significant statistical differences.

RESULTS AND DISCUSSION

The percentage yield of extraction of *Z. lotus* roots

Extraction results for roots of *Z. lotus* are shown in Table 1. According to the results obtained, the yields (*w/w*) of total polyphenol extract (13.9 %) was more important than of tannin extracts (5.5 %) and flavonoid extracts (ethyl acetate flavonoids : 1.8 % / butanol flavonoids : 1.1 %). This result can be explained by the fact that polyphenol extracts contain flavonoids, tannins and others chemical compounds like: phenolic acids, coumarins and constituents which have solubility in extraction solvents used. Finally, the presence of phenolic compounds (flavonoids, tannins, others phenolic compounds) in the roots of *Z. lotus*, indicates that this plant may have the ability as an antioxidant agent.

Table 1: Yields (%) of the extracts of *Ziziphus lotus* roots with respect to the dry matter *(w/w)*

Extract		Yield (%)
Total polyphenols (PolyR)		13.9
Flavonoids	Ethyl acetate fractions (EAR)	1.8
	Butanol fractions (ButR)	1.1
Tannins (TR)		5.5

Total phenolic, flavonoid and condensed tannin in the roots of *Z. lotus*

Phenolics or polyphenols have received considerable attention because of their physiological function, including antioxidant, antimutagenic and antitumour activities[39,40]. Phenolic compounds are widely distributed in plants[41], which have gained much attention, due to their antioxidant activities and free radical scavenging abilities, which potentially have beneficial implications for human health[41,42]. For that, total phenolic, flavonoid and proanthocyannidin contents of acetone – water (70/30, v/v) extract from roots of *Z. lotus* (total polyphenol extract) were assessed. Principal results showed (Table 2) that *Z. lotus* extract exhibited a significant amount of polyphenol content (20.09 mg PE/g DW), followed by proanthocyannidins (1.56 % :15.6 mg CA/g DW) and flavonoids (0.02 mg CE/g DW).

Although phenolic compounds are found in virtually all plants and plant parts, their quantitative distribution varies between and within plant species, and between different organs in a plant[43]. Plants with high levels of phenolic compounds have been shown to exhibit high antioxidant capacity[44]. Most of the antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers[45]. Flavonoids have been reported to be responsible for antioxidant activity[46]. The antioxidant, anti – inflammatory, antifungal and healing properties of some plant extracts have been attributed to the presence of tannins[47].

Table 2: Total phenolic, flavonoid and proanthocyannidin contents in the roots of *Ziziphus lotus*

Content	20.09	0.02	1.56

Antioxidant activity evaluation

Several methods have been used to determine antioxidant activity of *Z. lotus*. Our present study therefore involve four various established methods to evaluate antioxidative activity of root extracts, namely, DPPH radical scavenging activity, β-carotene bleaching assay (BCB), ferric reducing antioxidant power assay (FRAP) and total antioxidant capacity test (TAC). The results obtained from each analysis are considered below (Tables 3, 4, 5 and Figure 1). The activities were compared to the synthetic antioxidant: AA, GA, TA and BHA which were used as antioxidant references.

DPPH (Diphenyl-1-picrylhydrazyl) free radical scavenging assay

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples[48]. It is a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of extracts. As antioxidants donate protons to this radical, the absorption decreases. To evaluate the scavenging effects of DPPH of extracts of roots of *Z. lotus*, DPPH inhibition was investigated. These results are shown as relative activities against AA, GA, TA and BHA (Table 3). The best percentages of antioxidant activities were observed at 1 mg/mL, ButR extract (94.730 ± 0.056 %) and the synthetic antioxidants TA (94.130 ± 0.141 %), GA (93.760 ± 0.077 %) and AA (93.160 ± 0.552 %) indicating an excellent antioxidant activities on radical, followed by BHA (79.350 ± 0.120 %) and TR extract (77.575 ± 0.077 %). EAR (66.650 ± 0.296 %) and PolyR (58.535 ± 0.120 %) extracts had lower antioxidant activities and had possessed the lowest DPPH scavenging activities.

Antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutraliz-

Table 3: Percentage of DPPH radical scavenging as a function of solution concentration and IC_{50} for extracts extracted from the roots of *Ziziphus lotus*

Extract	C (mg/mL)					IC_{50} (mg/mL)
	0.10	0.25	0.50	0.75	1.0	
PolyR	9.975±0.190	24.005± 0.190	35.385 ± 0.106	46.880 ± 0.014	58.535±0.120	0.816± 0.007
EAR	14.185±0.233	30.170±0.282	42.075±0.077	53.900±0.000	66.650±0.296	0.611±0.072
ButR	23.155±0.049	56.490±0.282	79.640±0.028	87.006±0.004	94.730±0.056	0.211±0.017
TR	22.955±0.799	51.705±0.275	56.555±0.091	66.675±0.021	77.575±0.077	0.225±0.002
AA	8.550±0.106	21.630±0.021	91.120±0.021	92.180±0.113	93.160±0.552	0.413±0.041
GA	39.490±0.268	90.740±0.127	93.160±0.176	93.580±0.106	93.760±0.077	0.110±0.014
TA	22.150±0.233	67.623±0.141	93.980±0.403	94.050±0.127	94.130±0.141	0.214±0.019
BHA	23.260±0.289	56.237±0.273	78.600±0.106	78.73±0.091	79.350±0.120	0.214±0.004

Results are expressed in mean ± SD (n=3)

Table 4 : β-carotene bleaching activities (% of inhibition) as a function of solution concentration and IC_{50} for extracts extracted from the roots of *Ziziphus lotus*

Extract	C (mg/mL)					IC_{50} (mg/mL)
	0.10	0.25	0.50	0.75	1.0	
PolyR	32.07±0.155	90.818±0.200	99,478±0.751	100,000±0.205	100.000±0.113	0.175±0.002
EAR	4.385±0.091	8.046±0.005	37,065±0.120	46,335±0.162	55,550±0.113	0.850±0.002
ButR	24.920±0.183	33.532±0.045	49,470±0.056	54,765±0.091	60,210±0.296	0.501±0.001
TR	31.492±0.096	59.498±0.016	65,900±0.046	69,625±0.063	73,374±0.274	0.123±0.002
GA	3.534±0.262	26.500±0.084	58.330±0.042	61.223±0.082	64.470±0.082	0.433±0.001
BHA	58.060±0.268	70.080±0.028	77.205±0.134	78.825±0.190	80.745±0.106	0.044±0.002

Results are expressed in mean ± SD (n=3)

Table 5 : Total antioxidant capacities of different extracts of *Zizyphus lotus*

Extract	Equivalent to ascorbic acid (mg/mg extract)
PolyR	0.278 ± 0,007
EAR	0.073 ± 0,019
ButR	0.135 ± 0,033
TR	0.398 ± 0,105

Results are expressed in mean ± SD

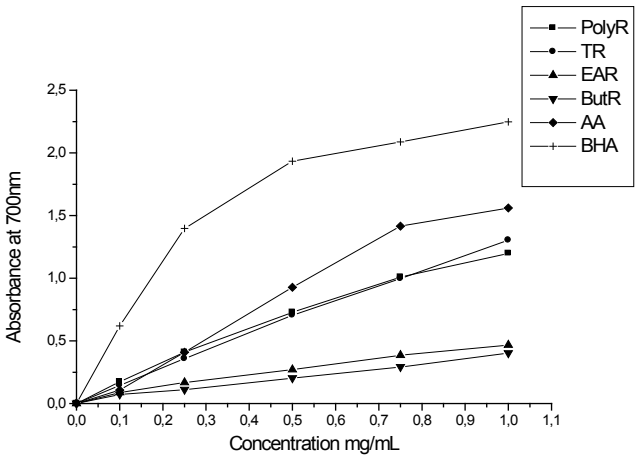

Figure 1: Ferric ion reducing power effects of *Ziziphus lotus* root polyphenol, flavonoid and tannin extracts, showing a dose dependant increase in absorbance. AA: ascorbic acid; BHA : Butylated - hydroxyanisol; PolyR : the polyphenol extract; EAR : ethyl acetate fraction of flavonoid extract; ButR : butanol fraction of flavonoid extract; TR : tannins extract.

ing its free radical character[49]. The colour changes from purple to yellow and its absorbance at wavelength 517 nm decreases. For these extracts: polyphenol, flavonoid and tannin fractions, the antioxidant capacity was dependent on the concentrations tested. As shown in Table 3, the antioxidant activity increased with the increase of their concentrations from 0.1 to 1 mg/mL after 60 min of incubation time at room temperature. We can conclude that the antioxidant activity depends to the concentration of sample, chemical composition of the extracts, the position of the CH_3 groups and the position of the OH groups on the benzene ring.

IC_{50} values (concentration of sample required to scavenge 50% free radical) were found to be the least in GA (0.110±0.014 mg/mL), followed by ButR (0.211±0.017 mg/mL), BHA (0.214±0.004 mg/mL), TA (0.214±0.019 mg/mL) and TR (0.225±0.002 mg/mL). EAR (0.611±0.072 mg/mL) and PolyR (0.816± 0.007 mg/mL) also showed good activity compared to AA (0.413±0.041 mg/mL). Tables 1, 2 showed that the roots of *Z. lotus* were markedly rich in phenolic compounds (polyphenols, tannins and other phenolic compounds) which may act as radical scavenging agents. Furthermore, some researchers showed that plant phenolic compounds have been found to possess potent antioxidant effects[50]. In addition, the flavonoids from plant extracts have been found to possess antioxidative properties in various studies[51]. The significant antioxidant activity of *Z. lotus* may be due to strong occurrence of polyphenol compounds (flavonoids, tannins, phenols…). Antioxidant activities of extracts from plants are mainly attributed to the compounds present in them. This can be due to the high percentage of main constituents or families of compounds, but also to the presence of others constituents or families of compounds in small quantities or to synergy among them.

According to the results of the study of Rached et al., the root methanolic extract of *Z. lotus*, exhibits moderate activity (9.14 ± 0.72 µg/mL) when compared to BHA used as a positive control (4.15 ± 0.25µg/mL)[52]. This result indicated that root extracts of *Z. lotus* have significantly strong antioxidant power.

β-Carotene Bleaching assay (BCB)

To the best of our knowledge, there are no available reports on the evaluation of antioxidant activity of *Z. lotus* using the β-Carotene bleaching assay (BCB). In the BCB assay, linoleic acid produces hydroperoxides as free radicals during incubation at 50°C. Linoleic acid hydroperoxides attack the β-carotene molecule and, as a result, it undergoes rapid decolorization. The corresponding decrease in absorbance can be monitored spectrophotometrically. The presence of antioxidants in the extract will minimize the oxidation of β-carotene by hydroperoxides. Hydroperoxides formed in this system will be neutralized by the antioxidants from the extracts. In this study, we evaluated the antioxidant activity of *Z. lotus* root extracts by the β-carotene linoleate bleaching method because β-carotene shows strong biological activity and is a physiologically important compound[53,54]. Thus, the degradation rate of β-carotene linoleate depends on the antioxidant activity of the extracts. There was a correlation between degradation rate and the bleaching of β-carotene; the extract with the lowest β-carotene degradation rate exhibited the highest antioxidant activity. The results obtained at different concentrations: 0.1 – 1 mg/mL are given in Table 4. These results are shown as relative activities against GA and BHA. The results showed that the inhibitory activity of the most samples was higher than 50% at 1 mg/mL, after 120 min of incubation time at 50°C. It is possible to conclude that the extracts of roots of *Z. lotus* were able to minimize the oxidation of β-carotene by hydroperoxides. The hydroperoxides formed in this system will be neutralized by the antioxidants from the extracts that is the principle of this method for the determination of antioxidant activity.

The best percentages of antioxidant activities at 1 mg/mL, were observed for the PolyR extract (100.000 ± 0.113%), the synthetic antioxidant BHA (80.745 ± 0.106 %) and TR extract (73,374 ± 0.274 %) (Table 4) indicat-

ing an excellent antioxidant activities. GA (64.470 ± 0.082 %) and ButR extract (60,210 ± 0.296 %) possessed good activity. EAR extract (55,550 ± 0.113 %) had lower antioxidant activity and had possessed the lowest percentage of inhibition.

The β-Carotene bleaching activity is usually expressed as percentage of inhibition but also by the antioxidant concentration required to 50% β-carotene bleaching inhibition. Basically, a higher percentage of inhibition is associated with a lower IC_{50} value.

The results show that BHA (0.044±0.002 mg/mL) was the most potent of all the samples, followed by TR (0.123±0.002 mg/mL). PolyR (0.175±0.002 mg/mL) showed similar activity to TR. The latters showed better activities than GA (0.433±0.001 mg/mL). ButR (0.501±0.001 mg/mL) also showed good activity compared to GA (0.433±0.001 mg/mL), while EAR (0.850±0.002 mg/mL) exhibits weak activity.

Ferric Reducing Antioxidant Power assay (FRAP)

The ferric reducing antioxidant power (FRAP) assay measures the reducing ability of antioxidants against the oxidative effects of reactive oxygen species. Electron donating antioxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. This assay is based on the ability of antioxidants to reduce the ferric form (Fe^{3+}) to the ferrous form (Fe^{2+}). Prussian blue colored complex is formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. Therefore, reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm[55,56]. In this assay, yellow color of the test solution changes to green or blue color depending on the reducing power of antioxidant samples. A higher absorbance indicates a higher ferric reducing power.

Increasing absorbance indicates an increase in reductive ability. Figure 1 presents the dose dependent ferric reducing powers of the sample extracts of *Z lotus*, AA and BHA. The reducing power of all the sample extracts, AA and BHA increased with increasing concentration. The reducing power of BHA was significantly more pronounced relative to the plant extracts and AA. However, the antioxidant potencies of AA and sample extracts were comparable at low concentrations. TR and PolyR possess the most important activities, while ButR had the lowest one. Generally, the reducing properties are associated with the presence in the roots of *Z. lotus*, compounds which exert their action by breaking the free radical chain by donating a hydrogen atom[57,58].

This activity may be due to strong occurrence of polyphenolic compounds such as flavonoids, tannins, phenols…..Due the high content of polyphenols and tannins of roots extracts, these phenolic compounds represent the primary source of this antioxidant activity.

Determination of Total Antioxidant Capacity

In the present study, the antioxidative properties of *Z. lotus* using the determination of total antioxidant capacity test were evaluated for the first time. Total antioxidant capacity of roots of *Z. lotus* is expressed as number of equivalents of ascorbic acid (Table 5). The assay is based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid [37]. TR (0.398 ± 0.105 mg/mg extract) had a higher capacity than had the other extracts, followed by PolyR (0.278 ± 0.007 mg/mg extract) and ButR (ButR (0.135±0.033 mg/mg extract), while EAR (0,073 ± 0,019 mg/mg extract) exhibits weak activity.

The results from various antioxidant activities revealed that some roots extracts of *Z. lotus* had significant antioxidant activity. The extracts were found to have different levels of antioxidant activity in the systems tested.

Statistical Analysis

Statistical correlations have been studied between total phenol, flavonoid, tannin contents and antioxidant activity determined by different assays, as shown in Table 6. Total flavonoid content (TFC) was shown to provide the highest association with TAC assay in the present study ($R^2 = 0.87$). Similar results were also found between total phenol content (TPC) and BCB assay ($R^2 = 0.85$) and FRAP test ($R^2 = 0.72$). This supports the results reported by Guo et al[59]. This result was also in agreement with Benzie and Stezo[60], who found a strong positive correlation between total phenolic content and FRAP assay. Leontowicz et al. reported a close correlation ($R^2 = 0.935$) of polyphenol content in extract measured by the Folin–Ciocalteu method and antioxidant activity determined by β-carotene bleaching assay (BCB)[61]. However, there was no significant correlations between total phenol, flavonoid and tannins contents and DPPH assay in our study. Our results indicated that high DPPH radical scavenging activity could not be due to phenolic compounds in the

Table 6: Correlation between total phenol, flavonoid, tannin contents and antioxidant activity determined by different assays (DPPH, BCB, FRAP and TAC)

	TPC	TFC	TTC	DPPH	BCB	FRAP	TAC
TPC	-						
TFC	0.00	-					
TTC	0.00	0.01	-				
DPPH	0.30	0.16	0.06	-			
BCB	0.85*	0.10	0.05	0.21	-		
FRAP	0.72*	0.07	0.12	0.11	0.39	-	
TAC	0.04	0.87*	0.01	0.00	0.01	0.21	-

TPC : total polyphenol content, TFC : total flavonoid content, TTC : total tannin content, DPPH : 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay, BCB : β-Carotene bleaching assay, FRAP : Ferric reducing antioxidant power assay, TAC : total antioxidant capacity test,* Significantly correlated at $P < 0.05$.

extracts. This may be due to other complex antioxidant compounds in different fractions rather than phenol content. Sun and Ho reported a significant correlation between total phenolics and scavenging ability of extracts on DPPH radicals[62]. By contrast, other studies found no correlation between scavenging activity and the total phenolic content[39–63].

CONCLUSION

Considering the results of the percentage yield of extraction of secondary metabolites (polyphenols, flavonoids and tannins), total phenolic, flavonoid and tannin contents, we can conclude that roots of Z. *lotus* contained appreciably high level of total phenolic compounds. The most sample extracts (polyphenols, flavonoids and tannins) from this plant also exhibited high antioxidant and free radical scavenging activities, and some even showed higher potency than the standard synthetic antioxidants in some instances.

These findings provide scientific evidence to support traditional medicinal uses and indicate a promising potential for the development of an antioxidant agent from *Z. lotus* plant. This medicinal plant by *in vitro* results appears as interesting and promising and may be effective as potential sources of novel antioxidant drugs.

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Pharmacognostic investigations of the aerial parts of *Chenopodium foliosum* Asch. and radical-scavenging activities of five flavonoids isolated from methanol extract of the plant

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ABSTRACT

Background: *Chenopodium foliosum* Asch. also known in Bulgarian folk medicine as "garliche" or "svinski yagodi" (swine's berries) has been used as a decoct of aerial parts for treatment of cancer, as antioxidant and immunostimulant. This study was undertaken to investigate its phytochemical, physicochemical and radical-scavenging activities of the aerial parts of the plant. **Materials and Methods:** Physico-chemical parameters: moisture content, foaming index, total ash, dichloromethane, alcohol and water soluble extractive, pleliminary phytochemical screening and TLC finger print profile were determined. Three new flavonol glycosides, 3-*O*-ß-gentiobiosides of 6-methoxykaempferol and gomphrenol and a new gomphrenol trioside as well as the known 3-*O*-ß-gentiobiosides of patuletin and spinacetin, previously isolated from the aerial parts of *Chenopodium foliosum Asch*., were analyzed for radical-scavenging activity using DPPH-, ABTS- free radicals. **Results:** The results of physico-chemical parameters showed moisture content- 6.05%, foaming index-250, total ash- 12.19% and dichloromethane, alcohol and water soluble extractive respectively 2.52%, 13.20% and 12.01%. Preliminary phytochemical screening of the aerial parts of *C. foliosum* reveals the presence of carbohydrates, flavonoids, phytosterols, saponins and alkaloids/amines. Patuletine-3-*O*-gentiobioside showed the highest DPPH (95.03 ± 0.09) and ABTS (87.20 ± 0.13) activity, compared to Vit C. 6-Methoxykaempferol-3-*O*-gentiobioside showed significant ABTS (81.09 ± 0.06) activity, but DPPH activity was lacking. The other flavonoids showed low DPPH activity, but moderate ABTS activity compared with BHT. **Conclusion:** The results of this study could be useful for correct identification of the plant and further standardization. In addition this study suggesting that the decoction prepared from *C. foliosum* could be a potential source of nutraceuticals with radical-scavenging activity.

Keywords: *Chenopodium*, phytochemical investigations, flavonoids, DPPH, ABTS.

INTRODUCTION

The genus *Chenopodium* L. comprises about 150 species, most of these are cosmopolites and are distributed mainly in subtropical and temperate regions[1]. *Chenopodium* is represented by 17 or 18 species in the Bulgarian flora, mostly ruderal plants and widely distributed in the country[2]. Many species of this genus have been used traditionally in indigenous systems of medicine for treatment of numerous ailments. The phytochemical investigations of genus *Chenopodium* have afforded compounds with vast variety of structural patterns. From the phytochemical point of view, the chenopods were reported to contain: flavonoids, saponins, terpenes, sterols, alkaloids and vitamins while the biological properties of chenopods include antimicrobial, cytogenetic, anthelmintic, cytotoxic, immunomodulatory, hypotensive, haemagglutination, trypanocidal and spasmolytic activity[3].

Chenopodium foliosum Asch. also known in Bulgarian folk medicine as "garliche" or "svinski yagodi" (swine's berries) is used as a decoct of aerial parts for treatment of cancer and as antioxidant. Quality control of herbs is a major problem, which effects the efficacy of the results.

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The present study will be helpful to practioners, researchers and and industries to indentify properly the drug.

Three new flavonol glycosides, namely 6-methoxy kaempferol-3-*O*-*β*-gentiobioside **2**, gomphrenol-3-*O*-*β*-gentiobioside **3** and gomphrenol-3-*O*-[6-*O*-(*β*-D-glucopyranosyl)-*β*-D-glucopyranoside] **4** as well as the known compounds patuletin-3-*O*-*β*-gentiobioside **1** and spinacetin-3-*O*-*β*-gentiobioside **5**, that have been previously isolated from the aerial parts of *C. foliosum* Asch[4]. were analyzed for radical-scavenging activity using DPPH-, ABTS- free radicals.

MATERIALS AND METHODS

Chemicals and equipment

1,1-Diphenyl-2-picrylhydrasyl (DPPH), potassium persulphate were from Sigma-Aldrich USA. 2,2,-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxytoluene (BHT) and ascorbic acid were purchased from Fluka, Germany. All other chemicals, including the solvents, were of anaytical grade. Moisture content was measured on a Sartorius Moisture Analyzers MA30. Total ash was determined on Muffle furnace Apro Term 1. Shimatdzu UV-1203 UV-Vis spectrophotometer (Shimatdzu, Japan) was used to measure the absorbance.

Plant material

The aerial parts of *Chenopodium foliosum* Asch. were collected from Beglika, Western Rhodopes, Bulgaria from June to September 2007, at an altitude of 1600 m. The plant was identified and a voucher specimen (No. SOM-Co-1207) was deposited at the National Herbarium, Institute of Biodiversity and Ecosystems Research, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Physico-chemical analysis

Moisture

Five grams of the aerial parts of *C. foliosum* were placed in a tared evaporating dish of the Sartorius Moisture Analyzers MA30 and was dried at 105 °C to a constant weight.

Foaming index

One gram of the aerial parts of *C. foliosum* accurately weight was boiling with 100 ml water for 30 min. After that it was cooled, filtered into a 100 ml volumetric flash and the sufficient water was added through the filter to dilute to volume. The decoction was poured into 10 stoppered test tubes (height 16 cm, diameter 16 mm) in successive portions of 1 ml, 2 ml, 3 ml etc. up to 10 ml and adjust the volume of the liquid in each tube with water to 10 ml. The tube were shaken for 15 seconds after 15 minutes the height of the foam was measured [5].

Determination of total ash

Two gram of the leaves and stem powder of *C. foliosum,* was taken in a tared silica crucible and incinerated by gradually increasing the heat to 500–600 °C until it is white, indicating the absence of carbon. The resultant ash was cooled and weighed. The percentage of the ash was calculated with in a reference to the air-dried drug[5].

Determination of alcohol soluble and dichloromethane soluble extractive

The powdered material was extracted with dichloromethane and methanol in a continuous extraction using a Soxhlet apparatus. The solvent was removed under reduced pressure. The extracts were concentrated under vacuum and yield a residue (% w/w), which was stored in a desicator at room temperature.

Determination of water soluble extractive

The powdered material was extracted with water in a continuous extraction using a warm maceration. The solvent was removed under reduced pressure. The extracts were concentrated under vacuum and yield a residue (% w/w), which was stored in a desicator at room temperature.

Preliminary phytochemical screening

The dried powder of the aerial parts of *C. foliosum* was used for preliminary phytochemical tests such as, Fehling´s test for carbohydrates; Liebermann-Burchard´s reactions for steroids; Borntrager´s test for anthaquinone glycosides; Foam test for saponine glycosides; Shinoda test for flavonoid glycosides; Tests with silicovolframic acid for alkaloids; sodium acetate/ferric ammonium sulfate test for tannins.

TLC finger print profile

Thin layer chromatography of the dichloromethane, alcohol and water extracts was studied and their Rf values were determined. All samples were performed on precoated silica gel 60 and silica gel 60 F_{254} aluminum plates. The mobile phase were EtOAc:AcOH:HCOOH:H_2O (25:3:3:7) and EtOAc:Hexane (6:4). The visualisation was

performed by spraying with Natural Product Reagent A and anisaldehyde/sulfuric acid.

The plates were observed under day light and UV 366 nm and the Rf values of the spots were determined.

Determination of radical-scavenging activity

DPPH radical-scavenging assay

Scavenging activity of flavonoids against DPPH radical was assessed according to the method of Blois with some modifications[6]. Briefly, 2 ml of each compound in MeOH (0.1 mM) was mixed with 2 ml of DPPH methanol solution (0.1 mM). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. Ascorbic acid and BHT were used as references. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity \%} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where $\text{Abs}_{\text{control}}$ is the absorbance of DPPH radical in methanol; $\text{Abs}_{\text{sample}}$ is the absorbance of DPPH radical solution mixed with sample/standard. All determinations were performed in triplicate (n=3).

