Advances in Pharmacological Sciences

The Treatment of Inflammation, Pain, and Fever Using Medicinal Plants

Guest Editors: Esra Küpeli Akkol, Srijit Das, Satyajit D. Sarker, and Lutfun Nahar

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Editorial Board

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Editorial

The Treatment of Inflammation, Pain, and Fever Using Medicinal Plants

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As the guest editors of the Journal, Advances in Pharmacological Sciences, we are glad to publish the special issue "The treatment of inflammation, pain, and fever using medicinal plants" receiving enough number of accepted papers. The major drawback of this special issue was *in vivo* and *in vitro* antiinflammatory, antinociceptive, and antipyretic evaluations of the medicinal plants which have ethnobotanical usage. Indeed, natural products have proved to be a rich source of therapeutic agents. Due to the side effects caused mostly by synthetic drugs, interest in natural products is growing rapidly and research into natural products has advanced tremendously in academia and pharmaceutical companies. Therefore, the papers accepted for publication in this special issue provide scientific evidence for the ethnomedicinal features and lead to the development of new drug candidates. For instance, A. Nazrun et al. demonstrated the anti-inflammatory role of vitamin E in prevention of osteoporosis in the research paper entitled "The Anti-inflammatory role of vitamin E in prevention of osteoporosis". In the study, vitamin E has been shown to inhibit COX-2, the enzyme involved in inflam-matory reactions. Of the two types of vitamin E studied, tocotrienol seemed to be better than tocopherol in terms of its ability to suppress bone-resorbing cytokines. In another study by M. Nadia et al., *Labisia pumila* was shown to have phytoestrogenic, anti-inflammatory, and antioxidative properties that make this plant an effective agent against osteoporosis. A mini review by Shilpi et al., was set out to compile and appraise the results on antinociceptive, anti-inflammatory, and antipyretic activity of mangrove plants that grow in the tidal coasts of tropic and subtropic region of the world. This paper finds that antinociceptive, anti-inflammatory, and antipyretic activity appears to be widespread in mangrove plants. According to the research paper by V. Shewale et al. anti-inflammatory activity of *Delonix regia* leaves was studied using carrageenan-induced rat paw edema and cotton pellet granuloma. The ethanol extract of *D. regia* leaves was reported to exhibit significant anti-inflammatory activity. Leaf methanol extract of *C. orbiculata* L. was investigated for antinociceptive and, anti-inflammatory activities using acetic acid writhing, hot-plate tests, and carrageenan-induced edema test by Amabeoku and Kabatende. The data obtained indicated that *C. orbiculata* has antinociceptive and anti-inflammatory activities, justifying the folklore use of the plant species by traditional medicine practitioners in the treatment of painful and inflammatory conditions.

Taking above mentioned studies into account, Advances in Pharmacology is pleased to publish the special issue "The treatment of inflammation, pain, and fever using medicinal plants".

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Review Article

Antinociceptive, Anti-Inflammatory, and Antipyretic Activity of Mangrove Plants: A Mini Review

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Mangrove plants are specialised plants that grow in the tidal coasts of tropic and subtropic regions of the world. Their unique ecology and traditional medicinal uses of mangrove plants have attracted the attention of researchers over the years, and as a result, reports on biological activity of mangrove plants have increased significantly in recent years. This review has been set out to compile and appraise the results on antinociceptive, anti-inflammatory, and antipyretic activity of mangrove plants. While the Web of Knowledge, Google Scholar, and PubMed were the starting points to gather information, other pieces of relevant published literature were also adequately explored for this purpose. A total of 29 reports on 17 plant species have been found to report such activities. While 19 reports were on the biological activity of the crude extracts, 10 reports identified the active compound(s) of various chemical classes of natural products including terpenes, steroids, and flavonoids. This review finds that antinociceptive, anti-inflammatory, and antipyretic activity appears to be widespread in mangrove plants.

1. Introduction

Mangrove forests are a special type of vegetation found in the coastal regions of the tropical and subtropical parts of the world. Global area that comprises mangrove forest is about 181000 square km. Majority of the mangrove forests is confined to the South East Asia and Australia, which accounts for 43% of the worldwide mangrove area (Table 1) [1, 2]. About 70 plant species of 27 genera have been reported from mangrove forests [2]. However, it should be noted that mangrove forests generally support the growth of non-mangrove plant species as well. For example, 334 plant species of 245 genera have been reported so far from the Sundarbans [3]. Flora of mangrove forests is unique from others in that their habitat extends along the border where the fresh and sea water merge. Therefore, unlike common terrestrial plants, they can withstand high salt concentration, can remain submerged in water, and maintain an efficient nutrient retention mechanism [1].

Mangrove forests are still quite unfamiliar to a vast population due to their limited distribution. However, the people inhabiting areas near mangrove forests heavily depend on these forests to meet their needs including their healthcare. During the early stage of human civilization, mangrove forests drew very little or no attention. This is to some extent because of the difficulty to access these areas. As the population continued to grow, people had to find new and unexplored sources including mangrove forests. In some parts of the world, mangrove forests are over utilised. As a result, human establishment grew in close proximity of

Table 1: Distribution of major mangrove forests around the world [2].

Region	Country
South and South East Asia	The Sundarbans, Bangladesh and India; Pichavaram, India; Balochistan, Pakistan; Estuarine mangroves, Thailand; Srilanka; The Philippines; East China, Taiwan; Japan; Malaysia; Borneo, Java and Eastern Indonesia
Middle East	Arabian Peninsula; Red Sea; Gulf including Bahrain, Qatar, UAE and Oman
Australasia	Western and Eastern Australia; South Pacific Islands; Papua New Guinea; Solomons Island
North and South America and the Caribbean	Florida and Bahamas, USA; Mexico; Puerto Rico; Eastern Venezuela; Trinidad; Guiana, Brazil
Africa	North West of Africa stretching from Mauritania to Sierra Leone; West of Africa from Liberia to Nigeria; South West Africa from Nigeria to Angola; East of Africa from Somalia to Tanzania; Mozambique; Madagascar and South Africa

these forests. For example, the density of population near the Sundarbans is as high as >500 per sq km [2]. Most of these people are directly or indirectly rely on the Sundarbans for their livelihood. In addition, natural disasters are putting these forests under the threat of extinction. For example, the mangrove forest in Tamil Nadu State of India was declared Reserve forests in 1880, but its protection ultimately failed [2].

Like other terrestrial plants, many mangrove plants have ethnopharmacological relevance and have also been exploited by the local people in the search for remedies for various ailments. However, only a few of the mangrove plants have so far been included in any books listing medicinal plants. This may be due to the difficulty in collecting and identifying these plant species and lack of adequate information available about their uses. As a part of our INSPIRE Project, funded by the British Council, a recent visit to the Sundarbans and subsequent interviews with people living nearby villages have revealed that the local people use a number of plants from the Sundarbans to treat various medical conditions.

With the introduction of rapid and reliable screening methods, researchers around the world have picked plant species of various origins including mangrove plants in the search for new medicine. This review aims to compile and appraise reports on the antinociceptive, anti-inflammatory, and antipyretic activity of mangrove plants.

2. Methodology

Web of knowledge, Google Scholar, and PubMed were used to search for the published reports since 1950. Other relevant publications, for example, books and journal articles, were also consulted. A total of 57 mangrove species were searched for the activity. The results are presented in three different tables; Table 2 gives a general outline of works that have been carried out so far on various mangrove plants for antinociceptive, anti-inflammatory, and antipyretic activity. It also describes the plant species, family, plant part used for the investigation, reported activity, and the screening method. Table 3 deals with those reports reporting the identification of active compound(s).

3. Antinociceptive, Anti-Inflammatory, and Antipyretic Activity

From the search, 29 hits were found with different mangrove species reporting one or more of these activities: antinociceptive, anti-inlfammatory, and antipyretic activity (Tables 2 and 3) [4–32]. Some of the reports coincide for a given species, and, therefore, a total of 17 plants were reported to have such activity. However, only one plant, *Pongamia pinnata* was studied for antipyretic activity. In nine cases, further phytochemical studies were carried out to find out the active constituent(s). One of the studies justified that the activity might be due to betulinic acid since betulinic acid is known for its anti-inflammatory activity and was present in the extract [8]. According to chemical classification, the active compounds, isolated from the mangrove plants, can be classified into diterpenes [11, 15], flavonoids [24], isoflavonoids [25, 29], monoterpenes [30], phenolics [30], steroids [32], triterpenes [29], xanthones [14], and a compound with unidentified structure [13] (Table 3).

The diterpenoids reported by Yodsaoue et al. [11] from the root extract of *Caesalpinia mimosoides* showed anti-inflammatory activity in micromolar range. The most potent activity was observed with mimosol D (Figure 1), which showed an IC_{50} for the inhibition of nitric oxide production at 3 μM and TNF-α production at 6.5 μM. Among the diterpenoids from the stems and twigs of the Chinese mangrove plant, *Excoecaria agallocha*, agallochaol O (Figure 2) at 100 μM showed 52.6% inhibition of interleukin-6 (IL-6) and other proinflammatory cytokines induced by lipopolysaccharide (LPS) [15]. Bio-assay guided phytochemical investigation of *Ipomoea-pes-caprae* resulted in the isolation of eugenol (Figure 3), a well-known analgesic, anti-inflammatory natural product [31, 33]. Some studies resulted in the isolation of steroids and triterpenes as the active compounds (Table 3) [32].

