Phytochemicals Having Neuroprotective Properties from Dietary Sources and Medicinal Herbs

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ABSTRACT

Many neuropsychiatric and neurodegenerative disorders, such as Alzheimer's disease, anxiety, cerebrovascular impairment, depression, seizures, Parkinson's disease, etc. are predominantly appearing in the current era due to the stress full lifestyle. Treatment of these disorders with prolonged administration of synthetic drugs will lead to severe side effects. In the recent years, scientists have focused the attention of research towards phytochemicals to cure neurological disorders. Nootropic herb refers to the medicinal role of various plants/parts for their neuroprotective properties by the active phytochemicals including alkaloids, steroids, terpenoids, saponins, phenolics, flavonoids, etc. Phytocompounds from medicinal plants play a major part in maintaining the brain's chemical balance by acting upon the function of receptors for the major inhibitory neurotransmitters. Medicinal plants viz. *Valeriana officinalis, Nardostachys jatamansi, Withania somnifera, Bacopa monniera, Ginkgo biloba* and *Panax ginseng* have been used widely in a variety of traditional systems of therapy because of their adaptogenic, psychotropic and neuroprotective properties. This review highlights the importance of phytochemicals on neuroprotective function and other related disorders, in particular their mechanism of action and therapeutic potential.

Key words: Neuroprotection, Phytochemicals, Medicinal herbs, Nootropics, Dietary sources.

INTRODUCTION

Neuroprotection refers to the strategies and relative mechanisms able to struggle down the Central Nervous System (CNS) against neuronal damage caused by various neuropsychiatric and neurodegenerative disorders such as Alzheimer's disease, anxiety, cerebrovascular impairment, seizures, Parkinson's disease, etc. Neurodegenerative diseases are estimated to be the second most common cause of death among elderly by the 2040s.[1] Among the strategies for neuroprotection, phytochemicals may represent a valuable remedy in preventing neurodegenerative diseases. Many categories of natural and synthetic neuroprotective agents have been reported, however, synthetic neuroprotective agents are believed to have certain side effects such as dry mouth, tiredness, drowsiness, sleepiness, anxiety or nervousness, difficulty with balance, etc. Herb based medicated products have drawn considerable awareness from research bases and industries in recent years at the national and international levels.[2] Hence, there has been intense interest focussed on the part of potential phytochemicals to modulate neuronal function and protective mechanism against neurodegeneration. As complementary and alternative therapy, herbal medicine or phytotherapy, refers to the medical utilization of plant components (leaves, stems, roots, flowers, fruits and seeds) for their curative properties. Generally, herbal products contain a variety of bioactive phytochemicals, including alkaloids, steroids, terpenoids, saponins, phenolics, flavonoids, etc. and it is difficult to specify which part of the herb has biologically active for special discourse. The researchers around the globe are searching for bioactive phytochemicals from the herbs which are being used as neuroprotective in traditional medicine like Chinese medicinal system, Indian Ayurvedic medicine system, Korean system of medicine, Mediterranean system of medicine, etc. This review will highlight the medicinal herbs from different traditional systems, their bioactive phytochemicals on neuroprotective function and other related disorders, in particular their mechanism of action

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and therapeutic potential.

Neurodegenerative diseases

Neurodegenerative diseases are progressive disorders of central nervous system that affect on the central and peripheral nervous systems. Gradual and progressive loss of neural cells may lead to neurodegeneration. Many factors are known to play a direct role in the initiation of neurodegeneration, free radical formation by the Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) is the main causative factor. Neuroinflammatory process is known to play a crucial role in the progress of several neurodegenerative diseases. According to the reports of the National Institute of Neurological Disorders and Stroke, there were more than 600 neurological disorders recorded worldwide.[3] Some of them were briefly discussed in this review at length.

Anxiety

Anxiety is characterised by psychological and physiological state in which cognitive, somatic, emotional, and behavioral components are involved. It can increase to an extent that may interfere with even normal routine of life and person may feel apprehensive regarding happenings of normal things in life. Anxiety disorders comprise seven clinical conditions[4] such as (a) Generalized Anxiety Disorder (GAD): In this condition person will have persistent fear and worry and become overly concerned with everyday matters. (b) Panic disorder: Person suffers from brief attacks of intense terror and apprehension, often marked by confusion, dizziness, trembling, shaking, nausea, difficulty breathing. (c) Phobias: In which fear and anxiety is triggered by a specific stimulus or situation. Agoraphobia: It is specific anxiety about being in a place or situation where escape is difficult or embarrassing or where help may be unavailable. (d) Social Anxiety Disorder (SAD): It is an intense fear of negative public scrutiny or of public embarrassment or humiliation. (e) Obsessive-Compulsive Disorder (OCD): It is primarily characterized by repetitive obsessions and compulsions. (f) Post-Traumatic Stress Disorder (PTSD): It is resulted from an extreme situation, such as major accident, child abuse, war situation, natural disaster, rape, hostage situations, etc. (g) Separation anxiety disorder: It is the feeling of inappropriate or excessive levels of anxiety over being separated from a place or person. Monoamines (dopamine, noradrenaline and serotonin), neuropeptides (galanin, neuropeptide Y, arginine vasopressin, tackykinin), neurosteroids and cytokines have been observed to play a modulators role in anxiety states.

Attention Deficit/ Hyperactivity Disorder

Attention Deficit/Hyperactivity Disorder (ADHD) is a loosely defined assemblage of neuropsychiatric symptom clusters that emerge in childhood and often persist into adulthood. ADHD is observed with increasingly employed as a diagnostic label for individuals who display a wide range of symptoms, such as inability to stay focused, restlessness, mood swings, temperament, problems in completing tasks, disorganization and inability to cope with stress.[5]

Depression

The term depression indicates a dampened mood and pervasive unhappiness. Depression is one of the most common emotional disorders, it may be manifested in varying degrees from feelings of slight sadness to utter misery and dejection. Changes in mood and the occurrence of depressive symptoms are determined by the biological as well as an environmental factor. According to World Health Organization (WHO), approximately 450 million people are suffering from a mental or behavioral disorder, and is predicted to increase significantly by 2020. Depression is one of the top ten causes of morbidity and mortality worldwide based on a survey by the WHO. In depressed conditions, there are several structural alterations of neurons that will take place such as, a decreased volume of the frontal cortex and hippocampus, dysfunctions of HPA axis, abnormalities in 5-HT and its receptors.[6] It is well established that the 5-HT is a monoaminergic neurotransmitter which is also dysregulated in other neurological disorder conditions such as, anxiety and schizophrenia.[6]

Dementia

Parkinson's Disease (PD) is a serious motor disorder and the most common brain degenerative disease in humans.[7] The loss of dopaminergic neurons in the substantia Nigeria is the most pervasive factor to contribute the characteristic symptoms of PD. It is characterized by four main symptoms: tremor, rigidity, bradykinesia and impairment of balance. The classic pathological findings are the presence of Lewy bodies in the substantia nigra, and loss of nerve cells in portions of its ventral tier.[7] Besides the neuropathologic symptoms, PD can be caused neurochemically by a consistent deficit in cholinergic neurotransmission. Alzheimer's Disease (AD) is a neurodegenerative disorder, which is characterized by deficit, learning and memory loss followed by cognitive disorders like depression, agitation and psychosis.

Epilepsy

Epilepsy is the most common neurological disorder, which affects almost 50 million worldwide.[8] It is a characterized by unpredictable occurrence of periodic epileptic seizures, i.e. involuntary contraction of striated muscle repeatedly. Seizures take place due to excessive and rapid discharge of cerebral neurons in the gray matter of the brain. In epilepsy, impaired voltage-sensitive sodium and calcium-channel functioning impaired GABA-mediated inhibition and excessive glutamate-mediated neurotransmission may trigger a cascade of events leading to neuronal damage and cell death.

Excitotoxicity

Excitotoxicity plays a key role in intoxication of some poisons like domoic acid (Amnesic shellfish poison by marine algae), kainic acid, etc. Excitotoxicity is the pathological process by which nerve cells are damaged and killed by excessive glutamate stimulation. Excitotoxicity may also express in different pathological conditions like, spinal cord injury, traumatic brain injury, multiple sclerosis, stroke, amyotrophic lateral sclerosis, and other neuronal disorders.[9] It is also involved in most aspects of normal brain functions including cognition, memory, and learning. Excitotoxicity can occur through over-activation of the NMDA receptor with the subsequent influx of Ca^{2+}, activation of both noise and iNOS, and excess generation of NO•.[10]

Schizophrenia

It is one of the most important forms of psychiatric illness. In this condition patient don't know what is happening at present. The symptoms of this disease are 2 types: 1) positive symptoms: delusions, hallucination, thought disorders, abnormal behavior. 2) Negative symptoms: withdrawal from social contact, flattening of emotional responses. The level of neurotransmitters such as dopamine, 5-HT, acetylcholine and norepinephrine levels upregulate in the brain in this condition.[11]

Neuroregeneration

Neuroregeneration is a concept which encompasses endogenous neuroprotection leading to neuroplasticity and neurite outgrowth.[12] It has been suggested that restoration of the neuronal and synaptic networks in the injured brain is necessary for the recovery of brain functions. It was once believed that nerve regeneration in the mammalian Central Nervous System (CNS) was irreversible, but recently it has become apparent that damaged neurons do regenerate in an active process under the presence of stimulatory substances such as Nerve Growth Factor (NGF) and Brain Derived Neurotrophic Factor (BDNF).[13] The role of neuroprotection involves a number of effects including a potential role against injury induced by neurotoxic species, an ability to suppress neuro-inflammation and to promote effective cognitive function. Recent studies suggest that different traditional neuroprotective herbal extracts/ isolated phytochemicals have potential role in prevention of neurodegenration. Evidence also exists for the neuromodulatory effects of phytochemicals and suggests that these phytochemicals may induce beneficial effects on the vascular system leads to change in cerebrovascular blood flow and modulation of neuronal function. Hence natural products may harmonize very well for the treatment of neuronal injury.[14] Lately, many compounds from natural sources have been demonstrated to possess neurotrophic and neuroprotective abilities.

Phytochemical based antioxidants in neuroregeneration

Phytochemical based antioxidants may have neuroprotective and neuroregenerative roles by reducing or reversing cellular damage and by slowing progression of neuronal cell loss.[15] In nature, antioxidants are grouped as endogenous or exogenous, the endogenous group includes enzymes like Superoxide Dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), and several proteins like albumin, ceruloplasmin, Haptoglobin and transferring. The most important exogenous antioxidants are dietary phytochemicals (polyphenols, phenolic acids, flavonoids, terpenoids, saponins etc.) and vitamins (ascorbic acid, alpha-tocopherol, and beta-carotene).[16] Antioxidants offer a promising approach in the control or slowing down progression of several neurodegenerative disorders leading to changes in cerbrovascular bood flow such as, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, ischaemic stroke, haemorrhagic stroke and Parkinson's disease.[16] In normal condition, a steady-state exists between pro-oxidants and antioxidants. However, when the rate of free radical generation exceeds the capacity of antioxidant defenses, oxidative stress promotes consequential severe damage to cellular machinery. Oxidative stress is associated with dysfunction in mitochondrial and endoplasmic reticulum, which includes apoptosis and protein misfolding in neurons. During neurodegenerative state, there would be decreased activities of antioxidant enzymes such as SOD, CAT, GPx, and GSH, which signify the role of antioxidants in neuroprotection.

An increase in Reactive Oxygen Species (ROS) and Reactive

Nitrogen Species (RNS) have also been proposed to play major role in many neurodegenerative disorders.[17] Redox regulation of neuronal survival at the level of transcription, which includes transcription factor alternations such as nuclear factor-ϰB (NF-ϰB), activating protein-1 (AP-1), nuclear factor erythroid 2 (Nrf2), and Hypoxia Inducible Factor (HIF) may affect overall oxidative status.[18] Furthermore, redox regulation of neuronal survival at the signal-transduction level influences the mitogen-activated protein kinase and PI3K/Akt pathways, as well as several redox sensitive apoptotic effectors such as caspases, Bcl-2, and cytochrome C, and their functions can be significantly affected by cellular ROS. There is an abundant evidence that flavonoids are effective in blocking oxidant induced neuronal damage by preventing the activation of caspase-3. The flavanols, epicatechin and 3'-O-methyl- epicatechin protect neurons against oxidative damage by suppressing JNK, C-gun and pro-caspase-3.[19] The flavanones, hesperetin, 5-Nitro-hespertin have observed to inhibit oxidant-induced neuronal apoptosis by activation of important protein and lipid kinase signalling pathways.[19]

Traditional herbs for neuroprotection

Nootropic is a term used for the medical drugs or nutritional supplements that have a positive effect on brain function.[20] A number of pharmaceutical compounds are in the market, which have been used for their neuroprotective property by altering the balance of particular brain functioning chemicals (neurotransmitters). Some of the drugs act on the enhancement of cerebral blood flow, metabolic rate of cerebral oxygen usage and metabolic rate of cerebral glucose in chronic impaired human brain function, i.e., stroke, poor brain blood flow, dementia and pseudo dementia. Several medicines are derived from the medicinal herbs and have shown memory enhancing properties by virtue of their proactive phytochemical constituents. One of the mechanisms suggested to dementia is decreased cholinergic activity in the brain. Therefore, cholinergic drugs (of plant origin) like: Muscarinic agonists (e.g. Arecoline, Pilocarpine etc.), nicotinic agonists (e.g. Nicotine) and cholinesterase inhibitors (e.g. Huperzine) can be employed for improving memory.[20] Some other classes of drugs used in dementia are: Stimulants or Nootropics (e.g. Piracetam, amphetamine), putative cerebral vasodilators (e.g. Ergot alkaloids, papavarine) and calcium channel blocker (e.g. Nimodipine).

In the present era, a sudden increase in neurological disorders and recognition of severe side effects associated with long term administration of synthetic drugs has alarmed the attention of researchers towards natural resources. Medicinal plants like *Bacopa monniera, Ginkgo biloba, Panax ginseng, Nardostachys jatamansi, Valeriana officinalis* and *Withania somnifera* have been used extensively in various traditional systems of therapy because of their adaptogenic, psychotropic and neuroprotecive properties. *Ginkgo biloba* has been prescribed in the Europe over the past two decades for the treatment of cerebral insufficiency, which is roughly equivalent to vascular dementia, includes memory loss, absent minds, confusion, depression, anxiety, dizziness and headache.[21] Recently, several traditional Chinese medicines and Indian herbal medicines (Ayurveda) have been reported to be neuroprotective via regeneration of the neuronal network. There are more than 120 traditional medicines that are being used for the therapy of CNS disorders in Asian countries.[22] In the Indian system of medicine the following medicinal plants have shown promising activity in neuropsychopharmacology: *Acorus calmus, Allium sativum, Angelica sinensis, Bacopa monniera, Centella asiatica, Celastrus paniculatus, Crocus sativus, Curcuma longa, Enhydra fluctuans, Ginkgo biloba, Glycyrrhiza glabra, Huperiza serrata, Hypericum perforatum, Nicotiana tabaccum, Physostigma venosum, Salvia officinalis, Terminalia chebula, Uncaria tomentosa, Valeriana wallichii, Withania somnifera* etc. Some of herbs being used in Chinese medicine are: *Angelica pubescens, Ledebouriella divaricata, Ligusticum chuanxiong, Morus alba, Salvia miltiorrhiza, Scutellaria baicalensis* and *Uncaria rhynchophylla*. Table-I describes an overview of some of the medicinal herbs with nootropic properties and their mechanism of action.

Biochemical action of some of the neuroprotective herbs

Bacopa monniera

"*Brahmi*," has been used in the Ayurvedic system of medicine for centuries as a brain tonic for learning, memory enhancement and anti stress agent in anxiety.[23] it contains many bio active phytochemicals like herpestine (alkaloids), d-mannitol, hersaponin and monnierin (saponins). Major active constituents have been identified as numerous bacosides and bacopasaponins (Figure.I-I). The constituents responsible for *Bacopa*'s cognitive effects are bacosides A and B.[24] The bacosides aid in repair of damaged neurons by enhancing neuronal synthesis, kinase activity, restoration of synaptic activity, and nerve impulse transmission.

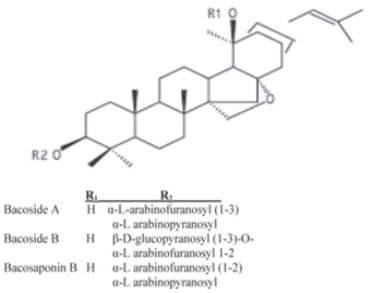
Centella asiatica

Centella asiatica is belonging to the family Apiaceae *(Umbelliferae)* is a psychoactive medicinal plant being used

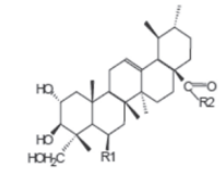
Table 1: Medicinal herbs, their active constituents and mechanism of action

Scientific name	Phytochemical group	Active constituents	Possible mechanism of action	References
Anxiolytic herbs				
Cassia siamea (Caesalpinaceae)	Phenoilcs	Barakol	Dopamine agonistic and 5-HT2 antagonist like effects	Sukma et al[68]
Magnolia officinalis (Magnoliaceae)	Polyphenols	Honokiol, Magnolol, Honokiol	Modulation of GABAA and GABAC receptors	Ai et al[69]
Passiflora incarnate (Passifloraceae)	Flavonoids	Chrysin	Enhancing GABAA/BDZ receptors	Dhawan et al[70]
Piper methysticum (Piperaceae)	Kavalactones	Methysticin, Kavain, Yangonin	Modulation of GABAA receptors	Feltenstein et al[71]
Scutellaria baicalensis (Labiatae)	Flavonoids	Wogonin, Baicalein, Baicalin	Modulation of GABAA/BDZ receptor complex	Liao et al[72]
Tilia tomentsa (Tiliaceae)	Flavonoids	Chrysin, Kaempferol	Modulation of BDZ receptor complex	Viola et al[73]
Withania Somnifera (Solanaceae)	Glyco withanolides	Sitoindoside IX and Sitoindoside X	Antioxidant action	Bhattacharya et al74
Ziziphus jujube (Rhamnaceae)	Saponins	Spinosin, Jujubosides	Inhibition of hippocampal hyperactivity	Shou et al[75]
Antidepressant herbs				
Apocynum venetum (Apocynaceae)	Flavonoids	-	Medicating central monoaminergic systems	Kim et al[76]
Areca catechu (Arecaceae)	Alkaloids	Arecaidine, Arecoline, Guvacine	Inhibition of MAO-A	Dar and Khatoon[77]
Bacopa monniera (Scorphularaceae)	Saponins	Bacoside A	Antioxidant action	Singh and Dhawan[24]
Cissampelos sympodialis (Menispermaceae)	-	Warifteine	Antioxidant action	Thomas et al[78]
Curcuma longa (Liliaceae)	Flavonoid	Curcumin	Inhibition of MAO-A	Park and Kim[79]
Hypericum perforatum (Hypericaceae)	Flavonoids	Hyperforin, Rutin	Inhibition of 5-HT, DA, MAO-A, MAO-B and NE reuptake	Cervo et al[80]
Morinda officinalis (Rubiaceae)	Oligosaccharides	-	Antioxidant action	Cui et al[81]
Perilla frutescens (Lamiaceae)	Poly phenols	Rosmarinic acid, Caffeic acid, Apigenin		Takeda et al[82]
Withania somnifera (Solanaceae)	Alkaloids	Withanolides and withanols	Mood stabilizing action	Bhattacharya et al[74]
Anti dementia herbs				
Angelica sinensis (Apiaceae)	Poly phenol	Ferulic acid	Antioxidant action	Yan et al[83]
Biota orientalis (Cupressaceae)	Ditetpenes	Pinusolides	Neuroprotection	Koo et al[84]
Centella asiatica (Apiaceae)	Saponins	Asiaticoside, oxyasiaticoside, centelloside, brahmoside, brahminoside	Antioxidant action; AChE inhibitor activity	Veerendra Kumar and Gupta[85]
Dipsacus asper (Dipsacaceae)	Saponins	-	Antioxidant action	Qian et al[86]
Galanthus nivalis (Amaryllidaceae)	Alkaloid	Galanthamine	A potent AChE inhibitor, enhances cholinergic nicotinic neurotransmission	Woodruff-Pak et al[87]
Gastrodia elata (Orchidaceae)		Gastrodin, Vanillin	Inhibition of GABA transaminase	An et al[88]
Ginkgo biloba (Ginkgoaceae)	Terpenoids	bilobolide, ginkgolides,	Inhibition of h-adrenoreceptors, antiagonistic effects on GABAA receptors	Hoyer et al[89]
Huperzia serrata (Huperziaceae)	Sesquiterpene alkaloid	Huperzine A	Potent AChE inhibitor	Wang et al[90]
Hypericum perforatum (Hypericaceae)	Flavonoids	Hyperforin	Facilitating adrenergic and 5-HT1A receptors	Klusa et al[91]
Paeonia lactiflora (Paeoniaceae)		Paeoniflorin	Modulating adrenergic systems	Ohta et al[92]
Panax ginseng (Araliaceae)	Triterpene saponins	Ginsenosides, Panaxynol	Inhibiting h-AP, enhancing Ach and neurotrophic activity	Hsieh et al[93]
Salvia miltiorrhiza (Lamiaceae)	-	Lithospermate B	Mediation of central DA, NE, and 5-HT Systems	Cheng et al[66]
Schizandrae chinensis (Magnoliaceae)		Schizandrin	Acting at ACh, 5-HT2, 5-HT1A and GABAA receptors	Zhang[94]
Uncaria				

Tomentosa (Rubiaceae) Uncaria	Alkaloids	Uncarine E, Rhynchophylline	Positive modulation of M1, 5-HT2 and NMDA receptors	Kang et al[95]
Angelica archangelica (Umbelliferae)	Methanol extract		AChE inhibitory activity	Yan et al[83]
Areca catechu	Alkaloids	Arecoline, talsaclidine	Muscarineic (M2) binding activity	Houghton and Seth [96]
Celastrus paniculatus (Celestraceae)	Seed oil		Inhibits the levels of noradranaline, dopamine and 5-hydroxy tryuptamine (5-HT)	Gattu et al [97]
Curcuma longa (Liliaceae)	Flavonoid	Curcumin	Reverse chronic stress-induced impairment of hippocampal neurogenesis and increase expression of brain-derived neurotrophic factor	Xu et al[47]
Huperzia serrata (Huperziaceae)	sesquiterpene alkaloid	Huperzine A	Neuroprotective against ß-amyloid peptide fragment	Wang et al [90]
Magnolia officinalis	Biphenolc lignans	Honokiol, magnolol	Inhibiting ROS formation	Ai et al[69]
Melissa officinalis	Flavonoids		Acetylcholine receptor activity in the central nervous system with both nicotinic and muscarinic binding properties	Perry et al[98]
Nardostachys jatamansi (Valirenaceae)	Sesquiterpenes lignans and neolignans,	Jatamansic acid, Jatamansone, Nardostachysin	Dopamine enhancing property	Rao et al[99]
Salvia lavandulaefolia	Cyclic monoterpenes	1,8-cineole, α-pinene, β-pinene	AChE inhibitory activity	Perry et al[98]
Anticonvulsing herbs				
Aconitum sp., (Ranunculaceae)	Alkaloids	Aconitine, Lappaconitine, Heteratisine, Mesaconitine	Activate NMDA receptor mediated responses and involvement of alpha receptor	Ameri et al[100]
Angelica dahurica (Apiaceae)	Furanocoumarin	Phellopterin	BZD receptors agonist	Bergendorff et al[101]
Berberis vulgaris (Berberidaceae)	Isoquinoline alkaloid	Berberine	Modulates neurotransmitter systems like NMDA, nitric oxide and serotonin	Bhutada et al[102]
Crocus sativus (Liliaceae)		Safranal	GABA(A)-BZD receptor complex	Hosseinzadeh and Khosravan[103]
Cymbopogon citratus (Gramineae)	Essential oil		GABA ergic neurotransmission	Blanco et al[104]
Cynanchum otophyllum (Apocynaceae)	Otophylloside	Alkaloids	Inhibition of tubulin synthesis and reduction in mossy fibre sprouting and neosynaptogenesis	Mu et al[105]
Ficus platyphylla (Moraceae)	Saponin		Suppresses excitatory and inhibitory synaptic traffic; blocks sustained repetitive firing and spontaneous action potential firing; impairs membrane excitability	Chindo et al[106]
Fraxinus griffithii (Oleaceae)		Ligustrosid	Increases GABA- facilitated inhibition in CNS through interaction with GABA-BZD-BARB/PICRO-CL receptor complex	Basori[107]
Ginkgo biloba (Ginkgoaceae)	Sesquiterpene	Bilobalide	Elevation in hippocampal GABA levels through potentiation of glutamic acid decarboxylase activity;	Rodriguez [108]
Nigella sativa (Ranunculaceae)	Anthraquinone	Thymoquinone	Opioid receptor mediated increase in GABAergic tone	Hosseinzadeh and Parvardeh[109]
Ocimum sanctum (Lamiaceae)	Essential oil		Involvement of dopaminergic neurons	
Panax ginseng (Araliaceae)	Ttriterpenes	Ginsenosides	Increases glutamate decarboxylase enzyme activity which increase the GABA level in brain	Choi et al[110]
Piper nigrum (Piperaceae)	Alkaloids	Piperine	Antagonist action at NMDA receptors	D'Hooge et al[111]
Rubia cordifolia (Rubiaceae)	Terpenoids		Raises GABA and 5- HT contents in brain	Kasture et al[112]



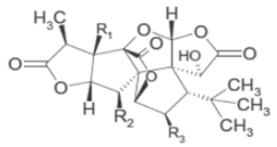
i) Bacosides (*Bacopa monniera*)



ASIATICOSIDE

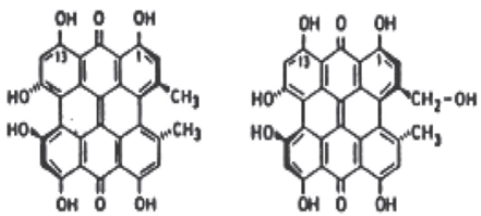
	R_1	R_2
1. Asiatic acid	-H	-OH
2. Madecassic acid	-OH	-OH
3. Asiaticoside	-H	-O-gluc-gluc-Rha
4. Madecassoside	-OH	-O-gluc-gluc-Rha

ii) Asiaticosides (*Centella asiatica*)

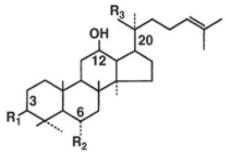


Ginkgolide A: R_1=OH, R_2=H, R_3=H
Ginkgolide B: R_1=OH, R_2=OH, R_3=H
Ginkgolide C: R_1=OH R_2=OH, R_3=OH
Ginkgolide J: R_1=OH, R_2=H, R_3=OH
Ginkgolide M: R_1=H, R_2=OH, R_3=OH

iii) Ginkgolides (*Ginkgo biloba*)

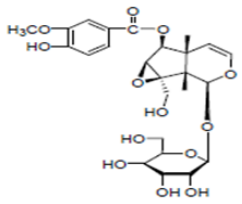


vi) Naphtodianthrones (*Hypericum perforatum*)

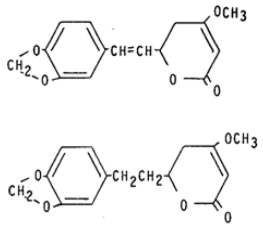


Ginsenosides	R1	R2	R3
Ginsenoside-Rg1	-OH	-O-Glc	-O-Glc
Ginsenoside-Rb1	-O-Glc2-Glc	-H	-O-Glc6-Glc

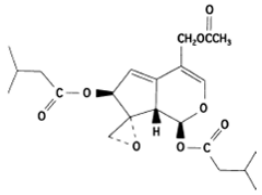
v) Ginsenosides (*Panax ginseng*)



vi) Picroside II (*Picrorhyiza scrophulariflora*)

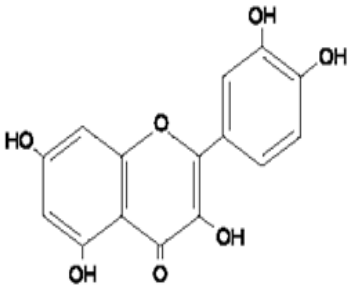


vii) Methysticin and dihydromethysticin (*Piper methysticum*)

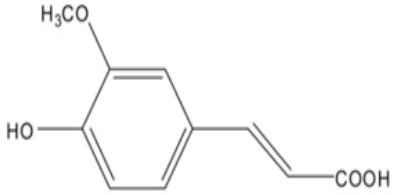


viii) Valepotriates (*Valeriana wallichii*)

Figure 1: Structures of bioactivate compounds of certain nootropic herbs

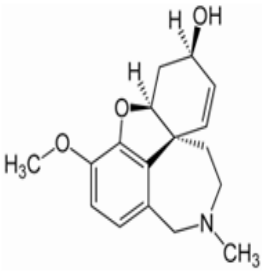


xviii) Quercetin

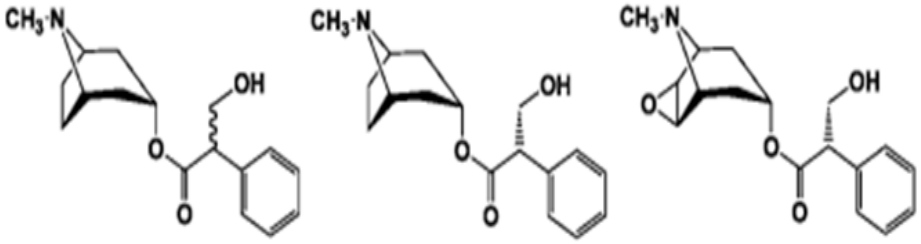


(*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylic acid

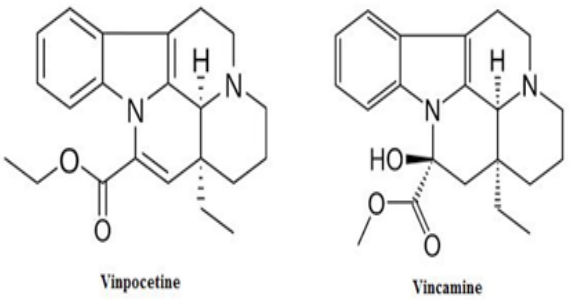
xix) Ferulic acid



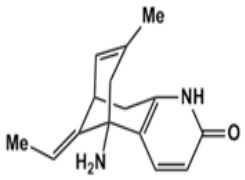
xxi) Galantamine
(*Galanthus nivalis*)



xx) Tropane alkaloids (*Datura*)



xxi) *Vinca minor*



xxiii) Huperzine A
(*Huperia serrata*)