ABTS radical-scavenging assay

For ABTS assay, the procedure followed the method of Zheleva-Dimitrova et al[7] with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12-14 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol. Fresh ABTS solution was prepared for each assay. 0.4 ml of each compound in MeOH (0.1 mM) was allowed to react with 2 mL of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid and percentage inhibition calculated as:

$$\text{ABTS radical scavenging activity \%} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where $\text{Abs}_{\text{control}}$ is the absorbance of ABTS radical in methanol; $\text{Abs}_{\text{sample}}$ is the absorbance of ABTS radical solution mixed with sample/standard. All determinations were performed in triplicate (*n*=3).

STATISTICS

Data were expressed as mean ± standard deviation (SD). Mann–Whitney rank-sum test was used to analyse the absorption of the samples of DPPH and ABTS tests. All samples were measured in triplicate (n=3). When p> 0.05 there is no statistically significant differences between the absorbtions of each sample that can compromise the final results.

RESULTS AND DISCUSSION

Physico-chemical analysis

The moisture content of the aerial parts of *C. foliosum* was 6.05%. The moisture content of a drug should be minimised in order to prevent decomposition or microbial contamination of a drug and also be controlled to make the drug of definite strength.

The ash of any organic material is composed of their non-valatile inorganic components. The controlled incineration of the crude drugs result in ash residue consisting of an inorganic material (metallic salts and silica). This parameters are used for determination of inorganic materials such as carbonates, silicates, oxalates and phosphates. Therefore, it is a criterion to judge the identity and purity of crude drugs.

The extractive value of the crude drug also determines the quality and purity of the drug material. The dichloromethane, alcohol and water soluble extractive value of the aerial parts of *C. foliosum* are 2,52%, 13.20% and 12.01% respectively (Table 1). The colour and consistency of the extracts from the aerial parts were also determined (Table 2).

Pleliminary phytochemical screening of the aerial parts of *C. foliosum* reveals the presence of carbohydrates, flavonoids, phytosterols, saponins and alkaloids/amins (Table 3). Ascaridole and other terpenoids were previously detected in the essential oil of *C. foliosum* by GC-MS analysis[8] while flavonol glycosides of patuletin, spinacetin, 6-methoxykaempferol and gomphrenol have been isolated from the methanol extract of the aerial parts[4].

TLC fingerprint profile of the dichloromethane, alcohol and water extracts of the aerial parts of *C. foliosum* were given in (Table 4).

Table 1: Physico-chemical parameters of the aerial parts of *C. foliosum*

Physico-chemical parameters	Value
Moisture content	6.05%
Total ash	12.19%
Foaming index	250
Dichloromethane soluble extractive of the aerial parts	2,52%
Alcohol soluble extractive of the aerial parts	13.20%
Water soluble extractive of the aerial parts	12.01%

Table 2: Colour and consistency of extracts from the aerial parts *C. foliosum*

Extracts	Colour	Consistency
Dichloromethane	dark-green	Waxy
Methanol	dark-brown	Semi solid
Water	dark-brown	Semi solid

Table 3: Phytochemical screening of the aerial parts of *C. foliosum*

Group of phytoconstituents	DE/A	ME/A	WE/A
Carbohydrates	-	+	+
Flavonoids	+	+	-
Antocians	-	-	-
Anthraquinones	-	-	-
Tannins	-	-	-
Phytosterols	+	-	-
Saponins	-	+	+
Carotenoids	-	-	-
Alkaloids/Amins	+	+	-

DE/A-dichloromethane extract of the aerial parts; **ME/A**-methanol extract of the aerial parts; **WEA**-water extract of the aerial parts;
"+" present; "-" absent

Table 4: TLC fingerprint profile of the extracts from the aerial parts of *C. foliosum*

Extracts of the aerial part	Solvent system	Detecting agents and number of spots	Rf values
Dichloromethane	EtOAc:Hexane (6:4)	Under day light 7 spots	0.28 (cream), 0.38 (lime -green), 0.47 (lime-green), 0.76 (lime-green), 0.78 (lime -green), 0.85 (forest-green), 0.93 (yellow)
		Anisaldehyde/sulfuric acid and heating (110 C°) 11 spots	0.28 (cream), 0.38 (lime -green), 0.47 (lime-green), 0.66 (light-pink), 0.76 (lime -green), 0.78 (lime-green), 0.81 (light-pink), 0.85 (forest-green), 0.88 (light-pink), 0.91 (light-pink), 0.93 (pink)
Methanol	EtOAc:AcOH:HCOOH:H_2O (25:3:3:7)	Naturstoffreagenz A (366nm) 7 spots	0.11 (light-brown), 0.22 (orange), 0.30 (light-brown), 0.43 (orange), 0.5 (light-brown), 0.7 (purple), 0.93 (light-blue)
		Anisaldehyde/sulfuric acid and heating (110 C°) 7 spots	0.13 (light-grey), 0.45 (blue), 0.6 (blue), 0.62 (blue), 0.76 (blue), 0.83 (blue), 0.93 (light-pink)
Water	EtOAc:AcOH:HCOOH:H_2O (25:3:3:7)	Naturstoffreagenz A (366nm) 4 spots	0.16 (blue), 0.22 (orange), 0.76 (orange), 0.93 (orange)
		Anisaldehyde/sulfuric acid and heating (110 C°) 5 spots	0.18 (blue), 0.25 (blue), 0.41, 0.57(blue), 0.60 (blue)

Determination of radical-scavenging activity with DPPH and ABTS assay

A phytochemical investigation of the aerial parts of *Chenopodium foliosum* Asch. led to the isolation and structural identification of three new flavonol glycosides, namely 6-methoxykaempferol-3-*O*-*β*-gentiobioside **2**, gomphrenol-3-*O*-*β*-gentiobioside **3** and gomphrenol-3-O-*a*-L-rhamnopyranosyl-(1→2)[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside **4** as well as the known compounds patuletin-3-*O*-*β*-gentiobioside **1** and spinacetin-3-*O*-*β*-gentiobioside **5**. The structures of the compounds were previously determined by means of spectroscopic methods (1D and 2D NMR, UV, IR, and HRMS) and DPPH free radical scavenging activity of the new compounds **2**, **3** and **4** at 100 μM were previously reported. Compounds **2** and **4** were not active while compound **3** showed weak DPPH free radical scavenging activity (18.0%) compared to vitamin C (97.4%) and BHT (48.8%) at the same concentration [3].

In these study radical-scavenging activity of the isolated compounds **1** and **5** at 100 μM were examined using 1,1 diphenyl-2-picrylhydrazyl (DPPH). 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) were used for measuring the radical-scavenging and antioxidant activity for all isolated compounds **1-5** at 100 μM.

The radical-scavenging activities of compounds (0.1mM) were compared with those of BHT and ascorbinic acid at the same concentrations and were expressed as percentage of inhibition of against DPPH and ABTS, respectively (Table 5).

Antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups and the effective radical-scavenging activity have flavonoids with 3′,4′-orthodihydroxy configuration in B ring and 4-carbonyl group in ring C. The presence of 3-OH group or 3- and 5-OH groups and C2-C3 double bond configured with a 4-keto arrangement also contribute to antioxidant activity[9]. Rice-Evans et al. also reported that glycosylation of flavonoids reduces their antioxidant activities when it is compared to the corresponding aglycones[10].

Patuletin-3-*O*-*β*-gentiobioside **1** showed the highest DPPH activity (95.03 % ± 0.09) and its activity is similar to that of ascorbic acid (97.42 %± 0.07) but higher than the radical-scavenging activity of BHT (48.80 % ± 0.07). In its moiety compound **1** has 3′,4′-orthodihydroxy configuration in B ring and 4-carbonyl group in ring C which determines its radical-scavenging activity. The other flavonoids demonstrate significantly low DPPH activity- **3** (18.02 % ± 0.06), **4** (2.31 % ± 0.03), **5** (3.94 % ± 0.05) and may be it is due to the absence of orthodihydroxy configuration. In these compounds 3′-OH is missing or

Table 5: DPPH, ABTS radical-scavenging activity of flavonol glycosides

Compounds	DPPH % ± SD	ABTS % ± SD
patuletin-3-*O*-*β*-gentiobioside **1**	95.03 ± 0.09	87.20 ± 0.13
6-methoxykaempferol-3-*O*-*β*-gentiobioside **2**	-	81.09 ± 0.06
gomphrenol-3-*O*-*β*-gentiobioside **3**	18.02 ± 0.06	29.22 ± 0.1
gomphrenol-3-O-*α*-L-rhamnopyranosyl-(1→2)[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside **4**	2.31 ± 0.03	28.30 ± 0.1
spinacetin-3-*O*-*β*-gentiobioside 5	3.94 ± 0.05	32.33 ± 0.06
Vit C	97.42 ± 0.07	91.60 ± 0.09
BHT	48.80 ± 0.07	26.49 ± 0.09

is replaced with -OCH_3 group. Gomphrenol glycosides, compounds **3** and **4** have 6, 7-methylenedioxy substitute, which reduced the number of -OH groups and decreased the antioxidant activity.

The highest ABTS radical-scavenging activity of isolated flavonoids possessed compounds **1** (87.20 % ± 0.13) and **2** (81.09 % ± 0.06). Activity of these compounds were similar to those of classic antioxidant ascorbic acid (91.60 % ± 0.09). With moderate ABTS radical-scavenging activity and comparable with the activity of BHT (26.49 % ± 0.09) were gomphrenol glycosides **3** (29.22 % ± 0.1) and **4** (28.30 % ± 0.1) and spinacetinegentiobioside compound **5** (32.33 % ± 0.06). With the lowest ABTS radical-scavenging activity were gomphrenol glycosides, compounds **3** and **4** and possibly it is due to the presence of 6,7-methylenedioxy substitute. The results show that diglycoside of gomphrenol **3** (29.22 % ± 0.1) has higher ABTS radical-scavenging activity than triglycoside **4** (28.30 % ± 0.1). The lower activity of gomphrenol triglycoside is probably due to the presence of another sugar.

CONCLUSION

The results of these study could be useful for correct identification of the plant and further standartization. With the highest radical-scavenging activity and perspective for further ivestigations of antioxidant activities were patuletin-3-*O*-*β*-gentiobioside **1** and 6-methoxykaempferol-3-*O*-*β*-gentiobioside **2.** Activity of these compounds were similar to those of classic antioxidant ascorbic acid. These results suggested that the decoction prepared from *C. foliosum* can be a potential source of flavonoids with a radical-scavenging activity.

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Sterols and triterpenes from the non-polar antitubercular fraction of Abutilon indicum

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ABSTRACT

Introduction: *Abutilon indicum Sweet* (Malvaceae) is used in Filipino folk medicine as demulcent, diuretic, sedative, aphrodisiac and antidiabetic remedy. It is also effective in the treatment of leprosy. It is also used as a laxative for patients having hemorrhoids and in the treatment of coughs, puerperal disease, urinary disorders, chronic dysentery and fever. **Methods:** Air-dried powdered leaves of *A. indicum* were initially extracted with DCM-methanol (1:1) to afford a crude extract which was fractionated on a silica gel column to afford four fractions. The first fraction which showed antimycobacterial activity was further chromatographed in a silica gel column to afford bioactive fractions from which four compounds were obtained. The crude extract, fractions and isolated compounds were screened for their inhibitory effect against *Mycobacterium tuberculosis* H_{37}Rv (ATCC) using the colorimetric Microplate Alamar Blue Assay (MABA) method. **Results:** The fractions obtained after silica gel chromatography of the crude DCM-methanol (1:1) revealed the first fraction (MIC = 64 μg/mL) to exert the highest inhibition against *M. tuberculosis* H_{37}Rv.Further separation of this fraction afforded sub-fractions with moderately strong inhibitory activity against the test organism (MIC up to 64 μg/mL). Chromatographic purification of sub-fraction 1 afforded four compounds which were spectroscopically identified as β-amyrin 3-palmitate **(1)**, squalene **(2)** and a 1:1 mixture of the sterols β-sitosterol **(3)** and stigmasterol **(4)**. Evaluation of the antimycobacterial activity of **1–4** showed insignificant inhibitory activity against the test organism (MIC = >128 μg/mL). **Conclusion:** The present results demonstrate the potential of *Abutilon indicum* as a plant source of compounds that may exhibit promising antituberculosis activity. While the known compounds isolated for this plant did not show antimycobacterial activity, the obtained results are considered sufficient reason for further study to isolate the metabolites from *A. indicum* responsible for the antitubercular activity.

Keywords: *Abutilon indicum*, Malvaceae, antitubercular, squalene, β-amyrin 3-palmitate, β-sitosterol, stigmasterol.

INTRODUCTION

Abutilon indicum Sweet or "*giling-gilingan*" in Filipino is a member species of the family Malvaceae and found throughout the Philippines in thickets and waste places at low and medium altitudes. It is a half-woody, erect, branched-plant 0.5 to 2.5 meters in height. The plant is a popular medicinal plant in the Philippines. The leaf decoction is used for cleansing wounds and ulcers, and for enemas or vaginal infection. The emollient leaf decoction is frequently used by Filipinos as demulcent, diuretic, sedative and aphrodisiac.[1] It has also a long medical history of being used as an antidiabetic remedy. The diuretic property can be traced from its root extract and can be taken for the relief of hematuria. It is also effective in the treatment of leprosy. The seeds from this plant are considered to be aphrodisiac and can be used as a laxative for patients having hemorrhoids and in the treatment of coughs, puerperal disease, urinary disorders, chronic dysentery, and fever.[2] Previous phytochemical studies reported the presence of fatty acids, carotenoid, sterols, triterpenes, sesquiterpenes, saponins, tannins, alkaloids,

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aromatic acids and flavonol glycosides.[3] Pharmacological studies reveal that the leaf extracts possess hypoglycemic and hepatoprotective properties.[4,5] The polyherbal formulations of this plant have also been reported effective in treating diabetes, hyperlipidemia, and as free radical scavengers.[6–8] Furthermore, this plant has been found to have antibacterial and antifungal properties.[9,10]

As part of our continued interest in exploring the antituberculosis constituents of Philippine medicinal plants,[11–16] we hereby report the isolation of **1–4** from the antimycobacterial fraction of *A. indicum*.

MATERIAL AND METHODS

Plant material

The leaves of *Abutilon indicum* were collected from Laoag, Ilocos Norte, Philippines in April 2006. Voucher specimens were authenticated by Asst. Prof. Rosie S. Madulid of the Herbarium of the Research Center for the Natural and Applied Sciences, University of Santo Tomas – Manila (USTH5034).

Extraction, fractionation and isolation of compounds

The air-dried leaves of *A. indicum* (1.3 kg) was soaked in 1:1 DCM-MeOH(17 L) for three days, and then filtered. The filtrate was concentrated under reduced pressure to afford the crude extract (151.0 g) which was chromatographed in increasing amounts of MeOH in DCM at 20% increments as eluents. Four fractions were obtained and the most antimycobacterial fraction (fraction one, 10.0 g) was further chromatographed in diethyl ether-ethyl acetate (20% gradients) to yield five fractions. Silica gel chromatography of the most active, sub-fraction one (7.0 g) in hexanes, and chloroform-acetonitrile-diethyl ether mixture (9:0.5:0.5) gave **1** (colorless solid, 104.2 mg), **2** (colorless oil, 11.3 mg) and a mixture of **3** and **4** (colorless crystals, 143.0 mg) (Figure 1).

Bacterial strains and growth conditions

M. tuberculosis H_{37}Rv ATCC 27294 (H_{37}Rv) obtained from the American Type Culture Collection (Rockville, Md.). For the first three (of four) replicate experiments, H_{37}Rv inocula were first passaged in radiometric 7H12 broth (BACTEC 12B; Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) until the growth index (GI) reached 800 to 999. For the fourth replicate experiment, H_{37}Rv was grown in 100 ml of Middlebrook 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.2% (v/v) glycerol (Sigma Chemical Co., Saint Louis, Mo.), 10% (vol/vol) OADC (oleic acid, albumin, dextrose, catalase; Difco), and 0.05% (v/v) Tween 80 (Sigma). The complete medium was referred to as 7H9GC-Tween. Cultures were incubated in 500-ml nephelometer flasks on a rotary shaker (New Brunswick Scientific, Edison, N.J.) at 150 rpm and 37°C until they reached an optical density of 0.4 to 0.5 at 550 nm. Bacteria were washed and suspended in 20 ml of phosphate-buffered saline and passed through an 8-mm-pore-size filter to eliminate clumps. The filtrates were aliquoted, stored at 280°C, and used within 30 days.

Microplate Alamar Blue Assay (MABA)[17]

Briefly, extracts, fractions and test compounds MICs against TB were assessed by MABA using rifampin as positive control. Compound stock solutions were prepared in DMSO at a concentration of 12.8 mM, and the final test concentrations ranged from 128 to 0.5 mM. 2-fold dilutions of compounds were prepared in Middlebrook 7H12 medium (7H9 broth containing 0.1% w/v casitone, 5.6 μg/mL palmitic acid, 5 μg/mL bovine serum albumin, 4 μg/mL catalase, filter-sterilized) in a volume of 100 mL in 96-well microplates (black viewplates). *Mtb* H_{37}RV (100 mL inoculum of 2 to 105 cfu/mL) was added, yielding a final testing volume of 200 mL. The plates were incubated at 37 °C. On the 7th day of incubation 12.5 mL of 20% Tween 80 and 20 mL of Alamar Blue (Trek Diagnostic, Westlake, OH) were added to the test plate. After incubation at 37 °C for 16 to 24 h, fluorescence of the wells was measured (ex 530, 590 nm). The MICs ware defined as the lowest concentration effecting a reduction in fluorescence of >90% relative to the mean of replicate bacteria only controls.

RESULTS

Silica gel fractionation of the DCM-MeOH (1:1) crude extract of the leaves of *A. indicum* (MIC= >128 μg/mL versus *Mycobacterium tuberculosis* H_{37}Rv) (Table 1) afforded four fractions with the first fraction being the most inhibitory according to the colorimetric MicroplateAlamar Blue assay (MABA)[17] (MIC = 64 μg/mL) (Figure 1). For comparison purposes, the known TB drug rifampin was used as positive drug standard for comparison purposes (MIC = 0.125 μg/mL). Further chromatographic separation of this fraction resulted into five fractions with MIC values up to 64 μg/mL. Chromatographic purification of the major constituents of the first sub-fraction gave β-amyrin 3-palmitate (**1**), squalene (**2**) and a 1:1 mixture of the sterols β-sitosterol (**3**) and stigmasterol (**4**).

The structures of **1–4** were established through NMR spectroscopic evidences and confirmed by comparison of their MS and NMR (^1H, ^{13}C) spectral data with those reported in the literature for β-amyrin 3-palmitate,[18] squalene[19] and mixture of β-sitosterol and stigmasterol[20] The MABA assay revealed no significant antitubercular activity for all isolated compounds (MIC = > 128 μg/mL). The result may indicate synergistic action of the constituents for marked antitubercular activity. To the best of our knowledge, this is the first report on the isolation of **1** and **2** from *A. indicum*.

Table 1: Minimum inhibitory concentration (MIC) values vs. *M. tuberculosis* $H_{37}Rv$ of *A. abutilon* fractions and isolated compounds 1–4

Fraction	MIC (μg/mL)*
Fraction 1	64 (91)
Fraction 2	>128 (58 @ 128)
Fraction 3	>128 (-24 @ 128)
Fraction 4	>128 (15 @128)
Fraction 1.1	64 (93)
Fraction 1.2	64 (94)
Fraction 1.3	>128 (82 @ 128)
Fraction 1.4	128 (90)
Fraction 1.5	128 (96)
Compound	
1	>128
2	>128
3 & 4 (1:1)	>128
Rifampin	0.125

*% Values in parentheses denote % inhibition.

DISCUSSION

Tuberculosis (TB) has re-emerged as the deadly plague by recent reports of outbreaks of drug-resistant cases. Mortality rates have escalated in number due to the HIV-1 epidemic and the inconsistent use of antibiotics, which has led to a rise of drug-resistant TB in areas of the world. This growing global health concern has led to renewed interest in natural product-inspired derivatives with promising activity against *Mycobacterium tuberculosis*. Literature reviews indicate natural products possessmarked growth inhibitory activity towards *M. tuberculosis* and some have been selected as prototype molecules for anti-TB drug development.[21–23] Relevant to this study, pentacyclic triterpenoids, sterols and polyenes have been reported as promising inhibitors of *M. tuberculosis* $H_{37}Rv$[24] Previous studies on *A. indicum* indicate the presence of several non-polar secondary metabolites *viz.* acyclic and pentacyclic triterpenes and sterols. An independent study on the methanolic extract of *A. indicum* showed activity against bacteria.[3] Based on our results, activity against *M. tuberculosis* is ascribed to the non-polar constituents of *A. indicum* in fraction one. However, purification of the major constituents yielding compounds **1–4** did not result into the isolation of the active constituents (MIC's >128 μg/mL). While a separate report indicates that squalene (**2**) is active against *M. tuberculosis*, our anti-TB assay data for **2** interestingly agreed with the result of Noro and co-workers.[25] Further purification of the antimycobacterial principles of *A. indicum* appears to be significant in order to clarify the bioactive components responsible for antitubercular activity.

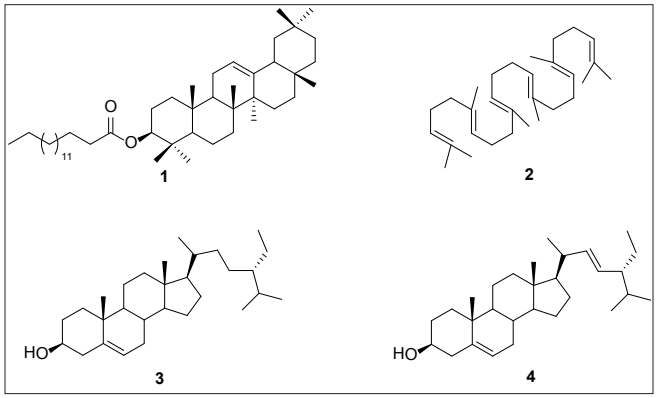

Figure 1: Compounds isolated from *A. indicum*.

CONFLICTS

All authors have none to declare.

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Estrogenic Effect of Methanolic Extract of *Avicennia alba* Blume. Aerial Parts in Female Wistar Albino Rats

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ABSTRACT

Objectives: To investigate the estrogenic effect of *Avicennia alba* (Blume) aerial parts as identified in the folklore recipes as a contraceptive **Methodology:** The estrogenic activity study of the methanolic extract was carried out by studying the histopathology of the uterus. The uterine weight and vaginal cornification were also observed. The diameter of uterus, thickness of endometrium and height of the endometrial epithelium were measured using a calibrated ocular micrometer. The estimation of total protein and cholesterol was carried out using a standard method described by Lowely et al. **Results:** The presence of alkaloids, anthraquinone glycosides, flavonoids, steroids, polyphenolics were detected in the aerial parts of *Avicennia alba*. The methanolic extract of *Avicennia alba* at 400 mg/kg body weight showed a significant ($p < 0.05$) increase in uterine weight, diameter of uterus, thickness of endometrium and height of the endometrial epithelium compared to the control. There was increase in vaginal cornification status. The histological examination of the uterus also showed estrogenic influence. A significant increase ($p < 0.05$) in total protein and cholesterol content in the uterus of standard and test drug treated rats was also observed. **Conclusion:** The methanolic extract of *Avicennia alba* at 400 mg/kg body weight showed significant estrogenic activity and the results are in consistent with the folkloric claim reports related to oral contraceptive effect of *Avicennia alba*

Keywords: *Avicennia alba*, contraceptive, estrogenic activity, uterus.

INTRODUCTION

Nowadays many women refuse the hormone replacement therapy such as estrogen replacement, due to adverse effects like vaginal bleeding and increased risk of developing breast and ovary cancer[1]. Therefore, there is a need of an alternative therapy, including herbal drugs. Medicinal plants are used worldwide for the treatment of various ailments, as well as for development of novel drugs. Over 20,000 species of medicinal plants are being used in traditional system of medicine and leading as the potential sources for discovery of new therapeutic agents[2]. Now in developing countries, the search for alternative medication for menopausal and postmenopausal women, based on plant extracts is increasing day by day[3]. Estrogen deficiency during menopause can develop many health problems, such as sleeping disorders, vaginal dryness, joint pain, mood swings, reduced bone density, cardiovascular disease etc[4]. The signaling of estrogens in the cells is mediated through the estrogen receptors like ERa and ERb. Binding of estrogen to ERa or ERb causes a conformational change in the receptor and promotes dimerization of receptors[5].

Phytoestrogens are compounds, which are produced from the natural source, having ability to interfere with estrogen action either interacting with the ERs or modulating the endogenous estrogen concentrations, possessing estrogen-like activity and can be used for the management of menopausal symptoms with few side effects[6]. Many phytoestrogens bind with a higher affinity to ERb than to ERa, suggesting that they may induce physiological effects through this ER subtype.