Plants often produce secondary metabolites under stressful conditions. Therefore, it is not surprising that mangrove plants, facing various ecological and environmental stresses, biosynthesise a wide range secondary metabolites of potential medicinal importance. The present literature survey has revealed that mangrove plants contain a wide range

Table 2: Antinociceptive, anti-inflammatory, and antipyretic activity of mangrove plant species.

No	Plant name	Family	Plant part tested	Observed activity	Test method	Refs
1	*Acanthus hirsutus* Boiss.	Acanthaceae	Aqueous extract	Antinociceptive	Acetic-acid-induced in mice	[4]
2	*Acanthus ilicifolius* Linn.		MeOH fraction of leaf extract	Anti-inflammatory	Carrageenan-induced rat paw oedema, COX (1 and 2) and 5-LOX activity	[5]
3	*Aegiceras corniculatum* (Linn.) Blanco.	Myrsinaceae	*n*-Hexane, EtOAc and MeOH extracts of stem	Antinociceptive, Anti-inflammatory	Acetic-acid-induced, formalin-induced paw licking and hot plate test in mice	[6]
4	*Aegiceras corniculatum* (Linn.) Blanco.		MeOH extract of stem	Anti-inflammatory	Rat paw oedema and peritonitis models were employed for *in vivo* studies. For *in vitro* studies, human platelets and rat neutrophils were stimulated with Ca(2+)-ionophore A23187 leading to the production of various proinflammatory metabolites, that is, 12-HTT, 12-HETE and LTB(4), and 5-HETE	[7]
5	*Avicennia officinalis* Linn.	Avicenniaceae	MeOH extract of leaves	Anti-inflammatory	Freunds adjuvant-induced arthritis, carrageenan-, and formalin-induced rat paw oedema	[8]
6	*Barringtonia racemosa* Linn.	Lecythidaceae	98% *n*-Hexane, 98% $CHCl_3$ and 95% EtOH extracts of leaf	Anti-inflammatory	Inhibition of nitric oxide formation in RAW 264.7 cells by Griess assay Amount of lipid peroxidation by ferric thiocyanate method	[9]
7	*Barringtonia racemosa* Linn.		Aqueous bark extract	Antinociceptive	Tail flick, hot plate, and formalin tests in rat	[10]
8	*Caesalpinia mimosoides* Lamk.	Leguminosae	CH_2Cl_2 and acetone extracts, pure compounds	Anti-inflammatory	Inhibition of lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW 264.7 cell lines	[11]
9	*Ceriops decandra* (Griff.) W. Theob.	Rhizophoraceae	EtOH extract of leaf and pneumatophore	Antinociceptive	Acetic-acid-induced in mice	[12]
10	*Calophyllum inophyllum* Linn.	Clusiaceae	EtOH extract of nut kernel	Anti-inflammatory	Carrageenan- and formalin-induced rat paw oedemas, cotton pellet implantation	[13]
11	*Calophyllum inophyllum* Linn.		(Pure compounds tested)	Anti-inflammatory	Carrageenan-induced hind paw oedema, cotton pellet granuloma and granuloma pouch techniques, in normal and adrenalectomized rats	[14]
12	*Excoecaria agallocha* Linn.	Euphorbiaceae	(Pure compounds tested)	Anti-inflammatory	Suppression of the expression of NF-κB and AP-1 targeted genes including TNF-alpha- and IL-6-induced by lipopolysaccharide (LPS) in mouse macrophages Raw 264.7 cells	[15]
13	*Nypa fruticans* Wurmb.	Arecaceae	MeOH extract of leaf and stem	Antinociceptive	Acetic-acid-induced in mice	[16]
14	*Pandanus foetidus* Roxb.	Pandanaceae	MeOH extract of leaf	Antinociceptive	Acetic-acid-induced in mice	[17]
15	*Pongamia pinnata* (L.) Pierre	Fabaceae	70% EtOH extract of leaf	Antinociceptive and antipyretic activity	Hotplate and tail flick, acetic acid writhing and Randall-Selitto nociceptive tests in mice and brewer's yeast-induced pyrexia in rats	[18]
16	*Pongamia pinnata* (L.) Pierre		70% EtOH extract of leaf	Anti-inflammatory	Carrageenin, histamine, 5-hydroxytryptamine and prostaglandin E-2-induced hind paw edema, kaolin-carrageenan and formaldehyde-induced hind paw oedema, cotton pellet granuloma models of inflammation	[19]

Table 2: Continued.

No	Plant name	Family	Plant part tested	Observed activity	Test method	Refs
17	*Pongamia pinnata* (L.) Pierre		70% EtOH extract of seed	Antinociceptive, Anti-inflammatory	Carrageenan-induced hind paw oedema and Randall-Selitto nociceptive test in rat	[20]
18	*Pongamia pinnata* (L.) Pierre		PE, $CHCl_3$, acetone and EtOH extracts of seed	Antinociceptive, Anti-inflammatory		[21]
19	*Pongamia pinnata* (L.) Pierre		70% EtOH extract of seed	Anti-inflammatory	Bradykinin and PGE-1-induced inflammation, histamine and 5-HT-induced inflammation	[22]
20	*Tamarix indica* Willd.	Tamaricaceae	80% MeOH extract of root	Antinociceptive, Anti-inflammatory	Acetic-acid-induced in mice, using carrageenan induced rat paw oedema	[23]
21	*Derris scandens* (Roxb.) Benth.	Fabaceae	$CHCl_3$ extracts of leaf and root and pure compounds	Anti-inflammatory	Carrageenan-induced paw oedema in rats	[24]
22	*Derris scandens* (Roxb.) Benth.		Aqueous extract of stem and pure compounds	Anti-inflammatory	Eicosanoid inhibition	[25]
23	*Ipomoea imperati* (Vahl) Griseb.		EtOH extract of whole plant	Antinociceptive	Acetic-acid-induced and hot plate test in mice	[26]
24	*Ipomoea imperati* (Vahl) Griseb.		MeOH-water extract of leaf	Anti-inflammatory	Mouse ear oedema induced by croton oil, arachidonic acid, cotton pellet-induced granulomas, inhibition of Phospholipase A(2) purified from *Apis mellifera* bee venom	[27]
25	*Ipomoea pes-caprae* (L.) R-Br.	Convolvulaceae	MeOH extract and two fractions of aerial part	Antinociceptive	Acetic-acid-induced and formalin test in mice	[28]
26	*Ipomoea pes-caprae* (L.) R-Br.		Pure compounds	Antinociceptive	Acetic-acid-induced and formalin test in mice	[29]
27	*Ipomoea pes-caprae* (L.) R-Br.		Crude extract and pure compounds	Anti-inflammatory	Inhibition of prostaglandin synthesis *in vitro*	[30]
28	*Ipomoea pes-caprae* (L.) R-Br.		Crude extract	Anti-inflammatory	Carrageenan-induced paw oedema and ear oedema induced in rats by arachidonic acid or ethyl phenylpropiolate, inhibition of prostaglandin synthesis *in vitro*	[31]
29	*Heritiera littoralis* Aiton	Sterculiaceae	Pure compounds	Anti-inflammatory	Nitric oxide (NO) inhibitory effects using RAW 264.7 macrophage cells	[32]

Table 3: Analgesic, anti-inflammatory compounds from mangrove plants.

No	Pure compound related to the observed activity	Refs
5	The anti-inflammatory activity of methanolic extract of *Avicennia officinalis* may be due to the presence of the phytoconstituent, betulinic acid	[8]
8	Mimosol D, taepeenin D, taepeenin L, (*E*)-7-hydroxy-3-(4-methoxybenzyl)chroman-4-one, (*E*)-7,8-dihydroxy-3-(4-methoxybenzyl)chroman-4-one, (*E*)-7-hydroxy-8-methoxy-3-(4-methoxybenzyl)chroman-4-one	[11]
10	Calophyllolide	[13]
11	Dehydrocycloguanandin and calophyllin-B	[14]
12	Agallochaol K, agallochaol O, agallochaol P, agallochaol Q, *ent*-17-hydroxykaur-15-en-3-one, *ent*-kaur-15-en-3b,17-diol, *ent*-15,18-dihydroxylabd-8,13*E*-diene	[15]
21	Ovaliflavanone and lupinifolin	[24]
22	3-γ,γ-dimethylallylweighteone, scandenin and genistein	[25]
26	Glochidone, betulinic acid, α-amyrin acetate, β-amyrin acetate, isoquercitrin	[29]
27	Eugenol and 4-vinyl-guaiacol	[30]
29	Ergosterol peroxide, 6-α-hydroxystigmast-4-en-3-one and stigmast-4-en-3-one	[32]

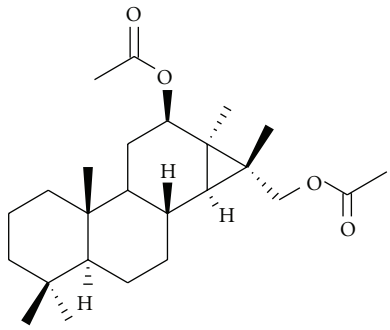

Figure 1: Mimosol D, an anti-inflammatory diterpene from the roots of *Caesalpinia mimosoides*.