Figure 1: Structures of bioactivate compounds of certain nootropic herbs

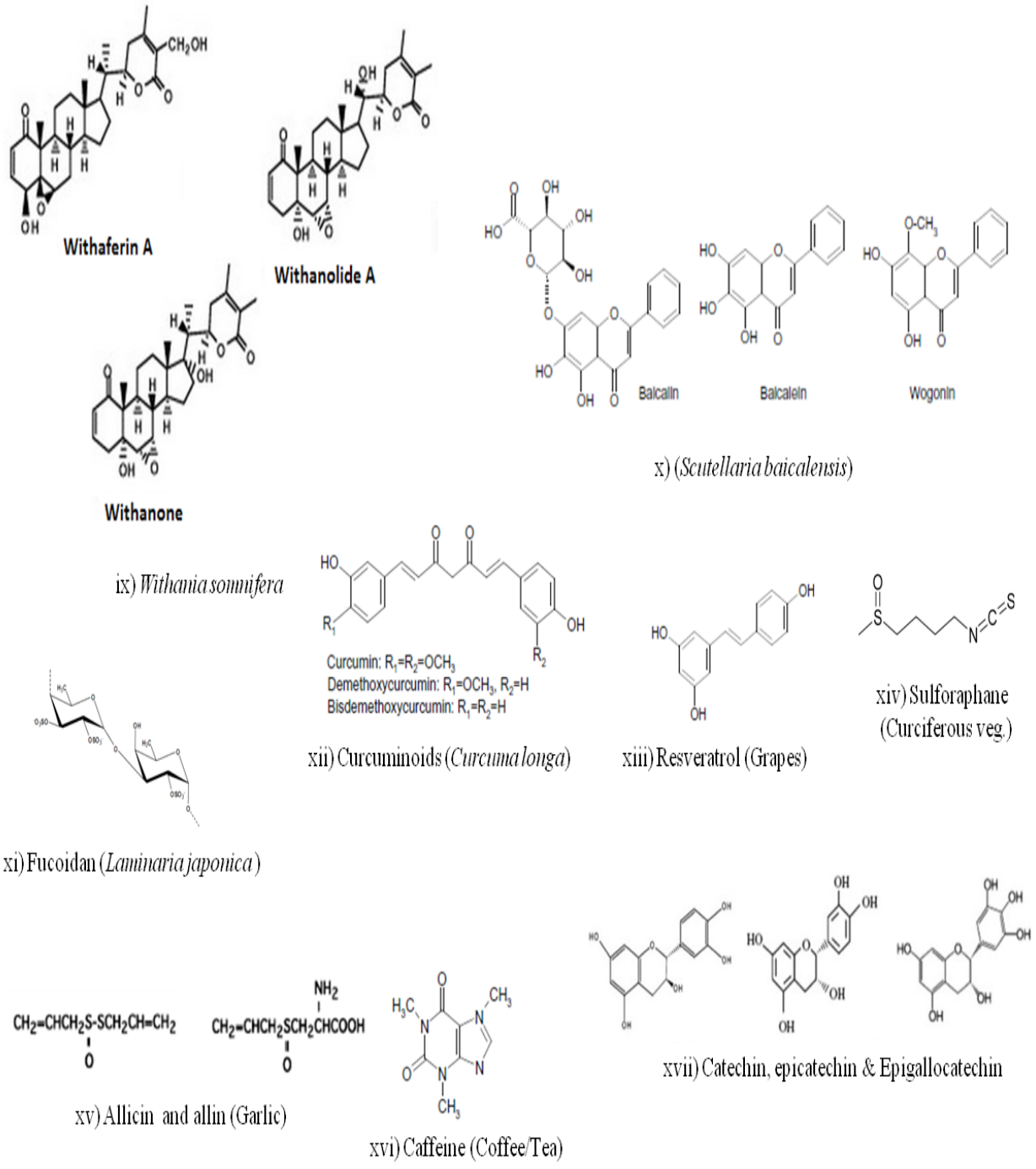


Figure 1: Structures of bioactivate compounds of certain nootropic herbs

for centuries in Ayurvedic system of medicine as a *media rasayna*[25]. Major bioactive compounds of this plant contain highly variable triterpenoid saponins, including asiaticoside, brahmoside, brahminoside, centralized, isothankunoside, oxyasiaticoside, thankunoside and other sapogenins. Triterpenoid acids in the plant consist asiatic acid, madecassic acid, brahmic acid, isobrahmic acid and betulic acid etc. (Figure.I-II). However, its exact mechanism of action in the treatment and management of neurodisorders has not been fully understood.[25]

Ginkgo biloba

Ginkgo biloba (Ginkgoaceae) is also known as maidenhair tree, Kew tree and is indigenous to East Asia. The herb has been reported to have memory enhancing property by increase the supply of O_2, and helps to eliminate free radicals from the system thereby improving memory. Phytoconstituents include terpenoids (bilobolide, ginkgolides), flavonoids (quercetin, keampferol and isorhamnetin), steroids (sitosterol and stigmasterol) and organic acids (ascorbic, benzoic shikimic and vanillic acid). Major bioactive constituents are diterpenic lactones, ginkgolides A, B, C, J and M (Figure.I-III), and a sesquiterpenic trilactone, the bilobalide.[26] The leaf extract showed a neuroprotective effect against Aβ and nitric oxide induced toxicity.[27] Bilobalide and ginkgolides present in *Ginkgo biloba* have been classified as nootropic agents.[28] The bilobalide increased Gamma-Amino Butyric Acid (GABA) levels and glutamic acid decraboxylase activity in mouse brains.[28]

Hypericum perforatum

It is also known as "St. John's Wort", the leaves are known to have bioactive compounds like, hypericin, pseudohypericin (Figure.I-IV), and related naphthodianthrones. This plant is a well known antidepressant agent in several traditional medicines and the mechanism of action initially was stated to be Mono Amine Oxidase (MAO) inhibited.[29]

Panax ginseng

Ginseng refers to a group of several species within the *Panax* genus (*Araliaceae*) growing in north-eastern Asia, with *Panax ginseng* (Asian ginseng), among the most commonly used species in Korean and Japanese traditional medicine.[28] *The ginseng root* is characterized by the presence of 13 ginsenosides (triterpenic saponin complex) (Figure.I-V). It is considered to be an adaptogenic herb and able to increase the body's resistance to various stresses like anxiety, trauma and fatigue by modulating the immune function. Ginsenosides from *Ginseng* may provide protection on the central cholinergic system, dopaminergic system and stimulates the hypothalamus pituitary-adrenal axis.[30] reported that the g*inseng* extract has the ability to protect SH-SY5Y human neuroblastoma cells against MPP^+ induced cytotoxicity and also observed ameliorative effect of ginseng water extract on various protective effects like, free radical scavenging activity, elevated Bax/Bcl-2 ratio, release of cytochrome C and activation of caspase-3 expression.

Picrorhiza scrophulariiflora

The roots of *Picrorhiza scrophulariiflora* (*Scrophulariaceae*) are rich in iridoid glycosides, terpenoids, phenylethanoid glycosides and phenolic glycosides.[31] The dried rhizomes of *Picrorhiza* have long been used in Southeast Asia to treat neuroprotective activities. Recently, picrosides I and II (Figure.I-VI) were shown to possess neuritogenic activity. The combination of picrosides showed a significant neurite outgrowth in PC12D cells through intracellular Mitogen-Activated Protein Kinase (MAPK)-dependent signalling pathway.[14]

Biochemical action of neuroprotective marine algae

Marine algae is a rich source of numerous beneficial health effects. They have been identified as valuable sources of structurally diverse bioactive compounds. Numerous *in vitro* and *in vivo* studies have documented with anti-oxidant and anti-inflammatory activities of marine algae.[32] *Ecklonia cava* (Phaeophyceae; Laminareaceae), also known as "sea trumpet", has been reported to possess anti-inflammatory activity.[33] *E. cava* was also able to suppress the levels of pro-inflammatory mediators such as nitric oxide (NO), prostaglandin-E2 (PGE2) and pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).[33] *Neorhodomela articulate* inhibited NO production, iNOS expression in interferon-gamma (IFN-γ) stimulated BV2 cells.[34] A number of bromophenols of *N. Ocelot* may be potential as anti-neuroinflammatory agents.[35] A phytochemical compound, fucoidan (Mwt. 7000 Dalton, consisting of 48% total sugar and 29% support). Isolated from *Laminaria japonica* has been reported with potent inhibitory effect against LPS-induced NO production in BV2 cells.[36,37] successfully showed that fucoidan (Figure.1-VII) could able to protect rat cholinergic neuronal death induced by Aβ1-42. Fucoidan pretreatment also blocked the activation of caspase-9 and caspase-3.[37] *Ulva conglobata* has been reported for its neural inflammatory, protective activity by suppression of pro-inflammatory enzyme expression such as ions and cyclooxygenase-2 (COX-2), which accounted for the

production of NO and PGE2, respectively.[38]

Some of the phytochemicals isolated from marine algae like, decal and phlorofucofluoroeckol possess memory enhancing and Acetyl Choline Esterase (AChE) inhibitory activity.[39] Furthermore,[40] screened several marine algae for AChE inhibitory activity and found that *Ecklonia stolonifera, Hypnea Valencia* and *Ulva reticulate* showed significant inhibitory activity. *Bryothamnion triquetrum* has been demonstrated to protect GT1-7 cell death produced by chemical hypoxia/aglycemia insult. The protective activity by *B. Triquetrum* seems to be due to its free-radical scavenging activity.[41]

Dietary sources of neuroprotective ingredients

Chronic diseases, such as coronary heart disease, diabetes, blood pressure and certain cancers are resulting from eating a diverse style, either in term of foods or food components. Importance to the dietary amino acids, melatonin and serotonin are supporting the several beneficial effects of Phyto foods associated with diet. Epidemiological studies on human populations suggest that phytochemicals in fruits and vegetables can protect against nervous system diseases. Oligomeric proanthocyanidins, naturally occurring antioxidants widely available in fruits, vegetables, nuts, seeds, flowers, roots and bark, have been reported to possess pharmacological and therapeutic activity against free radicals and oxidative stress. The grape seed proanthocyanidin extract provides significantly greater protection against free radicals and free radical-induced lipid peroxidation than vitamins C, E and &-carotene.[42] Reported that food supplementation with blueberry, spinach, spirulina attenuated stroke-induced cerebral infarction and general locomotor impairments. People who consume greater amount of vegetables and fruits may be at reduced risk for Alzheimer diseases.[43] Dietary supplementation with blueberries protected dopaminergic neurons in a rat model of PD and, improved learning and memory in mouse models of AD. Pomegranate juice improved behavior deficits[44] and apple juice inhibited cognitive impairments. Flavonoids are major constituents of beverages such as wine, tea, cocoa, and fruit juices like apple, grapes, strawberries, etc. Several dietary flavonoids are the neuroprotecive in nature include epigallocatechin, epigallocatechin gallate, catechin, epicatechin, condensed tannins and proanthocyanidins.

Garlic and onion has been shown to be neuroprotective, with the presence of organosulfur compounds (Allium and allicin).[45] Nuts are rich with nutrients and omega-3-fatty acids; hence the nuts may be helpful in activating the nerve cells from undergoing degeneration. Walnuts contain the monounsaturated fatty acid such as oleic acid, linoleic acid and a-linolenic acid.[46] Peanuts are useful in neuroprotection because they contain neuroprotective Resveratrol. Berries like blueberries, blackberries, cherries and strawberries are the major source of anthocyanins such as pelargonidin, cyaniding, melvidin. Flavonoid rich blueberry reported to have inhibitory activity of nitric oxide, TNF-α and IL-1β in activated microglia cells. Blueberries are also effective in running the age related deficits in motor function and memory enhancement by ERK pathway. Whole grains and flours are rich in fiber and known to delay the onset of dementia. Rice bran oil is rich in vitamin E (tocopherols and tocotrienols), which is excellent antioxidants and helps in neuroprotection. Bananas are super foods and contain important minerals and vitamins and possess dopamine, which is an important neurotransmitter. Homocysteine upregulation have been implicated in the destruction of the brain cells. Moreover, green leafy vegetables are rich in vitamin B, E and folate, which protect the brain by down regulating the levels of the amino acid homocysteine. Neuroprotective flavonoids such as apigenin, luteolin are found in artichoke, chives, celery and parsley. Dietary flavanones i.e. naringenin, hesperetin and taxifolin (citrus fruit and tomatoes) are observed with activation of ERK1/2 signaling in cortical neurons.

Curcumin

Curcuma longa (turmeric) is a common dietary supplement in the Indian culinary system. Several beneficial effects of curcumin for the nervous system have been reported. Curcumin (Figure.I-VII) treatment protected neurons against ischemic cell death and ameliorated behavioral deficits in experimental animal studies.[47] *In vitro* cell culture studies show that dietary curcumin is a strong dietary phytochemical for use in the prevention or treatment of age-related neurodegenerative diseases. Moreover, curcumin has been reported to reverse chronic stress-induced impairment of hippocampal neurogenesis and has the capacity to increase the expression of brain-derived neurotrophic factor (BDNF).[47] Its application also prevents the cultured rodent cortex cells against glutamate excitotoxicity.[48]

Resveratrol

Resveratrol (Figure.I-VIII), a phytophenol present in red grapes, which exhibits antioxidant activity. It is known that peripheral administration of Resveratrol could able to protect neurons in the brain and spinal cord against ischemic injury[49] reported that supplementation of

Resveratrol helped to reduce ischemic damage through spinal cord neuron protection in an experimental rat model of stroke. Similarly Resveratrol can protect cultured neurons against NO mediated oxidative stress induced death. It also protected dopaminergic neurons against metabolic and oxidative insults in midbrain slice cultures of Parkinson's disease model.[49] Resveratrol protected cells against the toxicity of mutant huntingtin in worm and cell culture models.[50] Resveratrol also protected neuronal cells by amyloid β-peptide and promoted the clearance of amyloid β-peptide from cultured cells of Alzheimer's disease model.[51]

Sulforaphane

Sulforaphane is an isothiocyanate present in broccoli, brussels sprouts and other vegetables belonging to family Cruciferaceae (Figure.I-IX). Previous studies of Zhao et al[52] reported that the neuroprotective effects of sulforaphane in reducing brain damage, brain edema and protective action of retinal pigment epithet. Sulforaphane has been reported to protect cultured neurons against oxidative stress and dopaminergic neurons against mitochondrial toxins.[53]

Allium and allicin

Organosulfur compounds, such as the allium and allicin are present in high amounts in *Allium sativum* (garlic) and *A. sepia* (onions). Allium and allicin are known to have neuroprotective and free radical scavenging activities (Figure.I-X). Beside theses activities, allyl-containing sulfides might activate the pathways related neuroprotection, resulting in the activation of mitochondrial uncoupling proteins.[54]

Several other dietary phytochemicals have neuroprotective in nature, such as: Caffeine, a methylxanthine is the most important bioactive constituent of this beverage (coffee) providing neuroprotection (Figure.I-XI). Other similar xanthine alkaloids found in tea and chocolate are theophylline and theobromine, respectively. Tea is prepared from the leaves of *Camellia sinensis*, also native to the Old World, whereas cacao derives from the seeds of the New World species *Theobroma cacao* L. (Sterculiaceae), used by the Aztecs as medicine. Other important sources of methylxanthine alkaloids are cola nuts (genus *Cola*, Sterculiaceae family) native to Tropical Africa and used to flavor carbonated beverages, guaraná (genus *Pauline*, Sapindaceae) from South America and a popular beverage in Brazil, and Mate (genus *Ilex*, Aquifoliaceae).

Major functional groups of phytochemicals in neuroprotection

Many phytochemicals have been shown to exert a neuroprotective action in animal and cell culture models. Poly phenols (phenolic acids, anthocyanins, proanthocyanidins, flavonols, tannins), isoprenoids (sesqiterpenes, diterpenes, triterpenes, steroids, saponins), alkaloids (indole alkaloids, lysergic acid diethylamide, tropane alkaloids, ergot group) and fatty acid are active components found in many medicinal plants and regulate a variety of enzymes as well as cell receptors.[55]

Polyphenols

Polyphenols are a group of plant secondary metabolites characterized by the presence of more than one phenolic unit which is linked directly to the aromatic ring. Numerous studies indicate that flavanols are of benefit for neuronal health. Catechin may protect against the brain injuries produced by endogenous neurotoxins involved in the onset of Parkinson's disease. Catechin, epicatechin and epicatechin gallate (Figure.I-XII) have also shown an ability to suppress neuroinflammation and can attenuate and inhibit activation of microglia and/or atrocities associated with the release of the mediators linked to the apoptotic death of neurons.[56] In addition, catechin derivatives may delay the onset of neurodegenerative disorders such as Alzheimer's disease through different mechanisms such as iron chelators, radical scavengers, and modulators of prosurvival genes.[56] Proanthocyanidins and condensed tannins are complex flavonoid polymers naturally present in cereals, legumes, and fruits.[14] Quercetin (Figure.XIII) has been shown to protect against ischemic injury, where calcium dysregulation is one of the main instigators of neuronal cell death and brain damage. It significantly attenuates Aβ-induced toxicity, protein oxidation and apoptosis in primary hippocampal cultures.[57] Indeed, treatment with quercetin reduced the spectrin breakdown products caused by ischemic activation of calcium-dependent protease cabin and inhibited the acid-mediated intracellular calcium level.[58] Some of the anthocyanins have the ability to cross the Blood-Brain Barrier (BBB) and diffuse through the central nervous system.[59] Anthocyanins have significant neuroprotective benefits in improving cognitive brain function and, defensive mechanism on oxidative stress and lipid peroxidation. There are few reports on the neuroprotective function of hydrolyzable tannins. Ellagic acid has been reported to reduce Aβ- induced neurotoxicity in human SH-SY5Y neuroblastoma cells through Aβ fibril formation.

The process of Aβ fibril formation may represent a protective mechanism of local Aβ clearance.[60] Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (Figure.I-XI) is an ample phenolic phytochemical found in plant components and can attenuate the stress-induced behaviour in the depression-like model in mice.[60]

Alkaloids

Alkaloids may affect the CNS, including nerve cells of the brain and spinal cord which control many direct body functions and the behaviour. They may also affect the autonomic nervous system, which includes the regulation of heartbeat, blood circulation and breathing. Indole alkaloids contain the indole carbon-nitrogen ring which is found in the fungal alkaloids ergine, psilocybin and lysergic acid diethylamide (LSD). These alkaloids may interfere in the action of serotonin in the brain.[61] Ergot alkaloids have marked effect on blood flow, which was originally thought to be the main mechanism of action. Tropane alkaloids (Figure.I-XX) from *Datura* (atropine, hyoscyamine and scopolamine) can affect on the spinal cord and Central Nervous System (CNS). Galantamine (Figure.I-XXI) belonging to the phenanthrene chemical class, obtained from *Narcissus tazetta, Galanthus nivalis* and *Leucojum aestivum*. It is capable to stimulate nicotinic receptors, inhibit cholinesterase activity and further enhance cognition and memory.[62] Vinpocetine (Figure.I-XXII) from *Vinca minor* is a highly potent vasodilator, which is useful to protect against hypoxia, ischaemia, enhancement of cerebral blood flow and glucose uptake.[62] Isoquinoline alkaloids are highly potent analgesic and a narcotic drugs isolated from *Papaver somniferum* (opium poppy), which results in increasing Gamma Amino Butyric acid (GABA) in the synapses of the brain.[62]

Isoprenoids

Many plant-derived essential oils, such as wormwood, have been known for anti convulsion over a century. The rhizome of valerian (*Valerian officinalis*) contains two pharmacologically active ingredients such are valepotriates and sesquiterpenes. Ortiz et al[63] reported the active effect of valerian extract on GABA (B) receptor binding property and GABA uptake inhibition in rat synaptosomes. Huperzine A, a sesquiterpene alkaloid extracted from the Chinese herb *Huperia serrata*, shows a broad range of neuroprotective properties. Huperzine A (Figure.I-XXIII) demonstrated in improved learning and spatial working memory. In *Centella asiatica* brahminoside (triterpenoid), β-pinene and γ-terpinene (monoterpenes) are active chemical substances in revitalizing and strengthening the nervous function.

Fatty acids

The structural arrangement of neurons is crucial to their function as the cells must uphold proper electrical gradients across the membrane and be able to release and reabsorb unmetabolized neurotransmitters. Fatty acid composition of the neuronal membrane maintains a key role in the structural arrangement of neurons.[64] Consuming Mono Unsaturated Fatty Acids (MUFAs) and Poly Unsaturated Fatty Acids (PUFAs) was shown to slow cognitive decline in animals and in humans. Omega-3 and omega- 6 fatty acids found in nuts, which are known to have neuroprotective activity. Numerous studies have shown that consuming diets deficient in n23 fatty acids will impair cognitive functioning. The fatty acid composition of neuronal membranes declines during aging process, however food supplementation with essential fatty acids was shown to improve membrane fluidity. In addition to affecting membrane biophysical properties, PUFAs in the form of phospholipids in neuronal membranes can also directly participate in signaling cascades to promote synaptic plasticity, neuronal function and neuroprotection.[35] Seabuckthorn seed oil is rich with omega-3: omega-6 (1:1) and also revealed that it has the cerebral cardiovascular functions by coordination of neurons.[65]

Mechanism of action of phytochemicals with respect to neuroprotection

Phytochemicals activate cellular stress-response pathways resulting in the upregulation of neuroprotective gene products. Increasing evidence has shown that the neuroprotective effects of the neurotrophic factors are mainly mediated by inhibiting the cell death/apoptosis pathways.[17] Approximately 50 neurotransmitters belonging to diverse chemical groups have been identified in the brain. Acetylcholine has been a special target for investigations because its insufficiency has been believed responsible for senile dementia and other degenerative cognitive disorders, including Alzheimer's disease. Phytochemicals can activate the transcription factor NF-kB by inducing the expression of antioxidant enzymes and Bcl-2. Some phytochemicals can activate multiple signalling pathways by ligands that bind to various receptors like G-Protein-Coupled Receptors (GPCR), Growth Factor Receptors (GFR) and Insulin Receptors (IR). These receptors activate kinase cascades which involve Protein Kinase C (PKC), Mitogen-Activated Protein Kinases (MAPK) and Phosphatidylinositol-3-Kinase (PI3K). Flavonoids activate ERE-CREB pathway and the P13 kinase-mTOR cascade

leading to changes in synaptic plasticity.

Phytochemicals including isothiocynates, insoles, terpenes, diallyl sulfides, curcuminoids and cytokines have been shown to activate one or more pathways upstream of nuclear factor erythroid 2-related factor 2 (NRF2). Ellison activates Transient Receptor Potential (TRP) ion channels in the plasma membrane of neurons, resulting in Ca^{2+} influx, which activates neuroprotective kinase cascades via Mitogen-Activated Protein Kinases (MAPK), Camp-Response-Element-Binding protein (CREB)[54]; CREB induces the expression of Brain-Derived Neurotrophic Factor (BDNF). BDNF is a major neurotrophic factor, which can initiate MAPK/ERK and PI-3K/Akt pathways, through binding to its tyrosine kinase TrkB receptor and thus activate the downstream molecules which can promote neurogenesis and cell survival.[66] Allyl-containing sulfides might activate other pathways related to stress, resulting in the upregulation of mitochondrial uncoupling proteins.[54]

Flavanones, such as hesperetin and its metabolite, 5-nitrohesperetin, have been observed to inhibit oxidant-induced neuronal apoptosis via a mechanism involving the activation/phosphorylation of signalling proteins important in the pro-survival pathways. Capsaicin activates specific Ca^{2+} channels (vanilloid receptors). A wide range of phytochemicals such are flavonoids, terpenes, and related substances are known to influence the function of ionotropic receptors for GABA, the major inhibitory neurotransmitter in the brain.[67] Such GABA modulators have been found in fruits, vegetables, various beverages, and herbal extracts such as *Ginkgo biloba* and *Ginseng*.

CONCLUSION

Many lifestyle factors endorse health of the nervous system in trouble by imposing a mild stress on neural cells. Phytochemicals in the form of dietary supplements, herbs and spices, comprise an unlimited source of molecules available for improving human health. It is likely that different phytochemicals produce *in vivo* additive and/or synergistic effects, thus amplifying (or reducing/inhibiting) their activities. Many of the phytochemicals have recently been reported to exert neuroprotective effects in various experimental models of neurological disorders. The information collected in this review on a large number of herbal extracts and constituents that possess therapeutic effects in animal models/*in vitro* cell culture models of neurological disorders may be used in a search for novel pharmacotherapies from medicinal plants for these illnesses.

The pharmacological actions of many phytochemicals and herbal extracts involve to some extent the mechanisms known to be responsible for convening neuroregeneration actions. For example, several anxiolytic agents have the capacity to enhance the inhibitory function of central GABA receptor. Many herbal antidepressant agents have the ability to inhibit MAO activity and modulate monoaminergic neurotransmission. Antidementia effects of many herbal agents are related to the inhibition of AChE activity. On the other hand, a considerable number of herbal extracts and constituents, most notably antidepressant and antidementia agents, possess antioxidant and neuroprotective actions, as evidenced by the protection against neuronal cell death induced by exposure to excessive free radicals, excitatory toxins, toxic derivatives of amyloid precursor protein, and other neurotoxins.

Although demand for phytotherapeutic agents is growing, there is a need for their scientific validation before plant derived extracts gain wider acceptance and use. Hence "natural" products may provide a new source of beneficial neuropsychotropic drugs. These studies provide a phytochemical basis for some of the effects that these herbal preparations have on brain function and neuroprotection. As implicitly outlined in this review, most of our current knowledge about CNS-active plants of cultural and traditional importance arose from ethnobotanical and ethnopharmaceutical studies, as for other natural active ingredients.

CONFLICT OF INTEREST

The authors have no conflict of interest

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Tannin components and inhibitory activity of Kakadu plum leaf extracts against microbial triggers of autoimmune inflammatory diseases

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ABSTRACT

Introduction: Autoimmune inflammatory diseases can be triggered by specific bacteria in susceptible individuals. *Terminalia ferdinandiana* (Kakadu plum) has documented therapeutic properties as a general antiseptic agent. However, the high ascorbic acid levels in Kakadu plum fruit may interfere with this activity. **Methods:** *T. ferdinandiana* leaf solvent extracts were investigated by disc diffusion assay against a panel of bacteria known to trigger autoimmune inflammatory diseases.Their MIC values were determined to quantify and compare their efficacies.Toxicity was determined using the *Artemia franciscana* nauplii bioassay. Non-targeted HPLC separation of crude extracts coupled to high resolution time-of-flight (TOF) mass spectroscopy with screening against 3 compound databases was used for the identification and characterisation of individual components in crude plant extracts. **Results:** Methanolic, aqueous and ethyl acetate *T. Ferdinandiana* leaf extracts displayed potent antibacterial activity in the disc diffusion assay against the bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis. The ethyl acetate extract had the most potent inhibitory activity, with MIC values less than 120 μg/ml against *P. mirabilis* and *A. baylyi* (both reference and clinical strains). The ethyl acetate extract had similar potency against *K. pneumonia* (both reference and clinical strains), but had higher MIC values (2733 μg/ml) against *P. aeruginosa*. The methanolic extract was also a potent inhibitor of bacterial growth, with MIC values generally < 1000 μg/ml. In comparison, the water, chloroform and hexane leafextracts were all substantially less potent antibacterial agents, with MICs values generally well over 1000 μg/ml. All *T. ferdinandiana* leaf extracts were either nontoxic or of low toxicity in the *Artemia fransiscana* bioassay.Non-biased phytochemical analysis of the ethyl acetate extract revealed the presence of high levels of tannins (exifone (4-galloylpyrogallol), ellagic acid dehydrate, trimethylellagic acid, chebulic acid, corilagin, punicalin, castalagin and chebulagic acid). **Conclusion:** The low toxicity of the *T. ferdinandiana* leaf extracts and their potent inhibitory bioactivity against the bacterial triggers of autoimmune inflammatory disorders indicates their potential as medicinal agents in the treatment and prevention of these diseases.

Key words: *Terminalia ferdinandiana,* rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis, *Proteus mirabilis, Klebsiella pneumoniae , Acinetobacter baylyi, Pseudomonas aeruginosa.*

INTRODUCTION

Autoimmune inflammatory disorders are a group of debilitating conditions including rheumatoid arthritis, ankylosing spondylitis, lupus and multiple sclerosis, which afflict genetically susceptible individuals. There is no common susceptibility profile for these disorders. Rheumatoid arthritis for example is most prevalent in middle aged to older women, whereas the onset of ankylosing spondylitis occurs most frequently in younger males.[1] There are no cures for any of these conditions. Instead, current treatment strategies aim to alleviate the symptoms (particularly pain, swelling and inflammation) with analgesics and anti-inflammatory agents and/or to modify the disease process through the use of disease modifying drugs. None of these treatments is ideal as prolonged usage of these drugs is often accompanied by unwanted side effects and toxicity.[2] There is a need

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to develop safer, more effective treatments for these conditions which will not only alleviate the symptoms, but may also cure or prevent the disease. A greater understanding of the onset and progression of these disorders should greatly assist in more relevant drug discovery and development.

The causes of the autoimmune inflammatory disorders are currently not well understood. However, it is generally accepted that they are immune disorders triggered in susceptible individuals by specific microbial infections. Recent serotyping studies have identified several of the bacterial triggers of these conditions and the bacterial antigens responsible for the induction of an immune response (Table 1). The major microbial trigger of rheumatoid arthritis has been identified as *Proteus mirabilis*,[3] a normal part of the human gastrointestinal flora. Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis[4] and *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.[5] *Borrelia burgdorferi* is linked with Lyme disease.[6] Whilst microbial triggers have also been postulated for lupus, the specific causative agents are yet to be identified. Similarly, members of the Enterobacteriaceae family are associated with Graves' disease and Kawasaki syndrome and *Mycoplasma pneumoniae* is associated with several demyelinating diseases.[7] The development of antibiotic agents targeted at the specific bacterial triggers of autoimmune inflammatory disorders would enable afflicted individuals to target these microbes and thus prevent the onset of the disease and reduce the severity of the symptoms once the disease has progressed.

Herbal medicines have been used for thousands of years in a wide variety of cultures to treat inflammatory disease. A re-examination of traditional medicines for the treatment of inflammation and rheumatic conditions is an attractive prospect as the antiseptic qualities of medicinal plants have also been long recognised and recorded. Furthermore, there has recently been a revival of interest in herbal medications due to a perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals. Antimicrobial plant extracts with high antioxidant contents are particularly attractive as they may treat the symptoms of inflammation as well as blocking the microbial trigger and thus have pleuripotent effects.

Terminalia ferdinandiana is an endemic Australian plant which has been reported to have an extremely high antioxidant content.[17, 18] Furthermore, it was reported that the fruit of this plant also has the highest ascorbic acid levels of any plant in the world, with levels reported as high as 6% of the recorded wet weight.[19,20] This is approximately 900 times higher (g/g) than the ascorbic acid content in blueberries (which were used as a standard). As a further comparison, oranges and grapefruit (which are considered good sources of ascorbic acid) only contain approximately 0.007% wet weight (0.5% dry weight).[21]

Terminalia ferdinandiana has strong antibacterial activity against an extensive panel of bacteria. In a recent study, solvent extracts of various polarities were tested against both Gram positive and Gram negative bacteria.[22] The polar extracts proved to be more effective antibacterial agents, indicating that the antibacterial components were polar compounds. Indeed, the polar extracts inhibited the growth of nearly every bacteria tested. Both Gram positive and Gram negative bacteria were susceptible, indicating that the inhibitory compounds readily crossed the Gram negative cell wall.