Since mixture of compounds, present in plant extracts, may exhibit better biological effects than isolated phytoestrogens, therefore, we evaluated the possible

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estrogenic effects of methanolic extract of *Avicennia alba* on immature rat model.

Avicennia alba (A. alba), belonging to the family- Acanthaceae, is one of the mangrove plants in southeast Asia mangrove forest[7] The plant has traditionally been used as the remedy for sexual disorders, scabies, rheumatism, paralysis, asthma and snake-bites, skin disease and ulcer[8]. Fruits are plastered on to boils and tumors, a poultice of unripe seed and leaves stop inflammation. and bitter resin used as contraceptive by women[9] The plant is reported to have a wide spectrum of medicinal properties, such as anti-cancer, anti-inflammatory, anti-microbial, antidiarrhoeal and analgesic[10] It contains high yield of steroids, triterpenes, flavonoids, alkaloids, saponins and tannins[11]

The new naphto-quinines and their analogues named Avicequinone A,B and C and Avicenol A,B and C from *A. alba* were isolated from stem bark of the plant[12] Due to the absence of scientific reports that corroborate the contraceptive property of *A. Alba*, the present investigation was focused to study the estrogenic effect of *A. Alba* on immature rat.

MATERIALS AND METHODS

Plant Materials

The aerial parts of plant *Avicennia alba* were collected from Sunderban area, West Bengal in month of October, 2011 and identified by Dr. K. Karthigeyan, Central National Herbarium, Botanical Survey of India, Howrah, West Bengal. A voucher specimen (CNH/128/2011/TECHII/637) has been deposited in the herbarium of the Department of Pharmacognosy, School of Pharmaceutical Sciences, Siksha O Anusandhan University, Odisha, India.

Preparation of plant extract

The air-dried plant materials (1 kg) of *A. alba* were coarsely powdered and extracted in a Soxhlet apparatus with methanol (2L) for 72 h. The methanolic extract was examined chemically to screen the presence of different phytoconstituents. The extract was concentrated to dryness in a flash evaporator (Buchi type) under reduced pressure and controlled temperature 50–60 °C. A total mass of 135 g of crude extract was obtained. The extract was stored in a refrigerator and a weighed amount of the extract was suspended in 10% aqueous Tween 80 solution and used for the present study.

ANIMALS

Colony-bred immature female albino rats (Wister strain), 21–23 days old and weighing between 30–45 grams were used. All the animals were maintained under standard husbandry conditions with food and water *ad libitum* in animal house. The temperature was 23±2°c and humidity was 50±5%. The experimental protocol was approved by Institutional Animal Ethics Committee, School of Pharmaceutical Sciences, Siksha 'O' Anusandhan University, Odisha, India. (Registration no. 1171/c/08/CPCSEA)

Acute toxicity study

Acute oral toxicity of methanolic extract of *A alba* was determined by using female, non pregment rat weighing 160–200 gm. The animals were fasted for 3 hours prior to the experiment. Organization for Economic Cooperation and Development (OECD) guideline 420[13] was adopted for toxicity studies. Animals were administered with single dose of extracts and observed for their mortality during 48 hrs study period. The dose up to 4000 mg/kg body weight was found tolerable as no death was found. Rats were observed individually after dosing for first 30 minutes periodically and daily thereafter, till 14 days for any toxicity sign of gross changes in skin and fur, eyes, mucous membrane, circulatory, central nervous system, autonomic and behavior pattern if any.

Estrogenic activity study

The uterine weight and vaginal cornification method was employed for the study[14,15]. Colony-bred immature ovariectomised female albino rats were divided into four groups consisting six animals in each group. The first group served as control and received the vehicles (Tween-80, 1%) only. The second group received a suspension of Ethinyl estradiol in distilled water using Tween-80 (1%) at a dose of 0.02mg/kg body wt. The third group received the methanol extract at 400 mg/kg body weight and Fourth group received methanol extract at 400 mg/kg body weight in an addition to 0.02mg/kg body wt of Ethinyl estradiol. Body weight and vaginal smears were recorded daily. All the above treatments were given orally for 7 days. All the animals were sacrified on 8th day of the experiment by decapitation under light ether anesthesia and the uteri were dissected out, surrounding tissues removed, blotted on filter paper and weighed quickly on a sensitive balance.

A portion of uterine tissues from control and treated animals were fixed in Bouins fluid for 24 hours, dehydrated in alcohol and then embedded in paraffin. The paraffin blocks were section at 6 µm and stained with haematoxylin-

eosin for histological examination for uterus. The diameter of uterus, thickness of endometrium and height of the endometrial epithelium were measured in 16 randomly selected sections using a calibrated ocular micrometer. The other portion of uterus was homogenized with ice-cold distilled water in a pre-cooled mortar and pestle to contain 10 mg of tissue/ml. The homogenated tissue was centrifuged in cold at 3000 rpm for 15 minutes and the supernatant was used for the estimation of total protein and cholesterol using standard method[16].

Statistical analysis

Statistical analysis of the differences between the group were analyzed by one-way analysis of variance (ANOVA) followed by Dunnet's t-test. $p<0.05$ was considered as statistically significant. All data are expressed as the mean value ± SD.

RESULTS

Phytochemical study

The preliminary phytochemical screening of the methanolic extract of *A. alba* revealed the presence of alkaloids, anthraquinone glycosides, flavonoids, steroids and polyphenolic compounds.

Estrogenic activity

Assessment of estrogenic activity of methanolic extract of *A. alba* was done by taking status of vaginal cornification, uterine weight, uterine biochemical estimation and uterine histology as parameters. The effect of the methanolic extract of *A. alba* on immature rat uterus is shown in Table 1, Table 2 and Table 3.

Table 1: Estrogenic activity of the methanolic extract of aerial part of *Avicennia alba*

Group	Treatment (Dose-mg/kg body weight)	Uterine weight mg/100gm body wt. (mean± SD)	Vaginal status	Vaginal cornification
i	Control (Tween 80, 1% v/v)	60.66±5.57	Closed	Nil
ii	Ethinyl estradiol (0.02 mg/kg)	146.83±5.26*	Open	+++
iii	Methanolic extract (400 mg/kg)	133.33±4.88*	Open	+ to ++
iv	Ethinyl estradiol (0.02 mg/kg) + Methanolic extract (400 mg/kg)	234.50±5.57*	Open	+++

No of animals used each group 6; + Nucleated epithelial cells, ++ Nucleated and cornified cells, +++ Cornified cells; * $p<0.05$ when compared to control

Table 2: Histological changes in the uterus and endometrium after treatment with methanolic extract of aerial part of *Avicennia alba*

Group	Treatment (Dose-mg/kg body weight)	Diameter of the Uterus (µm) (Mean±SD	Thickness of the endometrium (µm) (Mean±SD	Height of endometrial epithelium (µm) (Mean±SD
i	Control (Tween 80, 1% v/v)	356.33±7.33	99.83±6.76	19.83±5.26
ii	Ethinyl estradiol (0.02mg/kg)	684.50±7.17*	280.33±4.92*	35.83±4.35*
iii	Methanolic extract (400mg/kg)	544.50±3.61*	215.16±3.71*	28±4.33*
iv	Ethinyl estradiol (0.02mg/kg) + Methanolic extract (400mg/kg)	744±4.60*	341.50±5.46*	46.83±5.45*

No. of animals used each group 6; * $p<0.05$ when compared to control

Table 3: Biochemical changes in the uterus after treatment with methanolic extract of aerial part of *Avicennia alba*

Group	Treatment (Dose-mg/kg body weight)	Total protein mg/100mg (Mean±SD)	Cholesterol mg/100mg (Mean±SD)
i	Control (Tween 80, 1% v/v)	3.8±1.26	18.69±3.26*
ii	Ethinyl estradiol (0.02mg/kg)	7.45±1.09*	25.19±2.85*
iii	Methanolic extract (400mg/kg)	5.7±1.03*	22.11±3.60
iv	Ethinyl estradiol (0.02mg/kg) + Methanolic extract (400mg/kg)	9.26±1.63*	30.83±2.35*

No. of animals used each group 6; , $p<0.05$ when compared to control

Oral administration of the methanolic extract at 400 mg/kg body weight caused a significant increase ($p<0.05$) in uterine weight in immature ovariectomised rats. The standard drug, Ethinyl estradiol produced statistically significant ($p < 0.05$), 1.42-fold increase in uterine weight. The methanolic extract of *A.alba* -induced proliferative changes in the uterine endometrium as evidenced by increased height of luminal epithelium, with loose stroma and increased number of glands (Figure 3), compared to control. The control animals presented a typical infantile condition (Figure 1). Ethinyl estradiol induced proliferative changes (Figure 2). Methanolic extract in combination with Ethinyl estradiol also induced similar proliferative changes (Figure 4). It exhibit estrogenic activity as shown by the significant ($p <0.05$) increase in diameter of uterus and thickness of endometrium and vaginal epithelial cornification in immature rats when compared with control.

The number of cornified cells in the vaginal smears was considerably higher (+ to ++) in methanol extract treated rats and (+ to +++) in rats treated with combination of extract and Ethinyl estradiol than those of the controls (0 to +). Simultaneous administration of Ethinyl estradiol and the methanolic extract caused a highly significant increase in uterine weight (versus control, $p < 0.05$). There was also a significant increase in total protein and cholesterol content in the uterus of standard and test drug treated rats (versus control, $p < 0.05$).

Estrogenic activity of methanolic extract of aerial parts of *Avicennia alba*

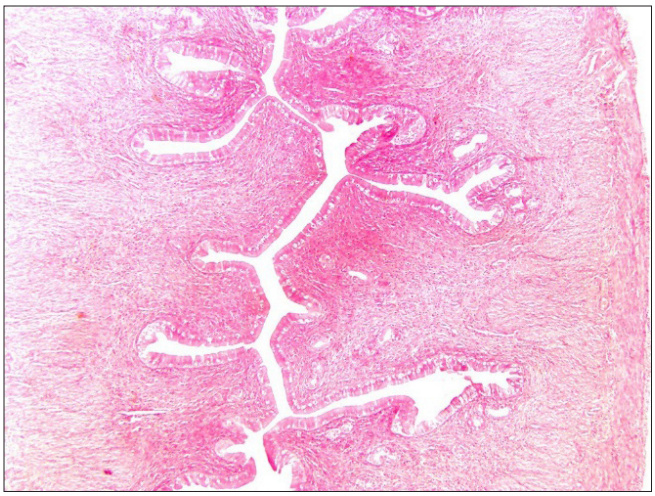

Figure 1: Section of immature rat uterus treated with Tween 80, 1% (Control) (H&E,100x), showing intact stroma.

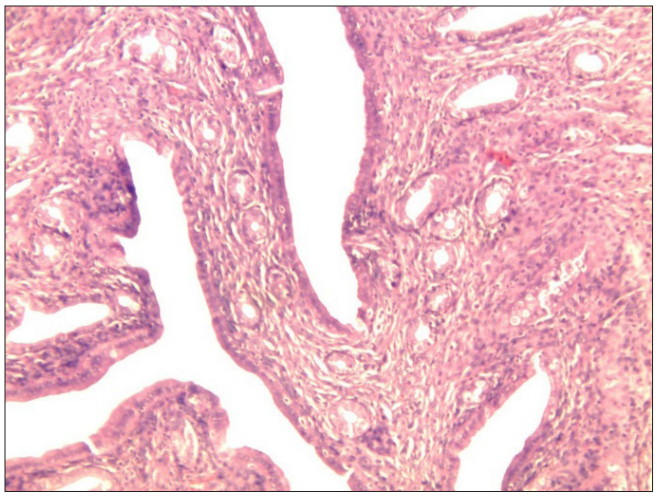

Figure 2: Section of immature rat uterus treated with (Ethinyl estradiol (0.02mg/kg bw) (H&E,100x), showing loose stroma and increased no. of glands.

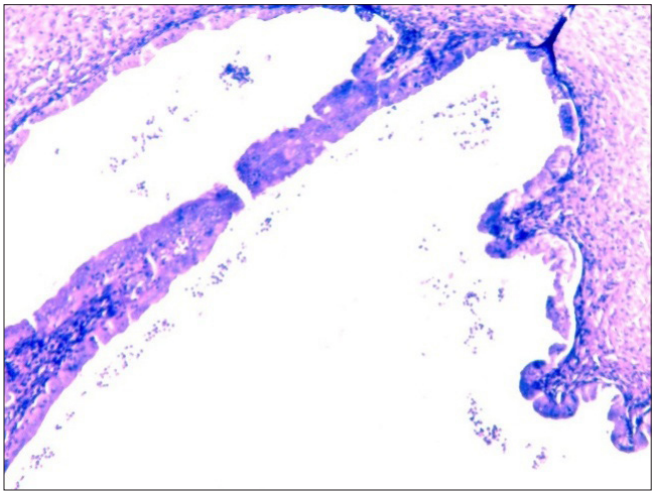

Figure 3: Section of immature rat uterus treated with methanolic extract 400 mg/kg bw of A. alba (H&E, 100x), showing loose stroma and increased no. of glands.

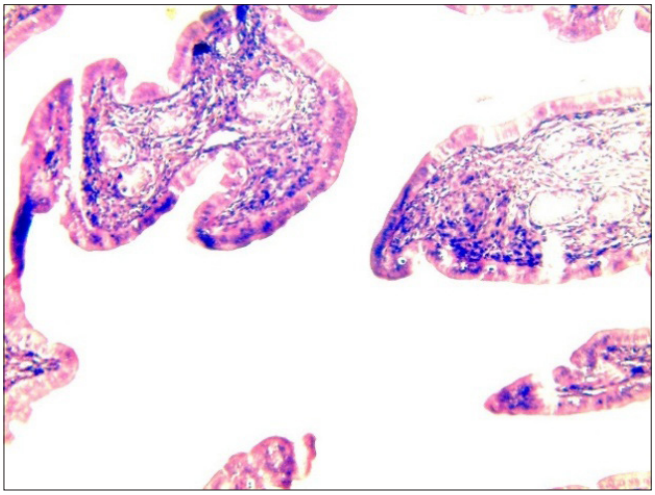

Figure 4: Section of immature rat uterus treated with methanolic extract 400 mg/kg bw of A. alba and with Ethinyl estradiol (0.02mg/kg bw) (H&E, 100x), showing loose stroma and increased no. of glands.

DISCUSSION

The ovarian hormone, estrogen causes various physiologic and biochemical changes in uterus and the female reproductive tract. When female rats are ovariectomized, the resultant lack of estrogen causes atrophy of the uterus and administration of estrogenic substances to ovariectomized rats causes uterotrophic effects, vaginal cornification and proliferative changes in uterine endometrium epithelium height[17].

Estrogen causes an increase in protein synthesis, uterine weight, water uptake and retention of fluid leading to ballooning of uterus[18]. Estrogen also causes vaginal opening which is a quality measure of estrogen potency. Presence of cornified cells in vaginal smears also indicates estrogenic activity[19]. In the ovariectomised rats, the uterine protein concentration in increased by estrogen[20,21].

Estrogen like steroid stimulates the synthesis of mRNA, protein and DNA[22]. In the present study, the increase in total protein concentration in uterus of female rats administered with aerial parts of methanolic extract of *Avicennia alba* may be attributed to steroid like substances in it. Cholesterol is a precursor molecule in steroidogenesis[23]. It was previously reported that estrogen administered increase the uterine cholesterol content in ovariectomised rat[24]. The methanolic extract increases the cholesterol level in ovariectomised rat uterus, which may be due to the presence of steroid like substances in the extract of *Avicennia alba* .

Methanolic extract of *A. alba* exhibited estrogenic activity as shown by significant increase in uterine weight, diameter of uterus, thickness of endometrium and vaginal epithelial cornification in immature rats. The histological examination of uterus of extract treated rats showed estrogenic influence, as evidenced by increased height of luminal epithelium with loose stroma and increased number of glands[25]. This confirms the estrogenic property of *A. alba*

Literature review on *A. alba* showed the presence of steroids, triterpenes, flavonoids, alkaloids, saponins and tannins[11]. Flavonoids and phenolic compounds are known to possess estrogenic activity[26]. Phytoestrogens like β-sitosterol, stigenosterol, stigmagmastanol etc possess estrogenic activity due to their affinity with estrogrnic receptors leading to infertility in animals[27,28] Thus the estrogenic activity shown by the extract *A. alba* may be attributed to the presence of flavonoids, phenolic compounds and phytosterols.

Simultaneous administration of Ethinyl estradiol and methanol extract caused significant increase in uterine weight when compared with control ($p<0.05$). The degree of uterotrophic potency was more than that produced by Ethinyl estradiol, when compared with control. These observations have also been confirmed when the uterotrophic changes such as diameter of uterus, thickness of endometrium were compared with control and standard Ethinyl estradiol treatment. This shows that the methanol extract has significant estrogenic activity when given alone. There was no anti estrogenic activity when given

along with Ethinyl estradiol at the tested dose. So the plant may be beneficial both as a contraceptive as well as in post menopausal hormonal replacement therapy.

Further, isolation of active constituents from the methanolic extract of *Avicennia alba* and estrogenic activity of such isolated compounds are in progress.

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Protective effect of *Cissampelos pareira* linn. Extract on gentamicin-induced nephrotoxicity and oxidative damage in rats

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ABSTRACT

Objective: To evaluate the potential nephroprotective and antioxidant activity of hydroalcoholic *Cissampelos pareira* (*C. pareira*) whole plant extract using gentamicin-induced rats. **Methods:** For studying acute toxicity study, single oral dose of 2g/kg hydroalcoholic extract of *C. pareira* was evaluated in rats by oral gavage. The nephroprotective activity was evaluated using gentamicin-induced model in rats. *In-vitro* antioxidant activity was evaluated by using DPPH assay and reducing power assay. *In-vivo* antioxidant activity was evaluated by using glutathione and lipid peroxidation estimations in gentamicin-induced rats. Hydroalcoholic *C. pareira* whole plant extract was given at a dose of 200 and 400 mg/kg *p.o.* **Results:** For acute toxicity testing rats administered with the extract at a dose 2 g/kg. the result showed no toxicity. Hydroalcoholic *C. Pareira* whole plant extract (200 and 400 mg/kg *p.o*) significantly decrease the elevated urinary glucose levels in the urine, decrease the elevated urea and creatinine levels in blood and increase the urinary creatinine levels in gentamicin-induced nephrotoxic rats. The extract had shown significant dose dependent increase in the DPPH and reducing power activity. There were a dose dependent decreasing and increasing of lipid peroxidation, glutathione levels in hydoalcoholic extract treated groups respectively. **Conclusion:** This study exhibits that hydroalcoholic *C. pareira* whole plant extract poses nephroprotective activity which may be due to its antioxidant activity.

Keywords Gentamicin, *Cissampelos pareira*, creatinine, urea, glucose, lipid peroxidation, glutathione, reducing power, DPPH.

INTRODUCTION

At present human beings are using different kinds of antibiotics to treat various infections. The aminoglycoside antibiotic like gentamicin is most frequently used drug for treating various infections caused by gram-negative organisms because of its rapid bactericidal activity and low resistance to those gram-negative organisms. But it is having major side effects like ototoxicity and nephrotoxicity. The nephrotoxicity is more frequent over ototoxicity[1]. Gentamicin has been confirmed to increase the generation of reactive oxygen species in the kidney which leads to renal anatomical and physiological damage. Furthermore, gentamicin treated rat kidneys are more prone to oxidative damage due to the generation of reactive oxygen species there by increased levels of lipid peroxidation and decreased levels of anti oxidant enzymes in gentamicin treated rat kidney[2]. Even though, gentamicin is having ototoxicity and nephrotoxicity still, it is using in clinical practices because of its potent bactericidal activity and less resistance, in those conditions a supportive therapy must be given to the patient to protect from the nephrotoxicity.

Therefore, the present work is to protect from the nephrotoxicity of the gentamicin by using naturally available antioxidants which are going to scavenge the reactive oxygen species. Antioxidants have been shown to improve signs of gentamicin induced nephrotoxicity[3]. A potential therapeutic approach to defend or reverse gentamicin induced oxidative damage and nephrotoxicity could have

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more importance for clinical consequences[4]. However, scientific reports on the exploration of *C. pareira* for its effects on renal function are scarce. So the present study was designed to investigate the antioxidant and nephroprotective activity of *C. pareira* hydroalcoholic extract in gentamicin induced rats.

MATERIALS AND METHODS

Preparation of whole plant extract

C. pareira whole plant was collected from the Chittoor district of Andhra Pradesh and it is authenticated by K. Madava Chetty, Assistant professor, Sri Venkateswara University, Tirupathi. The whole plant was dried under shade and pulverized separately into coarse powder by a mechanical grinder. The resulting powder was used for the extraction. The powder was extracted directly with 70% v/v ethanol, which was used for biological investigations and *in-vitro* antioxidant studies, after subjecting it to preliminary qualitative phytochemical studies[5]. The extract was concentrated under reduced pressure and stored in vacuum desiccators. Dried extract was suspended in distilled water by using tween 80.

Chemicals

Assay kits for the estimation of urinary glucose, urinary creatinine, serum urea, serum creatinine and DPPH were purchased from transasia bio-medicals ltd, coral clinical systems, span diagnostics ltd, coral clinical systems and aldrich respectively. All the chemicals used were of analytical grade.

Preliminary phytochemical investigation

The preliminary phytochemical screening was carried out for qualitative identification of phytoconstituents[6].

Experimental animals

Albino wistar rats weighing 150–250g was procured from Biogen, Bangalore. They were maintained in the animal house of Gautham College of Pharmacy. Animals were maintained under controlled condition of temperature at 27° ± 2° C and 12-h light-dark cycles. They were housed in polypropylene cages and a free access to standard pellets and ad libitum. All the studies conducted were approved by the Institutional Animal Ethical Committee (IAEC) of Gautham College of Pharmacy, Bangalore (REF-IAEC/021/12/2010) according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg No: 491/01/c/CPCSEA), Govt. of India.

Determination of acute toxicity

Experimental animals and procedure

Foe the determination of the dose $LD_{50,}$ albino rats of weighing 160–220g were used for the study. They were nulliparous and non-pregnant. These were acclimatized to laboratory conditions for one week prior to start of dosing. The *C. pareira* extract was suspended in distilled water by using tween 80, to prepare a dose of 2g/kg. The doses were selected according to the OECD guideline no.425. The procedure was divided into two phases. Phase I (observation made on day one) and phase II (observed the animals for next 14 days of drug administration). Two sets of healthy rats (each set of 3 rats) were used for this experiment. First set of animals were divided into three groups, each of one in a group. Animal were divided into three groups, each of one in a group. Animals were fasted overnight with water ad libitum. Animals received a single dose of 2g/kg was selected for the test, as the test item was a source from herb. After administration of extract, food was withheld for 3–4 hrs. if the animal dies, conduct the main test to determine the $LD_{50.}$ If the animal survives, dose four additional animals sequentially so that a total of five animals are tested. However, if three animals die, the limit test is terminated and the main test is performed. The LD_{50} is greater than 2g/kg, if three or more animals survive. If an animal unexpectedly dies late in the study, and there are other survivors, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period. Late deaths should be counted the same as other deaths. The same procedure was repeated with another set of animals to nullify the errors.

Evaluation of nephroprotective activity[7,8]

Rats were randomly assigned into 4 groups of each 6 animals each. Group-I was kept as normal control receiving saline (0.5 mL, i.p.) for 8 consecutive days and animals of groups II,III and IV were administered gentamicin 80 mg/kg/day, i.p. for 8 consecutive days. Group I and II received vehicle by oral gavage for 11 days and group III and IV were orally administered with hydroalcoholic extract of *C. pareira* suspended in tween 80 at a dose of 200 mg/kg and 400 mg/kg which was started 3 days prior to the gentamicin treatment and continued along with 8 days gentamicin treatment.

Group-I: Kept as normal control received saline (0.5 mL, i.p.) for 8 days.

Group-II: Gentamicin treated group, (80 mg/kg/day, i.p.) for 8 consecutive days.

Group-III: Gentamicin (80 mg/kg/day, i.p) nearly for 8 days in addition to this they also received 200 mg/kg, p.o. of hydroalcoholic extract of *C. pareira* which was started 3 days prior to the gentamicin treatment and continued with eight days gentamicin treatment.

Group-IV: Gentamicin (80 mg/kg/day, i.p) nearly for8 days in addition to this they also received 400 mg/kg, p.o. of hydroalcoholic extract of *C. pareira* which was started 3 days prior to the gentamicin treatment and continued with eight days gentamicin treatment.

At the end of the study, the animals were kept in individual metabolic cages for 24-hours urine collection. At the last day the animals were sacrificed under combination of ketamine (60 mg/kg) and xylazine (5 mg/kg) given intraperitoneally. Blood samples were collected via cardiac puncture in plain plastic tubes, kept aside for 1 hour at 4 ºC and centrifuged to separate serum. The serum was processed for the estimation of serum creatinine, serum urea. The urine samples were collected for the estimation of urinary glucose, urinary creatinine levels. The kidney samples were used for the *in-vivo* antioxidant studies.