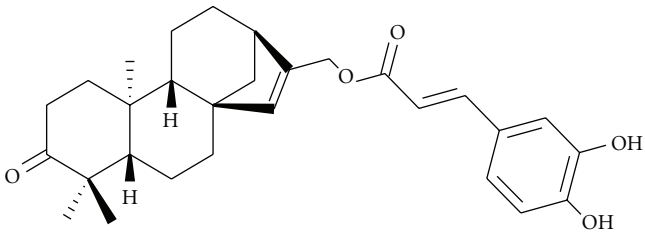

Figure 2: Agallochaol O, an anti-inflammatory diterpene from the stems and twigs of *Excoecaria agallocha*.

of compounds showing antinociceptive, anti-inflammatory and or antipyretic activity (Tables 2 and 3).

Pain itself is not any disease. It is manifested in certain disease or pathological conditions. Use of natural products in the management of pain goes back to thousands of years. Use of poppy by various civilizations or the use of willow bark to cure fever led to the isolation of morphine and salicylic acid, respectively [34]. These two drugs are still used extensively in modern medical practice. Present trend of the researchers to focus on mangrove plants has opened up an arena to find bioactive compounds from a source that has long been ignored or less explored. It is expected that research on mangrove plants will continue to rise in the coming days.

4. Possible Mechanism of Actions

It must be stressed that there are no or a few reports available on the possible mechanisms of action of the extracts or isolated compounds from the mangrove plants. However, exploring the methods applied in the published reports on evaluation of antinociceptive, anti-inflammatory, and/or antipyretic activity of mangrove plants [4–34], the following assumptions can be made about the possible mechanisms of actions. The sensation of pain can be initiated either peripherally or through the central nervous system. Peripherally mediated pain can be inhibited by NSAIDs which blocks the anti-inflammatory pathways responsible for pain. On the other hand, opioid analgesics are useful for the management of centrally acting pain in which opioid analgesics act by inhibition of opioid receptors. Acetic-acid-induced and formalin-induced paw licking represents peripherally acting pain sensation. Intraperitoneal administration of acetic acid

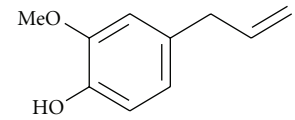

Figure 3: Eugenol, an analgesic and anti-inflammatory compound, from *Ipomoea-pes-caprae*.

or formalin mediates pain response through the release of inflammatory mediators, mainly prostacycline (PGI_2) [35, 36]. The hot plate test, the tail flick test, and the Randall-Selitto nociceptive test represent nociception through central mechanism [35, 37]. The rat paw oedema is an anti-inflammatory model that can be induced by carrageenan, formalin, kaolin, cotton pellet granuloma and granuloma, pouch. Inflammation of the rat paw can also be stimulated by administration of inflammatory mediators like histamine, or eicosanoids like 5-hydroxytryptamine and prostaglandin E-2 [22, 25]. Other anti-inflammatory models that have been used in the assessment include nitric oxide, TNF-α, and IL-6 induction by the administration of lipopolysaccharides in cell culture [14].

A wide range of methods were adopted by different research groups for the study of antinociceptive activity of mangrove plants. All these methods can be summed up to two major mechanisms, that is, centrally acting and peripherally mediated pain sensation. Different mangrove plants were able to inhibit pain sensation of both types. Therefore, it is possible to find opioid analgesics as well as analgesics in mangrove plants that act by inhibition of inflammatory pathways responsible for pain. Only in few cases, plants were investigated by methods that represent both of the mechanisms. Interestingly, articles that report the isolation of active compounds used methods representing peripherally acting pain sensation.

5. Conclusions

This review has revealed that antinociceptive, anti-inflammatory, and antipyretic activity appears to be widespread among mangrove plants, and thorough and systematic phytochemical and pharmacological studies are much needed to discover new antinociceptive, anti-inflammatory, and antipyretic medicinal entities from mangrove plants.

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Review Article

The Anti-Inflammatory, Phytoestrogenic, and Antioxidative Role of *Labisia pumila* in Prevention of Postmenopausal Osteoporosis

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Osteoporosis is characterized by skeletal degeneration with low bone mass and destruction of microarchitecture of bone tissue which is attributed to various factors including inflammation. Women are more likely to develop osteoporosis than men due to reduction in estrogen during menopause which leads to decline in bone-formation and increase in bone-resorption activity. Estrogen is able to suppress production of proinflammatory cytokines such as IL-1, IL-6, IL-7, and TNF-α. This is why these cytokines are elevated in postmenopausal women. Studies have shown that estrogen reduction is able to stimulate focal inflammation in bone. *Labisia pumila* (LP) which is known to exert phytoestrogenic effect can be used as an alternative to ERT which can produce positive effects on bone without causing side effects. LP contains antioxidant as well as exerting anti-inflammatory effect which can act as free radical scavenger, thus inhibiting TNF-α production and COX-2 expression which leads to decline in RANKL expression, resulting in reduction in osteoclast activity which consequently reduces bone loss. Hence, it is the phytoestrogenic, anti-inflammatory, and antioxidative properties that make LP an effective agent against osteoporosis.

1. Introduction

Plant has been one of the sources of medicine to treat various illnesses and diseases since ancient time. In the early 19th century, when chemical analysis first became available, scientists began to extract and modify the active ingredients from plants which later led to wide development of natural or traditional medicine that was mostly passed on orally from one generation to another. More than 35,000 plant species have been reported to be used in various human cultures around the world for their medical purposes [1]. Traditional medicine has been defined by the World Health Organization (WHO) as "health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination, to treat, diagnose and prevent illnesses or maintain well-being" [2].

Currently in Malaysia, over 2,000 species of lower plants with medicinal and therapeutic properties have been identified, and most of them have been used for many generations in various health care systems. About 17.1% of Malaysians used herbs to treat their health problems while 29.6% of them consumed herbs for their health maintenance [3]. The earliest report on medicinal plant research in Malaysia was carried out by Arthur in 1954 [4]. Subsequently, more plants were screened chemically for alkaloids, saponins, triterpenes, and steroids in the 90s [5, 6].

Amongst the famous herbs that are widely used in Malaysia by the locals are *Labisia pumila* (Kacip Fatimah), *Eurycoma longifolia Jack* (Tongkat Ali), *Orthosiphon stamineus* (Misai Kucing), *Quercus infectoria* (Manjakani), and *Piper sarmentosum* (daun kaduk). These plants are similar in terms of exhibiting phytochemical properties that are protective against various diseases. These herbs are known to exert antibacterial, antioxidant, and anti-inflammatory properties that make them beneficial against many types of diseases such as fever, asthma, joint pains, gastrointestinal diseases, bone disorders, and inflammatory disorders. [7–9]. This paper is a review which will be focusing on the content

and health benefits of one of the famous Malaysian herbs, Kacip Fatimah.

Kacip Fatimah or its scientific name *Labisia pumila* (LP) is a member of small genus of slightly woody plants of the family Myrsinaceae. There are four known varieties of *Labisia pumila* found in Malaysia but only three of them are widely used by the locals, which are recognized as *Labisia pumila* var. *pumila, Labisia pumila* var. *alata,* and *Labisia pumila* var. *lanceolata* [10, 11]. LP is found mainly in the lowland and hillforests of peninsular Malaysia at an altitude between 300 and 700 metres. It is also known by the locals as Selusuh Fatimah, Rumput Siti Fatimah, Akar Fatimah, Pokok Pinggang, and Belangkas Hutan [12, 13]. Of all the subtypes, *Labisia pumila* var. *alata* is the most widely used by the locals [10]. Its water extract is traditionally consumed especially by the Malay women to treat menstrual irregularities and painful menstruation, help contracting birth channel after delivery, and to promote sexual health function [14, 15]. It has also been used to treat dysentery, gonorrhoea, rheumatism, and sickness in bones [16, 17].

It is the phytoestrogen, anti-inflammatory, and antioxidative properties that make LP effective against various illnesses. LP was reported to exert estrogenic properties [18–20]. Theoretically, phytoestrogens can act as anti-estrogenic agents by blocking the estrogen receptors and exerting weaker estrogenic effect compared with the hormone [21]. The water extract of LP has been found to inhibit estradiol binding to antibodies raised against estradiol, suggesting the presence of estrogen-like compounds in the extract [22]. It also contains triterpene and saponins, including the compound ardisiacrispin A which were thought to be the reason behind the phytoestrogenic activity of LP [23].

LP has been widely used by the locals in Malaysia not only to ease menstrual pain, induce labor, and promote healthy sexual function but it is also used as an alternative to estrogen replacement therapy in postmenopausal women [24, 25]. Postmenopausal women are prone to osteoporosis due to the reduction in estrogen level. Estrogen acts on estrogen receptor-α (ERα) and receptor-β (ERβ) which has high affinity towards osteoblasts and osteoclasts [26]. Activation of estrogen-receptor complex is vital in maintaining bone remodelling processes [27]. Estrogen can induce osteoclasts apoptosis and inhibit osteoblasts apoptosis, which indirectly will reduce bone resorption and increase bone-formation activity [28]. Hence, reduction in estrogen is highly associated with bone loss. Dietary phytoestrogens such as LP can be an alternative to synthetic estrogen for hormone therapy to reduce side effects of prolonged hormone therapy such as risk of breast cancer, endometrial cancer, and cardiovascular diseases [29, 30]. This paper will focus on the role of *Labisia pumila* in offering protection against postmenopausal osteoporosis via its anti-inflammatory properties.

