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Review Article

Phytochemistry, Pharmacological Activities and Intellectual Property Landscape of *Gardenia jasminoides* Ellis: a Review

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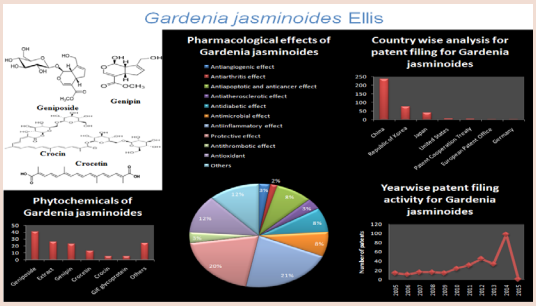
ABSTRACT

Gardenia jasminoides, the genus of Gardenia, a Chinese medicinal plant, which belongs to the family Rubiaceae is herb used since ancient times. It is also known as *Fructus Gardeniae* and *Gardenia augusta* as different synonyms, well known as Anant in Marathi language, Gandharaj in Hindi language and Zhi Zi in Chinese language. *Gardenia jasminoides* extracts and its main active phytoconstituents geniposide, genipin, crocin, crocetin have been reported for a wide range of pharmacological activities such as anti-hyperglycemic, anti–atherosclerotic, anti-inflammatory, anti-arthritis, anti-cancer, anti–apoptotic, anti-oxidant, anti-angiogenic, anti-thrombotic, anti-microbial and miscellaneous activities. Also it has been explored its protective effect through diverse mechanisms like neuroprotective for Alzheimer's disease, hepatoprotective, gastro-protective, retino-protective, nephro-protective, skin protective activities. This review will give new insights of *Gardenia jasminoides* relating to the ethnopharmacology, phytochemistry and pharmacological uses. This data will also highlight the patenting trends and different assignees involved in filing patents for *Gardenia jasminoides*.

Key words: Anant, Crocin, Crocetin, *Fructus Gardeniae*, *Gardenia jasminoides*, *Gardenia augusta*, Gandharaj, Geniposide, Genipin.

SUMMARY

- A number of phytochemicals isolated from *Gardenia jasminoides* Ellis in the structural characterization studies have been reported. Out of them, geniposide, genipin, crocin, crocetin are found as pharmacologically active principle. Geniposide is the major phytochemical extensively used in studies among all phytoconstituents.
- *Gardenia jasminoides* has been explored in a wide range of pharmacological activities such as anti-hyperglycemic, anti-atherosclerotic, anti-inflammatory, anti-arthritis, anti-cancer, anti-apoptotic, anti-oxidant, anti-angiogenic, anti-thrombotic, anti-microbial and miscellaneous activities. Its anti-inflammatory activity has been reported in the topmost rank.
- Out of these assignees, China is the main leading country to be assignee for filing highest number of patents for *Gardenia jasminoides* in the field of traditional Chinese medicine. In the year of 2014, patents for *Gardenia jasminoides* has been filed in the highest record.



PICTORIAL ABSTRACT

Abbreviations used: GJ: *Gardenia jasminoides* Ellis, FG: *Fructus Gardeniae*, TCM: Traditional Chinese Medicine, AD; Alzheimer's disease, WIPO : World Intellectual Property Organization, AP: Acute Pancreatitis, IDE: Insulin Degrading Enzyme, PPAR: Peroxisomal Proliferator-Activated Receptor, ROS: Reactive Oxygen Species.

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INTRODUCTION

Ethnopharmacology

Gardenia jasminoides Ellis (GJ), the genus of *Gardenia* which is belonging to the family Rubiaceae, an ancient medical herb, is noted for its medicinal properties in Chinese, Korean, pharmacopoeias. It is also known as *Fructus Gardeniae* and *Gardenia augusta* as different synonyms, well known as Anant in Marathi language; Gandharaj in Hindi language and Zhi Zi in Chinese language. *Gardenia jasminoides*, gardenia, cape jessamine, danh-danh, or jasmin is an evergreen flowering plant. Its origin is found in most Asian continents like Vietnam, Southern China, Taiwan, Japan, Myanmar and India. It is growing wild and also cultivated in garden in warm temperate and subtropical climates. It has heavily fragrant white summer flowers with its shiny green leaves. Traditionally the fruit of *Gardenia jasminoides* has been used in formulating folk medicine in treating inflammation, headache, edema, fever, hepatic disorders, and hypertension.[1] It is well known as *Fructus Gardeniae* (FG), namely the dried ripe fruits of *G. jasminoides*, has reported for its extensive pharmacological activities and widely used in Traditional Chinese Medicine (TCM).[2] Parmar *et al*[3] have been well documented the review of this plant species having various medicinal properties in 2000 however further reviewing of this plant species especially on *Gardenia jasminoides* has not been reported so far.

Literature survey and search strategies for patent applications

The aim of this study was to review the different pharmacological activities and to reveal newer phytoconstituents in *Gardenia jasminoides* through survey of several literatures. We have investigated scientific literature survey by applying search strategies "*Gardenia jasminoides* Ellis", "*Gardenia jasminoides*", "*Fructus Gardeniae*", "*Gardenia jasminoides* Extract" in the databases like PubMed, @sciencedirect, and Google scholar. Patent related information of *Gardenia jasminoides* was searched by using various databases such as @espacenet and WIPO.

Phytochemistry

Geniposide, genipin, geniposidic acid, crocin and crocetin are major phytoconstituents of *Gardenia jasminoides* which have been used exten-

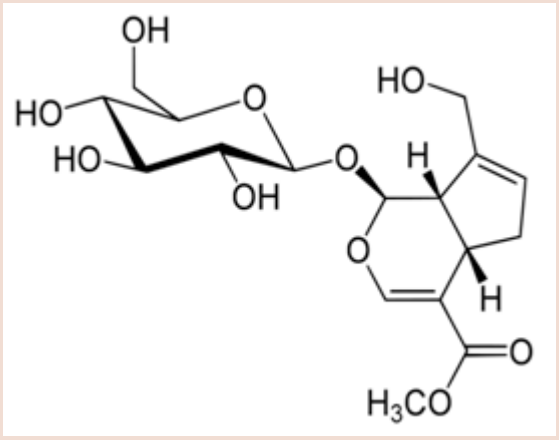

Figure 1: Structure of Geniposide

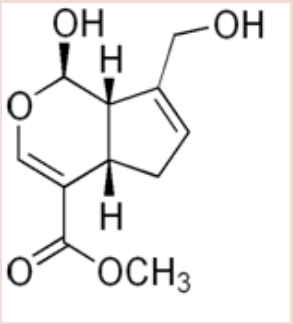

Figure 2: Structure of Genipin

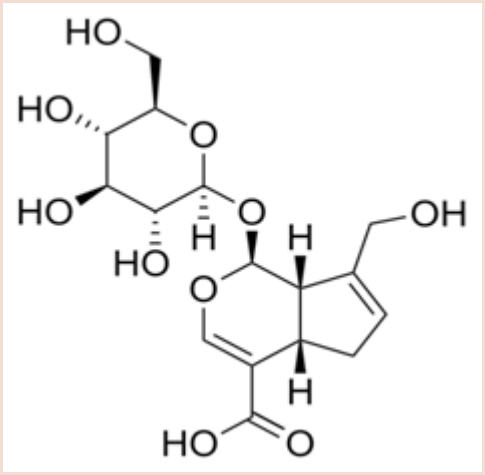

Figure 3: Structure of Geniposidic acid

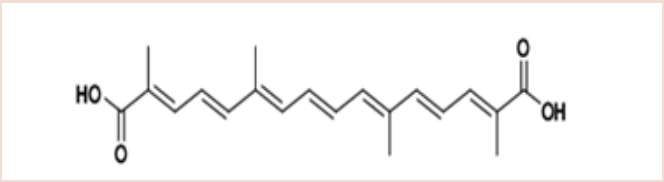

Figure 4: Structure of Crocetin

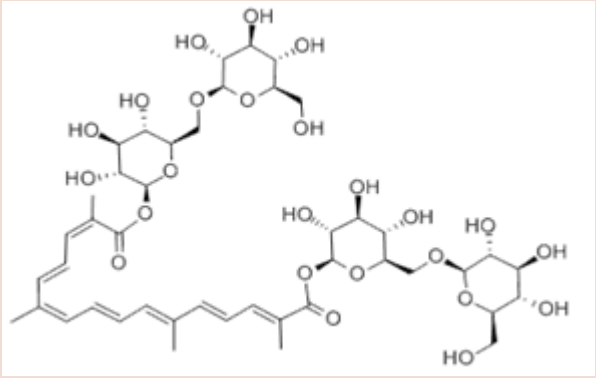

Figure 5: Structure of Crocin

sively in the phytochemical analysis and pharmacological studies (Figure 1-5). A number of newer phytochemicals have been isolated and many of them have been reported for anti-cancer, anti-inflammatory, anti-oxidant, anti-viral, anti-bacterial, anti-depressant, neuroprotective, anti-protozoal, useful for treatment of ankle sprain, osteoporosis and melanogenesis inhibitory effect as shown in Table 1.

Pharmacological activities

Gardenia jasminoides has been used for treatment of inflammation, folklore cure for different ailments, in the ancient traditional medicine system. Figure 6 depicts the overall scenario of the pharmacological activities of different phytoconstituents isolated from *Gardenia jasminoides.* Figure 7 overviews the pharmacological activities of *Gardenia jasminoides.*

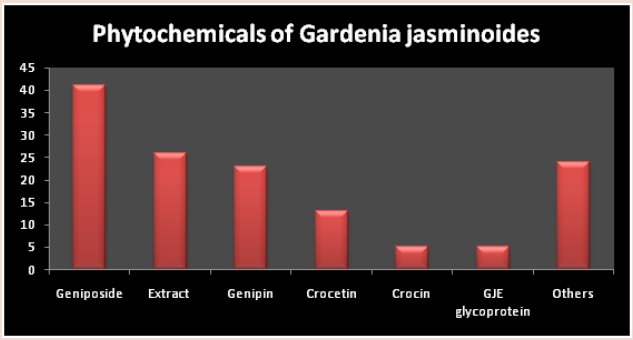

Figure 6: Pharmacological activities of different phytoconstituents isolated from *Gardenia jasminoides*

Table 1: Shows the different phytochemicals isolated from *Gardenia jasminoides* Ellis

Authors	Phytochemicals	Pharmacological effects
Wu *et al*[4]	geniposidic acid (1), chlorogenic acid (2), genipin-1-β-gentiobioside (3), geniposide (4), genipin (5), rutin (6), crocin-1 (7), crocin-2 (8)	-
Luo *et al*[5]	jasminoside I (1), gardenoside (2), gardaloside (3), 3-hydroxy-urs-12-ene-11-ketone(4), 5, 4'-dihydroxyl-7, 3', 5'-trimethoxyflavone (5), 5, 7, 3', 4', 5'-pentamethoxyflavone(6), 3, 5, 6, 4'-tetrahydroxy-3', 5'-dimethoxyflavone (7), shikimic acid (8), 1, 2, 4-benzenetriol (9), 3, 4-dimethoxy-benzoic acid (10), dibutyl phthalate (11) and diisobutyl phthalate (12)	-
Qin *et al*[6]	A novel triterpenoid 3α,16β,23,24-tetrahydroxy-28-nor-ursane-12,17,19,21-tetraen(1)	Anti-cancer effect
Jia-Ling *et al*[7]	garjasmine (1), dunnisin (2), α-gardiol (3), β-gardiol (4), diffusoside A (6), diffusoside B (7), genameside C (13), and deacetylasperulosidic acid (14)	-
Qin *et al*[8]	6″-O-trans-feruloylgenipin gentiobioside (1), 2'-O-trans-p-coumaroylgardoside (2), 2'-O-trans-feruloylgardoside (3)	-
Hai-Bo *et al*[9]	6″-O-trans-feruloylgenipin gentiobioside (1), 2′-O-trans-caffeoylgardoside (2), jasmigeniposide A (3), and one new bis-iridoid glucoside, jasmigeniposide B (4), along with six known analogues (5–10)	Anti-viral effect
Kwak *et al*[10]	Chlorogenic acid	Effect on osteoporosis
Liguo *et al*[11]	2-methyl-l-erythritol-4-O-(6-O-trans-sinapoyl)-β-d-glucopyranoside (1) and 2-methyl-l-erythritol-1-O-(6-O-trans-sinapoyl)-β-d-glucopyranoside (2), along with two known triterpenoids (3–4), four quinic acid derivatives (5–8) and one flavonoid (9)	Anti-inflammatory effect
Zuo *et al*[12]	syringic acid (1), syringaldehyde (2), vanillic acid (3), 3-hydroxy-vanillic acid (4), 3, 4, 5-trimethoxy-phenol (5), 4-hydroxy-3,5-dimethoxy-phenol (6), 4-methoxy-benzaldehyde (7), 7-hydroxy-5-methoxy-chromone (8), crocin-1 (9), crocin-2 (10).	-
Liguo *et al*[13]	ten iridoids (1–10) and ten pyronane monoterpenoids (11–20)	-
Song *et al*[14]	5, 7, 3'-trihydroxy-6, 4', 5'-trimethoxyflavone (1), 5, 7, 3', 5'- tetrahydroxy-6, 4'-dymethoxyflavone (2),kaempferol (3), quercetin (4), 3beta,23- dihydroxyurs-12-en-28-oic acid (5), 3beta,19alpha-dihydroxy-urs-12-en-28-oic acid (6), beta,19alpha,23-trihydroxy-urs-12-en-28-oic acid (7), emodin (8), physcion (9), crocin-I (10), beta-daucosterol (11), beta-sitosterol (12), stearic acid (13), palmitic acid (14), oleic acid (15)	-
Rao *et al*[15]	Gardenal-I (1), Gardenal-II (2), Gardenal-III (3), geniposide (4), 6-β-hydroxy geniposide (5), 6-α-hydroxy geniposide (6), 6-α-methoxy geniposide (7), feretoside (8),genipin-1-β-gentiobioside (9), shanzhiside (10), lamalbidic acid (11) picrocrocinic acid (12)	Anti-microbial and anti--protozoal effects
Li *et al*[16]	6″-O-trans-feruloylgenipin gentiobioside (1), 2'-O-trans-caffeoylgardoside (2), jasmigeniposide A (3), one new bis-iridoid glucoside, jasmigeniposide B (4), along with six known analogues (5-10)	Anti-viral effect
Yang *et al*[17]	2-methyl-L-erythritol-4-O-(6-O-trans-sinapoyl)-β-D-glucopyranoside (1) and 2-methyl-L-erythritol-1-O-(6-O-trans-sinapoyl)-β-D-glucopyranoside (2), along with two known triterpenoids (3-4), four quinic acid derivatives (5-8) and one flavonoid (9)	Anti-inflammatory effect
Zhang *et al*[18]	jasminoside A(1), epijasminoside A(2), 6-O-methylscandoside methyl ester (3), 6-O-methyldeacetylasperulosidic acid methyl ester (4), gardenoside (5), phenylmethol (6), 4-hydroxy-phenylmethol-O-beta-D-glucopyranosyl- (1-->6) -beta-D-glucopyranoside (7), 3,4-dihydroxy-phenylmethol-O-beta-D-glucopyranosyl-(1-6)-beta-D-glucopyranoside (8), 3-hydroxy4-methoxy-phenylmethol-O-beta-D-glucopyranosyl-(1-->6)-beta-D-glucopyranoside (9), 3-hydroxy-4-methoxyphenylmethol-O-beta-D-glucopyranoside (10)	-
Peng *et al*[19]	6"-O-trans-caffeoylgenipin gentiobioside (1), genipin 1-O-β-D-apiofuranosyl (1→6)-β-D-glucopyranoside (2), genipin 1-O-α-D-xylopyranosyl (1→6)-β-D-glucopyranoside (3), three new monocyclic monoterpenoids, jasminoside R (4), jasminoside S (5), jasminoside T (6)	Anti-inflammatory effects
Wang *et al*[20]	(Gardeniside A-C), 11α,12α-epoxy-3β-[(O-β-D-glucuronopyranoside-6'-O-methly ester)oxy]olean-28,13-olide (1),siaresinolic acid 3-O-β-D-glucuronopyranoside-6'-O-methly ester (2), 3-O-β-D-glucuronopyranoside-6'-O-methly ester-siaresinolic acid-28-O-β-D- glucopyranoside (3), oleanolic acid 3-O-β-D- glucuronopyranoside-6'-O-methly ester (4), oleanolic acid 3-O-β-D- glucuropyranoside (5), hederagenin 3-O-β-D- glucuronopyranoside-6'-O- methly ester (6), chikusetsusaponin IVa methyl ester (7), chikusetsusaponin (8), chikusetsusaponin IVa butyl ester (9), siaresinolic acid 28-o-β-d-glucopyranosyl ester (10)	Anti-cancer effect
Yu *et al*[21]	A new lignan glucoside, (+)-(7S,8R,8'R)-lyoniresinol 9-O-β-D-(6″-O-trans-sinapoyl)glucopyranoside (1), and a new iridoid glucoside, 10-O-trans-sinapoylgeniposide (2), together with eight known compounds	-
Kim *et al*[22]	protocatechuic acid (1), geniposide (2), 6'-O-trans-p-coumaroylgeniposide (3), 3,5-d-ihydroxy-1,7-bis (4-hydroxyphenyl) heptanes (4), and ursolic acid (5),	Anti-depressant effect
Huang *et al*[23]	geniposide (I), 6alpha-hydroxygeniposide (II), genipin-gentiobioside (III), adian-5-en-3alpha-ol (IV), (23Z) -cycloart-23-en-3beta,25-diol (V), 7alpha-hydroxy sitosterol (VI) and 5,8-epidioxystigmasta-6,22-dien-3-ol (VII)	-

Akihisa *et al*[24]	10-O-(4"-O-methylsuccinoyl)geniposide (7), and two new pyronane glycosides, jasminosides Q and R (13 and 14, resp.), along with nine known iridoid glycosides, 1-6 and 8-10, and two known pyronane glycosides, 11 and 12	Melanogenesis inhibitory effect
Yu *et al*[25]	(1R,7R,10S)-11-O-β-D-glucopyranosyl-4-guaien-3-one (1) and (1R,7R,10S)-7-hydroxy-11-O-β-D-glucopyranosyl-4-guaien-3-one (2)	-
Clifford *et al*[26]	three caffeoylquinic acids, three dicaffeoylquinic acids, three sinapoylquinic acids, four caffeoyl-sinapoylquinic acids, two feruloyl-sinapoylquinic acids, one p-coumaroyl-sinapoylquinic acid, three (3-hydroxy, 3-methyl) glutaroylquinic acids, two (3-hydroxy, 3-methyl) glutaroyl-feruloylquinic acids, one (3-hydroxy, 3-methyl) glutaroyl-dicaffeoylquinic acid, and one (3-hydroxy, 3-methyl) glutaroyl-caffeoyl-feruloylquinic acid. Six (3-hydroxy, 3-methyl) glutaroyl-caffeoylquinic acids were detected and two were tentatively assigned as 3-caffeoyl-4-(3-hydroxy, 3-methyl) glutaroylquinic acid and 3-caffeoyl-5-(3-hydroxy, 3-methyl) glutaroylquinic acid.	-
Jarubol *et al*[27]	Linalool, alpha-farnesene, z-3-hexenyl tiglate and trans-beta-ocimene	Anti-microbial and anti-oxidant effect
Chen *et al*[28]	genipin 1-O-beta-D-isomaltoside (1) and genipin 1,10-di-O-beta-D-glucopyranoside (2), together with six known iridoid glycosides, genipin 1-O-beta-D-gentiobioside (3), geniposide (4), scandoside methyl ester (5), deacetylasperulosidic acid methyl ester (6), 6-O-methyldeacetylasperulosidic acid methyl ester (7), and gardenoside (8)	Treatment of ankle sprain.
Yu *et al*[29]	6"-O-trans-sinapoylgenipin gentiobioside (1), 6"-O-trans-p-coumaroylgenipin gentiobioside (2), 6"-O-trans-cinnamoylgenipin gentiobioside (3), 6'-O-trans-p-coumaroylgeniposide (4), 6'-O-trans-p-coumaroylgeniposidic acid (5), 10-O-succinoylgeniposide (6), and 6'-O-acetylgeniposide (7), two new monoterpenoids, 11-(6-O-trans-inapoylglucopyranosyl) gardendiol (8) and 10-(6-O-trans-sinapoylglucopyranosyl) gardendiol (9), and three known ones, 6'-O-trans-sinapoylgeniposide (10), geniposide (11), and 10-O-acetylgeniposide (12),	Neuroprotective effect on Alzheimer's disease.
Li *et al*[30]	gardenia oil	Hypnotic and anti--seizure effects
Chen *et al*[31]	jasminodiol (1), jasminoside H (6), 6'-O-sinapoyljasminoside A (7), 6'-O-sinapoyljasminoside C (8), and jasminoside I (9)	Anti-inflammatory effect
Chen *et al*[32]	imperatorin (1), isoimperatorin (2), crocetin (3), 5-hydroxy-7, 3', 4', 5'-tetrainethoxyflavone (4), 2-methyl-3, 5-dihydroxychromone (5), sudan III (6), geniposide (7), crocin (8), crocin-3 (9)	-
Kim *et al*[33]	vanillic acid 4-O-beta-d-(6'-sinapoyl) glucopyranoside (1) and five new quinic acid derivatives, methyl 5-O-caffeoyl-3-O-sinapoylquinate (2), ethyl 5-O-caffeoyl-3-O-sinapoylquinate (3), methyl 5-O-caffeoyl-4-O-sinapoylquinate (4), ethyl 5-O-caffeoyl-4-O-sinapoylquinate (5), and methyl 3,5-di-O-caffeoyl-4-O-(3-hydroxy-3-methyl) glutaroylquinate (6)	Anti-oxidant effect, anti-viral effect
Chang *et al*[34]	gardaloside (1), jasminoside G (2), geniposide (3), 6alpha-hydroxygeniposide (5), ixoroside (7), and shanzhiside (8)	Immunosuppressive effect
Machida *et al*[35]	7 beta,8 beta-epoxy-8 alpha-dihydrogeniposide (1) 8-epiapodantheroside (2), were isolated, together with six known (3-8) and three artifact (9-11) iridoids	-
Machida *et al*[36]	gardenate A (1), 2-hydroxyethyl gardenamide A (2), (1R,7R,8S,10R)-7,8,11-trihydroxyguai-4-en-3-one 8-O-beta-D-glucopyranoside (3) and Jasminoside F (4)	-

Anti-diabetic and anti-atherosclerotic activities

Aqueous extract of *Gardenia jasminoides* in normal dose 200 mg/kg exerted a PPARγ-activating hypoglycemic effect by restoring insulin resistance therefore; it was proved as a potential agent for insulin-sensitizing in type 2 diabetes mellitus with insulin resistance[37] and also attenuated the severity of acute pancreatitis (AP) as well as pancreatitis-associated lung injury.[38] The main mechanism of hypoglycemic effect of geniposide was mediated by inhibiting the GP and G6Pase activities.[39] The fibril precursors of islet amyloid polypeptide (IAPP) are cytotoxic to pancreatic β cells which lead to β-cell dysfunction in type 2 diabetes mellitus (T2DM). The protective effects of geniposide exerted in pancreatic INS-1E cells by preventing human islet amyloid polypeptide (hIAPP)-induced cell damage in INS-1E cells and bacitracin, an inhibitor of IDE activity and involving up regulation of IDE expression a key degrading protein of (hIAPP).[40] Geniposide inhibited the phosphorylation of downstream target GSK3β which was counteracted by preincubation with LY294002 along with increased expression of GLUT2[41] and restraining the adhesion of monocytes to HUVECs and the expression of CAMs induced by high glucose in treatment for diabetic vascular injury.[42] Ethanolic extract of *Gardenia jasminoides* inhibited TNF-alpha-induced NF-kappaB activation, adhesion molecule expression, and monocyte-endothelial interaction in the mechanism of treating vascular diseases, such as atherosclerosis.[43] Geniposide up-regulated the expression of foxp3, promoted Treg-cell-associated cytokines (TGF-β1 and IL-10) cells and ameliorated the atherosclerotic lesions progression partly through lipids regulation and immunoregulation[44] while its metabolite genipin suppressed the intracellular lipid accumulation and also significantly increased the intracellular expression of a fatty acid oxidation-related gene (peroxisomal proliferator-activated receptor: PPARα) so it was confirmed its anti--obesity, insulin resistance-alleviating and abnormal lipid metabolism-alleviating effects.[45] Effects of geniposidic acid on protecting vascular endothelium and reversing plaque formation was elevated.[46] Genipin exerted anti-diabetic activity by improving insulin sensitivity through ameliorating insulin-stimulated glucose update and glycogen synthesis, inhibited overproduction of cellular reactive oxygen species (ROS); reversing hepatic oxidative stress-associated JNK hyperactivation and reduced Akt phosphorylation and alleviating mitochondrial membrane potential (MMP) and mitochondrial ATP dysfunction;[47] promoted glucose transporter 4 (GLUT4) translocation to the cell surface in sub-cellular membrane fraction and amplified the phosphorylation of insulin

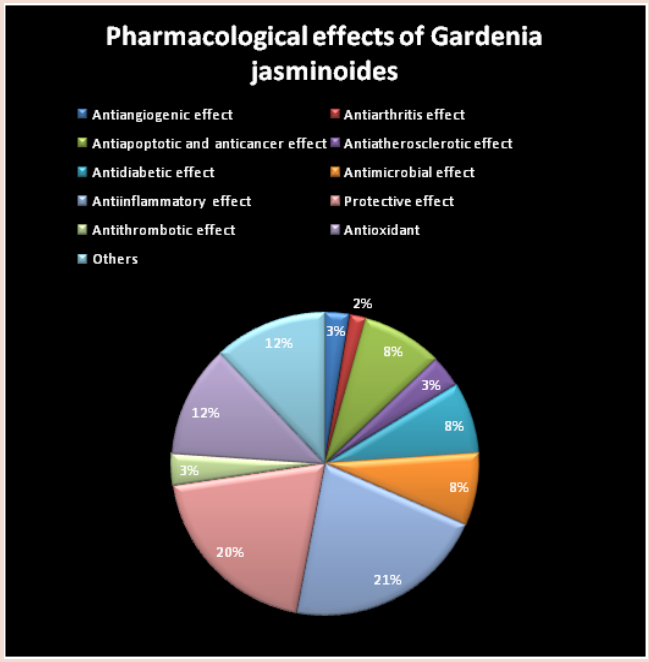

Figure 7: Overview of pharmacological activities of *Gardenia jasminoides* in scientific literature analysis
Source: WIPO Patent analysis

receptor substrate-1 (IRS-1), AKT, and GSK3β to augment ATP levels, closed K (ATP) channels, the concentration of calcium in the cytoplasm in C(2) C(12) myotubes with increases the level of ROS and ATP in myotubes.[48] Crocetin proved its anti-diabetic effect by restoring dexamethasone-induced insulin resistance and related abnormalities in rats[49] by inhibiting pancreatic lipase[50] and malabsorption of fat and cholesterol due to the inhibition of pancreatic lipase and its metabolite, crocetin improved hyperlipidemia.[51]

Anti-inflammatory activity

Geniposide markedly inhibited the lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α), IL-6 and IL-1β production both *in vitro* as well as *in vivo*. It neutralizes *in vitro* LPS through binding with LPS which significantly protected sepsis model mice[52] and significantly reduced the infiltration of inflammatory cells and down regulated the production of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) by suppressing the phosphorylation of inhibitory kappa B (IκBα), nuclear factor-κB (NF-κB), p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK).[53] Furthermore, it is not only down-regulating the expression of TLR4 up-regulated by LPS stimulated primary mouse macrophages but also LPS-induced IL-8 production in HEK293-mTLR4/MD-2 cells. It attenuated lung histopathologic changes in the mouse models *in vivo* which indicated for to be highly effective in inhibiting acute lung injury.[54] Anti-inflammatory effect of geniposide exerted by inducing the production of ROS and inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-stimulated N9 murine microglial cells through the p38, ERK1/2 and nuclear factor-κB (NF-κB) signaling pathways; attenuating the activation of N9 cells; inhibiting the overproduction of NO, intracellular ROS and the expression of iNOS induced by LPS in the cells and blocking the phosphorylation of p38, ERK1/2 and inhibited the drop-off of IκB induced by LPS in the cells.[55] Also geniposide acts as anti-asthmatic agent due to its anti-inflammatory properties which prevented eosinophilic pulmonary infiltration, attenuated the increases in interleukin (IL)-4, IL-5, and IL-13, and reduced eotaxin and vascular cell adhesion molecule 1 (VCAM-1) expression.[56] It substanti-ally inhibited LPS-induced alveolar wall changes, alveolar haemorrhage, and neutrophil infiltration in lung tissue, with evidence of reduced myeloperoxidase (MPO) activity by blocking nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinases (MAPK) signaling pathway activation.[57] It mainly exerts its anti-inflammatory effects through suppressing the expression mitogen-activated protein kinase (MAPK), activator protein (AP)-1and release of the LPS-induced production of the inflammatory factors such as cytokine, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), nitric oxide (NO) and prostaglandin E2 (PGE2), the mRNA and protein expression of the NO and PGE2 synthases, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).[58] It facilitates to restructure the ligament tears by proliferating ligament fibroblasts and promoting the synthesis of collagen in case of ankle sprain.[59] Genipin exhibited anti-inflammatory effects via downregulation of chemokine ligand, chemokine receptor, and IFN-induced protein productions in LPS-induced acute systemic inflammation.[60] Genipin prevented IL-1β-mediated CCL20 and IL-6 production in HPDLCs through suppressing nuclear factor kappa B (NF-κB) p65, extracellular signal regulated kinase (ERK) and MAPK/ERK kinase (MEK) phosphorylations.[61] Both genipin and geniposide inhibited production of exudate and nitric oxide (NO). However, genipin possessed stronger anti-inflammatory activity than geniposide.[1] Genipin increased production of the ROS and the ROS-producing NAPDH-oxidase (NOX) family oxidases, NOX2 and NOX3 by activating Akt, MAPKs and AP-1/NF-κB for ROS-dependent cyclooxygenase-2 (COX-2) expression up-regulation and prostaglandin E2 (PGE2) production.[62] It attenuates lipopolysaccharide (LPS)-induced sickness behavior in rodents due to changes of emotional behaviors through inhibition of neural activation and inflammatory responses in the paraventricular nucleus (PVN) of the hypothalamus and the central nucleus of the amygdala (CeA).[63] Anti-NO production and anti-inflammatory activities of *Gardenia jasminoides* were increased by suppression of the protein and m-RNA expressions of iNOS and COX-2 in LPS-activated macrophage and concluded that crocetin has greater anti-inflammatory activity than crocin.[64] Crocin markedly exerted the expression of heme oxygenase-1 (HO-1) leading to anti-inflammatory response by inhibiting inducible nitric oxide synthase (iNOS) expression and nitric oxide production via downregulation of nuclear factor kappa B activity in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages and inducing Ca(2+) mobilization from intracellular pools and phosphorylation of Ca(2+)/ calmodulin-dependent protein kinase 4 (CAMK4).[65] Crocin was found to inhibit the productions of prostaglandin E (2) (PGE (2)) in lipopolysaccharide (LPS)-challenged RAW 264.7 significantly, which is similar to its prevention of the nuclear translocation of the NF-kappaB p50 and p65 subunits.[66] Crocetin reduced the LPS-induced lung oedema and histological changes by increasing LPS-impaired superoxide dismutase (SOD) activity, and decreased lung myeloperoxidase (MPO) activity by significantly attenuating LPS-induced mRNA and protein expression of interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) in lung tissue.[67]

Gardenia jasminoides Ellis (GJE) has been used to cure inflammation in Korean folk medicine for a long time. Inhibitory effect of glycoprotein isolated from GJE (10 mg/kg, 27 kDa) was effective on inflammation mechanism in cadmium chloride-exposed ICR mice by decreasing the levels of lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and thiobarbituric acid-reactive substances (TBARS); attenuating c-Jun N-terminal protein kinase (JNK), heat shock protein 27 (Hsp27), activator protein (AP)-1, nuclear factor (NF)-κB and expression of inflammation-related mediators including pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 with increased activities of anti-oxidative enzymes viz; superoxide dismutase (SOD), gluthathione peroxidase (GPx)[68] and also suppressing intracellular ROS and intracellular

$Ca(2^+)$, activities of activator protein (AP)-1, cyclooxygenase (COX)-2, matrix metalloproteinase (MMP)-9, and arachidonic acid (AA).[68]

Protective effects

Neuroprotective activity for Alzheimer's disease (AD)

Microglia is the prime effectors in immune and inflammatory responses of the central nervous system (CNS). Brains of Alzheimer's disease (AD) patients are characterized by large deposits of amyloid beta peptide (Abeta). Abeta responsible to increase free radical production in nerve cells, leading to cell death that is characterized by lipid peroxidation, DNA/RNA and protein oxidation. Ethanolic extract of *Gardenia jasminoides* was effective significantly among hexane, chloroform, and ethyl acetate to ameliorate on Abeta-induced oxidative stress, by reducing oxidative stress.[69] Oxidative stress and mitochondrial dysfunction contribute to the disease progression in Alzheimer's disease (AD) which geniposide exerts protective effects on mitochondrial dysfunction in APP/PS1 mice through suppressing the mitochondrial oxidative damage to attenuate memory deficits and increasing the mitochondrial membrane potential and activity of cytochrome c oxidase through the suppression of mitochondrial oxidative stress. Thus, geniposide regarded to be a potential therapeutic reagent for halting and preventing AD progress.[70] Geniposide showed a 22.8% acetyl cholinesterase (AChE) inhibitory activity and a potent ameliorating effect on scopolamine-induced memory impairment in amnesic mice of 93.4% as compared to the control group.[71] It has protection to neuronal cells from damage in oxygen-glucose deprived hippocampal slice culture, the granule cell layer than on the pyramidal cell layer including CA 1 and CA 3.[72] Receptor for advanced glycation end products (RAGE) mediated Aβ-induced microglial activation leads to neuroinflammation through release of proinflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β). So it was proved to be a potent suppressor of neuroflammation by blocking significantly Aβ-induced RAGE-dependent signaling (activation of ERK and NF-κB) along with the production of TNF-α and IL-1β in cultured BV2 microglia cells;[73] by attenuating the oligomeric Aβ(1-42)-induced inflammatory response by blocking the ligation of Aβ to receptor for advanced glycation end products (RAGE); suppressing the RAGE-mediated signaling *in vitro* while the production of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and cerebral Aβ accumulation *in vivo*. Furthermore, geniposide augments synaptic plasticity by attenuating the Aβ-induced reduction of long-term potentiation and increasing the miniature excitatory postsynaptic current (mEPSC) amplitude and frequency in hippocampal neurons.[74] Neuroprotective potential of genipin exerted against hepatic damage from ROS and RNS production in organotypic hippocampal slice cultures (OHSC) by reducing S-nitroso-N-acetylpenicillamine (SNAP) induced cell death and nitrite to lower level.[75] Genipin repressed brain microglial activation effectively inhibiting LPS-induced nitric oxide (NO) release from cultured rat brain microglial cells as well as microglia stimulated with interferon-gamma and amyloid-beta; in turn to attenuate the release of tumor necrosis factor-alpha, interleukin-1beta, prostaglandin E(2), intracellular reactive oxygen species, and NF-kappaB activation.[76] Genipin induced neurite outgrowth in PC12h and protected Neuro 2a cells in rat primary hippocampal neurons from beta-amyloid peptide, serum deprivation, oxidative stress and through suppressing A23187 (calcium ionophore)-induced transcription of immunoglobulin-binding protein/glucose-regulated protein of 78 kDa (BiP/GRP78) protein, an endoplasmic reticulum (ER) stress marker protein and A23187-induced cytotoxicity in turn which significantly activated caspase3/7, as mediator of apoptosis, A23187. Therefore, genipin prevented neurodegeneration in Alzheimer's disease and Parkinson's disease involving ER stress.[77] Crocin and crocetin were effective in the inhibition of LPS-induced neurotoxic molecules like NF-κB activation, nitric oxide (NO) release from microglia, tumor necrosis factor-α, interleukin-1β, and intracellular reactive oxygen species from cultured rat brain microglial cells.[78]

Hepatoprotective activity

Gardenia jasminoides extract significantly reduced liver mRNA and/or protein expression of transforming growth factor β1 (TGF-β1), collagen type I (Col I) and α-smooth muscle actin (α-SMA) by suppressing the upregulation of TGF-β1, Col I and α-SMA in LX-2 exposed to recombinant TGF-β1and Smad2 phosphorylation in LX-2 cells.[79] Strong inhibitory action of *Gardenia jasminoides* extract on lipidosis and inflammatory injury in the rat model by enhancing serum ALT and AST activities, and expression of TNF-alpha and P-IkB proteins in liver tissue significantly led to inhibition of the free fatty acid metabolism pathway.[79] Hepatoprotective role of geniposide was initiated to acute alcoholic liver injury via up-regulating the expression of the main anti-oxidant enzymes.[80] Geniposide and genipin protected significantly to liver by potentiating increased hepatic heme oxygenase-1 protein expression; attenuating increased levels of tBid, Cytochrome C protein expression, caspase-3 activity; and reducing increased apoptotic cells in the hepatic ischemia/reperfusion (I/R) injured mice.[81] Glycine N-methyltransferase (GNMT) and glycogen phosphorylase (PYGL) were preferred for novel biomarker for hepatic injuries rather than convenient liver biomarkers.[82] Genipin increased hepatoprotectin markedly against d-galactosamine/lipopolysaccharides (GalN/LPS) induced hepatic damage related with its anti-oxidative, anti-apoptotic activities, and inhibition of NF-kappaB nuclear translocation and nuclear p-c-Jun expression.[83] Hepatoprotective effects of geniposidic acid alleviated GalN/LPS-induced liver injury through enhancing anti-oxidative defense system and involving apoptotic signaling pathways which was analogous to that of genipin.[81] Crocetin significantly restored the endothelium-dependent relaxation (EDR) of thoracic aorta by enhancing the vessel eNOS activity to lead the elevation of NO production.[84] GJE glycoprotein explored an inhibitory effect on glucose/glucose oxidase (G/GO)-induced cytotoxicity and intracellular reactive oxygen species production by blocking lactate dehydrogenase release; increasing nitric oxide production; activation of anti-oxidant enzymes accompanied by the inhibition of the cytotoxic-related signals hepatic cytochrome c, nuclear factor-kappaB and activator protein-1. In the way, GJE glycoprotein could ameliorate the liver function owing to its hepatoprotective and hypolipidaemic properties.[72]

Gastro-protective activity

Ethanolic extract of *Gardenia jasminoides* Ellis (GJE extract), exhibited potential anti-gastric diseases activity, such as gastritis and gastric cancer due to free radical scavenging activities Ursolic acid and crocin showed acid-neutralizing property by less inhibition of NaOH consumption amount whereas genipin inhibited approximately of HCl-ethanol induced gastric lesion in rats.[85] GJE extract, ursolic acid and genipin showed the acid-neutralizing capacities and inhibitory effects on the growth of *Helicobacter pylori* (H. pylori) in which the GJE extract and ursolic acid had cytotoxic activity against AGS and SUN638 gastric cancer cells while genipin and ursolic acid inhibited significant 97.1% HCl/ethanol-induced gastric lesions.[86]