In-vitro antioxidant studies

DPPH assay[9]

The free radical scavenging activity of hydroalcoholic extract was measured by 1,1 diphenyl-2-picryl-hydrazilDPPH), the slight modified spectrophotometric method. A solution of DPPH in methanol (6×10^{-5} M) was prepared freshly. A 3 ml aliquot of this solution was mixed with 1 ml *C. pareira* hydroalcoholic extract samples at varying concentrations (50–250 µg/mL). The solution in the test tube were shaken well and incubated in the dark for 15 min. at room temperature. The decrease in absorbance was measured at 517 nm. Ascorbic acid was used as reference material. All the tests were performed in triplicate and the graph was plotted with the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples.

The percentage inhibition was calculated by using the formula.

$$\% \text{ inhibition} = \{A_{control} - A_{sample} / A_{control}\} \times 100\ \%$$

Reducing power assay[10]

Different concentrations of the *C. pareira* hydroalcoholic extract (50–250 µg/mL) in 1 mL of distilled water were mixed in to the mixture of 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50º C for 20 min. following incubation, 2.5 mi of 10% trichloro acetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1 %) and the absorbance was measured at 700 nm. Ascorbic acid was used as the reference material. All the tests were performed in triplicate and the results averaged. Increased absorbance of the reaction mixture indicated the increased reducing power.

The % reducing power was calculated by using the formula

$$\% \text{ increase in absorbance} = \{A_{test} - A_{control} / A_{control}\} \times 100\ \%$$

In-vivo antioxidant studies

Glutathione estimation[11]

Tissue samples were homogenized in ice cold trichloroacetic acid (1 gm tissue plus 10 mL 10% TCA) in a tissue homogenizer. Glutathione measurements were performed using a modification of the Ellamn procedure (Aykae, et.all.,) Briefly, after centrifugation at 3000 rpm for 10 min. 0.5 mL supernatant was added to 2 ml of 0.3 M disodium hydrogen phosphate solution. A 0.2 mL solution of dithiobisnitrobenzoate (0.4 mg/mL in 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing.

% increase in absorbance is directly proportional to the increase in the levels of glutathione. Hence, % increase in absorbance is calculated.

Lipid peroxidation[11]

Stock solution of TCA-TBA-HCl reagent: 15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N hydrochloric acid. This solution may be mildly heated to assist in the dissolution of the thiobarbituric acid.

Combine 1.0 ml of biological sample (0.1–2.0 mg of membrane protein or 0.1–0.2 µmol of lipid phosphate) with 2.0 ml of TCA-TBA-HCl and mix thoroughly. The solution is heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. the absorbance of

the sample is determined at 535 nm against a blank that contains all the reagents minus the lipid. % decrease in absorbance is directly proportional to the decrease in the levels of lipid peroxidation. Hence, % decrease in absorbance is calculated.

Statistical analysis

The values were expressed as Mean ± SEM. The data analysed by using one way ANOVA followed by Dunnett's test using Graph pad prism software. Statistical significance was set at $P \leq 0.05$.

RESULTS

Phytochemical investigation

Phytochemical investigation revealed that the plant extract contains alkaloids, flavanoids, carbohydrates, glycosides, proteins, tannins but saponins are absent.

Acute toxicity study

In both phase I and II procedures, none of the animals did not show any toxicity upon single administration of *C. pareira* hydroalcoholic extract (2000 mg/kg, p.o). Thus, a low dose 200 mg/kg, p.o and a high dose 400 mg/kg, p.o were selected for the present study.

Effect on physical and biochemical parameters

Treatment of gentamicin, 80 mg/kg/day, i.p for 8 consecutive days, produced significant nephrotoxicity in rats. Change in body weight, there was significant decrease in body weight ($p<0.001$) (Table 1 and Figure 1). Urinary levels of glucose, creatinine and serum levels of urea, creatinine, there was significant increase in urinary glucose ($p<0.001$), serum urea ($p<0.001$), serum creatinine ($p<0.001$) and significant decrease in urinary creatinine ($p<0.05$) when compared with the normal control group (Table 2 and Figure 2, 3, 4 and 5) . Whereas co-administration of *C. pareira* hydroalcoholic extract at a dose of 200 and 400 mg/kg/day was found to significantly improved in change in body weight, urinary creatinine and significantly decreased urinary glucose, serum urea, serum creatinine when compared to gentamicin treated group.

In-vitro antioxidant

The *C. pareira* hydroalcoholic extract shown significant dose dependent increase in the DPPH radical scavenging

Table 1: Effect of *C. pareira* hydroalcoholic extract on physical parameter (change in body weight)

Group	Treatment	Change in body weight(g)
Group-I	Normal saline	9.833 ± 1.327
Group-II	Gentamicin 80 mg/kg. I.P	-10.50 ± 2.872***
Group-III	Gentamicin + HACP 200 mg/kg. P.O	1.500 ± 2.078**
Group-IV	Gentamicin + HACP 400 mg/kg. P.O	4.00 ± 2.543***

Values are mean ± SEM; n=6 in each group;*** significantly different at P<0.001,** significantly different at P<0.01.

Table 2: Effect of *C. pareira* hydroalcoholic extract on biochemical parameters (mg/dL)

Group	Treatment	Urinary glucose	Urinary creatinine	Serum urea	Serum creatinine
Group-I	Normal saline	1.987 ± 1.028	3.997 ± 0.487	26.35 ± 0.870	0.551 ± 0.204
Group-II	Gentamicin 80 mg/kg. I.P	95.55 ± 10.28***	1.775 ± 0.410*	66.94 ± 7.223***	2.108 ± 0.317***
Group-III	Gentamicin + HACP 200 mg/kg. P.O	27.81 ± 7.410***	1.775 ± 0.410**	35.56 ± 4.385***	1.218 ± 0.205*
Group-IV	Gentamicin + HACP 400 mg/kg. P.O	18.12 ± 4.450***	4.442 ± 0.612**	28.06 ± 3.632***	0.773 ± 0.204**

Values are mean ± SEM; n=6 in each group;*** significantly different at P<0.001,** significantly different at P<0.01,* significantly different at P<0.05.

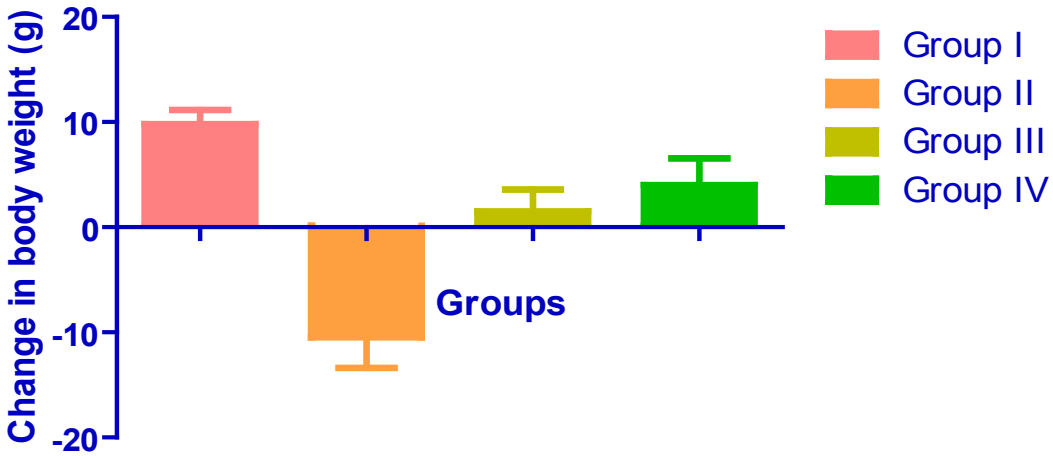

Figure 1: Effect of HACP on Change in Body Weights in Gentamicin Induced Nephrotoxic Rats.

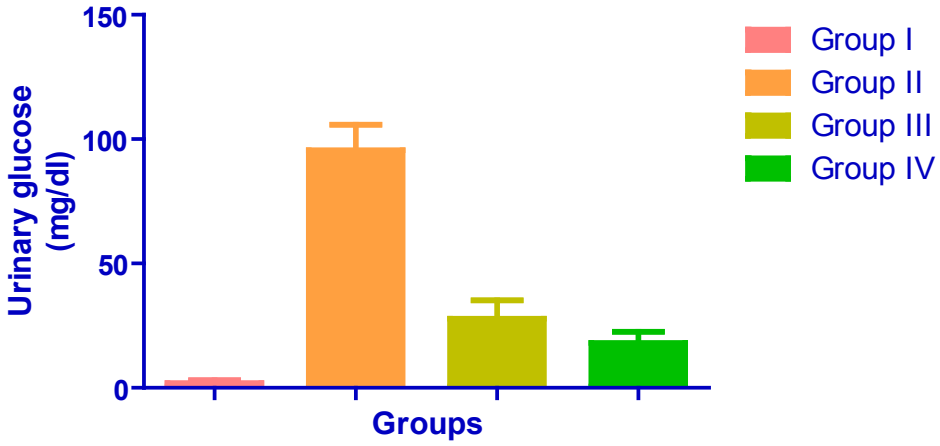

Figure 2: Effect of HACP on Urinary Glucose Levels in Gentamicin Induced Nephrotoxic Rats.

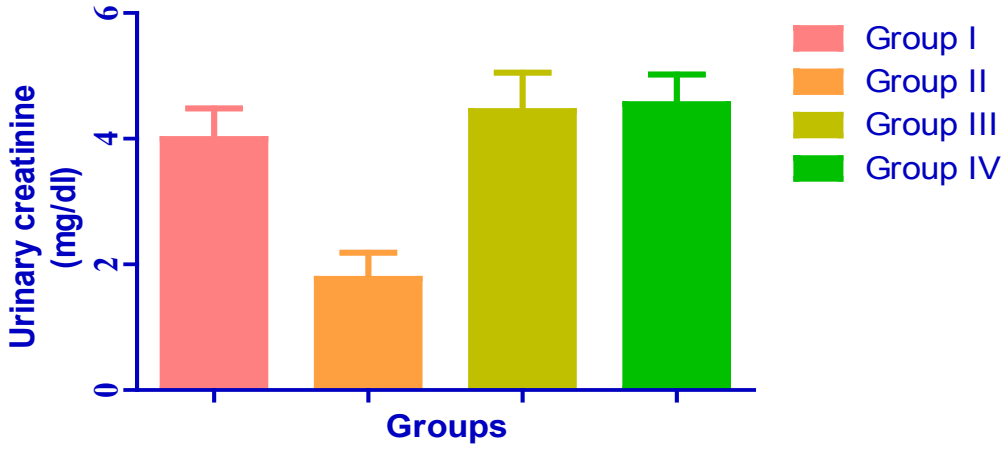

Figure 3: Effect of HACP on Urinary Creatinine Levels in Gentamicin Induced Nephrotoxic Rats.

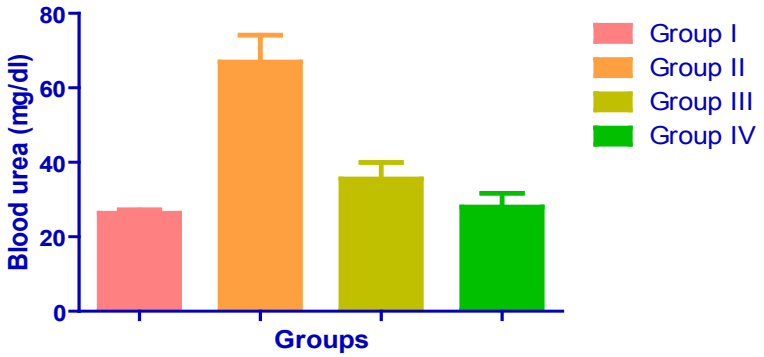

Figure 4: Effect of HACP on Blood Urea Levels in Gentamicin Induced Nephrotoxic Rats.

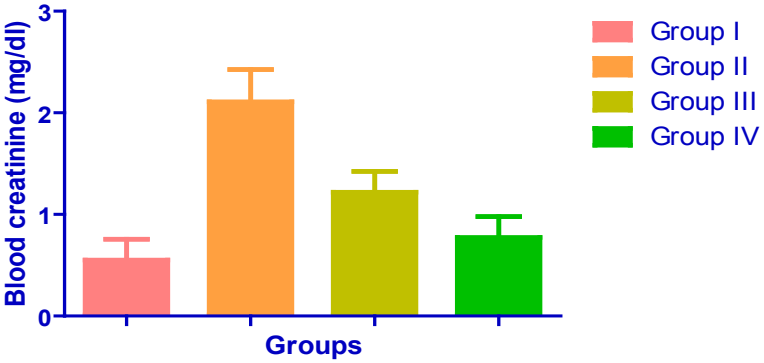

Figure 5: Effect of HACP on Blood Creatinine Levels in Gentamicin Induced Nephrotoxic Rats.

activity, and also significant dose dependent increase in the reducing property (Table 3 and Figure 6 and 7).

In-vivo antioxidant

There was a marked increase in lipid peroxidation in gentamicin treated rat kidneys whereas *C. pareira* hydroalcoholic extract had shown dose dependent inhibition of lipid peroxidation levels in rat kidneys, 200 mg/kg/day p.o and 400 mg/kg/day p.o had shown 58.91% , 72.00% inhibition respectively, There was a marked depletion of glutathione levels in gentamicin treated rat kidneys whereas *C. pareira* hydroalcoholic extract showed a dose dependent increase in the levels of glutathione, 200 mg/kg/day p.o and 400 mg/kg/day p.o had shown 36.01%,

Table 3: *In-vitro* antioxidant activity, DPPH and reducing power of *C. pareira* hydroalcoholic extract

Group	DPPH Absorbance	DPPH % Decrease	Reducing power Absorbance	Reducing power % Increase
Control	1.253 ± 0.023	–	0.276 ± 0.008	–
Ascorbic acid 50 µg	0.107 ± 0.003***	91.46	0.533 ± 0.002***	93.11
HACP 50 µg	1.166 ± 0.003***	6.94	0.340 ± 0.011***	23.18
HACP 100 µg	1.034 ± 0.005***	17.47	0.385 ± 0.007***	39.49
HACP 150 µg	0.917 ± 0.001***	26.81	0.424 ± 0.002***	53.62
HACP 200 µg	0.739 ± 0.002***	41.02	0.452 ± 0.004***	63.76
HACP 250 µg	0.519 ± 0.003***	58.57	0.486 ± 0.002***	76.08

Values are mean ± SEM; n=6 in each group;*** significantly different at P<0.001.

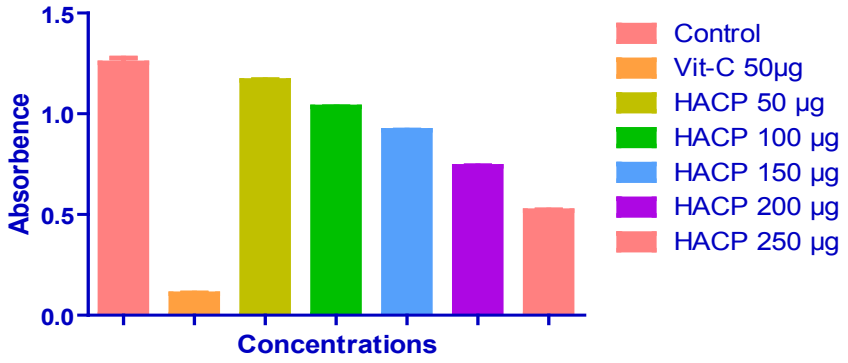

Figure 6: Effect of HACP on DPPH Radical Scavenging Activity

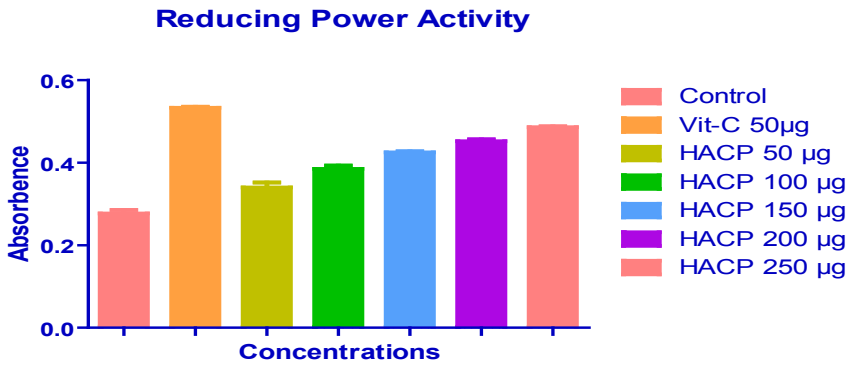

Figure 7: Effect of HACP on Reducing Power Activity

65.72% increase in glutathione levels respectively in rat kidneys (Table 4 and Figure 8 and 9).

DISCUSSION

Gentamicin is an important aminoglycoside antibiotic which is effective against gram negative microorganisms both in human and animals. Gentamicin is slightly bound to plasma proteins and is not metabolized in the body. It is excreted in unmodified form by the kidney[12,13]. Gentamicin, an effective and widely used aminoglycoside antibiotic against severe infections is known to be potentially nephrotoxic despite close attention to its pharmacokinetics and dosing schedules that limits its long term clinical use[14]. Several strategies, mechanisms and agents were utilized to prevent gentamicin nephropathy in animal model, however, their use to treat human subjects in clinical practice could not be achieved [15,16]. The real mechanism

Table 4: Effects of *C. pareira* hydroalcoholic extract on tissue lipid peroxidation (LP) and glutathione (GSH).

Group	Treatment	LP Absorbance	LP % Inhibition	GSH Absorbance	GSH % Increase
Group-I	Normal Saline	0.129 ± 0.006	–	1.885 ± 0.019	–
Group-II	Gentamicin 80 mg/kg. I.P	0.443 ± 0.015***	–	1.03 ± 0.033***	–
Group-III	Gentamicin + HACP 200 mg/kg. O.P	0.182 ± 0.009***	58.91	1.401 ± 0.016***	36.01
Group-IV	Gentamicin + HACP 400 mg/kg. O.P	0.124 ± 0.004***	72.00	1.701 ± 0.006***	65.72

Values are mean ± SEM; n=6 in each group; *** significantly different at P<0.001.

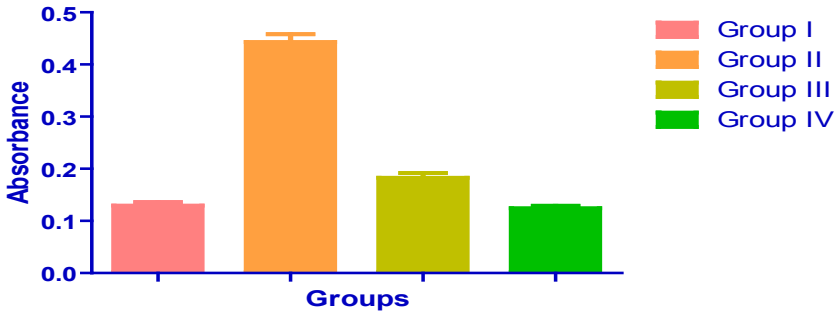

Figure 8: Effect of HACP on Tissue Lipid Peroxidation in Gentamicin Induced ephrotoxicity.

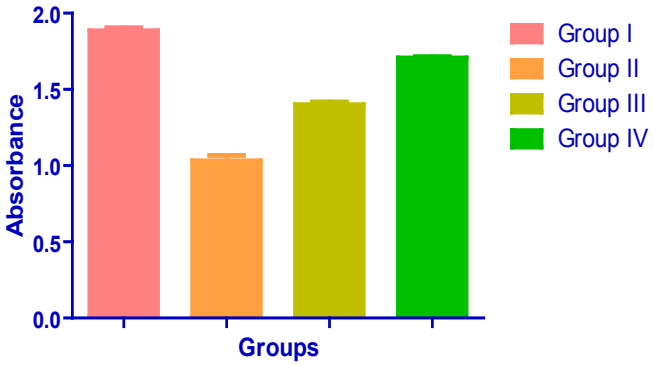

Figure 9: Effect of HACP on Tissue Glutathione in Gentamicin Induced Nephrotoxicity.

by which gentamicin has been shown both *in-vitro* and *in-vivo* studies to enhance the generation of reactive oxygen species (ROS). Abnormal production of ROS may damage some macromolecules, to induce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage [17,18]. Therefore, the diminution of nephrotoxicity would enhance its clinical value. Several agents that scavenge or interfere with the production of ROS have been used successfully to ameliorate gentamicin nephropathy[19]. In this present study, we identified that *C. pareira* hydroalcoholic extract had shown nephroprotective activity against gentamicin induced nephrotoxicity by its antioxidant activity. It was also found to be hepatoprotective[20], antinociceptive and antiarthritic[21], anti-inflammatory[22], and antifertility[23] activities besides other health benefits.

From the result of our study it was shown that administration of gentamicin elevated the levels of urinary glucose, serum urea, serum creatinine and lipid peroxidation levels in the kidneys whereas decreased urinary creatinine and glutathione levels in the kidneys. These results are in good agreement with those previously reported[24,3]. Our results also showed that treatment with different doses of *C. pareira* reduced the levels of urinary glucose, serum urea, serum creatinine and lipid peoxidation in kidneys whereas increases the urinary creatinine and glutathione levels in kidneys, dose dependently. *In-vitro* antioxidant study, DPPH radical scavenging activity and reducing power activity of the *C. pareira* hydroalcoholic extract had shown that the plan extract is having antioxidant activity. Hence natural and synthetic antioxidants and free radical scavengers are claimed to provide nephroprotective action against gentamicin induced nephrotoxicity. The probable mechanism of nephroprotection by *C. pareira* hydroalcoholic extract may be due to its antioxidant and free radical scavenging activity which increases renal mitochondrial antioxidant system[25], so thereby it can protect the kidneys from gentamicin toxicity. We are concluding that the *C. pareira* hydroalcoholic extract may have promising role in the treatment of gentamicin induced nephrotoxicity. However, further studies are needed to identify and isolate the active constituent which is responsible for the protective activity.

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Effect of *Tribulus Terrestris* on Learning and Memory in Wistar Rats

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ABSTRACT

Objectives: The present study was designed to evaluate the effect of aqueous extract of fruits of *Tribulus terrestris* on learning and memory in rodents. **Materials and Methods:** Thirty wistar rats were divided in 5 groups of 6 rats each. Baseline values for the time taken to reach reward chamber (TRC) in the Hebb William Maze and transfer latency (TL) in the T-maze were recorded on Day 1. Mean of 5 sessions was calculated for each rat. Group I was normal control, group II piracetam standard, group III, IV and V received *Tribulus terrestris* orally at 100mg/kg, 200mg/kg and 400mg/kg respectively for 14 days. At the end of 14 days, each rat was tested for TRC and TL and compared with the control group. **Results:** Group IV showed a significant decrease in TRC when compared to group I in Hebb William Maze ($p < 0.0001$). Group IV also showed a significant decrease in TL when compared to group I in T-maze ($p < 0.0001$). Group III showed a significant decrease in TL when compared to group I in the T-maze ($p = 0.035$), however there was no decrease in TRC in this group. **Conclusions:** The aqueous extract of fruits of *Tribulus terrestris* showed a dose dependent beneficial effect in learning and memory models in rats, with 200mg/kg being most beneficial.

Keywords: Hebb William Maze, Learning, Memory, T Maze, Tribulus terrestris

INTRODUCTION

Learning is defined as the acquisition of information and skills and subsequent retention of that information is called memory. Understanding the cellular basis of learning and memory is clearly one of the most challenging problems in science and is of enormous interest to every person. The possible benefits to everyone in general are almost unlimited. They might range from combating diseases such as Alzheimer's disease to helping an individual, store and recall information better. A number of agents have been tried on various behavioural and pharmacological models for evaluation of the process of learning and memory.

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The models can be broadly classified as exteroceptive and interoceptive aversion stimuli models[1]. The exteroceptive models consist of behaviour on mazes such as T maze and avoidance behaviour on shuttle box. Interoceptive models include scopolamine induced amnesia, electroshock induced amnesia and hypoxic stress induced learning deficits.

Tribulus terrestris is a flowering plant from the family Zygophallaceae and is native to warm and tropical regions such as southern Asia. The flowers are 4–10 mm wide, with five lemon yellow petals. A week after each flowerblooms, it is followed by a fruit that easily falls apart into four or five single-seeded nutlets. It is used as a tonic, aphrodisiac, analgesic, astringent, anti-hypertensive, diuretic, and urinary anti-infective[2]. Its aqueous extract has shown diminution of oxalate induced renal tubular epithelial cell injury and inhibition of calcium oxalate crystallization in vitro[3]. Tribulosin, a methanolic extract of *Tribulus terrestris* protects rat heart from ischemia/perfusion injury[4]. Despite its aphrodisiac activity, it has shown no effect on endocrine sensitive tissues like prostrate, seminal vesicle, uterus and vagina in wistar rat pointing to the lack of androgenic and estrogenic activity in vivo[5].

Since its effect on learning and memory hasn't been explored, the present study was designed to evaluate the effect of aqueous extract of fruits of *Tribulus terrestris* on learning and memory in rodent behavioral models of learning and memory.