2. Anti-Inflammatory Role of *Labisia pumila*

Osteoporosis is characterized by skeletal degeneration with low bone mass and destruction of microarchitecture of bone tissue. According to the National Institute of Health, osteoporosis is a skeletal disease which involves decline in mass and density which later leads to fracture [31]. Women, especially postmenopausal women, are more likely to develop osteoporosis than men due to tremendous decline in estrogen during menopause which will lead to decline in bone formation and increase in bone-resorption activity [32]. Osteoporosis is attributed to various factors, and there are evidences that inflammation also exerts significant influence on bone turnover, inducing osteoporosis [33, 34]. According to studies by Lorenzo and Manolagas and Jilka, certain pro-inflammatory cytokines play potential critical roles both in the normal bone remodeling process and in the pathogenesis of osteoporosis [34, 35]. For example, interleukin- (IL-) 6 promotes osteoclasts differentiation and activation [36]. IL-1 is another potent stimulator of bone resorption [37] that has been linked to the accelerated bone loss seen in postmenopausal osteoporosis [38].

Various epidemiologic studies reported an increase in the risk of developing osteoporosis in various inflammatory conditions such as rheumatoid arthritis, haematological diseases, and inflammatory bowel disease [39, 40]. Proinflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-6, IL-1, IL-11, IL-15, and IL-17 are elevated in these conditions [41]. IL-6 and IL-1 may influence osteoclastogenesis by stimulating self-renewal and inhibiting the apoptosis of osteoclasts progenitors [42, 43]. They promote osteoclasts differentiation which is an important stimulator of bone resorption that has been linked to accelerated bone loss seen in postmenopausal women [36]. Receptor activator of NF-$\kappa\beta$ ligand (RANKL) is a membrane-bound molecule of TNF ligand family which plays a crucial role in osteoclasts formation [44]. TNF is a cytokine that is involved in inflammation and is an important cofactor in bone resorption because this cytokine supports osteoclasts activation mediated by RANKL and c-Fms/macrophage colony-stimulating factor.

Estrogen is able to suppress the production of these proinflammatory cytokines [45, 46]. This is why estrogen withdrawal following menopause will lead to increase in these cytokines as proven in many studies. Studies on bone resorption demonstrated that the fall of estrogen level in postmenopausal women was able to stimulate local inflammation in the bone. Ovariectomy in rats was accompanied by increased production of IL-1 and TNF-α which later resulted in decrease in bone density. Hence, it is suggested that estrogen withdrawal can be associated with an increase in production of proinflammatory cytokines, which in turn increases osteoclasts activity resulting in profound bone loss [47]. Estrogen will stimulate production of osteoprotegerin (OPG), which is a potent antiosteoclastogenic factor. OPG acts as a decoy, blocking the binding of the RANK expressed in osteoblasts progenitors, to RANKL which is expressed in committed preosteoblastic cells [48]. This estrogen deficiency leads to upregulation of cytokines [49] and downregulation of OPG which will result in increase in inflammatory responses and increase in bone-resorption activity. In a study by Collin-Osdoby et al., [50] increases in RANKL and OPG mRNA expression were seen in endothelial cells

following an inflammatory stimulus. Therefore, suppression of these potent inflammatory mediators has been proposed to explain the deleterious effects of estrogen deficiency on the human skeletal system at menopause.

3. Phytoestrogenic Role of *Labisia pumila*

LP which has been opposed to exert phytoestrogen property can be used as an alternative to estrogen replacement therapy (ERT) in postmenopausal inflammation-induced osteoporosis. In contrast to ERT which can cause many harmful side effects, LP which originated from natural resources will not cause any side effect, if taken within its safe therapeutic dose. Toxicity testing of LP which was done by the Herbal Medicine Research Centre of Institute of Medical Research has shown that LD50 is safe at more than 5.0 g/kg [51]. LP extract was found to exhibit no-adverse-effect level (NOAEL) at the dose of 50 mg/kg in subacute toxicity study [52], 1000 mg/kg in subchronic toxicity study [53], and 800 mg/kg in reproductive toxicity study [51]. Therefore, LP is safe to be given at high dose as long as it does not outweigh the toxic dose.

Studies have shown that production of proinflammatory cytokines in response to estrogen withdrawal at menopause is responsible to the stimulation of osteoclastic bone resorption [54–56]. A study done by Choi et al. [57] indicated that the LP extract may have good potential to be developed as novel anti-inflammatory drug due to an experimental finding of treatment with LP extract which has markedly inhibited the TNF-α production and the expression of cyclooxygenase (COX)-2. COX-2 is an enzyme that is responsible for the production of mediators involved in inflammation. In vitro experiments have revealed increased COX-2 expression after stimulation with proinflammatory cytokines, such as IL-1 and TNF-α [58].

Pharmacological inhibition of COX can provide a relief from the symptoms of inflammation and pain. Studies have shown that COX-2 plays an important role in pathophysiology of osteoporosis by stimulating the production of prostaglandin (PGE_2). Excessive PGE_2 production might lead to increase in bone resorption, while deficient of its production might impair the bone-formation response, both to mechanical loading and remodelling [59]. Consequently, inhibition of the COX-2 enzyme in postmenopausal women may prevent menopausal bone loss [60]. Inhibition of the main proinflammatory cytokines has proven that LP extract could be a good material for the regulation of anti-inflammation process. TNF has been shown to stimulate osteoclast differentiation, increase its activation, inhibit its apoptosis, and inhibit osteoblast differentiation [61–63]. It also reduces bone formation in cultured osteoblast *in vitro* [64]. Similar to IL-1, TNF-stimulated induction of osteoclast-like-cell formation in bone marrow culture is mediated by increases in RANKL expression. However, in addition to increasing RANKL expression, TNF also inhibits OPG in an osteoblastic model [65]. Hence, inhibition of TNF will indirectly help in reducing bone loss.

4. Antioxidative Role of *Labisia pumila*

Based on previous studies, LP has been shown to exhibit antioxidative properties due to the presence of flavanoids, ascorbic acid, beta-carotene, anthocyanin, and phenolic compounds [66, 67]. According to Norhaiza et al. [68], there were positive correlations between the antioxidant capacities and the antioxidant compounds of LP extract with β-carotene having the best correlation, followed by flavonoid, ascorbic acid, anthocyanin, and phenolic content. β-carotene is one of the basic constituent of antioxidative effect. The chemical abilities of β-carotene to quench singlet oxygen and to inhibit peroxyl free radical actions are well established [69]. Flavonoid has been shown to be highly effective scavenger of free radicals that are involved in diseases such as osteoporosis and rheumatism which is associated with aging due to oxidative stress [70]. Anthocyanin and phenolic on the other hand, not only play a role as antioxidative agents, but also as anti-inflammatory agents [71–73]. These antioxidative and anti-inflammatory properties of LP extract explained the effectiveness of this medicinal plant against various diseases such as osteoporosis, rheumatism, and women sexual function.

Osteoporosis in postmenopausal women can also be explained in terms of oxidative stress mechanism. Ovariectomy has been proposed by many studies as a model of postmenopausal osteoporosis. Following ovariectomy, decline in estrogen level will result in significant bone loss due to bone resorption outweighing bone-formation activity [74]. Estrogen can be considered as an antioxidant as it was found to exhibit antioxidant protection of lipoproteins in the aqueous system [75] and was also shown to increase the expression of glutathione peroxidase in osteoclasts [76]. That is why decline in estrogen will lead to increase in osteoclasts activity resulting in bone loss. Free radicals are continuously produced in the body, mostly by biochemical redox reactions involving oxygen, which occur as part of normal cell metabolism. Free radicals, mainly reactive oxygen species (ROS), are efficiently scavenged, but oxidative stress occurs when there is an imbalance between increased ROS and inadequate antioxidant activity [77] which consequently accelerates aging process and leads to degenerative diseases such as osteoporosis, rheumatism, and cardiovascular disease.

ROS alter mitochondrial and nuclear DNA integrity by increasing the risk of mutations. When DNA repair mechanisms are overwhelmed, cells undergo apoptosis which will lead to tissue damage [78]. This can be applicable in postmenopausal osteoporosis mechanism. When body is subjected to high oxidative stress following estrogen reduction, lipid accumulation will occur. Lipid peroxidation will promote osteoblast apoptosis and simultaneously upregulating ROS production [79, 80]. ROS was shown to promote osteoclast resorption activity either directly or mimicking RANK signalling and stimulating osteoclast differentiation, or indirectly, by stimulating osteoblast/osteoclast coupling and subsequent osteoclast differentiation [81]. Oxidative stress has been acceded as a major contributor to the immune response. Activation of immune response mechanism is

characterized by establishment of an inflammatory response. Thus, osteoporosis can be associated with inflammatory mechanism.