Skin protective activity

Gardenia jasminoides extract (GJE) and its ethyl acetate fraction *Gardenia jasminoides* extract (GJE-EA) inhibited compound 48/80-induced histamine release from MC/9 mast cells. Topical application of GJE or GJE-EA to dermatophagoides farinae-exposed NC/Nga mice reduced the symptoms of atopic dermatitis (AD) by inhibiting the infiltration of inflammatory cells, and lowering serum levels of immunoglobulin E and histamine reducing the expression of cytokines (interleukin [IL]-4, IL-6, and tumor necrosis factor-alpha) and adhesion molecules (intercellular

adhesion molecule-1 and vascular cell adhesion molecule-1). Geniposide, but not crocin, inhibited the release of histamine from mast cells, which may contribute to the anti-allergic effect of GJE and GJE-EA.[87] Hydrolyzed gel of *Gardenia jasminoides* extract containing genipin was effective for the treatment of ecchymoses in a rat model.[88]

Nephro-protective activity

Potent uricosuric and nephro-protective effects activities of *Gardenia jasminoides* extract could also effectively reverse oxonate-induced alterations in renal urate transporter 1 (mURAT1), glucose transporter 9 (mGLUT9), organic anion transporter 1 (mOAT1), mOAT3, oncoprotein induced transcript 3 (mOIT3) expressions, as well as Tamm-Horsfall glycoprotein (THP) levels, resulting in the enhancement of renal uric acid excretion. *Gardenia jasminoides* extract significantly reduced serum urate levels and increased urinary urate levels and FEUA in hyperuricemic mice. It decreased serum creatinine, blood urea nitrogen (BUN), and fractional excretion of uric acid (FEUA) along with up-regulated expression of organic cation/carnitine transporters, improving renal dysfunction.[89]

Retino-protective activity

Protective effects of crocetin against retinal damage both of *in vitro* and *in vivo* by decreasing in caspase-3 and caspase-9 activities after retinal damage[90] and reducing oxidative stress in ischemia-induced retinal damage.[91]

Anti-arthritis activity

Geniposide healed arthritis through different mechanism like inhibiting the colonic inflammation damage in through decreasing the expression level of tumor necrosis factor-alpha (TNF-α), interleukin-1(IL-1) and interleukin-6 (IL-6), increasing the production of interleukin-10 (IL-10) and restraining the expression of phospho-p38 (p-p38) related proteins in fibroblast-like synoviocyte proliferation.[92] Geniposide relieved significantly paw swelling and arthritis index and exerted immunoregulatory effects through inducing Th17 cell immune tolerance and enhancing Treg cell-mediated activities by down-regulating the expression of p-JNK signaling in mesenteric lymph node lymphocytes (MLNL) and peripheral blood lymphocytes (PBL) of adjuvant arthritis (AA) rats and decreased the expression of phospho-JNK (p-JNK) in MLNL and PBL of AA rats in the pathogenesis of rheumatoid arthritis[92] and its potentiality in rheumatoid arthritis treatment proved in the previous study.[93]

Anti-oxidant activity

In terms of reducing power, free radical scavenging activities, aqueous extract of *Gardenia jasminoides* fruit exhibited higher anti-oxidant activity than that of its ethanolic extract [94] and its anti-oxidant potential of methanolic extract of *Gardenia jasminoides* contributed due to phenolics and flavonoids in leaves.[95] Geniposide possessed as a potential candidate for detoxification by inducing GST activity via increasing the transcription of GSTM1 and GSTM2 subunits[96] leading to the activation of GSH S-transferase (GST) acting through MEK-1 pathway by activating and increasing expression of Ras/Raf/MEK-1 signaling mediators.[97] Genipin quenched effectively 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, suggesting that genipin[74] and crocetin[64] act as a direct free radical scavenger. GJE glycoprotein showed anti-oxidant effect against the lipid peroxidation process in the Fe^{2+}/ascorbic acid system blocking the formation of thiobarbituric acid-reactive substances.[72] Increasing activities of anti-oxidative enzymes [catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx)], inhibition of inflammation related mediators (iNOS, COX-2, and NF-kappaB), production of nitric oxide (NO) and reactive oxygen species (ROS), myeloperoxidase (MPO) activity and thiobarbituric acid reactive substances (TBARS) levels, GJE glycoprotein (80 microg/ml) proved as a preventive and therapeutic agent for the ulcerative colitis. neutrophil infiltration and colonic lipid peroxidation due to its scavenging property.[98] A novel anti-oxidant water-soluble polysaccharide was isolated from *Gardenia jasminoides* Ellis proved significant scavenging abilities.[99]

Anti-apoptotic and anti-cancer activities

Gardenia jasminoides extract exhibited anti-oxidative and anti-apoptotic effects in HaCaT cells by attenuating the UVB induced mRNA expressions of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in HaCaT cells.[100] Dichloromethane fraction of *Gardenia jasminoides* extract was most efficient among n-hexane, ethyl acetate, n-butanol, and aqueous fractions in the mechanism of apoptosis led to the partial increase of caspase-3, caspase-8 and caspase-9 activities and the cleavage of poly (ADP-ribose) polymerase.[101] Cytoprotective of geniposide exhibited through novel strategy by up regulating the expression of heme oxygenase-1 (HO-1) to attenuate the cell apoptosis induced by 3-morpholinosydnonimine hydrochloride (SIN-1); inducing the nuclear translocation of nuclear factor-E2-related factor 2 (Nrf2) and activation of phosphatidylinositol 3'-kinase (PI3K) and both LY294002 (a specific inhibitor of PI3K) and Zinc protoporphyrin (ZnPP, an inhibitor of HO-1) to antagonize oxidative stress in hippocampal neurons.[102] Inhibitory effect of geniposide against formaldehyde-induced stress and apoptosis through increasing activity of intracellular anti-oxidants (superoxide dismutase and glutathione peroxidase); mRNA and protein levels of the anti-apoptotic gene Bcl-2 and geniposide protected SH-SY5Y cells by down regulating the expression of the apoptotic-related gene-P53, apoptotic executer-caspase 3 and apoptotic initiator-caspase 9.[103] Geniposide alleviated mammary gland apoptosis by down regulating Bax expression along with TLR4 expression; inhibiting Caspase-3 cleavage and preventing p53 phosphorylation and up-regulating Bcl-2 expression *in vivo*.[53] Anti-metastastic effect of Penta-acetyl geniposide [(Ac)(5)GP] exhibited an inhibitory effect on abilities of adhesion and motility by cell-matrix adhesion in the rat neuroblastoma line: C6 glioma cells by decreasing activity of matrix metalloproteinase-2 (MMP-2) and membrane type I matrix metalloproteinase (MT1-MMP) while enhancing the tissue inhibitor of matrix metalloproteinase-2 (TIMP-2), inhibiting phosphoinositide 3-kinase (PI3K) protein expression, phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and activation of transcription factor nuclear factor kappa B (NF-kappaB), c-Fos, c-Jun.[104] (Ac) 5GP decreased DNA damage and hepatocarcinogenesis induced by aflatoxin B1 (AFB1) by activating the phase II enzymes glutathione S-transferase (GST) and GSH peroxidase (GSH-Px); reducing the growth and development of inoculated C6 glioma cells; inducing sub-G1 peak through the activation of apoptotic cascades PKCdelta/JNK/Fas/caspase8 and caspase 3. (Ac) 5GP arrested cell cycle at G0/ G1 by inducing the expression of p21, thus suppressing the cyclin D1/cdk4 complex formation and the phosphorylation of E2F.[105] Genipin exhibited a strong apoptotic cell death effect in human non-small-cell lung cancer H1299 cells mediated by an increase in phosphorylated p38MAPK expression, activated downstream signaling by phosphorylating ATF-2 and leading to increased levels of Bax counteractive to p38MAPK signaling.[17] Genipin induced cell apoptosis in hepatoma cells and PC3 human prostate cancer cells due to increased significantly in the phosphorylated c-Jun NH(2)-terminal kinase (JNK) protein, phospho-Jun protein, p53 protein and bax protein which led to the accumulation of bax protein, further induced cell apoptotic death eventually.[106] Anti-proliferative activity of genipin in MDA-MB-231 exerted human breast cancer cells[22] by similar mechanism in the previous studies.[17,106] Genipin suppressed the constitutive STAT3 activation in U266 and U937 cells and stimulated Src homology 2 domain-containing phosphatase 1 (SHP-1), which dephosphorylates and inactivates STAT3 by blocking STAT3 activation via repressing the activation of c-Src, but

not Janus kinase 1 (JAK1) and also down regulated the expression of STAT3 target genes including Bcl-2, Bcl-x(L), Survivin, Cyclin D1, and VEGF. Furthermore, genipin effectively potentiated the cytotoxic effect of chemotherapeutic agents, such as bortezomib, thalidomide, and paclitaxel in U266 cells.[68] Genipin exhibited anti-tumor and anti-viral effects against Epstein-Barr virus (EBV) and EBV associated gastric carcinoma (EBVaGC) by significant cytotoxicity via inducing methylation on EBV C promoter and tumor suppressor gene BCL7A, arresting cell-cycle progress (S phases), up regulating EBV latent/lytic genes, stimulating EBV progeny production, activating EBV F promoter for EBV lytic activation in SNU719 cells and suppressed EBV infection.[107] Iridoid glycosides (IGs) exhibited anti-viral activity against influenza A virus via inhibition of intracellular acidification and Ca^{2+} influx during fusion and uncoating of influenza replication cycle.[41] Protective and anti--apoptotic activities of GJE glycoprotein in 100μg/ml exhibited significantly on the glucose/glucose oxidase (G/GO)-induced or hypoxanthine/xanthine oxidase (HX/XO)-induced cytotoxicity and apoptosis systems in NIH/3T3 cells, DNA fragmentation respectively by blocking activities against cytotoxicity and apoptosis; the activation of redox-sensitive signal mediators, protein kinase C alpha (PKCα) and nuclear factor-kappa B (NF-κB) in G/GO or HX/XO-induced apoptotic NIH/3T3 cells.[72]

Anti-angiogenic activity

Butanol fraction of *Gardenia jasminoides* Ellis fruit was most effective agent among successive hexane, ethyl acetate and aqueous fractions for their nti-angiogenic activity in the bioassay.[108] Geniposide showed anti-angiogenic activity in a dose-dependent manner by inibiting the growth of the transformed NIH3T3 cell line within the range of 25-100 microM.[2] Anti-angiogenic effects of crocetin suppressed on vascular endothelial growth factor (VEGF)-induced proliferation by inhibiting migration of human umbilical vein endothelial cells (HUVECs) and; human retinal microvascular endothelial cells (HRMECs) and phosphorylation of p38 significantly to protect VE-cadherin expression.[109]

Anti-thrombotic activity

Geniposide exhibited an anti-thrombotic effect via the suppression of platelet aggregation *in vivo* and inhibition of phospholipase-A(2) [(PLA (2)] activity acting as platelet antagonism. It inhibited activity resulting in significant decrease in EV71 virus infections, and internal ribosome entry site activity. Anti-enterovirus-71 (EV71) replication and viral IRES activity were inhibited by geniposide.[110] Anti-thrombotic action of iridoid glycosides (IGs) were assessed that it may potentially contribute to the treatment of cerebral ischemic diseases, including cerebral apoplexy.[111] Anti-hypertensive and anti-thrombotic effects of crocetin led to an increase in bioavailability of NO, possibly mediated by decreased inactivation of NO by reactive oxygen species.[112]

Anti-microbial activity

Bioassay-guided fractionation of 13 bioactive compounds from *Gardenia jasminoides* extracts exhibited anti-viral effects against influenza virus strain A/FM/1/47-MA *in vivo*.[113] Dichloromethane extract of the air-dried flowers of *Gardenia jasminoides* Ellis afforded moderately active *against Candida albicans*; slightly active against *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Trichophyton mentagrophytes*; and inactive against *Bacillus subtilis* and *Aspergillus niger*.[114] Methanolic extract of *Gardenia jasminoides* Ellis showed the highest level of anti-fungal activity against *Pleurotus ostreatus*, a wood-rotting fungus.[115]

Miscellaneous activities

Geniposide (GP) as an agonist of glucagon-like peptide-1 receptor (GLP-1R) through interaction of c-kit receptor with its ligand-SCF potent enhances norepinephrine (NE) induced hypopigmentation in the melanocytic melanogenesis.[116,117] Genipin inhibited RANKL-induced osteoclast differentiation in bone marrow macrophages (BMMs) during culture by suppressing RANKL-induced IκB degradation along with mRNA expression of osteoclastic markers such as NFATc1, TRAP, and OSCAR and inhibition of c-Fos protein proteolysis in RANKL-treated BMMs. Genipin could be qualified to be a candidate for the treatment of osteoporosis.[118] Genipin was useful for treating periodontal disease by preventing MMPs expression like release of MMP-1, MMP-3 from TNF-α-stimulated human periodontal cells.[61] Crocetin revealed its hypnotic effect.[119] Even a single administration of *Gardenia jasminoides* extract exhibited rapid anti-depressant effects in reducing the number of escape failures in the learned helplessness test significantly and decreased latency of food consumption in the novelty suppressed-feeding test with the elevated expression of brain-derived neurotrophic factor (BDNF) expression in the hippocampus.[120] Oil extract of *Gardenia jasminoides* used for depression therapy.[121]

Toxicity

Acute hepatotoxicity of geniposide has been proved in the recent studies when it was administrated above normal dose of 24.3 mg/kg or higher doses leads to hepatic injury via oxidative stress.[122,123] Genipin possesses genotoxicity.[124] Genipin possessed a significant induction on CYP2D6 and a remarkable inhibition on CYP2C19 and CYP3A4 not only from the expression of mRNA and protein but the level of enzyme activity. Caution should be exercised with respect to the induction or inhibition of genipin on CYP isoenzymes and the strong induction on P-glycoprotein.[125]

Patent review

Patents are the largest single source of technical information in the world. Literature carries poor objective information regarding the technological strategies being adopted by the commercial companies in their research laboratories because of proprietary secrecy and less accessible of that technologies during their development phase. Patent analysis provides good evidence for the degree of patents filed by firms and inventors. It can also show the technological advances and recent developments in the particular area.[126] Patents filed and granted on the use of *Gardenia jasminoides* alone or as active ingredient in the formulations were also considered for the review. Patent databases such as @espacenet and WIPO were searched and around 200 patents of interest retrieved in patent search and analysis which claimed for *Gardenia jasminoides*. However, analysis of these patents revealed that few of them mainly claim the method of extraction of active ingredient. Most of patents have been filed for TCM which were not included in the following table as these abstracts are difficult to interpret whether they are relevant to analyze.

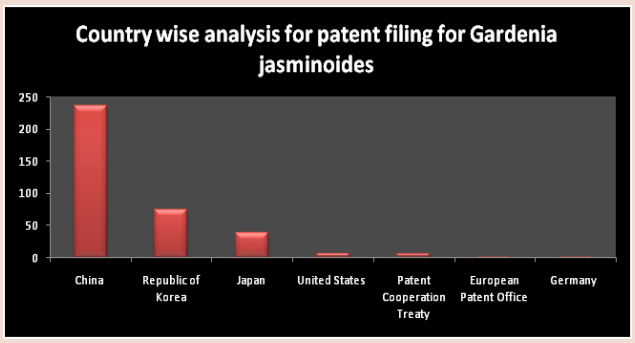

Figure 8: Country wise patent filling activity of *Gardenia jasminoides*

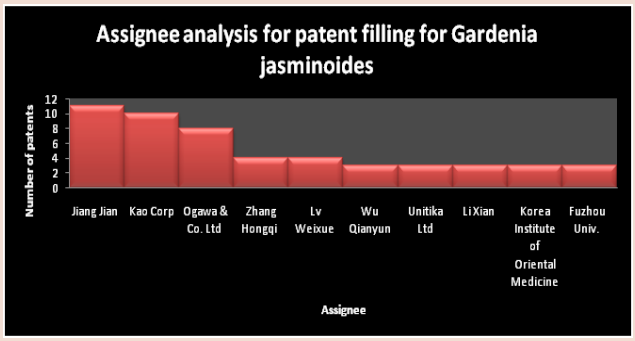

Figure 9: Assignee wise patent filling activity of Gardenia jasminoides
Source: WIPO Patent analysis

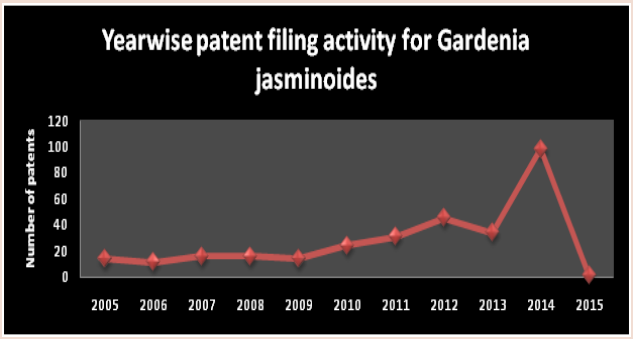

Figure 10: Year wise patent filling activity for *Gardenia jasminoides*

Table 2: **Patent overview of *Gardenia jasminoides***

Title	Publication number	Publication date date	Activity
Method for treating abnormal polyglutamine-mediated diseases	US2015064287	2015-03-05	Neuroprotective
Extraction method of *Gardenia jasminoides* volatile oil	CN104164302	2014-11-26	Extraction
A pharmaceutical composition comprising the hexane fraction of *Gardenia jasminoides* extract as an effective component for anti-platelet aggregation and a health functional food comprising the same	KR20140109099	2014-09-15	Anti-atherosclerotic activity
Method used for preparing high-purity gardenoside and crocin from *Gardenia jasminoides* Ellis	CN103951718	2014-07-30	Extraction
Perfume composition for expressing the fragrance of *Gardenia jasminoides* Ellis for. Grandiflora makino	KR20140030992	2014-03-12	Cosmetic
Rapid propagation method for *Gardenia jasminoides*	CN103461127	2013-12-25	Cultivation
Preparation method for *Gardenia jasminoides* gardenoside B	CN103435664	2013-12-11	Extraction
Production water recovery device used in extraction process of *Gardenia jasminoides* uranidin	CN203212364	2013-09-25	Extraction
Method for preparation of gardenia oil, gardenia green pigment and gardenia blue pigment through synchronous reaction	CN103060077	2013-04-24	Extraction
Gardenia jasminoides plant named Double Mint	USPP23507	2013-04-02	Taxonomy
Gardenia plant named 'BAB1183'	USPP22797	2012-03-08	Taxonomy
Processing principle-based individualized and characteristic quality evaluation method for *Gardenia jasminoides* Ellis decoction pieces	CN102335260	2012-02-01	Taxonomy
Gardenia jasminoides plant named 'leeone'	US2011162120	2011-06-30	Taxonomy
Interspecific hybidization of *Gardenia jasminoides* Ellis and *G. thunbergia* L.	USPP21541	2009-02-19	Taxonomy
Glycoprotein isolated from *Gardenia jasminoides* Ellis, and hepatoprotective, hypocholesterolemic and anti-inflammatory pharmaceutical composition containing the glycoprotein	KR100661481	2006-12-19	Hepatoprotective, hypocholesterolemic anti-inflammatory
Method for extracting genipin and geniposide from *Gardenia jasminoides*	CN101029066	2007-09-05	Extraction
Preparation of *Gardenia jasminoides* by membrane separation technology	CN1939459	2007-04-04	Extraction
Preparation of *Gardenia jasminoides* by macroporous adsorbing resin	CN1939458	2007-04-04	Extraction
Preparation of *Gardenia jasminoides* extracts	CN1939457	2007-04-04	Extraction

Country wise patent filing activity

Patent filed on *G. jasminoides* as alone or formulations of TCM worldwide were revealed that patent applications have been increased over the last two decades. Among countries, China is the most one leading country to file patent on *G. jasminoides* as shown in Figure 8.

Assignee analysis

In the patent activity total number of patents applied by assignee is a simple indicator.

According to Figure 9, companies like Jiang Jian attained highest patent applications and Kao Corp from Japan hold second position among assignees worldwide.

Year wise patent filing activity

Priority year was considered for the analysis. The year wise analysis as depicted in Figure 10 revealed that the highest numbers of patent applications has been filed in the year 2014 and observed steady increase in the overall filings over the years (2005-2012) while patents filed in 2013 and 2014 are not considered as the data would be incomplete due to the reason that the patent applications are only published after a period of 18

months from the date of filing.

Technology analysis

Pharmaceutical activity was considered as tool for technology strategies for the analysis. This technology analysis highlights that glycoprotein isolated from *Gardenia jasminoides* is effective for hepatoprotective, hypocholesterolemic anti-inflammatory activities while extract used as an anti-atherosclerotic and neuroprotective agent. Some of important patents related *G. jasminoides* have shown in Table 2.

CONCLUSION

Gardenia jasminoides has been used over many years in the Traditional Chinese Medicine (TCM). Till date it has been explored many pharmacologic activities and isolated many of active phytoconstituents using in the treatment of ailments and diseases. Apart from this, other countries has been increasingly curious attention in applying patents for specific isolated phytochemicals or *Gardenia jasminoides* in form of either aqueous or alcoholic extract exerting various pharmacologic activities like anti-inflammatory, anti-cancer, anti-oxidant, hepatoprotective, gastro-protective, etc. Out of these assignees, China is the main leading country to be assignee for filing highest number of patents for *Gardenia jasminoides* in the field of traditional Chinese medicine. Yet there is no found updating review in research knowledge as *Gardenia jasminoides* is currently holding an enormous significant position in the medical and pharmaceutical fields. So there is a need of hour to structuralize the comprehensive review of scientific literature related to *Gardenia jasminoides* and to analyze patents filed for *Gardenia jasminoides*. As per the presented review herewith-*Gardenia jasminoides* is the medicinal herb being used since ancient times. *Gardenia jasminoides* extracts and its main active phytoconstituents viz; geniposide, genipin, crocin, crocetin have been reported with extended pharmacological activities such as anti-hyperglycemic, anti-atherosclerotic, anti-inflammatory, anti-arthritis, anti-cancer, anti--apoptotic, anti-oxidant, anti-angiogenic, anti-thrombotic, anti-microbial and miscellaneous activities. Also it has been explored through different protective mechanisms like neuroprotective for Alzheimer's disease (AD), hepatoprotective, gastro-protective, retino-protective, nephro-protective, skin protective activities. Even though it is well documented of numerous health benefits of GJ, acute hepatotoxicity of geniposide has been reported in the recent studies when it was administrated in higher doses of geniposide. Pharmacokinetic and pharmacodynamic studies of geniposide should be investigated to prevent inducing hepatic injury due to overdoses. This data provides scientific scenario will helpful for developing research strategies and art of patent will also help in identifying the research drawbacks for generating intellectual property.

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CONFLICTS OF INTEREST

Author declares that there is no conflict of interest.

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Original Article

Iron Reducing and Radical Scavenging Activities of 13 Medicinal Plants From Côte d'Ivoire

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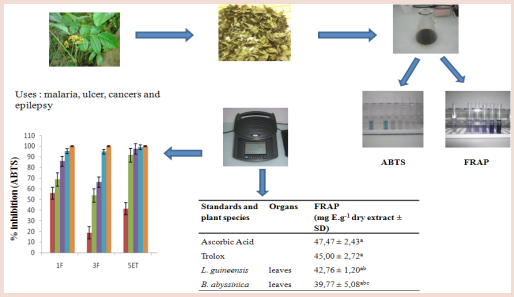
ABSTRACT

Objective: Oxidative stress has been involved in the development of varied human diseases. The aim of this study was to evaluate the iron reducing power and the antiradical activity of 13 plants traditionally used as medicinal plants in Côte d'Ivoire. **Materials and Methods:** FRAP (ferric reducing antioxidant power) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays were used to assess the antioxidant property of 80% methanol extracts prepared from the 13 plants. **Results:** A high iron reducing activity was exhibited by extracts from leaves of *Leea guineensis* (42.76 ± 28.54 mg of TE/gdry extract) and *Bersama abyssinica* (39.77 ± 31.29 mg of TE/g dry extract). *Smeathmannia pubescens* (% ABTS = 92.44 ± 12.93%), *L. guineensis* (%ABTS = 89.73 ± 15.10%), *Keetia venosa* (% ABTS = 88.78 ± 17.36 %) and *Sapium ellipticum* (%ABTS = 85.86 ± 25.10%), showed promising antiradical activity with IC_{50} values of 4.50, 5.00, 5.40 and 5.70 µg/mL respectively. These values are ($p < 0.05$) close to those of Trolox (CI_{50} = 4.10 µg/mL) and ascorbic acid (CI_{50} = 4.90 µg/mL). **Conclusion:** Our findings confirm the traditional use of the studied plants in treatment of various ailments. The results obtained provide promising baseline information for using these medicinal plants for improving the health status of the population.

Key words: ABTS, Antioxidants, Côte d'Ivoire, FRAP, Medicinal plants, Iron.

SUMMARY

- Studied plants of immense ethnomedical importance in West Africa.
- After several assays, it was found that *Leea guineensis* (leaves) and *Bersama abyssinica* (leaves) showed high iron reducing power.
- *Smeathmannia pubescens* (stem bark), *Leea guineensis* (roots), *Keetia venosa* (leaves) and *Sapium ellipticum* (Stem bark), exhibited promising radical scavenging activity.
- *Smeathmannia pubescens* efficient as Trolox. Hence, recommended as antioxidant.



PICTORIAL ABSTRACT

Abbreviations used: FRAP: Ferric reducing antioxidant power, ABTS-2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), TE: Trolox equivalence, IC: Inhibitory concentration, SD: Standard deviation.

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INTRODUCTION

Iron is a useful mineral for the metabolism of living organism. In the blood, it contributes to numerous physiological functions such as hematite maturation, oxygen transportation for cellular respiration, DNA and proteins synthesis.[1] However, the accumulation of iron in some essential organs such as liver, heart and spleen leads to free radical production via oxidative stress.[2]

This iron overload is called hemochromatosis and is implicated in the genesis or the complication of diseases like cancer, Alzheimer, malaria and diabetes.[3,4] Iron overload may contribute to the development of hepatocellular carcinoma[5] and about 85 % of the hepatocellular carcinoma occurs in developing countries.[6] According to WHO, cancers figure among the leading causes of death worldwide, accounting for 8.2 million of death. In Africa, cancer is a real issue for people due to the lack of facilities and the relative expensive cost of treatments.[7] In addition, iron overload can be cause by some heath conditions such as malaria which is endemic in Africa. This disease is a major cause of death especially for children under five years old, and more than 90% of malaria deaths occur in Sub-Saharan Africa.[8] One of the defense strategy of the host is to restrict iron availability to pathogens in order to reduce their virulence.[9] A major "trade-off" of this host defense strategy is accumulation of toxic iron in tissues and organs, which can act in a pro-oxidant and cytotoxic manner. This can lead to tissue damage, enhancing rather than preventing disease severity.

Synthetic iron chelators are used to facilitate iron overload elimination and are good antioxidants. However they are reported to be toxic[10] and naturally occurring compounds are needed as alternative treatments. Plants are good source of natural antioxidants and largely used as food and medicines. Phytocompounds such as flavonoids are well known for their antioxidant properties. As such they can strongly contribute to the treatment of iron and free radical-related diseases[11]

This study investigated 13 medicinal plants for their iron reducing antioxidant power and antiradical activity.

MATERIALS AND METHODS

Plant material

The studied plants were selected on the basis of ethnobotanical surveys carried out in Côte d'Ivoire and elsewhere in Africa. These plant species are used in traditional medicine in the treatment of cancer, malaria, diabetes and gastric ulcer. These plants were collected in August 2008 in the

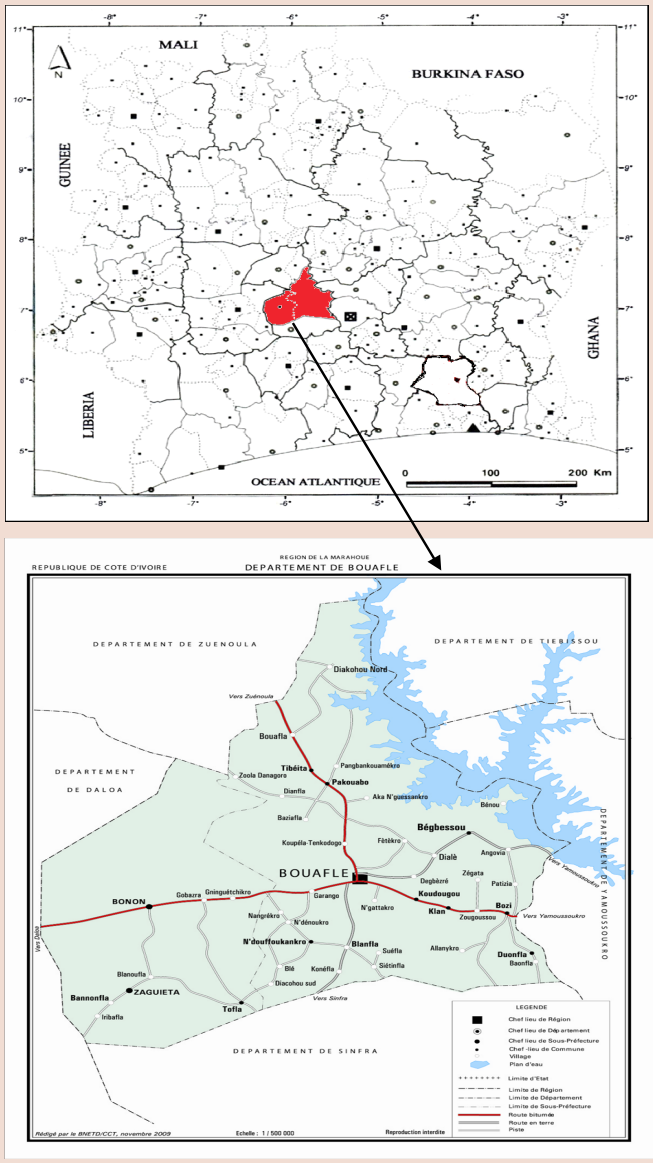

Figure 1: Map of the Department of Bouaflé showing the studying site, the Tibéita village (BNEDT/CCT; 2008)

Bouaflé savannah (Western-central Côte d'Ivoire) (Figure 1). Botanical identification of each species was performed at the herbarium of Centre Suisse de Recherches Scientifiques of Côte d'Ivoire and authenticated by Professor Aké-Assi Laurent† at the Centre National de Floristique of the University Félix Houphouët Boigny (Abidjan, Côte d'Ivoire).

Preparation of plant extracts

Parts of the selected plants were harvested, cleaned with tap water, dried in air-conditioned room at 18°C for two to three weeks, and then powdered. Fifteen grams of each powder was macerated in 150 mL of 80% methanol under mechanical stirring (200 rd/min) for 24 hours at room temperature. The filtrates were dried to obtained extracts. The yield for each extract was calculated using the formula: (weight of dry extract/ weight of powder plant material) x 100%.

FRAP assay

The ability of the plant extracts to reduce iron was assessed using the Ferric reducing antioxidant power (FRAP) assay.[12] In the presence of reductive compounds, iron ferric form (Fe^{3+}) non colored is transformed into iron ferrous form (Fe^{2+}) which is blue. The fresh FRAP reagent (10: 1: 1) was prepared from acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in HCl 400 mM) and ferric chloride (10 mM) and *kept* at 37°C. Plant extracts were serially diluted to obtain a range of concentrations (100, 50, 25, 12.5 and 6.25 µg/mL). Trolox (2.4, 1.2 and 0.6 µg/mL) was used as reference for standard curve Y = 0.065X + 0.043 where X is the value of absorbance and Y the Trolox equivalent value.

Subsequently, the FRAP reagent (2850 µL) was added to 150 µL of each concentration of plant extract. The mixture was maintained for 30 min at room temperature, and then absorbance was measured at 593 nm against methanol as blank. The results were expressed in mg of TE/g of dry extract according to the formula.[13]

FRAP value (mg of TE/g of dry extract) = $[(A_e - A_0) / (slope)]x[V/v)] / [w][1000]$

When: A_e= absorbance of sample; A_0= absorbance of blank; w=weight of the dry extract; V=total volume of extract; v=used volume of the extract and 1000=conversion factor.

ABTS assay

The ABTS radical scavenging activity of plant extracts was determined using the method described.[14] with slight modifications. Briefly, 2850 µL of ABTS reagent were added to 150 µL of the tested extracts, and mixed thoroughly at room temperature, for 2 hours in dark conditions. This reagent was obtained from 7 mM of ABTS solution and 2.42 mM potassium persulfate (1: 1 v/v), incubated at 23°C for 6 hours to 3 days prior use and absorbance was adjusted to 1.1 ± 0.2 at 734 nm by adding methanol. Then the absorbance was measured at 734 nm using a UV spectrophotometer, *methanol was used as blank.*

The percentage inhibition was calculated using the formula:

$$\% ABTS = [(A_0 - Ae)/(A_0)] \times 100$$

with A_0=absorbance of control; A_e= absorbance of sample. The different extracts were grouped according to their inhibition percentage: high activity, moderate activity and weak activity.

The IC_{50} was graphically determined using Trolox (100, 50 and 25 µM) as the reference compound for calibration. A low IC_{50} value indicates strong antioxidant activity in a sample.[15]

Statistical analysis

The software STATISTICA 7.1 was used for data analysis. Results obtained were reported as means ± SD of duplicate experiments. One-way analysis of variance (ANOVA 1) was performed to test the influence of concentrations on the percentage of inhibition and iron reducing power of extracts. The difference was significant at $p<0.05$. The least significant difference (LSD) test was used to determine the difference in the inhibition percentage of ABTS and the iron reducing power among the extracts.

The relationship between concentrations of extract and percentage of inhibition was determined, $R^2 \geq 0.90$ was considered as strong correlation.[16]

RESULTS

Of the 22 extracts, 10 (45.45%), showed iron chelating activity, and 13 (59.10%) exhibited antiradical activity.

Ferric reducing antioxidant activity

The FRAP values ranged from 42.76 ± 28.54 to18.06 ± 23.90 mg of TE/g of dry extract. There was a significant difference between the plant extracts tested (α=0.05, $p<0.001$ and F=6.04). The leaves extract of *Leea guineensis* showed the highest ferric reducing power (42.76 ± 28.54 mg of TE/g of dry extract). Moderate effect was obtained for leaves of *Bersama abyssinica* (39.77 ± 31.29 mg of TE/g of dry extract), roots of *L. guineensis* (37.60 ± 28.36 mg of TE/g of dry extract), stem bark of *Sapium ellipticum* (32.67 ± 27.44 mg of TE/g of dry extract) and root bark of

Table 1: Iron chelating potential (mg de TE/g of dry extract) of plant species tested

Plant species and standards	Families	Organs tested	FRAP (mg TE/g of dry extract) ± SD
Leea guineensis	Leeaceae	Leaves	42.76 ± 28.54^{ab}
Bersama abyssinica	Melianthaceae	Leaves	39.77 ± 31.29^{abc}
Leea guineensis	Leeaceae	Roots	37.60 ± 28.36^{abc}
Sapium ellipticum	Euphorbiaceae	Stem bark	32.67 ± 27.44 ^{bce}
Flacourtia indica	Flacourtiaceae	Root bark	31.92 ± 23.69^{bce}
Sapium ellipticum	Euphorbiaceae	Leaves	29.19 ± 23.88^{cde}
Vernonia guineensis	Asteraceae	Leaves	23.90 ± 19.64^{de}
Bersama abyssinica	Melianthaceae	Stem bark	21.83 ± 13.97^{de}
Cissus doeringii	Vitaceae	Leaves	21.41 ± 25.14^{de}
Pouteria alnifolia	Sapotaceae	Leaves	18.06 ± 23.90^{d}
Ascorbic acid	-	-	47.47 ± 37.80^{a}
Trolox	-	-	45.00 ± 27.65^{a}

TE: Trolox equivalence, F: Fisher statistical, FRAP: Ferric Reducing Antioxyant Power, SD: Standard deviation for 5 different concentrations. Values having the same letters are not statistically different according to LSD Fisher *post hoc* test.

Table 2: ABTS radical scavenging power of tested plants and standards

Plant species and Standards	Families	Organs	IC_{50} values (µg/mL)	R^2values
Smeathmannia pubescens	Passifloraceae	Stem bark	4.50	0.68
Leea guineensis	Leeaceae	Root	5.00	0.66
Keetia venosa	Rubiaceae	Leaves	5.40	0.72
Cissus doeringii	Vitaceae	Leaves	5.70	0.96
Sapium ellipticum	Euphorbiaceae	Stem bark	7.40	0.62
Pouteria alnifolia	Sapotaceae	Leaves	7.80	0.84
Keetia venosa	Rubiaceae	Root bark	9.10	0.88
Leea guineensis	Leeaceae	Stem	9.40	0.91
Leea guineensis	Leeaceae	Leaves	11.90	0.84
Vernonia guineensis	Asteraceae	Leaves	13.10	0.95
Smeathmannia pubescens	Passifloraceae	Leaves	18.30	0.94
Cuviera macroura	Rubiaceae	Leaves	19.60	0.97
Anthocleista nobilis	Loganiaceae	Stem bark	31.80	0.90
Ascorbic acid	-	-	4.10	0.75
Trolox	-	-	4.90	0.66

TE: Trolox equivalent, F: Fisher statistical, $IC_{50:}$ concentration of extract required to obtain 50% inhibition of ABTS radical.

Flacourtia indica (31.92 ± 23.69 mg of TE/g of dry extract). The leaves of *Sapium ellipticum* (29.19 ± 23.88 mg of TE/g of dry extract) showed weak iron reducing power (Table 1).

ABTS radical scavenging activity

The results of ABTS assay are presented in Table 2. The tested extracts showed antiradical activity ranging from 92.44 ± 12.93 to 52.08 ± 32.25%. There was a very high significant difference (α=0.05, p< 0,001, F=7.20) between ABTS inhibition of the extracts. The multiple comparison with the LSD test showed that the stem bark extract of *Smeathmania pubescens* (92.44 ± 1.97%) possessed the highest scavenging activity. The extract of *Leea guineensis* (roots), *Keetia venosa* (leaves) and *Sapium ellipticum* (stem bark) showed moderate scavenging activity with inhibitory percentage of 89.73 ± 15.10, 88.78 ± 17.36 and 85.86 ± 25.10% respectively. The remaining extracts showed low activity (Figures 2 and 3).

Of the studied plant extracts, *Smeathmania pubescens* showed the lowest CI_{50} value of 4.50 µg/mL which was lower than that of Trolox (CI_{50}=4.90 µg/mL), used as reference. The CI_{50} of *Leea guineensis* and *Keetia venosa* were slight low, with values of 5.00 and 5.40 µg/mL respectively (Table 2).