MATERIALS AND METHODS

Experimental animals

Wistar albino male rats weighing 180–220 grams inbred in the institutional animal house were used for the study. Rats were housed in clean polypropylene cages, three rats were kept in each cage, in a controlled environment (22°–24°c) with a 12 hour light and dark cycle with standard chow containing fat 4.15%, protein 22.15%, carbohydrates 4% (supplied by Amruth laboratory animal feed manufactured by Pranav Agro industries ltd., Sangli) and water *ad libitum*. The rats were allowed to acclimatise to these conditions for one week. Experiments were performed during the light phase of the cycle (10:00–17:00) in the Department of Pharmacology, Kasturba Medical College, Mangalore.

Study material

Tribulus terrestris fruit powder, obtained from Himalaya Health Care, Bangalore (gift sample), was dissolved in distilled water and administered to the rats in the doses of 100 mg/kg, 200 mg/kg and 400mg/kg.

Study procedure

The rodent behavioural models of learning and memory used for the study were Hebb William Maze and the T maze.

Hebb-William Maze is an incentive based exteroceptive behavioural model useful for measuring spatial and working memory of rats. It mainly consists of three compartments: chamber B, in which the rat is placed and has a sliding door that is opened to allow the rat to enter the middle chamber, (exploratory area of maze), the door of which is closed to prevent its back entry to chamber B. The animal has to explore the middle chamber and reach the reward chamber A, at the other end of the maze in which the reward (food) is kept. An electrical system provides indication when the rat is placed in chamber B, when it leaves it to enter the maze i.e. middle chamber and when it enters reward chamber A, thus enabling the reaction time to be noted without observing the animal. Time taken to reach the reward chamber (TRC) was recorded by the digital timer of the Hebb William Maze. Each rat was allowed to explore the maze for an additional 20seconds with all the doors opened before returning to its cage. Twelve hours fasted rats were employed in the study and the average of 5 sessions were recorded for each rat[6].

T - Maze serves as an exteroceptive behavioural model to evaluate learning and memory in rats. The apparatus consists of one open arm (50cm x 10cm) and two covered arms (50cm x 10cm x 40cm). The central platform (10cm x 10cm) and the maze is elevated to a height of 50cm from the ground. Each rat was placed at the end of the open arm, facing away from the central platform. Rats prefer to be in the covered arms. Transfer latency (TL) was taken as the time taken by the rat to move into the covered arms with all its four limbs. The rat was allowed to explore the maze for an additional 20 seconds before returning to its home cage[7–9]. The average of TL of 5 sessions was recorded.

Thirty wistar rats were divided in 5 groups of 6 rats each. Baseline values for the time taken to reach reward chamber (TRC) by Hebb William Maze and transfer latency (TL) by T-maze were recorded on Day 1. A mean of 5 sessions was calculated for each rat. Group I was normal control, group II piracetam standard intraperitoneally at 200mg/kg, group III, IV and V received *Tribulus terrestris* orally at 100mg/kg, 200mg/kg and 400mg/kg respectively for 14 days as shown in Table 1. At the end of 14 days, each rat was tested for TRC and TL and compared with the control group.

Statistical Analysis

The results were expressed as Mean ± Standard Deviation (SD). Statistical analysis was done using one way ANOVA followed by Tukey post hoc test, using SPSS version 11.5. The value of $p<0.05$ was considered significant. Analysis within groups between day 1 and day 14 in Hebb William Maze and T Maze was done by paired t test.

RESULTS

Hebb William Maze

In the analysis of the TRC in Hebb William Maze as shown in Table 2, all groups showed reduction compared to baseline. Group II receiving piracetam showed a significant reduction in TRC when compared to Group I, III and V ($p<0.001$). But no significant difference was seen when compared with Group IV. Group IV showed significant reduction in TRC when compared with Group

I

Table 1: Drugs along with the respective route administered to the animals

Group No.	Group	Test/standard drug treatment	Dose	Route	Duration of treatment
I	Normal control	2% Gum acacia	10 ml/kg	Oral	14 days
II	Piracetam control	Piracetam	200mg/kg	i.p	14 days
III	TT1	*Tribulus terrestris*	100 mg/kg	Oral	14 days
IV	TT2	*Tribulus terrestris*	200 mg/kg	Oral	14 days
V	TT3	*Tribulus terrestris*	400 mg/kg	Oral	14 days

TT- *Tribulus terrestris*. *Tribulus terrestris* was given at 3 different doses i.e. TT1, TT2 and TT3. i.p- intraperitoneal, ml- milli litre, mg- milli gram and kg- kilogram.

II

Table 2: Results of Hebb William Maze and T Maze

Group No.	Hebb William Maze: TRC (sec)		T Maze: TL (sec)	
	Day 1	Day 14	Day 1	Day 14
I	120.27 ± 14.51	76.63 ± 4.88	133.71 ± 7.93	139.46 ± 6.31
II	115.39 ± 4.18	47.46 ± 3.61*	132.85 ± 3.50	94.53 ± 2.04$^{£}$
III	123.42 ± 6.13	74.75 ± 5.86	133.95 ± 2.52	130.36 ± 7.39$^{€}$
IV	122.92 ± 10.89	54.18 ± 3.86$^{\$}$	135.16 ± 5.32	126.56 ± 3.29$^{\#}$
V	121.59 ± 9.30	73.19 ± 5.56	131.42 ± 5.43	133.40 ± 4.49

The data are expressed as Mean ± SD ; TRC- Time to reach Reward Chamber, TL- Transfer Latency ; *p<0.001 vs Group I, III, V ; $^{\$}$p<0.001 vs Group I, III, V ; $^{\#}$p<0.001 vs Group I ; $^{€}$p<0.05 vs Group I ; $^{£}$p<0.001 vs Group I, III, IV, V

III

Table 3 : Results of analysis within groups for the difference between Day 1 and 14 in Hebb William Maze and T maze

Group No.	Hebb William Maze: TRC (sec)	T Maze: TL (sec)
I	43.63 ± 13.68**	-5.75 ± 12.09
II	67.93 ± 2.93**	38.31 ± 2.65**
III	48.67 ± 9.77**	3.59 ± 7.57
IV	68.74 ± 13.61**	8.59 ± 6.93*
V	48.39 ± 6.42**	-1.98 ± 3.80

The data are expressed as Mean ± SD ; TRC- Time to reach Reward Chamber, TL- Transfer Latency ; **p<0.001, *p<0.05

I, III and V ($p<0.001$). As shown in Table 3, difference between day 1 and day 14 of TRC in all the groups was found to be statistically significant ($p<0.001$).

T Maze

As shown in Table 2, Group IV also showed a significant decrease in TL when compared to Group I in T-maze ($p<0.001$). Group III showed a significant decrease in TL when compared to Group I for the T-maze ($p=0.035$). Group II was found to be superior to all the other groups with regard to TL ($p<0.001$). As shown in Table 3, difference between day 1 and day 14 of TL in Group I ($p<0.001$) and Group IV ($p=0.029$) was found to be statistically significant.

DISCUSSION

Tribulus terrestris commonly known as "Gokshura" in India is a plant with multiple uses. It has been found to be useful in multiple conditions from erectile dysfunction and kidney dysfunction to urinary infections.

Hebb William Maze and T Maze behavioural models have been utilized for the assessment of learning and memory in rats in this study. A fall in TRC and TL on subsequent exposures to the Hebb William Maze and the T maze respectively, was taken as an index of successful retention[9].

Piracetam showed significantly reduced values of TRC when compared with all the groups except, the group that received *Tribulus terrestris* at a dose of 200mg/kg. This indicates that *Tribulus terrestris* at a dose of 200mg/kg was on par with piracetam with regard to reducing the TRC in the Hebb William Maze after 14 days. *Tribulus terrestris* at a dose of 200mg/kg also showed improved results when compared to the same being given at doses of 100mg/kg, 400mg/kg and also with the normal control.

Piracetam showed significantly reduced values of TL when compared to all the other groups. *Tribulus terrestris* at a dose of 200mg/kg showed significantly better results when compared to the normal control. *Tribulus terrestris* at a dose of 400mg/kg did not show significant difference in the reduction of TL. But, *Tribulus terrestris* at a dose of 100mg/kg also showed improved results when compared to normal control, but no decrease in TRC was noticed with this group. This indicates that *Tribulus terrestris* only at a dose of 200mg/kg could significantly reduce both TRC and TL.

In the study done by Martino Andrade AJ *et al*, *Tribulus terrestris* was used at a dose of 11, 42 and 110mg/kg/day and was unable to stimulate the endocrine sensitive tissues like prostate, seminal vesicle, uterus and vagina[5]. Another study, done by Jungmo Do *et al*, *Tribulus terrestris* showed a dose dependent increase in the Intracavernous Pressure after being administered for one month at a dose of 2.5, 5, 10, 50 and 100mg/kg in rabbits, thus demonstrating its use as a potential agent for erectile dysfunction[10].

Our study, is the first study exploring the effect of *Tribulus terrestris* on learning and memory in rats. The aqueous extract of fruits of *Tribulus terrestris* showed a dose dependent beneficial effect in learning and memory models in rats, with 200mg/kg being most beneficial.

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Physico Chemical Characterization and Anti Inflammatory Activity of Stem Extracts of *Berberis aristata* DC and *Cosinium fenestratum* Linn in Carrageenan Induced Wistar Rats

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ABSTRACT

An initial study on the physico-chemical properties of *Berberis aristata and Cosinium fenestratum*, both used as Daruharidra by the Ayurvedic drug industry, recorded all the tested parameters within the limits of Ayurvedic Pharmacopeia of India. Also, analysis of secondary metabolite of the plants was carried out focusing on the presence and quantification of berberine in the samples. Further, the potential difference in the anti-inflammatory activity of the aqueous and methanolic stem extracts of the two species was compared using carrageenan induced Wistar rats model. At a dose of 25 mg/kg, the aqueous and methanolic crude extracts of both the plant species showed significant inhibition of rat paw edema at various time intervals viz.,0, 30, 60, 120 and 240 minutes as against the control standard drug indomethacin ($p<0.01$, $p<0.001$).

Keywords: antiinflammation, Berberine, *Berberis aristata, Cosinium fenestratum*, Physico-chemical analysis.

INTRODUCTION

Herbal medicines are at great demand globally for primary healthcare due to their higher safety margins and cost effectiveness. Quality control of herbal medicines generates a lot of problems involving even the selection of the right kind of plant material for therapeutically efficacious compounds. Herbal medicines are being manufactured on large scale where manufacturers face many problems such as low-quality raw material, lack of authentication of raw material, non-availability of standards, lack of proper standardization methodologies of single drugs and formulations and lack of quality control parameters. Classical evaluation of herbal drugs is available based on Rasa, Guna, Virya, Vipaka and Karma etc.

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In the global view although there is shift towards the use of herbal medicine, consumers prefer to choose products with established standards. Therefore, it is a prime need to standardize Ayurvedic preparations to guarantee their purity, safety, potency and efficacy.

Herbal products represent a number of unique problems related to quality which are further complicated by the use of combination of herbal ingredients being used in traditional practice. Therefore, in case of herbal drugs and products the standardization should encompass entire field of study from cultivation of medicinal plant to its clinical application. WHO involves in standardization and quality control of herbal crude drugs[1] to monitor the physicochemical evaluation of crude drugs covering the aspects of selection and handling of crude material, safety, efficacy, stability assessment of finished product, documentation of safety and risk based on experience, provision of product information to consumer and product promotion.

Daruharidra, an important ingredient of traditional Indian ayurvedic system of medicine, is continuously used

for health care in India and other parts of the globe[2,3]. Ayurvedic Pharmacopeia of India correlates Daruharidra to *Berberis aristata* DC of family Berberidaceae, a spinous shrub native to mountains of North India and Nepal and commonly known as 'Daruhaldi'[4,5]. In South India, *Cosinium fenestratum* is traded as substitute for *B. aristata* in the name of Daruharidra[3–17] and native to Western Ghats as 'tree turmeric'. Daruharidra is useful in a vast range of disease conditions including inflammations, wounds, ulcers, jaundice, burns, skin diseases, abdominal disorders, diabetes, fever and general debility[2,6,7]. Both *B. aristata* and *C. fenestratum* are reported to possess the alkaloid called berberine, which is known to possess anti-inflammatory property for various ailments[8]. The ethnobotanical studies conducted on albino rats using alcoholic and aqueous root extracts of *B. aristata* showed significant activity on acute inflammation after two hours of carrageenan injection[9]. Studies also indicated that the topical instillation of aqueous extracts of *Curcuma longa* and *B. aristata* showed potent anti-inflammatory activity against endotoxin induced uveitis in rabbits[10]. Though the above studies report the anti-inflammatory property of *B. aristata* for various infections, no valid scientific evidence is available to legitimise the use of *C. fenestratum* as a substitute of *B. aristata.*

The present study was attempted to evaluate and compare the physico chemical properties of *B. aristata* and *C. fenestratum,* both traded as Daruharidra, and the anti-inflammatory activities of their aqueous and methanolic stem extracts in carrageenan induced Wistar rats for drug validation.

MATERIALS AND METHODS

Plant collection

B. aristata and *C. fenestratum* samples were collected and vouched by qualified taxonomists from the Institute of Ayurveda and Integrative Medicine (IAIM) Herbarium. Voucher specimens were deposited in the Repository of Medicinal Resources at IAIM, Bangalore, Karnataka, India.

Extraction of plant material

The extraction of stem samples was carried out using methanol[11].

Physiochemical Analysis

The following physicochemical parameters were analysed based on the protocols of Ayurvedic Pharmacopeia of India (API)[5]

Foreign Organic Matter: Foreign matter is a material consisting of parts of the medicinal plant material or materials other than those named, any organism, part of product of an organism, other than that named in the specification and description of the plant material concern. Foreign matter consists of mineral admixtures adhering to the medicinal plant materials, such as soil, stones, sand and dust. Known quantity of sample was weighed and spread in a thin layer and the foreign matter was sorted into groups either by visual inspection or with the help of magnifier. The sorted foreign matter was weighed and expressed as % foreign matter.

Moisture content: Moisture content of the samples was determined using infrared moisture balance model-M-3A Deluxe Voltage-230VAC (Advance Research Instrument Co).

Total Ash values: The total ash method is designed to measure the total amount of material remaining after ignition. This includes both "physiological ash", which is derived from the plant tissue itself, and "non physiological ash", which is residue of extraneous matter (e.g. sand and soil) adhering to the plant surface. When vegetable drugs are incinerated, they leave an inorganic ash. In some plants, the total ash is of importance and indicates the extent of care taken in the preparation of the drug. Carbon must be removed at as low a temperature (450°C) as possible because alkali chlorides, which may be volatile at high temperatures, would otherwise is lost. The total ash usually contains carbonates, phosphates, silicates and silica. 4g of the ground air dried samples are weighed and the content is spread into an even thin layer on a previously ignited, dried and tarred silica crucible. Silica crucibles were placed in the muffle furnace and the temperature was adjusted 450–500°C and allowed to ignite until it was white, indicating the absence of carbon. Crucibles were removed from muffle furnace, allowed to cool for 30 minutes in a dessicator and weighed without delay.

The content of total ash in mg/g of air dried material was calculated and expressed as % Ash by the following formula:

$$\text{Total Ash \%} = (B\text{-}C) \times 100/A$$

Where, A-Sample weight in (g); B-Weight of dish + contents after drying (g); C-Weight of empty dish (g)

Acid insoluble ash: Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter.

This measures the amount of silica present, especially as sand and siliceous earth. To the silica crucible containing the total ash obtained, 25 ml of hydrochloric acid was added, covered with a watch glass and boiled gently for 5 minutes on a burner. The insoluble matter was collected on an ash less filter paper by filtration and rinsed this filter paper repeatedly with hot water until the filtrate is neutral/free from acid. The filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to a constant weight in the muffle furnace at 450–500°C. The silica crucible was removed from the muffle furnace and allowed to cool in a dessicator for 30 minutes, weighed without delay. The content of acid insoluble ash was calculated using the formula

$$\text{Acid insoluble ash \%} = (B-C) \times 100/A$$

Where, A- Sample weight in g; B - Weight of dish + contents after drying (g); C - Weight of empty dish (g).

Comparision of HPTLC fingerprints and Quantification of Berberine

Sample preparation: 1 g of selected stems (Mesh no. 85) was extracted in water bath with methanol and filtered through Whatman No.1 filter paper. The bark was subjected to extraction until colorless with the same menustrum. The extracts were combined and evaporated to dryness. The residue obtained was redissolved in methanol (10 ml) from which 1ml was taken and made upto 10 ml and aliquots were taken for TLC analysis[11].

Standard berberine hydrochloride preparation: Berberine hydrochloride was purchased from Natural Remedies Pvt. Ltd., Bangalore (90% pure, HPLC Grade). Standard stock solution of berberine (0.1 mg/ml) was prepared in methanol in a 10 ml volumetric flask.

Analysis was performed on 20 cm X 10 cm TLC silica gel 60 F254 plates. Standard and the *B. aristata* and *C. fenestratum* sample solutions were applied to the plate using Linomat 5 (Camag, Switzerland) automated spray- on band applicator equipped with a 100 microlitre Hamilton syringe and operated with the Band length - 8 mm, Dosage speed -150 nL/s, Distance between bands - 12.5 mm, Distance from the plate edge -15 mm and Distance from the bottom of the plate - 10 mm. Development of the plates was carried out allowing 10 min for solvent saturation of the twin – trough chamber (Camag, Switzerland) at ambient temperature. A solvent system consisting of n – butanol:glacial acetic acid:water (14:3:4, v/v/v) for a total of 21ml of volume of solvent mixture for the migration distance was 80 mm. After development, the plate was dried using a hair dryer and evaluated visible light, 254 nm, 366 nm and after derivatisation with anisaldehyde-sulfuric acid reagent. The images were recorded using Reprostar 3 (Camag, Switzerland). Rf values of the markers and the compounds of interest were measured. Quantification of berberine was done at 343 nm using a calibration curve.

Method validation: Linearity was determined by using a standard solution of 100 mg/10 ml in methanol (n=3). For calibration curves, an appropriate volume of berberine stock solution was diluted in methanol, and using HPTLC concentration levels ranging between 20 and 100 ng / spot (n=3) were analyzed.

Experimental Animals

Wistar rats of either sex weighing 180–220g were procured from Central Animal House, J.S.S. College of Pharmacy, Ootacamund, Tamilnadu, India. The temperature in the experimental room was at 22°C (± 4°C) with 60% ±2 RH with appropriate lighting (12h light and dark cycle). Animals were housed in polycarbonate cages with stainless steel metal grades in bottom and were accessed to unlimited water supply and food. Experimental protocol was approved from Institutional Animal Ethical Committee (IAEC) and carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) by the J.S.S. College of Pharmacy under JSSCP/IAEC/PH.D/PH.COLOGY/01/2012–13.

Wistar rats of 180–220g were divided into 6 groups with 6 animals in each group as follows. Carrageenan indicates 1% w/v solution is prepared by dissolving 100mg of carrageenan in 10ml of 0.9% w/v NaCl and Indomethacin suspension was prepared accurately suspending 100 mg of Indomethacin in 10ml of distilled water using 1%w/v CMC (carboxy methyl cellulose) as suspending agent. A stock solution was prepared containing 10mg/ml of the drug.

Group-I: Carrageenan (Normal control)

Group-II: Carrageenan + Indomethacin 10 mg/kg

Group-III: Carrageenan + *Berberisaristata* (Aqueous extract – 25mg/kg)

Group-IV: Carrageenan + *Cosiniumfenestratum* (Aqueous extract – 25 mg/kg)

Group-V: Carrageenan + *Berberisaristata* (Methanolic extract – 25 mg/kg)

Group-VI: Carrageenan + *Cosiniumfenestratum* (Methanolic extract – 25 mg/kg)

Acute paw edema was produced by injecting carrageenan 1% w/w (0.1ml) into the sub plantar region of the left hind paw in the rats. The methanolic and aqueous extracts (200 mg/kg) and Indomethacin 10 mg/kg administered orally one hour before testing. The control group received vehicle 0.1 ml/100gm. The paw volume was measured by using digital plethysmometer (UGO Basile. Italy) at 0, 30, 60, 120 and 240 minutes after carrageenan challenge[12].

Statistical analysis

Data obtained from this study was performed using one-way ANOVA followed by Bonferroni multiple comparison test at 95% significance and expressed as mean ± SEM

RESULTS

Physicochemical characterization of B. aristata and C. fenestratum samples

Physicochemical analyses of both the plant species recorded that all the parameters comply with API limits. The details are given in Table 1.

Table 1: Physicochemical characterization of *Berberis aristata* and *Coscinium fenestratum*

Parameters	*Berberis aristata*	*Coscinium fenestratum*	API Limits
Foreign organic matter (% w/w)	NIL	NIL	NMT 2 %
Moisture content (% w/w)	7.60	8.20	NA
Total Ash (% w/w)	2.17	2.11	NMT 14%
Acid insoluble ash (% w/w)	0.39	0.62	NMT 5 %
Alcohol soluble extractive (% w/w)	6.67	6.54	NLT 6 %
Water soluble extractive (% w/w)	9.36	12.46	NLT 8 %

NA- Not available; NMT - Not more than; NLT – Not less than

Quantification of Berberine by HPTLC

The linearity curve of Berberine was calculated and graphically represented with standard berberine using which the berberine content in *B. aristata* and *C. fenestratum* was detected and represented in Table 2.

Table 2: Quantification of Berberine by High Performance Thin Layer Chromatography

Sample	Berberine content (%)
Berberis aristata	0.180
Coscinium fenetratum	0.380

HPTLC Fingerprinting of samples

The HPTLC fingerprinting of *B. aristata* and *C. fenestratum* was compared. Standard berberine showed Rf value of 0.47 which was noticed in both the species (Table 3). Hence, the method is valid and can be used to distinguish *B.aristata* and *C. fenestratum*. Although very large variations were not seen between the two species, it can be inferred that the geographical locations among these two plants do not affect the amount of berberine present.

Experimental mice study

The details of inflammation induced in the different animal groups treated with 1% w/v carrageenan, standard

Table 3: Rf values *Berberis aristata* and *Coscinium fenestratum* samples and standard Berberine

Sample	UV 254nm	UV 366nm
Standard Berberine	0.47 (Dark)	0.38, 0.47 (Yellow fluorescent)
Berberis aristata	0.22 (Blue) 0.38, 0.47 (all Dark)	0.22 (Blue), 0.38, 0.47 (Yellow fluorescent)
Coscinium fenestratum	0.02, 0.22 (Blue) 0.38, 0.47, 0.68 (all Dark)	0.02 (Blue fluorescent) 0.38, 0.47 (Yellow fluorescent), 0.58 (Green) 0.77 (Blue)

drug indomethacin 10 mg/kg, and aqueous and methanolic stem extracts of *B. aristata* and *C. fenestratum* 25 mg/kg are provided in Figure 1. The stem extracts of *B. aristata* and *C. fenestratum*, and the standard drug indomethacin had significant anti-inflammatory activity on paw edema as against the control from 60 minutes onwards which was maintained up to 240 minutes(*p<0.01, p<0.001*). Though at 60 minute, indomethacin treated group showed significantly higher anti-inflammation than the rest of the groups, at subsequent time intervals of 120 and 240 minutes, no such significant difference was observed. There was no significant difference in the anti-inflammatory activity of *B. aristata* and *C. fenestratum*. The results were comparable with that of the standard drug indomethacin @10 mg/kg.

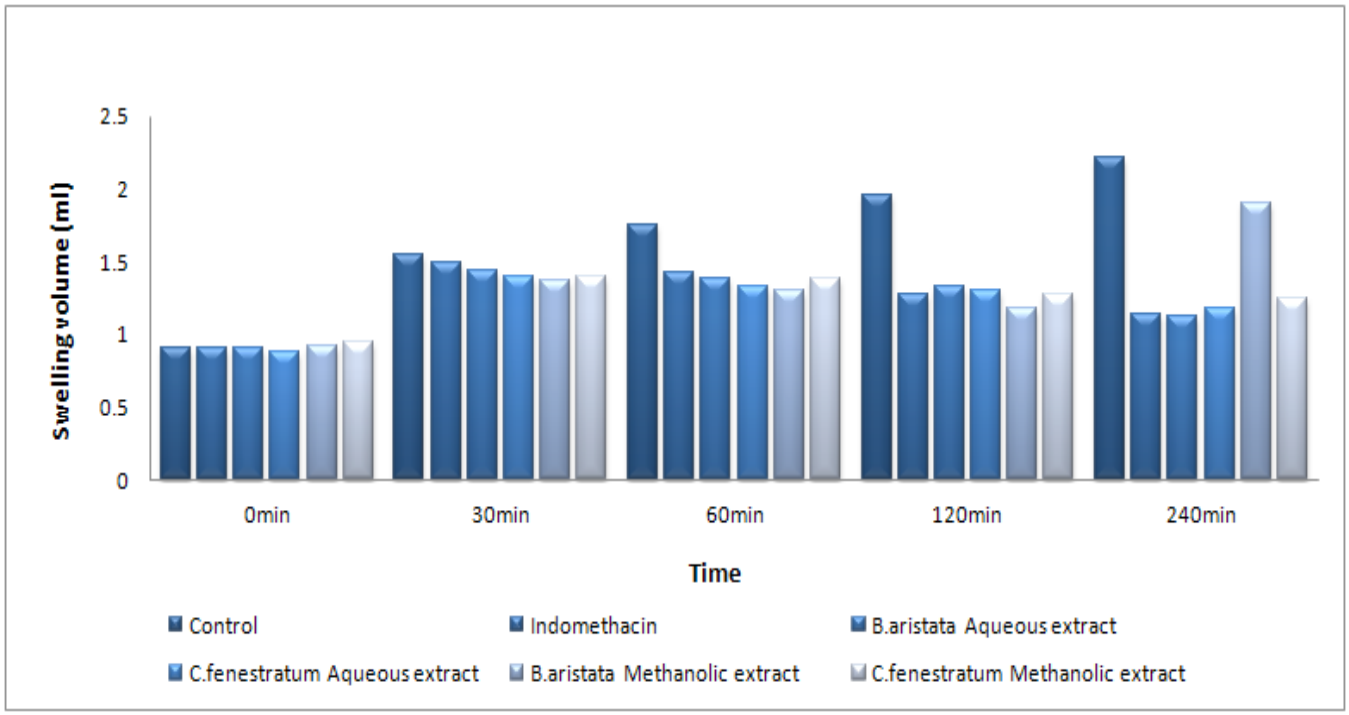

Figure 1: Inhibitory effect of Aqueous and Methanolic extracts of *Berberis aristata* and *Cosinium fenestratum* on carrageenan-induced edema of the hind paw in rats

DISCUSSION

Physicochemical analyses of plant extracts provide essential information regarding the chemical constituents for the pharmacological and pathological discovery of novel drugs[16]. The results of the physicochemical tests carried out in the present study confirmed that all the tested parameters of the samples were within the permitted levels of API and can be used for further phytochemical analysis. TLC and HPTLC profiling of the extracts confirmed the presence of phytochemicals. The Rf values of *B. aristata* and *C. fenestratum* were found to

be highly similar and were further addressed for animal model study to validate their respective anti inflammatory property.