Several studies have reported that high levels of vitamin C may inhibit eukaryotic cell death due to inhibition of oxidative stress.[23,24] Whilst similar studies are lacking for prokaryotic cells, it is possible that vitamin C may also have a protective effect, antagonising the antibacterial action of the extracts and protecting against bacterial cell death. Thus, whilst potent antibacterial activity has previously been reported for Kakadu plum fruit,[22] the growth inhibition may be more pronounced in extracts with lower vitamin C levels. The current study was undertaken to test the ability of Kakadu plum leaf extracts to inhibit the growth of bacterial associated with autoimmune inflammatory diseases.

MATERIALS AND METHODS

Plant source and extraction

T. ferdinandiana leaves were obtained from David Bohme of Northern Territory Wild Harvest, Australia. The leaves were thoroughly dried in a Sunbeam food dehydrator and the dried plant materials were subsequently stored at -30°C. Prior to use, the plant materials were thawed and freshly ground to a coarse powder. Individual 1 g quantities of the ground leaves were weighed into separate tubes and 50ml of methanol, water, ethyl acetate, chloroform or hexane were added. All solvents were obtained from Ajax and were AR grade. The ground leaveswere individually extracted in each solvent for 24 hours at 4 °C with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301.

Table 1: The bacterial triggers of the autoimmune inflammatory diseases examined in this study as well as the bacterial antigen and host susceptibility antigen sequences.

Disease	Bacterial Trigger	Bacterial Antigen	Bacterial Sequence	Host Antigen	Host Sequence	References
Rheumatoid arthritis	*Proteus mirabilis* and possibly also other Proteus spp.	haemolysin	ESRRAL	MHC class 2 allele HLA-DR4	EQ/KRRAA	3, 4, 8,9
		urease	IRRET	type XI collagen	LRREI	8,10
Ankylosing spondylitis	Klebsiella pneumoniae	nitrogenase reductase enzyme	QTDRED	MHC class 1 allele HLA-B27	QTDRED	3,11
		pullulanase	DRDE	MHC class 1 allele HLA-B27	DRED	12
		pullulanase	GxP	types I, III and IV collagen	GxP	13
Multiple sclerosis	Pseudomonas aeriginosa	γ-CMLD	TRHAYG	Myelin-neuronal antigen MBP	SRFSYG	14
	Acinetobacter spp.	4-CMLD	SRFAYG	Myelin-neuronal antigen MBP	SRFSYG	14
		3-OACT-A	LTRAGK	Myelin-neuronal antigen MOG	LYRDGK	14
		Acinetobacter regulatory protein	*KKVEEI	Neurofilament-M protein	*KKVEEI	14-16

MOG = myelin oligodendrocyte glycoprotein; MBP = myelin basic protein; 4-CMLD = 4-carboxy-muconolactone decarboxylase; 3-OACT-A = 3-oxoadipate CoA-transferase; γ-CMLD = γ-carboxy-muconolactone decarboxylase. * indicates the sequence likely to be responsible for cross-reactivity, although this is yet to be confirmed.

The resultant dry extract was weighed and redissolved in 10 ml deionised water.

Qualitative phytochemical studies

Phytochemical analysis of the *T. ferdinandiana* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[25-27]

Antioxidant capacity determination

The antioxidant capacity of each sample was assessed using the DPPH free radical scavenging method[28] with modifications. Briefly,DPPH solution was prepared fresh each day as a 400 µM solution by dissolving DPPH (Sigma) in AR grade methanol (Ajax, Australia). The initial absorbance of the DPPH solution was measured at 515 nm using a Molecular Devices, Spectra Max M3 plate reader and did not change significantly throughout the assay period. A 2 ml aliquot of each extract was evaporated and the residue resuspended in 2 ml of methanol.Each extract was added to a 96-well plate in amounts of 5, 10, 25, 50, 75 µl in triplicate. Methanol was added to each well to give a volume of 225 µl. A volume of 75 µl of the fresh DPPH solution was added to each well for a total reaction volume of 300 µl. A blank of each extract concentration, methanol solvent, and DPPH was also performed in triplicate.Ascorbic acid was prepared fresh and examined across the range 0-25 µg per well as a reference and the absorbance's were recorded at 515.All tests were performed in triplicate and triplicate controls were included on each plate. The antioxidant capacity based on DPPH free radical scavenging ability was determined for each extract and expressed as µg ascorbic acid equivalents per gram of original plant material extracted.

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd. Reference strains of *Acinetobacter baylyi* (ATCC33304), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA.All other clinical microbial strains were obtained from the School of Natural Sciences teaching laboratory, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4 °C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[29-32] Briefly, 100 µl of the test bacteria were grown in 10 ml of fresh nutrient broth media until they reached a count of approximately 10^8 cells/ml. An amount of 100 µl of bacterial suspension was spread onto nutrient agar plates.The extracts were tested for antibacterial activity using 5 mm sterilised filter

paper discs. Discs were impregnated with 10 µl of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 °C for 2 hours before incubation with the test microbial agents.Plates inoculated with the bacterial species *Klebsiella pneumoniae* , *Proteus mirabilis*, *Proteus vulgaris* and*Pseudomonas aeuroginosa*, were incubated at 30 °C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Acinetobacter baylyi*, were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (2 µg) were obtained from Oxoid Ltd. and served as positive controls for antibacterial activity. Filter discs impregnated with 10 µl of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the extracts were determined as previously described.[33,34] Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10 µl of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

Toxicity screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/mlsolution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[35-37] Briefly, 400 µl of seawater containing approximately 47 (mean 47.6, n = 128, SD 14.9) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400 µl of diluted plant extracts or the reference toxin were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A negative control (400 µl seawater) was run in triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 24h all nauplii were sacrificed and counted to determine the total % mortality per well. The LC50 with 95% confidence limits for each treatment was calculated using probit analysis.

Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments.

Non-targeted HPLC-MS/MS QTOF analysis

Chromatographic separations were performed as previously described.[28] Briefly, 2 µL of each sample was injected onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C18 column (2.1 x 100 mm, 1.8 µm particle size). The mobile phases consisted of (A) ultrapure water and (B) 95:5 acetonitrile/water at a flow rate of 0.7 mL/min. Both mobile phases were modified with 0.1% (v/v) glacial acetic acid for mass spectrometry analysis in positive mode and with 5 mM ammonium acetate for analysis in negative mode. The chromatographic conditions utilised for the study consisted of the first 5 min run isocratic ally at 5% B, a gradient of (B) from 5% to 100% was applied from 5 min to 30 min, followed by 3 min isocratic ally at 100%. Mass spectrometry analysis was performed on an Agilent 6530 quadrapole time-of-flight spectrometer fitted with a Jetstream electrospray ionisation source in both positive and negative mode.

Data was analysed using the Mass hunter Qualitative analysis software package (Agilent Technologies). Blanks using each of the solvent extraction systems were analysed using the Find by Molecular Feature algorithm in the software package to generate a compound list of molecules with abundances greater than 10,000 counts. This was then used as an exclusion list to eliminate background contaminant compounds from the analysis of the extracts. Each extract was then analysed using the same parameters using the Find by Molecular Feature function to generate a putative list of compounds in the extracts. Compound lists were then screened against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (650 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds). Empirical formula for unidentified compounds was determined using the Find Formula

function in the software package.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried *T. ferdinandiana* leaves with various solvents yielded dried plant extracts ranging from 59 mg (ethyl acetate extract) to 471 mg (water extract) (Table 2). Deionised water and methanol gave relatively high yields of dried extracted material, whilst all other solvents extracted lower masses. The dried extractswere resuspended in 10 ml of deionised water resulting in the extract concentrations shown in Table 2.

Qualitative phytochemical studies (Table 2) showed that methanol and water extractedthe widest range of phytochemicals.Both showed high levels of phenolics (both water soluble and insoluble phenolics)and tannins, as well as well as high to moderate to high levels of cardiac glycosides, saponins and flavonoids. Triterpenes and anthraquinones were also present in low levels in both extracts and alkaloids were detetected in the methanol extract. The ethyl acetate extract also had high levels of phenolics and moderate levels of flavonoids and tannins. Only low levels of phenolics, flavonoids and tannins were detected in the chloroform and hexane extracts.

Antioxidant content

Antioxidant capacity (expressed as ascorbic acid equivalence) for the *T. ferdinandiana* leaf extracts are shown in (Table 2). The antioxidant capacity ranged from a low of 0.4 mg ascorbic acid equivalence per gram of dried plant material extracted (hexane extract) to a high of 340 mg ascorbic acid equivalence per gram of dried plant material extracted (aqueous extract).Whilst significantly lower than the aqueous extract, the methanolic extract also had a high antioxidant capacity with 150 mg ascorbic acid equivalence per gram of dried plant material extracted (aqueous extract).

Antimicrobial activity

To determine the antimicrobial activity of the crude plant extracts, aliquots (10 µl) of each extract were tested in the disc diffusion assay against a panel of bacteriapreviously identified as microbial triggers of autoimmune inflammatory diseases. Both reference and clinical strains of *Proteus mirabilis* were strongly inhibited by the methanol, water and ethyl acetate *T. ferdinandiana* leaf extracts (Figure 1). Indeed, the methanol extract inhibited *P. mirabilis*growth of both strains more effectively than the ampicillin control, with zones of inhibition of >17.5 mm against both strains(compared to approximately 14 mm for the ampicillin control). The methanol, water and ethyl acetate extracts also inhibited *P. vulgaris* growth (Figure 2), with zones of inhibition of up to 15 mm (for the ethyl acetate extract), compared to 7mm for the ampicillin control.

The methanol and ethyl acetate extracts (but not the water extract) were similarly potent inhibitors of *K. pnuemoniae* growth (Figure 3). In general, the clinical strain was significantly more susceptible to the extracts, with the inhibition zone of the clinical *K. pneumonia* being nearly 3mm greater than for the ATCC reference strain. Whilst zones of inhibition were also noted for the water, chloroform and hexane extracts, these were only 6mm in diameter, indicating that the anti-Klebsiella compounds in these extractswere weak, in low concentrations and/or nonpolar.

In contrast(with the exception of the water extract), the *A. baylyi* reference strain was significantly more susceptible to the extracts than was the clinical strain (as determined by the zones of inhibition) (Figure 4). The methanol extract was the most potent bacterial growth inhibitor, with zones of inhibition of 19 and 16.3 mm for the reference and clinical strains respectively.The water and ethyl acetate extracts were also potent inhibitors of *A. baylyi* growth with >10 mm zones of inhibition.

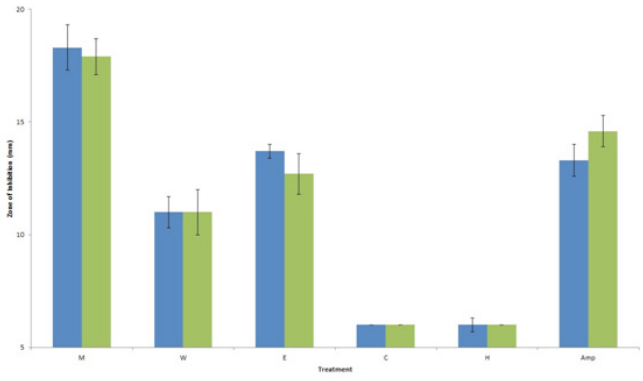

Figure 1: *Antibacterial activity of T. ferdinandia Leaf extracts against P. mirabilis measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC: 21721) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

Table 2: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant contents of *T. ferdinandiana* leaf extracts.

		M	W	E	C	H
	Mass of extract (mg)	331	471	59	59	58
	Concentration of extract (mg/mL)	33.1	47.1	5.9	5.9	5.8
Phenolics	Total phenolics	+++	+++	+++	+	+
	Water soluble phenolics	+++	+++	+++	-	-
	Water insoluble phenolics	+++	+++	+++	-	-
Alkaloids	Cardiac glycosides	+++	++	-	-	-
	Saponins	++	+++	-	-	-
	Triterpenes	+	++	-	-	-
	Polysteroids	-	-	-	-	-
	Meyer test	+	-	-	-	-
	Wagner test	+	-	-	-	-
Anthraquinones	Flavonoids	++	++	++	-	++
	Tannins	+++	+++	++	-	+
	Free	+	+	-	-	-
	Combined	+	+	-	-	-
	Antioxidant content by DPPH reduction (expressed as mg AA equivalence per g plant material extracted)	150	340	22	5	0.4

M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; +++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. AA = ascorbic acid.

P. aeruginosa growth was also susceptible to the methanol, water and ethyl acetate extracts (Figure 5). The clinical strain was significantly more susceptible to the extracts than was the reference strain. Zones of inhibition of 14.3 and 8.3 mm were noted for the clinical and reference *P. aeruginosa* strains respectively against the methanol extract. The zones of inhibition for the aqueous and ethyl acetate extracts were approximately 7.5 and 10.5 mm for the reference and clinical strains respectively.

The Antimicrobial efficacy was further quantified by determining the MIC values for each extract against the microbial species which were determined to be susceptible (Table 3). Most of the extracts were effective at inhibiting microbial growth, with MIC values against the susceptible bacteria generally <1000 µg/ml (<10 µg impregnated in the disc), indicating the potential of these extracts in

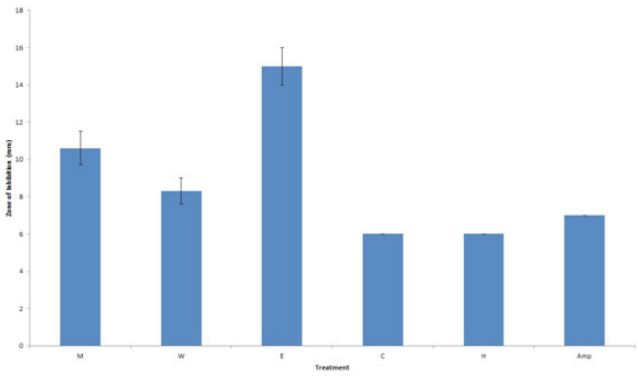

Figure 2: *Antibacterial activity of T. ferdinandia leafextracts against P. vulgaris measured as zones of inhibition (mm). M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

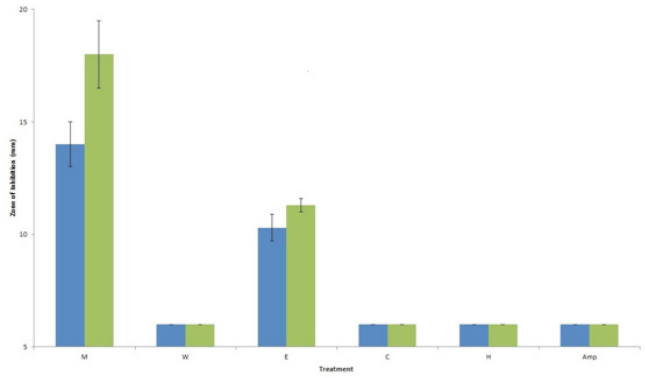

Figure 3: *Antibacterial activity of T. ferdinandia leafextracts against K. pneumoniae measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:31488) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

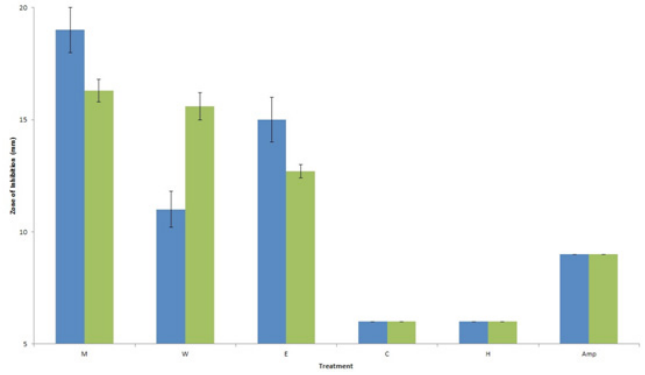

Figure 4: *Antibacterial activity of T. ferdinandia leafextracts against A. baylyi measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:33304) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

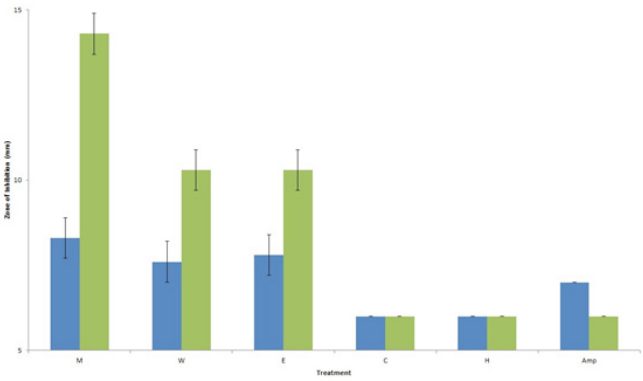

Figure 5: *Antibacterial activity of T. ferdinandia leafextracts against P. aeruginosa measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:39324) and the green bars represent the zones of inhibition against the clinical strain. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.*

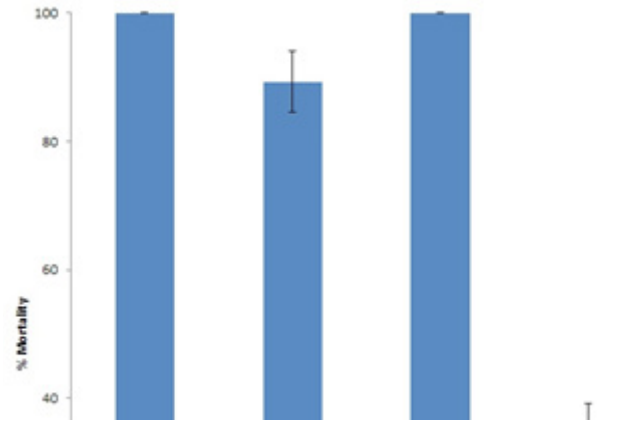

Figure 6: *The lethality of T. ferdinanianaleaf extracts (2000 µg/ml) and the potassium dichromate control (1000 µg/mL) towards Artemia nauplii following 24 hours exposure. M = methanolic extract; W = water extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.*

controlling multiple autoimmune inflammatory disorders. The MIC values determined for the ethyl acetate extract were particularly noteworthy, with most MIC values 100-200µg/ml (1-2 µg impregnated in the disc) against most of the bacterial triggers of autoimmune diseases tested.

Quantification of toxicity

T. ferdinandia leaf extracts were initially screened at 2000 µg/ml in the assay (Figure 6). For comparison, the reference toxin potassium dichromate (1000 µg/ml) was also tested in the bioassay. The potassium dichromate reference toxin was rapid in its onset of mortality, inducing mortality within the first 3 hours of exposure and 100% mortality was evident following 4-5 hours (unpublished results). The methanol, water and ethyl acetate extracts also induced significant mortality following 24 h exposure, indicating that they were toxic at the concentration tested. The chloroform and hexane extracts did not induce mortality significantly different to the seawater control and were therefore deemed to be nontoxic.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in

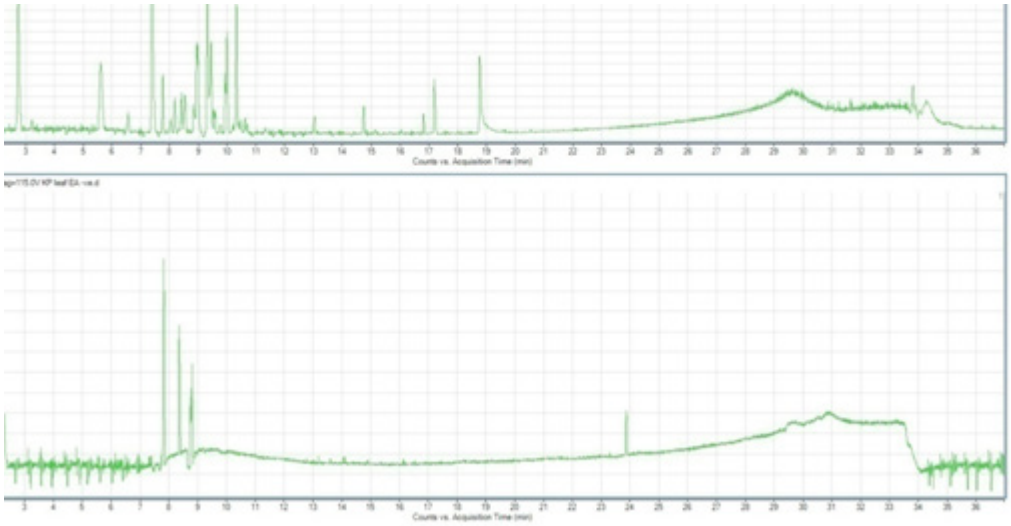

Figure 7:*(a) Positive and (b)negative ionRP-HPLC base peak chromatogram (BPC) of 2 µl injections ofT. ferdinandianaleafethyl acetate extract.*

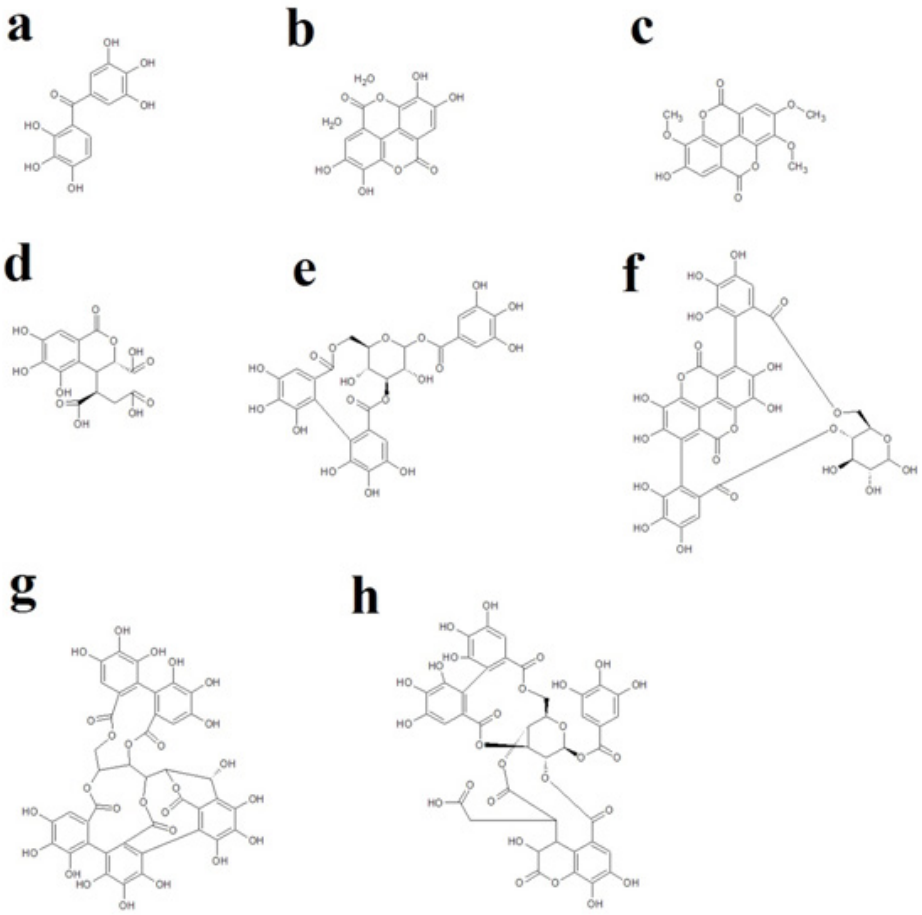

Figure 8:*Chemical structures of T. ferdinandiana leaf compounds detected in the ethyl acetate extract: (a) exifone (4-galloylpyrogallol); (b) ellagic acid dehydrate; (c) trimethylellagic acid; (d) chebulic acid; (e) corilagin; (f) punicalin; (g) castalagin; (h) chebulagic acid.*

artificial seawater to test across a range of concentrations in the *Artemia nauplii* bioassay at 24 hours. (Table 3) shows the LC50 values of the *T. ferdianadiana* leaf extracts towards *A. franciscana*. No LC50 values are reported for The chloroform and hexane extracts as less than 50 % mortality was seen for all concentrations tested. Extracts with an LC50 greater than 1000 µg/ml towards *Artemia* nauplii have been defined as being nontoxic in this assay.[38] As only the ethyl acetate extract had a LC50 <1000 µg/ml, all other extracts were considered nontoxic. Whilst the LC50 value for ethyl acetate is below 1000 µg/ml, the value of 767 µg/ml indicates low to moderate toxicity.

HPLC-MS/MS analysis

As the ethyl acetate extract had the greatest antibacterial efficacy (as determined by MIC) yet contained the least extracted material (Table 1), it was deemed the most promising extract for further phytochemical analysis. Optimised HPLC-MS/MS parameters were developed and used to determinethe ethyl acetate extract compound profile. The resultant base peak chromatograms for the positive ion and negative ion chromatograms are presented in (Figure 7a and 7b) respectively. The negative ion chromatograms had significantly higher background absorbance levels than the positive ion chromatogram, due to ionisation of the reference ions in this mode, possibly masking the signal for some peaks of interest.

The *T. ferdinandiana* ethyl acetate extract positive ion base peak chromatogram (Figure 7a) revealed numerous peaks, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds.Nearly all of the methanol extract compounds had eluted by 11 minutes (corresponding to approximately 30 % acetonitrile).Indeed, several peaks eluted in the first 1 minute with 5 % acetonitrile. However, several prominent peaks between 13 and 20 min, and a minor peak eluting later in the chromatogram (at nearly 34 min)indicates the broad spread of polarities of the compounds in this extract.

Qualitative mass spectral analysis of Kakadu plum leaf extracts

In total,63 unique masssignals were noted for the Kakadu plum leaf ethyl acetateextract (Table 4). Putative empirical formulas were achieved for all of these compounds. Of the 63 unique molecular mass signals detected,42 compounds (66.7 %) were putatively identified by comparison to the Metlin metabolomics, forensic toxicology (Agilent) and phytochemicals (developed in this laboratory) databases.

DISCUSSION

Previous studies within our laboratory reported broad spectrum antibacterial activity for *T. ferdianadiana* fruit extracts.[22] Whilst that study reported potent growth inhibitory properties for the fruit extracts, it did not determine the phytochemical(s) responsible for this activity. Whilst much of the phytochemistry of *T. ferdianadiana* has yet to be determined, the high antioxidant capacity and the extremely high levels of ascorbic acid in the fruit have been frequently reported.[17,18] Indeed, *T. ferdinandiana* fruit

Table 3: Minimum inhibitory concentration (µg/ml) of T. ferdinandiana leaf extracts and LC50 values (µg/mL) in the Artemia nauplii bioassay.

		Methanol	Water	Ethyl Acetate	Chloroform	Hexane
MIC (ug/mL)	*P. mirabilis* (reference strain)	356	925	119	1571	1571
	P. mirabilis (clinical strain)	494	1207	119	1571	1571
	P. vulgaris (reference strain)	734	1752	188	1571	1571
	K. pneumoniae (reference strain)	902	4667	192	1571	1571
	K. pneumoniae (clinical strain)	1352	4980	164	1571	1571
	A. baylyi (reference strain)	780	1188	125	1571	1571
	A. baylyi (clinical strain)	578	1779	108	1571	1571
	P. aeruginosa (reference strain)	739	1239	2733	1571	1571
	P. aeruginosa (clinical strain)	1020	1711	2733	1571	1571
	LC50 (ug/mL)	1133	1330	767	-	-

Numbers indicate the mean MIC and LC50 values of triplicate determinations. - indicates no inhibition.

has been reported to have ascorbic acid levels as high as 6% of the recorded wet weight.[19,20]

The high antioxidant capacity of *T. ferdinandiana* fruit has been postulated as being responsible (at least in part) to potential medicinal properties.[39,40] In particular, ascorbic acid has been linked to antibacterial, antifungal and antiviral activities, as well as anticancer properties.[39, 40] However, other studies have indicated that ascorbic acid may protect cells from oxidative stress and thus protect against cell death.[23,24] Whilst those studies examined the effects of ascorbic acid on eukaryotic cells, it is possible that ascorbic acid may have a similar protective effect against bacterial cell death. *T. ferdinandiana* leaves were examined in the current study in an attempt to minimise the effects of the high ascorbic acid contents present in the fruit.

The leaf extracts examined in this study displayed potent growth inhibitory activity against the bacterial species tested. Indeed, in several cases, we report similar or better antibacterial activities for the *T. ferdinandiana* leaf extracts compared to those previously reported for the equivalent fruit extracts.[22] MIC values <200 μg/ml are reported here for the leaf ethyl acetate extract against a bacterial trigger of ankylosing spondylitis (*K. pnuemoniae*). In the previous study, no inhibition of *K. pnuemoniae* growth by the fruit ethyl acetate extract was noted, indicating the greater potency of the leaf extract. The leaf ethyl acetate extract was also a much more potent inhibitor of *P. mirabilis* than the fruit ethyl acetate extract reported in the previous study (MIC values of 120 μg/ml and 500 μg/ml respectively).

As the ethyl acetate extract had the most potent antibacterial activity yet the least amount of extracted material, it was deemed the best extract for phytochemical analysis. A total of 63 compounds were detected in the ethyl acetate extract and 44 of these compounds were putatively identified. Of these compounds, 8 were identified as tannins. As well as having a wide diversity of tannin components, the tannins were present as major components. The corilagen, castalagin and the chebulagic acid chromatographic peaks accounted for approximately 11 %,1 % and 4 % of the total peak areas respectively for the negative ionisation mode chromatogram (Table 4). Puncialin accounted for approximately 6 % of the total chromatographic peak area in positive ionisation mode, with ellagic acid dehydrate, trimethylellagic acid and chebulic acid combined represent ingapproximately a further 5 % of the total peak area.

It is likely that the high tannin contents in the *T. ferdinandiana* ethyl acetate leaf extract contributes to the inhibitory activity against the microbial triggers of autoimmune inflammatory diseases. Gallotannins including exifone (4-galloylpyrogallol) (Figure 8a) have been reported to inhibit the growth of a broad spectrum of bacterial species[41] through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins,[42,43] and by inhibiting glucosyl transferase enzymes.[44] Elligitannins are also potent inhibitors of bacterial growth. Ellagic acid dehydrate (Figure 8b), trimethylellagic acid (Figure 8c), chebulic acid (Figure 8d), corilagin (Figure 8e), punicalin (Figure 8f) and castalagin (Figure 8g) have been reported to be highly potent antibiotics, with MIC values as low as 62.5 μg/ml.[41, 45, 46] Ellagitannins have also been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls.[41,45] Interestingly, the benzopyran tannin chebulagic acid (Figure 8h) has been reported to inhibit a multi-resistant strain of *Acinetobacter baumannii*,[47] a species taxonomically related to *A. baylyi*, examined in our study.

It is likely that other phytochemical classes may also contribute to the anti-inflammatory properties of these extracts. Alkaloids, anthraquionones, flavonoids, polyphenolics, phytosterols, saponins, stilbenes and terpenes have also been linked with anti-bacterial activity in different plant species and thus may be responsible (at least in part) for the bacterial growth inhibitory activities reported here. Several terpenoids previously reported in *T. ferdinandiana* fruit extracts have been reported to suppress NF-kB signaling (the major regulator of inflammatory diseases).[48] The monoterpenes limonene[49,50] and α-pinene[51] have been reported to inhibit NF-κBsignaling pathways. α-Pinene affects inflammation by inhibiting p65 translocation into the nucleus in LPS-induced NF-κB signalling.[51] Furthermore, many other sesquiterpenes and sesquiterpene lactones also have well established anti-inflammatory activities.[48] Whilst much work is still needed to characterize the mechanisms of action of these compounds, it appears that NF-kxxxxxxB inhibitory activities may be responsible.