Correlation

Strong and positive correlation (R^2≥0.90) was established between the concentrations of extracts and inhibitory percentage of ABTS radical for leaves of *Cissus doeringii*, *Vernonia guineensis* and *Cuviera macroura*, stems of *Leea guineensis*, leaves of *Smeathmannia pubescens* and stem bark of *S. pubescens* (Table 2). For the extracts of *Sapium ellipticum, L.*

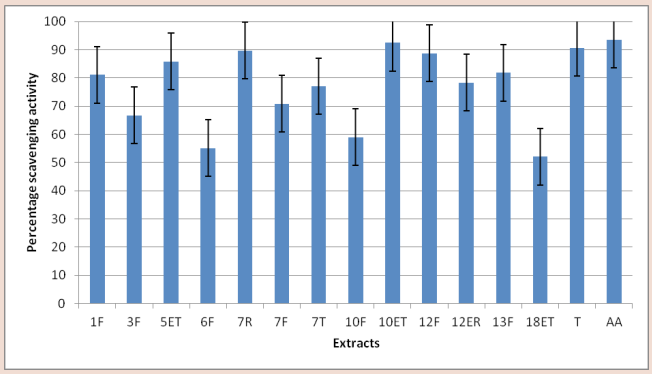

Figure 2: Mean of percentage of inhibition of extract for 5 different concentrations
For all the concentration of extracts, the difference was significant; bands having the same letters are not statistically different according to LSD Fisher *post hoc* test. 1 F: leaves of *Cissus doeringii,* 3 F: leaves *of Vernonia guineensis*, 5 ET: stem bark of *Sapium ellipticum*, 6 F: leaves of *Cuviera macroura*, 7 R: root of *Leea guineensis*, 7 F: leaves of *Leea guineensis*, 7 T: stem of *Leea guineensis*, 10 F: leaves of *Smeathmannia pubescens*, 10 ET: stem bark of *Smeathmannia pubescens*, 12 F: leaves of *Keetia venosa*, 12 ER: root bark of *Keetia venosa*, 13 F: leaves of *Pouteria alnifolia*, 18 ET: stem bark of *Anthocleista nobilis*, T: Trolox, AA: ascorbic acid.

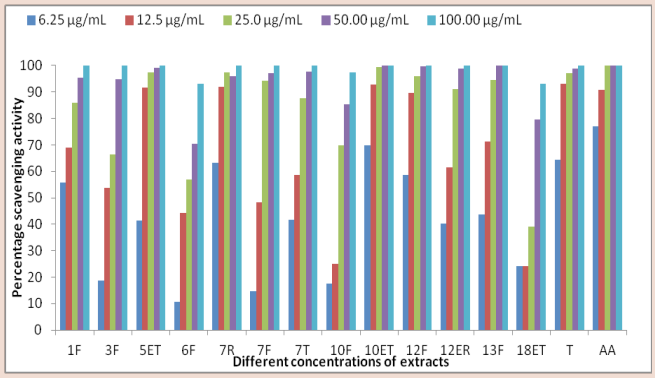

Figure 3: Percentage of inhibition for different concentrations of extracts 1 F: leaves of *Cissus doeringii,* 3 F: leaves *of Vernonia guineensis*, 5 ET: stem bark of *Sapium ellipticum*, 6 F: leaves of *Cuviera macroura*, 7 R: root of *Leea guineensis*, 7 F: leaves of *Leea guineensis*, 7 T: stem of *Leea guineensis*, 10 F: leaves of *Smeathmannia pubescens*, 10 ET: stem bark of *Smeathmannia pubescens*, 12 F: leaves of *Keetia venosa*, 12 ER: root bark of *Keetia venosa*, 13 F: leaves of *Pouteria alnifolia*, 18 ET: stem bark of *Anthocleista nobilis*, T: Trolox, AA: ascorbic acid.

guineensis, Keetia venosa, *Pouteria alnifolia*, and *Anthocleista nobilis*, low values (R^2=0.65-0.89) were obtained.

CONCULSION

Iron reducing and radical scavenging activities of these plants reveal that methanol extract of all the studied plants have either iron reduction or scavenging activities. Some of the plants such as Leea guineensis present the both activities. These results suggest that the active plants could be candidates to prevent oxidative stress related diseases. We plan to undergo further investigations such as study of the influence of season and location on antioxidant activity, elucidation of compounds responsible for the activity and toxicity in order to develop antioxidant nutraceutics.

DISCUSSION

This study investigated the antioxidant activity of 13 plants used in traditional medicine in Côte d'Ivoire. The most active plants were *S. pubescens*, *L. guineensis*, *Bersama abyssinica*, *K. venosa* and *C. doeringii*. *S. pubescens* showed the highest inhibitory percentage (% ABTS=92.44 ± 1.97 %). This strong antioxidant activity may explain some traditional uses of this plant, such as the treatment of toothache.[17] Free radicals are implicated in the genesis of pain and fatigue.[18] All the 13 plants studied were found to have good antioxidant power. The leaves of *L. guineensis* and *B. abyssinica* showed high iron reducing power. The stem bark of *S. pubescens* (4.50 µg/mL), root of *L. guineensis* (5.00 µg/mL), leaves of *K. venosa* (5.40 µg/mL) and *C. doeringii* (5.70 µg/mL) showed high antiradical activities close to that of Trolox and vitamin C. There is a good association between the traditional uses against chronic fatigue, malaria, cancers and diabetes of studied plants and their antioxidant properties.

In traditional medicine, *L. guineensis* is used to treat malaria, ulcer, cancers and epilepsy.[19] This plant also is used as analgesic. From what we discovered, it's worth mentioning that this plant possesses good iron reducing antioxidant power. Oxidative stress is aggravating factor of malaria, ulcer and cancer.[20] Iron overload can be observed during malaria due to host defense strategy.[9] The plant showed iron chelating activity revealing it may act by reducing the accumulation of iron during the treatment.

B. abyssinica is traditionally used for the treatment of malaria,[21] cancer, ulcer, rheumatism, wounds[22] and diabetes.[23] Free radicals are involved in the genesis of most of these diseases. Interestingly, the plant showed high activity in the current study. To the best of our knowledge, this is the first report of the iron reducing activity of *B. abyssinica*. Antioxidants are known to protect organisms against oxidative stress due to malaria.[24] Also iron overload can generate an oxidative stress responsible for inflammation in rheumatism.[25] The antioxidant properties of this studied plant may justify its traditional use for treating diseases and improving the health status of people. Our study revealed high antioxidant power for *Keetia venosa.* This plant is traditionally used to treat intercostals pains, rheumatism and is also used as tonic.[26] Muscular fatigue is strongly linked to the production of free radicals in the body.[27] The antiradical properties of *K. venosa* support its use in traditional medicine as a tonic.

Cissus doeringii showed a strong antiradical activity with IC_{50}=5.70 µg/mL. This plant has shown analgesic activity and antirheumatism property.[28,29] oxidative stress, due to iron overload may exacerbate rheumatism. Antioxidant compounds possess good anti-inflammatory properties useful in the treatment of rheumatism.[30]

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Original Article

Pharmacognostical and Phytochemical evaluation of *Ventilago calyculata* Tul. (Bark)

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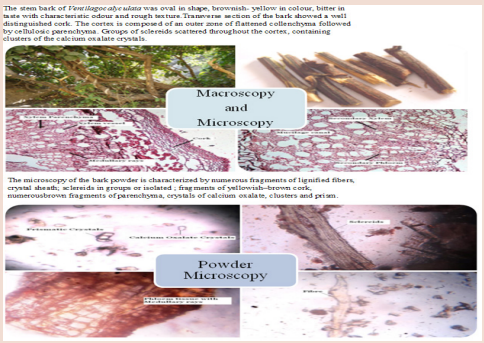
ABSTRACT

Background: *Ventilago calyculata* Tul. ('kevati'), is found throughout India as climbing shrub. It is widely used in various traditional system of medicine. **Objective:** In the present work pharmacognostical standardization has been developed for the systematic identification of the bark of *Ventilago calyculata*. Phenols and flavonoids were also quantified. **Materials and Methods:** Morphological, microscopical and phytochemical studies were performed. Various physicochemical parameters conforming the identity, quality, purity of the bark. The quantity of phenols and flavonoids were estimated. **Results:** The bark was oval, brownish yellow, bitter with characteristic odour and rough texture. The microscopical studies revealed the presence of cork with brownish contents, crimson inner cork, collenchyma, cellulosic parenchyma with cuboidal calcium oxalate crystals and schlereids. The total ash value, acid insoluble ash value and water soluble ash values of stem bark were found to be 15% w/w, 3.4% w/w and 11.6% w/w respectively. The percentage yields, total phenolic content and the total flavonoid content of the petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extracts were 2.77% w/w, 2.66% w/w, 3.8% w/w, 5.8% w/w, 11.0% w/w; 2.16 ± 0.04, 4.16 ± 1.04, 9.12 ± 1.14, 7.16±1.16, 1.16 ±1.02 mg/g (gallic acid equivalent) and 4.5 ± 0.55, 8.20 ± 1.12, 10.1 ± 0. 26, 6.5 ± 1.3, 0.66 ± 1.13 mg/g (rutin equivalent) respectively. **Conclusion:** There was a need to evaluate the extracts of the plant in order to provide scientific proof for its application and to explore the possibility of treating various diseases and disorders. Literature review indicates that very less work has been done on this plant and there is a wide scope for investigation.

Key words: Standardization, Total flavonoid content, Total phenolic content, *Ventilago denticulata* Willd, *Ventilago madraspatana* var. *calyculata* (Tul.) King.

SUMMARY

- *Ventilago calyculata* Tul. (*Rhamnaceae*) commonly known as 'kevati', 'raktavalli' in Sanskrit and 'pappili' in Siddha.
- It is found throughout India in hotter parts as climbing shrubs or lianas, on the trees.
- Different parts of this plant possess multifarious medicinal properties.
- Traditionally, its stem bark is used as a tonic (especially for stomach and liver as it is bitter and stimulates them to release enzymes). It's root, flower, and stem used in fever, night blindness, earache, urine retention, headache, rheumatism, dysuria, diabetes, eye diseases, abortifacient, syphilis, ulcer and stomachache. In Ayurveda, it is an ingredient of "mahā śyonāka taila" which is used in the form of drink, massage, inhalation and enema. This medicated oil is used in looseness of joints, chronic fever, gout, insanity, dysuria, vomiting, trembling etc.
- Until now, no scientific investigations had been carried out for the standardization of *Ventilago calyculata* Tul. bark. Hence, there was a need to provide scientific proof for standardization by the pharmacognostical and phytochemical evaluation of the bark of *Ventilago calyculata* Tul.



PICTORIAL ABSTRACT

Abbreviations used: Tul: Tulasne, var: variety, UV: Ultra Violet, nm: nano meter, SD: Standard deviation, µL: micro liter.

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INTRODUCTION

Herbal medicine is a major element of almost all traditional systems like Ayurvedic, Homeopathic, Naturopathic, Traditional Chinese medicine, and Native American medicine.[1] The commence of medicinal plants is flourishing globally day by day and besides there is also a huge demand of plant drugs all over the world, thus there might be a possibility of adulteration and substitution of the crude drugs with the genuine ones. Hence standardization of medicinal plants and natural products will provide useful information with regards to its correct identity and will help to differentiate from other closely related species as well as from other commercially available crude drugs.

Ventilago calyculata Tul. (*Rhamnaceae*) commonly known as 'Kevati', 'raktavalli' in Sanskrit and 'pappili' in Siddha. It is found throughout India in hotter parts as a climbing shrubs or lianas, on the trees.[2,3] Flowering from October to December and fruiting December to April of following year. Previous phytochemical studies of *Ventilago calyculata* Tul. have reported the presence of several anthraquinones, naphthoquinones, naphthalenes, xanthone and naphthoquinone lactones in root bark.[4-7] Different parts of this plant possess multifarious medicinal properties. In Ayurveda, it is an ingredient of "mahā śyonāka taila" which is used in the form of drink, massage, inhalation and enema. This medicated oil is used in looseness of joints, chronic fever, gout, insanity, dysuria, vomiting, trembling etc.[8] Traditionally, the stem bark powder mixed with sesame oil is used externally for the treatment of skin diseases and sprains. Sap obtained from the bark is utilized for the treatment of deafness. The root bark is used for the atonic dyspepsia, diabetes, mild fever and debility.[3] A paste of root is applied locally to excite granulation in wounds.[9]

Until now, no scientific investigation had been carried out for standardization of *Ventilago calyculata* Tul. Bark. Hence, there was a need to provide scientific proof for standardization by the pharmacognostical and phytochemical evaluation of the bark of *Ventilago calyculata* Tul.

MATERIALS AND METHODS

Plant collection and Authentification

The stem bark of *Ventilago calyculata* were collected from the local area of Bhopal, Madhya Pradesh, in the month of October 2010 and authenticated by Dr. Zia Ul Hassan, Botanist, Saifia College of Science and Education, Bhopal, Madhya Pradesh. The voucher specimen (248/Bot/Saifia/11) was deposited in Department of Pharmacognosy, Technocrats Institute of Technology, Pharmacy, Bhopal, Madhya Pradesh. The barks were washed thoroughly with tap water, shade dried, homogenized to coarse powder and stored in air tight bottle.

Pharmacognostic studies

Macroscopic characteristics

For morphological observations, fresh barks approx. 2-5 cm in lengths was used. The macromorphological features of the bark were observed under magnifying lens.[10]

Fluorescence analysis

Powdered material was analyzed under visible light, short ultra-violet light, long ultra-violet after treatment with organic and inorganic reagents was carried out for the powder.[11]

Microscopic characteristics

Bark sample was fixed and specimens were cast into paraffin blocks.[12] The paraffin embedded specimens were sectioned with the help of rotary microtome. The sections were stained[13] and glycerin mounted slides were observed. Photographs with different magnification were taken with Nikon Lab Photo 2 microscopic units. Powder microscopy was also carried out and the specific diagnostic characteristics were recorded.[15]

Physicochemical parameters

The physicochemical parameters like total ash value, loss on drying, water soluble ash value, acid insoluble ash value were determined as per WHO guidelines.[16] For physicochemical investigation, 10 g of powder was extracted by individual cold percolation method using alcohol and water as solvent. The solvent was evaporated to dryness and the dried crude extracts were stored in air tight bottle.

The dried and coarse powder of stem bark of *Ventilago calyculata* was extracted with the solvents of increasing polarity successively by hot extraction method using Soxhlet apparatus. The percentage yield of stem bark extracts viz. petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extract was calculated.

Phytochemical analysis

The dried and coarse powder of stem bark of *Ventilago calyculata* was subjected to qualitative phytochemical analysis.[17,18]

Quantitative Determination of Phytochemical Constituents

Determination of Total Phenolic Content[19]

For quantitative estimation of total phenolic component Folin-Ciocalteu phenolic reagent was used. 0.5 ml of sample (1mg/ml) solution was mixed with 2.5 ml of Folin Ciocalteu phenolic reagent (10%) and 2 ml of 7.5 % Na_2CO_3 solution and mixed. After that reacting mixture was incubated for 30 min at room temperature in dark condition and measured the absorbance at 760 nm. Here gallic acid was used as standard. The sample was tested in triplicate and a calibration curve for gallic acid was obtained. The results were compared to gallic acid calibration curve and the total phenolic content of extract was expressed as mg of gallic acid equivalents (GAE) per gram of dry extract.

Determination of Total Flavonoids Content[20]

The spectrophotometer assay for the quantitative determination of flavonoid content was carried out as described by Wang *et al*. with minor modifications using rutin as a standard. Briefly, extracts or standard solutions (0.25 mL) were mixed with 1.25 mL distilled water and 75 µL 5% $NaNO_2$. After 6 min, 75 µL of 10% $AlCl_3$ was added. After another 5 min, 0.5 mL of 1 M NaOH was added to the mixture. Immediately, the absorbance of the mixture was determined at 510 nm *versus* prepared water blank. Total flavonoids content was expressed as mg rutin equivalents (RE) per gram dry extract.

Statistical analysis: The data was expressed as mean ± SD. The significance of differences among the group was assessed using one way analysis of variance (ANOVA). $P \leq 0.05$ were considered as significance.

RESULTS AND DISCUSSION

Macroscopic characteristics

The stem bark of *Ventilago calyculata* is more or less oval or rounded in shape, outer bark is brownish yellow with crimson striations. It is bitter in taste with characteristic odour and rough texture. (Figure 1)

Figure 1: Macropscopy of *Ventilago calyculata*.
(A) *Ventilago calyculata* in the natural habitat; (B)Arial part showing the fruits; (C) Stem bark of *Ventilago calyculata*; (D) Powdered bark.

Fluorescence analysis

Fluorescence characteristic of powdered stem bark of *Ventilago calyculata* were observed for resolution of doubtful specimen. Powdered material was analyzed under visible light, short ultra-violet light, long ultra-violet after treatment with organic and inorganic reagents (Table 1).

Microscopic characteristics

Transverse section of the bark showed a well distinguished cork consists of numerous layers of small thin walled flattened cells arranged in radial rows and having yellowish brown contents which give a purple color with alkalies. The cortex is composed of an outer zone of flattened collenchyma followed by a large inner zone of thin walled flattened cellulosic parenchyma. Groups of sclereids scattered throughout the cortex. Some of them contain clusters of the calcium oxalate crystals. The secondary phloem is transversed by 1-5 seriate medullary rays, the sieve tubes are arranged in tangential bands alternating with the phloem fibers and phloem parenchyma. The fibers usually occur in small groups which are surrounded with parenchyma cells, each containing prism of calcium

Table 1: Fluorescence characteristics of dried powder of *Ventilago calyculata*

Powder + Reagent	Color observed in Ordinary light	Color observed under UV Short (254 nm)	Color observed under UV Long (365nm)
Powder as such	Brown	Green	Green
Powder+1N NaOH in water	Dark green	Brownish-green with black spots	Yellow with white spots
Powder +1N HCl	Yellow with pinkish spots	Yellow	Yellow with black spots
Powder+50% H_2SO_4	Brown	Yellow	Yellow with pink spots
Powder + Formaldehyde	White	Brown	Yellow
Powder + 5% Ferric chloride	Brown	Brown	Black
Powder + Iodine	Black	Yellow	Black

oxalate forming a crystal sheath. (Figure 2).

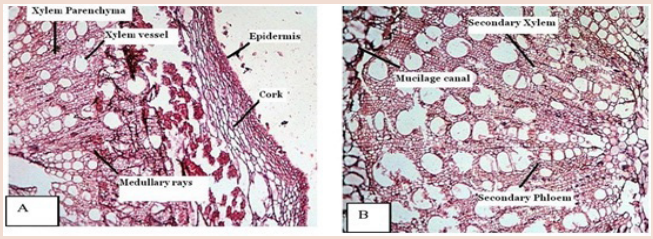

Figure 2: Photomicrograph of Transverse section (T.S.) of stem bark of Ventilago calyculata x400
(A) T.S. showing epidermis, cork, xylem parenchyma, xylem vessel, medullary rays; (B) T.S. showing secondary xylem, mucilage canal, secondary phloem

The powder of the bark powder is characterized by numerous fragments of lignified fibers, fibers in groups accompanied by crystal sheath; sclereids in groups or isolated ; fragments of yellowish–brown cork, numerous brown fragments of parenchyma, crystals of calcium oxalate, clusters and prism. The crystals are cuboidal in shape scattered in the powder. The crystals are in regular vertical fills or strands and vary in shape and sizes (Figure 3).

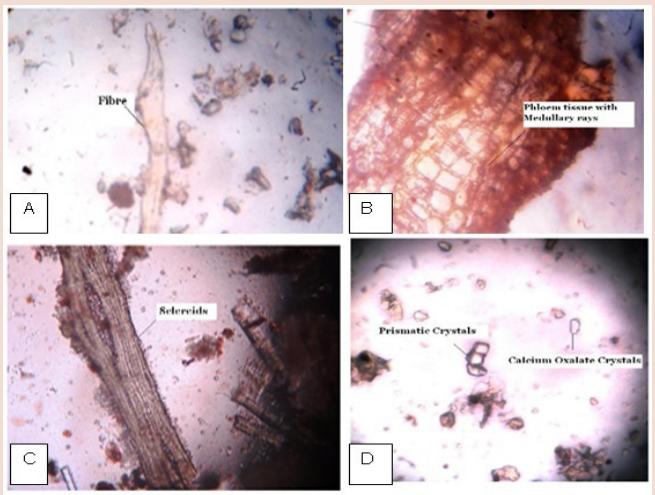

Figure 3: Photomicrograph of powder characteristics of stem bark of *Ventilago calyculata* x400
(A) Fibre; (B) Fragments of phloem tissue and medullary rays; (C) Sclereids; (D) Prismatic crystals of calcium oxalate

Physicochemical parameters

Physicochemical parameters of any drug give an idea of the earthy matter and/or inorganic composition and/or other impurities present along with a drug. The total ash value, acid insoluble ash value and water soluble ash value of stem bark were found to be 15% w/w, 3.4% w/w and 11.61% w/w respectively. The moisture content of the powdered drug was evaluated using loss on drying method and value observed was 3.18% w/w (Table 2).

Table 2: Physiochemical analysis of dried powder of *Ventilago calyculata*

S. No.	Values	Stem bark (% w/w)
Ash values		
1	Total ash value	not more than 11.61
2	Acid insoluble ash	not more than 4.2
3	Water soluble ash	not more than 7.41
Loss on drying		3.18

The dried and coarse powder of stem bark of *Ventilago calyculata* was extracted with the solvents of increasing polarity successively by soxhlet apparatus, while the aqueous extract was obtained by cold maceration method. The percentage yield of stems bark extracts *viz*. petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extract were found to be 2.77% w/w, 2.66% w/w, 3.8% w/w, 5.8% w/w, 8.0% w/w respectively (Table 3).

Table 3: Nature and percentage yield of various extracts of dried powder of *Ventilago calyculata*

Extracts	Colour & consistency of extract	Percentage yield (% w/w)
Petroleum ether	Reddish brown powder	2.77
Chloroform	Brownish powder	2.66
Ethyl acetate	Brownish powder	3.8
Ethanol	Dark brown with syrupy consistency	5.8
Water	Blackish brown powder	8.0

Phytochemical analysis

The dried and coarse powder of stem bark was tested for the presence of phytoconstituents using reported methods mentioned in the standards and results are given in Table 4. The preliminary phytochemical screening of various extracts was carried out and it was found that petroleum ether extract showed the presence of glycosides, flavonoids, phytosterols and phenolic compounds, chloroform extract showed the presence of glycosides, phenolic compounds, ethyl acetate extract showed the presence of alkaloids, flavonoids, glycosides, phenolic compounds and carbohydrates, ethanol extract showed the presence of carbohydrates, glycosides, phenolic compounds and flavonoids and aqueous extract showed the presence of alkaloids, carbohydrates, phenolic compounds,

Table 4: Qualitative chemical tests performed in the various extracts dried powder of *Ventilago calyculata*

Phytochemical tests	Petroleum Ether	Chloroform	Ethyl acetate	Ethanolic	Aqueous
Test for Alkaloids					
Mayer's reagents	-	-	-	-	+
Dragendorff's reagent	-	-	+	-	-
Hager's reagents	-	-	+	-	+
Wagner's reagent	-	-	-	-	+
Test for Carbohydrates					
Molisch's test	+	-	+	+	+
Fehling's test	+	-	+	+	+
Benedict's test	+	-	+	+	+
Barfoed's test	+	-	+	+	+
Test for Glycosides					
Legal's test	+	+	+	-	-
Borntrager's test	+	+	+	-	-
Baljet test	+	-	-	-	-
Keller-Killiani	+	-	-	+	-
Test for Phytosterol					
Libermann Burchard test	+	-	-	-	-
Salkowski test	+	-	-	-	-
Test for saponins					
Foam	-	-	-	-	+
Heamolysis	-	-	-	-	+
Test for Flavonoids					
Shinoda's test	+	-	+	+	+
Test for Mucilage	-	-	-	-	+
Test for Tannins/Phenols					
Lead acetate	+	+	+	+	-
Ferric chloride test	+	+	+	+	+

+ Present – Absent

Table 5: Total phenolic content of extracts of *Ventilago calyculata*

S.No.	Plant extract	Gallic acid equivalent (mg/g)
1	**Petroleum ether**	2.16±0.04*
2	**Chloroform**	4.16±1.04*
3	**Ethyl acetate**	9.12±1.14*
4	**Ethanol**	7.16±1.16*
5	**Aqueous**	1.16±1.02*

*Values are means of triplicate determination ± Standard deviation

Table 6: Total flavonoid content of extracts of *Ventilago calyculata*

S.No.	Plant extract	Rutin equivalent(mg/g)
1	**Petroleum ether**	4.5±0.55*
2	**Chloroform**	8.20±1.12*
3	**Ethyl acetate**	10.1±0.26*
4	**Ethanol**	6.5±1.3*
5	**Aqueous**	0.66±0.13*

*Values are means of triplicate determination ± Standard deviation

saponins and flavonoids. The total phenolic content of the petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extracts were 2.16 ± 0.04, 4.16 ± 1.04, 9.12 ± 1.14, 7.16 ± 1.16 and 1.16 ± 1.02 mg/g gallic acid equivalent respectively (Table 5). The total flavonoid content of petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extracts were 4.5 ± 0.55, 8.20 ± 1.12, 10.1 ± 0.26, 6.5 ± 1.3 and 0.66 ± 1.13 mg/g rut in equivalent respectively (Table 6).

The present study dealt with the pharmacognostic and phytochemical investigation of stem bark of *Ventilago calyculata*. Morphological and microscopic studies of the stem bark will enable to identify the crude drug. Ash values, extractive values can be used as reliable aid for detecting adulteration. The information obtained from preliminary phytochemical screening will be useful in finding out the genuineness of the drug. These simple but reliable standards will be useful to a lay person in using the drug as a home remedy. Also the manufacturers can utilize them for identification and selection of the raw material for drug production.

CONCLUSION

The plant *Ventilago calyculata* is widely used in various traditional system of medicine as a herbal remedy. It has been used since centuries as a tonic, in treatment of diarrhea, liver disorders, inflammation, leucorrhea, urinary tract infections, malarial fever and diabetes. Phytochemical screening assay is a simple, quick, and inexpensive procedure that gives the researcher a quick answer to the various types of phyto-

chemicals in a mixture and an important tool in bioactive compound analysis. Since bioactive compounds occurring in plant material consist of multi-component mixtures, their separation and determination still creates problems. Practically most of them have to be purified by the combination of several chromatographic techniques and various other purification methods to isolate bioactive compound(s). On the basis of physicochemical studies and preliminary phytochemical screening, this plant may give a significant effect against diseases and disorders, literature review indicates that very less work has been done on this plant and there is a wide scope for investigation.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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Original Article

Comparison *in vitro* of Antioxidant Activity between Fifteen *Campanula* Species (Bellflower) from Palestinian Flora

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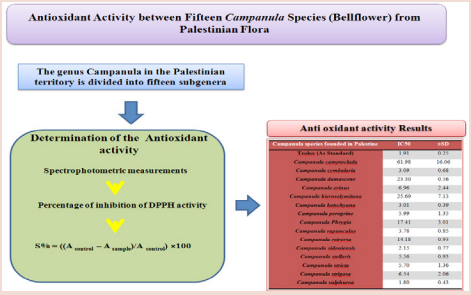
ABSTRACT

Background: The natural antioxidant products in the plant kingdom play an important role in the healthy life style and reduce the risk for various chronic diseases.**Objective:** The objective of this study was to investigate different antioxidant pharmacological property of methanol extract for fifteen species of *Campanula* plant from Palestinian flora. **Methods:** The antioxidant activity of fifteen *Campanula* species growing wildly in Palestine were studied using 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging activity and their antioxidant activity was compared to Trolox antioxidant activity. **Results:** The results clearly demonstrate a very high antioxidant activity of the *Campanula sulphurea* and *Campanula sidoniensis* and they showed almost the same antioxidant activity of Trolox. The other twelve species extracts also exhibited excellent antioxidant activity in DPPH radical scavenging activity in comparison with trolox standard. **Conclusion:** Methanol extract of *Campanula* can be valuable for treatment of different diseases and could be used as a possible new source of natural antioxidants in the food, nutraceuticals, pharmaceuticals and cosmetic industry.

Key words: Antioxidant, Bellflower, *Campanula* species, *Campanulaceae*, DPPH radical scavenging activity.

SUMMARY

- The results show a difference in the antioxidant activity in the fifteen *Campanula subgenera*.
- The results clearly demonstrate a high antioxidant activity of the *Campanula sulphurea* and *Campanula sidoniensis* which show the same antioxidant activity of Trolox.
- All *Campanula subgenera* show a moderate antioxidant activity.
- The *Campanula* species could be used as a possible new source of natural antioxidants.



PICTORIAL ABSTRACT

Abbreviations used: DPPH, IC_{50}, UV/Vis, S%, $A_{control}$ and A_{sample}

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INTRODUCTION

Palestine is unique and rich land in its natural flora diversity due to its geographical location as jointing point of Asia continent, Africa continent, and Europe continent, in addition to that it has mountains, hills, valleys, coastal plains, desert, Mediterranean Sea, Dead Sea and Rift Valley. Different climatic, phytogeographic and zoogeographic zones converges Palestine, creating great biological multi-diversity.[1,2] In many developed and developing countries, a huge section of the population relies on traditional herbal healers and their armamentarium of medicinal and non medicinal plants in order to provide health care needs.[3] Any therapeutic treatment or prevention of diseases began along ago with the utilization of plants, as well as the methods of preparations of folklore traditional healings throughout the world commonly used plants as part of their traditions and cultures.[4-6] The plant genus *Campanula* L. (Bellflower) belonged to the Campanulaceae family and contains about 300 species distributed widely in temperate regions of the northern part of Hemisphere. It includes perennial or annual. This plant is widely used for several ethano-medicinal purposes by tribal peoples and traditional practitioners.[7]

Most of all *Campanula* species grow wildly in the West and Central Asia, Black Sea, Mediterranean Sea, Eurasiatic Artic and North America regions.[8]

Campanula roots are fleshy and have a lot of fibers, the stems are simple, erect, stiff, slender, more or less covered with white stiff hairs, which disappeared when cultivated with 2 to 3 feet high. The leaves are oval or oblong and slightly crenate on long stalks, 1 to 3 inches long, the leaves on the stem are narrow, obscurely toothed or mostly entire. The flowers have purple, reddish purple, blue or white colors forming long, simple or slightly branched panicles on short peduncles which bloomed in July and August.[9,10]

The genus *Campanula* as represented in the Palestinian territory is divided into fifteen subgenera.[11,12]

- *Campanula camptoclada Boiss.*
- *Campanula cymbalaria Sm.*
- *Campanula damascena Labill.*
- *Campanula erinus L.*
- *Campanula hierosolymitana Boiss.*
- *Campanula kotschyana*
- *Campanula peregrina L.*
- *Campanula phrygia Jaub. & Spach*
- *Campanula rapunculus L.*
- *Campanula retrorsa Labill.*
- *Campanula sidoniensis Boiss. & Blanche*
- *Campanula stellaris Boiss.*
- *Campanula stricta L.*
- *Campanula strigosa Banks & Sol.*

- *Campanula sulphurea Boiss.*

Campanula plant genus contains flavonoids secondary metabolic compounds as kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside, rutin, lobetyol, coniferin, 4'-O-(p-hydroxybenzoyl)-isorhamnetin-3,7-di-O-b-D-glucopyranoside, lobetyolin, and quercetin-3-*O*-rutinoside and other phytochemical compounds as p-hydroxybenzoic acid, ethyl docosanoate, bis(2-ethylhexyl) adipate, sitosterolb-D-glucoside.[13-20] Bellflower different species have been used as a traditional medicine in form of decoctions for treatment of various diseases such as laryngitis, constipation, warts, tonsillitis and bronchitis for a long time in addition to their use as ornamental plants,[21,22] also as emetics, spasmolytic, antiallergic, antioxidant, antimicrobial, antiviral and antiphlogistic properties, as well as they possess refreshing and stimulant properties.[23] The roots have been chewed fresh for treatment of lung and heart problems while the roots infusion has been used as ear drops for ears inflammations. A decoction of the entire plant has been used as a wash in the treatment of sore eyes.[24]

METHODOLOGY

Materials and reagents

Trolox ((S)-(-)-6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) and 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich. Methanol was of analytical grade. All other chemical reagents that are used in the research were purchased from reliable commercial sources.

Instrumentation

The following instrumentations were used: Shaker device (LabTech Shaking Incubator), rotatory evaporator (Heidolph VV2000), heater and stirrer [Heidolph OB2000], Spectrophotometer (Jenway 6505 UV/Vis Spectrophotometer).

Plant material

Campanula's fifteen species were collected from different regions in Palestine during the spring session (June- August, 2013and 2014) and authenticated by Dr. Nidal. A. Jaradat. A voucher specimen was deposited in the Herbarium of the Laboratory of Pharmacognosy at An-Najah National University as presented in (Table 1).

The plants species under study were washed twice with distilled water, dried in the shade at an average temperature of 20-30°C, for 72 hours and stored in a dry place.

Preparing of plants extracts

For evaluating of the antioxidant capacity of studied fifteen *Campanula* species plants, the entire plants were powdered separately using a grinder. The extraction was performed at room temperature. About 100 g of the each *Campanula* species were soaked in 1 Liter of methanol (99%) and put in a shaker device at 100 rounds per minute for 72 hours and stored in refrigerator for 4 days. The extracts were then filtered using filter papers. The extract was then concentrated under vacuum on a rotatory evaporator. The crude extract was stored at 4°C for further use, and this procedure repeated for all fifteen *Campanula* species.

Anti oxidant activity

Trolox standard and plant working solutions

A stock solution of a concentration of 1mg/1ml in methanol was firstly prepared for all samples of plant extracts and the standard trolox. The working solutions of the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 µg/ml) were prepared by suitable dilution with methanol from the stock solution.

Table 1: A voucher specimen codes for all Palestinian *Campanula* species

All *Campanula* species founded in Palestine	Voucher specimen herbarium code
Campanula camptoclada	Pharm-PCT-476
Campanula cymbalaria	Pharm-PCT-477
Campanula damascene	Pharm-PCT-478
Campanula erinus	Pharm-PCT-479
Campanula hierosolymitana	Pharm-PCT-480
Campanula kotschyana	Pharm-PCT-481
Campanula peregrine	Pharm-PCT-482
Campanula Phrygia	Pharm-PCT-483
Campanula rapunculus	Pharm-PCT-484
Campanula retrorsa	Pharm-PCT-485
Campanula sidoniensis	Pharm-PCT-486
Campanula stellaris	Pharm-PCT-487
Campanula stricta	Pharm-PCT-488
Campanula strigosa	Pharm-PCT-489
Campanula sulphurea	Pharm-PCT-490

Spectrophotometric measurements

2, 2-diphenylpicrylhydrazyl (DPPH) was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above prepared working concentration in a ratio of 1:1:1 respectively. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentration was DPPH with methanol only.

The solutions were incubated in dark place for 30 minute at room temperature before the absorbance readings were recorded at 517 nm.

Percentage of inhibition of DPPH activity

The percentage of antioxidant activity of the fifteen *Campanula* species and the Trolox standard were calculated using the following formulaThe DPPH radical scavenging activity (S %) was calculated using the following equation:

$$S\%=((A_{control}-A_{sample})/A_{control})\times 100$$

Where $A_{control}$ is the absorbance of the blank control (containing all reagents except the extract solution) and A_{sample} is the absorbance of the test sample. The antioxidant half maximal inhibitory concentration (IC_{50}) for all plant samples and the standard were calculated using Bio Data Fit edition 1.2 (data fit for biologist).

Data analysis

The antioxidant activity was reported as a percentage of DPPH reduction. The inhibition of the *Campanula* plants and Trolox standard at different concentration were plotted and tabulated and the IC_{50} for each of them was calculated using the Bio Data Fit fitting program. The best fit for the data was the adapted model to calculate the IC_{50}.

RESULTS AND DISCUSSION

Antioxidant activity

There are a lot of clinical studies suggesting that the antioxidant compounds in the plants leaves, fruits and vegetables, are the main factors for the observed efficacy of these products in reducing the incidence of chronic diseases including heart disease and some cancers. The free radical scavenging activity of antioxidants in foods has been substantially investigated and reported.[25] The free radical scavenging activity of the

Table 2: Maximum inhibitory concentration of the fifteen Campanula species and trolox standard and ±SD

Campanula species founded in Palestine	IC_{50}	±SD
Trolox (As Standard)	1.91	0.25
Campanula camptoclada	61.98	16.06
Campanula cymbalaria	3.09	0.68
Campanula damascene	23.30	0.56
Campanula erinus	6.96	2.44
Campanula hierosolymitana	25.69	7.13
Campanula kotschyana	3.01	0.39
Campanula peregrine	5.99	1.35
Campanula Phrygia	17.41	3.01
Campanula rapunculus	3.78	0.85
Campanula retrorsa	14.18	0.93
Campanula sidoniensis	2.15	0.77
Campanula stellaris	5.56	0.93
Campanula stricta	5.70	1.36
Campanula strigosa	6.54	2.06
Campanula sulphurea	1.80	0.43

methanolic extract of the entire fifteen *Campanula* species have been tested by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1–100 µg/ml. The zero inhibition was considered for the solution which contained only DPPH without any plant extract.

The results show a difference in the antioxidant activity for all samples. The more potent activity was for *Campanula sulphurea* extract was comparatively relative with IC_{50} of trolox standard. Moreover, the antioxidant activity for the other plants were comparative with slight difference in the antioxidant activity a summary of the antioxidant activity is presented in (Table 2).

The results clearly demonstrate a high antioxidant activity of the *Campanula sulphurea* and *Campanula sidoniensis* which show the same antioxidant activity of Trolox. *Campanula cymbalaria, Campanula rapunculus* have a high antioxidant activity that is equal to about half the antioxidant activity of trolox. Moreover, *Campanula strigosa, Campanula stellaris Campanula peregrine Campanula erinus* have an antioxidant activity that is three fold less than the Trolox antioxidant activity. All the other plants show a moderate antioxidant activity that is less than 25 µg/ml with an exceptional to only one plant namely *Campanula camptoclada* which have an antioxidant activity of about 62 µg/ml.

CONCLUSION

The results clearly show that the *Campanula* species in general have high free radical scavenging activity of antioxidants. The *Campanula sulphurea* in comparison with the other twelve *Campanula* species showed the highest antioxidant activity which was almost equal to the Trolox. Thus *Campanula* species could be used as a possible new source of natural antioxidants in the food, nutraceuticals, pharmaceuticals and cosmetic industry.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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Original Article

In vitro Acetyl Cholinesterase Inhibitory assay of *Acacia catechu Willd* Ethanolic Seed Extract

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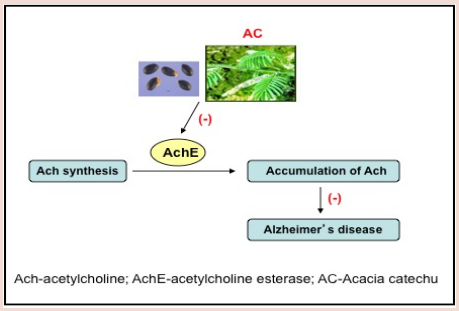
ABSTRACT

Aim & objective: The aim of this study was to evaluate acetyl cholinesterase inhibitory activity of *Acacia catechu* ethanolic seed extract to introduce a new source for management of Alzheimer's disease. **Background:** Alzheimer's disease is a complex, multifactorial, progressive, neurodegenerative disease primarily affecting the elderly population and is estimated to account for 50–60% of dementia cases in persons over 65 years of age. It is likely that the inhibition of acetyl cholinesterase by *Acacia catechu* ethanolic seed extract that is rich in flavonoids and antioxidants may aid in the protection of neurodegenerative disorders and ultimately Alzheimer's disease. **Methods:** Anti cholinesterase activity is determined by adopting *In vitro* standard protocol. **Results:** According to the obtained results, the inhibitory activity (IC_{50} values, µg /ml) of extracts was 204.38 ± 2.54 µg/ml. **Conclusion:** The results indicated and confirmed the traditional use of *Acacia catechu* ethanolic seed extract for management of central nervous system disorders. It showed the moderate activity in inhibition of acetyl cholinesterase at various concentrations. However, further investigations on identification of active components in the extracts are needed.