The results of the animal study demonstrated the anti-inflammatory property of both *B. aristata* and *C. fenestratum* extracts that significantly inhibited paw oedema induced by carrageenan in rats. Carrageenan induced paw edema model is generally used to study anti-inflammatory activity of drugs[15]. The inflammatory condition, a biphasic response, induced by carrageenan, could be attributed to the step-wise release of vasoactive substances such as histamine, bradykinin and serotonin in the early phase and prostaglandins in the acute phase[13,14]. These chemical substances probably increase the vascular permeability, thereby promoting accumulation of fluid in tissues that accounts for oedema. Similar inference has been obtained in studies conducted on anti inflammatory activities of root and leaf extracts of *B.aristata*[9]. The ability of the extracts to reduce the size of oedema produced by carrageenin suggests that they contained chemical components that may be active against inflammatory conditions.

CONCLUSION

The present study scientifically validates the anti-inflammatory property of *B. aristata* and *C. fenestratum*. Similarity observed in the anti-inflammatory activity of both the herbal drugs appears to fulfil the necessary criteria expected to use *C. fenestratum* as equivalent/substitute to *B. aristata*.

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Phytochemical Investigation, Antioxidant and Antifungal Activities of Rhizomes of *Euphorbia Fusiformis*

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ABSTRACT

Objective: Isolation of compounds from *Euphorbia fusiformis* rhizomes and evaluation of extracts and each isolates for its antioxidant and antifungal activities. **Results:** Five compounds were isolated including Euphol **1**, β-Sitosterol **2**, Caudicifolin **3**, Scoparone **4** and Scopoletin **5**. The occurrence of the compounds **2**, **4** and **5** in the herb reported here for the first time. The ethyl acetate extract showed significant antioxidant activity (IC_{50} = 2.781 μg/ml) and its yielded compound **3** showed moderate antioxidant activity (IC_{50} = 3.25 μg/ml) using DPPH method. The ethyl acetate extract showed better antifungal activity against *Aspergillus niger and Candida albicans.* **Conclusions:** *Compounds* **2, 4, 5** *were isolated first time from this plant.* The ethyl acetate extract showed significant antioxidant and antifungal activity against *A. nigar* and *C. Albicans.* Thus, this study concludes by demonstrating the isolation, characterization, antioxidant and antifungal properties of *E. fusiformis*, which may have further therapeutic value.

Keywords: *Euphorbia fusiformis*, coumarins, antioxidant activity, antifungal activity.

INTRODUCTION

The genus Euphorbia is the largest in the plant family Euphorbiaceae, comprising about 2000 known species[1] and ranging from annuals to trees. All contain latex and have unique flower structures, many of which are used in folk medicine. Over the past twenty years, they have received considerable phytochemical and biological attention. *Euphorbia fusiformis* Buch.-Ham.exD. Don is a rare medicinal flora, found in the Western and Eastern Ghats of India. The local name in Telangana is paala gaddalu[2]. The ethnobotanical value of this plant is due to its action as a remedy for several diseases like rheumatism, gout, paralysis and arthritis, liver disorders and diarrhea[3–6]. Earlier pharmacological studies have reported that the species possess antibacterial,[7] hepatoprotective,[8] anti-arthritic, anti-inflammatory,[4] antinociceptive,[9] and anti-fungal activities[10]. There is no scientific report available in support of the antioxidant and antifungal activities of *n*-hexane and ethyl acetate extracts of this plant. In the present work, we report the isolation and identification of compounds **1–5** along with the antioxidant and anti-fungal activity (against *Aspergillus niger* and *Candida albicans*) of rhizomes of *E. fusiformis*.

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MATERIALS AND METHODS

Chemicals

Potato Dextrose Agar (PDA) and Fluconazole were purchased from HiMedia Laboratories, Mumbai. L-Ascorbic acid, DPPH and DMSO were procured from Sigma-Aldrich, India. *A. Niger* (M9687) and *C. albicans* (M7253) were obtained from Microbial Type Culture Collection & Gene Bank (MTCC), Chandigarh.

Collection of plant material

The rhizomes of *E. fusiformis* were collected from Chengicherla forest reserve, Chengicherla Village, Ranga Reddy District of Andhra Pradesh, India, during the month of August-2012. The plant was taxonomically identified and

authenticated by Dr. Venkat Ramana, Assistant Professor, Department of Botany, Nizam College, Osmania University, AP, India. A voucher specimen (CRC-EF-001) was deposited in the CSIR-CIMAP, Research Centre, Hyderabad.

Extraction and isolation

The rhizomes were sliced in to small pieces, dried in the shade and ground to powder (wt: 750 gr.). The powdered material was extracted successively with n-hexane (3L × 3), ethyl acetate (3L × 5) and methanol (3L × 5) by using hot percolation method. Later, the solvents were filtered and evaporated at reduced pressures to obtain crude extracts.

The crude n-hexane extract **EHX** (18 g) was subjected to column chromatography over silica gel (100–200 mesh) and eluted successively with solvents n-hexane and mixtures of ethyl acetate in hexane. The fractions eluted in 2% ethyl acetate in n-hexane yielded compound **1** (520 mg) and the fractions collected in 5% ethyl acetate in hexane separated compound **2** (30 mg) in pure form. The concentrated ethyl acetate extract **EET** (35 g) was subjected to column chromatography over silica gel (100–200 mesh) and eluted successively with solvents *n*-hexane and mixtures of ethyl acetate in *n*-hexane. The fractions eluted with 10% ethyl acetate in n-hexane yielded compound **3** (450 mg) in pure form. The fractions collected in 20% and 30% ethyl acetate in *n*-hexane were combined and further purified by repeated column chromatography by eluting with chloroform and mixture of methanol in chloroform. The similar fractions collected in chloroform were combined and concentrated to get compound **4** (125 mg) and fractions collected in 1% methanol in chloroform gave compound **5** (35 mg) in pure states.

DPPH assay

Antioxidant activity of extracts *n*-hexane, ethyl acetate and compounds **1–5** were determined by DPPH radical scavenging method[11]. DPPH radicals have an absorption maximum at 517 nm, which disappears with reduction by an antioxidant compound. The DPPH solution in methanol (0.1 mM) was prepared and 1 mL of this solution was mixed with 4 mL of test compound/standard methanolic solution (different concentrations ranging from 10 to50 µg/mL). The samples were incubated for 30 min at room temperature in a dark chamber and then the decrease in absorbance at 517 nm was measured (A_E). A blank sample containing 1mL DPPH and 4 mL methanol was prepared and its absorbance was measured (A_B). Radical scavenging activity was calculated using the following formula: Percentage of inhibition = $[(A_B - A_E) / A_B] \times 100$. Percentage of radical scavenging activity was plotted against the corresponding concentration of the extract to obtain IC_{50} value which is defined as the amount of antioxidant standard required to scavenge 50% of free radical of DDPH in the assay system. The IC_{50} values are inversely proportional to the antioxidant activity, calculated from data obtained graphically.

Antifungal activity

The plant extracts *n*-hexane, ethyl acetate and compound **1–5** were assayed for antifungal activity against *Aspergillus niger* (M9687) and *Candida albicans* (M7253). These microbes were grown on PDA plate (39 g/L) at 28 °C and maintained with periodic sub-culturing at 4 °C and the control is fluconazole. Different concentrations (100 and 150 µg/ml in DMSO) of test solutions/control were tested.

The extracts and compounds **1-5** were screened for antifungal activity by agar well diffusion method with sterile cork borer of size 6.0 mm[12]. The cultures of 48 hours old grown on PDA were used for inoculation of fungal strain on PDA plates. An aliquot (0.02 ml) of inoculum was introduced to molten PDA and poured in to a petri dish by pour plate technique. After solidification, the appropriate wells were made on agar plate by using cork borer. In agar well diffusion method, 0.05 ml of sample/standard was introduced serially after successful completion of one plant analysis. Incubation period of 24–48 h at 28 °C was maintained for observation of antifungal activity. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the plant extracts. The complete antifungal analysis was carried out under strict aseptic conditions. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in duplicates.

RESULTS AND DISCUSSION

Chemistry

Based on the physical, chemical and Spectroscopic properties and previous reported literature, compound **1** was confirmed as a tritenpene, Euphol,[13] compound **2** as β-Sitosterol, compound **3** as a diterpenoid, Caudicifolin,[14] compound **4** and **5** as coumarins, Scoparone and Scopoletin respectively[15,16]. Compounds **2**, **4** and **5** were isolated for the first time from *E. fusiformis*. The Structures are shown in figure 1.

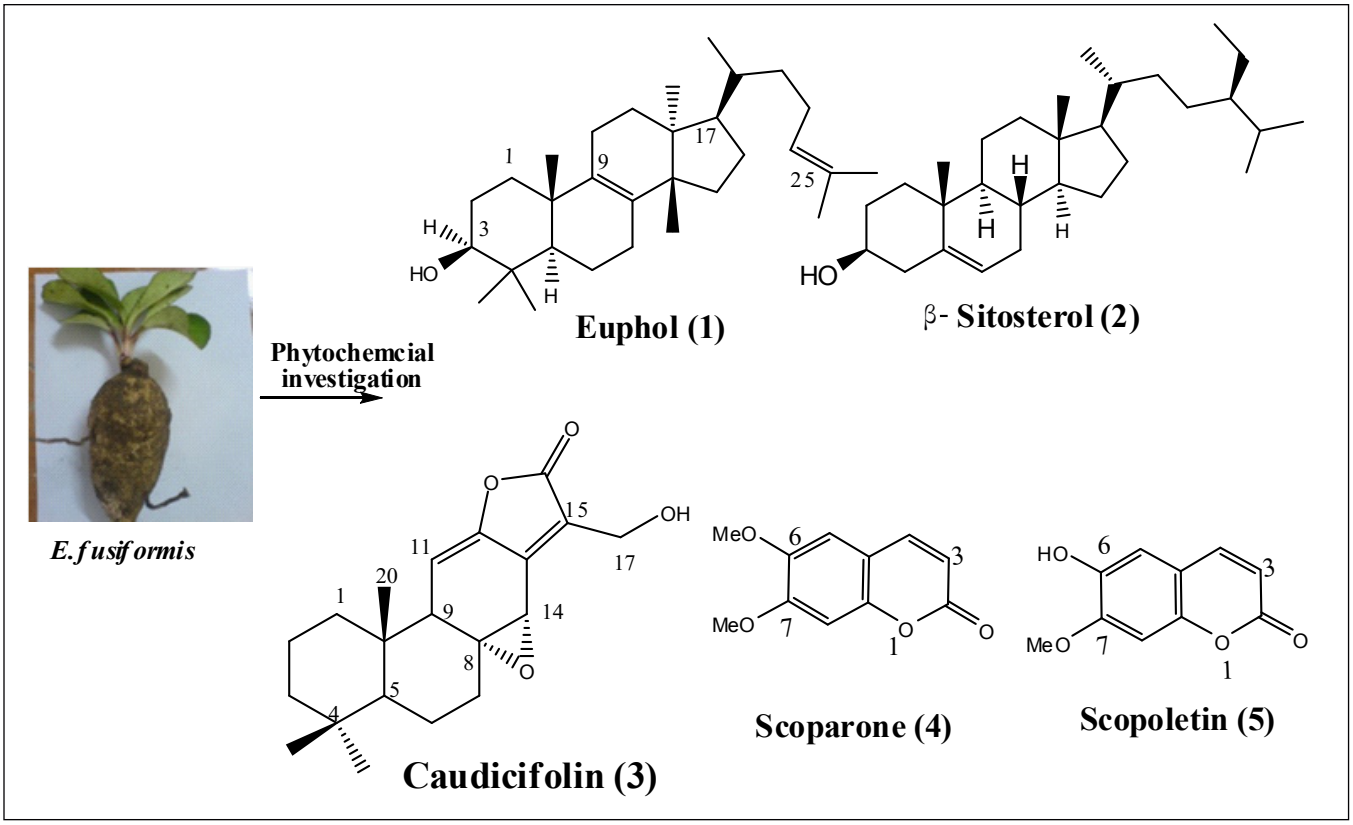

Figure 1: Chemical structures of isolated compounds 1–5 from *E. fusiformis*

Spectral data

Euphol 1: White needles, mp 100-102°C (reported 105 °C), ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.09 (1H, *t*, *J* = 5.7 Hz, H-24), 3.23 (1H, *dd*, *J* = 4.5, 11.4 Hz, H-3), 1.68, 1.60, 1.00, 0.95, 0.87, 0.80, 0.75 (each 3H, s, CH_3×7), 0.84 (3H, *d*, *J*= 6.3 Hz, CH_3). ESI-MS (positive mode) m/z: 427 $[M+H]^+$ (calculated mass for $C_{30}H_{50}O$ is 426.7).

Caudicifolin 3: White solid; M.P. 177-179 °C (lit. 177-182 °C); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 5.58 (1H, d, *J* = 6 Hz, H-11), 4.64 (2H, s, H-16), 4.07 (1H, s, H-14), 2.79 (1H, brs, OH), 2.66 (1H, d, *J* = 6 Hz, H-9), 0.75, 0.88, 0.97 (9H, s, H-18, H-19, H-20); ^{13}C NMR (300 MHz, $CDCl_3$):): δ (ppm) 169.2 (C-16), 147.3 (C-12), 146.6 (C-13), 127.5 (C-15), 106.4 (C-11), 61.3 (C-8), 56.2 (C-16), 54.4 (C-14), 53.5 (C-5), 51.8 (C-9), 41.5 (2C, C-10 and C-3), 39.9 (C-1), 33.9 (C-18), 33.5 (C-7), 33.4 (C-4), 21.9 (C-19), 20.8 (C-6), 18.4 (C-2), 15.1 (C-20); ESI-MS (positive mode) m/z: 331.13. $[M+H]^+$, 353.09 $[M+Na]^+$ 683.30 $[2M+Na]^+$ (calculated mass for $C_{20}H_{26}O_4$ is 330.42).

Scoparone 4: Yellow solid; M.P. 148-150 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.64 (1H, d, *J* = 9.6 Hz, H-4), 6.87 (1H, s, H-5), 6.86 (1H, s, H-8), 6.30 (1H, d, *J* = 9.6 Hz, H-3), 3.97, 3.94 (each 3H, s, 2 × CH_3O-); ^{13}C NMR (300 MHz, $CDCl_3$): δ (ppm) 161.4 (C-2), 152.8 (C-7). 150.0 (C-6), 146.3 (C-9), 143.3 (C-4), 113.5 (C-10), 111.4 (C-3), 108.0 (C-5), 100.0 (C-8), 56.3 (2 × CH_3O-); ESI-MS (positive mode) m/z: 207 $[M+H]^+$, 229 $[M+Na]^+$ (calculated mass for $C_{11}H_{10}O_4$ is 206.19).

Scopoletin 5: Yellow solid; M.P. 199-202 °C (lit. 203-205 °C); ^{1}H NMR (300 MHz, $CDCl_3$,): δ (ppm) 7.60 (1H, d, *J* = 9.6 Hz, H-4), 6.89 (1H, s, H-5), 6.83 (1H, s, H-8), 6.24 (1H, d, *J* = 6 Hz, H-4), 3.94 (3H, s, CH_3O-); ESI-MS (positive mode) m/z: 193.3 $[M+H]^+$, 215.3 $[M+Na]^+$ (calculated mass for $C_{10}H_8O_4$ is 192.17).

Study of antioxidant capacity

The extracts and its isolated compounds **1-5** were tested for antioxidant activity and their potencies shown as IC_{50} values in mg/ml compared to standard ascorbic acid (table 1 and figure 2). Among the extracts, ethyl acetate extract **EET** was most potential (IC_{50} 2.781 μg/ml) and amongst compounds compound **3** was most potential antioxidant active (IC_{50} 3.25 μg/ml).

Table 1: DPPH radical scavenging capacity of *E. fusiformis* plant extracts and isolated compounds

S. no.	Sample	IC_{50} (μg/ml)	S. no.	Sample	IC_{50} (μg/ml)
1	n-hexane extract **EHX**	3.377	5	Caudicifolin **3**	3.25
2	Ethyl acetate extract **EET**	2.781	6	Scoparone **4**	4.625
3	Euphol **1**	9.39	7	Scopoletin **5**	3.56
4	β-Sitosterol **2**	9.00	8	Ascorbic acid	2.67

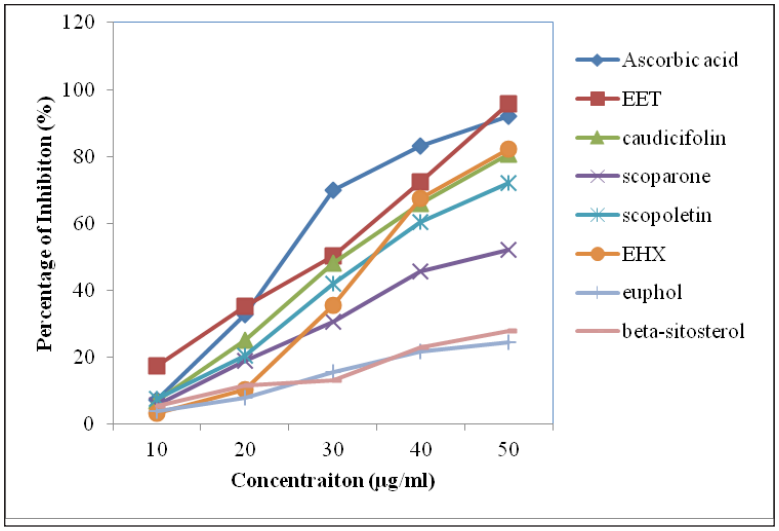

Figure 2: The DPPH radical scavenging activity for extracts EHX, EET, compound 1–5 and ascorbic acid of *E. fusiformis*

Studies on antifungal activity

Antifungal activity of *n*-hexane and ethyl acetate extracts and compounds **1–5** assayed and the data on effect on the growth of *C. Albicans* and *A. niger* presented in table 2. The data revealed that ethyl acetate showed significant reduction in growth of *C. albicans* and *A. niger* when compared to the reference compound flucanozol. The compounds isolated from this extract **3–5** showed significant differences in their efficacy. The n-hexane extract and its isolated compounds **1** and **2** not exhibited the antifungal activity against both fungi.

Table 2: Antifungal activity (zone of inhibition in mm) on the growth of *A. niger* (M9687) and *C. albicans* (M7253).

S. no.	Extracts and isolated compounds	Zone of inhibition at different concentrations (mm)			
		C. albicans		*A. niger*	
		100 (μg/ml)	150 (μg/ml)	100 (μg/ml)	150 (μg/ml)
1	n-Hexane	--	--	--	--
2	Ethylacetate	12	15	13	15
3	Euphol **1**	--	--	--	--
4	β-sitosterol **2**	--	--	--	--
5	Caudicifolin **3**	7	9	2	4
6	Scoparone **4**	5	7	6	7
7	Scopoletin **5**	4	5	3	4
8	fluconazole	9	11	11	13

CONCLUSION

Chromatographic fractionation of *E. fusiformis* resulted in the isolation of five compounds; compounds **2**, **4** and **5** are isolated for the first time from this herbal source. All the extracts and compounds were screened for antioxidant and antifungal activities in *in vitro* mode. The ethyl acetate extract showed significant antioxidant and antifungal activity in both *C. Albicans* and *A. nigar* (better than the standard fluconazole). Thus, this study concludes by demonstrating the isolation, characterization, antioxidant and antifungal properties of *E. fusiformis*, which may have further therapeutic value.

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Isolation of Phytochemicals From Anticancer Active Extracts of *Syzygium alternifolium* Walp. Leaf

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ABSTRACT

Objective: The aim of the present study was to isolate the phyto molecules from the leaf of endemic medicinal pant, *Syzygium alternifolium*. The phytochemical investigation of the leaf of the plant yielded a flavonoid Eucalyptin **1** and a triterpinoid Epibetulinic acid **2** in pure state. **Results:** The compound **1** is being reported for the first time from this plant. The anti-cancer activity showed leaf hexane extract (IC_{50} values 8.177 and 2.687 μg/ml) was significantly active, when compared to extracts and compounds, against human cancer cell lines MCF-7 and DU-145. Also, hexane extract potentially inhibited the growth of DU-145 cell lines when compared with the reference compound doxorubicin. Amongst the isolated compounds, **1** was better cytotoxic than **2**. **Conclusions:** The hexane extract of leaves of *S. alternifolium* yielded compounds **1** and **2** and the structure elucidation, based on spectroscopy, revealed them as Eucalyptin and Epibetulinic acid respectively. The compound **1** is being reported for the first time from this plant. The anti-cancer activity showed leaf hexane extract (IC_{50} values 8.177 and 2.687 mg/mL) was significantly active, when compared to extracts and compounds, against human cancer cell lines MCF-7 and DU-145. Also, hexane extract potentially inhibited the growth of DU-145 cell lines when compared with the reference compound doxorubicin. Amongst the isolated compounds, **1** was better cytotoxic than **2**.

Keywords: *Syzygium alternifolium*, Myrtaceae, Eucalyptin, Epibetulinic acid, anticancer activity.

INTRODUCTION

Syzygium alternifolium is an endangered tree species belongs to family Myrtaceae, which is very rare and its existence is confined to a few places in the Tirumala hills of Chittoor district in Andhra Pradesh. There are few reports available on the bio-activity and phytochemistry of the plant. The whole plant is an excellent source of secondary metabolites which have several medicinal values like Antimicrobial,[1] Anti-diabetic,[2] Anti-inflammatory,[3] Hypoglycemic and Anti-Hyperglycemic[4].

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MATERIALS AND METHODS

Collection of plant material

S.alternifolium leaves were collected from Thirumala Hills of Andhra Pradesh, and was identified by Taxonomist, Prof. V.S. Raju of Kakatiya University, Warangal, Andhra Pradesh. A voucher specimen was deposited at the CIMAP-Research Centre, Hyderabad, India under the accession number CP-SA-1/2012.

Extraction and isolation

Leaves of *S. alternifolium* were collected, shade dried and powdered. The powdered plant material (1 kg) was extracted successively with Hexane and Methanol using sohxlet apparatus at reflux temperatures. The extracts were filtered and evaporated at reduced pressures to obtain crude mass. The hexane extract (20 g) was purified by column chromatography by using silica gel 100-200 mesh size as stationary phase. The fractions collected in 20 % ethyl acetate in hexane resulted in the isolation of a yellow coloured compound (100 mg) which was further

refined with cold hexane washings to yield compound **1** (50 mg). Similarly, fractions collected in 30 % ethyl acetate in hexane fractions gave a colourless compound **2** (200 mg). The structures of the compound **1** and **2** were elucidated on the basis of $^{1}H/^{13}C$/2D-NMR, and Mass spectra. The methanol extract did not yield any pure compound.

Anticancer Activity

Cell culture

Human breast cancer cell line (MCF-7) and human prostate cancer cell lines (DU-145) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) the cell lines were grown in DMEM medium supplemented with 10% FBS, 0.3% sodium bicarbonate, 10 mL/L antibiotic antimycotic solution (10,000 U/ml penicillin, 10 mg/L streptomycin and 25µg/mL amphotericin B), 1 mL/L of 4mM L-glutamine and 1 mL/L of 100 mM sodium pyruvate culture was maintained in CO_2 incubator at 37°C with a 90% humidified atmosphere and 5% CO_2.

Preparation of samples for MTT assay

Test compounds, extracts and isolated compounds, were taken in 10 mg/ml of DMSO and various dilutions were made with sterile PBS (1X) to get desired concentrations. All formulations were filtered with 0.22 µm sterile filter and 20 minutes of UV eradication before adding to the 96 well plates containing cells.