Estrogen can prevent osteoblast cell death and RANKL stimulation by suppressing ROS. Estrogen deficiency is a key step in ROS-mediated stimulation of bone loss via TNF-α signalling pathway. Stimulation of this proinflammatory cytokine will induce bone resorption by indirectly affecting production of essential osteoclast differentiation factor, thereby enhancing proliferation of osteoclast lineage [82]. Glutathione peroxide (GPx) and superoxide dismutase (SOD) are the main antioxidative enzymes that play a pivotal role in counteracting oxidative stress [83]. These enzymes were found to be lowered in postmenopausal women with osteoporosis. This failure of antioxidant defences will result in deleterious effect of hydrogen peroxide on bone health [84]. Studies of antioxidant supplementation such as vitamin E on postmenopausal rat model have shown that lipid peroxidation was successfully inhibited and antioxidative enzymes were restored to acceptable level. In study by Norazlina et al. (2007), IL-6 level was high in ovariectomised rats showing high bone resorption rate, and this level was significantly reduced after three months of tocotrienol (vitamin E) supplementation. In the same study, vitamin E-deficient rats given palm vitamin E showed an improvement in bone calcium content and reduced bone resorption marker [85]. Hence, it is shown that antioxidant is effective in reducing bone-resorption activity as well as improving bone calcium content.

Main antioxidative compound in LP such as flavonoid and β-carotene has been shown in previous studies to inhibit production of nitric oxide and expression of inducible nitric oxide synthase (iNOS) [86] most likely by suppression of NF-κB [87]. NF-kB is an oxidative stress-responsive transcription factor which is activated by free radicals, inflammatory stimuli, and other cytokines. Thus, free radicals may increase bone resorption through activation of NF-kB. It has previously been shown *in vitro* and in rodents that free radicals are involved in osteoclastogenesis and in bone resorption [88]. Oxidative stress may increase bone resorption through activation of NF-κB which plays an important role in osteoclastogenesis [89, 90]. Hence, supplementation of LP which contains antioxidative properties can reduce oxidative stress level which indirectly prevents bone loss.

According to a recent study by Nazrun et al. (2011), osteocalcin, a bone formation marker, was found to be lowered in ovariectomised rats. After being treated with LP results showed an increase in osteocalcin to the level seen in sham-operated group indicating normalisation of bone formation. Bone resorption marker, CTX on the other hand, was found to be reduced after the rats were treated with LP [91]. CTX is sensitive and specific in detection of osteoporosis [92]. This result showed that LP was as effective as estrogen in preventing changes in bone markers induced by ovariectomy.

Based on its positive effects on the bone markers of ovariectomised rats which are comparable to estrogen and its safety profile, LP has the potential to be used as an alternative treatment for postmenopausal osteoporosis. All in all, it is the anti-inflammatory, phytoestrogenic, and antioxidative properties of LP that make it an effective natural medicine in treatment and prevention of osteoporosis.

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Review Article

The Anti-Inflammatory Role of Vitamin E in Prevention of Osteoporosis

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There is growing evidence that inflammation may be one of the causal factors of osteoporosis. Several cytokines such as IL-1, IL-6, RANKL, OPG, and M-CSF were implicated in the pathogenesis of osteoporosis. These cytokines are important determinants of osteoclast differentiation and its bone resorptive activity. Anticytokine therapy using cytokine antagonists such as IL-receptor antagonist and TNF-binding protein was able to suppress the activity of the respective cytokines and prevent bone loss. Several animal studies have shown that vitamin E in the forms of palm-derived tocotrienol and α-tocopherol may prevent osteoporosis in rat models by suppressing IL-1 and IL-6. Free radicals are known to activate transcription factor NFκB which leads to the production of bone resorbing cytokines. Vitamin E, a potent antioxidant, may be able to neutralise free radicals before they could activate NFκB, therefore suppressing cytokine production and osteoporosis. Vitamin E has also been shown to inhibit COX-2, the enzyme involved in inflammatory reactions. Of the two types of vitamin E studied, tocotrienol seemed to be better than tocopherol in terms of its ability to suppress bone-resorbing cytokines.

1. Introduction

Osteoporosis is a bone disease, characterized by low bone mass and increased risk of fractures [1]. It is well accepted that osteoporosis can be caused by various endocrine, metabolic, and mechanical factors. However, recently, there are opinions that there may be an inflammatory component in the etiology of osteoporosis [2, 3]. There is plenty of evidence linking inflammation to osteoporosis. Epidemiological studies have identified higher incidence of osteoporosis in various inflammatory conditions such as ankylosing spondylitis, rheumatoid arthritis, and systemic lupus erythematosus [4–7]. This association was also observed clinically whereby the degree of osteoporosis was equivalent to the extent of inflammation. If the inflammation was systemic, bone loss will occur at all skeletal sites, whereas if the inflammation was only restricted to a site, bone loss will only occur locally at that site of inflammation [3]. Elderly patients are more prone to osteoporosis, and this was believed to be connected to the elevated production of proinflammatory cytokines with aging [8, 9].

The occurrence of inflammation is indicated by the presence of inflammatory markers such as cytokines and C-reactive protein. Biochemical studies have demonstrated elevation of proinflammatory cytokines TNF-α and IL-6 in arthritic disease such as gouty arthritis, rheumatoid arthritis, and psoriatic arthritis [10, 11]. An obvious relationship between inflammation and osteoporosis was seen in rheumatoid arthritis, whereby proinflammatory cytokines were released causing bone loss around the affected joints [12]. The level of C-reactive protein, a sensitive marker of systemic inflammation, was also found to be associated with bone mineral density [13]. Inflammation may contribute to bone loss by affecting the bone remodeling process, favouring bone resorption activity by osteoclasts rather than bone formation activity by osteoblasts [14, 15]. Bone resorption is determined by the balance between two cytokines, receptor activator of nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG) [16]. RANKL is crucial for the differentiation and activation of osteoclast [17]. Higher RANKL levels were associated with lower bone mineral density in men [18]. Administration of serum

RANKL to mice promoted osteoclast growth and activation, leading to osteoporosis [19]. On the other hand, OPG antagonizes RANKL by binding with RANKL and preventing it from binding to RANK receptors. By doing that, OPG was able to inhibit osteoclastogenesis and bone resorption [20]. Macrophage colony stimulating factor (MCSF) is another important determinant of osteoclastogenesis, but its mechanism to modulate osteoclastogenesis is still not clear [20].

The "upstream" cytokines such as IL-1, IL-6, and TNF-α [21, 22] and "downstream" cytokines such as RANKL, OPG, and M-CSF [23–25] played an important role in bone remodeling. Imbalance in their bioactivity may lead to bone loss and osteoporosis. Cytokines are small- to medium-sized proteins or glycoproteins with molecular weight ranging from 8 to 40,000 dalton. They act as the biological mediator for most cells and function at low concentrations between 10^{-10} and 10^{-5} molar. They have a short half-life of less than 10 minutes, and their serum level can be as low as 10 pg/mL. The cytokine levels increase dramatically during inflammation and infection. The measurement of cytokine levels in close vicinity to bone such as the bone marrow is important for studies on osteoporosis and other bone diseases. In postmenopausal women, cytokine production by the peripheral monocytes correlated well with cytokines secreted by monocytes in the bone marrow. Therefore, cytokine levels in the serum are representative of the local monocytes [26]. Stromal cells and osteoblasts produce interleukin-1, interleukin-6, and tumor necrosis factor-α. These proinflammatory cytokines are also known as the bone-resorbing cytokines or proosteoclast cytokines as they promote osteoclast differentiation and activity [27–30]. The bone resorption activity of these cytokines in ovariectomised rats was reduced with anticytokine therapy such as IL-1 receptor antagonists and TNF-binding protein [31]. Vitamin E, a potent antioxidant vitamin, was also found to inhibit or suppress cytokine production [32, 33]. This vitamin E action may be responsible for its ability to prevent inflammation and osteoporosis, seen in several studies on osteoporosis using animal models [34].

Vitamin E is a group of potent, lipid-soluble, chain-breaking antioxidants. It can be classified into tocopherol and tocotrienol based on the chemical structure. Palm oil, which is extracted from the pulp of the fruit of the oil palm *Elaeis guineensis*, is abundant in tocotrienols. Tocotrienol has an unsaturated farnesyl (isoprenoid) side-chain, while tocopherol has a saturated phytyl side chain [35].

Vitamin E occurs in eight isoforms of α-, β-, γ-, and δ-tocopherols or tocotrienols. It was thought that both the γ and δ isomers of tocopherol have better antioxidant and anti-inflammatory activities than the α isomer [36, 37]. Once vitamin E is absorbed in the intestine, it will enter the circulation via the lymphatic system and be transported to the liver with the chylomicrons [38]. Vitamin E is metabolized by cytochrome P450 and then excreted in the urine [39].

In human subjects and animal models, high doses of vitamin E were found to exhibit anti-inflammatory effects by decreasing C-reactive protein (CRP) and inhibiting the release of proinflammatory cytokines [40]. These were evident in a study on patients with coronary artery disease, whereby the CRP and tumor necrosis factor-α (TNF-α) concentrations were found to be significantly lowered with α-tocopherol supplementation compared to placebo [41]. Since vitamin E was also found to inhibit cyclooxygenase-2 activities, it was thought to be able to exert anti-inflammatory and anticarcinogenic activities, especially in the colon [42]. This was demonstrated by Yang et al. [43], who found that vitamin E was able to significantly lower colon inflammation index and reduced the number of colon adenomas in mice given azoxymethane.