Key words: *Acacia catechu* seed, Acetyl cholinesterase inhibitor, Alzheimer's disease, Neurodegenerative, Spectrophotometric analysis.

SUMMARY

- *Acacia catechu* Willd. Belongs to the family *fabacaae* Commonly known as khadira in Sanskrit and karungali in Tamil is a very potent medicinal plant with diverse pharmacological actions.
- Alzheimer's disease (AD) is an neurodegenerative disorder affecting people all over the world. It is associated with loss of cholinergic neurons in the brain and decreased level of Ach.
- *Acacia catechu* seed is rich in Catechin, Epicatechin and it also exhibits significant antioxidant property.
- *Acacia catechu* seed inhibits Acetyl cholinesterase level hence, recommended for management of Alzheimer's disease.



Ach-acetylcholine; AchE-acetylcholine esterase; AC-Acacia catechu

PICTORIAL ABSTRACT

Abbreviations used: Ach: Acetyl choline, Ache: Acetyl cholinesterase, Ac: *Acacia catechu.*

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INTRODUCTION

Alzheimer's disease (AD) is an neurodegenerative disorder associated with loss of neurons in the certain areas of brain which leads to cognitive impairment, neurological disturbances, behavioral abnormalities, even leads to death. The pathophysiology of AD is not clear, but it is believed to be associated with cholinergic pathway impairment, leading to reduction in acetylcholine level in certain regions of the brain.[1] Acetylcholine, a neurotransmitter, which is hydrolyzed by acetyl cholinesterase (AChE) and butyryl cholinesterase (BuChE) is considered to play an important role in the pathology of AD.[2]

Neuritic plaques (Neurofibrillary tangles, amyloid plaques) are the major structural abnormality in seen commonly in AD patients. Amino acid Beta-amyloid peptide is an chief protein substance of amyloid in AD individuals.[3] The therapy of AD depends on decreasing the progression of the disease by improving the quality of life. Inhibition of acetyl choline level may aid in control of AD.

Cognitive enhancers are used in management of AD, Few of the cognitive enhancers are only approved by FDA in united states.[4] few drugs that have received regulatory approval currently includes donepezil, rivastigmine and galantamine, these drugs acts through increasing the concentration of acetylcholine at the neurotransmitter sites or directs by regulating activity at nicotinic receptors.[5]

Various side effects of medications reported in clinical trials causes nausea, vomiting, diarrhea, syncope and bradycardia.[6] Absolutely, there requires a fundamental need for an alternative to anti-cholinesterase compounds with fewer side effects that leads to investigation on plants as a novel source of treatment of AD.[7]

Natural sources have been used since antiquity in the treatment of various diseases including cognitive disorders, such as AD.

Considering the importance of plant based compounds in drug discovery, the present study was undertaken to evaluate the anti-cholinesterase activity of a number of selected medicinal plants with various ethno botanical uses, aiming to discover new compounds for anti-cholinesterase activity to be used in management of AD.[8-10]

Acacia catechu (Family: *Fabacaae*) is an indigenous tree grown in all the parts of the world. Commonly known as karungali in Tamil and khadira in Sanskrit. Similarly to Neem, Turmeric, *Aloe Vera*, People in Kerala used this karungali leaves in boiling water, extracted juice for management of digestive disorders.[11] It has been used in Ayurveda (Indian Medicinal System) for years extensively as an anti-inflammatory agent. It has been proven to possess antioxidant, anticancer, antiulcer, hepato protective, anti diabetic effects.[12-17] It also has a role in dye industry the heartwood of khadira is employed for this purpose. Study reported that

Table 1: Anti cholinesterase inhibitory assay of *Acacia catechu* ethanolic seed

AchE inhibitory assay	
Concentration (ug/ml)	% inhibition
50	5
100	10
150	18
200	25
250	35
300	45
IC50 *Acacia catechu* = 204.38 = ± 2.54 (ug/ml)	-
IC_{50} Galantamine = 2.727 = ± 0.08 (ug/ml)	-

Acacia catechu Catechin produced promising result in cognitive enhancement in AD patients.

Hence, Ethanolic extract of *Acacia catechu* seed is investigated for its anti-cholinesterase inhibitory activity for the first time.

MATERIALS AND METHODS

Plant material

Acacia catechu seeds were collected from Hosur, Tamilnadu and was authenticated by Dr. H.B Singh, NISCAIR, New Delhi and the voucher specimen were stored for further use in Green Chem lab, Bangalore.

Ethanolic Extraction

Seeds were shade dried for a week. Dried seeds were milled to fine powder. Powder was passed through 100 mesh sieve and stored in a sealed polythene bag. 2.5 kg of powdered *Acacia catechu* seeds were extracted with 10 liters of Ethanol, at 65°C temperature, for 1 hour, in a 20 liter round bottom flask with Graham condenser attached. Condenser was cooled circulating with chilled water. After 1 hour of extraction, round bottom flask was cooled to room temp and the extract were filtered and collected. The Marc was extracted repeatedly with 10 liters of Ethanol, twice. The extracts were filtered and collected. The combined extracts was evaporated to dryness under reduced pressure in a Buchi Rotary Evaporator (Switzerland) at 65°C, to obtain 150 g of powder extract. The w/w yield of the prepared extract was 6%. The extract were stored at 4°C until used.

Chemicals

Acetylthiocholine iodide (ATCI), 5,5'–dithio-bis-2-nitrobenzoic acid (DTNB), bovine serum albumin (BSA) were purchased from Sigma (USA). Buffers and other chemical were of extra pure analytical grade. The following buffers were used: Buffer A:50 mM Tris–HCl, pH 8, containing 0.1% BSA; Buffer B:50 mM Tris-HCl, pH 8 containing 0.1 M NaCl, 0.02 M $MgCl_2 \times 6H_2O$.

Anti-cholinesterase inhibitory assay

The AChE activity was measured according to the method developed by Eldeen *et al.*[18,19] with slight modifications. To various concentrations of sample extracts (50–250 µg/ml), 25 µl of 15 mM acetylthiocholine iodide (ATCI), 125µl of 3 mM DTNB (5-5'-thiobis-2-nitrobenzoic acid) in Buffer C (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_{20}$) and 50 µl of Buffer B (50 mM Tris-HCl, pH 8, containing 0.1% bovine serum albumin) were added. Thereafter, AChE (0.2 U/ml) was added and the final mixture was read at 405 nm immediately. Galantamine was used as a standard drug.

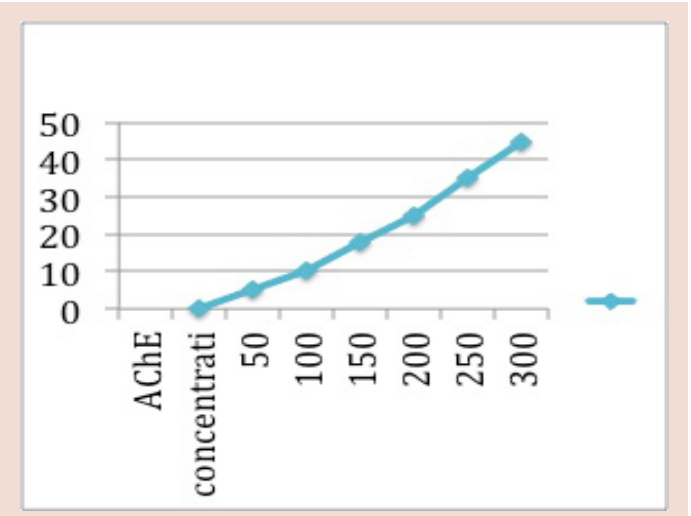

Figure 1: Acetyl choline inhibitory assay of *A. catechu* seed extract

Determination of IC_{50}

The percentage inhibition was then calculated using the following formula;

% inhibition = [(Control OD – Sample OD)/Control OD] x 100

The experiment was done in triplicate and concentrations of the test extract that inhibit the hydrolysis of the substrate (acetylcholine) by 50% (IC_{50}) were determined by linear regression analysis between the inhibition percentage versus the extract concentration.

RESULT AND DISCUSSION

Neurodegenerative disease is a term applied to a variety of conditions arising from a chronic breakdown and deterioration of the neurons, particularly those of the central nervous system.

Alzheimer's disease is associated with loss of cholinergic neurons in the brain and the decreased level of AChE. The major therapeutic target in the AD treatment strategies is the inhibition of brain AChE.[20] Cholinesterase inhibitor drugs, inhibiting AChE activity, maintain ACh level by decreasing its breakdown rate.[21] Although the underlying patho physiological mechanisms are not clear, AD is firmly associated with impairment in cholinergic pathway, which results in reduced level of acetylcholine (Ach) that is hydrolysed by cholinesterase (ChE) in certain areas of brain.

Medications currently approved by regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to treat the cognitive manifestations of AD and improve life quality of the patients are Donepezil, rivastigmine and galantamine as reversible AChE inhibitors, and memantine as an NMDA receptor antagonist.

Tacrine was the first of the AChE inhibitors approved for the AD treatment in 1993, but its use has been abandoned because of a high incidence of side effects including hepatotoxicity.[22-24]

Plant based compounds have been used as cognition enhancers herbs like *Withania somnifera*, *Centella asiatica*, *Curcuma longa, Bacopa monnieri, Convolvulus pluricaulis, Celastrus paniculatus, Nardostachys jatamansi* have been reported to be used in management of cognitive impairment in Ayurvedic system of medicine.[25,26]

Acetyl cholinesterase inhibitory activity is dose dependently increasing at a concentration ranging from 50 µg/ml to 300 µg/ml and when compared to standard Galanthamine the inhibitory activity is moderate even though the tested extract shows significant activity. The results are depicted in Table 1 and Figure 1 and can be used for treating neurodegenerative disorders.

It was reported that the presence of Catechin, Epicatechin in *Acacia catechu* is responsible for treating cognitive impairment and the observed anti-cholinesterase activity found in the study could be due to the presence of Catechins, Quercetin and Epicatechin.[27]

CONCLUSION

The results concluded that *Acacia catechu* ethanolic seed extract exhibited potential acetyl cholinesterase inhibitory activity, further *In vivo* studies are required to prove its cognitive efficacy.

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Original Article

Anti-hyperglycemic and Anti-lipidemic activities of Diabac (a polyherbal formulation) in Streptozotocin-nicotinamide induced type 2 diabetic rats

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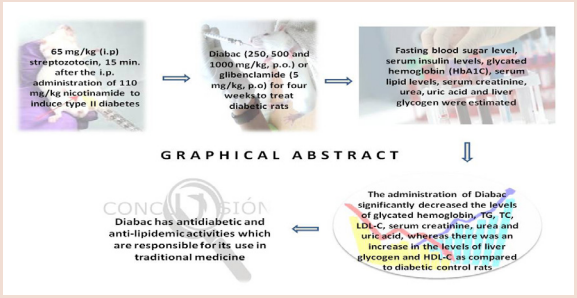
ABSTRACT

Aim: The objective of the work was to investigate the antidiabetic activity of Diabac (a polyherbal formulation) in streptozotocin-nicotinamide induced type 2 diabetic rats. **Methods:** Oral glucose tolerance test (OGTT) was performed to evaluate effect of Diabac on elevated glucose level. The type 2 diabetes was induced by overnight fasted rats by a single intraperitoneal (i.p.) injection of 65 mg/kg streptozotocin, 15 min. after the i.p. administration of 110 mg/kg nicotinamide. The diabetic rats were treated with Diabac (250, 500 and 1000 mg/kg, p.o.) or glibenclamide (5 mg/kg, p.o) for four week. Various parameters were studied such as fasting blood sugar level, serum insulin levels, glycated hemoglobin (HbA_{1C}), serum lipid levels, se rum creatinine, urea, uric acid and liver glycogen. **Results:** Treatment with Diabac significantly reduced the blood sugar levels in OGTT. Diabetic rats showed a significant increase in the levels of glycated hemoglobin, serum lipids, serum creatinine, urea and uric acid, whereas there was a decrease in serum insulin, liver glycogen and HDL-C levels as compared to normal control rats. The administration of Diabac or glibenclamide significantly decreased the levels of glycated hemoglobin, TG, TC, LDL-C, serum creatinine, urea and uric acid, whereas there was an increase in the levels of liver glycogen and HDL-C as compared to diabetic control rats. However, the treatment with Diabac did not show any significant change in serum insulin levels as compared to diabetic control rats. **Conclusion:** These results of present study concluded that Diabac has anti-diabetic and anti-lipidemic activities which are responsible for its use in traditional medicine.

Key words: Diabac, Glycated hemoglobin, Liver glycogen, Serum lipids, Streptozotocin.

SUMMARY

- Administration of Diabac (250, 500 and 5000 mg/kg), a polyherbal formulation to the STZ-nicotinamide induced diabetes resulted in a decrease the levels of fasting blood sugar, glycated hemoglobin, TG, TC, LDL-C, serum creatinine, urea and uric acid, while there was an increase in the levels of liver glycogen and HDL-C as compared to diabetic control rats.



PICTORIAL ABSTRACT

Abbreviations used: HbA_{1C}: Glycated hemoglobin, TC: Total cholesterol, TG: Triglycerides, LDL-C: Low density lipoprotein-cholesterol, HDL: High density lipoprotein-cholesterol.

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INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from the defects in insulin secretion, insulin resistance or both.[1] It is considered to be one of the five foremost causes of death in the world.[2] There are reports that incidence of diabetes mellitus was 2.8% in 2000 and is expected to be increase to 4.4% in 2030.[3] For a long time, diabetes mellitus has been treated with a number of medicinal plants or their extracts based on folklore medicine.[4] The oral hypoglycemic agents (sulfonylurea, biguanide, thiazolidinedione, α-glycosidase inhibitor and DPP-IV inhibitor) can produce several undesirable side effects and in addition, they are not suitable for use in pregnancy.[5] Thus, the management of diabetes without any side effects is still a challenge. Therefore, the search for more effective and safer hypoglycemic agents has continued to be an important area of active research. Since, the oral hypoglycemic agents cause several side effects it has become a need to search for a new herbal drug with little side effects.[6] Diabac contains herbal extract with known antidiabetic action (Table 1).[7-16]

In present study, we try to investigate antidiabetic effect of Diabac (a polyherbal formulation from Bacfo Pharmaceuticals India Limited, Noida) Since, there is no scientific evidence of this herbal formulation in experimentally induced type II diabetes.

MATERIALS AND METHODS

Drugs and chemicals

Diabac (Bacfo Pharmaceuticals India Limited, Noida), Streptozotocin, nicotinamide (Himedia, Mumbai, India) and Glibenclamide (USV Limited) are the main drugs used in present study. All biochemical kits were purchased from Span diagnostics Ltd, Surat, India and other chemicals and reagents used in the study were of analytical grade.

Experimental animals

Albino Wistar rats (200-250 g) of either sex were obtained from Zydus Research Centre, Ahmedabad. All animals were maintained under standardized condition (12-h light/dark cycle, 24 ± 2°C & humidity 35-60 %) and they were provided with standard pellet diet and water *ad libitum*. The rats were left for 48 h for adaptation prior to the beginning of the experiment. The study was approved by Institutional Animal Ethics Committee (IAEC) and carried out in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animal) guidelines.

Table 1: Composition of Diabac (A polyherbal formulation)

Ingredients	Botanical name	Part used	Weight (mg)
Gurmar	*Gymnema sylvestre*	Whole Plant	100
Jamunmingi	*Eugenia jamolana*	Whole Plant	100
Bilvapatra	*Aegle maemelos*	Whole Plant	100
Nyagrodha	*Ficus bengalensis*	Whole Plant	100
Shilajeet	*Asphaltum*	Whole Plant	100

Bhavna Dravya: Processed with the extracts of Karela (*Memordica charantia*), Bhumi Amla (*Phyllanthus niruri*), Neemswaras (*Azadirachta indica*), Triphla & Vijaysar (*Pterocarpus masrsupium*)

Acute toxicity study

At the basis OECD guideline no. 423, the acute oral toxicity was carried out in albino Wistar rats of either sex weighing 200-250g.[17] Diabac was given at the dose of (100, 200, 500, 1000, 2000 and 5000 mg/kg, p.o.) for 3 animals and the signs and symptoms were observed after 0, 30, 60, 120, 180, 240 min and then once a day for next 14 days.

Oral glucose tolerance test (OGTT)

Oral glucose tolerance test was performed in overnight (12h) fasted normal rats.[18] Rats were divided into five groups of six of each. Groups 1 received drinking water, Groups 2 received glibenclamide (5 mg/kg, p.o.), group 3-5 received Diabac (250, 500 and 1000 mg/kg, p.o.). Glucose (2 g/kg, p.o.) was fed 30 min prior to the administration of above-mentioned treatments. Blood glucose levels were measured by collecting the blood samples from the tail vein and they were collected at 0, 30, 60, 90 and 120 minutes after the glucose loading and blood glucose levels were measured.

Induction of type 2 diabetes

Sreptozotocin (65 mg/kg, i.p.) and nicotinamide (110 mg/kg, i.p.) were administered to overnight fasting albino Wistar rats (200-250 g) to induce Type 2 diabetes. Nicotinamide (dissolved in normal saline) was given first and 15 minutes later streptozotocin (dissolved in citrate buffer, pH 4.5) was administered.[19,20] Hyperglycemia was confirmed by elevated blood glucose levels at 72 h and then on day 7 after injection and only animals with fasting blood glucose level greater than 200 mg/dl were selected for antidiabetic study.

Experimental design

The rats were divided into six groups each consisting of six animals.

Group I: Normal control rats (distilled water 1 ml/kg, p.o.).

Group II: Diabetic control rats.

Group III: Diabetic rats treated with glibenclamide (5 mg/kg, p.o.).

Group IV: Diabetic rats treated with Diabac (250 mg/kg, p.o.)

Group V: Diabetic rats treated with Diabac (500 mg/kg, p.o.)

Group VI: Diabetic rats treated with Diabac (1000 mg/kg, p.o.)

All the aforementioned treatments were started one week (7 days) after induction of diabetes and treatments continued for 28 days.

The fasting blood sugar levels were measured on 1, 7, 14, 21 and 28 days periodically. Urine volume and urine glucose contents were estimated. At the end of the experiments, blood samples were collected from the retro orbital plexus of rats under light ether anesthesia, using glass capillaries and stored in with or without disodium ethylene diamine tetra-acetate for estimation of biochemical parameters. After, allowing the blood to clot for serum separation for 15 minutes, it was centrifuged at 5000 rpm for 20 minutes for separation of serum. Then the serum was stored at -20°C until further estimation.

Blood glucose, glycated hemoglobin (HbA_{1C}), hemoglobin (Hb) were estimated using whole blood. The total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL-C), high density lipoprotein (HDL), serum creatinine, urea and uric acid were estimated from serum using standard diagnostic kit. The serum insulin was determined by radioimmunoassay method.[21] Glycogen level in liver was determined as according to the method of Roe *et. al.*[22]

Histopathology

After sacrifice, pancreas tissues of each group were rapidly dissected out and washed immediately with saline and fixed in 10% phosphate buffered formalin. Paraffin-embedded specimens were cut into 5 µm-thick sections and stained with hematoxylin and eosin (H&E). The sections were examined under the light microscope (Olympus BX10, Tokyo, Japan) for the presence of histopathological changes and photomicrographs (Olympus DP12 camera, Japan) were taken. The observer performing histopathological evaluation was blinded to the animal treatment groups.

Statistical analysis

All the data are expressed as mean ± SEM (n=6). The statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons post test using a computer-based fitting program (Prism, GraphPad version 5, GraphPad software, Inc). The significance level was set at $P<0.05$ for all tests.

RESULTS

Acute oral toxicity

The administration of Diabac up to a dosage 5000 mg/kg did not show any sign of toxicity and no mortality for 14 days. Therefore, the pharmacological studies were carried out using $1/20^{th}$ (250 mg/kg), $1/10^{th}$ (500 mg/kg) and $1/5^{th}$ (1000 mg/kg) dose levels of Diabac.

Effect of Diabac on oral glucose tolerance test (OGTT)

Glucose challenge to normal rats increased blood glucose levels with maximum at 60 min and slight reduction in blood glucose was observed at 90 min onwards. The treatment with Diabac or glibenclamide improved glucose tolerance significantly at 60 min to 120 min compared to normal control animals (Table 2).

Effect of Diabac on fating blood glucose levels

The effect of Diabac on fasting blood glucose level of diabetic rats is shown in Table 3. Diabetic rats showed a significant increase in the fasting blood glucose levels as compared to normal control rats. At day 14 onwards, treatment with Diabac (250, 500 and 1000 mg/kg) showed a significant dose dependent decrease in blood glucose levels as compared

Table 2: Effect of Diabac on oral glucose tolerance test in non-diabetic rats

Groups	Treatment	Blood glucose (mg/dl)				
		0 min	30 min	60 min	90 min	120 min
1	Normal control	102.3 ± 3.25	127.2 ± 3.60	147.0 ± 7.90	115.7 ± 7.04	107.5 ± 2.99
2	Glibenclamide (5 mg/kg)	95.83 ± 2.18	104.5 ± 4.07**	96.83 ± 4.84***	85.67 ± 5.14***	91.33 ± 4.95*
3	Diabac (250 mg/kg)	102.3 ± 4.21	113.8 ± 5.69	137.5 ± 5.57*	117.0 ± 2.28	111.3 ± 2.18
4	Diabac (500 mg/kg)	98.17 ± 3.71	126.2 ± 5.24	125.3 ± 4.75**	112.3 ± 3.13*	103.0 ± 3.36*
5	Diabac (1000 mg/kg)	96.50 ± 2.66	109.2 ± 2.88*	121.0 ± 2.76***	107.7 ± 2.31*	99.50 ± 1.60*

Values are expressed in mean ± S.E.M (n=6), where *P<0.05, **P<0.01, ***P<0.001 as compared to normal control.

Table 3: Effect of Diabac on fasting blood glucose levels in STZ-nicotinamide induced type II diabetes

Groups	Treatment	Fasting Blood glucose (mg/dl)				
		Day 1	Day 7	Day 14	Day 21	Day 28
1	Normal control	73.67 ± 1.706	76.83 ± 1.92	80.17 ± 1.77	74.67 ± 3.373	87.33 ± 5.80
2	Diabetic control	243.7 ± 12.73	254.2 ± 15.13###	279.5 ± 17.61###	311.8 ± 24.17###	319.2 ± 23.96###
3	Glibenclamide (5 mg/kg)	297.7 ± 26.70	268.7 ± 16.52	223.2 ± 6.45**	189.8 ± 15.54***	132.7 ± 10.24***
4	Diabac (250 mg/kg)	322.5 ± 33.77	314.2 ± 30.27	217.8 ± 10.5**	208.3 ± 9.03***	207.5 ± 8.21***
5	Diabac (500 mg/kg)	293.0 ± 19.90	304.8 ± 23.45	202.2 ± 8.69***	189.5 ± 7.63***	169.8 ± 8.99***
6	Diabac (1000 mg/kg)	288.8 ± 15.75	301.2 ± 21.99	200.3 ± 5.06***	185.2 ± 11.87***	119.2 ± 6.4***

Values are expressed in Mean ± S.E.M (n=6), where ###P<0.001 as compared to normal control; *P<0.05, **P<0.01, ***P<0.001 as compared to diabetic control.

Table 4: Effect of Diabac on body weight, urine volume and urine glucose in STZ-nicotinamide induced type II diabetes

Groups	Treatment	Initial body weight (g)	Final body weight (g)	Urine Volume (ml)	Urine glucose
1	Normal control	275.0 ± 10.25	266.7 ± 11.45	20.83 ± 1.24	Nil
2	Diabetic control	216.7 ± 10.54	173.3 ± 8.02###	54.33 ± 2.81###	+++
3	Glibenclamide (5 mg/kg)	213.3 ± 6.146	203.3 ± 7.149	25.00 ± 1.34***	Nil
4	Diabac (250 mg/kg)	211.7 ± 8.333	185.0 ± 9.91**	43.17 ± 1.27***	+
5	Diabac (500 mg/kg)	220.0 ± 10.65	204.2 ± 7.79	35.00 ± 0.93***	Nil
6	Diabac (1000 mg/kg)	216.7 ± 8.433	209.2 ± 8.40	28.33 ± 1.02***	Nil

Values are expressed as Mean + S.E.M (n=6), Where, ###P<0.001 as compared to normal control; **P<0.01, ***P<0.001 as compared to diabetic control.
(+) - Trace elements of sugar and (+++) - more than 2% of sugar.

to diabetic control rats.

Effect of Diabac on body weight, urine volume and urine glucose

Diabetic rats showed a significant decrease (P<0.001) in body weight compared to normal control rats. However, treatment with Diabac (500 and 1000 mg/kg, p.o.) or glibenclamide did not show any significant reduction in body weight as compared to diabetic control, while Diabac (250 mg/kg) treated rats showed a significant reduction in body weight. Administration of Diabac or glibenclamide to diabetic rats showed a significant (P<0.001) reduction in urine volume as compared to diabetic control rats. An effect of Diabac or glibenclamide on urine glucose is shown in Table 4.

Effect of Diabac on Hemoglobin, Glycated hemoglobin, serum insulin and liver glycogen levels

Diabetic rats showed a significant (P<0.001) elevation in the level of glycated hemoglobin (HbA_{1C}) and reduction in the level of hemoglobin (Hb), serum insulin as compared to normal control rats. The treatment with Diabac (250, 500 and 1000 mg/kg, p.o.) or glibenclamide (5 mg/kg, p.o.) showed a significant (P<0.001) reduction in levels of glycated hemoglobin and an increase in the level of Hb at dose dependant manner. In contrast, treatment with Diabac did not show any significant alteration in serum insulin levels as compared to diabetic untreated rats. Liver glycogen levels of diabetic rats were significantly decreased as compared to normal control rats. The treatment with Diabac (500 and 1000 mg/kg) showed a significant (P<0.001) increase in levels of liver glycogen as compared to diabetic control rats. In addition, Diabac (250 mg/kg) treated rats did not show any significant alteration in liver glycogen content as compared to diabetic control rats (Table 5).

Effect of Diabac on serum lipid levels

Diabetic rats showed a significant (P<0.001) increase in the level of TG, TC and LDL-C and a reduction in level of HDL-C as compared to normal control rats. Treatment with Diabac (500 and 1000 mg/kg) showed a significant reduction in level of TG (P<0.001), TC (P<0.001), LDL-C (P<0.001) and an increase in HDL-C (P<0.01; P<0.001) levels in diabetic rats when compared to diabetic control rats. Moreover, treatment with Diabac (250 mg/kg) showed a significant decrease in levels of TG (P<0.001), TC (P<0.05), LDL-C (P<0.01) and did not show any sig-

Table 5: Effect of Diabac on hemoglobin (Hb), glycated hemoglobin, serum insulin and liver glycogen levels in STZ-nicotinamide induced type II diabetes

Groups	Treatment	Hb (g/dl)	Glycated hemoglobin (HbA_{1C} %)	Serum insulin (mU/L)	Liver glycogen (mg/g)
1	Normal control	14.67 ± 0.28	6.183 ± 0.17	1.01 ± 0.12	55.66 ± 2.38
2	Diabetic control	8.16 ± 0.56$^{###}$	11.75 ± 0.26$^{###}$	0.27 ± 0.04$^{###}$	7.86 ± 1.04$^{###}$
3	Glibenclamide (5 mg/kg)	12.48 ± 0.30***	6.78 ± 0.11***	0.76 ± 0.03**	48.33 ± 4.58***
4	Diabac (250 mg/kg)	11.27 ± 0.62***	9.31 ± 0.10***	0.35 ± 0.04	16.11 ± 0.92
5	Diabac (500 mg/kg)	12.93 ± 0.28***	8.27 ± 0.11***	0.39 ± 0.03	30.04 ± 1.80***
6	Diabac (1000 mg/kg)	14.10 ± 0.52***	7.18 ± 0.13***	0.40 ± 0.03	47.21 ± 3.47***

Values are expressed as Mean + S.E.M (n=6). Where, $^{###}$P<0.001 as compared to normal control; **P<0.01, ***P<0.001 as compared to diabetic control.

Table 6: Effect of Diabac on serum lipid levels in STZ-nicotinamide induced type II diabetes

Groups	Treatment	TG (mg/dl)	TC (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)
1	Normal control	99.29 ± 2.76	124.4± 6.58	44.49 ± 8.14	60.0 ± 3.65
2	Diabetic control	181.6 ± 3.53$^{###}$	294.0 ± 12.26$^{###}$	237.7 ± 10.53$^{###}$	20.0 ± 1.82$^{###}$
3	Glibenclamide (5 mg/kg)	106.7 ± 2.06***	137.0 ± 5.86***	62.32 ± 3.73***	53.33 ± 5.27***
4	Diabac (250 mg/kg)	162.6 ± 1.11***	255.7 ± 4.78*	197.4 ± 5.11**	25.83 ± 2.38
5	Diabac (500 mg/kg)	142.5 ± 1.61***	203.0 ± 6.04***	133.3 ± 5.55***	41.17 ± 1.53**
6	Diabac (1000 mg/kg)	119.4 ± 1.86***	138.0 ± 5.92***	55.75 ± 2.97***	58.33 ± 4.01***

Values are expressed as Mean + S.E.M (n=6). Where, ### P < 0.001 as compared to normal control; *P<0.05, **P<0.01, ***P<0.001 as compared to diabetic control.

Table 7: Effect of Diabac on serum creatinine, urea and uric acid in STZ-nicotinamide induced type II diabetes

Groups	Treatment	Serum creatinine (mg/dl)	Urea (mg/dl)	Uric acid (mg/dl)
1	Normal control	0.84 ± 0.03	30.63 ± 1.23	2.88 ± 0.27
2	Diabetic control	1.85 ± 0.05###	59.93 ± 1.49###	8.26 ± 0.16###
3	Glibenclamide (5 mg/kg)	0.87 ± 0.02***	36.64 ± 1.43***	3.30 ± 0.20***
4	Diabac (250 mg/kg)	1.65 ± 0.02*	51.32 ± 0.88***	7.86 ± 0.17
5	Diabac (500 mg/kg)	1.55 ± 0.02***	42.97 ± 1.50***	7.19 ± 0.29*
6	Diabac (1000 mg/kg)	0.89 ± 0.03***	35.86 ± 1.09***	4.12 ± 0.22***

Values are expressed as Mean + S.E.M (n=6). Where, $^{###}$P<0.001 as compared to normal control; *P<0.05, **P<0.01, ***P<0.001 as compared to diabetic control.

nificant changes in HDL-C level as compared to diabetic untreated rats (Table 6).

Effect of Diabac on serum creatinine, urea and uric acid

There was a significant (P<0.001) increase in the level of serum creatinine, urea and uric acid in diabetic control rats as compared to normal control rats. The administration of Diabac (500 and 1000 mg/kg) showed a significant decrease in the level of serum creatinine (P<0.001), urea (P<0.001) and uric acid (P<0.05; P<0.001) as compared to diabetic control rats. In addition, treatment with Diabac (250 mg/kg) showed a significant decrease in levels of serum creatinine (P<0.05), urea (P<0.001) and did not show any significant differences in uric acid level as compared to diabetic untreated rats (Table 7).

Histopathological study

In normal control rats, appearance of pancreas was shown normal. Pancreas of diabetic control rats showing reduced islet cells. However, the treatment with Diabac showed a recovery of islet cells to near normal appearance (Figure 1).

DISCUSSION

Administration of streptozotocin-nicotinamide caused diabetes, which may be because of destruction of beta cells of the islet of Langerhans of the pancreas.[23] Excessive production and decreased utilization of glucose by the tissue are the fundamental basis of hyperglycemia in diabetes mellitus.[24] When Diabac was administered to glucose loaded overnight fasted normal rats, hypoglycemia was observed after 60 min. The reduction in blood glucose levels was reached at its maximum at 2 h. In current study, administration of Diabac showed a significant reduction in blood glucose levels in streptozotocin-nicotinamide induced hyperglycemia. This effect might be due to diminish hepatic glycogenolysis, gluconeogenesis and increased utilization of glucose by the tissues.

In diabetic control rats, glycated hemoglobin levels were found to be increased as compared to normal control rats due to the persistent hyper-

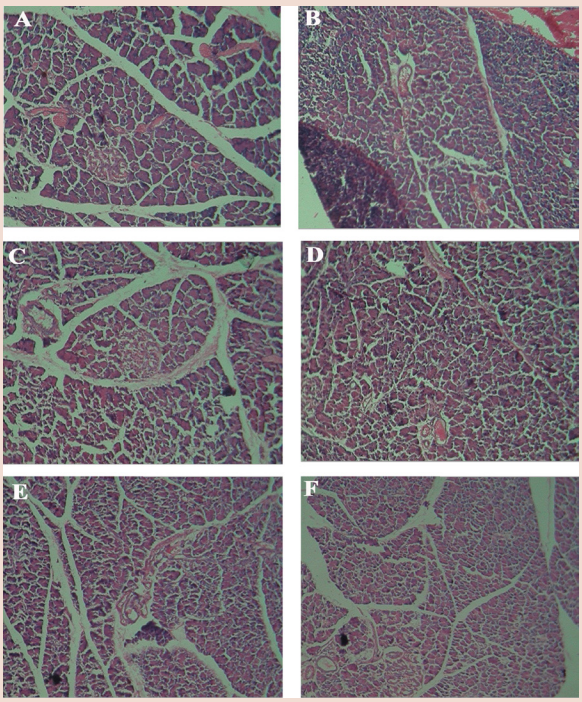

Figure 1: Photomicrograph of pancreas of rats
(A) Normal control group; (B) Diabetic control group; (C) Diabetic + glibenclamide; (D) Diabetic + Diabac (250 mg/kg); (E) Diabetic + Diabac (500 mg/kg); (F) Diabetic + Diabac (1000 mg/kg).

glycemia. It was previously reveled that elevation in non- enzymatic and autooxidation glycosylation in one of the probable mechanisms concerning the hyperglycemia and the vascular complications.[25] Treatment with Diabac showed a significant reduction in the glycated hemoglobin levels. The ability of Diabac to reduce glycated hemoglobin levels in diabetic rats showed it's potentiality to prevent the diabetic associated complication. In diabetes, insulin deficiency leads to decrease in protein synthesis in all tissue and thus the synthesis of hemoglobin is also reduced.[26] Administration of Diabac significantly increased hemoglobin levels in diabetic rats.

Streptozotocin-nicotinamide induced diabetes is characterized by severe reduction in body weight due to increased the muscle destruction or degradation of structural proteins.[27] When diabetic rats were treated with Diabac, it showed an improvement in body weight as compared to untreated diabetic rats, which may be due to its protective effect in controlling the muscle wasting i.e. reversal of gluconeogenesis and the improvement in glycemic control.

Coronary heart disease and cerebrovascular disease are more common in diabetes. The atherogenic situation is proceeding at a more rapid rate in diabetic than non-diabetic subjects.[28] The elevation in levels of triglycerides, total cholesterol, LDL-C and decreased HDL-C levels were reported in diabetic condition.[29] In current study, administration of Diabac significantly decreased elevated levels of triglycerides, total cholesterol, LDL-C and increased level of HDL- C in diabetic rats as compared to diabetic control rats. Lipid lowering effect of Diabac might be helpful in controlling diabetic linked complication.

Protein glycation in diabetes might be accountable for muscle wasting and increased release of purine, major source of uric acid, as well as increased the activity of xanthine oxidase.[30,31] Administration Diabac significantly reduced the serum creatinine, urea and uric acid levels in diabetic rats. These finding supports that Diabac improved kidney function in diabetic condition and for that reason, it helps to prevent diabetic related early renal damage.

In earlier study, it has been observed that hepatic glycogen is decreased during diabetes.[32] In the study, deficiency of insulin in the diabetic condition may result in the inactivation of glycogen synthase. The significant increase in hepatic glycogen content of Diabac treated diabetic rats may be because of the reactivation of glycogen synthase system. Histopathological studies also supported our results. Diabetic rats showed reduced islet cells, which were restored to normal upon treatment with Diabac. There were no alterations found in normal rats.

CONCLUSION

From this study, it was concluded that Diabac has a significant antidiabetic effect. The Diabac also showed improvement in lipid profile, body weight and renal function in diabetic condition. Therefore it might be helpful in preventing diabetic associated complication. Our present investigation supports the conventional use of Diabac in the management of diabetes.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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Original Article

Anti Hypertensive Activity of the Ethanolic Extract of *Lantana camara* leaves on high salt loaded wistar albino rats

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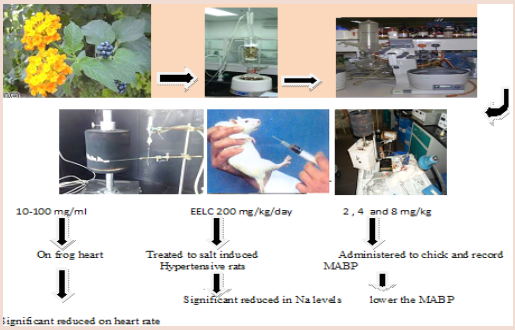
ABSTRACT

Background: Hypertension is the most common and major cardiovascular disease. There is an urgent need for treament of hypertension by exploration of several medicinal plants having potent anti hypertensive activity as the modern medicines are having many side effects. **Objective:** The study focus on scientific evaluation of antihypertensive activity of ethanolic extract of *Lantana camara leaves* (EELC) in different experimental models. **Material & methods:** Antihypertensive activity was conducted on wister albino rats by determining serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), creatinine and Sodium levels by using Semi Autoanalyser and Flame photometer; chick mean arterial blood pressure by using condon's mercury manometer and isolated frog heart for recording cardic responses using student kymograph. **Results:** EELC produced negative inotropic and negative chronotropic effect, antagonised by atropine on isolated frog heart. EELC shows dose dependent ($p<0.05$) decreased mean arterial blood pres¬sure (MABP) in anaesthetic chick. Salt treated rats displayed significant ($p<0.05$) increase in blood level of SGOT, SGPT, Creatinine and sodium, de¬crease in pottassim levels in comparision with normal rats. Treatment with EELC (200 and 400 mg/kg) significantly balanced the ionic levels such as lower the sodium and elevate the potassium levels. Creatinine levels were signifi-cantly ($p<0.05$) reduced by the treatment with EELC. There are no significant changes occurred in serum SGOT and SGPT upon EELC administration. The present study suggests that treatment of salt hypertensive rats with EELC protects against renal injuries. **Conclusion:** It was concluded that ethanolic extract of *Lantana camara* leaves reduces work load of heart, maintain inotonic levels by negative chronotropic effect, relaxes the smooth muscles in chick and salt hypertensive rats against renal and vascular injuries is proved.