Cytotoxicity evaluation (MTT assay)

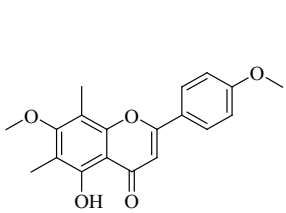
Cytotoxicity of formulations was assessed using MTT assay to determine the cell viability according to a reported method[5]. The assay is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells into purple formazan crystals which gets dissolved in DMSO and read at 570 nm. Briefly, 1x104 exponentially growing cells were seeded into each 96 well plate (counted by Trypan blue exclusion dye method) allowed to grow till 60-70% confluence then compounds(name of the compounds if applicable) were added to the culture medium with the final concentrations ranging from of 10, 25, 50 and 100 µg/ml and along with controls(negative(without compound) and positive (Doxorubicin)) incubated for 24 hours CO_2 incubator at 37°C with a 90% humidified atmosphere and 5% CO_2. Then the media of the wells were replaced with 90 µl of fresh serum free media and 10 µl of MTT (5mg/ml of PBS), plates were incubated at 37°C for 2h, there after the above media was discarded allow to dry for 30 minutes. Add 100µl of DMSO in each well at 37°C for 5min. The purple formazan crystals were dissolved and immediately read absorbance at 570nm was measured using Spectra Max plus 384 UV-Visible plate reader (Molecular Devices, Sunnyvale, CA, USA). IC_{50} values were determined by probit analysis software package of MS-excel. % Cell viability (from control) versus concentration.

RESULTS AND DISCUSSION

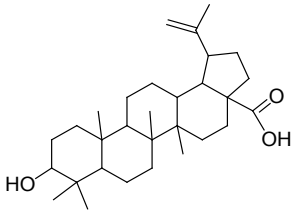
Chemistry

Structure elucidation of compounds **1** and **2** were done using IR, 1D/2D NMR and Mass spectral data. Basing on the spectroscopy and also comparison from the literature[6] compound **1** was identified as Eucalyptin (Figure 1). Compound **1** was obtained as a yellow powder (Eucalypitin); TLC Rf: 0.41 in *n* Hexane/ethylacetate (1:2); 1H-NMR (300 MHz, $CDCl_3$, δ ppm) 6.560 (s), 12.804 (s), 2.156 (s), 3.740 (s), 2.336 (s), 7.816 (d), 6.976 (d), 3.84 (s); 13C-NMR: (75 MHz, $CDCl_3$, δ ppm): 163.07, 103.27, 182.45, 152.20, 113.30, 7.47, 59.70, 161.80, 104.41, 7.76, 157.30, 107.98, 123.10, 127.19, 113.77, 156.51, 54.73. ESI-MS of **1** gave molecular ion peak at m/z 327 (M+1) corresponding to the molecular formula $C_{19}H_{18}O_5$.

Basing on the various spectroscopy data and comparison from the literature,[7] compound **2** was identified as epibetulinic acid (Figure 1). White amorphous solid (m. p. 279–280°C); TLC Rf: 0.41 in *n* Hexane/ ethylacetate (1:3); IR In KBr (nue max cm^{-1}): 3448 (OH), 2943 (C–H), 1687 (C=O), 1641(C=C), 1450. 1H-NMR (300 MHz, $CDCl_3$, δ ppm) (ppm): 4.53 (2H, d, J = 39 Hz), 3.17 (m), 3.00 (m), 2.29 (q), 2.23 (d), 2.21 (s), 0.75-1.61 (m, rest of protons); 13C-NMR: (75 MHz, $CDCl_3$, δ ppm): 38.77, 27.44, 78.05, 38.90, 55.41, 18.33, 34.38, 40.76,50.58, 37.26, 20.90, 25.56, 38.45, 42.49, 30.61, 32.20,57.23, 46.93, 49.34, 150.41, 29.74, 37.06, 28.02, 15.36, 16.06, 16.15, 14.73, 179.40, 109.71, 19.41; ESI-MS *m/z*: 455 [M-H] corresponding to the molecular formula $C_{30}H_{48}O_3$.



1 Eucalypitin



2 Epibetulinic acid

Figure 1: Structures of the isolated compounds

Anticancer activity

Anticancer activity, in *in vitro* mode, was performed on leaf hexane and methanol extracts and on the isolated compound **1** and **2** respectively (Table 1). The data from table 1 infers that leaf hexane extract (IC_{50} values 8.177 and 2.687 µg/mL) was significantly active when compared with others against human cancer cell lines MCF-7 and DU-145. Hexane extract potentially inhibited the growth of DU-145 cell lines when compared with the reference compound doxorubicin. Amongst the isolated compounds, **1** was better cytotoxic on the above cell lines than **2**.

Table 1: Anticancer activity of leaf extracts and isolated compounds

Extract and Compound	IC_{50} in µg/mL	
	MCF-7	DU-145
Hexane	8.177±0.035	2.687±0.009
Methanol	36.238±0.011	55.746±0.003
1	51.466±0.023	31.081±0.035
2	>100	>100
Doxorubicine	1.856±0.003	13.707±0.02

CONCLUSION

The Hexane extract of leaves of *S. alternifolium* yielded compounds **1** and **2** and the structure elucidation, based on spectroscopy, revealed them as Eucalyptin and Epibetulinic acid respectively. The compound **1** is being reported for the first time from this plant. The anti-cancer activity showed leaf Hexane extract (IC_{50} values 8.177 and 2.687 mg/mL) was significantly active, when compared to extracts and compounds, against human cancer cell lines MCF-7 and DU-145. Also, Hexane extract potentially inhibited the growth of DU-145 cell lines when compared with the reference compound doxorubicin. Amongst the isolated compounds, **1** was better cytotoxic than **2**.

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Protective role of *Helicteres isora* plant extract on plasma and tissue glycoprotein components in streptozotocin induced hyperglycemic rats

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ABSTRACT

The present investigation was carried out to evaluate the protective role of *Helicteres isora* plant extract on glycoprotein metabolism in streptozotocin (STZ) induced type 2 diabetic rats. *Helicteres isora* plant extract was administered orally (200 mg/kg body weight) for 45 days to normal and diabetic rats. The effects of *Helicteres isora* plant extract on plasma and tissue glycoproteins (hexose, hexosamine, sialic acid and fucose) were determined. The levels of plasma glycoproteins containing hexose, hexosamine, sialic acid and fucose were significantly increased in diabetic rats when compared with normal control rats. There was a significant decrease in the level of sialic acid and elevated levels of hexose, hexosamine and fucose in the liver and kidney of STZ induced diabetic rats. On oral administration of *Helicteres isora* plant extract to diabetic rats showed decreased levels of plasma glycoproteins. The level of tissue sialic acid was increased whereas the levels of tissue hexose, hexosamine and fucose were reversed to near normal. The present study indicates that the *Helicteres isora* plant extract possesses a significant protective effect on glycoprotein metabolism in addition to its anti-diabetic effect.

Keywords: *Helicteres isora*, Diabetes, Plasma Glycoproteins, Streptozotocin.

INTRODUCTION

Medicinal plants played an important role in Indian culture since Rig Veda (5600 BC) where about 67 medicinal plants were recorded. It is estimated that 80% of population rely on traditional medicines due to high cost of modern medicines, lack of availability of required medicines and personal preferences. It is identified that about 20,000 plants have good medicinal value and 7500 species are used by traditional communities[1]. Diabetes is becoming the third killer of mankind, after cancer and cardiovascular diseases, because of its high prevalence, morbidity and mortality[2]. Diabetes is widely recognized as one of the leading causes of death in the world[3].

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People suffering from diabetes are not able to produce or properly use insulin in the body, so they have a high level of blood glucose. Diabetes is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced, thus results in decreased glucose transport into muscle and fat cells, and increased hepatic glucose output. These defects contribute to hyperglycemia, resulting in the impairment of the metabolism of glucose, lipids, proteins and glycoproteins[4]. In the diabetic state, glucose is used by the insulin independent pathways, leading to the synthesis of oligosaccharide moieties of glycoprotein; hexose, hexosamine, fucose, and sialic acid have an important role in protein stability, function, and turnover[5]. Glycoproteins are carbohydrate - l inked protein macromolecules found in the cell surface, which is the principle component of animal cell. The oligosaccharide moieties of glycoproteins, hexose, hexosamine, fucose and sialic acid have an important role in protein stability, function and turnover,[6] membrane transport, cell differentiation and recognition, secretion and absorption of macromolecules and the adhesion of

macromolecules to the cell surface[7]. Glycoproteins play a major role in the pathogenesis of diabetes mellitus due to impaired metabolism[8]. Insulin deficiency and high levels of plasma glucose in diabetic condition may result in an increased synthesis of glycoproteins[9].

The level of glycoproteins has been associated with severity and duration of diabetes. At the cell surface or inside the cells, fucose and sialic acid form specific structures, called glycanic chains covalently linked to lipids or proteins. An increase in the biosynthesis and or decrease in the metabolism of glycoproteins could be related to deposition of these materials in the basal membrane of pancreatic cells.

MATERIALS AND METHODS

Animals

Male albino Wistar strain rats (weighing 180–200 g b.w.) were procured from the Central Animal House, Rajah Muthiah Medical College (RMMC), Annamalai University. They were acclimatized to animal house conditions, and fed with standard pellet diet (Hindustan Lever Limited, Mumbai, India) and water *ad libitum*. The rats used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research Hyderabad, India and the study approved by the ethical committee (Vide. No: 845), Annamalai University.

Drugs and Chemicals

All the chemicals used in this experiment were obtained from Sigma Chemical Company (St Louis, MO, USA), Hi Media (Mumbai, India), and SD-Fine Chemicals (Mumbai, India). All chemicals used were of analytical grade.

Experimental induction of type 2 diabetes in rats

Non-insulin dependent diabetes mellitus was induced by the method of Masiello *et al*., (1998)[10], in overnight fasted rats by a single intraperitonial injection of 45 mg/kg body weight STZ, STZ was dissolved in citrate buffer (0.1M, pH 4.5) and nicotinamide was dissolved in normal saline. Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined at 72 h and then on day 7 after injection. The animals with plasma glucose concentration more than 250 mg/dl were used for the study.

Experimental procedure

In the experiment, a total of 24 rats (12 diabetic surviving rats and 12 normal rats) were used. The rats were divided six in each group. *Helicteres isora* was dissolved in vehicle solution (corn oil) and administered orally using an intragastric tube for a period of 45 days.

Group 1: Normal control rats (vehicle treated)

Group 2: Normal rats + *Helicteres isora* (200 mg/kg b.w)

Group 3: Diabetic rats

Group 4: Diabetic rats + *Helicteres isora* (200 mg/kg b.w)

At the end of the experimental period, the rats were deprived of food overnight and sacrificed by decapitation. Blood sample was collected in a tube containing potassium oxalate and sodium fluoride (3:1) for the estimation of plasma glycoproteins. Liver and kidney were dissected out, washed in ice-cold saline, patted dry and weighed.

Biochemical assays

Extraction of glycoproteins

To 0.1 ml of plasma, 5.0 ml of methanol was added, mixed well and centrifuged for 10 min at 3000×g. The supernatant was decanted and the precipitate was again washed with 5.0 ml of 95% ethanol, recentrifuged and the supernatant was decanted to obtain the precipitate of glycoproteins. This was used for the estimation of hexose and hexosamine.

For extraction of glycoproteins from the tissues, a known weight of the tissue was homogenized in 7.0 ml of methanol. The contents were filtered and homogenized with 14.0 ml of chloroform. This was filtered and the residue was successively homogenized in chloroform-methanol (2:1v/v) and each time the extract was filtered. The residue (defatted tissues) was obtained and the filtrate decanted. A weighed amount of defatted tissue was suspended in 3.0 ml of 2N HCl and heated at 90°C for 4h. The sample was cooled and neutralized with 3.0 ml of 2N NaOH. Aliquots from this were used for estimation of fucose, hexose, hexosamine and sialic acid.

Determination of glycoproteins

Plasma and tissue hexose and hexosamine were estimated by the method of Dubois and Gilles, 1933[11] with slight modifications by Niebes, 1972[12] respectively. Sialic acid

and fucose were estimated by the method of Dische and Shettle, 1948[13] respectively.

Statistical analysis

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT) using a statistically software package (SPSS for Windows, V.13.0, Chicago, USA). Results were presented as mean ± S.D. $p<0.05$ were considered as statistically significant.

RESULTS

In the present study we have reported that plant extract 230mg/kg body weight showed better effects. Therefore 230mg/kg body weight was used in the present study.

Effect of H.isora extract on plasma and tissue glycoproteins

Figure. 1 shows the changes in the levels of plasma glycoproteins of control and diabetic rats. Significantly higher levels of glcoproteins were observed in the plasma of the diabetic rats when compared with normal control rats. Treatment with *H.isora* extract to diabetic rats resulted in a significant reduction of glycoproteins in the plasma when compared with diabetic control rats.

The levels of liver and kidney glycoprotein of control and experimental rats were shown in Figures 2–5. The levels of glycoproteins, hexose, hexosamine and fucose were significantly increased whereas the level of sialic acid was significantly decreased in diabetic rats. Oral administration of *H.isora* extract significantly reversed these changes in the liver and kidney of diabetic rats.

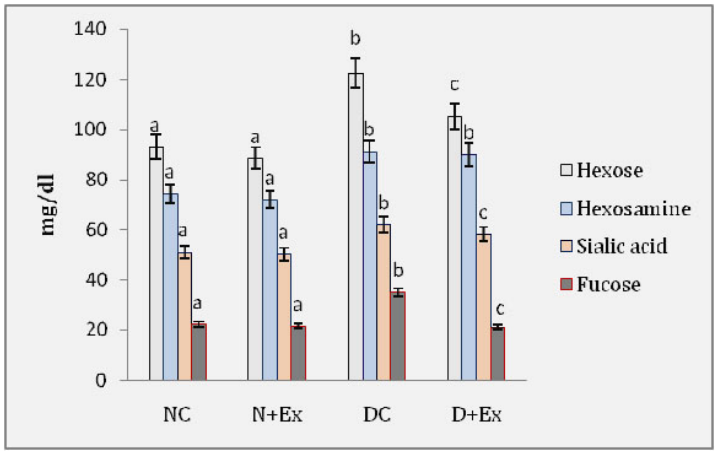

Figure 1: Changes in the levels of plasma glycoproteins in control and experimental rats. NC-Normal control, N+Ex- normal + extract, DC-diabetic control, D+Ex-diabetic + extract; Values are given as mean ±S.D. for 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$(DMRT).

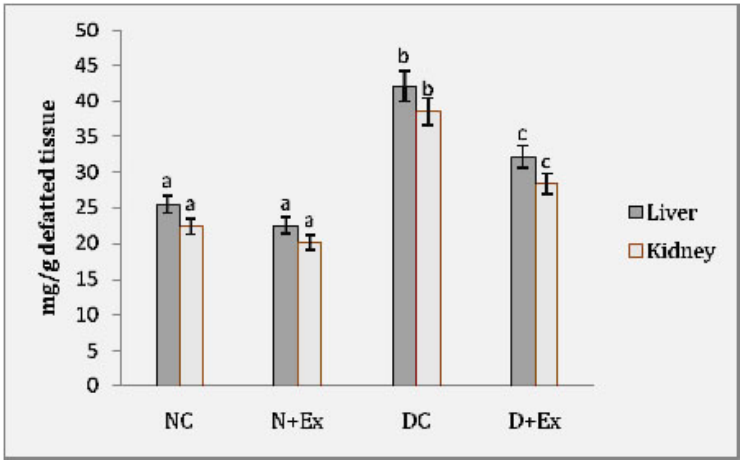

Figure 2: Changes in the levels of tissue hexose in control and experimental rats. NC-Normal control, N+Ex- normal + extract, DC-diabetic control, D+Ex-diabetic + extract; Values are given as mean ±S.D. for 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$(DMRT).

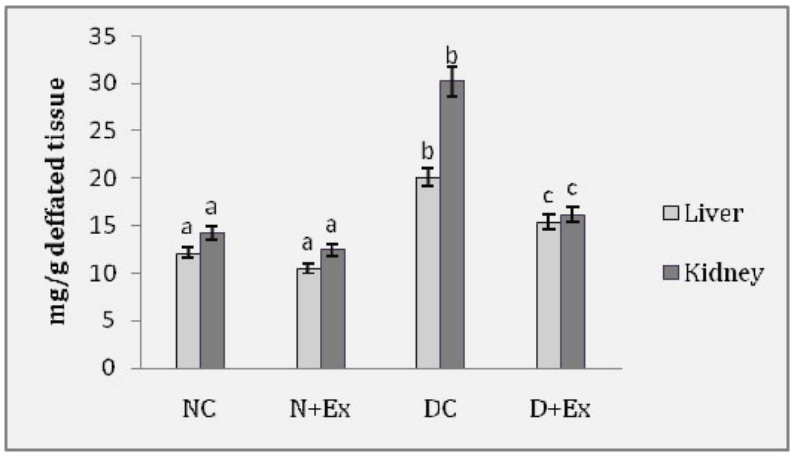

Figure 3: Changes in the levels of tissue hexosamine in control and experimental rats. NC-Normal control, N+Ex- normal + extract, DC-diabetic control, D+Ex-diabetic + extract; Values are given as mean ±S.D. for 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

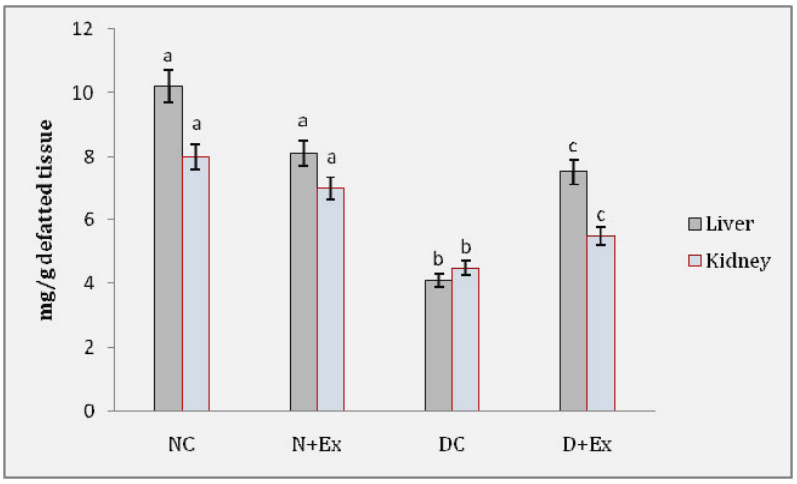

Figure 4: Changes in the levels of tissue sialic acid in control and experimental rats. NC-Normal control, N+Ex- normal + extract, DC-diabetic control, D+Ex-diabetic + extract; Values are given as mean ±S.D. for 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

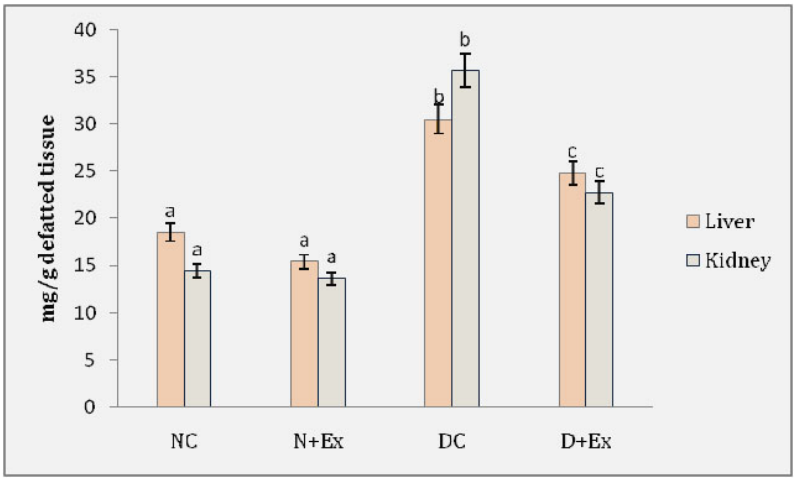

Figure 5: Changes in the levels of tissue fucose in control and experimental rats. NC-Normal control, N+Ex- normal + extract, DC-diabetic control, D+Ex-diabetic + extract; Values are given as mean ±S.D. for 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$(DMRT).

DISCUSSION

The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed[14]. Furthermore, an increasing reliance on the use of medicinal plants in the society has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedy[14]. Diabetes mellitus is a chronic metabolic disease with the highest rates of prevalence and mortality worldwide that is caused by an absolute or relative lack of insulin and or reduced insulin activity, which results in hyperglycemic and abnormalities in carbohydrate, protein and fat metabolism[15]. In diabetes, synthesis of glycoproteins was decreased because of reduced incorporation of glucose caused by insulin deficiency. Several studies have emphasized the multiplicity of disturbances affecting the metabolism of carbohydrates, proteins and lipids in diabetes[16,17].

In this study, we have observed altered levels of hexose, hexosamine, fucose and sialic acid in plasma and tissues of STZ induced diabetic rats. Glycation is a nonenzymatic reaction of glucose and the saccharide derivatives with proteins, nucleotides and lipids[18]. In hyperglycemia, the reactions occur between reducing sugars and amino groups of proteins to yield a Schiff's base intermediate. These schiff's base intermediate undergoes rearrangement to form a relatively stable Amadori product. The Amadori product further undergoes a series of reactions through dicarbonyl intermediates to form AGE (advanced glycation endproducts). Glycation occurs inside and outside the cells. Glycation of cellular proteins produces changes in structure and loss of enzymatic activity. These effects are countered by protein degradation and renewal.

In extracellular matrix the glycation produces changes in macromolecular structure affecting matrix-matrix and matrix cell interactions associated with decreased elasticity and increased fluid filtration across the arterial wall and endothelial cell adhesion[19].

When the concentration of AGEs increased above a critical level, cell surface AGE receptors become activated. Abnormalities in the metabolism of glycoproteins are observed in both naturally occurring and experimental diabetes[20]. The increases in plasma glycoprotein components have been reported to be associated with the severity and duration of diabetes.

Fucose is member of a group of essential sugars that the body requires for functioning of cell to cell communication and its metabolism appear to be altered in various disease conditions such as diabetes mellitus[21]. Due to increased glycosylation in the diabetic state the fucose levels could be increased. Experiments conducted in our laboratory showed elevated levels of fucose in diabetic animals[22]. Our results suggest that the increased fucosylated proteins in diabetic rats could be due to increase in the synthesis and/or decrease in degradation of these proteins. Sialic acid is a terminal component of the non-reducing end of the carbohydrate chains of glycoproteins and glycolipids, which are essential constituents of many hormones and enzymes present in serum and tissues. Sialic acid is an important constituent for the characteristic changes of transformed cells; the liver is the major site involved in the synthesis of sialic acid and other glycoproteins[9].

The synthesized glycoproteins are made to circulate in blood. There is a pronounced increase in serum rather than in other organs. The decrease in the content of sialic acid in tissues may be due to the utilization for the synthesis of fibronectin, which contains sialic acid residues in the core structure[23].

Agents with antioxidant or free radical scavenging property may inhibit oxidative reactions associated with glycation. In this context, previous studies have shown that decrease in hyperglycemia could lead to a decrease in glycoprotein levels[24]. Administration of *Helicteres isora* plant extract to diabetic rats resulted in a significant reversal of all these changes to near normal.

CONCLUSION

In conclusion, oral administration of *Helicteres isora* plant extract exhibits a protective effect on the carbohydrate moieties of glycoproteins in STZ induced diabetic rats.

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Screening of Antioxidant & Antidiabetic Potential of Polyphenol rich fraction from *Cichorium intybus*

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ABSTRACT

Introduction: *Chicory* (*Cichoriumintybus*) commonly known as kasni has health benefits such as improving liver health, digestion, boosting immune system possibly because of its water soluble dietary fibres and polyphenols. A diet rich in polyphenols and fibers helps in loweringcholesterol leading to less plaque formation, preventing blockage and risk of stroke. Owing to the fact that these polyphenols may reduce the elevated glucose the present research work was undertaken to extract the polphenols from *Chicory* roots and evaluate their antioxidant and hypoglycemic potential. **Materials and Methods:** Polyphenol rich fraction of root extract was subjected to estimation of total phenolic estimation and free radical scavenging activity using various antioxidant assays. The antidiabetic potential of the polyphenol rich fraction was also estimated using Alloxan Induced Diabetic Rat model. **Results:** The total phenolic content of the polyphenolic fraction was found to be 30.23μg/ml. The IC_{50} values for DPPH and ABTS assay for the polyphenolic fraction were found to be 60μg/ml and 4.2mg/ml respectively. The TEAC value was found to be 1.178 mM. Administration of polyphenol rich fraction (30 mg/kg, p.o.) decreased elevated SG from 122mg/dl to78.66mg/dl on 14th day (***$p<0.001$). Also a significant reduction in triglycerides (**$P<0.01$), total cholesterol (**$P<0.01$), serum LDL (**$P<0.01$) and serum alkaline phosphatase (**$P<0.01$) was observed at 30 mg/kg, p.o. However, serum HDL was found to be elevated with no change in serum insulin (*$P<0.05$). **Conclusion:** It can be concluded that the polyphenol rich fraction of Chicory roots possess a strong hypoglycemic potential probably due to their antioxidant activity.