This paper will focus on the effects of vitamin E on bone-resorbing cytokines with special attention on IL-1 and IL-6.

2. Interleukin-1 (IL-1)

IL-1 plays an important role in various reactions towards infection, inflammation, and immune activation. This cytokine is produced by various cells but the main producer is the monocyte. In the physiological condition, monocytes do not secrete IL-1 but, under pathological conditions such as septic shock, IL-1 is rapidly released and acts directly on the blood vessels. Other cytokines such as TNF-α and interferon, bacterial endotoxin, virus, and antigen can also stimulate the release of IL-1. Reactive oxygen species such as superoxide radicals have been shown to induce IL-1 production [32, 44]. IL-1 is involved in the pathogenesis of various diseases associated with bone loss such as osteoporosis [45, 46], cancer-induced osteolysis [47], rheumatoid arthritis [48], and osteolysis of orthopedic implants [49]. IL-1 is also an important factor in both *in vivo* and *in vitro* bone resorption [50, 51]. It stimulates the formation and activity of osteoclasts, leading to excessive bone resorption. Suda et al. [52] demonstrated that the presence of osteoblast and stromal cells was crucial in the formation of osteoclasts by IL-1. Thomson et al. [53] also reported that osteoblasts secrete a factor that stimulates the bone-resorbing activities of rat osteoclasts. However, Xu et al. [54] demonstrated that rat osteoclasts expressed mRNA to IL-1 receptors, while Yu and Ferrier [55] found that osteoclast is one of the target cells for IL-1. These studies proved that IL-1 can act directly on osteoclasts without the presence of osteoblasts or stromal cells. IL-1 may also promote formation of osteoclasts [56]. It acts by activating nuclear factor κB (NFκB) in osteoclast and prevents its apoptosis [57]. It was found that the estrogen-deficient state in postmenopausal women or ovariectomised rats resulted in increased production of IL-1 by monocyte and other bone marrow cells [58, 59]. Estrogen replacement or IL-receptor antagonist was able to prevent the elevation of IL-1 in ovariectomised rats [60, 61]. Vitamin E was also found to have the ability to suppress IL-1 production by activated monocytes [62]. In a different study, combination of superoxide dismutase and vitamin E was effective in inhibiting IL-1 production by human monocytes [32]. The ability of vitamin E to inhibit IL-1 in the bone environment may have prevented bone loss.

3. Interleukin-6 (IL-6)

IL-6 is another cytokine that is associated with various pathophysiological processes in humans. It is produced by the haematopoetic and nonhaematopoetic cells when they were exposed to various types of stimulation. During bone remodeling, IL-6 is produced in nanomolar concentrations by stromal cells and osteoblasts under the influence of parathyroid hormone, vitamin D_3, growth factor, and other cytokines [63]. IL-6 was also reported to be produced by osteoblasts when stimulated by IL-1, TNF-α, and lipopolysaccharide [64]. McSheeny and Chambers [65] reported that osteoblasts were stimulated by local IL-1 to produce IL-6, which was responsible for the activation of osteoclasts. IL-6 promoted the differentiation of osteoclasts from its precursor and played an important role in the pathogenesis of osteoporosis due to estrogen deficiency [66, 67]. The IL-6 elevation in postmenopausal women was reduced by estrogen replacement therapy [68]. The elevation of IL-6 may be related to free radical activities especially reactive oxygen species. Reactive oxygen species was found to elevate the IL-6 levels directly via activation of nuclear factor κB (NFκB) [69]. High cytokine levels would also result in activation of NFκB and promotion of osteoclastogenesis [70].

4. Vitamin E as Anticytokine Agent

The effects of vitamin E on bone resorbing cytokines for prevention and treatment of osteoporosis have been studied using FeNTA and nicotine rat models [34, 71]. These models represent osteoporosis caused by oxidative stress and smoking, respectively. However, similar studies in humans are still lacking. Ferric nitrilotriacetate (FeNTA) is an oxidizing agent which produces free radicals via the Fenton reaction [72, 73]. Oxidative stress can be induced in rats by injecting them with FeNTA, allowing the hazardous effects of free radicals on various organs and tissues including bone to be studied. The bone resorbing cytokines, IL-1 and IL-6, were found to be elevated in this oxidative stress rat model, indicating inflammation. This was accompanied by osteoporotic changes as indicated by the measurement of bone markers and histomorphometric parameters [34]. The elevation of cytokines was probably achieved through the activation of cytokine-encoding genes like STAT3 or nuclear factor-kappaB by the free radicals [74, 75]. Therefore, there exist relationships between free radicals, inflammation, and bone loss which can lead to osteoporosis. When vitamin E in the form of tocotrienols and α-tocopherol were supplemented to these rats, IL-1 and IL-6 elevations were suppressed. Concurrent with this, the osteoporotic changes were also inhibited [34, 71, 76]. Therefore, there is a possibility that vitamin E, a potent antioxidant, has prevented free radicals from causing inflammation and osteoporosis. Tocotrienols seemed to be more superior than α-tocopherol in suppressing proinflammatory cytokines in the FeNTA rat model and in protecting their bone against osteoporosis [34]. Both the tocopherol and tocotrienol may have achieved this by scavenging the free radicals generated by FeNTA before they could activate the monocytes and osteoblasts, cells that produce IL-1 and IL-6.

Cigarette smoking is a modest risk factor for osteoporosis [77]. Nicotine is among the 4,700 chemicals found in the tar phase of cigarette smoke [78]. Nicotine injected into rats can be used as a model for osteoporosis related to smoking. Various animal studies have confirmed the deleterious effects of nicotine on bone remodeling [79–85]. Nicotine inhibited osteoblast activity and growth [86, 87] but stimulated osteoclast activity [83]. Nicotine has also been shown to induce oxidative stress in both *in vitro* and *in vivo* animal studies [88, 89]. Crowley-Weber et al. [90] had reported that other than oxidative stress, nicotine also activated nuclear transcription factor-κB (NF-κB) in the tissues of smokers. The activation of NF-κB-signaling pathway may be the mechanism for bone loss as it is responsible for osteoclast differentiation [76, 91]. Nicotine has been shown to significantly elevate the proinflammatory cytokines IL-1 and IL-6 in rats. Using the same model, tocotrienol was able to prevent nicotine-induced elevation of IL-1 and IL-6, while tocopherol had no significant effects on both cytokines [71]. Tocotrienol was more effective compared to tocopherol in terms of its action on bone resorbing cytokines and therefore was more effective in reducing inflammation and bone loss.

5. Anti-Inflammatory Action of Vitamin E in Prevention of Osteoporosis

Results from studies on cytokines have given us some insight on the mechanisms involved in the protection of vitamin E against osteoporosis. Free radicals are known to activate transcription factor NFκB which leads to the production of bone resorbing cytokines interleukin-1 and interleukin-6. These proinflammatory cytokines were believed to provide the link between inflammation and osteoporosis. Vitamin E may scavenge and neutralize free radicals before they could activate transcription factor NFκB. This was seen in an oxidative stress model (FeNTA model) in which vitamin E had reduced the levels of bone-resorbing cytokines [34]. Alternatively, vitamin E may have prevented the activation of NFκB by enhancing the internal antioxidative enzymes within the bone. This was demonstrated by Maniam et al. [92], whereby vitamin E supplementation reduced the femoral thiobarbituric acid-reactive substance (TBARS) and increased the glutathione peroxidase activity.

Since osteoporosis is associated with inflammation, there is also a possibility that Vitamin E may have some anti-inflammatory action. Yam et al. [93] found that tocotrienol was able to suppress cyclooxygenase-2 (COX-2) expression in RAW 264.7 cells that were exposed to lipopolysaccharide. COX-2 is an inducible enzyme expressed during inflammation. A RAW cell is a macrophage-like cell which transformed into preosteoclasts when RANKL is added. This suggested that vitamin E may act as anti-inflammatory agent in protecting bone against excessive osteoclastic activity. Previous study has shown that aspirin or other nonsteroidal anti-inflammatory drugs (NSAID) inhibited NFκB [94]. Similar

to tocotrienol, these anti-inflammatory drugs inhibit COX-2. As the activation of NFκB is linked to proinflammatory cytokines and inflammation, it further provides evidence of the anti-inflammatory role of tocotrienol in preventing osteoporosis.

Based on the results from the studies above, tocotrienol was more superior than tocopherol in terms of its ability to suppress bone resorbing cytokines. The more superior tocotrienol action may be contributed by its more potent antioxidant property. It has better interaction with lipoprotein in membrane lipids and is uniformly distributed in the membrane layer compared to tocopherol [35, 95]. Tocotrienol was also better at maintaining the antioxidant status within the rat bone compared to tocopherol [92]. Thus, the antiosteoporotic effect of tocotrienol may be partly explained by its anti-inflammatory as well as antioxidative effects.