Key words: EELC (Ethanolic extract of *Lantana camara* leaves), Wistar albino rats, Hypertension, Mean arterial blood pressure (MABP) and Normotensive rats (NTR).

SUMMARY

- *Lantana camara* is belongs to family verbenaceae, biologically *Lantana camara linn.*
- EELC exhibited biphasic response, initially negative ionotropic and negative chronotropic effect followed by sharp positive ionotropic effect, the early response, such as negative inotropic and chronotropic effect.
- EELC exhibited a dose dependent decrease in the mean arterial blood pressure (MABP) of anaesthetized chick.
- EELC 400 mg/kg significantly maintain ionic level (increased the potassium levels and decrease Sodium levels) on salt induced hypertensive rats.
- In the salt hypertensive rats, the kidney exhibited pathological abnormalities such as cortical degeneration, tubular degeneration, hydrophilic swelling and necrotic changes were observed. In the control, standard and plant extract treated rats, the kidney exhibited a normal architecture.



PICTORIAL ABSTRACT

Abbreviations used: EELC: Ethanolic extract of *Lantana camara* leaves, MABP: Mean arterial blood pressure,Na: Sodium, mg: milligrams, ml: milliliters, kg: kilogram.

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INTRODUCTION

Hypertension is most common cardiovascular disease and is a major public health issue. Recent studies have reported an increasing trend in the prevalence of hypertension in Indian subcontinent. This increase was found to be about 30% in urban population and 10% in rural habitants in last three decades.[1] *Lantana camara* is belongs to family verbenaceae, biologically *Lantana camara linn*. The *Lantana camara* was screened for anticancer activity, Oleanonic acid exhibited promising cytotoxicity against A375 cells.[2-3] Antimicrobial activity on *E. Coli*, *S. aureus*,[4-5] Antifungal activity,[6] Hypoglycemic activity,[7] Anti-inflammatory activity,[8] Antimotility activity,[9] Wound healing effect.[10-11] Even though they are several modern medicines for treatment of hypertensive but the same times having equal side effects. Hence there is an urgent need for treament of hypertensive activity by exploration of they are several medicinal plants having potent anti hypertensive activity.[12] The present study was done to evaluate the anti hypertensive activity of EELC using various animal models.

EXPERIMENTAL DESIGN

Materials and methods

Collection of plant and preparation of extract

Lantana camara was collected from shri Vishnu botanical garden, authenticated by the botanist. Mrs. P. Prasanna Kumari. Head of the Department of Botany, D.N.R (A) College, Bhimavaram, Andhra Pradesh, India. Powdered plant material of leaves of *Lantana camara* were first defatted using petroleum ether (60-80^{0}C). Preparation of etanolic extract by 30 g of powdered plant material was packed in soxhlet apparatus and subjected to continuous hot percolation for 8 hours. The percentage yield of the ethanolic extract was 10.80% w/w.

Experimental design for study the effect EELC on Isolated Frog Heart

Frog was sacrificed with a head-blow using a steel rod and pith it. The skin and abdomen were cut and opened. Cut the pectoral girdle using a

bone cutter and remove pericardium. Syme's cannula, connected to the reservoir of frog Ringers solution and immediately into the Sinus venous of the heart through posterior vena cava. Cut the connecting blood vessels and take out the heart from the animal and mounted on a stand. Covered the heart with a thin layer of cotton wool to prevent drying. Wet the heart frequently with the physiological solution to prevent drying, connect the heart to the Starling lever, which in turn is connected to the smoked drum for recording the responses of the heart. The level of frog Ringers solution in the Syme's cannula was maintained by a fixing a glass tube into the cork fixing to the reservoir (Marriotte's bottle) tightly. The heart was allowed to stabilize and when the heart rate and cardiac output were taken, the recordings were made on slow rotating drum kymograph paper. The extract was dissolved in Frog ringer's solution to obtain appropriate concentrations of 1, 2, 4, 8 and 10 mg/ml and studied heart rate (HR), Cardiac output (CO) and force of contraction presence of Atropine.[13]

Experimental design for the hypotensive activity of EELC on anaesthetized chick model

Chick of either sex weighing between (0.5-1.5 kg) were anaesthetize with Phenobarbital sodium (60-90 mg/kg) given intramuscularly. Rat and chick are the only species of experimental animals which require such a heavy dose of Phenobarbital sodium. When corneal reflexes are lost, shift the animal to an operation table. Secure the neck and the limbs properly to the nails using a twine. Then animal may be kept warm with heat from an electric bulb, expose the trachea with a vertical cut on the neck and retract the muscles around the trachea with vertical teasing of the muscles using the iris-dissecting forceps. Cannulate the trachea with a polythene cannula to put the chick on artificial ventilation if required.[13]

The tracheal secretions which normally cause respiratory embarrassment, particularly in older animals, cleared by sucking out using a 2 ml syringe with a hypodermic needle whose level is cut and a polythene cannula sleeved over it. Cannulate the right external ischiadic vein with a polythene cannula, for the administration of the agonists and plant extract. Carefully free the ischiadic artery (IA), located much deeper below to the side position (above the femoral bone), from the adhering tissues carefully free the associated nerve and a fine blood vessel running parrel to IA. Pass two threads bellows the IA and make two loose loops around it. Tie off the distal loop at a site as close to the head as possible. Keeping the retracted forceps below the blood vessels often help in tying the distal loop sufficiently distal to the proximal loop. Apply a bulldog clip very close to the proximal clip. Introduce the arterial cannula carefully in IA. Heparin of 500 IU after dilution was administrated into the arterial cannula. Tie the proximal loop over the arterial cannula, which is connected to a Condon's mercury float manometer in which a positive pressure (normally 120 mmHg) is maintained. Release the clamp on the link tube slowly. Record the Mean arterial blood pressure (MABP) on a slow moving smoked paper, administration of the agonist the various concentration of EELC (2,4 and 8 mg) 20 minutes after the cannulating of the IA. If there any blood loss during the dissection, warm the normal saline or dextrin solution can be administered slowly through the venous cannula.

Experimental design for the antihypertensive activity of EELC on salt induced hypertensive rats

Three months old male Wristar albino rats, weighting between 180 and 200 g were used. Animals were acclimate in the Animal House (Regd. No:439//PO/01/a/CPCSEA) at Shri Vishnu College of Pharmacy, Bhimavaram, and Andhra Pradesh, INDIA). Rats were fed with standard diet and water ad-libitum. Normotensive rats were randomly divided into five groups, and each group group consists six animals.[14-15]

- Group I, Normal control, received distilled water served as normotensive rats.
- Group-II, Disease control, received 18% NaCl (10 ml/kg/day), served as salt hypertensive rats (SHR).
- Group III, Received 18% NaCl (10 ml/kg/day) + Spironolactone (0.71 mg/kg).
- Group IV, Received 18% NaCl (10 ml/kg/day) + EELC 200 (mg/kg/day).
- Group V, Received 18% NaCl (10 ml/kg/day) + EELC 400 (mg/kg/day).

This experimental protocol follows up to 28 days. On 0, 7, 14, 21 and 28 days to estimate Creatinine, Serum glutamic oxaloacetic transaminase (SGOT) Serum glutamic pyruvic transaminase. (SGPT), Sodium and Potassium levels. At the end of this experimental period, all the animals were sacrificed and isolate the kidneys and trimmed of any fatty tissue and stored in 10% neutral phosphate buffered formalin solution for histological studies.[16]

Table 1: Preliminary Qualitative Phytochemical study of the *Lantana camara* plant extracts

Various extracts of *Lantana camara*				
Type of extract → / Test for ↓	Pet ether	Chloroform	Ethanol	Aqueous
Carbohydrate	-	+	+	+
Alkaloids	+	-	+	-
Glycoside	-	+	+	-
Polypeptides	+	-	+	-
Saponins	-	+	+	+
Tannins	+	+	+	-
Fats and Oils	-	-	-	-
Flavonoids	-	+	+	-
Terpenoids	+	+	-	-
Steroids	+	+	+	+

Note: pet. Ether – petroleum ether, + - Present, - - Absent.

Histological studies of kidney in different groups of rats

Histopathology examination of renal slices from different groups of rats were examined.

RESULT

Preliminary Qualitative Phytochemical studies of the plant extracts

Preliminary phytochemical investigation reports the presence of various phytoconstiuents and are summarized in Table 1. Major constituents are Glycosides, Saponins, Tannins, Flavonoids, Terpenoids and Steroids.

Effect of EELC on isolated frog heart

Table 2, Figure 1a,1b, 2a and 2b shown that the EELC extract produced dose dependent negative inotropic and negative chronotropic effect on isolated frogs heart. The observed responses antagonized by atropine.

Effect of EELC on mean arterial blood pressure using anaesthetized chick

Table 3, Figure 3a, 3b Shown that the extract produced significantly ($P< 0.05$) reduced mean arterial blood pressure on anaesthetized chick in a dose dependent manner.

Table 2: Effect of EELC on heart rate presence of Atropine (150 µg/ml) using isolated frogs heart

EELC (mg)	Heart rate (beats/min)		Force of contraction	
	EELC	EELC + ATP (150 µg)	EELC	EELC + ATP (150 µg)
Normal	85	100	7.5	7.5
1	69	80	7	7.6
2	68	76	6.5	7.9
4	63	73	6.1	8.3
8	60	68	5.1	8.9
10	56	69	1.3	8.5

Table 3: Effect of EELC on mean arterial blood pressure using anaesthetized chick

Z	Drug (Con.)	MABP (mm of Hg)
1	Normal	88
2	Adrenaline (50 µg)	132
3	Adrenaline (100 µg)	159
4	EELC (1 mg)	74
5	EELC (2 mg)	66
6	EELC (3 mg)	59

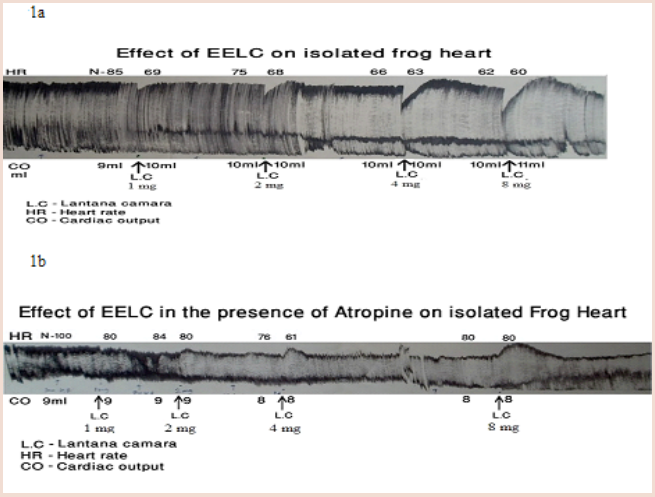

Figure 1: Effect of EELC on isolated frog heart

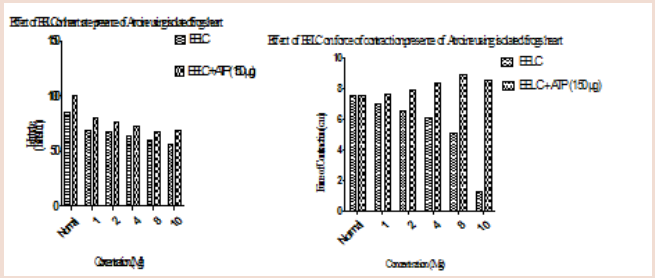

Figure 2: Effects of EELC on force of contraction in the presence of Atropine on isolated frog heart.

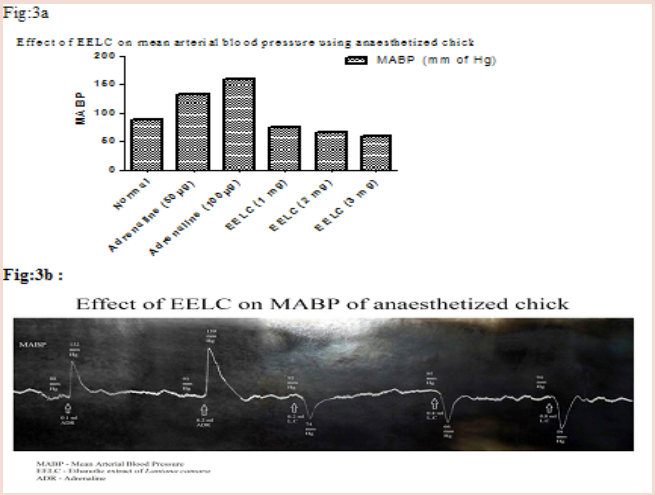

Figure 3: Effect of EELC on mean arterial blood presure on anaesthesised chick

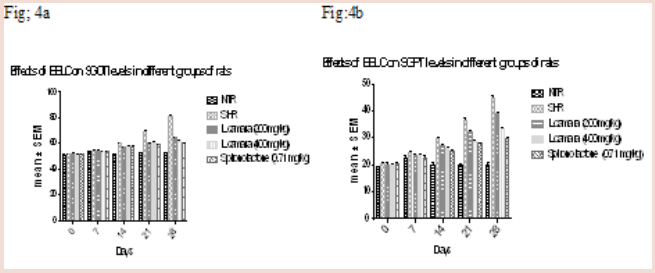

Figure 4: Effect of EELC on SGOT and SGPT in wistar albino rats

Effects of EELC on SGOT and SGPT on salt induced hypertensive rats

Table 4, Figure 4a, 4b Salt loaded rats showed a significant **(P<0.05) increase in blood levels of SGOT and SGPT, as compared to NTR. *EELC* (200 and 400 mg/kg/day) less significantly to reduced the rise of concentrations of SGOT and SGPT.

Effects of EELC on Creatinine levels on salt induced hypertensive rats

Table 5, Figure 5a shows Salt loaded rats displayed a significant **(P < 0.05) increase in blood levels of Creatinine, as compared to NTR. EELC (200 and 400 mg/kg/day) less significant reduced the rise of concentrations of creatinine. Similar results were observed in animals treated with

Table 4: Effects of EELC on SGOT and SGPT on salt induced hypertensive rats

Days	Group-I	Group-II	Group-III	Group-IV	Group-V
Serum glutamic oxaloacetic transaminase (SGOT) U/L					
0	51.00±0.44	51.20±0.58	52.00±0.70	51.20±0.58	51.60±0.50
7	53.80±0.37	53.20±0.37	53.80±0.66	54.40±0.50	53.80±0.37
14	51.60±0.50	60.40±0.50	56.60±0.50	58.20±0.37	57.60±0.50
21	52.20±0.58	68.60±1.02	60.40±0.50	60.60±0.74	58.80±0.37
28	52.20±0.66	81.40±1.02**	64.40±0.50	61.80±0.58	60.00±0.31**
Serum glutamic pyruvic transaminase (SGPT) U/L					
0	19.00±0.44	20.40±0.50	20.00±0.70	20.00±0.54	20.40±0.50
7	22.40±0.92	24.60±0.50	23.40±0.50	23.60±0.50	22.60±0.60
14	20.00±0.70	29.60±0.50	27.20±0.37	26.00±0.70	25.00±0.31
21	19.60±0.50	36.40±0.92	32.20±0.37	28.40±0.50	27.80±0.37
28	20.00±0.70	44.40±1.12**	39.20±0.37	33.00±0.54	29.80±0.58**

Values are expressed in MEAN ± S.E.M of five animals. One way analysis of variance followed by Dunnetts multiple comparisions test ; **(P<0.05) significance between salt treated rats vs EELC (400 mg/kg).

Table 5: Effects of EELC on Creatinine levels on salt induced hypertensive rats

Days	Group-I	Group-II	Group-III	Group-IV	Group-V
Creatinine mg/dl					
0	0.26±0.04	0.30±0.04	0.32±0.03	0.32±0.03	0.28±0.03
7	0.28±0.03	0.72±0.03	0.54±0.050	0.52±0.03	0.48±0.03
14	0.32±0.03	1.14±0.05	0.82±0.03	0.86±0.04	0.58±0.03
21	0.34±0.04	1.54±0.05	1.08±0.03	0.96±0.02	0.70±0.04
28	0.28±0.03	2.20±0.07**	1.44±0.02	1.18±0.03	0.98±0.03**

Values are expressed in MEAN ± S.E.M of five animals. one way analysis of variance followed by Dunnetts multiple comparisions test ; **(p<0.05) Significantly between salt treated rats and EELC (400 mg/kg).

Fig:5a

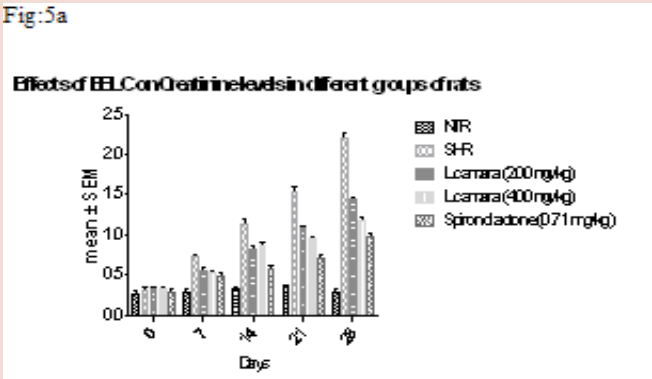

Figure 5: Effect of EELC on Creatinine in wistar albino rats

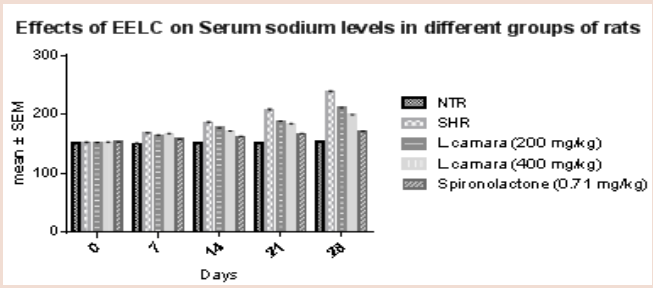

Figure 6: Effect of EELC on Serum Sodium levels in wistar albino rats

and Spironolactone (0.71 mg/kg/day).

Effects of EELC on Serum Sodium levels in different groups of rats

Salt loaded rats displayed a significant **(P<0.05) increase in serum levels of Sodium, as compared to NTR. EELC (200 and 400 mg/kg/day) significantly reduced the rise of concentrations of serum Sodium levels. As Similar to Spironolactone (0.71 mg/kg/day). The results were summarized in Table 6 and depicted in Figure 6.

Effects of EELC on Serum Potassium levels in diiferent groups of rats

Salt loaded rats displayed a significant **(P < 0.05) decrease in serum levels of Potassium, as compared to NTR. EELC (400 mg/kg/day) significantly increased the concentrations of serum Potassium. The results were summarized in Table 7 and depicted in Figure 7.

Table 6: Effects of EELC on Serum Sodium levels in different groups of rats

Days	Group-I	Group-II	Group-III	Group-IV	Group-V
Sodium mEq/L					
0	151.46±0.96	152.12±0.94	151.80±1.01	152.48±1.19	152.80±0.89
7	150.10±1.1	168.06±1.10	164.08±1.39	166.72±1.42	157.72±0.67
14	151.78±1.10	186.12±1.59	176.84±0.83	171.40±0.81	162.22±0.83
21	151.10±0.65	207.28±2.34	188.28±0.74	183.28±1.54	167.08±0.92
28	153.42±1.59	238.88±1.41**	211.20±1.02	199.12±0.98	171.66±0.53**

Values are expressed in MEAN ± S.E.M of five animals. One way analysis of variance followed by Dunnett's multiple comparisions test ; **($p<0.05$) significance between salt treated rats vs EELC (400 mg/kg).

Table 7: Effects of EELC on Serum Potassium levels in diiferent groups of rats

Days	Group-I	Group-II	Group-III	Group-IV	Group-V
Potassium mEq/L					
0	6.18±0.22	5.92±0.08	5.96±0.16	5.88±0.08	6.10±0.08
7	6.22±0.10	5.98±0.06	5.62±0.12	5.94±0.05	5.74±0.08
14	6.20±0.07	5.86±0.08	5.54±0.16	5.96±0.17	5.62±0.13
21	6.14±0.14	5.10±0.08	5.58±0.18	6.28±0.16	6.24±0.15
28	6.28±0.29	4.54±0.09**	5.52±0.25	6.56±0.18	6.20±0.07**

Values are expressed in MEAN ± S.E.M of five animals. one way analysis of variance followed by Dunnetts' multiple comparisions test ; **($p<0.05$) significance between salt treated rats vs EELC (400 mg/kg).

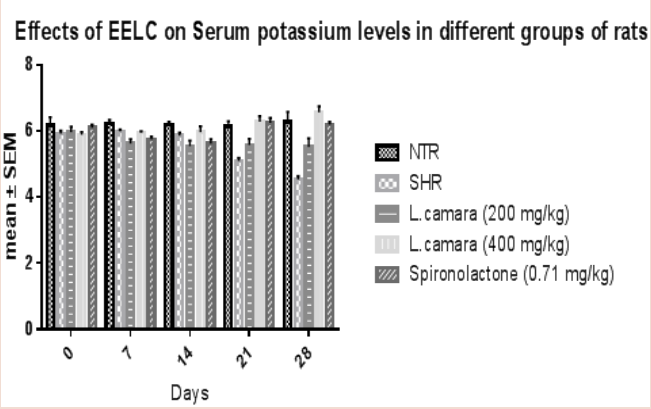

Figure 7: Effect of EELC on Serum Potassium levels in wistar albino rats

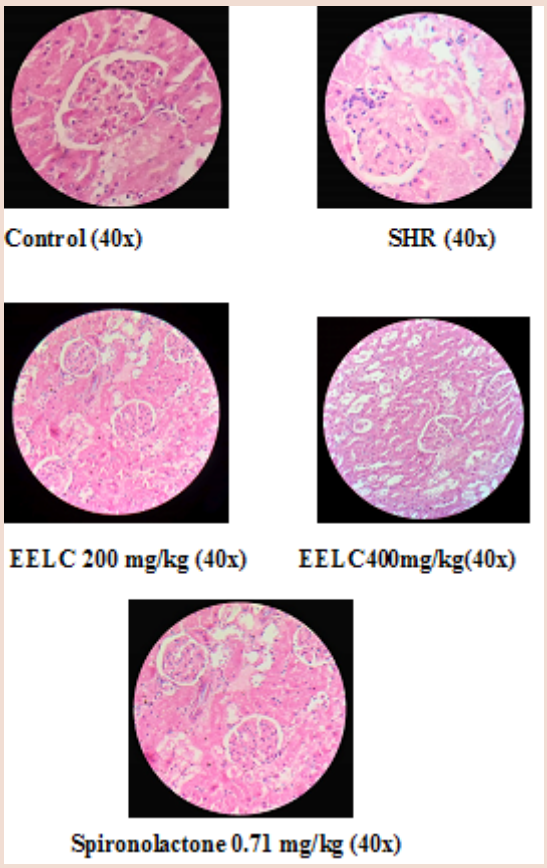

Figure 8: Histopathological changes of kidneys in various groups of salt hypertensive rats

Histological studies of different groups of rats kidney

Histopathology examination of renal slices from different groups of rats were examined. Less cortical tubular degeneration observed with treatment of EELC (400 mg/kg). In the salt hypertensive rats, the kidney exhibited pathological abnormalities such as cortical degeneration, tubular degeneration, hydrophilic swelling and necrotic changes were observed. (Figure 8).

DISCUSSION

EELC exhibited biphasic response, initially negative ionotropic and negative chronotropic effect followed by sharp positive ionotropic effect, the early response, such as negative inotropic and chronotropic effect are antagonized by atropine. Hence these response are mediating through muscaranic receptors. Few reported are suggested the cholinomimetic drugs are reduces workload of heart, hence these agents are benefit in the treat-

ment of cardio vascular complication. such as hypertension.[17] This result might represent evidance for the antihypertensive activity of EELC (Table 2, Figure 1a, 1b, 2a & 2b) EELC exhibited a dose dependent decrease in the mean arterial blood pressure (MABP) of anaesthetized chick (Table 3, Figure 3a & 3b). It is interesting to note that have similarly reported that the effectiveness of extracts of Tribulus terrestris in inhibiting ACE activity.[18] However other mechanisms including a vasodilator effect mediating via a direct effect on the arterial smooth muscle or interfering with other neuroeffective mechanism such as adrenergic system could not be ruled out.[19] Some scientific studies are reported that the saponins contribute to vasorelaxant effect.[20] In this study, hypertension was induced by administering 18% NaCl to normal rats. It is worth noticing that they also urinated consequently. This diuretic behaviour accounts for an obvious homoeostatic need of their organism frequently associated with increase in oxidative stress and hardening of blood vessels.[13,21] High salt intake induced increase of plasma SGOT.[22] Concomitant administration of NaCl 18% with extract (200 and 400 mg/ kg/day) permitted to reduce less significantly the increase of SGOT and SGPT in hypertensive rats. This result suggests that plant extract might partially protect various tissues, including hepatic tissues, from high salt-induced injuries.[21-23] As *Lantana camara* extract prevented that hypercreatinemia, it might prevent any eventual disruption of glomerular filtration. Electrolyte imbalance and alteration in renin-angiotensin system plays a key role in the pathogenesis of hypertension.[24] Numerous epidemiological and intervention studies have demonstrated a positive correlation between sodium intake and elevated blood pressure.[21] Dietary intake of K, Ca and Mg has been reported to lower blood pressure. Kidney plays a central role in the regulation of the balance of body salt and water, and disordered regulation of renal functions is responsible for the altered balance of hypertension.[25] Dietary supplementation of KCl significantly increased GFR in hypertensive rats. The increase in GFR in hypertensive rats treated with potassium may relate to the increase in NO production.[24] *EELC* 400 mg/kg significantly increased the potassium levels on salt induced hypertensive rats. This result suggest that the plant extract might have the effect on potassium levels. In the salt hypertensive rats, the kidney exhibited pathological abnormalities such as cortical degeneration, tubular degeneration, hydrophilic swelling and necrotic changes were observed. In the control, standard and plant extract treated rats, the kidney exhibited a normal architecture. These results suggests that the plant extract might represent support for the antihypertensive activity. EELC having high amount of alkaloids, glycosides, polypeptides, saponins and terpinoids. so this plant having the anti hypertensive property.[26]

CONCLUSION

The Ethanolic extract of *Lantana camara* leaves shown hypotensive properties in the anaesthetized chick, which might be involved in smooth muscle relaxation. The present study suggests that treatment of salt hypertensive rats with ethanolic extract of *Lantana camara* leaves protects against renal and vascular injuries, possibly through the antioxidant activity and maintaining the normal ionic composition. It was concluded that ethanolic extract of *Lantana camara* leaves reduce work load of heart, and maintain ionic levels by negative chronotropic effect.

These results suggests that the plant extract support for the antihypertensive activity. However, long-term, randomized controlled trials are needed to evaluate the antihypertensive activity.

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CONFLICTS OF INTEREST

We declare that we have no conflict of interest.

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Original Article

Phytopharmacological evaluation of aerial parts of *Woodfordia fruticosa* (L.) Kurz in Cough Variant Asthma

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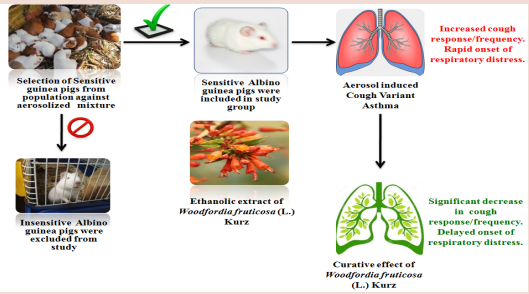
ABSTRACT

Background: Cough variant asthma (CVA) is characterized by prolonged non productive cough which responds to bronchodilator therapy. None of herbal drug is reported to possess pharmacological activity against CVA. **Objective:** To investigate the pharmacological potential of ethanolic extract of *Woodfordia fruticosa* (L.) Kurz (EEWF) against CVA as well as to develop an efficient screening model for CVA. **Material and Method:** Anti-tussive effect of EEWF was evaluated against nebulized aqueous solution of 0.1 g/ml of citric acid to determine the cough response. EEWF potential was finally accessed against aerosolic mixture of 0.3 g/ml of citric acid mixed with 0.1% histamine and 2% acetylcholine chloride to evaluate the convulsive latency, percentage protection and cough frequency against CVA. **Results:** EEWF at aerosolic dose of 6% w/v exhibit decrease in of the average coughs frequency (4.83 ± 0.30) which is quite significant effect as compared to standard drug codeine. EEWF against aerosol induced CVA was found to exhibit a significant bronchoprotection of 41.75% and decreases number of coughs (7.16 ± 0.47) at 200 mg/kg as compared to control (14.16 ± 0.60). **Conclusion:** EEWF at 200 mg/kg dose exhibited bronchoprotective and anti-tussive effects against aerosol induced CVA.

Key words: Acetylcholine, Anti-tussive, Bronchoprotection, Citric acid, Cough, Cough variant asthma (CVA), Ethanolic extract of *Woodfordia fruticosa* (L.) Kurz (EEWF), Histamine.

SUMMARY

- The present study investigated a potential curative effect of ethanolic extract of *Woodfordia fruticosa* (L.) Kurz in Aerosol induced Cough Variant Asthma.
- As none of any model exists to assess the activity of the drug for CVA, so our study also aimed to develop an effective pharmacological screening model for CVA.
- The extract not only protects the animals from bron¬choconstriction and bronchospasm but also suppress the cough frequency, which almost covers the basic etiological relevance for CVA, which pharmacologically corroborates its effective treatment against CVA.



PICTORIAL ABSTRACT

Abbreviations used: CVA: Cough Variant Asthma, EEWF: Ethanolic Extract of *Woodfordia Fruticosa (L.) Kurz*, OECD: Organization for Economic Cooperation and Development.

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INTRODUCTION

Cough variant asthma (CVA), one of the most common causes of chronic cough,[1-4] is considered a precursor and a variant form of classic asthma with typical symptoms of wheezing and dyspnea.[5-9] The etiological basis for effective management of asthma needs bronchiodialator action as reliever medication, anti-inflammatory as preventive medication and long acting β2 agonist as symptom controller.[10-11] Cough variant asthma (CVA) is one of the commonest forms of asthma characterized by an unproductive dry cough as the main or only symptoms and CVA patients has a more sensitive cough reflex. Complications like excessive mucous production and cough reflex may synergize the complication as bronchiolar chocking and asthmatic attack. [12]

Woodfordia fruticosa (L.) Kurz is a straggling leafy shrub of family Lythraceae, locally known as Dhatki or Dhai, having the bright red color flowers is distributed abundantly throughout the India, as well as in the majority of the South and East Asian countries.[13-14] Phytochemical studies of *Woodfordia fruticosa* can be ascribed for its important bioactive phytoconstituents such as flavonoids, sterols, anthraquinones, saponins and tannins.[15-17] Earlier studies indicates, that *Woodfordia fruticosa* possess important pharmacological activities including antipyretic, anti-inflammatory,[18] immunomodulatory,[19] etc. None of herbal origin drug posses combine effect as anti-asthmatic, and anti-tussive activity. So as ideal management aerial parts of *Woofordia fructucosa* could be explored against asthma specific cough reflex i.e. cough variant asthma.

MATERIAL AND METHODS

Plant material

The aerial parts of *Woodfordia fruticosa* (L.) Kurz for the present study were collected in the month of January locally from, Bhopal, Madhya Pradesh, India. The plant was identified and authenticated by Dr. Zia Ul Hasan, Head of Department, Department of Botany, Safia Science College, Bhopal, (M.P.) India, and a specimen voucher (334/Bot/Saifia/12), deposited in the Herbarium of the Department of Pharmacognosy, Truba Institute of Pharmacy, Bhopal, (M.P.), India, for future reference.

Extraction

The aerial parts of *Woodfordia fruticosa* were shade dried for 2 weeks, then pulverized to a coarse powder, passed through sieve No. 20 to maintain uniformity. Coarsely dried powder was first defatted with petroleum ether (60-80°C) for 72 hours to remove fatty materials and then extracted with ethanol (95%) using soxhlet apparatus for 36 hr., obtained orange, brown extract was collected and concentrated in vacuum under reduced pressure using a rotary flash evaporator and the dried crude extract was stored in airtight container at 4°C for further study. The yield of the extract was 13.72%.

Phytochemical screening

Ethanolic extract of *Woodfordia fruticosa* (EEWF) was subjected to various phytochemical screening tests for the identification of the phytoconstituents presents in the aerial parts of *Woodfordia fruticosa* using standard procedures.[20]

Animals

The experiment was carried out on Healthy albino guinea pigs (400-600 g). Animals were provided from the authorized animal house of Truba Institute of Pharmacy, Bhopal, Madhya Pradesh, India. The animals were acclimatized to the standard laboratory conditions in cross ventilated animal house at temperature 25 ± 2°C relative humidity 44-56% and light and dark cycles of 12:12 hours, fed with standard pellet diet and water *ad libitum* during experiment. The experiment was approved by the institutional animal ethics committee (IAEC) as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (Approval No. 1196/a/08/CPCSEA).

Acute oral toxicity study

The acute oral toxicity study was evaluated as per Organization for Economic Cooperation and Development (OECD) guidelines no. 425, on guinea pigs of either sex, weighing between 400-600 g. Before the experiment, animals were fasted overnight with water *ad libitum*. Three animals were selected which receives a dose of 2000 mg/kg. All three animals were received a single dose of 2000 mg/kg body weight of EEWF by oral gavage. Animals were observed individually for any sign of toxicity, behavioral changes, and mortality after dosing, with special attention given during the first 4 hours, and thereafter for 24 hours, for a total period of 7 days.

In-vivo citric acid induced anti-tussive evaluation[21]

Guinea pig (400-600 g) of either sex was selected and divided into four groups, i.e. control, standard (codeine, 0.03GM/ml) and test groups (EEWF, 3% w/v and 6% w/v, body weight, respectively) consisting of six animals in each group. Each unanaesthetized and unrestrained animal was individually placed in a transparent chamber of (dimensions, 30 cm x20 cm x20 cm) and exposed to nebulized aqueous solution of 0.1 g/ml of citric acid (nebulizing rate 0.7 ± 0.04 ml/min) for continuous 7 minutes. During the last 5 minutes of exposure each animal was observed continuously and closely to determine the number of cough responses. The above protocol was performed for every animal from each group for 10 minutes after exposing animals to aerosol solutions of normal saline (for baseline measurement), codeine solution (0.03 gm/ml, standard), EEWF (3% w/v and 6% w/v).

In-vivo aerosol induced cough variant asthma (CVA)

The effect of ethanolic extract of *Woodfordia fruticosa* against cough variant asthma was evaluated by a modified method. Guinea pigs (400-600 g) of either sex were screened out by challenging the animals by putting them one by one in an aerosolized chamber nebulized with mixture of 0.3 g/ml of citric acid mixed with an equal volume solution of 0.1% histamine and 2% acetylcholine chloride (nebulizing rate 0.7±0.04 ml/min) to determine the pre-convulsive time[22] as well as appropriate cough response and was observed for maximum 4 mins. Within that period of time the animal was regarded as insensitive or not suitable if they do not show any respiratory distress (convulsions) and tussive response. The sensitive guinea pigs after screening were selected and grouped into three groups, i.e. control and test groups (EEWF, 100 mg/kg and 200 mg/kg, body weight, p.o.) consisting of six animals in each group. Animals of test groups were treated with a single oral dose of EEWF extract (EEWF, 100 mg/kg and 200 mg/kg, body weight, p.o. respectively) daily for 15 days prior to bronchial challenge. On last day extract was administered 1 h before the bronchial challenge and after 30 minutes, animals were individually placed in a specified polystyrene transparent chamber (dimension, 30 cm x 20 cm x 20 cm) nebulized with an aerosolic mixture of 0.3 g/ml of citric acid mixed with an equal volume solution of 0.1% histamine and 2% acetylcholine chloride to evaluate the convulsive latency, percentage protection and cough frequency.

RESULTS

Preliminary phytochemical investigation of EEWF revealed the presence of alkaloids, glycosides, flavonoids, tannins, triterpenoids, polyphenols, carbohydrates and proteins. Acute toxicity studies of EEWF were performed in accordance with OECD 425 and extract found to exhibit a great margin of safety up to dose of 2000 mg/kg and there was no change in the behavioral pattern and not any sign of toxicity and mortality observed during the overall toxicity studies. Accordingly 1/10 of this dose was considered to be the experimentally safe dose. EEWF at 3% and 6% w/v aerosolic dose against citric acid induced tussive reaction was found to exhibit a significant ($P<0.001$) reduction in cough response as compared to control (Table 1). After accessing the anti-tussive activity separately, the EEWF potency was evaluated against aerosol (an aerosolic mixture of 0.3 g/ml of citric acid mixed with an equal volume solution of 0.1% histamine and 2% acetylcholine chloride) induced cough variant asthma (CVA) and was evaluated for convulsive latency, percentage protection and cough frequency. As no any drug was reported as standard treatment for CVA for pharmacological screening models, so here we are comparing the efficacy of the EEWF with the control group. EEWF at 100 and 200 mg/kg was found to exhibit a significant bronchoprotection of 36.77% and 41.75% respectively as compared to control and significant ($P<0.01$ and $P<0.001$) decreases in cough response at the dose level of 100 and 200 mg/kg respectively as compared to control (Table 2).

DISCUSSION

The present investigation was attempted to evaluate the bronchoprotective and anti-tussive effect of EEWF against aerosolized cough variant

Table 1: Anti-tussive effect of EEWF against citric acid induced tussive reaction

Treatment groups	Dose	No. of cough
Control	-	12.5 ± 0.56
Standard	0.03 gm/ml	3.33 ± 0.21***a
EEWF	3% w/v	7.16 ± 0.47***a, ***b
EEWF	6% w/v	4.83 ± 0.30***a

All values are represented as mean ± SEM, n = 6 animals in each group, Data were analyzed by one-way ANOVA, followed by Tukey-Kramer Multiple Comparisons Test, a- Significant difference as compared to control group, b- Significant difference as compared to standard group, and *P<0.05, **P<0.01, ***P<0.001.

Table 2: Effect of EEWF against aerosolized induced CVA

Treatment groups	Dose (mg/kg)	Latency (in seconds)		% protection	No. of coughs
		Before treatment	After treatment		
Control	-	88.83 ± 0.83	89.66 ± 0.95	-	14.16 ± 0.60
EEWF	100	70.5 ± 1.05	111.5 ± 1.47***	36.77	9.66 ± 0.33***
EEWF	200	86 ± 1.29***	147.66 ± 0.66***	41.75	7.16 ± 0.47**

All values are represented as mean ± SEM, n=6 animals in each group, Data were analyzed by one-way ANOVA, followed by Tukey-Kramer Multiple Comparisons Test. Results are considered significant as compared to control group and *P<0.05, **P<0.01, ***P<0.001.

asthma (CVA). Therefore evaluation was first undertaken for anti-tussive effect of EEWF against citric acid induced tussal response. Finally the ability of the extract against aerosolized (aerosolic mixture of 0.3 g/ml of citric acid mixed with an equal volume solution of 0.1% histamine and 2% acetylcholine chloride) induced CVA has been evaluated.