Keywords: *Cichoriumintybus*,polyphenols, antioxidant, antidiabetic, total cholesterol.

INTRODUCTION

Diabetes is one of the most common non-communicable diseases found globally (2.8%) and is the fourth leading cause of death in most developed countries. Almost 3.2 million people die of diabetes across the world every year[1]. World Health Organization (WHO) has estimated approximately 160,000 diabetics worldwide which would double in the year 2025[2]. Ageing, highcaloric rich diet, obesity and stressful life style have further severed diabetes patients[3,4]. The modern treatment includes administration of insulin or oral hypoglycemic agents like sulphonylureas, biguanides etc. However these oral hypoglycemic agents are found to be less effective in insulin-dependent diabetes and their side effects such as allergic skin reactions (photosensitivity), gastrointestinal disturbances, blood dyscrasis, hepatic dysfunction and hypoglycemia etc further limit their use[5].Thus despite considerable progress in the treatment of diabetes in modern and traditional systems of medicine search for newer drugs continues[6–8].

The present research work is one such attempt to investigate the antidiabetic potential ofChicory,a leafy vegetable with a broad therapeutic potential. Hydroalcoholic seeds extract of the plant acts as hepatoprotectant. Its root extracts is found to lower liver lipids, triglycerides and cholesterol content. Chicoryis reported to contain more than 10% of total polyphenols, the most dominant being dicaffeoylquinic acids (71% of total polyphenols) with marked antioxidant and anti-hyperlipidimic activity[9,10].

The plant also contains Chicoric acid which was found to stimulate insulin release from INS-1 E insulin secreting

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cell lines and rats islet of Langerhans as well as glucose uptake[11].

Two new anthocyanin have been isolated from flowers of *Cichoriumintybus* and identified as delphinidin 3, 5-di-O-(6-O-malonyl_beta glucoside) and delphinidin 3-O_(6-O-malonyl-beta-D-glucoside)-5-O-beta-D-glucoside[12].

The Leaves contain coumarines, esculetin, cichoriin andsesquiterpene lactones. A new coumaringlucoside ester Cichoriin-6'-*p*-hydroxyphenyl acetate, was isolated from chicory leaves along with cichoriin. New sesquiterpene lactone like cichoridiol, Cichosterol (seco-sterol), Lactucin, Lactucopicrin, 11,13-dihydrolactupicrin were also reported in roots and leaves of *Cichoriumintybus*[13,14].

The plant is reported to possess anti-hyperlipidemic, antioxidant, Hypoglycemic, anti-inflammatory, Antibacterial-anti-hepatotoxicactivity[15–20].

It is now a worldwide accepted truth that diet rich in polyphenols helps in lowering cholesterol levels in the blood leading to less plaque formation, preventing the blockage and hence reducing the risk of hypertension, stroke[10] Owing to the fact that these polyphenols may also reduce the elevated glucose concentration and since very few research papers pertaining to their hypoglycemic potential are reported, the present research work was undertaken to extract the polyphenols from*Chicory* roots and evaluate their antioxidant and hypoglycemic potential. Various pathophysiological parameters such as lipid profile (HDL, LDL), body weight, serum alkaline phosphatase level were also monitored which are generally elevated with the onset of diabetes.

MATERIAL AND METHODS

Plant material collection, Authentication and extraction

The dried roots of Chicoriuminty bus family *Asteraceae* was collectedfrom Ayurvedicstore and authenticated from Botanical Survey of India, Koregaon park, Pune (BSI/WRC/Tech/2010/1008).

Air dried roots of *Cichoriumintybus* were coarsely pulverized and passed through 120 sieves to remove fines. The coarse root powder was exhaustively extracted with ethanol, filtered and the filtrate was concentrated in a rotary evaporator. The ethanolic extract was redissolved in water, filtered and the filtrate was concentrated to get a polyphenol rich aqueous fraction[10,11].

Determination of Total Phenolic Content

A 100µl of extract was mixed with 0.5ml FC reagent (diluted 10 times with distilled water). The solution was mixed with 7ml of distilled water and allowed to stand at room temperature for 5 minutes. 1.5ml sodium bicarbonate (60mg/ml) solution was added to the mixture and left at room temperature in dark for 2 hours. Absorbance was read at 725nm against blank using UV-Visible spectrophotometer (Perkin Elmer Lambda 35, USA). A calibration curve was prepared, using a standard solution of gallic acid (0.2, 0.4, 0.6, 0.8 and1mg/ml). Results were expressed as gallic acid equivalents (GAE) mg /ml[17].

In - Vitro Antioxidant Activity

1. DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay

A solution of 3.3mg DPPH in 100ml methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02 - 0.1 mg of the extract. The reaction mixture was vortexed thoroughly and left in the dark at 25°C for 30 min. The absorbance of the mixture was measured at 517 nm. BHT was used as reference. The ability to scavenge DPPH radical was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{A_{(control)} - A_{(test)}}{A_{(control)}} \times 100$$

where $A_{(control)}$is the absorbance of DPPH radical + methanol; A(test) is the absorbance of DPPH radical + test sample.

IC_{50} value i.e. the half maximal (50%) inhibitory concentration was calculated using the % Inhibition values graphically[18].

2. ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) scavenging activity

The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution

was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The $ABTS^{+}$ scavenging capacity of the extract was compared with that of BHT and percentage inhibition was calculated using the same equation as that for DPPH method[19].

3. FRAP (Ferric Reducing Antioxidant Potential) Assay:

The stock solutions included 300 mM acetate buffer (3.1 g sodium actete and 16 ml acetic acid), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mMHCl, and 20 mM $FeCl_3$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3$. The temperature of the solution was raised to 37°C before using. Plant extracts (150 µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µMTrolox. The FRAP scavenging capacity of the extract was compared with that of BHT and the percentage inhibition was calculated using the same equation as that for DPPH method[19].

Biological Screening

Healthy adult Wistar albino rats aging between 2 and 3 months and weighing 150–200 g were used for the study. Housed individually in polypropylene cages, maintained under standard conditions (12 h light and 12 h dark cycle, 25±30°C, 35–60% relative humidity), the animals were fed with standard rat pellet diet (Hindustan Lever Ltd., Mumbai, India) and water ad libitum.

Acute Oral toxicity

Acute toxicity test was performed according to the OECD guideline for testing of chemicals 425 (OECD 2001) on healthy Wistar albino rats of either sex. They were divided into two groups, of five rats each and were orally fed with the polyphenol rich fraction in increasing dose levels of body weight as follows100, 500, 1000, 3000 and 5000 mg/kg. The animals were observed continuously for 24 h for their behavioral and Psychological response[20].

After a period of 24 h, 72 h and 14 days they were observed for any lethality or death. Approval number: (ACP/2551(G)/2009)

Oral Glucose Tolerance Test

The rats were fasted for 18 hours and later fed with distilled water. Rats were divided into four groups as follows:

Group I- Glucose (2.5 g/kg, p.o.)

Group II- Glibenclamide (0.4 mg/kg)

Group III- polyphenol rich fraction (15 mg/kg)

Group IV- polyphenol rich fraction (30 mg/kg)

All the groups were glucose loaded (2.5 g/kg, p.o.), 30 minute after test and standard drug administration. Blood samples were withdrawn from all animals at 0, 30 and 60 minutes after glucose loading and glucose levels were estimated using Glucose oxidase–peroxidase reactive strips and a Glucometer (ArkayPiramel Medical Pvt. Ltd. INDIA)[19,20].

Evaluation of Serum glucose in Alloxan induced Diabetic rats

The acclimatized animals were fasted for 24 hours after which they were administered with water*ad libitum* and injected subcutaneously with 120 mg/kg b.w. of alloxan monohydrate freshly prepared in 0.9% normal saline solution. After 72 hrs blood samples was withdrawn by retro orbital puncture and serum glucose (SG) was estimated by enzymatic colorimetric method. The animals which showed SG more than 200 mg/dl were selected for study.

Group I: Normal Control (Saline solution, 10 ml/kg, p.o.)

Group II: Glibenclamde (0.4 mg/kg)

Group III: polyphenol rich root extract (15 mg/kg)

Group IV: polyphenol rich root extract (30 mg/kg)

The drug solutions were prepared and administered orally according to the body weight of the animals. Blood samples were withdrawn from all animals at baseline, 7^{th} and 14^{th} day by retro orbital puncture method and were subjected to estimation of various biochemical parameters using enzymatic calorimetric method. The effects of test and standard drugs in normal and diabetic rats were observed by evaluating fasting blood glucose levels using the BIO LAB Diagnostics Kit (505nm) on CHEM-7 semi auto-analyzer along with measure of serum insulin at 7^{th} and 14^{th} day of the study[21].

Analysis of Biochemical Parameters

The biochemical parameters associated with the diabetic disease were determined on 14th day of treatment on CHEM-7 semi auto-analyzer at specific wavelengths (λ)[22, 23].

i. Serum Triglycerides: ERBADiagnostic kit (λ: 505 nm)

ii. Serum Total Cholesterol: ERBADiagnostic kit (λ: 546 nm)

iii. Serum HDL: ERBADiagnostic kit (λ: 600 nm)

iv. Serum LDL: ERBADiagnostic kit (λ: 600 nm)

v. Serum Alkaline Phosphatase: MBK SPAM Diagnostics Kit(λ: 510 nm)

vi. Serum Insulin: ELISA reader

Statistical analysis

Data were statistically evaluated using one-way ANOVA, followed by Dunnett test. The values were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

The Total Phenolic content of *Cichoriumintybus* root extract was found to be 30.23 µg/ml

2. *DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay:*

The 50% Inhibition of DPPH radical for standard antioxidant was found at the concentration of 60µg/ml(**IC_{50}** 60µg/ml). The polyphenol rich fraction showed a

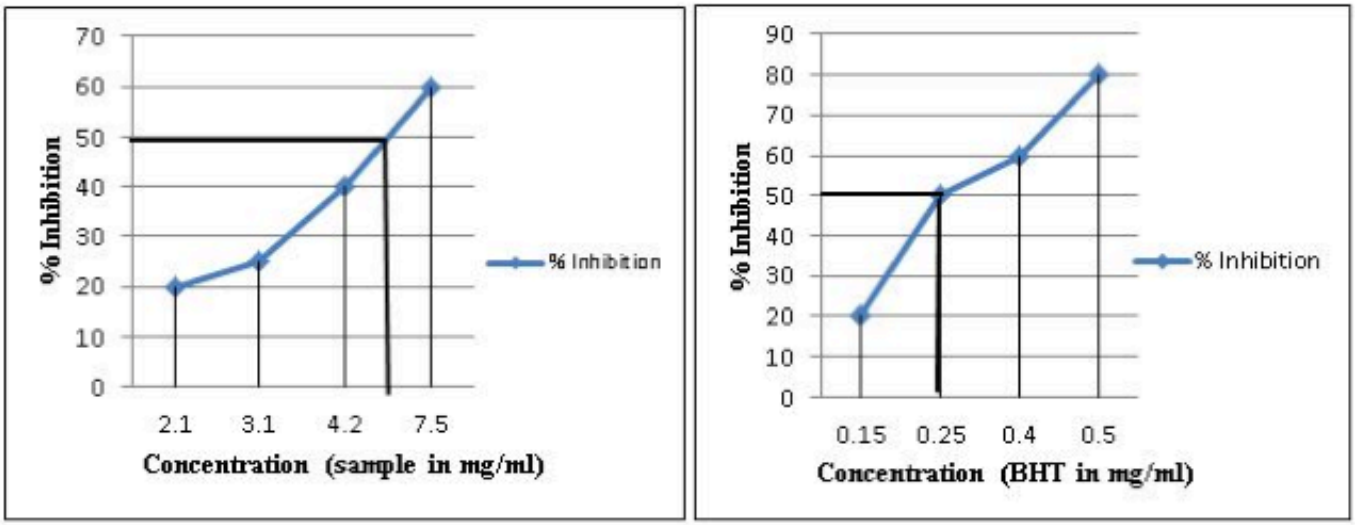


Figure 1: IC_{50} values for DPPH Assay

potential antioxidant capacity with This was comparatively similar to the standard antioxidant compound BHT which showed IC_{50} value of 60µg/ml. (Table 1).

3. *ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) scavenging activity:*

The 50% inhibition of the reactive oxygen species was found for the isolated polyphenols at 4.5 µg/ml (***P<0.001) concentration. The 50% inhibition of standard antioxidant compound BHT was found at the concentration of 0.25mg/ml (***P<0.001).

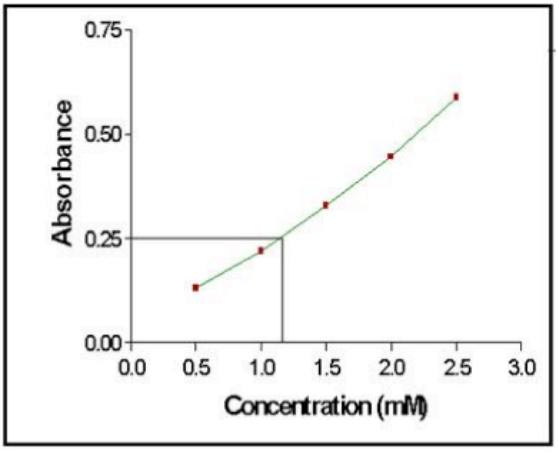


Figure 2: IC_{50} values for ABTS Assay

Table 1: IC_{50} value for Antioxidant Assays

Sr. No.	Sample	IC_{50} value for DPPH Assay	IC_{50} value for ABTS Assay
1	Test	60µg/ml	4.2mg/ml
2	Standard (BHT)	50 µg/ml	0.25mg/ml

3. *FRAP Antioxidant Assay:*

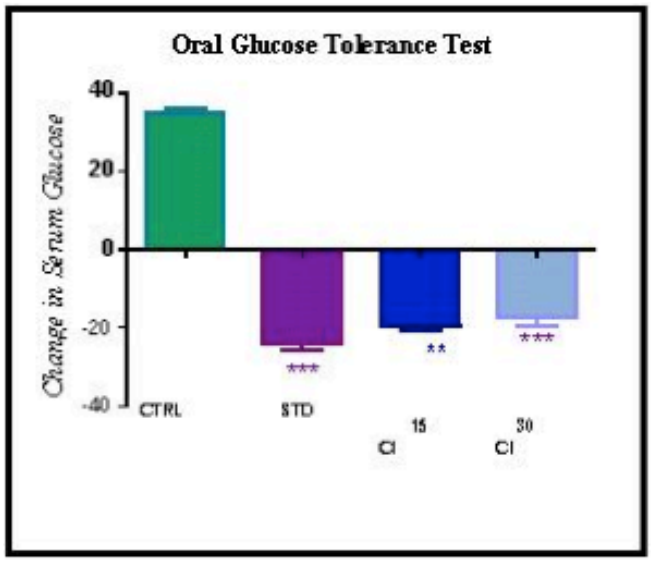


Figure 3: Trolox Equivalent curve

The TEAC value was found to be 1.178 mM for the polyphenol rich fraction of the root extract of*Cichoriumintybus*.

Biological Screening

Acute Oral Toxicity

The LD_{50} value was found to be greater than 5000 mg/kg with no mortality.

Oral Glucose Tolerance Test

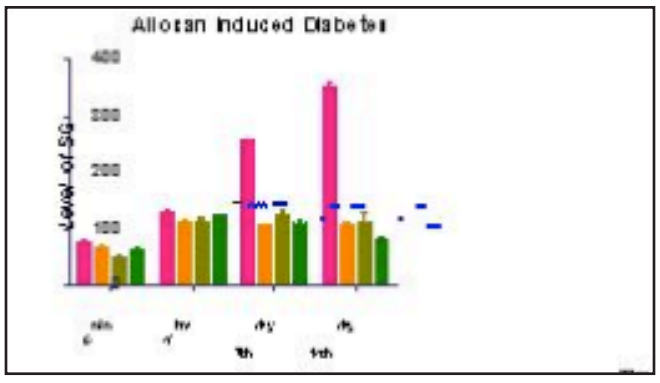
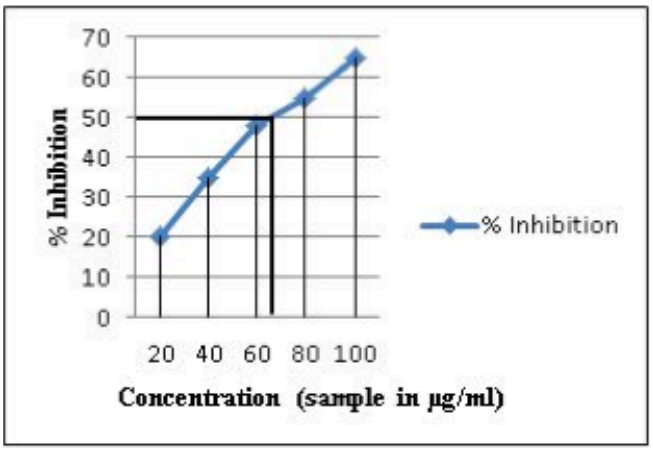


Figure 4: Effect of Drug treatment on glucose loaded rats after 90 min

In the present study glucose (2.5 g/kg) was administered to rats after pretreatment with polyphenol rich fraction. Administration of 15 mg/kg, p.o. dose caused change in SG by 19.33, 16 and 9.5 at 30, 60 and 90 minutes respectively. Peak reduction was observed at 90 minutes. Administration of 30 mg/kg of polyphenol rich fraction caused a significant change in SG by 24, 13.5 and 7 at 30, 60 and 90 minutes respectively. Peak reduction was observed at 90 minutes.

Alloxan Induced Diabetes in Rats



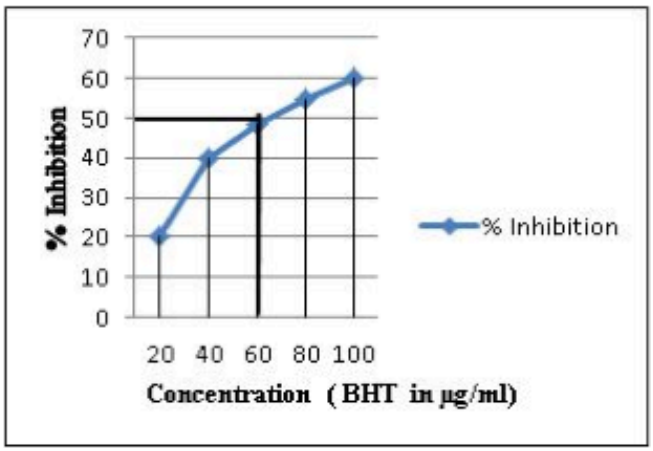


Figure 5: Effect of Serum Glucose (SG) at different time interval

Administration of standard Metformin (150mg/kg, p.o) resulted in decrease in elevated SG 112mg/dl to 103mg/dl on 7th day (***p<0.001) and 112mg/dl to 104.66mg/dl on 14th day (**p<0.01). Administration of

polyphenol rich fraction of *Cichoriumintybus* (CI) (15 mg/kg, p.o.) resulted in increase in elevated SG 111mg/dl to 124.66mg/dl on 7th day(**p<0.01) and decrease in elevated SG 111mg/dl to 108.33mg/dl on 14th day (**p<0.01).

Administration of polyphenol rich fraction of *Cichoriumintybus* (CI) (30 mg/kg, p.o.)resulted in decrease in elevated SG 122mg/dl to 109.66mg/dl on 7th day (***p<0.001) and 122mg/d to78.66mg/dl on 14th day (***p<0.001).

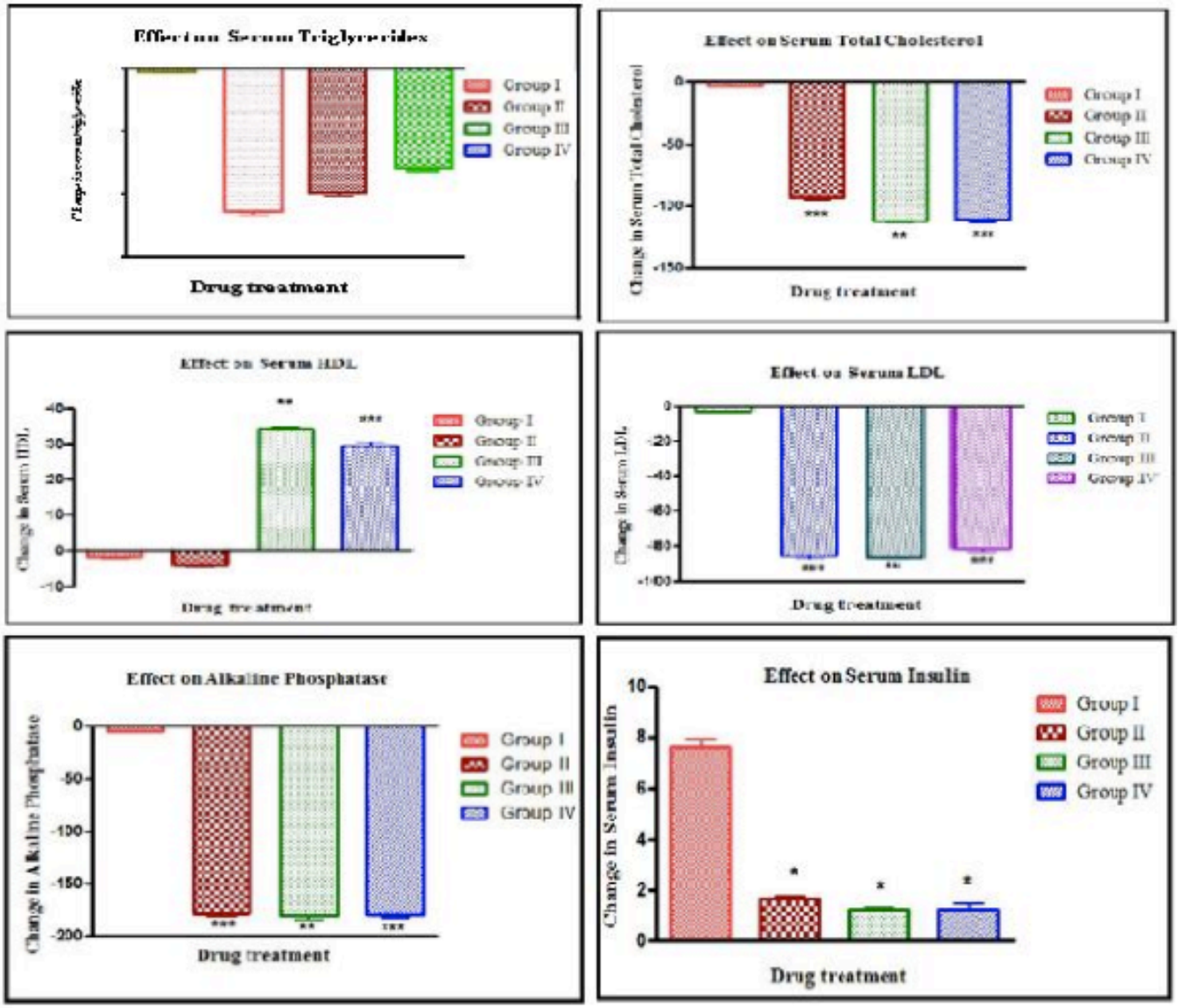


Figure 6: Effect of Polyphenol rich fraction and standard drug on various biochemical parameters

Chronic heart disease in diabetic patients is associated with increased plasma cholesterol levels, reduced HDL in non insulin dependent diabetic patients and the most common lipid abnormality i.e. raised triglyceride levels. The efficacy of conventional anti-diabetic drugs on lipid metabolism has been reported to decrease over a period of time. When treated with Cichorium intybus-polyphenol rich fraction (30mg/kg), there was a significant reduction in serum triglycerides (**P<0.01), total cholesterol concentrations (**P<0.01) (Figure 6) and significant increase in serum HDL level in diabetic rats (**P<0.01) respectively as compared to untreated group. (Figure 6) 30mg/kg dose also caused significant decrease (**P<0.01) in Serum LDL and alkaline phosphatase level. (Figure 6) However, there was no significant change in serum insulin of all drug treated animals (**P<0.05). (Figure 6) While the serum triglyceride and cholesterol levels decreased significantly

in drug treated diabetic rats, the HDL cholesterol level was found to be improved. Thus the weight loss associated with drug treated diabetic rats may be attributed directly to its lipid lowering activity or indirectly to its influence on various lipid regulation systems. The comparable effect of Cichoriumintybus polyphenol rich fraction (30mg/kg) with Glibenclamide may suggest similar mode of action since it was able to lower blood sugar level in alloxanised rats where the pancreatic β-cells were permanently damaged indicating that the polyphenol rich fraction possesses extra pancreatic effects.

CONCLUSION

Thus it can be concluded that the polyphenol rich fraction of Cichoriumintybus has a promising hypoglycemic potential which might be accounted for its abundant water soluble polyphenol content which are proven to scavenge the harmful free radicals. However the confirmation can be drawn from the isolation and structural elucidation of the polyphenols from the polyphenol rich fraction and its screening in the diabetic human population to elucidate its mechanism of action.

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