Whilst none of these terpenoid compounds were detected in our study, it is possible that they may be present and may contribute to the antibacterial activity of the *T. ferdinandiana* leaf extracts. Our study examined the phytochemical composition of the extracts using HPLC-MS/MS, whereas the previous studies used GC-MS analysis. Generally, HPLC-MS/MS is a good choice for metabolomic profiling studies as it detects a larger amount of compounds of varying polarities than does GC-MS. However, HPLC-MS/MS analysis is limited to studies of the mid-highly polar compounds

Table 4: Qualitative HPLC-MS/MS analysis of the T. ferdinandiana leaf extracts, elucidation of empirical formulas and identification (where possible) of the compound.

Putative identification	Empirical formula	Molecular mass	Retention time	% peak area	
				Negative ionisation mode	Positive ionisation mode
protocatechuic acid	$C_7 H_6 O_4$	154.0272	0.522	2.34	
Ethyl 4-hydroxybenzoate	$C_9 H_{10} O_3$	166.063	0.632	0.69	
(1S,5R)-4-Oxo-6,8-dioxabicyclo [3.2.1]oct-2-ene-2-carboxylic acid	$C_7 H_6 O_5$	170.0219	0.417	7.22	
Gabapentin	$C_9 H_{17} NO_2$	171.126	8.996		1.3
shikimic acid	$C_7 H_{10} O_5$	174.0539	0.395	11.54	
2-tert-Butyl-4-methoxyphenol	$C_{11} H_{16} O_2$	180.1153	14.724		1.18
Mannitol	$C_6 H_{14} O_6$	182.0793	0.505		0.56
Valdipromide	$C_{11} H_{23} NO$	185.1785	0.91		1.41
Benzenemethanol, 2-(2-aminopropoxy) -3-methyl-	$C_{11} H_{16} O_3$	196.1106	9.988	1.31	
11-amino-undecanoic acid	$C_{11} H_{23} N O_2$	201.1734	0.691		6.67
jasmonic acid	$C_{12} H_{18} O_3$	210.1257	11.536		0.15
vanilpyruvic acid	$C_{10} H_{10} O_5$	210.0529	0.632	0.58	
	$C_9 H_6 O_7$	226.0117	0.871	0.59	
Heptylheptanoate	$C_{14} H_{28} O_2$	228.2092	20.989	0.51	
	$C_{11} H_8 O_7$	252.0272	1.808	0.93	
Diprophylline	$C_{10} H_{14} N_4 O_4$	254.1012	0.512		1.5
palmitic acid	$C_{16} H_{32} O_2$	256.2412	23.868	3.38	
2-cyclohexylpiperidine oxalate	$C_{13} H_{23} N O_4$	257.1631	3.205		0.7
	$C_{15} H_{21} N_5$	271.1794	7.743		1.66
Exifone	$C_{13} H_{10} O_7$	278.0433	9.32	1	
	$C_{12} H_{10} O_8$	282.0382	0.406	0.39	
N-(2,3-Dimethylphenyl)-1,3,5-triazaspiro[5.5]undeca-1,4-diene-2,4-diamine	$C_{16} H_{23} N_5$	285.1943	4.277		0.56
	$C_{14} H_4 N_4 O_4$	292.0228	7.434		1.51
	$C_{14} H_4 N_4 O_4$	292.0229	2.254	5.15	
gingerol	$C_{17} H_{26} O_4$	294.1836	13.017	1.25	
	$C_{17} H_{25} N_5$	299.211	8.158		1.09
TEGASEROD	$C_{16} H_{23} N_5 O$	301.19	2.726		9.12
	$C_{13} H_8 O_9$	308.017	0.751	1.04	
	$C_{14} H_4 N_4 O_5$	308.0173	0.978	1.02	
9,13-dihydroxy-11-octadecenoic acid	$C_{18} H_{34} O_4$	314.2462	20.261	0.26	
	$C_{17} H_{25}$ N5 O	315.2059	7.359		15.57
Naphtho[2'',3'':4',5']imidazo[2',1':2,3] [1,3]thiazolo[4,5-b]quinoxaline	$C_{19} H_{10} N_4 S$	326.0647	0.555	1.42	

9,12,13-trihydroxy-10,15-octadecadienoic acid	$C_{18}H_{32}O_5$	328.2251	11.03	0.25	
ellagic acid dihydrate	$C_{14}H_{10}O_{10}$	338.0281	1.085		0.77
trimethylellagic acid	$C_{17}H_{12}O_8$	344.0538	14.237	1.3	0.85
chebulic acid	$C_{14}H_{12}O_{11}$	356.0391	0.356	1.84	2.99
	$C_{15}H_{38}N_{10}O_2$	390.3178	29.379	3	
Val His Phe	$C_{20}H_{27}N_5O_4$	401.2059	7.411		0.67
PheLeu His	$C_{21}H_{29}N_5O_4$	415.222	8.951		5.37
Pro Trp Lys	$C_{22}H_{31}N_5O_4$	429.238	10.297		4.1
vitexin	$C_{21}H_{20}O_{10}$	432.1064	9.397	0.77	5.59
1alpha,25-dihydroxy-26,27-dimethyl-22,22,23,23-tetradehydro vitamin D3 / 1alpha,25-dihydroxy-26,27-di	$C_{29}H_{44}O_3$	440.3262	6.929		0.14
luteolin	$C_{21}H_{20}O_{11}$	448.1025	8.73	2.84	7.99
	$C_{21}H_{12}N_4O_9$	464.0606	8.394		4.73
Meclocycline	$C_{22}H_{21}ClN_2O_8$	476.0962	13.012		0.78
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.1112	9.854	0.27	
	$C_{20}H_{18}O_{14}$	482.0701	0.593	0.7	
	$C_{26}H_{16}O_{16}$	584.0442	2.249	0.74	
	$C_{31}H_{48}O_4$	484.3525	4.256		0.66
	$C_{31}H_{48}O_4$	484.3529	6.545		1.18
	$C_{29}H_{20}N_4O_{10}$	584.1174	10.667		0.57
	$C_{28}H_{14}N_{14}O_3$	594.1377	12.355	0.26	
NORSTICTIC ACID PENTAACETATE	$C_{28}H_{24}O_{15}$	600.1113	9.468	0.37	0.48
	$C_{26}H_{16}N_{10}O_{10}$	628.1062	14.188	0.3	
corilagin	$C_{27}H_{22}O_{18}$	634.0819	7.803	10.74	0.69
Diethyl 1,7-bis(2,4-dinitrophenyl)-1,7-dihydrodipyrazolo[3,4-b:4',3'-e] pyrazine-3,5-dicarboxylate	$C_{24}H_{16}N_{10}O_{12}$	636.0976	8.345	9.67	
	$C_{23}H_{16}N_{10}O_{13}$	640.0908	1.623	0.15	
	$C_{32}H_{56}N_{20}$	720.5001	31.075		1.2
Diadenosine triphosphate	$C_{20}H_{27}N_{10}O_{16}P_3$	756.0815	11.293		0.41
punicalin	$C_{34}H_{22}O_{22}$	782.0621	9.363		5.65
castalagin	$C_{41}H_{26}O_{26}$	934.0715	8.692	1.1	
chebulagic acid (isomer 1)	$C_{41}H_{30}O_{27}$	954.0957	7.654	1.32	
chebulagic acid (isomer 2)	$C_{41}H_{30}O_{27}$	954.0965	9.878	2.25	

and is not as useful for studies aimed at highly non-polar compounds. The terpenoids are relatively nonpolar compounds and it is possible that our analysis protocol was unable to detect them. However, this is unlikely as we have used this protocol routinely in our lab and have previously detected low polarity compounds including several of these terpenoids. It is more likely that if present, their levels were below the threshold of detection in our system and that they did not contribute significantly to the potent antibacterial activity reported here.

Our findings also indicate that *T. ferdinandiana* leaf extracts displayed low toxicity towards *Artemia franciscana*. Indeed, the methanol, water, chloroform and hexane extracts all had LC50 values well in excess of 1000 μg/ml. Only the ethyl acetate extract displayed significant toxicity with an LC50 value of 767 μg/ml. As an LC50of ≥ 1000 μg/ml is defined as nontoxic,[38] this extract is considered to be of only low to moderate toxicity.

CONCLUSION

The results of this study demonstrate the potential of *T. ferdianadiana* leaf extracts to block the growth of bacterial species associated with the onset of several autoimmune inflammatory diseases. Thus, *T. ferdianadiana* leaf extracts have potential in the prevention and treatment of rheumatoid arthritis, ankylosing spondylitis and multiple sclerosis in genetically susceptible individuals. Further studies aimed at the purification and identification of the bioactive components are needed to examine the mechanisms of action of these agents.

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Evaluation of cytotoxic, DNA protecting and LPS induced MMP-9 down regulation activities of *Plectranthus amboinicus* (Lour) Spreng. essential oil.

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ABSTRACT

Introduction: *Plectranthus amboinicus*(Lour) Spreng is a known medicinal plant used in Siddha and Ayurveda medicines in India. It has enormous medicinal potential to treat various diseases. **Methods:** The present study focused on the use of essential oil obtained from the leaves of Plectranthusamboinicusto test cytotoxicity against breast (MCF-7) and colorectal (HT-29) cancer cell lines, to protect DNA from H_2O_2 induced genotoxicity through comet assay and to treat inflammation in lipopolysaccharide (LPS) induced over expression of matrix metalloproteinase-9 (MMP-9) in human peripheral blood mononuclear cells (PBMCs) by gelatin zymogram and inhibition at transcriptional level confirmed using RT-PCR (reverse transcriptase polymerase chain reaction). **Results:** Cytotoxicity of essential oil against MCF-7 and HT-29 cancer cell lines revealed the IC50 values of 53 ± 0.01 and 87 ± 0.01 µg/mL respectively. At 200µg/mL essential oil protected against 75% of DNA damage in 3T3-L1 fibroblast cells. Essential oil showed significant reduction in production of MMP-9 in a concentration dependent manner. **Conclusion:** Overall, the results showed that essential oil of *P. amboinicus* is a potent bioactive substance and it could be used in herbal medicine preparations.

Keywords: *Essential oil , Cytotoxicity , Antigenotoxicity, Zymogram, RT- PCR*.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of endopeptidases which digests proteins of the extracellular matrix. They are important for regulating normal tissue development in morphogenesis, angiogenesis and wound healing. However, unregulated activities of MMPs can play a role in many disease states including cancer expansion, invasion and metastasis, arthritis and atherosclerosis.[1] Numerous factors, namely tissue inhibitors of matrix metalloproteinases (TIMP), plasminogen activator inhibitors and general protease inhibitors such as 2 macroglobulins and 1 antitrypsin may limit or block the enzymatic activity of the activated MMPs.[2] The use of synthetic inhibitors of MMPs and antibiotics were effectively found to reduce the vascular expression of MMPs, in particular MMP-2 (72kDa) and MMP-9 (92kDa) and delay the progression of atherosclerotic plaque.[3] Long term use of synthetic drugs may cause side effects that limit their clinical use. Natural products are an enormous reservoir of structurally diverse secondary metabolites that potentially inhibit the inflammatory process by affecting different molecular targets.[4] *Plectranthus amboinicus* is cultivated in home gardens throughout India

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for use in traditional medicine, being used to treat cough, chronic asthma, hiccough, bronchitis etc.The leaves have been reported to have antiinflammatory and antitumor activities.[5]Antifungal, antibacterial and larvicidal activities of essential oil were reported in the literature.[6-9] Based on the medicinal uses of *P. amboinicus*, the present study was focused on cytotoxic, antigenotoxic and MMP-9 down regulation activities of essential oil under *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Acrylamide, bisacrylamide, gelatin, sodium sulphate anhydrous, trisHcl, sodium dodecyl sulphate (SDS), bromophenol blue, glycine, methanol, coomassie brilliant blue R250, tryphan blue, ethidium bromide and tetramethylethylenediamine (TEMED), glycerol, EDTA, isopropyl alcohol, RPMI-1640, dulbecco's modified eagle medium (DMEM) medium with sodium bicarbonate without L-glutamine and phenol red, phosphate buffered saline (PBS), fetal calf serum (FCS), high melting point agarose (HMP), low melting point agarose (LMP) and triton-X100 were purchased from Hi Media Pvt. Ltd, India. Dimethyl sulfoxide (DMSO) and diethyl pyro carbonate (DEPC) treated water was purchased from Ranbaxy Fine Chemicals, Pvt. Ltd, India. Histopaque-1077, lipopolysaccharide (*E.coli* 0111:B4) and ammonium persulfate were purchased from Sigma Aldrich, USA and trizol reagent from GeneiPvt. Ltd, India.

Plant material

The leaves of *Plectranthus amboinicus* (Lour) Spreng. was collected from healthy green plants growing in the medicinal plant garden, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India.

Hydrodistillation of leaves

Freshly collected leaves of *P. amboinicus*(1kg) were hydrodistilled for 3 hours using Clevenger apparatus for essential oil extraction. Extracted essential oil was treated with sodium sulphate anhydrous to remove water. The purified oil was then filled in small vials, tightly sealed and stored in a refrigerator (4ºC) for further analysis.

Cell lines and culture

The human breast (MCF-7) and colorectal cancer (HT-29) cell lines were cultured in a T25 cm^2 cell culture flask containing DMEM supplemented with 10% FBS, penicillin (100U/mL) and streptomycin (100µg/mL). The cell lines were incubated in humidified incubator at 37° C with 5% CO_2.

MTT assay

The cell line culture in the T25cm^2 flask was harvested using trypsin and the cell number was counted using a hemocytometer. 1x 104 cells/100mL medium was added in each well of 96 well plate and incubated for 24h. Then the cells were treated with various concentrations of essential oil dissolved in medium and further incubated for 48h. A 20 µl of MTT (5mg/mL) in phosphate buffered saline was added to each well and the plate was incubated at 37º C for 4 h. The medium was removed and 100µL of DMSO was added to each well. After 10 minutes of incubation at 37º C the plate was read at 570 nm using a microplate reader.10 The percentage of cell viability was calculated as 100 (absorbance of test/absorption of the control).

Comet assay

Cell culture and treatment

The 3T3-L1 fibroblasts (4-5x106) were cultured in DMEM supplemented with 10% fetal bovine serum in 25 cm^2 tissue culture flasks. The cells in culture were treated with various concentrations of oil (25, 50,100, 150 and 200 mg/mL) for 60 min in a CO_2 incubator. After pretreatment, the cells were exposed to 100 mM of H_2O_2 for 30 min on ice. The cells were harvested, centrifuged for 5 min at 1500 rpm and resuspended in phosphate buffered saline (PBS).

Single cell gel electrophoresis (Comet assay)

A 25 mL of cell suspension was mixed with 75 mL of 0.6% low melting agarose. The suspension was spread on a frosted microscopic slide precoated with 0.8% of normal melting agarose. The cell suspension was covered with a cover slip and kept on ice for 10 min. The cover slips were removed and the slides were incubated overnight in lysis solution containing 1% SDS, 2.5 M NaCl, 100 mM Na_2EDTA, 1% Triton X-100 and 10% DMSO at 4°C. The slides were arranged in an electrophoresis tank filled with prechilled electrophoretic buffer (1mM Na_2EDTA and 300 mMNaOH) and incubated for 20 min. Electrophoresis was carried out at 25 V (300 mA) for 20 min using a power supply (CBS Scientific company, USA). After electrophoresis, the slides were washed with 0.4 M Tris (pH 7.5) and stained with ethidium bromide (20 mg/mL). The slides were viewed using an Olympus BX50 fluorescence microscope. The comet tail length was measured using an

eyepiece micrometer and the DNA damage was calculated as follows:

Comet tail length (lm) = (maximum total length) - (head diameter).[11]

MMP-9 down regulation

Isolation of mononuclear cells

Human lymphocytes were isolated from fresh whole blood of healthy volunteers by adding RPMI1640 with 10% of fetal calf serum on ice for 30 min, then underlaid with 2.5mL of Ficoll Histopaque 1077 followed by centrifuge at 200g for 3 min at 4°C. Isolated lymphocytes were stained with 0.4% trypan blue and viable cells were counted using Haemocytometer under microscope. Cell viability of more than 95% was taken in to further studies.

Stimulation of inflammation and sample treatment

Cells (1×10^5 cells/well) cultured in 96 well plates were induced inflammation using 1 µg/mL LPS.[12] After 24h incubation, *P. amboinicus* essential oil in different concentration (50, 100, 150, 200 and 250 µg/mL) were added to each well and incubated for 24 h. At the end of incubation, the cell free media was collected and assayed for MMP-9 inhibition by gelatin zymography and reverse transcriptase polymerase chain reaction **(**RT-PCR) method.

Gelatinzymography

SDS-PAGE was carried out according to the method of Laemmli[13] using 7.5% gel. Zymogram gel consisted of 7.5% polyacrylamide gel copolymerized with gelatin (1 mg/mL). Following electrophoresis, the gel was washed successively with 50 mL of 2.5% (v/v) Triton X-100 in distilled water (for an hour) to remove SDS. The gel was then incubated with developing solution ($CaCl_2$ 10mM, Triton X-100 1% and Tris buffer, 50mM pH 7.4) at 32°C for 18 h. Further, the gel was stained with coomassie brilliant blue R250 for 2 h and destained overnight to reveal the bands.

RNA isolation and RT-PCR analysis

The cells were harvested after LPS and essential oil treatment and transferred to fresh 1.5 mL centrifuge tubes and centrifuged at 1,400rpm for 10min at 4ºC. The supernatant was discarded and the pellet was suspended in 1mL of trizol to lyse the cells. About 200 µL of chloroform was added and mixed by pipetting for 30sec and centrifuged at 14,000rpm for 15min at 4ºC. Aqueous phase was carefully transferred to a fresh microfuge tube and 500 µL of isopropyl alcohol was added and incubated at room temperature for 10min. The tubes were then centrifuged at 1,400rpm for 10min at 4ºC and the supernatant was discarded. RNA pellet was washed with 75% ethanol by spinning at 1,400 rpm for 5min at 4ºC. The supernatant was discarded, the pellet was air dried and suspended in 9µL of deionised autoclaved diethyl pyro carbonate (DEPC) treated water.

Reverse transcription was carried out as follows. To the above sample, 2µL of dNTP, 5µL of cDNA synthesis buffer, 1µL of oligo d (T), 1µL of reverse transcriptase enzyme mix (Thermo Fischer Scientific, India) and 9µL of nuclease free water were added and reverse transcription was carried out in a thermocycler (Eppendorf) to synthesis cDNA. The synthesized cDNA was further used for PCR. Primers for human MMP-9 and β-actin (Helini, India) were as follows forward primer: 5´-AAG ATG CTG CTG TTC AGC GGG-3´ and reverse primer 5´-GTCCTCAGGGCACTGCAGGAT-3´ for MMP-9[12] and forward primer 5´-CGGATGTCCACGTCACACTT-3´ and reverse primer: 5´-GTTGCTATCCAGGCTGTGCT-3´ for β-actin. PCR conditions for MMP-9 and β-actin, initial denaturation (94°C for 5 min), denaturation (94°C for 30 sec), annealing (57°C for 30 sec for MMP-9) and (60°C for 30 sec for β-actin), extension (72°C for 1 min) and final extension (72°C for 10 min).

RESULTS AND DISCUSSION

Essential oil extraction

Essential oils are complex mixtures of biologically active substances used since a long time as flavouring agents and constituents of a number of commercial products. Scientific literatures have revealed the cytotoxicity, anticholinesterase, antimicrobial, antiinflammatory and antioxidant activities of essential oils. Hydrodistillation of *Plectranthus amboinicus* (Lour) Spreng leaves yielded light yellow colour oil with strong aromatic smell. Out of 1.0 kg leaves 1500µL of oil was extracted. The collected oil was treated with sodium sulphate anhydrous and excess water was removed and kept at 4 ºC for further use.

Cytotoxicity

The cytotoxic activity of essential oil against breast (MCF-7) and colorectal (HT-29) cell lines were significant and IC50 value calculated as 53±0.01 and 87±0.01mg/mL respectively. Plant essential oils and their individual components have anticancer activity when tested on a number of human cancer cell lines including colon, breast, leukemia, lung and gastric cancer. The observed

cytotoxicity of oil may be due to bioactive phenolic compound, carvacrol. Similar result is reported in the literature that carvacrol has cytotoxic effect against various cell lines.14-16 At the same time other constituents of the oil such as γ- terpinene, p-cymene, β- caryophyllene and α-humulene could also be taken into account for their possible synergistic effects.

DNA protecting activity of oil

*DNA p*rotecting effect ofoil on H2O2 induced toxicity was studied. Cells (3T3-L1) treated with 100mM H2O2 produced the maximum tail length of 10.63±0.70mm (Table 1). Cells pretreated with different concentrations of oil showed decrease in tail length. The antigenotoxic activity of oil was observed by the reduction in tail length with increasing concentration and the optimum concentration was observed as 200mg/mL (Figure 1). H2O2 is a natural source of oxidative damage in cells causing a spectrum of DNA lesions, including single and double strand breaks. According to Collins17, DNA damage due to H2O2 results from the production of hydroxyl radicals in the presence of transition metal ions such as iron via Fenton reaction. The observed DNA protecting activity of oil may thus be due to hydroxyl radical scavenging activity. In addition, the protective activity of oil may also be attributed to electrons donated for neutralization of free radicals.

MMP-9 inhibition and down regulation

The optimum concentration of 1µg/mL of LPS was used to induce inflammation. After incubation, different concentration of essential oil was added (50, 100, 150, 200 and 250 µg/mL) and gelatinase activity was measured by gelatin zymography. Cells treated with oil showed significant reduction in MMP-9 expression. While increasing the concentration the intensity of the band decreased (Figure 2), 200 µg/mL of oil observed as optimum concentration needed to inhibit MMP-9 expression. The total RNA of the cells was isolated and RT-PCR was performed to test the down regulating activity of oil on MMP-9 production

Table 1: Protection of H_2O_2 induced DNA damage by by P.amboinicus essential oil

Concentration of oil (mg/mL)	Tail length (mm)[a] (Mean ± SD)
Control	0.51 ±0.41*
H_2O_2 100 (mM)	10.63 ±0.70*
25	7.42 ±0.47*
50	6.22 ±0.50*
100	5.9 ±0.48*
150	4.0 ±0.40*
200	3.17 ±0.38*

[a]Represents p < 0.05 vs. control, as tested by the Student's '*t*'- test

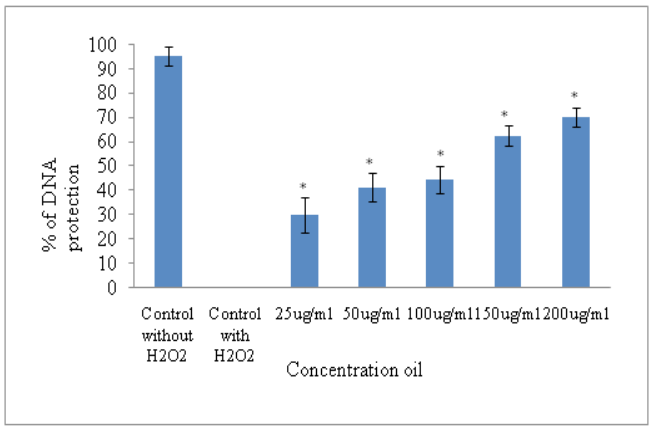

Figure 1 DNA damage induced by H_2O_2 in 3T3-L1 fibroblasts

The cells were incubated with 100 µM H_2O_2 and 25, 50, 100, 150 and 200 µg/mL oil on ice for 4h and then used comet assay. At 100 µM, a maximum tail length of 10.63 ±0.70 µm was observed. Means ± standard deviations are presented

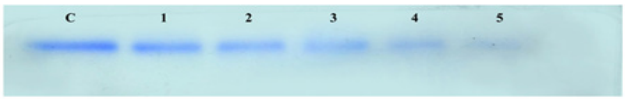

Figure 2: Gelatinzymogram of MMP-9

MMP-9 inhibition of different concentrations of P.amboinicus essential oil after LPS treatment (C) Control, (1) 50, (2) 100, (3) 150, (4) 200 and (5) 250 µg/mL of essential oil

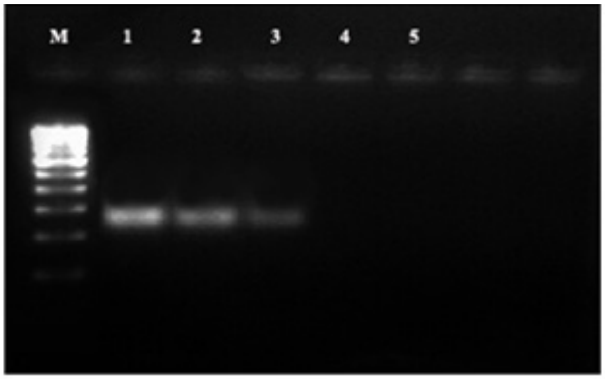

Figure 3 : Effect of *P.amboinicus* essential oil on down regulation of MMP-9

Lane M: marker ;Lane 1: 50 ; Lane 2: 100 ;Lane 3: 150 ;Lane 4: 200 and Lane 5: 250 µg/mL of essential oil

at transcriptional level. The results of RT-PCR clearly indicated that cells treated with LPS alone showed over expression of MMP-9 but cells treated with different concentration of oil showed significant reduction of MMP-9 expression. The down regulation activity of oil was time and concentration dependent (Figure 3). β-actin served as an internal marker (Figure 4). Inhibition of inflammatory cytokine and mediator production or function serves as a key mechanism in the control of inflammation and agents that suppress the expression of these inflammation

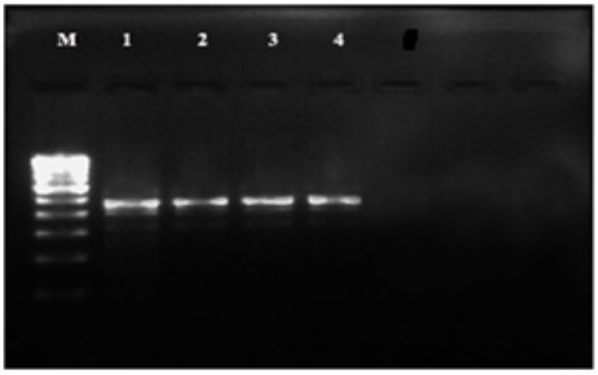

Figure 4 β-actin served as an internal marker

associated genes have therapeutic potential in the treatment of inflammatory diseases.[18] Result obtained in the present study shows, P.amboinicus essential oil is a potent MMP-9 inhibitory agent under in vitro. The observed activity may be due to the presence of bioactive mono and sesquiterpenes. However the known anticancer and antiinflammatory agent carvacrol which is the major compound[8] in oil might be the reason for observed MMP-9 down regulation. In human macrophage like U937 cells, carvacrol suppressed LPS induced COX-2 mRNA expression, suggesting that carvacrol regulates COX-2 expression through its agonistic effect on PPARγ.[19] We assumed that mechanism of down regulation may be associated with the inhibition of nuclear factor kappa B (NFKB).[12] using Aloe vera where down regulation of MMP-9 either by directly inhibiting the activation of NFKB and its subsequent binding to MMP-9 or by inhibiting the production of mediators such as prostaglandins which induce MMP-9 expression.

CONCLUSIONS

P.amboinicus essential oil is a good source of bioactive mono and sesquiterpenes. It is clear that oil may be considered as a potential natural product and could be used as a part of daily supplement in food to prevent health related problems.

ACKNOWLEDGEMENT

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CONFLICTS OF INTEREST

All authors have none to declare.

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Immunomodulatory Activity of *Atalantia monophylla* DC. roots

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ABSTRACT

Objective: The objective of the present study was to evaluate the immunomodulatory activity of the roots of *Atalantia monophylla.* In the present study Pet. Ether, Chloroform and Methanolic fractions of the ethanolic extract of the roots of *Atalantia monophylla*, were administered orally, in the doses of 10 mg/kg and 30 mg/kg, to evaluate the immunomodulatory activity. **Materials and Methods:** The Methanolic fraction showed most significant effect when compared with control group, in the dose of 30 mg/kg, in the *E.Coli* induced abdominal sepsis and Carbon Clearence Test as models for non-specific immune response. In the models of the Specific immune response, cell mediated immune response to SRBC - delayed type of hypersensitivity (DTH) the methanolic fraction in the dose 30 mg/kg, when compared with control group, showed most significant effect on decrease in footpad edema after treatment. **Results:** Humoral immune response was assessed by Sheep erythrocyte agglutination test, in which the Methanolic fraction of *Atalantia monophylla* in the dose of 30 mg/kg showed most significant ($p<0.05$) increase in antibody titer after treatment when compared with control group. **Conclusion:** The present investigation reports that the Methanolic fraction of the ethanolic extract of the roots of *Atalantia monophylla*, in the dose of 30 mg/kg showed most significant immunomodulatory activity.

Key words: *Atalantia monophylla*; Immunomodulatory; Phagocytosis; Cell-mediated immune response; Humoral immune response.

INTRODUCTION

In coming years, use of indigenous drugs have increased to a very great extent as an alternative medicine. So, there is increasing need of undertaking studies for development of parameters of assessing these drugs scientifically. The main concepts of Rasayana drugs of Ayurvedic medicines are that these drugs increase the resistance of body as rejuvenating agents. Many plants under this class are reported for promoting and restoration of health. In many cases natural products are used as an alternative to the conventional chemotherapy against a variety of diseases. Immune system plays an important role in contributing to maintenance of homeostasis and establishment of body's integrity. In the therapy immunomodulators are used to stimulate or suppress the immune response of the host. These are now recognized as an alternative to conventional chemotherapy in a variety of diseased conditions, especially when host's defense mechanisms have to be activated because of impaired immune response. A selective immunomodulators have to be induced in situations where it acts by stimulating specific and nonspecific responses and may be even useful for prevention and treatment of immunodeficiency related disorders allergic reactions, organ transplantation and AIDS.[1]

The scientifically unexplored plants have tremendous potential for developing new pharmaceutical products containing patient friendly phytochemicals coupled with lesser chances of drug resistance. In this regard, the objective of the present study is to investigate Immunomodulatory activity of the Roots of *Atalantia monophylla* was undertaken.

Atalantia monophylla DC, is a large, thorny shrub or small tree. It is found in peninsular India, Orissa, Assam, Meghalaya and in the Andamans. In the local language it is called as 'Jungli Limbu' or 'Makad-limbu'. The roots possess anti-spasmodic, stimulant and antirheumatism property.[2,3] The plant have been reported to have

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Antioxidant activity, Antifungal activity[4] and Mosquitocidal activity.[5] The drugs acting on rheumatoid arthritis are also proved to be immunomodulators.[6,7] It is also reported to have anti-oxidant property, which also justified its role as an immunomodulator,[8] thatswhy the roots of the plant *Atalantia monophylla* were selected to investigate its immunomodulatory activity.