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Research Article

Antinociceptive and Anti-Inflammatory Activities of Leaf Methanol Extract of *Cotyledon orbiculata* L. (Crassulaceae)

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Leaf methanol extract of *C. orbiculata* L. was investigated for antinociceptive and anti-inflammatory activities using acetic acid writhing and hot-plate tests and carrageenan-induced oedema test in mice and rats, respectively. *C. orbiculata* (100–400 mg/kg, i.p.) significantly inhibited acetic acid-induced writhing and significantly delayed the reaction time of mice to the hot-plate-induced thermal stimulation. Paracetamol (300 mg/kg, i.p.) significantly inhibited the acetic acid-induced writhing in mice. Morphine (10 mg/kg, i.p.) significantly delayed the reaction time of mice to the thermal stimulation produced with hot plate. Leaf methanol extract of *C. orbiculata* (50–400 mg/kg, i.p.) significantly attenuated the carrageenan-induced rat paw oedema. Indomethacin (10 mg/kg, p.o.) also significantly attenuated the carrageenan-induced rat paw oedema. The LD_{50} value obtained for the plant species was greater than 4000 mg/kg (p.o.). The data obtained indicate that *C. orbiculata* has antinociceptive and anti-inflammatory activities, justifying the folklore use of the plant species by traditional medicine practitioners in the treatment of painful and inflammatory conditions. The relatively high LD_{50} obtained shows that *C. orbiculata* may be safe in or nontoxic to mice.

1. Introduction

Pain and inflammation are some of the most common manifestations of many diseases afflicting millions of people worldwide [1, 2]. Even though there are effective orthodox medicines used to alleviate these manifestations [3], traditional medicine practitioners in, mainly, developing countries have used herbal medicines to treat various ailments including pain and inflammation [4]. The dependence of the population especially in the rural communities in South Africa on plant medicines as well as traditional medicine practitioners for their healthcare needs is cultural. One of such plants used by traditional medicine practitioners to treat various ailments is *Cotyledon orbiculata* L. [5, 6]. It belongs to the family Crassulaceae. It is a small shrub with fleshy leaves and widely distributed in Southern Africa. It is known locally as "Seredile" in Sotho and Tswana, "Plakkie" in Afrikaans, and "Imphewula" in Xhosa [5, 6]. *C. orbiculata* is used in the treatment of various ailments in different parts of South Africa. The fleshy leaves have been used to treat corn and warts. The juice of the leaves is used as drops for earache and toothache and as hot poultice for boils and inflammation [5–7]. Infusion of the fleshy leaves of *C. orbiculata* has also been used by traditional medicines practitioners in South Africa for the treatment of epilepsy, inflammation, and aches (Oral communication).

According to the literature, very limited evaluation has been done on the pharmacological activities of the plant species despite the wide folklore use [8]. This study was, therefore, intended to investigate the antinociceptive and anti-inflammatory activities of *C. orbiculata* in mice and rats, respectively. The acute toxicity and HPLC studies of the plant species were also carried out.

2. Materials and Methods

2.1. Plant Material. The fleshy leaves of *C. orbiculata* were collected from Kirstenbosch National Botanical Garden, Cape Town, in September, 2010. The plant material was identified by the curator of the Gardens as well as a taxonomist in the Department of Biodiversity and Conservative Biology,

University of the Western Cape and the voucher specimen (COT 25) deposited in the University's Herbarium.

2.2. Preparation of Plant Extract. The fleshy leaves (10.5 kg) of *C. orbiculata* were washed with water, sliced into pieces, and dried in a ventilated oven at 40°C for 120 h. The dried plant material (640 g) was ground into fine powder using Waring Commercial laboratory blender and passed through 850 μm sieve. For the preparation of the methanol extract, the dried powder (120 g) was extracted in a soxhlet extractor with methanol for 72 h. The methanol filtrate was evaporated to dryness using a Buchi RE11 rotavapor and Buchi 461water bath. A yield of 55.4 g of crude methanol extract was obtained and preserved in a dessicator. Fresh solution of the crude leaf methanol extract was prepared by dissolving a given quantity of the methanol extract in a small volume of dimethylsulfoxide (DMSO) and made up to the appropriate volume with physiological saline. The methanol solution was administered intraperitoneally (i.p.) to mice and rats in a volume of 1 mL/100 g of body weight.

2.3. Animals. Male albino mice bred in the Animal House of the Discipline of Pharmacology, School of Pharmacy, University of the Western Cape, South Africa, weighing 18–30 g were used for the antinociceptive activity and acute toxicity studies. Young adult male Wistar rats, bought from the University of Cape Town, South Africa, and weighing 160–210 g were used for anti-inflammatory activity study. The animals were housed in a quiet laboratory with an ambient temperature of 22 ± 1°C and a12 h light/12 h dark cycle was maintained. They all had access to food and water *ad libitum*. All the animals were fasted for 16 h during which they had access to water prior to the commencement of the experiments. Each animal was used for one experiment only.

2.4. Drugs and Chemicals. Indomethacin (Sigma Chemical Co.) was dissolved in a minimum amount of dimethylsulfoxide (DMSO, Sigma Chemical Co.) and adjusted to the appropriate volume with physiological saline. Carrageenan (Sigma Chemical Co.) and morphine sulphate (Bodene) were dissolved in physiological saline to an appropriate volume. Acetic acid (Merck) was dissolved in physiological saline to an appropriate strength. Paracetamol (Sigma Chemical Co.) was dissolved in a minimum volume of propylene glycol 400 (BDH, UK) and adjusted to the appropriate volume with physiological saline. DMSO solution was prepared by dissolving an equal amount of DMSO used to dissolve the plant extract, in an appropriate volume of physiological saline. Indomethacin was given orally to rats by means of a bulbed steel needle. Carrageenan was injected into the subplantar surface of the right hind paws of the rats.

Morphine, acetic acid, and paracetamol were administered intraperitoneally (ip) to mice. Fresh drug solutions were prepared each morning of the experiment. All drugs were administered in a volume of 1 mL/100 g of body weight, while constant volumes of carrageenan, DMSO, physiological saline, and acetic acid were used. Control animals received equal volume injections of the appropriate vehicles. The doses and pretreatment times of the leaf methanol extract of *C. orbiculata* and standard drugs, indomethacin, morphine, paracetamol, and the vehicles, physiological saline and DMSO, were obtained from preliminary studies in our laboratory.

3. Assessment Pharmacological Activities

3.1. Antinociceptive Activity of Cotyledon orbiculata

3.1.1. Acetic Acid Writhing Test. The methods of Koster et al. [9] and Williamson et al. [10] were used for the assessment of the antinociceptive activity of *C. orbiculata*. Mice were used in groups of 8 per dose of plant extract, standard drug, paracetamol, or DMSO. They were placed singly in a transparent perspex mouse cage and allowed to acclimatize to their environment for 30 min prior to the commencement of the experiment. In the control experiment, the animals were pretreated with 0.25 mL of physiological saline (i.p.) for 15 min and then given intraperitoneal injection of 0.20 mL of 3% acetic acid solution, an irritant, used to induce writhing (pain). The mice were then left for 5 min, and the writhes were counted for the next 20 min. A writhe is defined as contraction of the abdominal muscles accompanied by elongation of the body and the hind limbs.

In the test experiment, a group of 8 mice were pretreated for 15 min with either the plant extract (i.p.) or the standard analgesic drug, paracetamol (i.p.), after which they were injected with 0.20 mL of the 3% acetic acid intraperitoneally, allowed to stand for 5 min and then the number of writhes counted for 20 min as for the control experiment. The experiment was repeated with another group of 8 mice pretreated with 0.25 mL of DMSO solution (i.p.) for 15 min, after which they were injected with 0.20 mL of the 3% acetic acid intraperitoneally, allowed to stand for 5 min, and then the number of writhes counted for 20 min. All experiments were performed in a quite laboratory with an ambient temperature of 22 ± 1°C. The ability of the plant extract to prevent or significantly reduced the number of acetic acid-induced writhes was an indication of an antinociceptive activity.

3.1.2. Hot-Plate Test. The methods of Williamson et al. [10] and Eddy and Leimback [11] were used in the hot-plate test for the antinociceptive activity of *C. orbiculata*. Mice were used in groups of 8 per dose of plant extract, standard drug, morphine, or DMSO. Control animals were individually placed in a 21 glass beaker placed on a thermostatically controlled hot plate (model HC500, Bibby Sterilin Ltd., England) set at 50–55°C, before and 15 min after intraperitoneal injection of 0.25 mL of physiological saline. The pain threshold is considered to be reached when the animals lift and lick their paws or attempt to jump out of the beaker. The time taken for the mice to exhibit these characteristics, also known as the reaction or response time, was noted by means of a stopwatch. The animals were tested before and 15 min, 30 min, 45 min, and 60 min after intraperitoneal injection of 0.25 mL of physiological saline. The experiments

were repeated using other groups of animals, which were tested before and 15 min, 30 min, 45 min, and 60 min after the intraperitoneal administration of either the plant extract, morphine, or DMSO. All experiments were performed in a quite laboratory with an ambient temperature of 22 ± 1°C. A cutoff time of 60 s was used to avoid harm to the mice. The ability of the plant extract to delay the reaction time was taken as an indication of an antinociceptive activity.