Histamine is an important mediator of bronchial muscle contraction and the obstruction of these may occur via H1 receptors. In addition, acetylcholine released from efferent nerve endings of the inner bronchus results in the excessive formation of inositol 1,4,5-triphosphate (IP3) in bronchial muscles that lead to the intracellular release of calcium and initiate bronchoconstriction. It has been reported that bronchial acetylcholine and H1 receptor blockade results in bronchodilation, which is considered as vital in the treatment of asthma.[23] A prominent effect caused by both leads to varied degree of bronchoconstriction that causes asphyxia and death. Bronchodilators can delay the occurrence of these symptoms.[24]

In animals, coughing has been elicited by number of methods.[25-28] In the present study, the anti-tussive activity of EEWF has been compared with that of standard drug codeine against coughing induced by chemical stimulation (citric acid). The extract showed significant inhibition of cough as compare to standard drug codeine in dose dependent manner. Thus the extract might be acting via the central nervous system cough suppressant action, but the exact mechanism of action cannot be withdrawn from the preliminary study. In the conclusive study against aerosolized induced CVA, we exposed the animals against acetylcholine, histamine and citric acid, and the oral dose of EEWF was found to possess significant bronchoprotective and anti-tussive activity that might suggested that the extract possess histaminic and cholinergic receptor antagonistic property along with the central cough suppressant, this observed activity may be correlated with the presence of saponins and polyphenols in EEWF.[29]

CONCLUSION

In conclusion, results suggested the potential role of EEWF for the treatment against CVA. The extract not only protects the animals from bronchoconstriction and bronchospasm but also suppress the cough frequency, which almost covers the basic etiological relevance for CVA, which pharmacologically corroborates its effective treatment against CVA.

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Original Article

GC-MS Analysis of Bioactive Phytochemicals Present in Ethanolic Extracts of Leaves of *Annona muricata:* A Further Evidence for Its Medicinal Diversity

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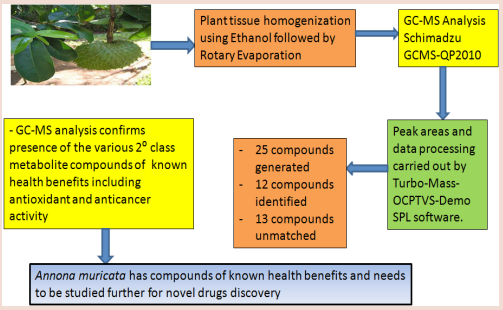
ABSTRACT

Background: Folk medicine has taken an important place especially in developing countries where limited health services are available. However, the absence of scientific evaluation of medicinal plants may cause serious adverse effects. **Objective:** To analyze the phytochemical composition of the ethanolic extracts of leaves of *Annona muricata* using gas chromatography mass spectroscopy (GC-MS). **Materials and methods:** GC-MS Analysis was used. **Results:** The GC-MS Analysis revealed 25 constituents of which 12 of the compounds were identified. The major constituents were two unidentified compounds with percentage peak areas of 23.51% and 16.8%. Of the identified compounds, the outstanding in composition were 7-Tetradecenal, (Z) (peak area 9.39%), n-Hexadecanoic acid (peak area 7.12%), Oleyl Alcohol (peak area 6.15%), Phytol (peak area 5.61%), cis, cis, cis-7,10,13-Hexadecatrienal (peak area 4.26%), 2-Pentadecanol (peak area 3.93%), 9,12-Octadecadienoic acid, ethyl ester (peak area 3.21%), 1,2-Benzenedicarboxylic acid, butyl octyl ester (peak area 2.67%), and 1,E-11,Z-13-Octadecatriene (peak area 2.15%), while the rest had less than 2% composition by peak area. **Conclusion:** The current study suggests that ethanolic extracts of leaves of *Annona muricata* are a potent therapeutic agent and paves the way for the development of several treatment regimens based on compounds from this extract.

Key words: *Annona muricata*, Ethanolic extracts, GC-MS, Medicinal diversity, Phytochemicals.

SUMMARY

- *Annona muricata* is widely used to treat various diseases.
- **Objective:** To conduct a GC-MS Analysis on ethanolic leaves extracts 25 compounds were generated, 12 compounds were identified, 13 were not.
- The major constituents were two unidentified compounds with percentage peak areas of 23.51% and 16.8%.
- Identified compounds included: 7-Tetradecenal, (Z) (peak area 9.39%), n-Hexadecanoic acid (peak area 7.12%), Oleryl Alcohol (peak area 6.15%), Phytol (peak area 5.61%) cis, cis, cis-7,10,13-Hexadecatrienal (peak area 4.26%), 2-Pentadecanol (peak area 3.93%), 9,12-Octadecadienoic acid, ethyl ester (peak area 3.21%), 1,2-Benzenedicarboxylic acid, butyl octyl ester (peak area 2.67%), 1,E-11,Z-13-Octadecatriene (peak area 2.15%). The rest had less than 2% composition by peak area.
- All identified compounds of known health benefits. Further studies needed on the plant's therapeutic value.



PICTORIAL ABSTRACT

Abbreviations used: GC-MS: Gas Chromatography Mass Spectroscopy, TIC: Total Ion Chromatogram.

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INTRODUCTION

Plant use in treatment of diseases is as old as civilization,[1,2] and complementary medicine is still a major part of habitual treatments of different maladies.[2,3] Generally, complementary medicine has a long history of serving people all over the world.[4,5] In recent times and due to historical, cultural, and other reasons, folk medicine has taken an important place especially in developing countries where limited health services are available. However, the absence of scientific evaluation of medicinal plants may cause serious adverse effects.[3,6,7]

Natural products are extremely an important source of medicinal agents. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer based molecular modeling design, none of them can replace the importance of natural products in drug discovery and development.[8] Many non-natural, synthetic drugs cause severe side effects that were not acceptable except as treatments of last resort for terminal diseases such as cancer and that the metabolites discovered in medicinal plants may avoid the side effect of synthetic drugs, because they must accumulate within living cells.[5]

Annona muricata L commonly known as Graviola or Soursop belongs to the family of Annonaceae and is the most tropical semi deciduous tree with the largest fruits of the *Annona* genus. It is a typical tropical tree with heart shaped edible fruits and widely distributed and native to Sub-Saharan Africa countries that lie within the tropics including Uganda.[7] The leaves are lanceolate with glossy and dark green in color and had been traditionally used to treat headaches, hypertension, cough, asthma and used as antispasmodic, sedative and nervine for heart condition,[9,10] as well as cancer. It is widely used for complementary treatment in many

countries such as Amazonia, Barbados, Borneo, Brazil, Cook Islands, Curacao, Dominica, Guatemala, Guam, Guyana, Haiti, Jamaica, Madagascar, Malaysia, Peru, Suriname, Togo and West Indies,[11,12,13] as well as in Uganda.[7] It is hoped that traditional medicine will in future provide the cure to many tropical diseases that have defied orthodox prescriptions. The objective of this study was therefore to undertakephytochemical composition analysis using GC-MS on the ethanolic extracts of leaves of *Annona muricata*.

MATERIALS AND METHODS

Sample collection and authentication

Fresh leaves of *Annona muricata L.* were collected from the wild in Eastern Uganda in the district of Kaliro during the month of August 2013. The plant was identified and authenticated in the Makerere University Botanical Herbarium (MHU) by Ms. Olivia Wanyana Mangeni. A voucher specimen was deposited in the herbarium under the collection number GY 021- 10/13- MB 300-0007/12-001.

Samples preparation and Extraction

The leaves of *Annona muricata*were washed with water and cut into small pieces, drying was done at room temperature, and the dried leaves were powdered. 150 g of powdered leaves were extracted using 500 ml ethanol for three days by the plant tissue homogenization method as previously described.[14] The extract was then concentrated using a rotary evaporator and kept at 4°C until used.

Chemicals and reagents

All chemicals and reagents were procured from certified suppliers and were of the highest analytical standard.

Gas Chromatography Mass Spectroscopy

Gas chromatography mass spectroscopy (GC-MS), a hyphenated system which is a very compatible technique and the most commonly used technique for the identification and quantification purpose was used. The unknown organic compounds in the complex mixture can be determined by interpretation and also by matching the spectra with reference spectra.

Preparation of extract

The ethanolic extract of the leaves was analyzed using Gas Chromatography Mass Spectroscopy for the identification of the phytochemical compounds present. A solvent blank analysis was first conducted using 1 µl of absolute ethanol. Then 1 µl of the reconstituted ethanolic extract solution was employed for GC-MS analysis as previously described with modifications.[15,16]

Analysis

GC-MS analysis was carried out on a GC system comprising a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) instrument; Schimadzu GCMS-QP2010, employing the following conditions: Column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) as carrier gas at a constant flow of 1ml/minute and a sample injection volume of 1 µl which was employed (split ratio of 10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 minutes), with an increase of 10°C/minute, to 200°C, then 5°C/minute to 280°C, ending with a 9 minutes isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da. Total run time was 30 min. The compounds were then identified from the GC-MS peaks, using library data of the corresponding compounds. GC-MS was analyzed using electron impact ionization at 70 eV and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library using NISP Search. The relative % amount of each component was calculated by comparing its average peak area to the total areas. Measurement of peak areas and data processing were carried out by Turbo-Mass-OCPTVS-Demo SPL software.

RESULTS

Our previous study,[7] reported that ethanolic leaves extracts of *Annona muricata* L showed anticancer and antioxidant activities. The results obtained from this study represented an important step towards the effective characterization of the secondary class metabolite compounds from this plant using GC-MS analysis. Preliminary qualitative phytochemical analysis of extracts revealed it to be rich in secondary class metabolite compounds of alkaloids, saponins, terpenoids, flavonoids, coumarins and lactones, anthraquinones, tannins, Cardiac glycosides, phenols and phytosterols as shown in the Figure 1.

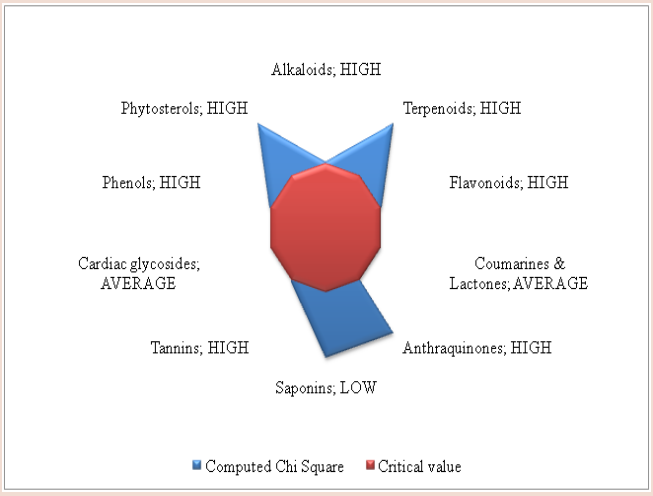

Figure 1: Phytochemicals present in Ethanolic leaves extracts of *Annona muricata* with relative abundance computed from the χ^2 Test.[7]

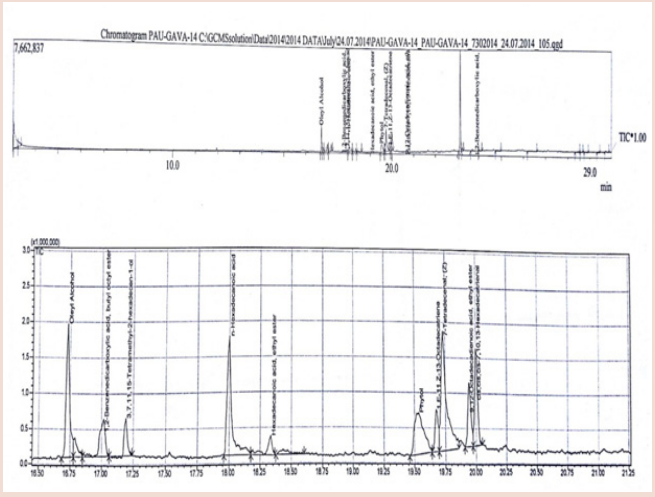

Figure 2: Total Ion Chromatogram (TIC) of ethanolic extract of leaves of *Annona muricata*

Table 1: Phyto-components generated in the ethanolic leaves extract of *Annona muricata* by GC-MS Peak Report TIC

Peak #	Retention time/ minutes	% Composition by Area	Matched compound IUPAC Name	Chemical Formula	Comment
1	3.034	16.8	-	-	Unidentified
2	3.342	3.93	2-Pentadecanol	$C_{15}H_{32}O$	Matched
3	16.732	6.15	Oleyl Alcohol	$C_{18}H_{36}O$	Matched
4	16.792	1.04	-	-	Unidentified
5	17.015	2.67	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	Matched
6	17.19	1.37	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	Matched
7	17.998	7.12	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	Matched
8	18.331	1.29	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	Matched
9	18.431	0.73	-	-	Unidentified
10	19.531	5.61	Phytol	$C_{20}H_{40}O$	Matched
11	19.678	2.15	1,E-11,Z-13-Octadecatriene	$C_{18}H_{32}$	Matched
12	19.733	9.39	7-Tetradecenal, (Z)	$C_{14}H_{26}O$	Matched
13	19.946	3.21	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	Matched
14	20.003	4.26	cis, cis, cis-7,10,13-Hexadecatrienal	$C_{16}H_{26}O$	Matched
15	23.143	23.51	-	-	Unidentified
16	23.225	1.08	-	-	Unidentified
17	23.695	1.74	-	-	Unidentified
18	24.025	0.75	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	Matched
19	24.75	1.25	-	-	Unidentified
20	26.402	1.54	-	-	Unidentified
21	28.35	1.12	-	-	Unidentified
22	28.567	0.74	-	-	Unidentified
23	28.875	0.85	-	-	Unidentified
24	29.331	0.74	-	-	Unidentified
25	29.704	0.95	-	-	Unidentified

The total ion chromatogram (TIC) of the ethanolic extract, showing the GC-MS profile of the compounds identified is given in Figure 2. The peaks in the chromatogram were integrated and were compared with the database of spectrum of known components stored in the GC-MS NISP library. Phytochemical analysis by GC-MS analysis of the ethanolic extract of leaves of *Annona muricata* revealed the presence of different fatty acids, heterocyclic compounds, esters among others. 25 peaks were generated.

The detailed tabulations of GC-MS analysis of the extracts are given in Table 1. From the analysis, 25 compounds have been elucidated for the first time in this study on *Annona muricata*, of which 12 compounds were effectively matched and identified. The ethanolic extract of the plant generated 25 constituents, the major constituents were at peaks 15 (peak area 23.51%), Peak 1 (peak area 16.8%), 7-Tetradecenal, (Z) (peak area 9.39%), n-Hexadecanoic acid (peak area 7.12%), Oleryl Alcohol (peak area 6.15%), Phytol (peak area 5.61%), cis, cis, cis-7,10,13-Hexadecatrienal (peak area 4.26%), 2-Pentadecanol(peak area 3.93%), 9,12-Octadecadienoic acid, ethyl ester (peak area 3.21%), 1,2-Benzenedicarboxylic acid, butyl octyl ester (peak area 2.67%), and 1,E-11,Z-13-Octadecatriene (peak area 2.15%), while the rest had less than 2% composition by peak area.

DISCUSSION

The presence of various secondary class metabolites identified puts these results in line with earlier studies that were carried out on the ethanolic seeds extract of *Annona muricata*, and the phytochemical tests showed that ethanol soursop seeds extract contains secondary metabolites compounds group of saponins, alkaloids and triterpenoids, flavonoids, anthraquinones, tannins, and cardiac glycosides, which they noted that they are defense chemical compounds of plants produced in the plant tissue.[16,17] The plant could thus be used for the management of various healthy conditions associated with the metabolites screened.

Using GC-MS Analysis, 25 compounds have been elucidated for the first time in our study on *Annona muricata*, of which 12 compounds were effectively matched and identified.

1,2-Benzenedicarboxylic acid, butyl octyl ester is a plasticizer compound with antimicrobial, antifouling, antioxidant and hypo-cholesterolemic activities.[18] 3,7,11,15-Tetramethyl-2-hexadecen-1-ol is recorded to have anti-tuberculosis, insecticidal, anti-inflammatory, antioxidant and antimicrobial activities. n-Hexadecanoic acid on the other hand which is commonly known as Palmitic acid has nematicide, pesticide, lubricant, anti-androgenic, flavor, hemolytic 5-alpha reductase inhibitor, antioxidant and hypo-cholesterolemic properties.[16]

Hexadecanoic acid, ethyl ester is a fatty acid ester with nematicide, pesticide, lubricant, anti-androgenic, flavor, and has hemolytic 5-alpha reductase inhibitor properties.[19,20]

Phytol is a diterpene with antimicrobial, anticancer, anti-inflammatory, anti-diuretic, immune-stimulatory and anti-diabetic properties. 9,12-Octadecadienoic acid, ethyl ester is a linoleic acid which has hypocholesterolemic, 5-alpha reductase inhibitor, antihistaminic, insectifuge, anti-eczemic, and anti-acne properties. Finally, 1,2-Benzenedicarboxylic acid, diisooctyl ester is a plasticizer compound with antimicrobial and antifouling properties.[15,19,20]

It is worth noting that of the major constituents identified in our extract, the compounds with the highest composition at peaks 15 (peak area 23.51%) and Peak 1 (peak area 16.8%) have not been matched in the library. These two could be very novel compounds that need to be analyzed further in order to elucidate their nature. The same applies to the remaining 11 compounds which have not been identified irrespective of their percentage composition being less than 2%.

CONCLUSION

GC-MS analysis of the ethanolic extract of leaves of *Annona muricata* revealed the presence of different fatty acids, heterocyclic compounds, esters among others. This confirms the results on presence of the various secondary metabolite compounds detected by the qualitative procedures. These mass spectra are fingerprint of the compound which can be identified from the data library. Hence, the identified phytocomponents using GC-MS can be used as a pharmacognostical tool for the identification of adulterants. The current pioneering study suggests that ethanolic extract is a potent therapeutic agent. It paves the way for the development of several treatment regimens based on this extract. In addition, further research is necessary to identify and purify the active compounds responsible for therapeutic activity, as well as the unidentified compounds.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR'S CONTRIBUTIONS

All authors contributed immensely towards the research work and development of the manuscript. YG–designed experiment, collected samples, conducted laboratory analysis, analyzed data, participated in write-up of manuscript. FA–participated in the design, analysis, write up and reviewed drafts of the manuscript. FW–participated in the design,coordination, analysis, write up and reviewed drafts of the manuscript. EHA -participated in the design and coordination of the study, data interpretation and write-up of manuscript. All authors read and approved the final manuscript.

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Original Article

In vivo Antimalarial Evaluation of Embelin and its Semi-Synthetic Aromatic Amine Derivatives

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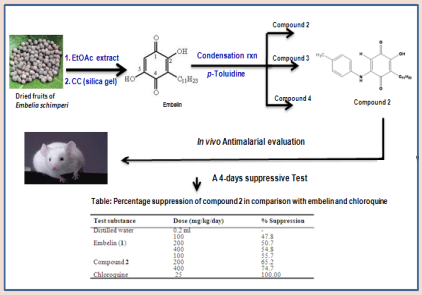
ABSTRACT

Background: In less developed countries like Ethiopia, malaria is traditionally treated by remedies prepared from medicinal plants. One such plant that falls in this category is *Embelia schimperi* Vatke whose fruits are employed for the treatment of a variety of ailments including taeniasis and malaria. **Objective:** In the present study, the *in vivo* antimalarial activity of embelin isolated from the fruits of *Embelia schimperi* Vatke and its semi-synthetic aromatic amine derivatives was evaluated. **Methods:** Silica gel column chromatography was used to isolate embelin from the ethyl acetate extract of the fruits of *E. schimperi*. Aromatic substituted embelin derivatives were semi-synthesized by using a one-step condensation reaction of embelin with aromatic amines. The compounds were characterized based on their UV, IR, HR-ESIMS, ^{1}H and ^{13}C NMR and DEPT-135 spectral data. Antimalarial activity was evaluated using a modified Peter's 4-day suppressive test against chloroquine sensitive *Plasmodium berghei* infection in mice. **Results:** Embelin and the semi-synthetic derivatives showed significant ($p<0.05$) *in vivo* antimalarial activity in a dose-dependent manner with 47.8-74.7% parasite suppression at tested doses of 100-400 mg/kg. Among the compounds semi-synthesized, 5-(*p*-tolylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione showed maximum antimalarial activity (74.7 % suppression) at a dose of 400 mg/kg. No major signs of toxicity were observed when either embelin or the semi-synthesized derivatives were administrated to mice at the highest tested dose (2 g/kg). **Conclusion:** The results underline that the antimalarial activity of embelin can be improved by preparing its aromatic semi-synthetic amine derivatives without affecting the safety of the parent molecule.

Keywords: Antimalarial, Aromatic substituted embelin, A 4-Day suppressive test, *Embelia schimperi*, Embelin.

SUMMARY

- Embelin is the major constituent of the fruits of *Embelia schimperi* Vatke.
- It has a long alkyl chain (undecyl) as a substituent, which confers solubility in the non-polar phase and cell permeability.
- Aromatic amine derivatives of embelin were semi-synthesized and one of the compounds obtained by condensation of embelin with p-toludine showed improved antimalarial activity.



PICTORIAL ABSTRACT

Abbreviations used: LC-MS: Liquid Chromatography-Mass Spectrometry, DEPT: Distortionless Enhancement by Polarization Transfer.

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INTRODUCTION

Malaria is a parasitic disease caused by *Plasmodium* species transmitted from the blood of an infected person and passed on to a healthy human by a female *Anopheles* mosquito. Although the disease was eliminated in most western countries more than 50 years ago, it is still rampant in many African countries with all estimated malaria cases occurring in just five African countries, Ethiopia being one of them.[1] It is estimated that three-fourths of the land below 2000 meters in Ethiopia is malarious with two-thirds of the country's population at risk. *Plasmodium falciparum* and *P. vivax* account for 60-70 % and 30-40 % of malaria cases in the country, respectively.[2] Despite the availability of various malaria control strategies none of them appears to be appropriate and affordable in all contexts.[3-4] Current treatment for malaria relies on chemotherapy, as no efficient vaccine is available. The burden of this disease is getting worse, mainly due to the increasing resistance of *Plasmodium* species against the widely available antimalarial drugs.[5] Although, in recent years artemisinin and its analogues have provided much needed drugs for the treatment of chloroquine-resistant malaria, these are unavailable and/or unaffordable to many people who live in malarious areas.[6] Effective novel therapeutics are, therefore, needed to combat the parasite.

Srinivas *et al.*[7] reported that the methanolic extract of the fruits of *Embelia schimperi* possesses *in vitro* activity against chloroquine resistant *P. falciparum*. Embelin (**1**) is the major constituent of *E. ribes*.[8] It has been reported that embelin possesses wide spectrum of biological activities, such as antibacterial, antitumor, anti-inflammatory, antimalarial and analgesic.[9-10] In the present study embelin (**1**) isolated from the fruits of *E. schimperi* and its semi-synthesized aromatic amine derivatives (**2**-**4**) have been evaluated for their *in vivo* antimalarial activity against mice infected with *P. berghei*.

MATERIALS AND METHODS

General

Mass spectra (MS) were recorded on a Dionex Ultimate 3000 LC-MS. The measurement was carried out by an electrospray ionization (ESI) method with negative mode. The source voltage and temperature were fixed at 3kV and 250°C, respectively. NMR spectra (^{1}H, ^{13}C, DEPT-135) were recorded on a Bruker Avance DMX 400 FT-NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C at room temperature

using deuterated chloroform. A region from 0 to 12 ppm for ^{1}H and 0 to 205 ppm for ^{13}C was employed for scanning. Signals were referred to an internal standard tetra methylsilane (TMS). Chemical shifts are reported in δ units. Multiplicities of ^{1}H NMR signals are indicated as *s* (singlet), *brs* (broad singlet), *d* (doublet), *t* (triplet), *m* (multiplet) and *nr* (not well resolved). $CHCl_3$ (E. Merck, Stockholm), EtOAc (Research-Lab-Fine, India); MeOH (Reagent Chemical Ltd, UK); silica gel G6 F_{254} (E. Merck, Darmstadt), aniline. *o*-toluidine, and *p*-toluidine (Sigma-Aldrich Labor chemikalien GmbH, Germany), acetic acid glacial (Supreme Enterprises, India) have all been used as received.

Experimental animals and test organisms

Swiss albino mice of either sex weighing 20-30 g and age 6-8 weeks were obtained from the Department of Biology (DoB), College of Natural Sciences (CoNS), AAU animal house. All animals were housed in an air-conditioned room and were allowed to acclimatize for one week before the study. The animals were kept at room temperature and were exposed to a 12 h light/dark cycle. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline.[11] Before and during the experiment, the mice were allowed free access to standard pellets and water *ad libitum*. *P. berghei* ANKA strain (chloroquine sensitive), was obtained from the DoB, CoNS, AAU. The parasite was subsequently maintained in the laboratory by serial blood passage from mouse to mouse on weekly bases. Chloroquine phosphate (EPHARM, Ethiopia) was used as a reference antimalarial drug.

Plant material

Fruits of *E. schimperi* were purchased from local market in March 2012 in Addis Ababa, Ethiopia and authenticated by Mr. Melaku Wondafrash, the National Herbarium, Addis Ababa University (AAU).

Extraction and Isolation of embelin

Dried and powdered fruits of *E. schimperi* (0.5 kg) were macerated in EtOAc at room temp for 72 h, filtered and the solvent evaporated to afford a dark brown solid (25 g). The crude extract was subjected to silica gel column chromatography and eluted with hexane and EtOAc gradients. Embelin (**1**) was isolated from the *n*-hexane/EtOAc fraction (1:10 v/v).

Preparation of 5-(p-tolylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (2)

A mixture of embelin (**1**) (1 mmol) and *p*-toluidine (**5**) (1 mmol) was dissolved in glacial acetic acid (30 ml) and refluxed on a water-bath for 4 h as shown in Figure 1. The reaction mixture was cooled to room temperature and poured into ice-cooled diluted HCl. The solid separated was filtered, washed with ethanol, dried and purified by re-crystallization from chloroform/ethanol mixture (4:1) to yield a deep violet prism (**2**).

Preparation of 5-(o-tolylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (3)

o-Toluidine (**6**) (1 mmol) was mixed with embelin (**1**) (1 mmol) in the presence of glacial acetic acid (40 ml) and refluxed for 4 h on a water-bath. After cooling, the reaction mixture was poured into ice-cooled diluted HCl to obtain a solid product. It was then filtered, dried and purified by re-crystallization from chloroform/ethanol (4:1) to yield a reddish brown prism (**3**).

Preparation of 5-(phenylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (4)

Embelin (**1**) (1 mmol), aniline (**7**) (1 mmol) and glacial acetic acid (40 ml) were mixed and refluxed for 4 h on a water-bath. After cooling, the reaction mixture was poured into ice-cooled diluted HCl and the precipitate collected. The product was filtered, dried, and purified by recrystallization from chloroform/ ethanol (2:1) to yield a violet amorphous compound **4**.

Acute toxicity test

Acute toxicity study was conducted as per the internationally accepted protocol drawn under OECD Guideline 425.[12] Twenty healthy Swiss female albino mice weighing 23-25 g were randomly divided into 4 groups (Groups A-D) of 5 mice per group. After fasting for 3-4 h, on day one, four mice (one mouse from each group) were given 2 g/kg of compounds **1-4,** respectively. The mice were then kept under strict observation for physical or behavioral changes for 24 h, with special attention during the first 4 h. Following the results from each of the mice, other four mice were recruited per group and fasted for 3–4 h and administered a single dose of 2 g/kg of each of the test compounds and were observed in the same manner. These observations continued for further 14 days for any signs of overt toxicity such as hair erection, lacrimation and mortality.

In vivo antimalarial activity

Parasite inoculation

P. berghei infected albino mice with parasitaemia level of 20-30% were used as donor. Parasitized erythrocytes were obtained from a donor infected mouse by cardiac puncture with a sterile needle and syringe. The blood was then diluted with physiological saline in such a way that 1 ml blood contains 5×10^{7} infected erythrocytes. Sufficient male Swiss albino mice were selected and each inoculated intra peritonealy with 0.2 ml of the infected blood.

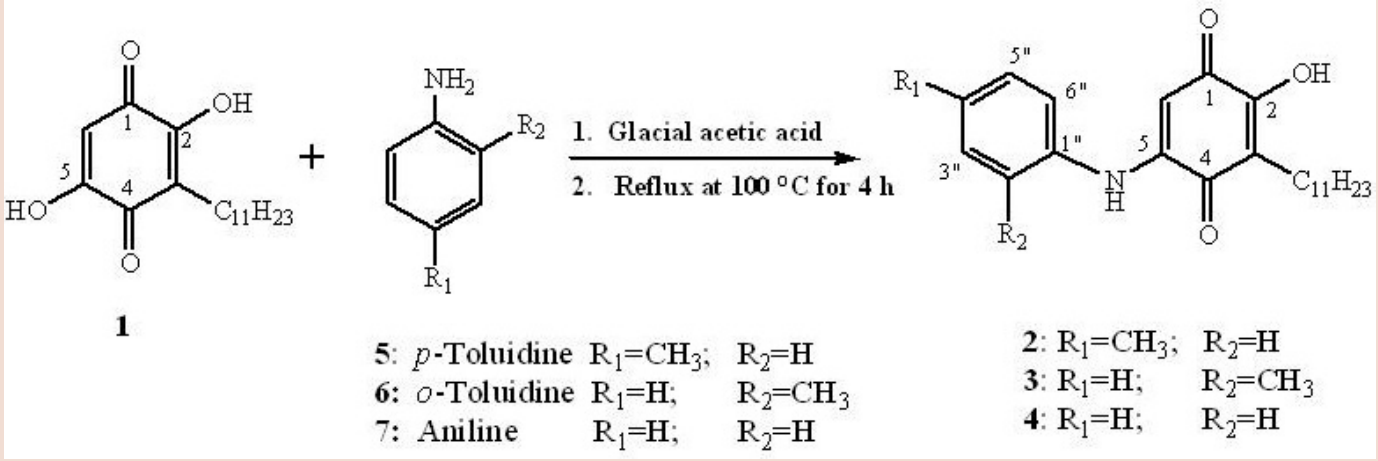

Figure 1: Synthesis of embelin derivatives **(2-4)** by treating embelin **(1)** with aromatic amino reagents **(5-7)**

4-Day suppressive test

A modified Peter's 4-day suppressive test against chloroquine sensitive *P. berghei* infection in mice was employed. The mice were divided into fourteen groups (five mice per group). Group I: negative control, Groups IIa, IIb IIc, IIIa IIIb IIIc IVa IVb IVc and Va Vb Vc, receiving embelin (**1**) and its derivatives **2**-**4** at doses of 100, 200, and 400 mg/kg/ of body weight per day, respectively, while group VI (positive control) were treated with chloroquine at a dose of 25 mg/kg/day in a volume of 0.5 ml. All the test substances were administered through oral route by using oral gavages. Treatment was started 3 h after infection on day 0 and then continued daily for four days (i.e. from day 0 to day 3). On the fifth day (D_4) thin blood smears were prepared fixed in MeOH and stained with 10 % Geimsa solution to be examined under the microscope with an oil immersion objective of 100x magnification power to evaluate percent suppression.[13-14]

Percentage growth inhibition of the parasites was calculated by the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{Parasitaemia in negative control} - \text{Parasitaemia in study group}}{\text{Parasitaemia in negative control}} \text{x100}$$

Monitoring of body weight and mean survival time

Body weight of each mouse was measured before infection (day 0) and on day 4 on healthy mice at the tested doses using digital weighing balance. Mean survival time was recorded from day 1–28 after infection.

Data Analysis

Results of the study were expressed as mean ± standard error of mean (M ± SEM). Data were analyzed using Windows SPSS Version 20. Comparison of parasitaemia among groups and statistical significance was determined by one-way ANOVA and student t-test at a 95 % confidence interval (α = 0.05). The results were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Structure elucidation

Compound (**1**) was isolated as a bright orange crystal from *n*-hexane/ethyl acetate (1:10; v/v). The negative-mode HR-ESI mass spectrum of compound **1** gave a pseudomeolecular ion at *m/z* = 293.17527 ([M-H]$^-$), indicating a molecular formula of $C_{17}H_{26}O_4$ (calcd. 293.175285 [M-H]$^-$). ^{1}H NMR spectrum of compound **1** showed a singlet at 6.03 ppm, which was assignable to H-6. In addition, a triplet at 0.90 ppm (3H, H-11'), a broad singlet at 1.21 ppm (14H, H-4' to H-10'), a broad singlet at 1.31 (2H, H-3′), a multi plet at 1.51 (2H, H-2′) and a broad singlet at 2.46 ppm (2H, H-1') showed the presence of the undecyl chain as part of the structure of compound **1**. The ^{13}C NMR spectrum of compound **1** revealed signals corresponding to 13 carbon atoms only, which does not fit with the number of carbons proposed in the molecular formula above. This is because oxygen bearing ring carbons of 2, 5-dihydroxy-3-alkyl-1,4-benzoquinones do not appear in the ^{13}C NMR spectrum because of fluxional effect caused by intra-molecular hydrogen bonding. This effect leads to long spin relaxation time which in turn leads to the saturation of oxygen–carbon signals as it was also indicated by Mahendran *et al.*[15] Hence, compound **1** was unequivocally identified as the known compound embelin (**1**) by comparing its ^{1}H and ^{13}C NMR data (listed below) with those reported in literature for the same compound.[10,15]

Embelin (1)

A bright orange crystal (hexane/EtOAc; 1:10); mp: 140-142°C; Rf: 0.3 (*n*-propanol/*n*-BuOH:NH_4OH;7:1:1); HR-ESIMS (-ve): *m/z*=293.17527 ([M-H]$^-$) (calcd. 293.17529 for $C_{17}H_{26}O_4$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.28 (*s*, OH), 6.03 (1H, *s*, H-6), 2.46 (2H, *t*, H-1′), 1.51 (2H, *m*, H-2′), 1.31 (2H, *brs*, H-3′), 1.21 (14H, *brs*, H-4′/H-10′), 0.90 (3H, *t*, H-11′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 117.0 (C-3), 102.2 (C-6), C-1, C-2, C-4, C-5 (signals not observed)), 32.0 (C-1′), 29.7 (C-2′), 29.7 (C-3′), 29.6 (C-4′), 29.6 (C-5′), 29.5 (C-6′), 29.4 (C-7′), 28.0 (C-8′), 22.7 (C-9′), 22.6 (C-10′), 14.2 (C-11′).

In an attempt to improve the antimalarial activity of embelin (**1**), its aromatic amino derivatives **(2-4)** were semi-synthesized (Scheme 1) by using a one-step condensation reaction of embelin (**1**) with *p*-toluidine (**5**), *o*-toluidine (**6**) and aniline (**7**), respectively, and tested against mice infected with *P. berghei* parasites. Condensation reaction of the benzoquinone embelin (**1**) with aromatic amines (**5**-**7**) involves three stage processes: in the first stage the more nucleophilic amino group of the reagents attacks the carbonyl carbon of embelin (**1**), followed by dehydration and ketoenol tautomersim which would lead to the formation of the target products.[16] Our assumption was that the more basic derivatives of embelin could improve antimalarial activity by facilitating localization and accumulation of the compounds within the acidic plasmodial vacuole where they undergo protonation and possibly inhibit GSH dependent heme degradation.

Compound **2** was obtained as deep violet prisms when embelin (**1**) was heated with *p*-toluidine (**5**) in the presence of glacial acetic acid. The negative-mode HR-ESI mass spectrum of compound **2** gave a pseudomeolecular ion at *m/z* = 382.23983 ([M-H]$^-$), corresponding to a molecular formula of $C_{24}H_{33}NO_3$ (calcd. *m/z* = 382.238219 [M-H]$^-$). The ^{1}H NMR spectrum of compound **2** exhibited two sets of *ortho*-coupling doublets that resonated at δ7.14 and 7.23 ppm (*J* = 8.4 Hz), corresponding to *p*-tolyl-H2′′,6′′ and *p*-tolyl-H3′′,5′′, respectively, in addition to signals comparable to those of embelin (**1**). The presence of 24 carbon atoms in compound **2** was evident from in the ^{13}C NMR spectrum. It was also noted that compound **2** contains 1,4 *para*-disubstitued benzene ring in the ^{13}C NMR spectrum [δ 122.85 (C-2′′,6′′), 130.27 (C-3′′,5′′), 134.23 (C-4′′) and 136.42 (C-1′′)]. Both ^{1}H and ^{13}C NMR data indicated that the reaction took place at C-5 position, not at the C-2 position of embelin (**1**) and this might be due to steric factor that is apparent in the latter. Further confirmation of the reaction site was obtained from ^{13}C NMR data, where +8.06 ppm upfield shift of C-6 in **2** (δ 94.15) was observed compared to that of the same carbon in **1** (δ 102.21) due to high electron donating nature of the amine group. Thus, compound **2** was finally characterized as 5-(*p*-tolylamino)-2-hydroxy-3-undecyl-cyclohexa-2,5-diene-1,4-dione as indicated below.

5-(p-Tolylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (2)

A deep violet prism ($CHCl_3$/EtOH; 4:1); Yield: 98.3 % (w/w); Mp: 150-151°C; Rf: 0.48 ($CHCl_3$/pet ether; 1:1); HR-ESIMS (-ve): *m/z*=382.23983 ([M-H]$^-$) (calcd. 382.23822 for $C_{24}H_{33}NO_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 5.96 (1H, *s*, H-6), 2.47 (2H, *t*, H-1′), 1.19-2.11 (18H, H-2′-H-10′), 0.89 (3H, *t*, H-11′), 7.14 (2H, *d*, H-2′′/H-6′′), 7.23 (2H, *d*, H-3′′/H-5′′), 2.37 (3H, *s*, 4′′-CH_3), 7.28 (1H, *brs*, NH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 183.0 (C-1), 154.8 (C-2), 116.4 (C-3), 180.3 (C-4), 146.4 (C-5), 94.2 (C-6), 32.0 (C-1′), 20.8-29.7 (C-2′-C-10′), 14.2 (C-11′), 136.4 (C-1′′), 122.9 (C-2′′/C-6′′), 130.3 (C-3′′/C-5′′), 134.2 (C-4′′), 24.3 (4′′-CH_3).