MATERIALS AND METHODS

Plant material and it's Authentication

The roots of the plant *Atalantia monophylla* DC were collected in the month of August from the local area of Tirupati, Dist. Chitoor (A.P.) India. It was then shade dried. The roots of the plant *Atalantia monophylla* DC. were authenticated from the Botanist and Taxonomist, Dr. K. Madhava Chetty, Asst. Prof., Dept. of Botany, Sri Venkateswara University, Tirupati-517502. (Reference no. 2008-09/135).

Preparation of Extracts & Fractions

The dried roots of *Atalantia monophylla* DC (AM) were extracted with ethanol as a solvent by using maceration technique. 2 kgs of the powdered crude drug was kept in a jar containing sufficient amount of ethanol for around three weeks. Vigorous intermittent shaking with the help of a mechanical shaker was done frequently. It was then filtered. Filtrate was then evaporated to dryness with the help of Rota evaporators and Vacuum oven. The dried ethanolic extract of AM was then processed for fractionation by using different solvents like Pet. Ether (40-60), Chloroform and Methanol. All the fractions were evaporated to dryness.

Preparation of Drug Solution

All the powdered fractions of the ethanolic extract were accurately weighed, and then dispersed in distilled water using a suspending agent TWEEN 80. The appropriate stock suspensions of the drugs were prepared. The doses were administered orally by selecting the appropriate concentration of the stock solution**.** The plain suspension of the distilled water with TWEEN 80 served as control.

Animals

Swiss male albino mice (18-22 gm) and wistar rats of either sex (150-200 gm) were used. They were maintained at 25 ± 2° C and relative humidity of 45 to 55% and under standard environmental conditions (12 h light: 12 h dark cycle). The animals had free access to food (Amrut feed, Chakan oil mills, India) and water *ad libitum* throughout study. Institutional Animal Ethical Committee approved the protocol (Proposal No. 03/IAECCOP/2009). All the experiments were carried out between 9:00-16:00 hour.

Preliminary Phytochemical Screening of the Fractions

All the fractions of the ethanolic extract were investigated for preliminary phytochemical analysis using various tests to determine the presence of different phytoconstituents in different fractions.[9]

Acute Toxicity Studies

In the Acute Toxicity Study, Oral administration of all the fractions of the ethanolic extract of crude drug at the doses of 175, 550 and 2000 mg/kg in mice showed no adverse effect or mortality was observed in wistar rats up to 2000 mg/kg, p.o of extracts during the 24 hours and 14 days observation. From pilot study data and review of literature, two different doses 10 mg/kg and 30 mg/kg were selected for further study.[10,11]

Pharmacological Screening of Different Fractions

All the fractions of ethanolic extracts of drugs were screened for their immunomodulatory effect by using following pharmacological screening models.

Models for Non- specific Immune Response

E. coli Induced Abdominal Sepsis (Determination of Host Resistance)

Four groups each consisting of 06 animals. All animals were injected with 1.0 mg of E.coli serotype 0111:B4 LPS in a volume of 500µl of sterile solution. Immediately after this, group I to IV received isotonic NaCl solution, (10mg/kg), test drug dose I.

There after quantities of bacteria in the intra abdominal fluid (10ml of sterile saline was injected into the abdominal cavity and then sample of peritoneal levage fluid was collected under anaesthesia and subjected to quantitative evaluation of the bacteria.) and rate of lethality was observed at every 24 hour for the period of 72 hours.[12]

Carbon Clearance Test (Phagocytic Response)

In this test three groups of animals were used with 6 rats in each group. Group I served as a control and received vehicle i.e. plain suspension of sterile water with TWEEN

80 (10ml/kg) only. On the other hand, animals of group II received doses D1 mg/kg of particular fraction of drug extract orally daily for the period of 05 days. Carbon ink suspension was injected via the tail vein to each rat 48 hours after the five-days treatment. Blood samples (25 µl) were then withdrawn from the retro-orbital plexus under mild ether anesthesia at 0 and 15 minutes after injection of colloidal carbon ink and lysed in 0.1% sodium carbonate solution (03 ml). The optical density was measured spectrophotometrically at 660 nm. The phagocytic index was calculated using the following formula;

$$K = \log OD\ 1 - \log OD\ 2 / t_2 - t_1$$

Where, OD1 and OD2 are the optical densities at time t_1 and t_2.[13,14]

Models for Specific Immune Response

Cell-mediated Immune Response to SRBC (DTH)

Cell mediated immune response was assessed by T - cell population test and delayed type hypersensitivity (DTH).

T - cell Population Test

In this test, three groups of rats were used with 6 rats in each group. Group I served as a control and received vehicle i. e. plain suspension of sterile water with TWEEN 80 (10 ml/Kg) only. Rats of group II received doses D1 mg/kg of particular fraction of drug extract orally daily for 10 days. On 11th day blood was collected from retro orbital plexus and anticoagulated with sodium citrate in separate test tubes. The test tube containing blood was placed in a left sloping position 45^0 at 37 ^{0}C for 01 hour. Supernatant fluid containing lymphocytes and leukocytes were removed using micropipette.[15,16]

Delayed Type Hypersensitivity (DTH)

The mice were divided into 04 groups, each containing 06 animals. Group I served as normal control Group and received plain suspension of sterile water with TWEEN 80 orally (01 ml/Kg) for the period of 21 days. Group II served as negative control Group receives Cyclosporine 100 µg/mouse, i.p. on 14th day of study. Animal of group III were administered doses D1 mg/kg of particular fraction of drug extract orally daily for the period of 21 days. Mice were immunized with 0.1ml of 20% SRBC's in normal saline intraperitonially on 14th day of study.

On day 21st, animal from all group get challenged with 0.03 ml of 1% SRBC's in sub plantar region of right hind paw. Footpad reaction was assessed after 24 hours i.e. on 22nd day. Increase in foot paw edema was measured using digital plethysmometer – LE7500 (Panlab, USA).[15]

Humoral Immune Response (Haemagglutination Antibody Titer)

Humoral immune response was assessed by Sheep erythrocyte agglutination test.

Sheep Erythrocyte Agglutination Test

Rats were divided into three groups with 06 rats in each group. Group I served as a control and received vehicle i.e. plain suspension of sterile water with TWEEN 80 (10 ml/Kg) only. Rats in groups II were administered two doses D1 mg/kg of particular fraction of drug extract orally daily for 10 days.[15]

Preparation of Sheep Red Blood Cells (SRBC's)

Sheep blood was collected from local slaughter house in sterile Alsever's solution in 1:1 proportion of Alsever's solution (freshly prepared). RBC's were counted microscopically (5 x 10^9/ml). Blood was kept in the refrigerator at 4 ^{0}C and processed, for the preparation of Sheep RBC (SRBC's) batch, by cetrifugating (3000rpm for 05minutes) and washing with physiological saline 4-5 times followed by suspending into buffered saline for further use.[13]

Formula of Alsever's Solution

Sodium chloride	:	0.42 gms/100 ml
Sodium citrate	:	0.80 gms/100 ml
Glucose	:	2.05 gms/100 ml

All the rats were injected with 0.25ml of 5 x 10^9 SRBC/ml on 6th, 8th, and 10th days for achieving maximum titer of antibody. On day 11, blood was collected and serum was separated by centrifuging at 200 rpm for the period of 15 minutes. 100 l of serum diluted serially with normal saline in separate test tubes. Dilutions were made i.e.20, 40, 60 up to 1280. To this, 50 l of SRBC added and incubated at 37 ^{0}C for 18 hours. All the tubes were then subjected to examine under microscope for agglutination and compared with control. The highest dilution giving hemagglutination was taken as the antibody titer. The antibody titers were expressed in the graded manner, the minimum dilution (01/02) being ranked as 01, and mean ranks of different groups were compared for statistical significance.[17]

Data Analysis

Data obtained were subjected to statistical analysis using one way ANOVA followed by Dunnetts 't' test using graphpad software.

RESULTS AND DISCUSSION

Plant Extraction & Fractionation

The dried roots of *Atalantia monophylla* DC were extracted with the help of ethanol by maceration method. The yield was 5.78%.

The ethanolic extracts of the roots of *Atalantia monophylla* DC was fractionated by using different solvents like Pet. Ether (40-60), Chloroform and Methanol with increasing polarity. The yields of the fractions were calculated and have been shown in the Table 1.

Preliminary Phytochemical Screening of the Fractions

The results of the Preliminary phytochemical screening of the Pet. Ether, Chloroform and Methanol fractions of the ethanolic extract of the roots of *Atalantia monophylla* R.Br. have been shown in Table 2.

Models for Non- specific Immune Response

E. coli Induced Abdominal Sepsis (Determination of Host Resistance)

The Methanolic fraction of AM 30 mg/kg showed most significant ($p<0.05$) effect when compared with control group for 24 hrs, 48 hrs and 72 hrs. Other doses of AM fractions do not showed any significant effect. The results have been shown graphically in the Figure 1.

Carbon Clearance Test (Phagocytic Response)

The Methanolic fraction of AM 30 mg/kg showed most significant ($p<0.05$) and ($p<0.01$) dose dependent increase in carbon clearance when compared with control. The Methanolic fraction of AM 10mg/kg showed significant ($p<0.05$) increase in carbon clearance when compared with control. AMC10 mg/kg, AMC 30mg/kg, AMPE 10mg/kg and AMPE 30mg/kg also showed some significant activity by increase in carbon clearance when compared with control. The results have been shown graphically in the Figure 2.

Models for Specific Immune Response

Cell-mediated Immune Response to SRBC (DTH)

Cell mediated immune response was assessed by T - cell population test and delayed type hypersensitivity (DTH).

T - cell Population Test

None of the doses of fractions of the crude drug showed any significant change. As no significant changes were observed during the T-cell Population test so the data have not been Presented.

Delayed Type Hypersensitivity (DTH)

The Methanolic fraction of AM 30 mg/kg showed most significant effect ($p<0.01$) decrease in footpad edema after treatment when compared with control group. The Methanolic fraction of AM 10 mg/kg also showed significant effect ($p<0.01$). The other fractions and doses of AM did not show any significant activity. The results have been shown graphically in the Figure 3.

Humoral Immune Response (Haemagglutination Antibody Titer)

Humoral immune response was assessed by Sheep erythrocyte agglutination test.

Sheep Erythrocyte Agglutination Test

The Methanolic fraction of AM 30mg/kg showed most

Table 1: Yield of the fractions of the ethanolic extract of crude drug

Crude Drug	Pet. Ether Fraction % Yield	Chloroform Fraction % Yield	Methanol Fraction % Yield
Atalantia monophylla DC	16.75	21.15	18.50

Table 2: Preliminary phytochemical screening of the fractions of the ethanolic extract of *Atalantia monophylla* DC

Phyto constituents	Pet. Ether fraction	Chloroform fraction	Methanolic fraction
Carbohydrates	-	-	+
Glycosides	-	-	-
Saponins	-	-	-
Flavonoids	-	-	+
Alkaloids	-	+	-
Tannins	-	-	+
Steroids	+	-	-
Amino Acids	-	-	-
Proteins	-	-	-

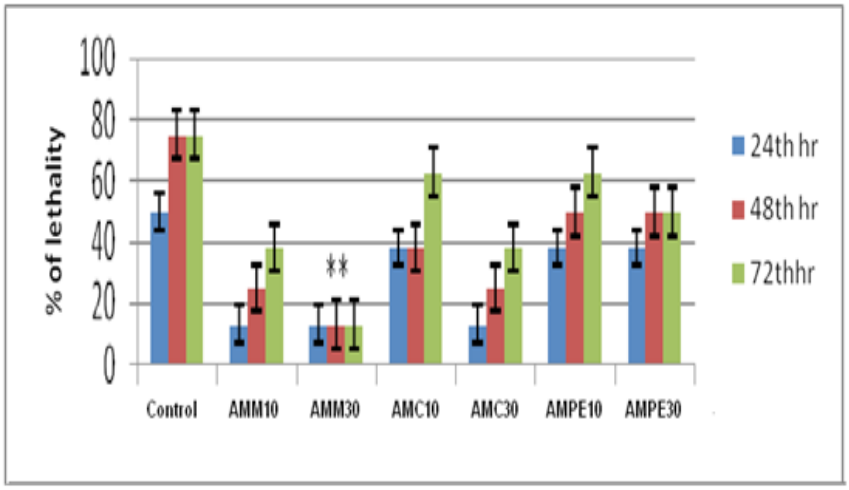

Figure 1: Effect of *Atalantia monophylla* fractions treatment on E. coli induced abdominal sepsis

*Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's t test. *P<0.05, **P<0.01*

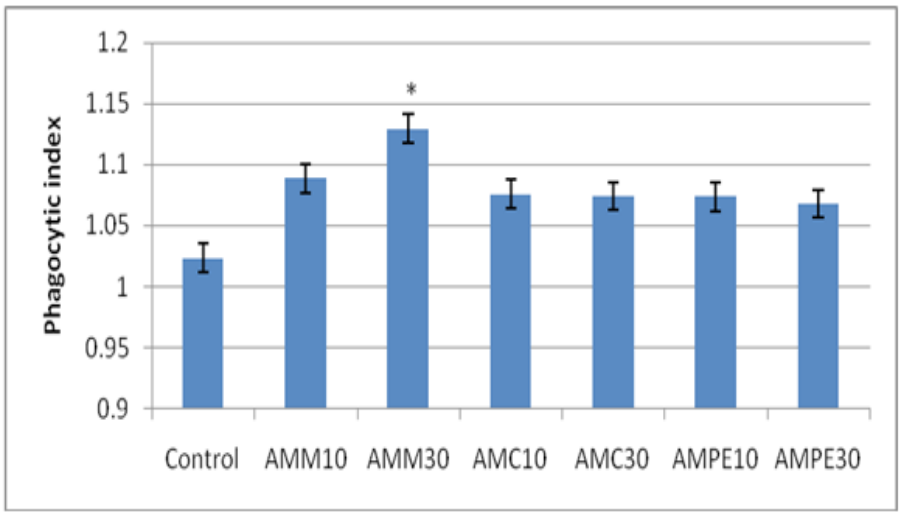

Figure 2: Effect of *Atalantia monophy*lla fractions treatment on carbon clearance test

*Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's t test. *P<0.05, **P<0.01*

significant (p<0.05) increase in antibody titer after treatment when compared with control group. The other fractions AMM 10mg/kg, AMC 10mg/kg, AMC 30mg/kg, AMPE 10mg/kg, AMPE 30mg/kg did not show any significant increase in antibody titer after treatment when compared with control group. The results have been shown graphically in the Figure 4.

CONCLUSION

The roots of *Atalantia monophylla* DC. were selected to investigate the immunomodulatory activity. Traditionally the crude drug was used in the treatment of chronic rheumatoid arthritis[2,3] and crude drug was scientifically reported for having anti-oxidant property.[4] As the anti-oxidant property and anti-rheumatism drugs can be used as

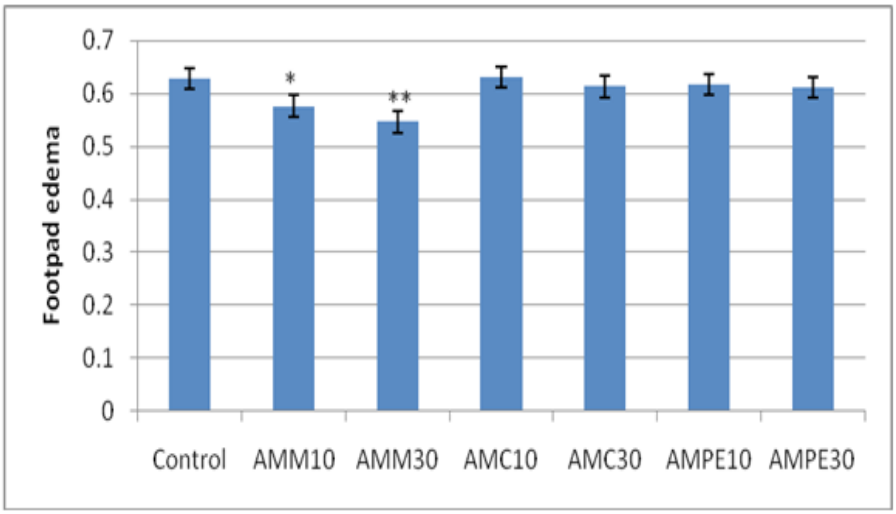

Figure 3: Effect of *Atalantia monophylla* fractions treatment on foot pad edema
*Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's t test. *P<0.05, **P<0.01*

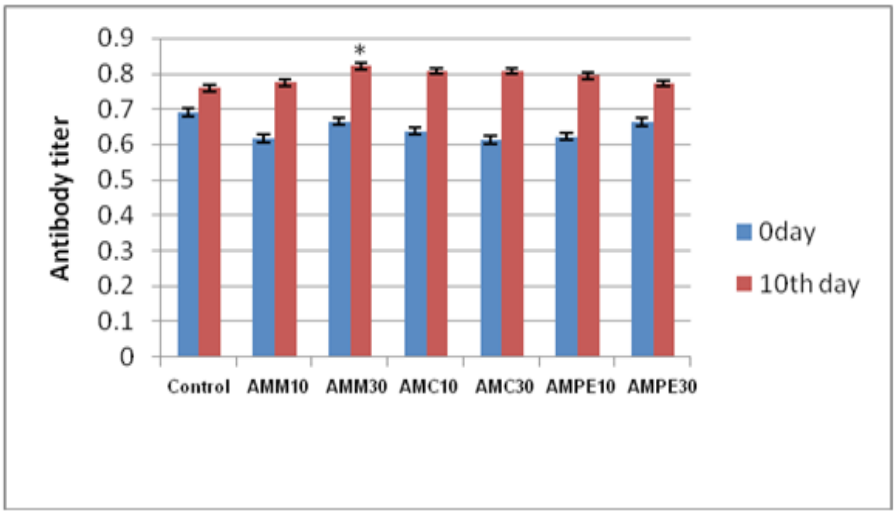

Figure 4: Effect of *Atalantia monophylla* fractions treatment on sheep RBC
*Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05, **P<0.01*

immunomodulators.[6-8] So the selection of the crude drug is justified.

The present investigation indicates that the methanolic fraction of the ethanolic extract of the roots of *Atalantia monophylla* DC., exert a significant immunomodulatory activity by enhancing Specific Immune response as well as Non-specific immune response. The Preliminary phytochemical screening of the methanolic fraction of the crude drugs revealed the presence of flavonoids which may be the phytoconstituents responsible for immunomodulatory activity.[18] Thus the crude drug showed significant immunostimulant property, and which may be due to the flavonoids present in it. Further investigation is underway to find out the pytoconstituents present in the fraction which is responsible for this action.

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Pharmacognostic Evaluation of Indigenous Medicinal Plant *Kedrostis foetidissima* (Jacq.) Cogn.

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ABSTRACT

Introduction: Ingeneral, members of Cucurbitaceae are well exploited for their medicinal, economic and culinary values,however few of them are less exploited.The present study deals with pharamcognostical evaluation ofone such plant namely Kedrostis foetidissima(Jacq.)Cogn.called as Appakovai in Tamil. **Methods:** Pharmacognostic evaluationswere carried out by organoleptic evaluation, anatomical studies and powder microscopy of leaves, roots and root tubers. **Results:** Organoleptic evaluation revealed that the characteristic white tubers, as storage organs,leaves exhibited a foetidodour when crushed.Anatomy of leaves showed lithocysts and anomocytic stomata,root showed the presence of fissured periderm and cleaved vascular bundlesand that of root tubers showed 3 or 4 exarch xylem. Powder microscopy of root tubers showed narrow and wide fibers; long, narrow and drum shaped vessels and scattered sclereids. **Conclusion:** The present Pharmacognostic and Powder microscopic studiesreveals that, the characteristic features of this plantparts resembles, the other members of cucurbitaceae .

Keywords: *Kedrostis foetidissima, Pharmacognosy, Powder microscopy.*

INTRODUCTION

Human beings depend on plant resources for food, shelter, clothing and medicine. Many members of Cucurbitaceae are exploited for their medicinal, economic and culinary values, However *Kedrostis foetidissima (*Jacq.*)* Cogn. is less exploited.[20] The plant is widely distributed in Africa and Asian countries. Most species of the genus Kedrostis are distributed chiefly in Africa. However, one species present in India is found in Gujarat, Konkan, Malabar, Deccan, and Carnatic regions of India.[5] In Tamil Nadu, this it is distributed in Cuddalore, Salem, Dharmapuri and Coimbatore districts.[12] The present study aims to evaluate the pharmacognosy of *Kedrostis foetidissima (*Jacq.*)* Cogn. (Figure 1), which has got wonderful medicinal values on cough,cold, asthma and piles.[14]

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Figure 1: Kedrostis foetidissima–Habit

MATERIALS AND METHODS

Chemicals and solvents

All chemicals and solvents used for the study were AR grade and was purchased from SRL- India.

Collection of Plant

The plant material used in this study was collected from wild area of Thittagudi (Tk) in Cuddalore district of Tamil Nadu, India (Latitude11.45; Longitude 79.75) Roots, tubers and leaves were washed thoroughly in tap water and then rinsed in distilled water for further use.

Phenology

Periodical phonological survey was conducted from September 2011 to March 2011. As the root tubers remain dormant under the soil during unfavorable season (April to September). The survey was limited to the above mentioned period.

Pharmacognostic Studies

The plant was identified on the basis of organoleptic, macroscopic and microscopic (anatomy) observations as summarized below.

(a)Organoleptic Characters

Study of Organoleptic characters of the plant was carried out using physical examination of the plant parts and light microscopic evaluations.[22]

(b)Anatomy

The anatomy of root tubers, leaves and stem were studied using the protocols of Subha et al (2011). Briefly, fresh plant parts were fixed in FAA (5% Formalin, 5% Acetic acid and 90% of 70% Ethanol) for 24 hours and subsequently dehydrated using series of t-Butyl alcohol. Fixed samples were embedded in paraffin wax blocks, sectioned using rotary microtome, stained with toluidine blue, safranin, fast-green, IKI as per protocols of O'Brien et al(1964), viewed under Nikon Labphoto2polarized microscope (Nikon , USA).

(c) Powder Microscopy

For powder microscopic observations, a coarse powder of root tuber was spread in a glass slide and viewed in bright field and polarized light microscope (Nikon Labphoto2 polarized microscope, USA. [3]

Deposition of voucher specimen

A voucher specimen of identified plant was authenticated and deposited in National Institute ofHerbal Science – Plant Anatomy Research center (PARC), Chennai- 45.

RESULTS AND DISCUSSION

According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for basic health care.[23] Medicinal plants are source of raw material for modern and traditional systems of medicine. In India, Siddha and Ayurvedic medicines are widely used to treat many common ailments. The treatments, in most cases, are administered by traditional healers and generally consist of crude plant extracts. Recently, biologically active compounds are isolated from such plants and used as herbal medicine.[21]

Botanical description

It is a scandent, monoecious climber with perennial root system. Stem -slender, angled, branched and sparsely hairy. Tendrils - axillary, simple, filiform and glabrous.Leaves-simple, bright green, hairy and more or less scabrid on both sides, margins distantly toothed or 5-angled.Petioles short and hairy.[14] **Male flowers**: yellow,2 to 4 at the apex, pedicels capillary, long with hairy calyx and campanulate (Figure 2a). **Female flowers**: pale yellow and pubescent, peduncles longer than male flowers, calyx 5, united, hairy and campanulate (Figure 2b). Ovary inferior, oblong, beaked and pubescent. Fruits sub-sessile, deep red and tapering into a long narrow beak. Seeds ***brown with a narrow sharp wing.***[10,19]

Figure 2a: Female Flower

Figure 2b: Male Flower

Pharmacognostic Studies

Organoleptic observations

*Kedrostis foetidissima (*Jacq.*)* Cogn. is a climber with root tubers. The root tuber is thick, fleshy and a white storage organ which is 4 mm in thickness. Surface appearance, size, odour and texture of the leaves were done by physical examinations. The leaves were slightly bitter; they look fleshy and hairy in appearance. It gives a foetidodour when crushed, the surface of the leaves are rough due to the presence of hairy out growths.

Anatomy of Plant Parts

Microscopic Features of Leaves

Lamina

The leaf is fairly thick, soft and has thin, less prominent midrib. The midrib is slightly projecting on abbatial side and more or less flat on the adaxial side. The midrib portion is about 300 µm thick. The vascular system of the midrib consists of two juxtaposed collateral bundles which include wide, angular thick walled xylem elements. The lamina is 220 µm thick. It consists of thick adaxial epidermis comprising thin walled cylindrical cells. At frequent intervals, a group of epidermal cells dilate extensively and possess calcium carbonatecystoliths. The cystolith bearing cells are called lithocysts.[3]

The epidermal cells as seen in surface view of the paradermal sections, are wide, thick walled and highly wavy rendering the cells amoeboid in outline. The cell surface is smooth. The stomata occur only on the abaxial epidermis. They are anomocytictype having no specific subsidiary cells. The guard cells are wide and elliptic, measuring 25 X 20 µm in size (Figure 3).

Root tuber

The tap root becomes modified into thick, fleshy, white storage organ. It is about 4mm thick. In sectional view, the tuberous rootconsists of outer thin periderm, wide

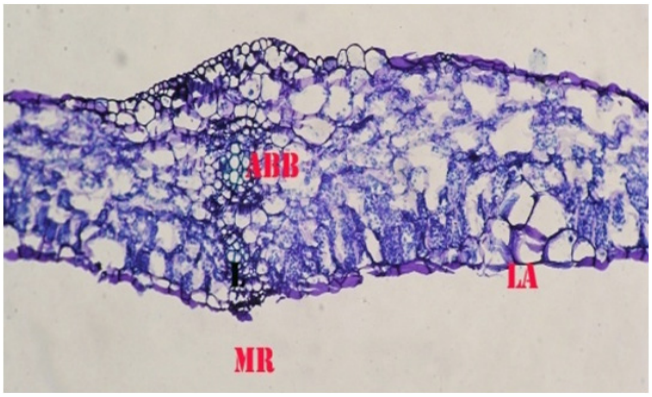

Figure 2: Lamina T.S MR-Mid rib, LA-Lamina, ABB-Abaxial bundle

Table 1: Phenology of the plant

MONTHS	SEPTEMBER				OCTOBER				NOVEMBER				DECEMBER				JANUARY				FEBRUARY				MARCH			
WEEKS	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
GERMINATION	------																											
VEG. PHASE	----------				-------																							
FLOWER BUDS							---------																					
FLOWERS									----------				-----															
YOUNG FRUITS															------		--											
MATURE FRUITS																	----------				----------							
LEAF / FRUIT FALL																									----------			

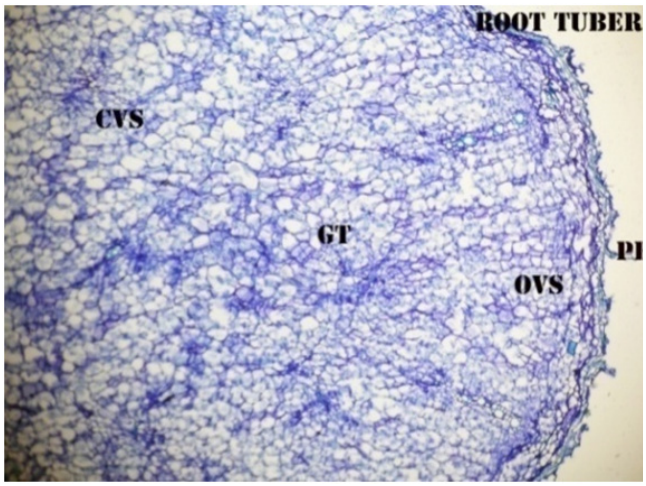

Figure 3: Root tuber - T.S (CVS-Central vascular system, GT-Ground tissue, OVS-Outer vascular system PE-Periderm)

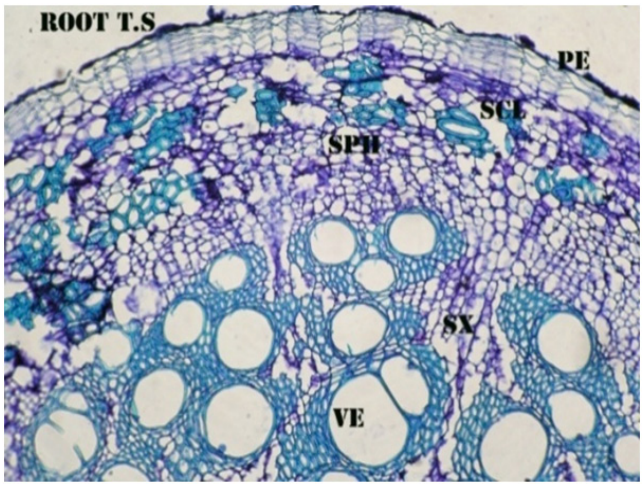

Figure 4: Root T.S(PE-Periderm, SPH-Secondary phloem, SX-Secondary xylem, VE-Vessel element)

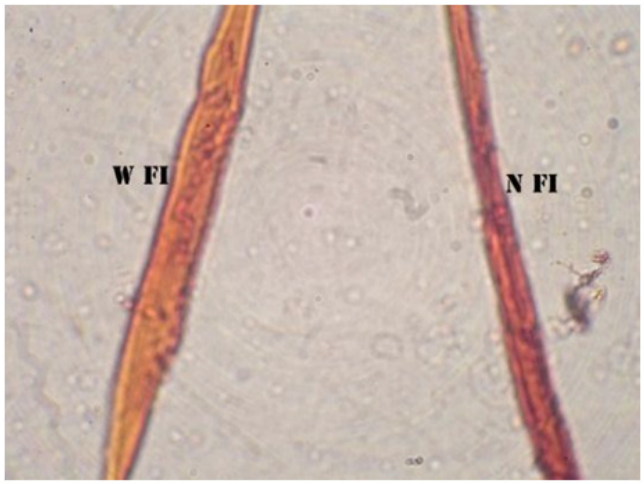

Figure 5: Powder microscopy of Root tuber (WFI-wide fibre, NFI- Narrowfibre)

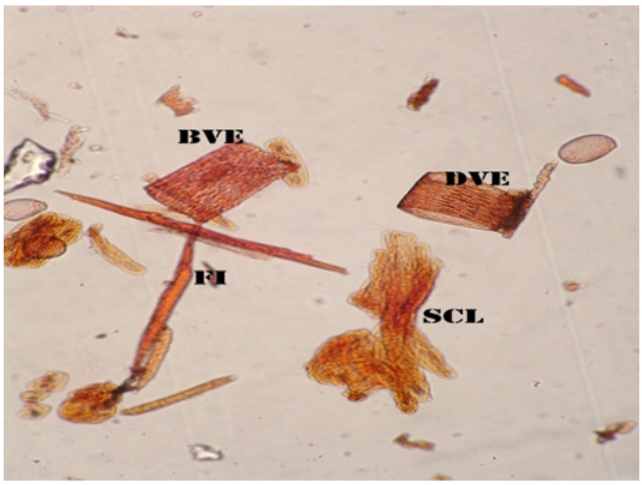

Figure 6: Powder microscopy of Root tuber (BVE-Broad vessel element,SCL-Sclereids, DVE-Drum shaped vessel elements, FI-Fibres)

parenchymatous ground tissue and isolated small vascular strands (Figure 4).

Periderm is a thin outermost zone of cells comprising two or three layers of cells. The periderm surface is irregularly fissured.The ground tissue occupies the major portion of the tuber and consists of large thin walled irregular and compact parenchyma cells. The ground parenchyma contains dense accumulation of starch grains. The starch grains are mostly compound type, simple parenchyma being less frequent. Many prismatic crystals are grouped into spherical ball compound type. The compound star grains are about 100µm thick.

Vascular strands are found in discrete units; some strands are seen along the periphery of the ground tissue. These outer vascular strands are in narrow radial files, the xylem elements are narrow and thin walled. In the central core also seen small nests of vascular elements. These strands have three or four xylem strands.[3]

Root

The root is 1.6 mm thick. It consists of outermost rough, fissured and narrow periderm. It is superficial in position and includes 5 to 7 layers of suberised phellem cells. Inner to it is the presence of cortical zone where brachysclereids and fiber elements are scattered. The vascular cylinder is cleaved into many fan shaped segments which are wider at the periphery and narrow at the centre. On the outer ends

of the xylem wings phloem mass occur which includes sieve elements which are random in alignment (Figure 4).[3]

Powder Microscopy

The powder/ macerated preparation of the root-tuber exhibit the following inclusions as seen under the microscope. Fibres: Xylem fibres are abundant and are thin walled. There are two types of fibres, one is narrow type and another is wide type. Narrow fibres are thick walled with reduced lumen;up to 500µm long. The wide fibres have thick wall and wider lumen;up to 400µ long (Figure 5).

The vessel elements of different shape and size are common in the powder. Some of the vessel elements arelong, narrow and cylindrical, measuring 450µm long.Another type of vessels includes wider, shorter vessel elements measuring 160-180µm long. A third category of cells are much wider and drumshaped,they are 100µm long and 150µm wide. There are scattered sclereids which are short, wide and lumened cells. They have thick walls and abundant pits. The sclereids are 150µm long (Figure 6).[22]

Phenology

As for as phenology is concerned , the germination of root tubers started in the first and second week of September, vegetative phase remained from first week of September to second week of October, Flower buds were given off during the third week of October and transformed into flowers in first week of November and continued till the second week of December, the flowers were converted into young fruits during third week of December and continued till first week of January, matured fruits were formed in the second week of January and continued till the fourth week of February, lastly the leaves were turned yellow and withering of leaves and ripened fruits took place during the month of march (Table -I).

CONCLUSION

The earlier ethno botanical study revealed the usage of this plant as medicine. In spite of its medicinal values it is not very commonly used as other members of Cucurbitaceae. The pharmacognostic investigations of leaves, roots and root tubers revealed the characteristic anatomical features of Cucurbitaceae. Powder microscopic results of root tubers showed the presence of narrow and wide fibers, narrow, wide and drum shaped vessels. Though there is a scarce reference about the medicinal values of roots of this plant in the text, the leaves have got wonderful curative effects against cough, cold, asthma and piles. The phonological study revealed the availability of this plant only for a period of six months in a year. Thus pharmacognostical studies were carried out to ensure the identity of the plant and a little effort is taken to explore the uses of this plant.

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Phytochemical and analytical evaluation of *Cordia dichotoma* Linn. leaves

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ABSTRACT

Background: An ethnomedicinally important plant, *Cordia dichotoma* Linn is practiced in various indigenous systems of medicine and popular among the various ethnic groups in India for the cure of variety of ailments as an astringent, anthelmentic, diuretic, demulcent, anti-diabetic and expectorant. Because of the increasing demand, maintaining quality standards is the need of the day. **Aims and Objectives:** The present study was designed to set standard pharmacognostical, physicochemical, phytochemical, fluorescence and HPTLC chromatographic profile of the leaves of *Cordia dichotoma* Linn (CD). **Materials and Methods:** CD, which was previously authenticated, was subjected to pharmacognostical, physicochemical, fluorescence and high performance thin-layer chromatography (HPTLC) analysis as per standard protocol. **Results and Conclusion:** The final observations were recorded. The loss on drying at 105°C was found to be 8.5% w/w, total ash value 13% w/w, acid-insoluble ash 5.07% w/w, water-soluble ash 5.49% w/w, water-soluble extractive 9.2% w/w, alcohol-soluble extractive 5.81% w/w and pH (1% aqueous extract) 6.88. Phytochemical screening showed the presence of steroid, carbohydrate, alkaloid, saponin, cardiac glycosides, flavonoid and phenolic compounds in methanolic extract. The CD fluorescence was seen in UV light and it was of different colour in different solvents. HPTLC analysis revealed 5 peaks at wavelength 366 nm with max R_f values in the range of 0.3 to 0.93. The purity and quality of the leaves of *Cordia dichotoma* or pharmaceutical preparations prepared from it can be tested by pharmacognostical, physicochemical, fluorescence and HPTLC observations of the present study.

Keywords: *Cordia dichotoma,* Fluorescence analysis, Physicochemical parameters, HPTLC chromatogram.

INTRODUCTION

Cordia dichotoma Linn belonging to the family Boraginaceae is a small to moderate-sized deciduous tree with a short bole and spreading crown widely distributed in India and Srilanka.[1] It is commonly named as Indian cherry (English), Lasura/Bhokar/Borla (Hindi), Vadgundo/Gunda (Gujarati). The various parts of the plant viz., stem bark and leaves are practiced in various indigenous systems of medicine viz., Ayurveda and Unani and popular among the various ethnic groups in India for the cure of variety of ailments as an astringent, anthelmentic, diuretic, demulcent, anti-diabetic and expectorant. Their leaves are traditionally used for the treatment of jaundice at Dandakaranya area, Andhra Pradesh in India. It is reported to have antioxidant, juvenomimetic, antifertility, anti-inflammatory and various other pharmacological activities.[2-4] Carotenoids are mainly present in their leaves which have potent antioxidant activity.[5] Present study deals with the detailed pharmacognostical study of its leaves, physicochemical evaluation, fluorescence analysis and HPTLC chromatographic fingerprint profiling. Microscopic method is one of the simplest and cheapest methods to start with for establishing the identity of the source materials despite of the availability of sophisticated modern research tools for evaluation of the plant and plant derived crude drugs. This study will provide standardized parameters for the leaves of *Cordia dichotoma* Linn to future scientists for its correct identification and adulteration, if any.

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MATERIALS AND METHODS

Collection and authentication of plant specimen

Leaves of the plant *Cordia dichotoma* Linn for the study were collected from nearby region of Kukrail forest, Lucknow, Uttar Pradesh and authenticated by National Botanical Research Institute, Lucknow (authentication reference number NBRI/CIF/306/2012 dated 18/06/2012).

Materials and reagents

Soxhlet apparatus, rotavapor, CAMAG HPTLC system (Muttenz, Switzerland) equipped with Linomat5 applicator, Reprostar3, TLC scanner3, twin trough plate development chamber, Hamilton syringe (100µl, Reno, Nevada, USA), win-CATS software, analytical grade chemicals (Fischer Scientific and E. Merck, India), HPLC grade methanol (E. Merck, India).

Pharmacognostical evaluation

Pharmacognostical evaluation of CD comprises of organoleptic characters [i.e., color, odor, taste, and texture] and microscopic studies. Organoleptic characters were recorded by observing with naked eyes. Microscopic studies, i.e., transverse section (T.S.) of leaf by treating with saffaranin and mounting with glycerin water over glass slide was observed under Carl Zeiss Binocular microscope attached with camera and microphotographs were taken.[6]

Physicochemical evaluation

CD was analyzed through physicochemical parameters i.e., loss on drying, total ash value, acid-insoluble ash, water-soluble ash, pH value and extractive values.[7,8]

Phytochemical screening

Phytochemical screening for carbohydrate, protein, alkaloid, steroid, glycoside, etc., had been carried out.[9]

Fluorescence analysis

Finely powdered CD after treatment with different chemicals was analyzed through fluorescence analysis. It was performed for the different extracts too but without any chemical treatment.[10,11]

HPTLC chromatographic analysis

Sample solution of methanolic extract of *Cordia dichotoma* leaves (MECD) was spotted in the form of band using a Hamilton syringe on precoated silica gel GF 60_{254} aluminium plate by means of CAMAG Linomat 5 sample applicator. Toluene: ethyl acetate: formic acid (5:4:1) was used as the mobile phase. After development, plate was kept in CAMAG Reprostar 3 and densitometric scan was performed with a Camag TLC scanner3 in reflectance absorbance mode at UV detection as 254 nm and 366 nm under the control of win-CATS software.[12,13]

OBSERVATIONS AND RESULTS

Pharmacognostical evaluation

Organoleptic features of CD are shown in **Figure 1**. Shape of leaf is almost ovoid with dentate margin. Upper and lower surfaces are rough with light green in color and appearance. Odor is pleasant and taste is mucilaginous. Microscopy of CD showed features like scattered vascular bundles having patches of perimedullary phloem, unicellular and multicellular covering trichomes as shown in **Figure 2**. Xylem vessels and calcium oxalate crystals were seen too in powder microscopy.

Physicochemical evaluation

CD was analyzed through physicochemical parameters. pH of 1% (w/v) aqueous solution of powdered leaves was found to be 6.88, approximately of neutral pH. Other observations are presented in **Table 1 and Table 2**.

Table 1: Loss on drying and ash values for the powdered leaves of the plant *Cordia dichotoma*

Physicochemical parameters		*% (with reference to air dried drug)*
Loss on drying		8.5
Ash value	Total ash	13
	Acid insoluble ash	5.07
	Water soluble ash	5.49

Phytochemical screening

Methanolic extract showed the presence of steroid, carbohydrate, alkaloid, saponin, cardiac glycosides, flavonoid and phenolic compounds. The intensity of presence is shown in **Table 3**.

Fluorescence analysis

Fluorescence characteristics of powdered leaves of the plant *Cordia dichotoma* and its various successive solvent extracts under day and ultraviolet light are presented in **Table 4 and Table 5**.

Figure 1: Leaves of *Cordia dichotoma* Linn

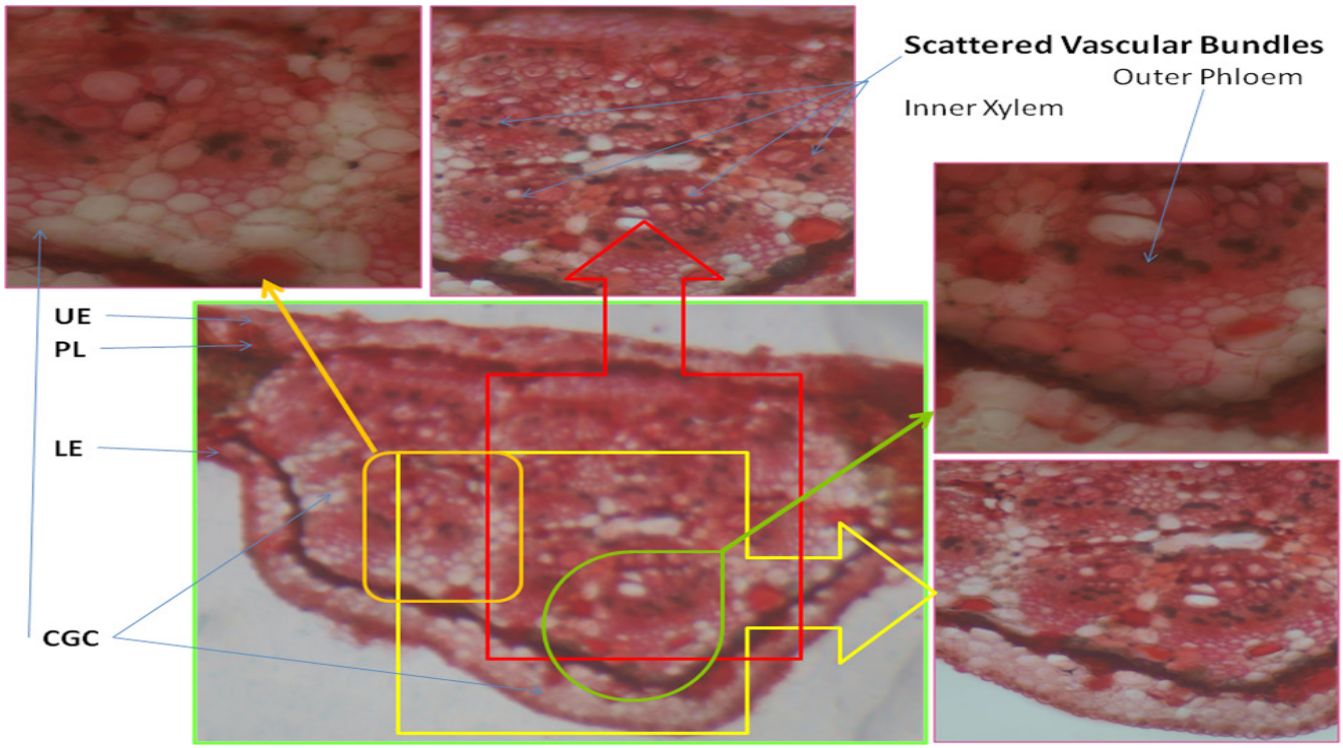

Figure 2: T.S. of leaf of *Cordia dichotoma* Linn in midrib region under 4X, 10X and 28X views of microscope showing scattered vascular bundles [UE: Upper Epidermis, LE: Lower Epidermis, PL: Palisade Layer, CGC: Collenchymatous Ground Cells].

Table 2: Extractive values with color, consistency and solubility in water of different extracts for the powdered leaves of the plant *Cordia dichotoma*

Solvent extracts	*Color*	*Consistency*	*Solubility in water*	*Extractive values (% yield)*
Petroleum ether extract	yellowish green	sticky	insoluble	0.60
Chloroform extract	dark yellowish green	non-sticky	soluble	0.80
Methanol extract	dark green	non-sticky	highly soluble	5.81
Aqueous extract	brown	dry powder	highly soluble	9.20

Table 3: Preliminary phytochemical screening of the methanolic extract of *Cordia dichotoma* leaves

Chemical Tests	Results	Chemical Tests	Results
1. Tests for phenolics and flavonoids		C. Cardiac Glycosides	
a) Lead acetate test	++	a) Legal test	–
b) Ferric chloride test	++	b) Keller-Killiani test	+
c) Sodium hydroxide test	++	**D. Test for steroids** Salkowski test	++
d) Shinoda test	++	**4. Test for Carbohydrates:**	
2. Test for Alkaloids		**A. Reducing sugar**	
a) Mayer's test	–	a) Molisch's test	–
b) Dragendorff's test	–	b) Fehlings test	+
c) Wagner's test	++	**B. Starch** (iodine test)	–
d) Hager's test	++	**5. Test for Proteins and free amino acids**	
3. Test for Glycosides:		a) Biuret test	+
A. Saponin Glycosides		b) Millions test	++
a) Foam Test	++	c) Xanthoprotein test	++
b) Sodium bicarbonate test	+	d) Ninhydrin test	+
B. Anthraquinone Glycosides Borntrager's test	–	*(++) indicates medium presence, (+) weak and (–) absence.*	

Table 4: Fluorescence characteristics of powdered leaves of the plant *Cordia dichotoma*

Chemical treatment	Fluorescence		
	Day light	UV light	
		254 nm	366 nm
Powder as such	greenish brown	purple	black
Powder + water	brown	light green	brown
Powder + 1 N HCl	brown	light green	brown
Powder + 5% NaOH	light green	dark green	purplish brown
Powder + 1 N NaOH (Alc.)	green	green	black
Powder + 50% HNO_3	reddish green	green	purplish brown
Powder +1M H_2SO_4	brown	green	brown
Powder +25% liquid ammonia	green	green	purplish black
Powder +acetic acid	light green	dark green	purple
Powder+iodine solution	yellow	dark green	black
Powder + 5% $FeCl_3$ in ethanol	light green	dark green	black

HPTLC chromatographic analysis

On analyzing under densitometer at UV 254 nm and 366 nm, the HPTLC chromatogram revealed several peaks **(Figure 3, Figure 4, Table 6 and Table 7)**. It revealed 3 peaks at UV 254 nm. While, It revealed 5 peaks at UV 366 nm with max R_f values in the range of 0.3 to 0.93 indicating the occurrence of atleast 5 different components in 5 µl of MECD. Four of the components with max R_f values 0.3, 0.53, 0.60 and 0.65 were found to be predominant as the percentage area was more with 30.97%, 13.18%, 26.23 and 20.91% respectively. One of the components with max R_f value of 0.93 was found to have the percent area less than 10%.

Table 5: Fluorescence characteristics of various successive solvent extracts of powdered leaves of the plant *Cordia dichotoma*

Chemical treatment	Day light	Fluorescence	
		UV light	
		254 nm	366 nm
Petroleum ether extract	yellowish green	green	Black
Chloroform extract	light green	light green	greenish black
Methanolic extract	green	dark green	black
Aqueous extract	reddish brown	chocolate colored	black

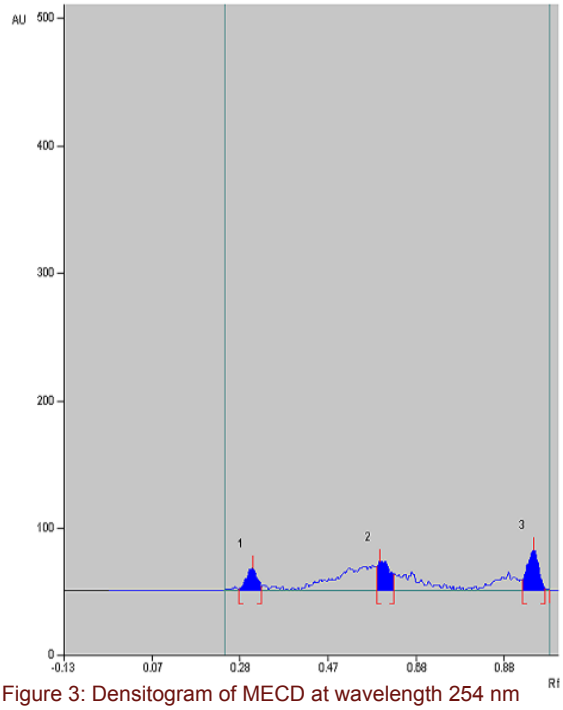

Figure 3: Densitogram of MECD at wavelength 254 nm

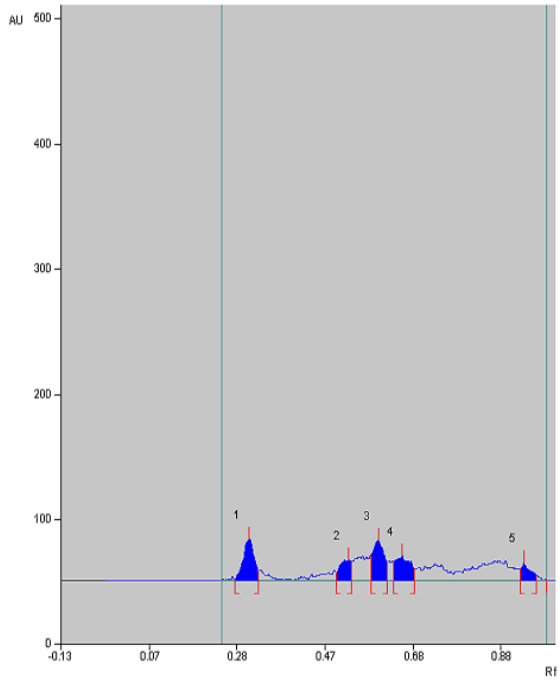

Figure 4: Densitogram of MECD at wavelength 366 nm

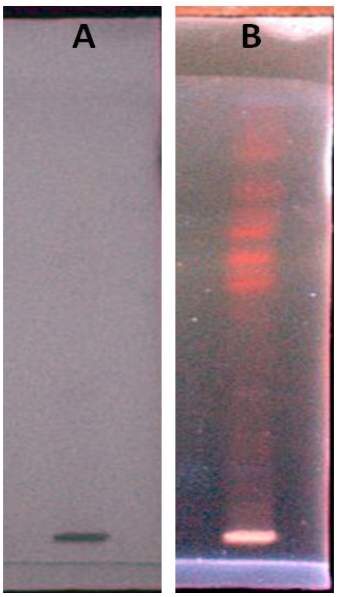

Figure 5: TLC photographs of MECD [under UV 254 nm (A), and UV 366 nm (B)].

DISCUSSION

Standardization of plant material is a complex task due to their heterogeneous composition, which is in the form of whole plant, plant part or extracts obtained thereof. Thus, to solve the task, there has been a rapid increase in the standardization of selected medicinal plants of potential therapeutic significance in recent years. To ensure reproducible quality, proper identification and authentification of starting material is first and essential step. Thus, care was given during collection of plant material for proper plant part and proper time of collection. Then, it was authenticated by expert scientist of the field. Physicochemical parameters were generally employed for deciding the identity, purity and adulterants, if any, present in the plant materials. Despite the modern techniques, identification of plant materials by the pharmacognostic evaluation and HPTLC chromatography analysis is

Table 6. **Densitogram table of MECD for measurement at wavelength 254 nm**

Peak	Start R_f	Start Height	Max R_f	Max Height	Max %	End R_f	End Height	Area	% Area
1	0.27	0.8	0.30	17.4	24.29	0.33	2.9	412.8	23.47
2	0.59	18.2	0.59	23.1	32.26	0.63	12.8	594.9	33.83
3	0.92	7.8	0.94	31.1	43.46	0.97	0.8	751.0	42.70

Table 7. **Densitogram table of MECD for measurement at wavelength 366 nm**

Peak	Start R_f	Start Height	Max R_f	Max Height	Max %	End R_f	End Height	Area	% Area
1	0.27	1.2	0.30	33.5	29.10	0.34	4.0	876.9	30.97
2	0.50	6.2	0.53	16.5	14.33	0.54	14.6	373.1	13.18
3	0.58	19.6	0.60	31.7	27.50	0.62	14.6	742.8	26.23
4	0.63	15.4	0.65	20.0	17.32	0.68	9.1	592.1	20.91
5	0.92	8.9	0.93	13.5	11.75	0.96	3.3	246.5	8.71

more reliable. As a part of standardization, organoleptic evaluation was carried out. Organoleptic evaluation is a technique of qualitative analysis based on the study of morphological and sensory parts like trichomes of drugs. The organoleptic characters of the leaf serve as diagnostic tools. The microscopic and physicochemical evaluation had been carried out. The percent extractives in different solvents like petroleum ether, chloroform, alcohol, water indicate the quantity and nature of constituents in the extracts. Extractive values are also helpful in estimation of specific constituent soluble in particular solvent. HPTLC chromatographic analysis was carried out which is particularly most valuable because, chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment to Indian traditional medicine and Chinese traditional herbal medicine. The final observations were recorded. The loss on drying at 105ºC was 8.5% w/w, total ash value 13% w/w, acid-insoluble ash 5.07% w/w, water-soluble ash 5.49% w/w, water-soluble extractive 9.2% w/w, alcohol-soluble extractive 5.81% w/w and pH (1% aqueous extract) 6.88. The CD fluorescence was seen in UV light and it was of different colour in different solvents. HPTLC analysis revealed 5 peaks at wavelength 366 nm with max R_f values in the range of 0.3 to 0.93. This finding is useful to supplement existing information with regard to identification and standardization in the powdered form of plant drug to distinguish it from adulterants.

CONCLUSION

From the present analysis, it can be concluded that the pharmacognostical characters along with their physicochemical parameters, fluorescence characteristics and HPTLC chromatographic profiling of the MECD leaf yielded a set of standards that may serve as an important source of information with regard to its standardization and identification to ascertain the identity and purity of the leaves of CD or pharmaceutical preparations prepared from it in further research studies.

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Antioxidant and metal chelating activities of *Lagenaria siceraria* (Molina) Standl peel, pulp and aerial parts in relation to their total phenol and flavonoid content

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ABSTRACT

Objective: The aim of the present study was to evaluate the antioxidant activity and total phenol and flavonoid content of different parts (peel, pulp and aerial parts) of *Lagenaria siceraria.* **Method:** Successive extraction was done by cold percolation method using solvents of different polarity viz. petroleum ether, toluene, ethyl acetate, acetone, water. Total phenol content was determined by Folin-Ciocalteu's reagent method and flavonoid was determined by aluminium chloride colorimetric method. The antioxidant assays evaluated were 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, 2, 2′-Azino-bis-(3-ethyl) benzothiazoline-6-sulfonic acid (ABTS) radical cation scavenging activity and Ferric reducing antioxidant power. **Results:** *L. siceraria* peel possessed maximum phenol and flavonoid content in acetone and toluene extracts respectively. FRAP was maximum in acetone extract of peel. The peel extract demonstrated stronger DPPH activity with IC_{50} value of 111 μg/ml followed by pulp extract. The same extract was effective in scavenging ABTS radical with an IC_{50} value of 39 μg/ml while other parts were ineffective. **Conclusion:** This work demonstrated good antioxidant activity of *L. siceraria* vegetable cultivated in India and recommends that the peel of this vegetable may be of interest from a functional point of view as a major source of natural antioxidant.

Key words: *Lagenaria siceraria*, antioxidant activity, DPPH, ABTS, FRAP, total phenol.

INTRODUCTION

Oxidation is essential to many living organisms for the production of energy to fuel biological process. However, oxygen-centre free radicals and other reactive oxygen species (ROS) which are continuously produced *in vivo,* results in cell death and tissues damage. Free radicals are derived from two sources: endogenous source such as nutrient metabolism, aerobic respiration, stimulated polymorphonuclear leucocytes and macrophages, ageing process, action of peroxisomes and activation of inflammatory cells[1] and exogenous sources such as environmental agents (tobacco smoke, ionizing radiation, air pollution, organic solvents, pesticides, etc.). Scientific evidence has suggested that under oxidative stress conditions, oxygen radicals such as superoxide anions ($O2^-$), hydroxyl radical (OH) and peroxyl radicals (H_2O_2) are produced in biological system. These reactive oxygen species can damage DNA which causes mutation and chromosomal damage. It also oxidizes cellular thiols and extracts hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membranes lipids.[2] Moreover, the production of excessive free radicals stimulates the oxidative damage and such situation contribute to more than one hundred disorder in humans including atherosclerosis, coronary heart disease, neurodegenerative disorder, cancer and they play major role in the aging process.[3]

Antioxidants have the ability to protect organisms from

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damage caused by free radical-induced oxidative stress. A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials. The commercial development of plants as source of antioxidants to enhance health is of current interest. It has been suggested that there is an inverse relationship between dietary intake of antioxidant rich foods and the incidence of human disease. Various herbs and spices have been reported to exhibit antioxidant activity, for example *Ocimum sanctum, Piper cubeba* Linn., *Allium sativum* Linn., *Terminalia bellerica, Camellia sinensis* Linn., *Zingiber officinale* and several Indian and Chinese plants. Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer. Therefore, antioxidants are vital substances which possess the ability to protect the body from damage caused by free radicals induced oxidative stress.[4] Synthetic antioxidants also available in market, such as butylated hydroxy anisole (BHA), butylated hyroxy toluene (BHT) but they are gaining less importance due to their side effects and carcinogenicity for this reasons increasing attention has been directed towards natural antioxidants.[5]

Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. *Lagenaria siceraria* is well documented as a food source but there is still not much known about the antioxidant potential of its different parts. Therefore, in the present study we have evaluated the free radical scavenging activity of different parts of *Lagenaria siceraria* in different solvents like petroleum ether, toluene, ethyl acetate, acetone and water and also estimated total phenol and flavonoid contents of the crude extract.

Lagenaria siceraria (Molina) Standl. Belongs to the family Cucrbitaceae. It is widely cultivated as a vegetable crop in tropical countries such as India, Japan and Thailand. The fruit is reported to contain the tritereperiodecucurbitacine B, D, G, H and 22-deoxy cucurbitacin the bitter principal of cucurbitaceae. Two sterols i.e., fucosterol and campesterol, aerpenebyonolic acid (an allergic compounds, flavones-c glycosides, ribosome inactivating protein) .Lagenin (antiproliferative, mmunosuppressive, antifertility).[6] reported a new a new phenolic glycoside from fruit. They are widely used in Ayurveda and other folk medicines; traditionally used for its cardioprotective, cardiotonic, general tonic, diuretic, aphrodisiac, antidote to certain poisons, scorpion strings, alternative purgative, scorpion strings, and cooling effects. It cures pain, ulcers and fever and used for pectoral cough, asthma and other bronchial disorders especially syrup prepared from the tender fruits.[7] Some of the reported activities are Hypolipidemic and antihyperlipidemic effects,[8] Diuretic activity,[9] Cardioprotective effect,[10,11] Cytotoxic activity,[12] Antioxidant activity,[13-16] Antimicrobial activity,[17] Lipid-lowering and antioxidant activity,[18] hepatoprotective activity,[19] antinociceptive and antioxidant from root,[20] antithrombotic potential of the fruits.[21]

MATERIALS AND METHODS

Plant Collection

The seeds, peel and aerial part of *Lagenaria siceraria (*Molina) standl. were collected from Jamnagar, Gujarat, India in August, 2011. The various parts were washed thoroughly with tap water, shade dried and homogenized to fine powder and stored in air tight bottles.

Successive extraction method

Successive extraction[22,23] was done by cold percolation method. 10 g of dried powder was taken in 100 ml of petroleum ether in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 24 h, it was then filtered through eight layers of muslin cloth, centrifuged at 5000 g for 15 minutes and the supernatant was collected and air dried under reduced pressure to obtain the dried residue. Petroleum ether was evaporated from the powder. This dry powder was then taken successively in 100 ml of each solvent (toluene, ethyl acetate, acetone, water) and was kept on a rotary shaker at 120 rpm for 24 h. Then the procedure followed was same as above, and the residues were weighed to obtain the extractive yield of all the extracts and were stored in air tight bottles at 4°C.

Quantitative phytochemical analysis

Determination of total phenol content

The amount of total phenol content of different solvent extracts was determined by Folin-Ciocalteu's reagent method.[24] The extract (0.5 ml) and 0.1 ml of Folin-Ciocalteu's reagent (0.5 N) were mixed and the mixture was incubated at room temperature for 15 min. Then, 2.5 ml of saturated sodium carbonate solution was added and further incubated for 30 min at room temperature and the absorbance was measured at 760 nm using a digital spectrophotometer (Systronic, India), against a blank sample. The calibration curve was made by preparing gallic acid (10 to 100 μg ml^{-1}) solution in distilled water. Total phenol content is expressed in terms of Gallic acid equivalent (mg g^{-1} of extracted compounds).

Determination of flavonoid content

The amount of flavonoid content of different solvent extracts was determined by aluminium chloride colorimetric method.[25] The reaction mixture (3.0 ml) consisted of 1.0 ml of sample (1 mg ml^{-1}), 1.0 ml methanol, 0.5 ml of aluminium chloride (1.2%) and 0.5 ml potassium acetate (120 mM) and was incubated at room temperature for 30 min. The absorbance of all samples was measured at 415 nm using a digital spectrophotometer (Systronic, India), against a blank sample. The calibration curve was made by preparing a quercetin (5 to 60 μg ml^{-1}) solution in methanol. The flavonoid content is expressed in terms of standard equivalent (mg g^{-1} of extracted compound).