Compound **3,** obtained as violet prisms (98.5 % yield), showed a [M-H]$^-$ at *m/z*=382.23857 in the negative-mode HR-ESI mass spectrum suggesting a molecular formula of $C_{24}H_{33}NO_3$ (calcd. *m/z*=382.238219 [M-H]$^-$). The ^{13}C NMR and DEPT-135 spectra of compound **3** showed signals for 24 different carbon atoms corresponding to 10 methylenes, 5 methines, 2 methyls and 7 quaternary carbon atoms including two carbonyls (δ 180.06 and δ 183.01). Further evidence was obtained from the ^{1}H NMR spectrum, which showed signals consistent with the structure proposed for compound **3**: an olefinic proton H-6 (δ 5.62, *s*), two methyl groups (δ 0.87, *t* and δ 2.29, *s*) and four aromatic protons H-3′′-H-6′′ (δ 7.25-7.32). The remaining assignments are shown below). From the data presented above, the structure of compound **3** was deduced as 5-(*o*-tolylamino)-

Table 1: Percentage suppression of *Plasmodium berghei* in mice after four days of treatment with embelin (1) and its semi-synthetic aromatic amine derivatives (2-4)

Test substance	Dose (mg/kg /day)	% Parasitaemia ± SEM	% Suppression
Distilled water	0.2 ml	47.12 ± 3.21	-
Embelin (1)	200	23.22 ± 2.64*b	50.7
	400	21.32 ± 3.82*c	54.8
	100	24.60 ± 1.73*a	47.8
Distilled water	0.2 ml	51.53 ± 8.32	-
Compound 2	100	22.84 ± 1.49*d	55.7
	200	17.92 ± 5.46*e	65.2
	400	13.06 ± 3.19*f	74.7
Distilled water	0.2 ml	51.53 ± 8.32	-
Compound 3	100	26.20 ± 2.79*a	49.2
	200	25.16 ± 2.44*b	51.2
	400	22.31 ± 4.98*c	56.7
Distilled water	0.2 ml	51.53 ± 8.32	-
Compound 4	100	24.00 ± 1.87*g	53.4
	200	23.80 ± 1.78*h	53.8
	400	21.80 ± 2.16*i	57.7
Chloroquine	25	0.00	100.00

Values are presented as M ± SEM; n =5; *p < 0.001 compared to the negative control; values with different letters are significantly different (p<0.05).

2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione.

5-(o-Tolylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (3)

A violet prism ($CHCl_3$/EtOH; 4:1); Yield: 98.5 % (w/w); Mp: 120-121°C; Rf=0.49 ($CHCl_3$/pet ether; 1:1); HR-ESIMS (-ve): *m/z*=382.23857 ([M-H]$^-$) (calcd. 382.23822 for $C_{24}H_{33}NO_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 5.62 (1H, *s*, H-6), 2.47 (2H, *t*, H-1′), 1.12-2.20 (18H, *m*, H-2′-H-10′), 0.87 (3H, *t*, H-11′), 7.25-7.32 (4H, *nr*, H-3″-H-6″), 2.29 (3H, *s*, 2″-CH_3), 7.81 (1H, *brs*, NH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 183.0 (C-1), 154.6 (C-2), 116.2 (C-3), 180.1 (C-4), 147.1 (C-5), 94.1 (C-6), 32.0 (C-1′), 17.8-29.7 (C-2′-C-10′), 14.2 (C-11′), 135.0 (C-1″), 133.1 (C-2″), 127.3 (C-3″), 127.1 (C-4″), 131.5 (C-5″), 124.5 (C-6″), 17.8 (2″-CH_3).

Compound **4**, a reddish brown prism, was obtained by reaction of embelin (**1**) with aniline (**7**) in the presence of glacier acetic acid. A close analysis showed characteristic signals for a substituted 1,4-benzoquinone moiety in the ^{1}H NMR (δ 6.03, *s*, H-6) and ^{13}C NMR (δ 94.48, C-6; δ 182.93, C-1; δ 180.37, C-4) spectra.

The molecular formula of compound **4** was deduced as $C_{23}H_{31}NO_3$ by HR mass spectrometry (observed: *m/z*=368.22259 ([M-H]$^-$; calcd. *m/z*=368.222569). The ^{13}C NMR, along with the DEPT spectra, revealed the presence of one CH_3, ten CH_2, six CH and six quaternary carbons, including two carbonyl carbons. The complete assignments of ^{1}H and ^{13}C NMR data are summarized below. Thus, the structure of compound **4** was elucidated as 5-(phenylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione.

5-(Phenylamino)-2-hydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione (4)

A reddish brown prism ($CHCl_3$/EtOH; 2:1); Yield: 96.1 % (w/w); Mp: 157-159°C; Rf: 0.53 ($CHCl_3$/pet ether; 1:1); HR-ESIMS (-ve): *m/z*=368.22259 ([M-H]$^-$) (calcd. 368.22257 for $C_{23}H_{31}NO_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.03 (1H, *s*, H-6), 2.47 (2H, *t*, H-1′), 1.12-2.21 (18H, *m*, H-2′-H-10′), 0.89 (3H, *t*, H-11′), 7.27 (2H, *nr*, H-2″/H-6″), 7.42 (2H, *t*, H-3″/H-5″), 7.28 (1H, *nr*, H-4″), 7.99 (1H, *brs*, NH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 182.9 (C-1), 154.3 (C-2), 116.3 (C-3), 180.4 (C-4), 146.0 (C-5), 94.5 (C-6), 32.0 (C-1′), 22.7-29.7 (C-2′-C-10′), 14.2 (C-11′), 136.9 (C-1″), 122.8 (C-2″/C-6″), 129.8 (C-3″/C-5″), 126.3 (C-4″).

Acute toxicity study

Acute toxicity study indicated that embelin and its derivatives caused no mortality up to the highest tested dose of 2 g/kg within the first 24 h as well as for the following 14 days. Also, the compounds did not cause overt toxicity such as lacrimation, tremors, hair erection, salivation, diarrhoea. Thus, the median lethal dose (LD_{50}) of all compounds is said to be greater than 2000 mg/kg, indicating a good safety margin.

Antimalarial activity

In the present study, a 4-day suppressive test indicated that embelin (**1**) possesses *in vivo* antimalarial activity against *P. berghei,* with maximum parasitaemia suppression of 54.8 % at a dose of 400 mg/kg. It has been reported that malaria parasite degrades up to 80 % of the haemoglobin in the host cell and releases heme which is toxic to parasite and their host.[17] Neutralization of heme occurs mainly due to hemozoin formation by the parasites. Inhibition of hemozoin formation is, therefore, an important drug target to kill the parasite cell.[18] Inhibition of hemozoin formation takes place through different routes *via* drug binding to the heme or inhibition of glutathione (GSH) dependent heme degradation. According to Huang *et al*,[19] embelin acts as an antimalarial with a mechanism of action similar to that of the well-known 4-aminoquinoline chloroquine, by inhibiting hemozoin formation. The presence of hydroxyl groups in chloroquine bind iron of heme and lead to the formation of π–π adducts, which inhibit hemozoin formation. Similarly, Basilico *et al.*[20] proposed that the hydroxyl groups present in embelin may bind to the iron of heme and inhibit the formation of hemozoin which is essential for survival of parasites.

As shown in Table 1, embelin (**1**) and its derivatives (**2**-**4**) have produced a dose dependent chemosuppression, with maximum parasite suppres-

Table 2: Mean survival time of *Plasmodium berghei* infected mice after treatment with embelin (1) and its semi-synthetic aromatic amine derivatives (2-4)

Test substance	Dose (mg/kg /day)	Mean survival time (days)
Distilled water	0.2 ml	5.60 ± 0.55
Embelin (1)	100	7.80 ± 0.44*
	200	8.00 ± 0.71**
	400	9.00 ± 0.00***
Compound 2	100	7.80 ± 0.44*
	200	7.90 ± 0.54*
	400	8.00 ± 0.00*
Compound 3	100	7.00 ± 1.26*
	200	7.00 ± 1.00*
	400	7.40 ± 0.89**
Compound 4	100	6.50 ± 0.57*
	200	6.50 ± 1.01*
	400	7.70 ± 0.67**
Chloroquine	25	ND

Values are presented as M ± SEM; n = 5; *p>0.05; **=p<0.01; ***=p<0.001 when compared to the negative control; ND=No death within the follow-up 28-days.

sion of 74.7 % for compound **2** at a dose of 400 mg/kg/day. The semi-synthesized compounds are more basic than embelin due to the presence of amine substituent which enhances pKa values. This could be important for accumulation of the compounds in the acidic digestive vacuole of the parasite where they undergo protonation. Whilst the electron-donating ability of the *para*-substituted methyl group makes compound **2** to be relatively more basic, the *ortho*-substituted methyl group in compound **3** imparts steric hindrance to protonation of the amine group. Thus, as shown in Table 1, compound **2** is the most active while compound **3** is the least active among the synthesized compounds, with compound **4** showing intermediate activity due to the presence of the phenyl group which has neither electron-donating nor withdrawing effect. According to Rudrapal and Cheita,[21] replacement of 4′-OH group in 4-amino-quinolines by several amino substituent, provides interesting antimalarial activity. 4-Amino-quinolines accumulate at high concentrations into the parasite's acidic food vacuole, which is their site of action. Thus, stronger basicity of a molecule increases antimalarial activity due to better uptake in the vacuole owing to the pH gradient between the cytosol and the acidic vacuole.

The results of the present study revealed that the antimalarial effect of the semi-synthetic derivatives (**2** and **4**) is more than that of their parent compound (**1**), justifying our assumption that it would be possible to enhance activity by preparing the more basic amino derivatives of embelin (**1**). The antimalarial efficacy of embelin (**1**) and its derivatives was further evaluated by a mean survival time testing.[22] The mean survival time of mice treated with embelin (**1**) and its derivatives was longer when compared with vehicle treated animals. As shown in Table 2, all the tested compounds prolonged mean survival time of the experimental animals in a dose dependent manner.

The mean weight of each group, with the exception of the vehicle and compound **3** (200 mg/kg/day) treated groups, declined on the fifth day of infection (Table 3). The observed decline in weight might be explained by the direct effect of the compounds to cause appetite loss of the experimental animals.

CONCLUSION

In conclusion, the results of the present study underline that the antimalarial activity of embelin can be improved by preparing its aromatic semi-synthetic amine derivatives without affecting the safety of the parent molecule.

ACKNOWLEDGMENTS

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Table 3: Body weight of *Plasmodium berghei* infected mice after the administration of embelin (1) and its semi-synthetic aromatic amine derivatives (2-4)

Test substances	Dose (mg/kg/day)	Wt D_0 ± SEM	Wt D_4 ±SEM	Percent change
Distilled water	0.2 ml	24.34 ± 0.34	23.04 ± 1.21	-5.6
Embelin (1)	100	22.30 ± 0.70	20.94 ± 1.57	-6.5
	200	22.94 ± 0.13	21.46 ± 1.61	-6.9
	400	24.04 ± 0.20	23.24 ± 1.75	-3.4
Compound 2	100	23.00 ± 0.21	21.66 ± 0.59	-6.2
	200	23.20 ± 0.16	22.52 ± 1.22	-3.0
	400	24.12 ± 0.15	24.64 ± 1.07	2.1
Compound 3	100	22.24 ± 0.23	21.20 ± 1.48	-4.9
	200	22.28 ± 0.17	22.62 ± 1.08	1.5
	400	22.56± 0.13	20.98 ± 1.16	-7.5
Compound 4	100	22.06 ± 0.89	20.22 ± 0.83	-9.1
	200	22.10 ± 0.17	20.24 ± 0.93	-9.2
	400	23.60 ± 0.89	22.40 ± 0.90	-5.4
Chloroquine	25	24.18 ± 0.17	24.28 ± 2.02	0.4

Values are presented as M ± SEM; n = 5; Wt D_0: weight pre-treatment on day zero; Wt D_4: weight post-treatment on day five.

CONFLICT OF INTERESTS

Authors do not have any conflict of interest.

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Original Article

Hepatoprotective effect of Livplus-A polyherbal formulation

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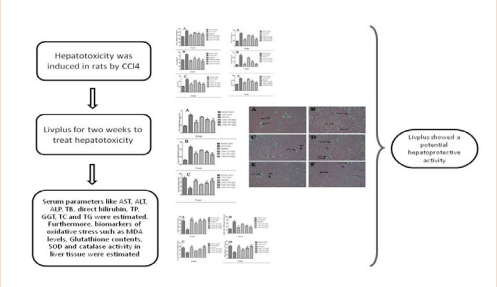
ABSTRACT

Objective: The aim of the present study was to investigate the hepatoprotective effect of Livplus (a polyherbal formulation) against CCl_4-induced hepatotoxicity in rats. **Methods:** Hepatotoxicity was induced in rats by i.p. injection of CCl_4 once three days for 14 days. Livplus or Silymarin was administered along with CCl_4 and the biochemical parameters like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkalinephosphatase (ALP), total bilirubin (TB), direct bilirubin, total protein (TP), gamma-glutamyl transferase (GGT), total cholesterol (TC) and triglycerides (TG) were estimated. Furthermore, biomarkers of oxidative stress such as MDA levels, Glutathione contents, SOD and catalase activity in liver tissue were estimated. **Results:** Treatment with Livplus significantly reduced the elevated levels of ALT, AST, ALP, bilirubin (direct and total), GGT, TC, TG and increased levels of TP compared to CCl_4 control rats. The treatment with Livplus also showed a significant increase in glutathione contents, SOD and catalase activity and a decrease in MDA levels compared to CCl_4 control rats. **Conclusion:** The finding of present study indicates that Livplus showed a potential hepatoprotective activity. These results support the traditional use of Livplus in the treatment of liver disorders.

Key words: Livplus, CCl_4, Hepatotoxicity, GGT, Hepatic enzymes.

SUMMARY

- Administration of Livplus (100, 200 and 400 mg/kg), a polyherbal formulation to the CCl4-induced hepatotoxicity resulted in a decrease the elevated levels of ALT, AST, ALP, bilirubin (direct and total), GGT, TC, TG, MDA and an increase-levels of TP, GSH, SOD, catalase activity compared to CCl_4 control rats.



PICTORIAL ABSTRACT

Abbreviations used: AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkalinephosphatase, TB: Total bilirubin, TP: Total protein, GGT: Gamma-glutamyl transferase, TC: Total cholesterol, TG: Triglycerides. MDA: Malondialdehyde SOD: Super oxide dismuatse.

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INTRODUCTION

Liver is the most essential organ concerned with the biochemical activities in human body. The most important role is to detoxicate the toxic substances.[1] The management of liver disorders is still a challenge. Therefore, the search for more effective and safer hepatoprotective drugs has continued to be an important area of active research. Since, there is no effective synthetic and conventional drugs are available, it has become a highly essential to search new drugs from herbal origin with little side effects. For a long time, herbal drugs are used for the treatment of liver diseases.[2]

At present there are various polyherbal formulations available in the market for the treatment of liver diseases. Livplus is one of the polyherbal formulations (Bacfo Pharmaceuticals India Limited, Noida) that consist of several herbal extracts mentioned in Table 1. It is used as hepatoprotective, hepato-stimulant and offers a compressive coverage of the treatment of liver diseases.[3-9] But, this drug has not been proved as hepatoprotective drug in any experimental set up. Therefore, in present study we try to investigate hepatoprotective effect of Livplus against CCL_4- induced hepatotoxicity in rats.

MATERIALS AND METHODS

Drugs and Chemicals

Livplus was gifted by Bacfo Pharmaceuticals India Ltd., Noida (India). Silymarin was purchased from commercial market. All biochemical kits were purchased from Span Diagnostics Ltd., Surat (India). All other chemicals and reagents used in the study were of analytical grade.

Experimental Animals

Albino Wistar rats (200-250 g) of either sex were obtained from Zydus Research Centre, Ahmedabad. All animals were maintained under standardized condition (12-h light/dark cycle, 24 ± 2°C & humidity 35-60 %) and they were provided with standard pellet diet and water *ad libitum*. The rats were left for 48 h for adaptation prior to the beginning of the experiment. The study was approved by Institutional Animal Ethics Committee (IAEC) and carried out in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animal) guidelines.

Acute toxicity study

On the basis OECD guideline no. 423, the acute oral toxicity was carried out in albino Wistar rats of either sex weighing 200-250 g.[10] Livplus was given at the dose of (100, 200, 500, 1000 and 2000 mg/kg, p.o.) for 3 animals and the signs and symptoms were observed after 0, 30, 60, 120, 180, 240 min and then once a day for next 14 days.

Experimental design

Carbon tetrachloride (CCl_4)-induced acute hepatotoxicity in rats

Albino Wistar rats were divided into six groups, each group having six animals. Group I: Normal control animals were administered carboxy

Table 1: Composition of Livplus (a polyherbal formulation)

Ingredients	Botanical name	Part used	Weight (mg)
Bhringraja	*Eclipta alba*	Whole plant	100
Bhumiamla	*Phyllanthus niruri*	Whole plant	100
Kasni	*Cichorium intybus*	Whole plant	75
Katuka	*Picrorhiza kurroa*	Root	75
Punarnava	*Boerhaavia diffusa*	Whole plant	50
Daruharidra	*Berberis aristata*	Whole plant	50
Kalmegha	*Andrographis paniculata*	Whole plant	50

Bhawana Dravya: Processed in the fruit extract of *Piper longum*, fruit extract of *Pipernigrum*, rhizome extract of *zingiber officinale* (Trikatu), whole plant extract of *Boerhaavia diffusa* (punarnava) and whole plant extract of *Cichorium intybus* (Kasni).

methyl cellulose (1 mL/kg of 1 %, w/v, p.o.); Group II: Carbon tetrachloride (1 mL/kg, i.p.); Group III: Silymarine (100 mg/kg, p.o.); Group IV, V and VI: Livplus (100, 200 and 400 mg/kg, p.o. in 1 % w/v of CMC), respectively. Livplus or silymarin was given daily for two weeks of respective groups, while carbon tetrachloride was given simultaneously every 72 h for 14 days except in group I.[11]

At the end of the experiments, blood samples were collected from the retro orbital plexus of rats under light ether anaesthesia, using glass capillaries. For separation of serum blood was allowed to clot for 15 minutes and it was then centrifuged at 5000 rpm for 20 minutes. The serum was stored at -20°C until further biochemical estimation. Serum was used for analysis of various biochemical parameters including, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphate (ALP), total bilirubin (TB), direct bilirubin, total protein (TP), gamma-glutamyl transferase (GGT), total cholesterol (TC) and triglycerides (TG).

Estimation of biomarkers of oxidative stress

Liver was removed and kept in autoclaved inverted petridis in cold conditions. The tissues were cross chopped with surgical scalpel into fine slices in chilled 0.25 M sucrose, quickly blotted on a filter paper. They were minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10 %w/v) with 25 strokes of tight Teflon pestle of glass homogenizer at a speed of 10,000 ×g at 0˚C using the Remi cooling centrifuge. The clear supernatant obtained was used for assay of lipid peroxidation MDA (malondialdehyde) content, endogenous anti-peroxidative enzymes such as superoxide dismutase (SOD), catalase and GSH (glutathione). Lipid peroxidation or MDA formation[12] SOD activity,[13] catalase activity [14] and GSH[15] were estimated.

Histopathology

After sacrifice, liver tissue of each group was rapidly dissected out and washed immediately with saline and fixed in 10% phosphate buffered formalin. Paraffin-embedded specimens were cut into 5 µm-thick sections and stained with hematoxylin and eosin (H&E). The sections were examined under the light microscope (Olympus BX10, Tokyo, Japan) for the presence of histopathological changes and photomicrographs (Olympus DP12 camera, Japan) were taken. The observer performing histopathological evaluation was blinded to the animal treatment groups.

Statistical analysis

All of the data are expressed as mean ± SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test as appropriate using computer based fitting program (Prism, Graphpad 5). The significance level was set at P<0.05 for all tests.

RESULTS

Acute oral toxicity

The oral administration of Livplus in rats up to the dose 2000 mg/kg did not show any sign of toxicity and no mortality for 14 days. It was shown that Livplus was safe up to oral dose of 2000 mg/kg of body weight. The experimental protocol was carried out using 1/20th (100 mg/kg), 1/10th (200 mg/kg) and 1/5th (400 mg/kg) dose based on toxicity study.

Effect of Livplus on AST, ALT and ALP in CCl_4-induced hepatotoxicity in rats

There was a significant (*P < 0.001*) increase in the levels of AST, ALT and ALP in CCl_4 control rats as compared to normal control rats. In contrast, the treatment with Livplus at the dose of 100 mg/kg showed a significant (*P<0.05*) reduction in AST and ALT levels as compared to CCl_4 control rats. However, the treatment with Livplus at the dose of 200 and 400 mg/kg or silymarin (100 mg/kg) showed a greater significant (*P<0.001*) reduction in AST and ALT levels as compared to CCl_4 control rats (Figure 1A-B).

The treatment with Livplus (200 mg/kg) showed a significant (*P < 0.01*) decrease in ALP levels as compared to CCl_4 control rats, while the CCl_4 control rats treated with Livplus (400 mg/kg) or silymarin (100 mg/kg) showed more significant (*P<0.001*) reduction in ALP levels as compared to CCl_4 control group. However, Livplus (100 mg/kg) treated rats did not show any significant difference in the levels of ALP as compared to CCl_4 control group (Figure 1C).

Effect of Livplus on total bilirubin, direct bilirubin and total protein in CCl_4-induced hepatotoxicity in rats

CCl_4 control rats showed a significant (*P<0.001*) increase in the levels of TB and direct bilirubin in CCl_4 control rats as compared to normal control rats. However, the treatment with Livplus (200 and 400 mg/kg) showed a significant reduction in TB (*P<0.05; P<0.001*) and direct bilirubin (*P<0.01; P<0.001*) levels as compared to CCl_4 control rats. In contrast, the treatment with Livplus (100 mg/kg) did not show any significant change in TB and direct bilirubin levels as compared with CCl_4 control group (Figure 2A-B).

There was a significant (*P<0.001*) reduction in the levels of TP in CCl_4control rats as compared to normal control rats. In contrast, the treatment with Livplus (200 and 400 mg/kg) showed a significant (*P< 0.05; P<0.001)* increase in TP levels as compared to CCl_4 control rats, but Livplus (100 mg/kg) treated rats did not show any significant alteration in the levels of TP as compared with CCl_4 control group (Figure 2C).

Effect of Livplus on total cholesterol, triglycerides and GGT in CCl_4-induced hepatotoxicity in rats

In CCl_4 control rats, TC and TG levels were significantly (P<0.001) increased when compared to normal control rats. The treatment with Liv-

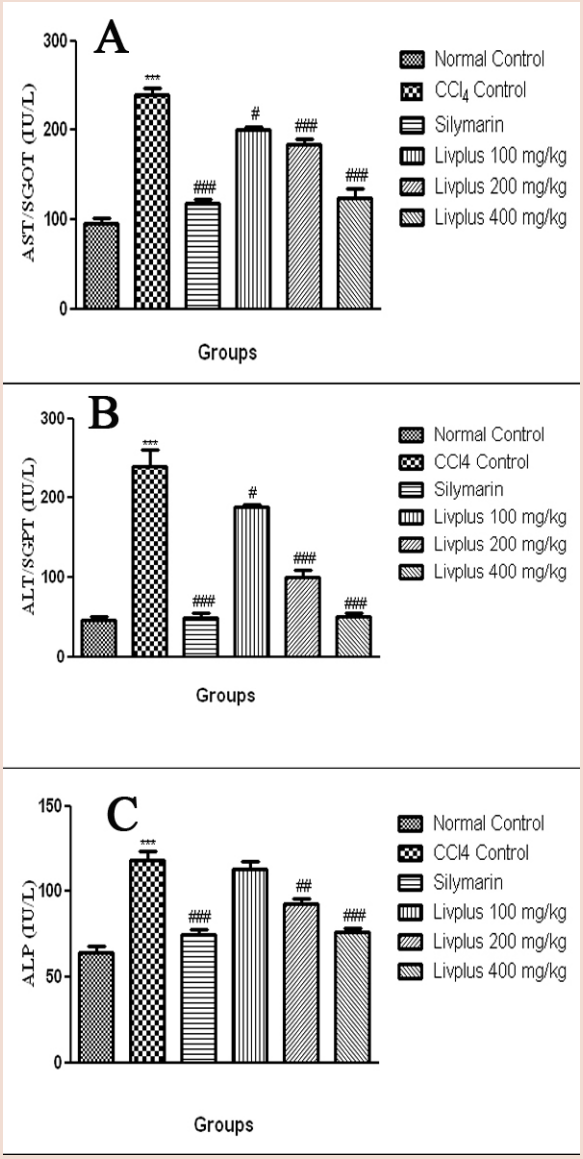

Figure 1: Effect of Livplus on serum (A) AST, (B) ALT and (C) ALP in CCl_4-induced hepatotoxicity in rats
Values are expressed as Mean + S.E.M (n=6). Where, ***P < 0.001 as compared to normal control; #P<0.05, ##P<0.01, ###P<0.001 as compared to CCl_4 control

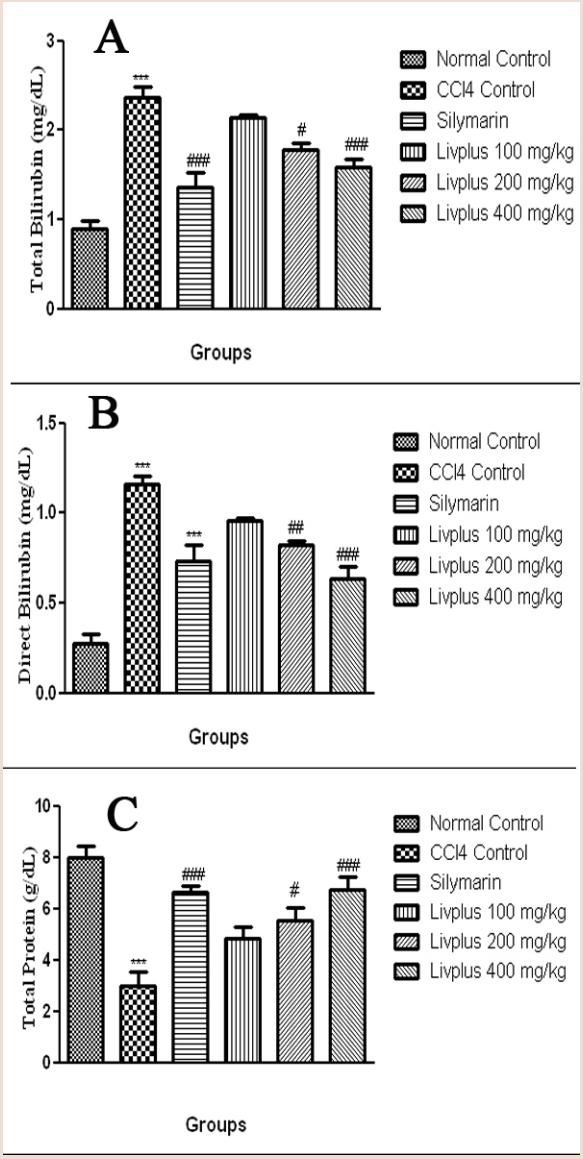

Figure 2: Effect of Livplus on (A) total bilirubin, (B) direct bilirubin and (C) total protein in CCl_4-induced hepatotoxicity in rats
Values are expressed as Mean + S.E.M (n=6). Where, ***P < 0.001 as compared to normal control; #P<0.05, ##P<0.01, ###P<0.001 as compared to CCl_4 control

plus (200 and 400 mg/kg) showed a significant (*P<0.05; P<0.001)* reduction in TC levels as compared to CCl_4 control rats. The treatment with Livplus (100 mg/kg) did not show any significant change in the levels of TC as compared with CCl_4 control group. Moreover, the treatment with Livplus (100, 200 and 400 mg/kg) showed a significant (*P<0.05; P <0.001; P<0.001*) reduction in TG levels as compared to CCl_4 control rats (Figure 3A-B).

CCl_4 control rats showed a significant (*P<0.001*) increase in GGT level as compared to normal control rats. In contrast, the treatment with Livplus (200 and 400 mg/kg) showed a significant (*P<0.01; P<0.001*) reduction in GGT levels as compared to CCl_4 control rats, while rats treated with 100 mg/kg did not show any significant difference in the levels of GGT as compared with CCl_4 control group (Figure 3C).

Effect of Livplus on SOD, MDA, catalase and GSH in CCl_4-induced hepatotoxicity in rats

In CCl_4 control group, SOD, catalase and GSH levels were significantly (*P<0.001*) decreased as compared to normal control rats. In contrast, the treatment with Livplus (100, 200 and 400 mg/kg) showed a significant increase in SOD (*P<0.01; P<0.001; P<0.001) and GSH (P<0.01; P<0.001; P<0.001)* levels as compared to CCl_4 control rats. In addition, treatment with Livplus (200 and 400 mg/kg) showed a significant *(P<0.05; P<0.001)* increase in catalase activity as compared to CCl_4 control rats, while animals treated with 100 mg/kg did not show any significant effect in the levels of catalase as compared with CCl_4 control group.

The content of MDA, end product of lipid peroxidation was significantly (P<0.001) increased in liver tissue of CCl_4 control rats as compared to normal control rats. The treatment with Livplus (200 and 400 mg/kg)

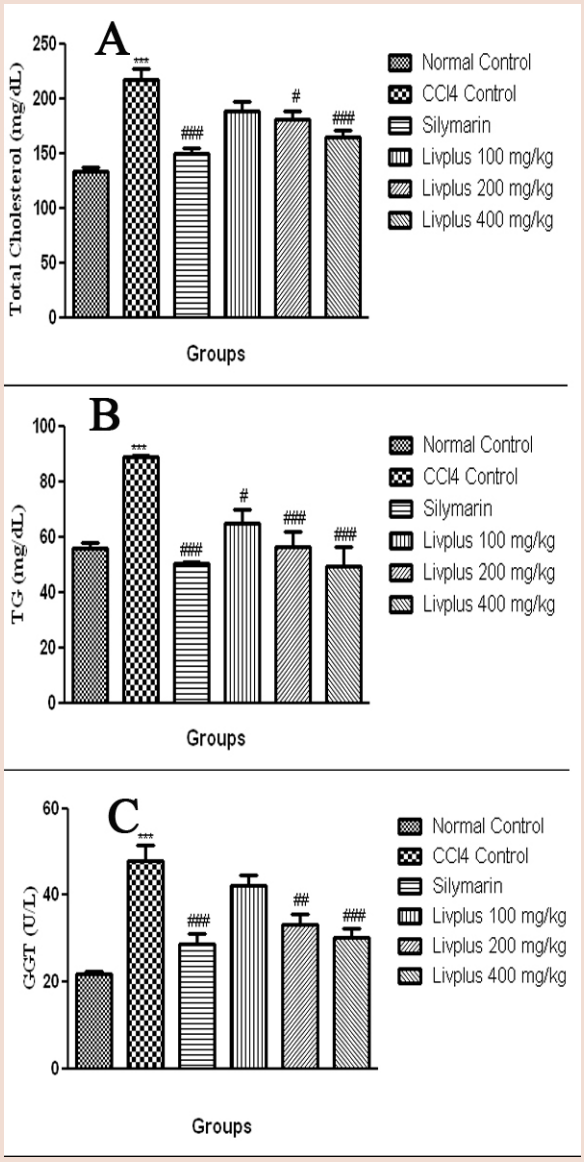

Figure 3: Effect of Livplus on (A) total Cholesterol, (B) triglycerides and (C) GGT in CCl_4-induced hepatotoxicity in rats
Values are expressed as Mean + S.E.M (n=6). Where, ***P < 0.001 as compared to normal control; #P<0.05, ##P<0.01, ###P<0.001 as compared to CCl_4 control

showed a significant (*P<0.05; P<0.001*) reduction in MDA levels as compared to CCl_4 control rats, but Livplus (100 mg/kg) treated rats did not show any significant reduction in the levels of MDA as compared to CCl_4 control group (Figure 4A-D).

Histopathological observation

The histological profile of the hepatic tissue of the normal control animals showed a normal lobular architecture. Normal hepatocytes were arranged in single cell cords radiating away from a central vein (A). CCl_4 treated rats showed disturbed liver architecture, exhibiting central lobular necrosis with tiny vacuoles, and fatty infiltrations (B). CCl_4 control rats treated with silymarin and Livplus (400 mg/kg) retained normal hepatic tissue architecture, so received significant protection from CCl_4-induced hepatic damage (C and F). CCl_4 control rats treated with Livplus (200 mg/kg) showed minimal inflammatory cellular infiltration, regeneration of hepatocytes around central vein was also observed and almost near normal liver architecture (E), while Livplus (100 mg/kg) treated did not show any significant hepatic tissue architectural changes (D) (Figure 5A-F).

DISCUSSION

Liver is one of the vital organs in animal body and plays a central role in transforming and clearing the chemicals, but it is susceptible to toxicity of several agents including drugs and chemicals. More than 900 drugs have been reported to cause liver injury.[16] Carbon tetrachloride is one of the most commonly used chemical for the screening of hepatoprotective drugs. Therefore, administration of CCl_4 can lead to enzymatic activation, mainly by CYP_2E_1, into trichloromethyl free radicals (CCl_3) inside the membrane of the endoplasmic reticulum. This is followed by chloromethylation, peroxidation and progressive destruction of the unsaturated fatty acid of the endoplasmic reticulum membrane phospholipids. These methods are known as lipid peroxidation, ultimately it is responsible for functional and structural disruption of hepatocytes.[17] In liver damage, cellular enzymes such as AST, ALT, ALP, bilirubin (direct and total) will escape into the serum resulting in elevation of the their serum concentration. Histology of liver showed disturbed liver architecture, exhibiting central lobular necrosis with tiny vacuoles, and fatty infiltrations.[18] Reduction of glutathione content, SOD activity, catalase activity and increased in lipid peroxidation is a markers for the hepatic damage.[19-21]

The treatment with Livplus (200 and 400 mg/kg, p.o.), silymarin (100 mg/kg, p.o.) for 14 days showed a significant protection against CCl_4 – induced liver damage by in virtue of reduction in cellular enzymes like AST, ALT, ALP, bilirubin (direct and total). It's hepatoprotective effect is also confirmed by prevention of histological changes caused by CCl_4. The possible mechanism of action may be associated with inhibition of CYP_2E_1 activity. In present study, CCl_4 control rats showed a significant increase in MDA levels and a decrease in glutathione content, SOD activity, catalase activity as compared to normal control rats. However, the treatment with Livplus (200 and 400 mg/kg, p.o.) or silymarin (100 mg/kg, p.o.) showed a significant reduction in MDA levels and an increase in glutathione content, SOD activity and catalase activity as compared to CCl_4 control rats.

It was previously reported that administration of CCl_4 caused the decrease in number of hepatocytes which in turn might result into decreased hepatic capacity to synthesize protein and glycogen. But, when Livplus was given along with CCl_4, there was a significant increase in total protein which may be due to the hepatoprotective effect.[22] In previous study, it was also reported that Gamma GT (Gamma-Glutamyl Transpeptidase) was significantly increased in rat intoxicated with CCl_4 in comparison with normal control group.[23] GGT which is present in the membrane of endoplasmic reticulum of the hepatocytes. When it is released extensively from damaged hepatic cell to the bloodstream is considered a good diagnostic profile for hepatic damage. In present study, there was a significant increase in the level of GGT in CCl_4 control group, while Livplus treated animals restored the levels of GGT. Administration of CCl_4 control rats caused a significant increase in TC and TG levels.[24] The treatment with Livplus showed a significant reduction in TC and TG levels. In current study, a comparative histopathological study of the liver from various treatments further supported the hepatoprotective potential.

CONCLUSION

These results showed that Livplus (200 and 400 mg/kg) showed a significant protection in dose dependant manner against experimentally induced hepatotoxicity. The possible mechanism behind the hepatoprotective effect of Livplus might be associated with inhibition of CYP_2E_1 activity and stimulation of antioxidant defense mechanism against the free radicals generated by CCl_4. Therefore, it was concluded that Livplus has a significant hepatoprotective effect. Our present investigation sup-

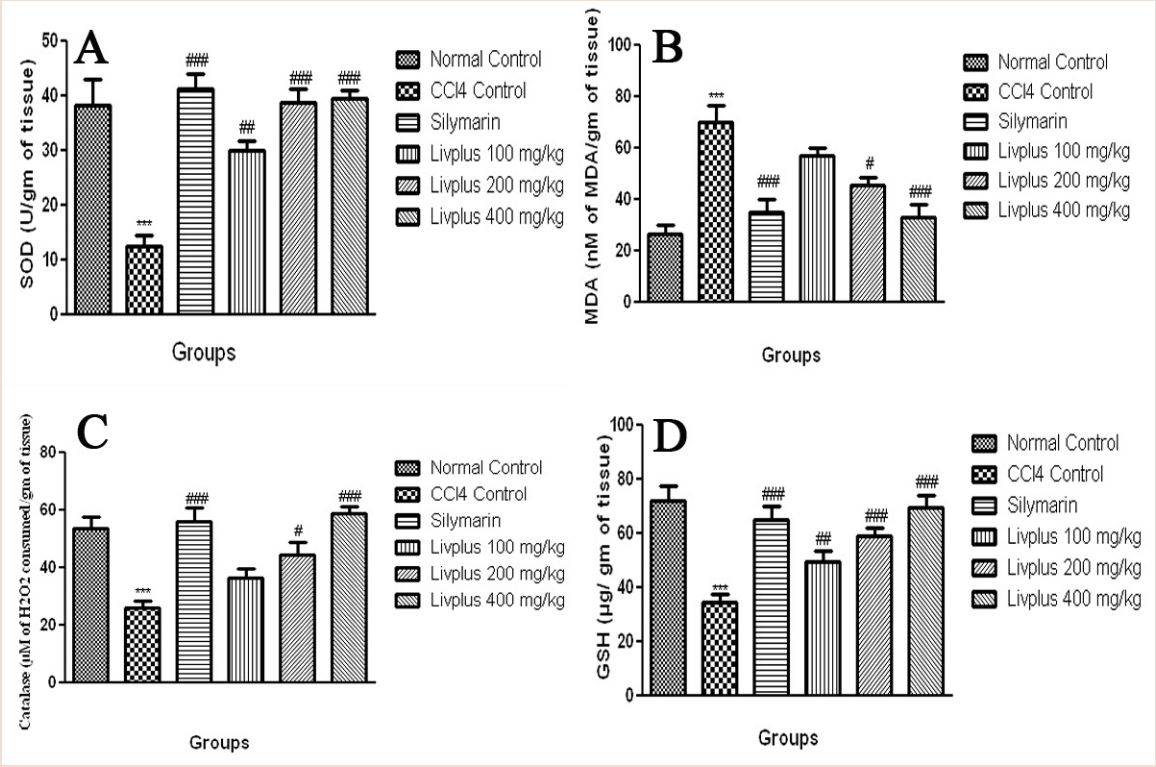

Figure 4: Effect of Livplus on (A) SOD, (B) MDA, (C) Catalase and (D) GSH in CCl_4-induced hepatotoxicity in rats
Values are expressed as Mean + S.E.M (n=6). Where, ***P<0.001 as compared to normal control; #P<0.05, ##P<0.01, ###P<0.001 as compared to CCl_4 control

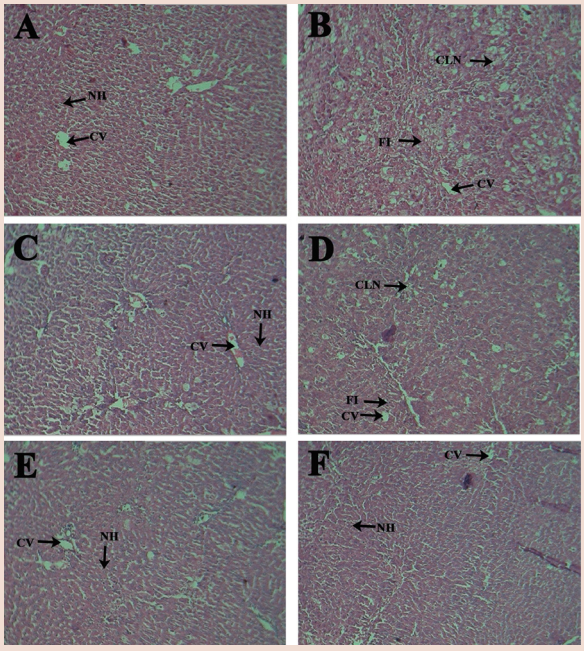

Figure 5: Effect of Livplus on histopathological changes in albino Wistar rat liver
(A) Normal control; (B) CCl_4 control; (C) Silymarine + CCl_4 treatment; (D) Livplus 100 mg/kg + CCl_4 treatment; (E) Livplus 200 mg/kg + CCl_4 treatment; (F) Livplus 400 mg/kg + CCl_4 treatment. Hematoxylin and eosin stain, CLN: central lobular necrosis; CV: central vein; FI: fatty infiltration; NH: normal hepatocytes

ports the traditional use of Livplus in the treatment of hepatotoxicity.