Antioxidant activity

Ferric reducing antioxidant power (FRAP)

The reducing ability of different solvent extracts s of *Lagenaria siceraria* was determined by FRAP assay.[26] FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). 0.1 ml extract is added to 3.0 ml FRAP reagent [10 parts 300 mM sodium acetate buffer at pH 3.6, 1 part 10 mM TPTZ (2,4,6- tripyridyl-s-triazine) in 40 mM HCl and 1 part 20 mM $FeCl_3$] and the reaction mixture is incubated at 37°C for 10 min and then the absorbance was measured at 593 nm. $FeSO_4$ (100 to 1000 μM ml^{-1}) was used as a positive control. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as M $FeSO_4$ equivalents per gram of extracted compound.

Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The free radical scavenging activity of different solvent extracts was measured by using DPPH by the modified method described by McCune and Johns.[27] The reaction mixture 3.0 ml consisting of 1.0 ml methanol, 1.0 ml DPPH (0.3 mM) and 1.0 ml of solvent extracts of different concentrations of the *Lagenaria siceraria* diluted by methanol, was incubated for 10 min, in dark, after which the absorbance was measured at 517 nm using digital spectrophotometer (Systronic, India), against a blank sample. Ascorbic acid (2 to 16 μg ml^{-1}) was used as positive control (Liu *et al.,* 2011). The percentage inhibition was determined by comparing the results of the test and the control. Percentage of inhibition was calculated using the formula:

$$\% \text{ Inhibition} = [1 - (A/B)] \times 100$$

Where, B is the absorbance of the blank (DPPH plus methanol) and A is absorbance of the sample (DPPH, methanol, plus sample).

Determination of superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of different solvent extracts of *Lagenaria siceraria* was measured following the method described by Robak and Gryglewski.[28] Superoxide radicals are generated by oxidation of NADH and assayed by the reduction of NBT. The reaction mixture 3.0 ml consisted of 1.0 ml of the solvent extracts of different concentrations of the *Lagenaria siceraria,* diluted by distilled water, 0.5 ml Tris HCl buffer (16 mM, pH 8), 0.5 ml NBT (0.3 mM), 0.5 ml NADH (0.93 mM) and 0.5 ml PMS (0.12 mM). The superoxide radical generating reaction was started by the addition of PMS solution to the mixture. The reaction mixture was incubated at 25°C for 5 min and then the absorbance was measured at 560 nm using digital spectrophotometer (Systronic, India), against a blank sample. Gallic acid (50 to 225 μg ml^{-1}) was used as a positive control (Robak and Gryglewski, 1988). Percentage of inhibition was calculated as described earlier.

Determination of 2, 2'-Azino-bis-(3-ethyl) benzothiazoline-6-sulfonic acid (ABTS) radical cation scavenging activity

The ABTS radical cation scavenging activity of different solvent extracts of *Lagenaria siceraria*, determined by the method described.[29] ABTS radical cations are produced by reaction of ABTS (7 mM) with potassium persulfate (2.45 mM) and incubating the mixture at room temperature in the dark for 16 h. The working solution obtained was further diluted with methanol to give an absorbance of 0.85 ± 0.20. 1.0 ml of different concentrations of solvent extracts and fractions of the *Lagenaria siceraria* diluted by methanol was added to 3.0 ml of ABTS working solution. The reaction mixture was incubated at room temperature for 4 min and then the absorbance was measured at 734 nm using digital spectrophotometer (Systronic, India), against a blank sample. Ascorbic acid (1 to 10 μg ml^{-1}) was used as a positive control. Percentage of inhibition was calculated as described earlier.

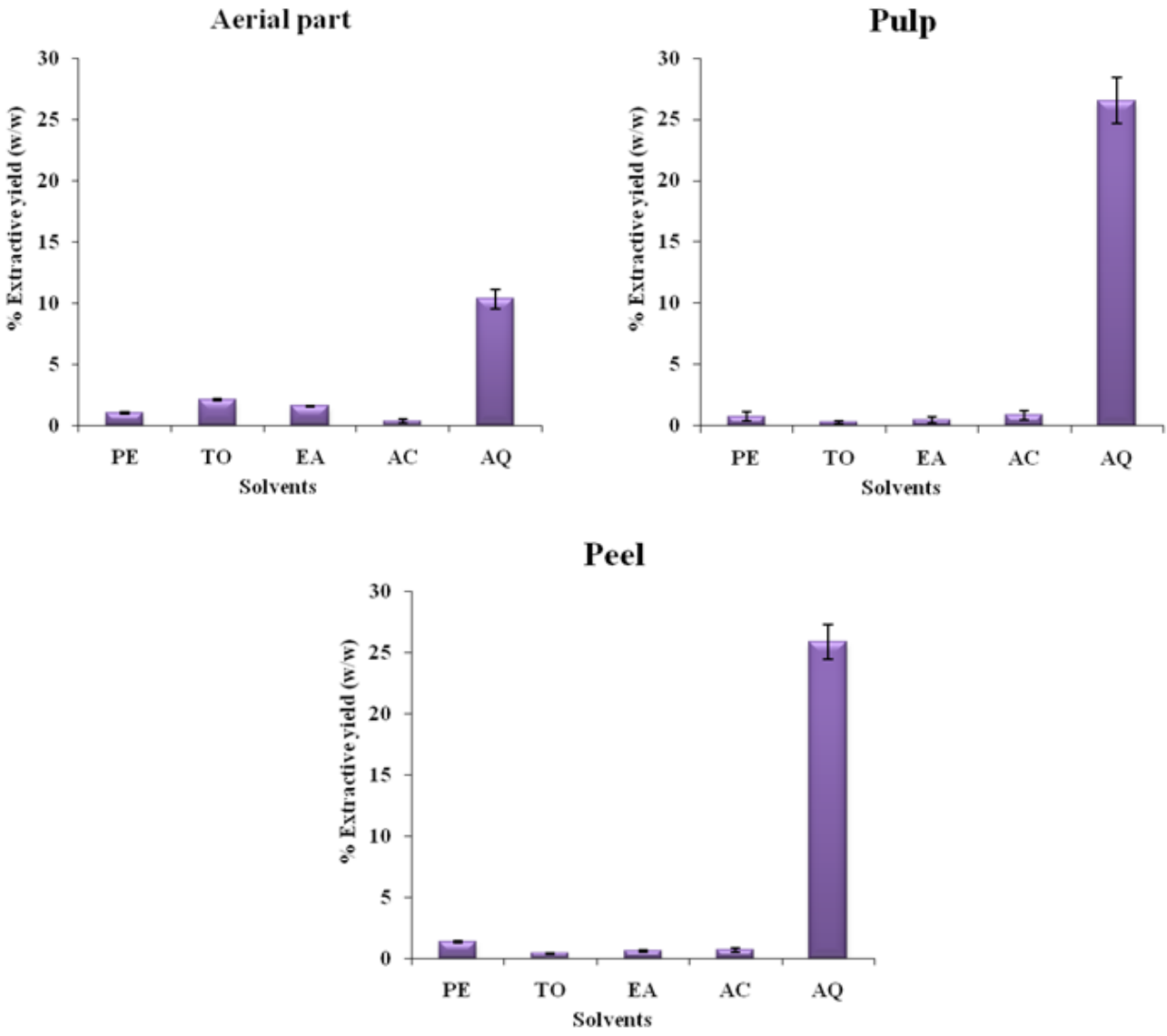

Figure 1: Extractive yield of different solvent extracts of different part of *L. siceraria*

RESULTS AND DISCUSSION

Extractive yield

The extraction yield of antioxidant compounds from plant materials is influenced mainly by the conditions under which the process of liquid solid extraction is achieved, the type of the solvent used to separate the soluble fraction from the permeable solid the degree of polymerization of phenolics and their interaction with the other components. The selection of an appropriate solvent is one of the most important factors in this operation.

The dry powder of different parts of *L. siceraria* was extracted in water (aqueous) and organic solvents like petroleum ether, toluene, ethyl acetate, acetone by cold percolation successive method. The results are shown in (Figure 1). In aerial part, maximum extractive yield was in aqueous extract and minimum was in acetone extract (Figure 1a). In pulp, maximum extractive yield was in aqueous and minimum was in toluene (Figure 1b). In peel, maximum extractive yield was in aqueous and minimum was in toluene (Figure 1c). The extractive yield depends on solvents time and temperature of extraction as well as the chemical nature of the sample. Under the same time and temperature condition, the solvent used and the chemical property of sample are the two most important factors.[30] There are many reports in the literature where extractive

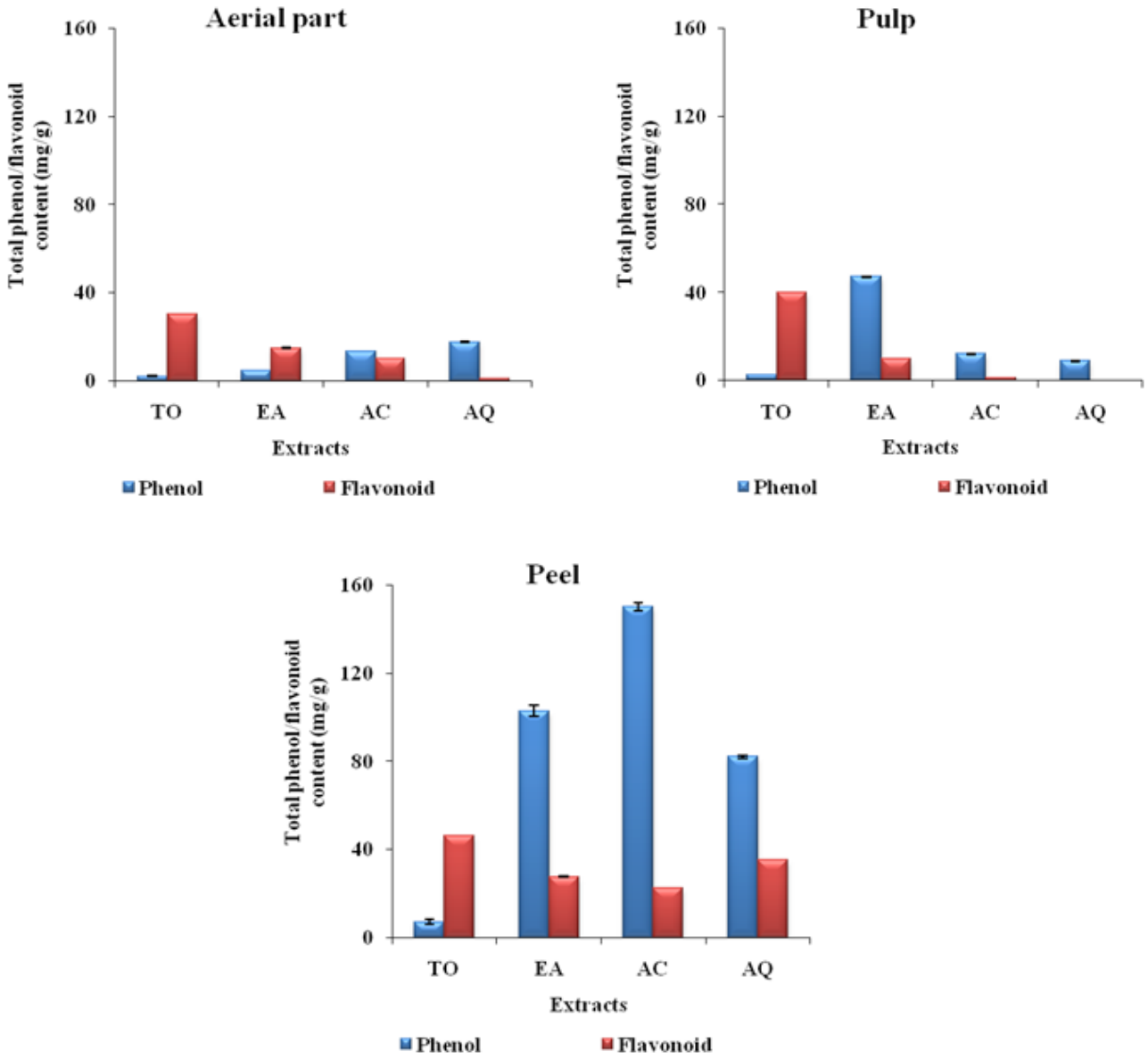

Figure 2: Total phenol and flavonoid content of different parts of *L. siceraria*

yield varied with different solvents .[31-35]

Total phenol and flavonoid content

Phenolic compounds such as flavonoids or phenolic acids are responsible for antioxidant activity due to their free radical scavenging capacity, although, there are several other mechanisms that contribute to the antioxidant activity like electron donor, metal chelating and scavenger of singlet oxygen.[36] In aerial part, maximum phenol content was in aqueous extract and minimum was in toluene extract while maximum flavonoid content was in toluene extract and minimum was in aqueous extract (Figure 2a). In pulp, maximum phenol was in ethyl acetate extract and minimum was in aqueous extract while maximum flavonoid content was in toluene extract and minimum was in acetone extract (Figure 2b). In peel, maximum phenol content was in acetone extract and minimum was in toluene extract while maximum flavonoid content was in toluene extract and minimum was in acetone extract (Figure 2c). When different parts are compared both phenol and flavonoid content was maximum in peel; maximum phenol content was in acetone extract while flavonoids was in toluene extract. Similar reports are found in guava,[37] *Mangifera pajang*,[38] *Annona cherimola* ,[39]etc.

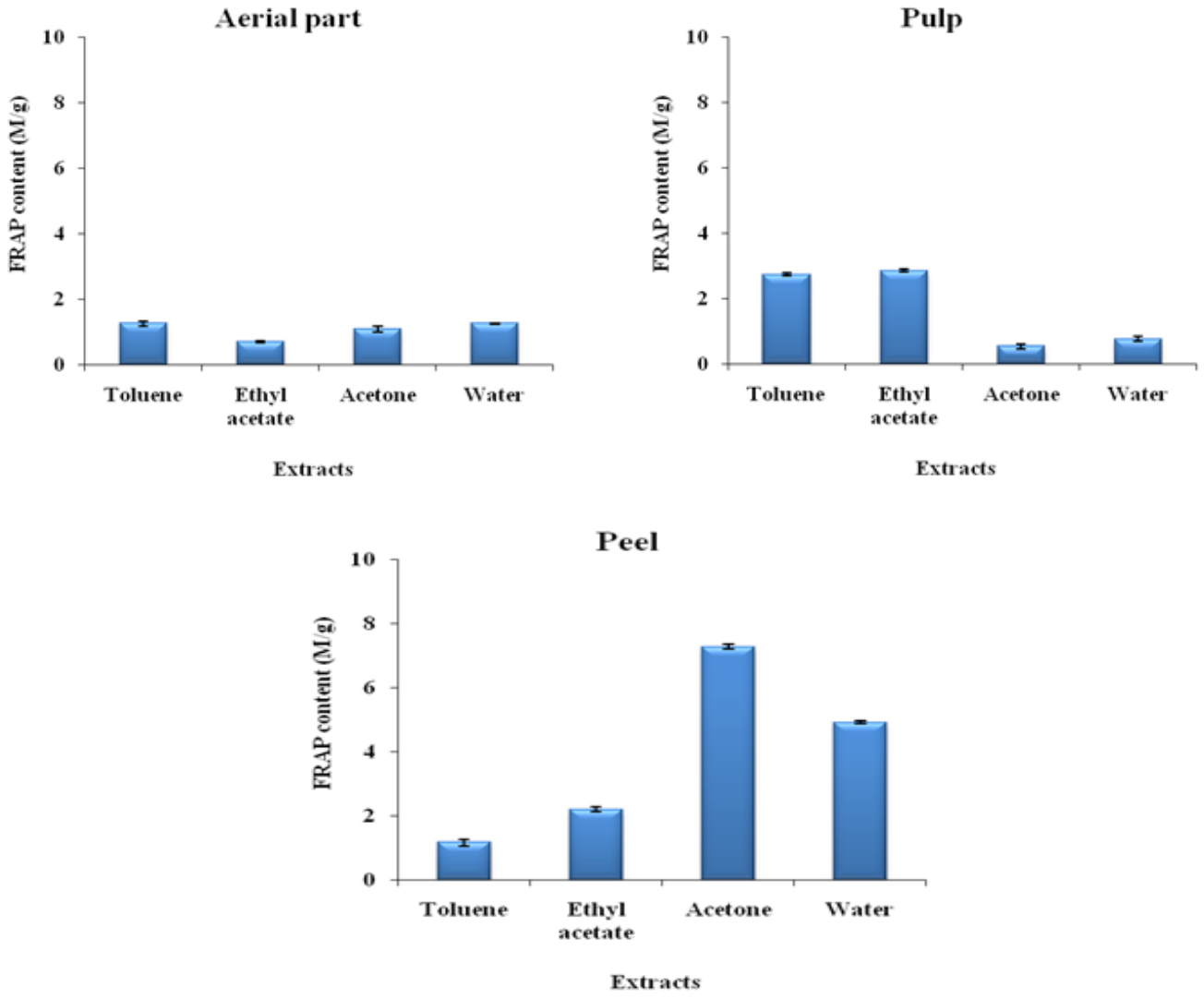

Figure 3: FRAP of different solvent extracts of different parts of *L. siceraria*

Antioxidant

Primary antioxidants, when present in trace levels, the response of antioxidants to different radical or oxidant sources may be different. Therefore, no single assay accurate reflects the mechanism of action of all radical sources or all antioxidants in a complex system, at least two methods should be employed in order to evaluate the total antioxidant activity, due to various oxidative processes. In this study, antioxidant activity was determined by three different *in vitro* antioxidant assays.

FRAP assay

The principle of the FRAP method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous coloured form in the presence of antioxidants. The reducing power property indicates that the antioxidant compounds are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process, so that they can act as primary and secondary antioxidants. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. In aerial part, maximum FRAP was in aqueous extract and minimum was in ethyl acetate extract (Figure 3a). In pulp, maximum FRAP was in ethyl acetate extract and minimum was in acetone extract (Figure 3b). In peel, maximum FRAP was in acetone extract and minimum was in toluene extract (Figure 3c). There was a direct correlation between phenol content and FRAP. Acetone extract of peel had maximum phenol content and highest FRAP activity.

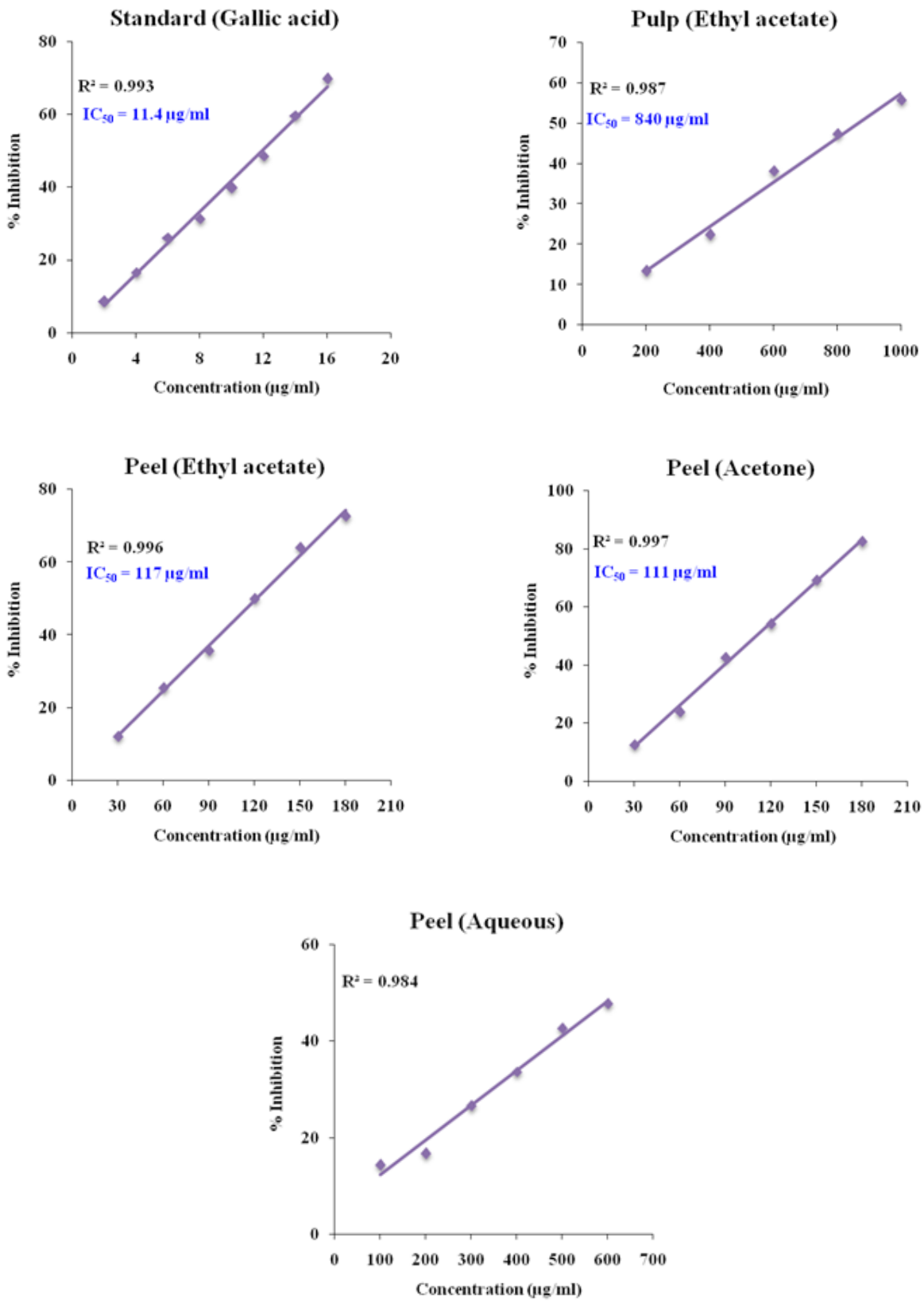

Figure 4: DPPH free radical scavenging activity of standard and different extracts of *L. siceraria pulp* and peel

DPPH assay

DPPH has been extensively used as a free radical to evaluate antioxidant substances that reduce DPPH. by donating hydrogen to form the non-radical DPPH – H. - DPPH• has an intense violet colour with a maximum absorbance at 517 nm, but turns colourless as unpaired electrons are scavenged by antioxidants.40 DPPH activity was present in L. siceraria peel (ethyl acetate, acetone, and aqueous extracts) and pulp (ethyl acetate extract) while it was absent in aerial part. The IC_{50} value of standard ascorbic acid was 11.4 µg/ml (Figure 4a). In pulp, maximum IC_{50} value was in ethyl acetate extract (Figure 4b) while in peel, minimum IC_{50} value was in acetone extract (Figure 4c). Aerial parts had low phenol content and it did not show any DPPH activity. In pulp, amongst four solvent extracts, ethyl acetate extract had maximum phenol content and it showed some DPPH activity. Amongst three parts evaluated, peel had maximum phenol content and acetone extract of peel had maximum phenol content (Figure 2) and it showed best DPPH activity (Figure 4d). The results support the popular view that there is a direct correlation between phenolic content and antioxidant activity, further supporting that phenols play an important role in beneficial effect of medicinal plants.[41-44]

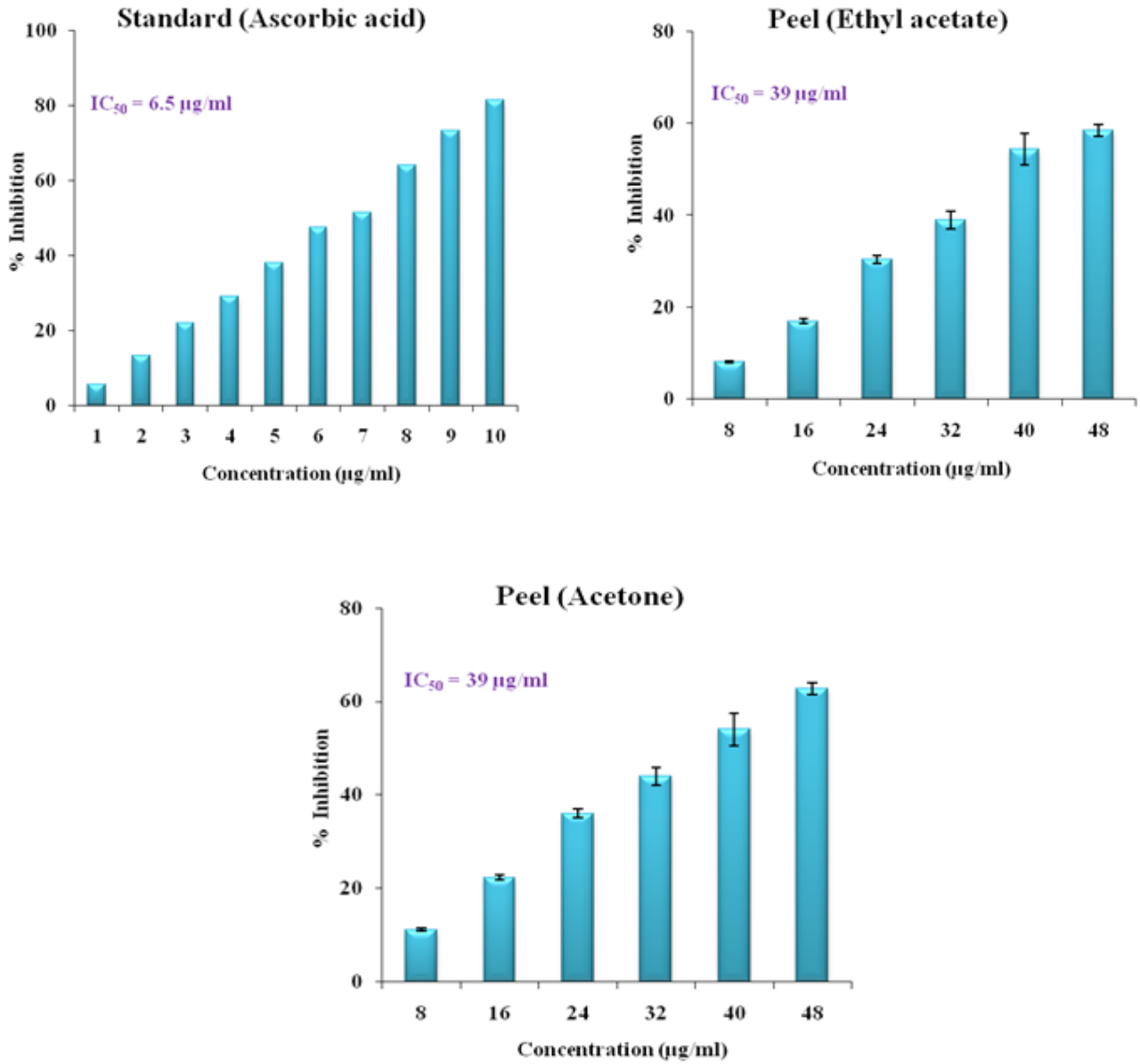

Figure 5: ABTS radical cation scavenging activity of standard and different extracts of *L. siceraria* peel

ABTS assay

ABTS assay is better to assess the antiradical capacity of both hydrophilic and lipophilic antioxidants because it can be used in both organic and aqueous solvent systems as compared to other antioxidant assays.[45] $ABTS^{+\bullet}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate and in the presence of the plant extract, preformed cation radical gets reduced and employs a specific absorbance at 734 nm, a wavelength remote from the visible region and it requires a short reaction time. The ABTS radical cation scavenging activity of different solvent extracts of *L. siceraria* and standard ascorbic acid is shown in (Figure 5). The IC_{50} value of standard ascorbic acid was 6.5 µg/ml (Figure 5a). ABTS activity was found only in ethyl acetate and acetone extracts of peel. None of the solvent extracts of other two parts i.e. pulp and aerial parts of *L. siceraria* showed ABTS activity. The IC_{50} value of both the solvent extracts of peel was 39 µg/ml. (Figures 5b-5c). There was a direct correlation between phenol content and ABTS activity. Acetone extract of peel had maximum phenol content and highest ABTS activity.

There are no universal criteria for presence or absence of antioxidant activity in different plants or plant parts. It is imperative that one should evaluate more than one antioxidant methods and in more than one solvent in a single plant. This is necessary because plant is rich in secondary metabolites and it is not known which one predominates; and also the mechanism of action of different antioxidant assays is different.[46] Therefore in the present study different parts of the same plant were evaluated for their antioxidant potential in different solvents and different antioxidant assays were done. The peel of this commonly consumed vegetable has excellent potential as an antioxidant additive in foods because it showed good DPPH activity, FRAP, reducing capacity and excellent ABTS activity indicating multiple modes of antioxidant activity. It can be suggested that it has the ability to scavenge free radicals responsible for many chronic diseases and disorders. However, the phytochemicals responsible for antioxidant activity has to be identified and determines which may help in drug development.

CONCLUSION

Searching plant sources may bring new natural products into pharmaceutical, cosmetic and food production. In the present work, the high antioxidant capacity observed for acetone extract of L. siceraria peel suggest that it may play a role in preventing human diseases in which free radicals are involved, such as cancer, ageing and cardiovascular diseases. Therefore, it is suggested that this plant could be used as an additive in the food industry providing good protection against oxidative damage. The plant is easily accessible in high quantities and therefore its application could be beneficial. These are novel, natural and economic sources of antioxidants which can be used in the prevention of diseases caused by free radicals. Therefore, our study will definitely open, scope for future utilization of these waste products for therapeutic purpose. However, conformation of its activity in in vivo models should be carried out. Such antioxidants could replace synthetic toxic antioxidants.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest

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Quality Control Standardization and *In-Vitro* Antioxidant Activity of *Aganosma dichotoma* K. Schum Root

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ABSTRACT

Introduction: *Aganosma dichotoma* K. Schum (AD) is a large climber with very stout stem belonging to the family Apocynaceae. The Plant has significant medicinal value as described in traditional system of medicine. The objective of the present study is to scientifically develop a standard monograph for AD on the basis of its pharmacognostical and phytochemical aspects. **Methods:** The study includes quality control standardization as per the standard methods provided in World Health Organization for standardization of medicinal plants. Fluorescence drug analysis, preliminary phytochemical screening of different fractions, quantification of some phytoconstituents and in-vitro antioxidant activity were also carried out. Quantification of Quercetin in the ethanolic extract of *A. dichotoma* was determined by HPTLC analysis. The ethanolic extract of root of *A. dichotoma* was subjected to in-vitro antioxidant activity. **Results:** The diagnostic characters of *A.dichotoma* root were evaluated on the basis of macroscopical and microscopical characters. Physicochemical parameters were evaluated such as 6.7% w/w loss on drying with; ash values (in % w/w): 13.75 total ash, 5.75 acid-insoluble ash, 3.6 water-soluble ash; Extractive values (% w/w): 12.75 water, 11.82 ethanol, 2.26 ethyl acetate, 3.13 chloroform, and 3.16 pet ether; foaming index 181.81; swelling index 3.2 ml/g; hemolytic activity 227.89 unit/gm of powder drug and crude fiber content was 19.4%. Total numbers of starch grain in 1 mg of root powder were 2,49,981. Quantification of quercetin in the ethanolic extract was assessed by HPTLC analysis and was found to contain 2.40%, w/w. **Conclusion:** The parameters determined in the present study may provide necessary information for identification and authentication of plant material.

Key words: *Aganosma dichotoma*, HPTLC, *in-vitro* antioxidant activity, Pharmacognosy.

INTRODUCTION

From time immemorial plants, parts of plant and isolated phytochemicals have been used in prevention and treatment of various health ailments as different formulations especially in traditional system of medicine like Ayurveda, Siddha and Unani.[1] According to the World Health Organization (WHO), almost 80% of the world's population depends on traditional medicine as their primary healthcare needs.[2] The active researches in the field of herbal traditional medicines have gained considerable momentum worldwide during the past decade. A part from incredible therapeutic potentials, herbal drugs formulations are prone to contamination, deterioration and variation in composition of constituents, which gives rise to little or no therapeutic efficacy.[3] In order to obtain good quality herbal formulations, it becomes extremely important to make an effort towards standardization of the plant material (used as traditional medicine) for proper marketing authorization and approval.

A. dichotoma is commonly known as Malati in Hindi and in Sanskrit it is known as Jati. Eight species of *A. dichotoma* are widely distributed from India to China Philippines and Indonesia. In India, it is mainly distributed across Assam, Bihar, West Bengal, Orissa, Andhra Pradesh and Tamil Nadu.[4] The plant is a large climber with very stout stem and belongs to family Apocynaceae. This plant produces milky latex which is a characteristic of Apocynaceae family. *Aganosma* was first described by Blume (1826) as a section of Echites and raised to generic status by G. Don (1837) with a number of species based on specimens in Wallich's herbarium.[5] Traditionally, *A. dichotoma* is used as emetic, anthelmintic, in bronchitis, leprosy, skin diseases, ulcer,

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inflammations, arthritis, purulent discharges from the ear and diseases of the mouth. Flowers are good for eye diseases and leaves cure biliousness.[4] Some reported phytoconstituents in flowers includes β-sitosterol, ursolic, vanillic and ferulic acids, quercetin and its glycosides, rutin, hyperin, isoquercetin and quercetin-3-arabinosides.[6] Leaves contain quercetin, kaempferol, glycoflavones, leucoanthocyanins and vanillic, syringic, protocatechuic, ferulic and sinapic acid.[7] Even though the plant is used traditionally for ailment of various diseases, however, there is no data available on its quality control profile.

The basis of nomenclature system used in ancient time attributing different names to the same plant and same name for different plants together with the time, have caused considerable confusion in selection of medicinal plants in various parts of the country. One of the examples is **Parpat** which is important drug of the Indian system of medicine. Nine different plants i.e., *Fumaria indica* (Fumariaceae), *Polycarpaea corymbosa* (Caryophyllaceae), *Justicia procumbens* (Acanthaceae), *Rungia repens* (Acanthaceae), *Rungia parviflora* (Acanthaceae), *Peristrophe bicalyculata* (Acanthaceae), *Glossocardia linearifolia* (Compositae), *Mollugo stricta* (Ficoidaceae) and *Oldenlandia corymbosa* (Rubiaceae) are known as parpat but all of them are totally different from each other and having different medicinal uses.[8-10] Vernacular name of *A. dichotoma* is Malati in Hindi and there are some other plants which are also known as Malati in different languages, for example *Combretum indicum* (Combretaceae) known as malati in Hindi[11] and *Jasminum grandiflorum* (Oleaceae) (Malati in Telugu).[12] Due to same common name of the medicinal plants there are chances of misidentification resulting in ambiguity. Hence standardization will provide guideline for proper identification. Keeping this in view was performed pharmacognostical standardization of *A. dichotoma* root.

MATERIALS AND METHODS

Plant Material

A. dichotoma roots were collected from Tumbura Kona Kshetram at Seshachalam hills and Tirumela hills, Chittor District, Andhra Pradesh, South India in the month of April. It was authenticated by Dr. K. Madhava Chetty, Taxonomist, S. V. University, Tirupati. A voucher specimen (COG/AD/17) has been retained in Department of Pharmaceutics, Indian Institute of Technology-BHU, Varanasi, India for further reference.

Macroscopic and Microscopic Evaluation

The dried roots were studied for their macroscopic character such as color, odour, taste, size, shape and texture. The roots were cleaned and fixed in formalin, acetic acid and 70% ethanol mixture for 24 hr prior to experimentation. The specimens were dehydrated with graded series of tertiary butyl alcohol.[13] Infiltration of specimen was carried out by gradual addition of paraffin wax until the solution attained super saturation. Then the specimens were cast into paraffin blocks and sectioned with the help of rotary microtome (at 10 μm to 12 μm thickness) followed by dewaxing.[14] Finally the sections were stained with toludine blue, and phloroglucinol & HCl solution (1:1). Sections were photographed with a Nikon trinocular microscopic unit, Model E-200, Japan. For powder microscopy, fine powder of root was cleared with chloral hydrate and stained with phloroglucinol and conc. HCl solution (1:1) and mounted with glycerin.

Determination of Physicochemical Parameter

From dried root the presence of any foreign matter was resoluted manually by using magnifying lens. Some more physicochemical parameters such as total ash value, acid insoluble ash value, water soluble ash value, loss on drying, extractive value in different solvents (petroleum ether, chloroform, ethyl acetate, ethanol and water), foaming index, swelling index and hemolytic activity of *A. dichotoma* were determined. The crude fiber content was determined by boiling the plant material with 10% nitric acid and treating it with 2.5% NaOH solution.[15] Total number of starch grains in *A. dichotoma* root was estimated by using the lycopodium spore method.[16] Fluorescence analysis is an important qualitative diagnostic tool for the presence of chromophore in crude powdered drug. Fluorescence powder drug analysis was performed under daylight, short UV, and long UV.[17]

Preliminary Phytochemical Screening

The coarsely powdered root of *A. dichotoma* was extracted by hot soxhlation using 95% ethanol (3L v/v) as solvent for extraction till the powdered drug was exhausted. The resulting extract was filtered and concentrated under reduced pressure to obtain the crude ethanolic extract of *A.dichotoma* (EAD). After the extraction process, successive fractionation was done by suspending the ethanolic extract (EAD) in aqueous media then partitioning with solvents of varying polarity such as Petroleum ether, chloroform

and ethyl acetate in order of their ascending polarity. Further, the extract (EAD) and its successive fractions were subjected to preliminary phytochemical tests to check the presence of various phytochemical classes.[18]

Quantitative Estimation of Phytoconstituents

Total Phenolic content

Total phenolic content present in the ethanolic extract of root of *A. dichotoma* was estimated by using Folin ciocalteau reagent method.[19] In a test tube aliquots of varying concentration of the extract were taken and volume was made up to 1 ml with distilled water. 0.5 ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Then the tubes were vortexed, placed in the dark for 40 min and the absorbance was recorded at 725 nm. The amount of total phenolics was calculated as tannic acid equivalents from the calibration curve.

Total Tannin content

Tannins are separated from rest of the mixture by adsorption on insoluble matrix polyvinylpolypyrrolidone (PVPP), and the total tannin content was determined by Folin–Ciocalteu procedure (mentioned as above). Insoluble, cross-linked PVPP (100 mg) was taken in a test tubes and 1.0 ml distilled water and 1 ml tannin containing extract was added. Tubes were maintained at 4 °C for 15 min, then subsequently vortexed and centrifuged for 10 min and the supernatant was collected. Aliquots of supernatant (0.2 ml) were transferred into test tubes, and then non-absorbed phenolics were determined. Finally observed values were subtracted from total polyphenol contents, and the total tannin content is expressed as; mg Tannic acid/100 g dry plant material. All measurements were done in triplicate.[20]

Total Flavonoid and Flavonol content

In the ethanolic root extract total flavonoid content was estimated by using aluminum chloride, standard compound Rutin was used as reference. The method for the estimation of flavonoids content is based on the formation of a flavonoid-aluminium complex (λmax 415 nm). In a test tube 1 ml of alcoholic plant extract, 1 ml of aluminium chloride and 3 ml of sodium acetate were mixed. After 2.5 hours, absorbance was measured at 415 nm.[21] The total flavonoid and total flavanol content were estimated by formula;

$$X = (A.m_o) / (A_o.m)$$

Here

X: the flavonoid content, mg/gm plant extract in root extract

A: the absorption of plant extract solution

A_o: the absorption of standard solution

m: the weight of plant extract (mg)

m_o: the weight of Rutin in solution (mg)

Total Alkaloid content

Total alkaloid content was estimated by gravimetric method.[22] 5 gm powdered drug was extracted repeatedly with 0.1N H_2SO_4 in an ultrasonic bath (3x50 ml). Then the solution was filtered and washed the acidic solution with chloroform in four successive quantities of 25 ml. The chloroform washing was rejected and acid solution was basified with dilute ammonia solution and then extracted with diethyl ether (20 ml x 5). Diethyl ether extract was washed with 5 ml distilled water, and then residue was dried to constant weight at 105^{0}C.

Total Saponins content

Total saponin content was estimated using Diosgenin as reference compound. The plant material was initially centrifuged with aqueous methanol and subsequent treatment with anisaldehyde-ethyl acetate reagent and H_2SO_4 and finally its absorbance is measured at 430 nm.[23]

Pesticide Residue

For estimation of pesticides like (aldrin, HCH, DTH, dieldrin, and malthion *etc.*) in crude drugs, Florisil R (grade 60/100) column chromatographic technique was used. Acceptable daily intake of aldrin and dieldrin is not more than 0.0001mg/kg body weight. As per the WHO guidelines for determination of pesticide residues, HCH and DTH should be no more than 0.005 mg/kg body weight.[15]

WHO and FAO (Food and Agricultural Organization) set the maximum permissible limits of pesticides frequently present in the herbs. Most of these pesticides are mixed with the herbs during the time of cultivation. Usually pesticides like DDT, BHC, toxaphene, aldrin cause various serious side-effects in human beings. The pesticide content

of the powdered root of *A. dichotoma* was determined by treating with acetonitrile: water (65:35) mixture followed by blending and filtration. To the filtrate (250 ml), 100 ml light petroleum, 10 ml sodium chloride (40%) and 600 ml water were added with constant shaking for 35 to 45 sec. The solvent layer was separated and washed twice with 100 ml portions of water to which 15 g of anhydrous sodium sulfate was added with vigorous shaking. The extract was separated, and its volume was reduced to 5 ml to 10 ml, this extract is then passed through column (packed with Florisil R grade 60/100 PR, activated at 650^{0}C). Three elutes were obtained after running the column with three ratios of ether: light petroleum mixture containing 6%, 15%, and 50% of ether, respectively. Elutes were evaporated to dryness, transferred to a sample holder, and burned in a combustion flask flushed with oxygen containing the solvent (water for chloride and H_2SO_4 in case of phosphate pesticides). For determining the chloride pesticides, 15 ml of this solution was mixed with 1 ml of ferric ammonium sulfate (0.25 mol/l) and 3 ml of mercuric thiocyanate and allowed to stand for 10 min after swirling and absorbance was measured at 460 nm. Phosphate pesticides were determined after mixing 7 ml solution to 2.2 ml sulfuric acid (300 g/l), 0.4ml ammonium molybdate (40g/l), and 0.4 ml aminonaphtholsulfonic acid followed by swirling, heating at 100^{0}C for 12 min, and absorbance was measured at 820 nm.[15]

Quantification of Quercetin in A. dichotoma by HPTLC

Ethanolic Extract of root of *A. dichotoma* (EAD) was standardized with quercetin using high performance thin layer chromatography (HPTLC). A stock solution of EAD (10 mg/mL), and quercetin (0.2 mg/mL) was prepared in methanol. The mobile phase for developing the chromatogram consisted of chloroform, methanol and formic acid mixture in the ratio 75:15:10 (v/v/v). The study was carried out using Camag- HPTLC instrumentation (Camag, Mutten, Switzerland) equipped with Linomat V sample applicator, Camag TLC scanner 3, Camag TLC visualizer and WINCATS 4 software for data interpretation. The R_f values was recorded and the developed plate was screened and photo-documented at ultra violet range with wavelength (λmax) of 254 nm.

In-vitro Antioxidant Study

In-vitro antioxidant activity of extract was evaluated due to presence of phenolic and flavonoid compounds in extract. The ethanolic extract of root of *A. dichotoma* was then subjected to *in-vitro* antioxidant activity by using; DPPH method, total antioxidant capacity, scavenging of hydroxyl radical by deoxyribose method, scavenging of nitric oxide radical, assay of reducing power and scavenging of hydrogen peroxide. DPPH assay method is based on the reduction of ethanolic solution of colored free radical DPPH by free radical scavenger. Absorbance was measured at 517 nm and ascorbic acid was used as standard.[24] Total antioxidant capacity of ethanolic extract of plant was evaluated by using phosphomolybdenum. This assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of a green phosphate/Mo(V) complex at acidic Ph.[25] The hydroxyl radical scavenging activity of plant extract was evaluated by deoxyribose method using butylated hydroxyl anisole (BHA) as a standard and absorbance was measured at 532 nm.[26]

In nitric oxide scavenging assay; sodium nitroprusside in aqueous solution at physiological pH spontaneously generated nitric oxide, which interacted with oxygen to produce nitrite ions, that can be estimated by using modified Griess Ilosvay reaction and the absorbance of chromophore formed was measured at 540 nm.[27] Potassium ferricynide method was used for the assay of reducing power using ascorbic acid as standard and absorbance was measured at 700 nm. H_2O_2 scavenging activity of plant extract was evaluated, and absorbance was recorded at 230 nm and percent inhibition calculated.[28]

RESULTS

Macroscopic and Microscopic evaluation

Dried roots are cylindrical with slightly tapered in shape,

Figure 1: *Aganosma dichotoma* G.DON root

6-10 cm long and 0.5-1.5 cm in width. Outer surface of root is dark reddish brown, much shriveled and wrinkled longitudinally while internally root is buff to light yellow in color. Outer layer is easily exfoliated, separating from the wood in large, papery flakes or strips (Figure 1). It has no perceptible odor and taste.

Transverse section of *A. dichotoma* root shows exfoliating wavy cork, consisting of 3-4 layered brick shaped cork cells filled with tannins (Phellem). Next to phellem, phelloderm is present, made up of 8-10 layered parenchymatous wide cells. Single layered pericyclic sclerenchymatous cells are situated in between the phellem and phelloderm. Periderm is followed by secondary cortex which is made up of 10-12 layered thin walled parenchymatous cells. A number of parenchymatous cells of secondary cortex contain starch grains, which are simple, round to oval in shape but the hilum and striation are indistinct. Solitary calcium oxalate crystals are also found in cortex region. Anomalous structures are reported, formation of xylem and phloem is irregular, which shows furrowed xylem and phloem is situated in furrows. The secondary xylem consists of narrow vessels and vasicentric tracheids, both having simple pits. The medullary rays become more deep and monoseriate in the section. Additional arcs of phloem more deeply seated in the pith known as intraxylary phloem which is a main characteristic of *Aganosma* (*Echites*) genus. Pith is frequently containing sclerosed elements (Figure 2).

The macerated powdered characteristics of *A. dichotoma* roots were expressed in terms of µm (minimum-mean-maximum; length x width) and showed the presence of a large number of fibres having slender shape and tapering ends with septa (131.4–158.45-183.55 x 3.6-5.4-7.2 µm), tracheids are pitted thickening with tapering ends, measuring (102.90-126.35-149.80 x 4.7-6.5-8.3 µm), xylem vessels of varying size and shape measuring (58.23-72.79-97.85 x 17.90-24.67-28.90 µm) and have pitted thickenings. Group of cork cells and lignified sclerenchymatous cells were appeared in the powder microscopy. Parenchymatous cells were also visible which are round, oval and elongate in structure having size varying between (24.62-31.81-67.42 x 15.90-24.63-37.67). Solitary calcium oxalate crystals with varying size and shape (25.48-35.67-36.18) were also present (Figure 3).

Physicochemical Parameters

The plant material had 0.837% w/w foreign matter and showed 6.7% w/w loss on drying with; ash values (in % w/w): 13.75 total ash, 5.75 acid-insoluble ash, and 3.6 water-soluble ash; Extractive values (% w/w): 12.75 water, 11.82 ethanol, 2.26 ethyl acetate, 3.13 chloroform, and 3.16 pet ether; foaming index 181.81; swelling index 3.2 ml/g; hemolytic activity 227.89 unit/gm of powder drug and crude fiber content was 19.4%. Total numbers of starch grain in 1 mg of root powder were 2,49,981. Fluorescence powder drug analysis of root of *A. dichotoma* varied with

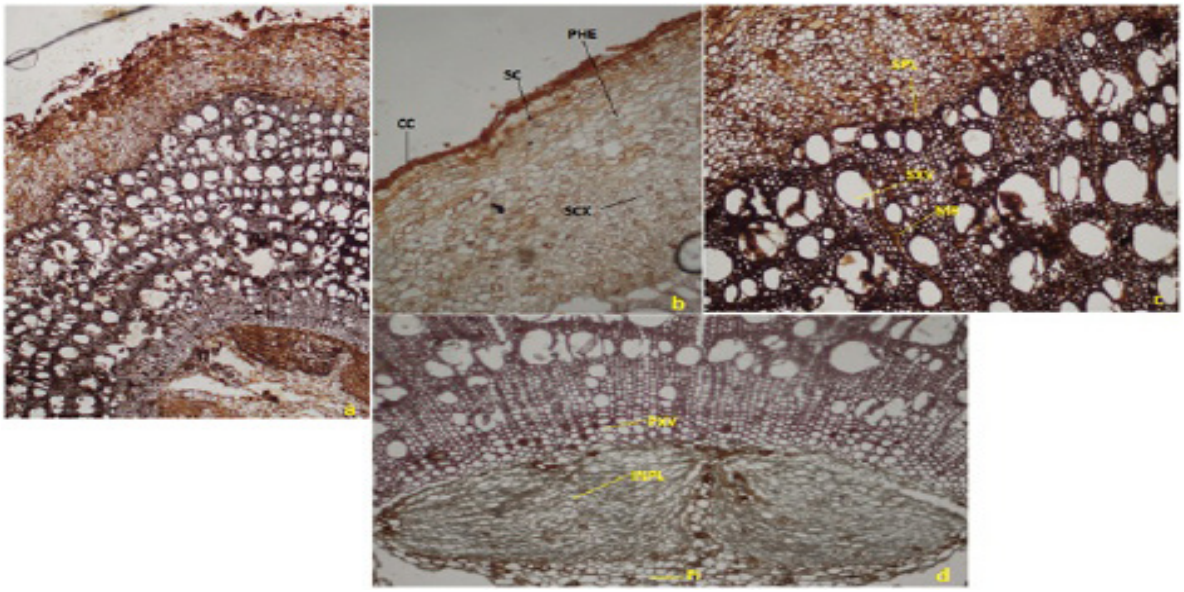

Figure 2: Histological study of A.dichotoma root. [a]: Transverse section of root [b]: T.S showing Periderm Region [c]: T.S. showing Secondary Phloem and Secondary Xylem [d]: T.S. showing intraxylary Phloem and Pith region [CC –cork cell layer, SC- Sclerenchymatous cell layer, PHE- Phelloderm, SCX- Secondary Cortex, SPL- Secondary Phloem, MR- Medullary Rays, SXV- Secondary Xylem Vessel, PXV- Primary Xylem Vessel, INPL- Intraxylary Phloem, PI- Pith

TABLE 1: Fluorescence Analysis of A. dichotoma Root Powder Drug

Test	Day light	Short UV	LongUV
Powder + 1 N NaOH in methanol	Peach Puff	Light green	Honey dew
Powder + 1 N NaOH in water	Dark golden rod	Green yellow	Lime green
Powder + 1 N HCL in methanol	Saddle Brown	Yellow green	Daark Salmon
Powder + 1 N HCL in water	Rosy Brown	Dark sea green	No Fluorescence
Powder + 1 N HNO3 in methanol	Wheat color	Lawn green	Light green
Powder + 1N HNO3 in water	Tan	Light green	No fluorescence
Powder + 5% iodine	Maroon	Dark red	No fluorescence
Powder + 5% FeCl3	Sienna	Dark green	No fluorescence
Powder + 50% KOH	Khaki	Yellow green	Lime
Powder + 25% ammonia	Corn silk	Green yellow	Olive drab
Powder + picric acid saturated	Gold	Green yellow	No fluorescence
Powder + acetic acid	Antique white	Dark sea green	Dark olive green

TABLE 2: Preliminary Phytochemical Screening of Ethanolic Extract of A. dichotoma and its Successive Fractions:

Phytoconstituents	EAD	PEF	CF	EAF	AF
Flavanoids	+	-	-	-	-
Phenolics & Tannins	+	-	-	-	+
Steroids	+	+	+	+	-
Coumarins	-	+	+	+	-
Cardiac Glycosides	+	-	-	-	+
Anthraquinone Glycosides	+	-	-	-	-
Alkaloids	-	-	+	-	+
Saponin	-	+	-	-	+
Carbohydrate	+	-	+	+	+
Reducing Sugar	+	-	+	+	-

(EAD- Ethanolic extract of A. dichotoma root powder, PEF-Petroleum ether fraction, CF-Chloroform fraction, EAF-Ethyl acetate fraction, AF-Aqueous fraction)

TABLE 3: Quantitative Estimation of Phytoconstituents of Ethanolic Extract of Aganosma Root

Phytoconstituent Class	Total Content in mg/gm of Plant Extract
Total Phenolic Content	125.65 ± 2.58 (Equivalent to Tannic Acid)
Total Tannin Content	104.96 ± 1.35 (Equivalent to Tannic Acid)
Total Flavanoid Content	62.20 ± 2.01 (Equivalent to Rutin)
Total Flavanol Content	1.97 ± 0.06 (Equivalent to Rutin)
Total Alkaloid Content	0.2% w/w
Total Saponin Content	49.2 ± 1.92 (Equivalent to Rutin)

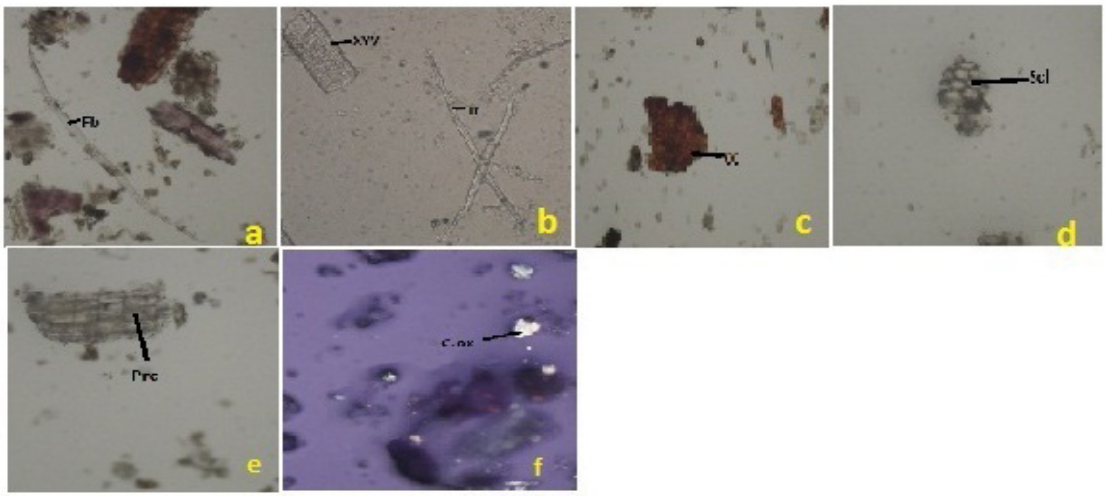

Figure 3: Powder characteristics of Aganosma root powder, [a]: Fiber (Fb), [b]: Pitted Xylem Vessel (XYV) and Tracheids (Tr), [c]: Cork Cells (CC), [d]: Sclerenchymatous Cells (Scl), [e]: Parenchymatous Cells (Prc), [f]: Solitary Calcium Oxalate Crystals (C.ox)

the extract (Table 1).

Preliminary Phytochemical Screening

The extracts of root of *A. dichotoma* varied in the phytochemicals profile (Table 2).

Quantification of Phytoconstituents

Quantification of different phytoconstituents (Total phenolic, Total tannin, Total flavanoid, Total flavanol, Total alkaloid and Total saponin) were reported in (Table 3).

Determination of Pesticide Residue

Chlorinated pesticide obtained in first and second elute from column were 0.1426 and 0.2794 mg/kg of the crude drug respectively. Phosphated pesticide obtained in first, second and third elute from column were 0.0032, 0.0215 and 0.0107 mg/kg of the crude drug respectively.

Quantification of Quercetin in A. dichotoma by HPTLC

HPTLC study for the quantification of quercetin in EAD was analyzed in Fig 4 for the first time by scanning at wavelength (λmax 254 nm). Quantity of quercetin present in EAD was found to be 2.40% (w/w).

Determination of In-vitro Antioxidant Study

The total antioxidant capacity of ethanolic extract of root of *A.dichotoma* showed potential antioxidant activity compared with the Ascorbic acid used as a standard. The absorbance of ethanolic extract of plant (100 µg/ml) was 0.356 at 695 λ_{max}. Thus the result of Total antioxidant capacity was found to be 57.75 µg/ml of ethanolic extract of *A.dichotoma* which was equivalent to 100 µg/ml of standard. Ascorbic acid. The result of DPPH scavenging activity in this study indicates that the plant was potentially active. Ethanolic extract showed IC_{50} value (104.41 ± 0.2) while standard. Ascorbic acid showed IC_{50} value (98.20 ± 4.11). Nitric oxide scavenging activity was estimated by using Griess reagent, which showed a very moderate scavenging activity of plant extract (IC_{50} 137.07 ± 0.72) in comparison to rutin (IC_{50} 71.32 ± 1.91). The scavenging potential of hydrogen peroxide by ethanolic extract was also found to be considerably moderate with IC_{50} value of 143.80 ± 0.81 compared to rutin (IC_{50} 99.92 ± 1.18). Hydroxyl radical production was assessed by the iron (II)–dependent deoxyribose damage assay following the Fenton reaction. The result demonstrated a very good scavenging activity with an IC_{50} value of 127.63 ± 0.56 compared to positive control BHA (IC_{50} 103.14 ± 2.53). Assay of reducing power is a concentration-dependent reaction. The assay of reducing power depicted a very moderate reducing capacity of the extract (0.198 ±0.01 µg/mL) as compared to standard ascorbic acid (0.419 ± 0.006 µg/mL).

DISCUSSION

Currently, there is a great demand on the quality control profile and standardization of medicinal plant drug/plant

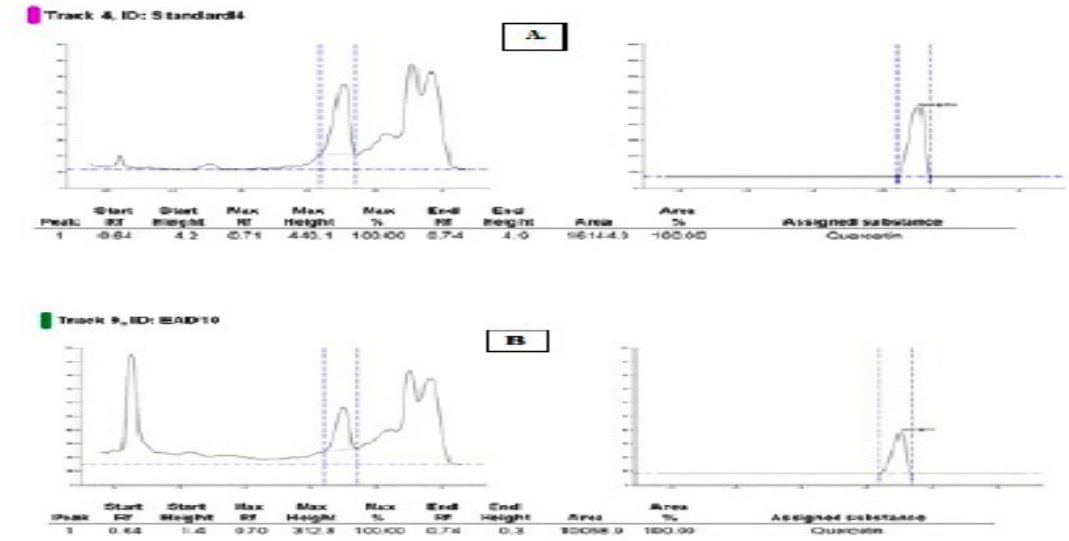

Figure 4: HPTLC densitogram of quercetin in ethanol extract of A.dichotoma (EAD). In figure A: Standard peak of quercetin B: Peak of quercetin present in EAD

parts for their therapeutic potentials. According to the World Health Organization, modern pharmacognostic techniques are available for the identification and evaluation of crude drugs which are more reliable, accurate and inexpensive. Pharmacognostical evaluation of a particular drug of a plant/plant parts provides valuable information in terms of its morphological, microscopical, and physical characteristics and is therefore, preferred as a primary step in standardization of a plant. Therefore some diagnostic features have been evolved to identify and to differentiate *A. dichotoma* root from the other crude drugs and its adulterants. From the microscopical studies, it was observed that the T.S. of root showed the presence of various unique indicative characters which are the intraxylary phloem, solitary calcium oxalate crystals *etc.*

The physicochemical evaluation is an imperative parameter which is useful in detection of adulteration or improper handling of the drug. The significance of ash values are quantitative standards that correspond to the presence of various impurities like carbonate, oxalate and silicate which may be naturally occurring or intentionally added to crude drug as a form of adulterant. Total ash includes both physiological as well as non–physiological ash, while acid insoluble ash consist mainly silica and indicate contamination with earthy material. The water soluble ash is used to estimate the amount of inorganic elements present in drugs.[29] Extractive values are valuable to evaluate the amount of active chemical constituents present in the plant/plant parts using different solvents. Loss on drying indicates that the drug is safe regarding any growth of bacteria, fungi and yeast.[30] The foaming index parameter is the ability of plant material and their extracts to form importunate foam. The swelling index is carried out due to therapeutic or pharmaceutical value of many medicinal plants may be attributed to its swelling property, which is due to the presence of gums, mucilage, pectin, and hemicelluloses. Hemolytic activity is seen in plants containing mainly saponins that diffuse hemoglobin into the surrounding medium[15]. Preliminary phytochemical screening revealed the presence of anthraquinone and cardiac glycosides, alkaloids, flavonoids, tannins, steroids, saponin and coumarins in different fractions of *A. dichotoma*. Such phytochemical screening is helpful in the prediction of natural phytoconstituents present in the tested drugs, which further leads to the isolation of compounds since phytochemicals are proven to be responsible for the activity of the drugs. Quantitative estimation of phytoconstituents is efficient parameters to set up standards for crude drugs. Moreover, the chemical standardization of EAD was also ascertained with the help of HPTLC and the amount of quercetin was quantified as a chemical marker.

The results obtained from the *in vitro* antioxidant studies showed that ethanolic extract of *A. dichotoma*, possess potent antioxidant activity, which may be attributed to the good availability of phenols, tannins, and flavonoids. The total antioxidant capacity and reducing power of a plant play a dominant role in depicting its antioxidant activity. All these plant based constituents should be well standardized and documented and their limits present in the plants should be estimated. Hence, to avoid the misuse of harmful plant material it is necessary to scientifically develop a pharmacognostical and physicochemical standards of a particular plant material which may ensure and maintain its quality, efficacy and safety profile. The parameters, which are being reported first time in this work, could be useful in the preparation of the herbal monograph for proper identification and authentication of *A. dichotoma*.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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