ACKNOWLEDGEMENT

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CONFLICTS OF INTEREST

We declare that we have no conflict of interest.

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Original Article

Comparative Pharmacognostic, Phytochemical and Biological evaluation between five *Chlorophytum* species

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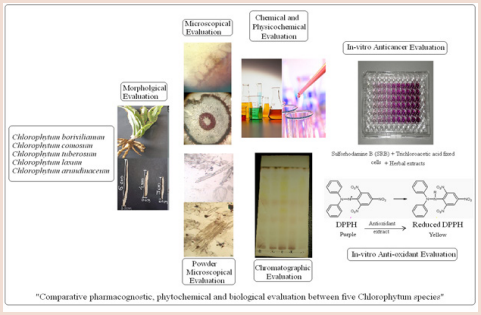
ABSTRACT

Objective: To establish comparative pharmacognostic, phytochemical and biological evaluation parameters between five *Chlorophytum species* i.e. *Chlorophytum borivilianum* Santapau and Fernades, *Chlorophytum comosum* (Thunb.) Jacq., *Chlorophytum tuberosum* Br., *Chlorophytum laxum* R. Br. and *Chlorophytum arundinaceum* Baker, of very popular Ayurvedic plant *Safed Musali*. **Materials and methods:** Comparative evaluations of Macro and microscopical, physico-chemical parameters of tubers of all five species were investigated and preliminary phytochemical analysis, estimation of major phytochemicals and TLC profiles were also carried out for qualitative phytochemical evaluation. *In-vitro* antioxidant and anticancer activity was carried out for extract of tubers of all five species. **Results:** Macro, micro, powder microscopical parameters of tubers of five species were examined and recorded the result. Tubers of all the five species are distinct in their morphology as well as anatomical characters. Physicochemical characters (Ash values, Loss on drying (LOD), swelling index and foaming index) as well as total saponin content shows great variability among five species. Results of *In-vitro* antioxidant by DPPH method shows difference in antioxidant potential between tubers of all five species. Extract of tubers of all five species do not show any type of *In-vitro* anticancer activity by SRB method against HL 60 leukemia cell line. **Conclusion:** All of the evaluated parameters are very good pharmacognostic standards for future comparative identification and authentication of specific species because all five species shows morphological, anatomical, chemical differences as well as varies in antioxidant potential.

Key words: *Arundinaceum*, *Borivilianum*, *Chlorophytum* Comosum, DPPH, HL-60, Laxum, SRB, Tuberosum.

SUMMARY

- *Safed musli* is very popular Ayurvedic drug and ingredient of many herbal formulations.
- There are almost 215 species that have been reported in the genus *Chlorophytum*.
- Unfortunately, most of these species are indistinguishable.
- Incorrect identification affects quality and efficacy of medicinal products containing this plant.
- Evaluated parameters in this work are very good pharmacognostic standards for future comparative identification and authentication of specific species.



PICTORIAL ABSTRACT

Abbreviations used: LOD: Loss on drying, DPPH: 2,2-diphenyl-1-picrylhydrazyl, SRB: Sulforhodamine B, CB: *Chlorophytum borivilianum*, CC: *Chlorophytum comosum*, CT: *Chlorophytum tuberosum*, CL: *Chlorophytum laxum*, CA: *Chlorophytum arundinaceum*, AE: Aqueous extract, EE: Ethanolic extract, WHO: World Health Organisation, ACTREC: Advanced Center for Treatment, Research and Education in Cancer.

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INTRODUCTION

A number of species belonging to the genus Chlorophytum are noted for their medicinal benefits in Ayurvedic, and Unani system of medicine are popularly known as safed musli.[1] Traditionally, roots of these species are reputed to possess various pharmacological utilities like immunemodulation, adaptogenic, aphrodisiac and anti-stress properties due to saponins as one of the important phytochemical constituents. Safed musli is one of the ingredients of Chyawanprash, a very popular and useful Ayurvedic rasayana (rejuvenator) formulation. There are almost 215 species that have been reported in the genus Chlorophytum.[2,3] All are perennial rhizomatous herbs. Rhizomes are often short and inconspicuous while roots are usually thicker or slightly fleshy. The important plants which have so far been explored include *C. adescendens, C. borivilianum, C. laxum, C. tuberosum and C. comosum*.[4] Unfortunately, most of these species are indistinguishable morphologically in the field or from photographs as well as from literature and hence chances of incorrect identification are more which surely affects quality and efficacy of medicinal products containing this plant. So we aimed to establish comparative pharmacognostic (macroscopical, histological, powder microscopical, physicochemical and World Health Organisation (WHO) parameters) and phytochemical (preliminary phytochemical evaluation, saponin estimation and TLC profile of extract) diagnostic parameters.

Chlorophytum species are rich in both monodesmosidic saponins (oligosaccharide chain attached at C3 position) and bidesmosidic saponins (an additional sugar moiety at the C26 or C28 positions).[5,6] Saponins are a group of naturally occurring plant glycosides, characterized by their strong foam forming properties in aqueous solution. The presence of s ponins has been reported in more than 100 families of plants out of which at least 90 kinds of natural saponins have been found to possess significant anti-cancer properties. There are more than 11 distinguished classes of saponins including dammaranes, tirucallanes, lupanes, hopanes, ole-

ananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes and steroids.[7] Due to the great variability of their structures, saponins always display anti-tumorigenic effects through varieties of antitumor pathways.[8] In addition, there are a large amount of saponins that still either remain to be trapped or studied in details by the medicinal chemists. Free radicals are major cause of cancer cell development. Saponin, flavonoids and alkaloids are very well known to have immunomodulater, antioxidant and anticancer properties.[9] So in present thesis it is decided to evaluate saponin fractions of different *Chlorophytum* species for their probable *in vitro* antioxidant and *in vitro* anticancer potential.

MATERIALS AND METHODS

Material collection, identification and authentication

The plant materials were collected from in and around Amravati and Akola district (Maharashtra) during the rainy season of year 2012 and 2013 for correct botanical identification. Herbarium specimens were prepared of collected species of *Chlorophytum* and authenticated from Dr. Prabha Y. Bhogonkar (Director, Government Vidarbha Institute of Science and Humanities, Amravati) and Dr. Arvind S. Dhabe Professor, Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad.

Pharmacognostic evaluation, Macroscopic and Microscopic evaluation

Macroscopy of tubers was studied by observing the organoleptic characters such as color, size, texture and surface characteristics. Thin hand cut sections were taken from the fresh tubers of all collected species, transferred to a watch glass containing water with the help of brush. Collected species were also subjected to powder microscopy. Thin uniform sections and powder of collected species were mounted on a slide and were treated with different reagents such as Phloroglucinol and hydrochloric acid (to establish lignifications in cells and tissues), Iodine solution (to determine starch grains content), Sudan red Solution (to determine the presence of oil globules).[10]

Physicochemical evaluation

Tubers of collected species were dried under the shade so as to avoid the decomposition of chemical constituents, powdered in a multi-mill and stored in dry air tied containers for phytochemical screening. Ash values, LOD, swelling index, and foaming index was measured by following the standard pharmacopoeial techniques. Powder materials of collected species were subjected to fluorescence analysis. Qualitative phytochemical tests were carried out by standard methods. Quantitative phytochemical analysis was carried out for estimation of saponin content by gravimetric analysis.[11]

Phytochemical evaluation

Qualitative phytochemical tests were carried out by standard methods. Quantitative phytochemical analysis was carried out for estimation of saponin content by gravimetric analysis.[11]

Extraction and fractionation

The root powder of *C. borivilianum*, *C. comosum*, *C. laxum*, *C. tuberosum* and *C. arundinaceum* were extracted by microwave assisted extraction in methanol. In microwave extraction, microwave power was 20%, irradiation temperature was 40°C and extraction is carried out for 10 min. Methanolic extract of *C. borivilianum*, *C. comosum*, *C. laxum*, *C. tuberosum* and *C. arundinaceum* are subjected to thin layer chromatography to confirm the presence of saponin. Saponin of *C. borivilianum*, *C. comosum*, *C. laxum*, *C. tuberosum* and *C. arundinaceum* were isolated by precipitation method. Dissolve methanolic extract in methanol, and add diethyl ether dropwise until complete precipitation of saponin is obtained. Air dry saponin fraction and store in tightly closed container.[12]

Chromatographic Studies[13]

TLC of methanolic extracts of all five *Chlorophytum* species for 2 mobile phases done to evaluate qualitative difference for the presence of saponins in theses selected five species. All chromatographic parameters are given in Table 4.

In vitro antioxidant activity by DPPH method[14]

DPPH free radical scavenging method is used for determination of *in vitro* antioxidant activity. 1 ml different concentration of extract solution and standard (Ascorbic acid) were taken in different vials. To this 5 ml of methanolic solution of DPPH was added, shaken well & mixture was incubated at 37°C for 20 min. The absorbance was measured against methanol as blank at 516 nm. Absorbance of DPPH was taken as control. Percentage antiradical activity calculated by using following formula;

$$\% \text{ Anti-radical activiity} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} \times 100$$

In-vitro anticancer screening by SRB assay method

The anticancer activities of extracts were studied at Advanced Center for Treatment, Research and Education in Cancer (ACTREC), Mumbai. The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x 105 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100 µl of different test compound concentrations were added to the cells in microtitre plates. The plates were then incubated at 37°C for 72 hours in 5% CO_2 incubator, microscopic examination was carried out, and observations recorded every 24 hours. After 72 hours, 25 µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form overall concentration 10%. The plates were incubated at 4°C for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air-dried. The air-dried plates were stained with 100 µl sulforhodamine-B (SRB) and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. The air-dried plates were stained with 100 µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100 µl of 10mM Tris base was then added from wells to solubilize the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using formula;

$$\%cell\ inhibition = 100-\{(Ti-Tz)/(C-Tz)\} \times 100$$

Where, Ti = Absorbance value of test compound, Tz = Absorbance value of blank, C = Absorbance value of control.

RESULT AND DISCUSSION

Material collection, identification and authentication

Total five species of *Chlorophytum i.e. borivilianum, tuberosum, laxum, comosum* and *arundinaceum* were collected from different locations and identified as well as authenticated from Dr. Prabha Bhogonkar, Ex-HOD, Botany, Vidarbha Institute of Science and Humalities College, Amravati and Dr. Arvind S. Dhabe Professor, Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. Authenticated species further evaluated comparatively for their morphological, micro-

Figure 1: Comparative morphological evalaution between selected five *Chlorophytum* species

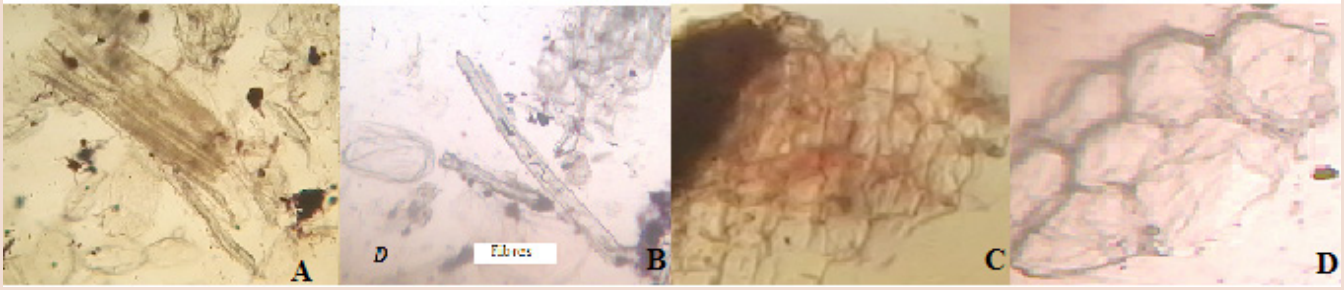
Table 1: Comparative morphological evaluation parameters of selected five *Chlorophytum* species

	Features	CB	CC	CT	CL	CA
Tubers	Color	White to off white	White to cream	Off white to cream	White to off white	Off white to cream
	Odour	Slight characteristics	Slight characteristic pleasant	Slight characteristic	Slight characteristic	Slight characteristic
	Size	2 to 4 cm in length and 0.5 cm in width	10 to 15 cm in length and 0.1-0.3 cm in width	3 to 7 cm in length and 0.9 to 1.5 cm in width	0.9 to 2.5 cm in length and 0.3 to 0.6 cm in width	3 to 8 cm in length and 0.3 to 0.6 cm in width
	Shape	Elongated, hard, tapering at both the ends, fracture is short	Elongated, hard, tapering towards bottom, fracture is short	Elongated, thick fleshy and tuber like, hard, tapering at both ends and short fracture	Elongated, hard, tapering at bottom with branching tread like structure, short fracture	Elongated, hard, tapering at both the ends, fracture is short
	No. of roots	8-10	10-15	15-20	14-16	8-10
Leaf	Length	25±1.5 cm	20±5.0 cm	22±5.0 cm	10±5 cm	12±4 cm
	Width	1.0±1.5 cm	1.0±0.5 cm	10±5 cm	0.4±0.2 cm	0.4±0.2 cm
	Color	Green colored	Green colored	Green colored	Light green colored with white margin at the edges	White colored midrib with green edges
	No. of leaves	5-12	5-12	5-10	6-10	5-12
Stem	-	Reduced with root stock	Reduced with root Stock	Reduced with root Stock	Reduced with root Stock	Reduced with root Stock
Flowers	-	White, arranged in alternate clusters bracts liner papery and purplish	Small single white with white petals in an alternating pattern	White, with Elliptic petals and erect stamens with yellow anthers.	Small paired white with greenish white petals	Small paired white with greenish white petals
Seeds	-	Black in colour, orbicular.	Flattish, black in color and shiny.	Lack in color with angular edges.	Black in colour with oval shaped	Black in colour with oval shaped

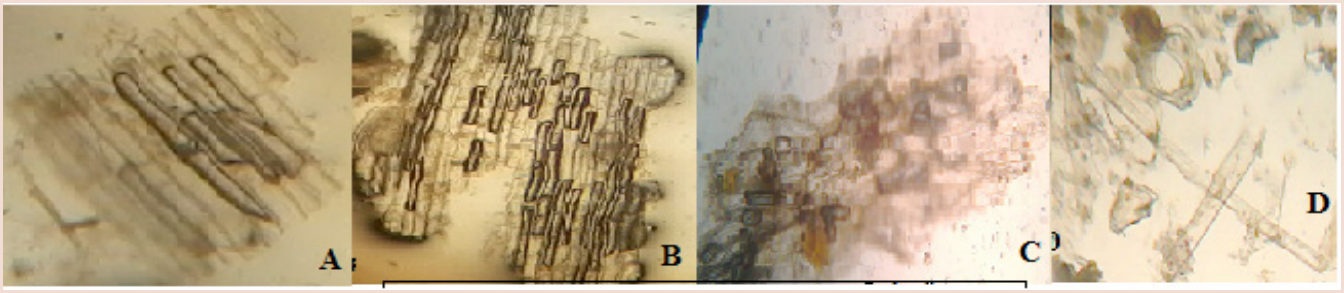
scopical, physicochemical and phytochemical parameters and also evaluated for their antioxidant and anticancer potential by *in-vitro* methods.

Pharmacognostic evaluation, Macroscopic and Microscopic evaluation

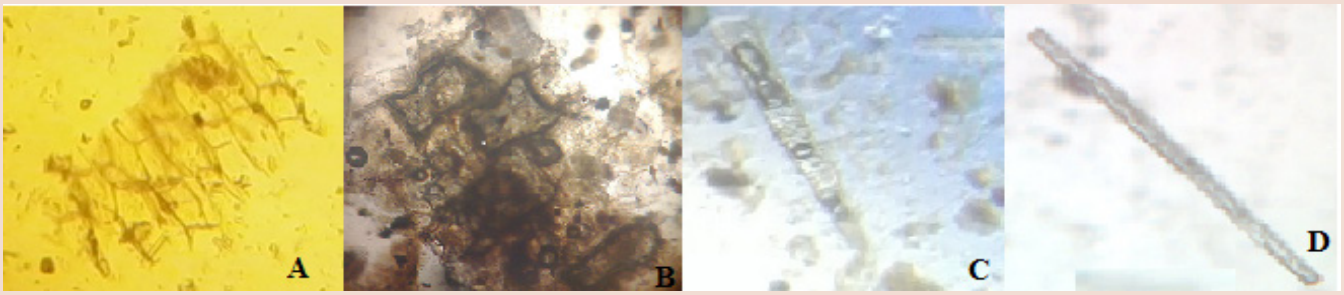
Macroscopy of whole plant of all five species of *Chlorophytum* was studied and summarised in Figure 1 and Table 1. Powder microscopy of tubers of all species of *Chlorophytum* was studied in detailed. It was examined under microscope first with low power (10X) and then magnified with 45 X. (Figure 2) Microscopical examination of *C. borivilianum* shows fragments of lignified reticulate vessels, fibres with thin heavily lignified, pointed or bifurcated ends, thin walled elongated large packed parenchymatous cells, and thin walled colorless cork cells. Microscopical examination of *C. comosum* shows sclerenchymatous fibres, thin walled cork cells filled with few colorless and few are with yellowish brown matter, tracheids with thin walled, pitted lignified xylem vessels. Microscopi-



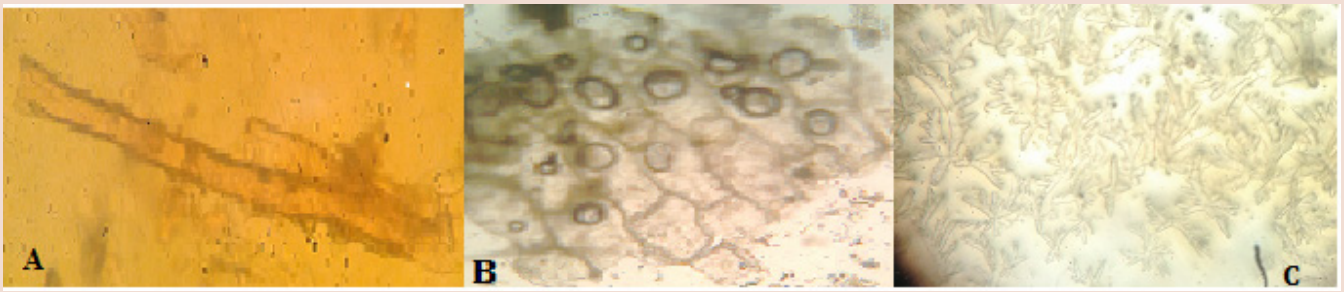
Chlorophytum borivilianum-A: Vessels-Fragments of lignified reticulate vessels; B: Fibre-Thick, heavily lignified with pointed or bifurcated ends; C: Parenchyma-Thin walled, elongated large cell, packed; D: Cork cells-Thin walled, colorless cork cells



Chlorophytum comosum-A: Vessels; B: Xylem- Tracheids with thin walled, pitted lignified cells; C: Cork cells- Thin walled, few colorless and few are with yellowish brown matter; D: Fibre-Sclerenchymatous fibres



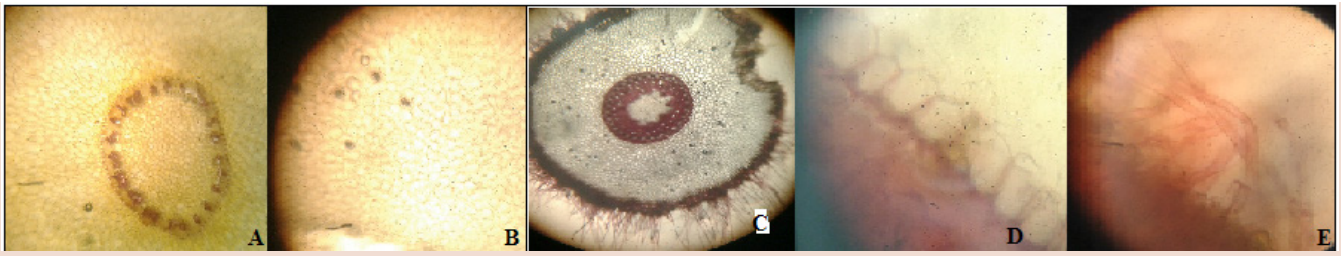
Chlorophytum tuberosum-A: Parenchyma- Lignified, pitted xylem parenchymatous cells, filled with some yellowish brown matter; B: Cork cells- Brownish thick walled, wavy cells containing brown matter (saponin, mucilage); C: Xylem vessels- Spiral tracheids with tapering end. D: Fibre Sclerenchymatous fibres



Chlorophytum laxum-A: Fibre- Sclerenchymatous fibres; C: Cork cells- Thin walled filled with brownish matter; C: Calcium oxalate crystals

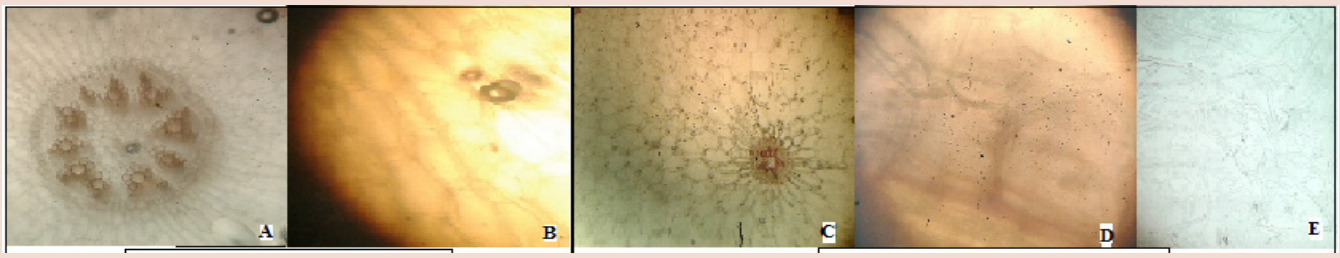
Chlorophytum arundinaceum-A: Fibres- Numerous, stratified thin walled lignified fibres; B: Cork cells -Thick walled, few colorless and few are with yellowish brown matter; C: Calcium oxalate crystals: Needle, oval shaped

Figure 2: Comparative powder microscopical evalaution between tubers of selected five *Chlorophytum* species



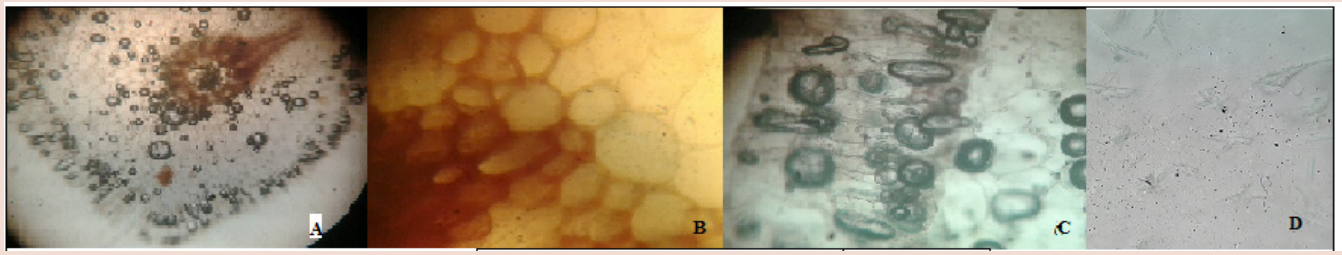
Transverse section of *C. borivilianum* **Transverse section of *Chlorophytum comosum***

A: T. S. of *C. borivilianum* root showing presence of pith i. e. parenchymatous cells, endodermis; B: cortex region showing polygonal cells, cork cells which are present at outer layer; C: T. S. of root *C. comosum* showing presence of pith, parenchymatous cells, endodermis; D: cork cells which are present at outer layer; E: covering trichomes



Transverse section of *C. tuberosum* **Transverse section of *Chlorophytum laxum***

A: T. S. of *C. tuberosum* root showing presence of pith, parenchymatous cells, endodermis; B: cork cells which are present at outer layer; C: T. S. of C. laxum root showing presence of pith, parenchymatous cells, endodermis; D: cork cells which are present at outer layer; E: Calcium oxalate crystals.



Transverse section of *C. arundinaceum*

A: T. S. of root showing presence of pith, parenchymatous cells, endodermis, cork cells; B: cortex region; C: cork cells which are present at outer layer; C: Calcium oxalate crystals; D: calcium oxalate crystals needle shaped.

Figure 3: Comparative histological evalaution between tubers of selected five *Chlorophytum* species

cal examination of *C. tuberosum* shows sclerenchymatous fibres, lignified, pitted xylem parenchymatous cells, brownish thick walled cork cells containing brown matter, spiral tracheids of xylem vessels with tapering ends. Microscopical examination of *C. laxum* shows sclerenchymatous fibre, thin walled cork cells filled with brownish matter, feather shaped calcium oxalate crystals. Microscopical examination of *C. arundinaceum* shows sclerenchymatous fiber, thick walled cork cells few colorless and few are with yellowish brown matter, some are needle and some are oval shaped calcium oxalate crystals. Transverse section of tubers (Figure 3) is observed under microscope first with low power i. e. 10 X and then magnified with 45 X. On microscopical examination it shows presence of:

- **Cortex:** This is followed by a very large zone of cortex. The outermost layer of the cortex (which is outermost boundary in most pieces) just below the epidermis consist of cells which are of mostly rectangular, appearing much longer than wide. The rest of the cortical cells are rounded of polygonal, parenchymatous and have little or no intercellular spaces (probably due to swelling).
- **Vascular bundle:** The vascular tissue is not very elaborate. Xylem is consisting of joined vessels, 3-5in number in each group. There are about 30-35 groups of xylem. However fibres are quite abundant, surrounding the vessels are joined to form more or less continuous irregular ring. Phloems are arranged just above the xylem.
- **Trichomes:** An elongated outgrowth of an epidermal cell is termed as trichome or plant hair.

Physicochemical evaluation

Total ash includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological" ash, which is the residue of the extraneous matter adhering to the plant surface. Acid insoluble ash measures the amount of silica and siliceous earth. Water-soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water. These values are useful for detecting low grade products, exhausted drug and excess of sandy or earthy matter. The LOD can help in deciding the percentage of moisture should be allowed in the dried crude drug which will not affect its shelf-life. Swelling index is determined to calculate the amount of mucilage present in drug. Foaming index is calculated to know the frothing property of drug. Total saponin content is determined for quantitative analysis of saponin. All determined physicochemical constants are given in Table 2 which can further used for standardisation of selected plants.

Phytochemical evaluation

Powder of *C. borivilianum*, *C. comosum*, *C. tuberosum*, *C. laxum* and *Chlorophytum arundinaceum* species extracted with ethanol and water. The aqueous and ethanolic extracts of collected species are tested for dif-

Table 2: Physicochemical parameters for selected five *Chlorophytum* species

Physical constants	CB	CC	CT	CL	CA
Total ash value [% w/w]	4.08	8.22	7.17	6.61	6.81
Acid insoluble ash [% w/w]	0.15	0.91	0.66	0.45	0.59
Water soluble ash [% w/w]	0.61	1.89	1.16	0.90	0.92
Loss on drying [% w/w]	20.1	21.9	27.5	16.1	10.8
Swelling index	6.00	4.00	6.50	6.50	7.00
Foaming index	250	333.33	333.33	>100	142.82
Total saponin content	4.5 %	6.5 %	6.0 %	1.0 %	1.5 %

CB: *Chlorophytum borivilianum*, CC: *Chlorophytum comosum*, CT: *Chlorophytum tuberosum*, CL: *Chlorophytum laxum*, CA: *Chlorophytum arundinaceum*

Table 3: Preliminary Phytochemical Evaluation for selected five *Chlorophytum* species

Test	Inference									
Species ⇨	CB		CC		CT		CL		CA	
Extracts ⇨	AE	EE	AE	EE	AE	EE	AE	EE	AE	EE
Phytochemical class ⇩										
Carbohydrates	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Proteins	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Alkaloids	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
Cardiac Glycoside	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Anthraquinone Glycoside	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Cynogenetic Glycoside	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Coumarin Glycoside	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Saponin Glycoside	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Flavonoids	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Tannins	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve
Steroid or Triterpenoid	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Saponin	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
For Powder Drug										
Gum	-ve	-	-ve	-	-ve	-	-ve	-	-ve	-
Mucilage	+ve	-	+ve	-	+ve	-	+ve	-	+ve	-
Fatty Oil	-ve	-	-ve	-	-ve	-	-ve	-	-ve	-
Essential Oil	-ve	-	-ve	-	-ve	-	-ve	-	-ve	-

CB: *Chlorophytum borivilianum*, CC: *Chlorophytum comosum*, CT: *Chlorophytum tuberosum*, CL: *Chlorophytum laxum*, CA: *Chlorophytum arundinaceum*, AE: Aqueous extract, EE: Ethanolic extract. +ve: Present, -ve: Absent.

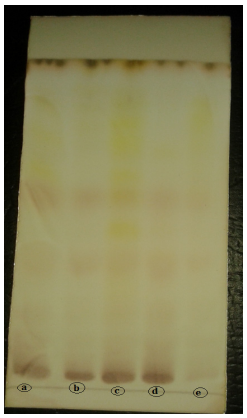
ferent phytoconstituents like carbohydrates, proteins, alkaloids, glycosides, saponins, tannins, terpenoids, flavonoids, protein, mucilages and volatile oils. (Table 3) The Knowledge of the chemical constituents of plants is desirable because such information will be valuable for synthesis of complex chemical substances and to screen for biological activities. A preliminary phytochemical evaluation of *C. borivilianum* reveals presence of carbohydrates, alkaloids, saponin, flavonoid, tannin, saponin glycosides and mucilages. A preliminary phytochemical evaluation result of *C. comosum* reveals presence of carbohydrates, saponin, flavonoid, steroids or triterpenoid, saponin glycosides and mucilages. A preliminary phytochemical evaluation result of *C. tuberosum* reveals presence of carbohydrates, alkaloids, saponin, flavonoid, steroids or triterpenoid, saponin glycosides and mucilages. A preliminary phytochemical evaluation result of *C. laxum* reveals presence of carbohydrates, alkaloids, saponin, flavonoid, tannin, saponin glycosides and mucilages. A preliminary phytochemical evaluation result of *Chlorophytum arundinaceum* species reveals presence of carbohydrates, alkaloids, saponin, flavonoid, tannin, saponin glycosides and mucilages. *C. comosum* contain large amount of alkaloids, saponins as compared to other 4 species. The saponins, phenolic and flavonoids are widely distributed secondary metabolites in plants having anti-oxidant activity and wide range of biological activities as anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammatory, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities.

Chromatographic Studies

In Thin Layer Chromatographic (TLC) studies, it is observed that maximum saponins are separated in mobile phase A. Maximum numbers of saponins (6) are observed in tuberosum species. On the basis of RF (Retention factor) values, few saponin bands are same in all species ex-

Table 4: Details of TLC evaluation of all *Chlorophytum* species

	Mobile Phase A Chloroform: Methanol: Glacial acetic acid: water (6.5:3.2:1.2:0.8)		Mobile Phase B Chloroform: methanol: water (7.0:3.0:0.4)	
Species	**Total Spot**	**Spot details**	**Total Spot**	**Spot details**
C. borivilianum	4	Light brown 0.191, Light brown 0.375, Light brown 0.559, Yellow 0.632.	2	Light brown 0.100 Light brown 0.319
C. comosum	3	Light brown 0.194, Light brown 0.353, Light brown 0.568	2	Light brown 0.100 Light brown 0.319
C. tuberosum	6	Light brown 0.180, Light brown 0.360, Yellow 0.461, Light brown 0561, Yellow 0.626, Yellow 0.763	2	Light brown 0.100 Light brown 0.308
C. laxum	4	Light brown 0.201, Light brown 0.360, Light brown 0.561, Yellow 0.691	2	Light brown 0.100 Light brown 0.310
C. arundinaceum	4	Light brown 0.209, Light brown 0.216, Light brown 0.554, Yellow 0.763	2	Yellow 0.100 Light brown 0.310



TLC plate of Mobile Phase-A

TLC plate of Mobile Phase-B

a: Spot of methanolic extract of *C. borivilianum*, b: Spot of methanolic extract of *C. comosum*, c: Spot of methanolic extract of *C. tuberosum*, d: Spot of methanolic extract of *C. laxum*, e: Spot of methanolic extract of *C. arundinaceum* plant.

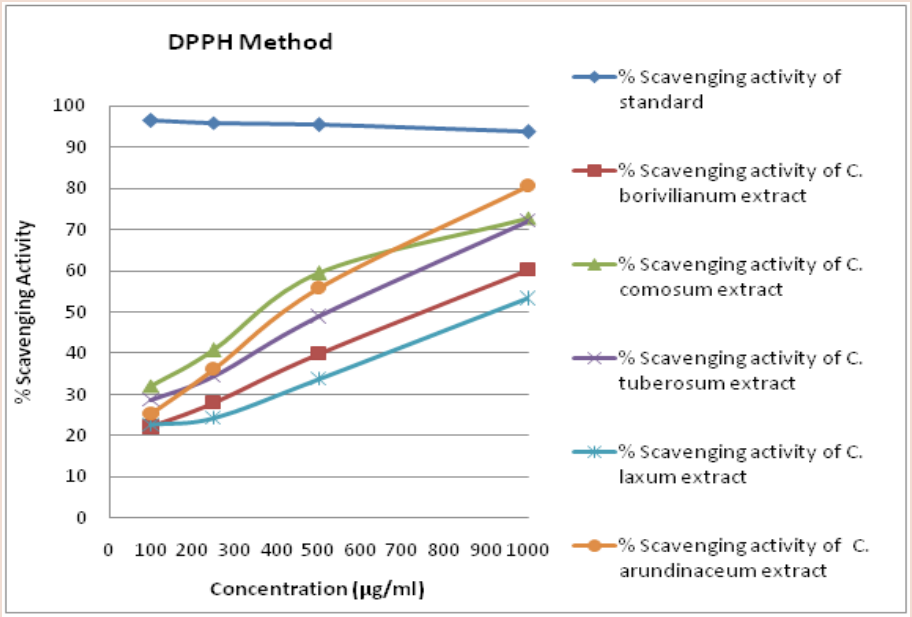


Figure 4: Percentage scavenging activity of selected five species of *Chlorophytum* species

cept *tuberosum* and *arundinaceum* species where extra bands are also observed at different RF values. (Table 4) It is observed that maximum sapnins are separated in mobile phase A. Maximum number of saponins (6) are observed in tuberosum species. Same saponin pattern is observed for few saponins in all species except tuberosum and arundinaceum species.

In-vitro Antioxidant Activity

In-vitro antioxidant activity of methanolic extract of C. *borivilianum*, *C. comosum*, *C. tuberosum*, *C. laxum* and *C. arundinaceum* species was performed by DPPH scavenging radical activity. Result demonstrates that all plant extract have antioxidant activity. (Figure 4) Among all species, *C. arundinaceum* shown strong antioxidant activity, while *C. comosum* and *C. tuberosum* shown significant activity, *C. borivilianum* shown moderate activity and *C. laxum* shown weak activity.

In-vitro anticancer screening by SRB assay method

Saponins belong to a group of naturally derived compounds, which have demonstrated substantial cytotoxic activity through different mechanisms. With this background we decided to evaluate probable anticancer effect of saponins of *Chlorophytum* species on leukemia cell lines. In-vitro anticancer activity of methanolic extract and saponin fraction of *C. borivilianum, C. comosum, C. tuberosum, C. laxum* and unidentified *Chlorophytum* species was studied by SRB assay method on HL60 leukemia cell line. Result demonstrates (Table 5) that *C. comosum* methanolic extract inhibited the growth of HL 60 cells to certain extent but not potent in effect. It is also found that remaining species extracts and fractions do not possess anticancer effect against leukemia cell line, but may act as anticancer agent against other types of cancer. Hence future scope involves screening Saponins of *Chlorophytum* species on different cancer cell lines.

Table 5: *In-vitro* anticancer activity results by SRB method

Cell line	Drug concentration (µg/ml) calculated from graph		
HL 60	LC50	TGI	GI150
Test 1	>80	>80	>80
Test 2	>80	>80	57.2
Test 3	>80	>80	>80
Test 4	>80	>80	>80
Test 5	>80	>80	>80
Test 6	>80	>80	>80
Test 7	>80	>80	>80
Test 8	>80	>80	>80
Test 9	>80	>80	>80
Test 10	>80	>80	>80
ADR	>80	32.0	<10

Where Test 1: *C. borivilianum* extract, Test 2: *C. comosum* extract, Test 3: *C. tuberosum* extract, Test 4: *C. laxum* extract, Test 5: *C. arundinaceum* extract, Test 6: *C. borivilianum* saponin fraction, Test 7: *C. comosum* saponin fraction, Test 8: *C. tuberosum* saponin fraction, Test 9: *C. laxum* saponin fraction, Test 10: *C. arundinaceum* saponin fraction and ADR: Adriamycin (Doxorubicin). Known drug GI50 : Growth inhibition of 50 % (GI50) calculated from [(Ti-Tz)/(C-Tz)] x 100 = 50, drug concentration resulting in a 50% reduction in the net protein increase TGI: Drug concentration resulting in total growth inhibition (TGI) will calculated from Ti = Tz LC50 : Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of 50% cells following treatment is calculated from [(Ti-Tz)/Tz] x 100 = -50.

CONCLUSION

In conclusion all collected species extract have antioxidant activity may be due to presence of saponin, tannins or flavonoids. Unknown *Chlorophytum* species plant is more efficient antioxidant agent as compared to other collected (four) species while *C. comosum* and *C. tuberosum* significant antioxidant activity and *C. borivilianum* and *C. laxum* give moderate antioxidant activity. While methanolic extract and saponin fraction of *C. borivilianum*, *C. comosum*, *C. tuberosum*, *C. laxum* and *C. arundinaceum* do not possess *in-vitro* anticancer effects against HL 60 leukemia cell line, but may act as anticancer against other types of cancer due to presence of saponins, flavonoids and alkaloids. Finally it can be concluded that all five species have morphological, microscopical, chemical differences and even same for antioxidant activity. But all species do not found anticancer against HL-60 except *C. comosum* which have IC_{50} is 57.2% and other above 80%.

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