3.2. Anti-Inflammatory Activity of Cotyledon orbiculata

3.2.1. Rat Paw Oedema Test. Modified method of Williamson et al. [10] and Winter et al. [12] were used to assess the anti-inflammatory activity of *C. orbiculata*. Rats were used in groups of 8 per dose of plant extract, standard drug, physiological saline, or DMSO. The rats were divided into five groups. Rats in Group I (control) were given 0.25 mL (i.p.) of physiological saline. Group II rats received plant extracts (50–400 mg/kg, i.p.). Group III rats were given the standard anti-inflammatory drug, indomethacin (10 mg/kg, p.o.), and Group IV rats received 0.25 mL (i.p.) of DMSO (vehicle). Group V rats were untreated. Oedema or acute inflammation was induced in Group I or control rats pretreated for 15 min with 0.25 mL (i.p.) of physiological saline by injecting 0.1 mL of carrageenan (1% dissolved in 0.9% saline solution) into the subplantar surface of the right hind paw. The oedema following the carrageenan injection was noticeable within 30–40 min. The volume of the right hind paw was measured before and then after the injection of carrageenan at 30 min intervals for 4 h by volume displacement method using plethysmometer (IITC Life Sciences, USA). Group II rats were pretreated for 15 min with plant extracts intraperitoneally (i.p.), Group II rats for 1 h with indomethacin orally (p.o.) and Group IV rats for 15 min with DMSO (i.p.) prior to the injection 0.1 mL of carrageenan into the subplantar surface of the right hind paws of the rats in each group. The experiments were repeated with the volumes of the rats' right hind paws measured before and then after the injection of carrageenan at 30 min intervals for 4 h using the plethysmometer. The volumes of the untreated rats' right paws were also measured at 30 min intervals for 4 h. Oedema was expressed as a mean increase in paw volume with respect to physiological saline control. Inhibition was expressed as a percentage increase or decrease in oedema volume. The ability of the plant extract to inhibit the foot oedema was taken as an indication of an anti-inflammatory activity. All experiments were performed in a quite laboratory with an ambient temperature of 22 ± 1°C.

3.2.2. HPLC Analysis. Chromatographic system: Beckman HPLC system consisting of double pump Programmable Solvent Module model 126; Diode Array detector Module model 168; Samsung computer 386 with management System Gold (Gold V601) software supplied by Beckman; Column, C18 Bondapak 5 μm and dimensions (250 × 4.6 mm).

Chromatographic conditions: Mobile phase: solvent A: 1% acetic acid; solvent B: methanol; Mode: gradient; flow rate, 1 min/min; injection volume, 10 μL; detector, UV at 350 nm. The HPLC operating conditions were programmed to give the following: 0 min, solvent B: 20%; 5 min, solvent B: 40%; 15 min, solvent B: 60%; 20 min, solvent B: 80% and 27 min, solvent B: 20%. The run rate was 30 min.

3.2.3. Acute Toxicity Testing. The method described by Lorke [13] and modified by Hilaly et al. [14] was used to determine the median lethal dose (LD_{50}) of the leaf methanol extract. Mice were fasted for 16 h and then randomly divided into groups of eight mice per cage. Graded doses of the plant extract (100, 200, 300, 400, 600, 800, 1600, 2000, 2400, 2800, 3200, 3600, and 4000 mg/kg) were separately administered orally by means of a bulbed steel needle to mice in each test group. The control group was administered with 0.25 mL (p.o.) of physiological saline by means of a bulbed steel needle. The mice in both the test and control groups were then allowed free access to food and water and observed for over 5 days for signs of acute toxicity including death. The median lethal dose (LD_{50}) of the leaf methanol extract of *C. orbiculata* would be calculated if applicable, from a plot of log dose-response curve which would be constructed for the plant species.

3.3. Statistical Analysis. The data on the number of writhes exhibited by the mice and the effect of carrageenan on the rat's right hind paw were analysed using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (GraphPad Prism, version 5.0, GraphPad Software, Inc., SanDiego CA p2130, USA) and presented as mean ± standard error mean (SEM). P values of less than 5% ($P < 0.05$) were considered statistically significant.

3.4. Ethical Considerations. The experimental protocol used in this study was approved (07/04/31) by the Ethics Committee of the University of the Western Cape, Bellville 7535, South Africa, and conforms with the University's Regulations Act concerning animal experiments.

4. Results

4.1. Pharmacological Activities: Antinociceptive Activity of Cotyledon orbiculata

4.1.1. Acetic Acid Writhing Test

Effect of Leaf Methanol Extract of Cotyledon orbiculata on Acetic Acid-Induced Writhing. 0.20 mL (i.p.) of 3% acetic acid produced a substantial number of writhes in control mice pretreated with 0.25 mL (i.p.) of physiological saline. Leaf methanol extract of *C. orbiculata* (100–400 mg/kg, i.p.) in a dose-dependent manner, significantly reduced the number of acetic acid-induced writhes. 100 mg/kg (i.p.) of the plant species reduced the writhes by 51%. 200 mg/kg (i.p.) and 400 mg/kg (i.p.) of *C. orbiculata* produced 67% and 76% reduction in writhes produced by 0.20 mL of 3% acetic acid in mice, respectively. Similarly, paracetamol (300 mg/kg, i.p.) profoundly reduced the number of writhes elicited by

0.20 mL of 3% acetic acid by 93%. DMSO (0.25 mL, i.p.) did not significantly alter the acetic acid-induced writhes in mice (Table 1).

Effect of Leaf Methanol Extract of Cotyledon orbiculata on Hot-Plate-Induced Nociception. Mice pretreated with physiological saline reacted to hot-plate thermal stimulation at 50°C–55°C either by lifting and licking their paws or attempting to jump out of the beaker. This manifestation occurred within 6.63 ± 0.60 sec in the first 15 min after intraperitoneal administration of 0.25 mL of physiological. saline and within 2.75 ± 0.31 sec, 60 min later after the injection of 0.25 mL of physiological saline. Leaf methanol extract of *C. orbiculata* (100–200 mg/kg, i.p.) significantly delayed the reaction times of the animals to hot-plate thermal stimulation 30 min after treatment. *C. orbiculata* (400 mg/kg, i.p.) significantly delayed the pain reaction time of the mice to the hot-plate-induced thermal stimulation over the 1 h period of measurement. Similarly, morphine (10 mg/kg, i.p.) significantly delayed the reaction time of the mice to the hot-plate-induced thermal stimulation over the 1 h period of measurement. DMSO (0.25 mL, i.p.) did not significantly alter the reaction time of the mice to the hot-plate-induced thermal stimulation over the 1 h period of measurement (Table 2).

Effect of Leaf Methanol Extract of C. orbiculata on Carrageenan-Induced Right Hind Paw Oedema. Carrageenan (1%) injected into the subplantar of the right hind paws of the rats pretreated with physiological saline induced oedema or acute inflammation in the paws within 30–40 min. The oedema reached its maximum intensity 3 h after injection. 50 mg/kg (i.p.) of the leaf methanol extract of *C. orbiculata* significantly reduced the carrageenan-induced oedema from 60 min up to the 4 h period of measurement. *C. orbiculata* (100–400 mg/kg, i.p.) significantly reduced the carrageenan-induced oedema over the 4 h period of measurement. Indomethacin (10 mg/kg, p.o.) profoundly reduced the carrageenan-induced oedema in the right hind paws of rats over the 4 h period of measurement (Table 3).

4.1.2. Acute Toxicity Test. There were no deaths or signs of acute toxicity observed after oral administration of 100–4000 mg/kg of the leaf methanol extract of *Cotyledon orbiculata* with the highest dose tested (4000 mg/kg, p.o.) being the no-adverse-effect-level (NOAEL). That is, the LD_{50} was probably greater than 4000 mg/kg (p.o.) in mice.

4.1.3. HPLC Analysis. The chromatographic spectrum of the leaf methanol extract of *C. orbiculata* obtained revealed major peaks at the following retention times (minutes): 6.983, 10.521, 12.088, 12.838, and 13.342 (Figure 1).

5. Discussion

In the present study, the leaf methanol extract of *C. orbiculata* significantly inhibited the acetic acid-induced writhing and significantly inhibited the nociception produced by hot plate. *C. orbiculata* also significantly attenuated carrageenan-induced rat right hind paw oedema. Satyanarayana et al. [15] has shown that acetic acid produced writhing or nociception by stimulating the production of prostaglandin. Paracetamol, a standard analgesic drug [2], has been shown to inhibit prostaglandin synthesis in the brain [16]. It is, therefore, not surprising that paracetamol significantly attenuated acetic acid-induced nociception in this study. The effect of paracetamol on prostaglandin in relation to acetic-acid-induced writhes may be direct or indirect. Since *C. orbiculata* also attenuated acetic acid-induced writhing, it is probable that the plant species may be producing its antinociceptive activity by affecting the prostaglandin system. Morphine, a standard centrally acting analgesic

Table 1: Effect of leaf methanol extract of *Cotyledon orbiculata* on acetic acid-induced writhing in mice.

Treatment groups	Dose (mg/kg)	Number of writhes Mean ± SEM		Percentage reduction (%)
PS	0.25 mL	28.13	3.92	
C. orbiculata	100	13.83*	3.17	51
	200	9.20**	3.04	67
	400	6.75***	1.97	76
Paracetamol	300	2.10***	0.24	93
DMSO	0.25 mL	29.04	2.73	0

*$P < 0.025$, **$P < 0.005$, ***$P < 0.001$ versus 3% acetic acid (0.20 mL, i.p.) control, ANOVA ($n = 8$). Writhes are expressed as number of counts per 20 minutes.
PS: physiological saline.
DMSO: dimethylsulfoxide.

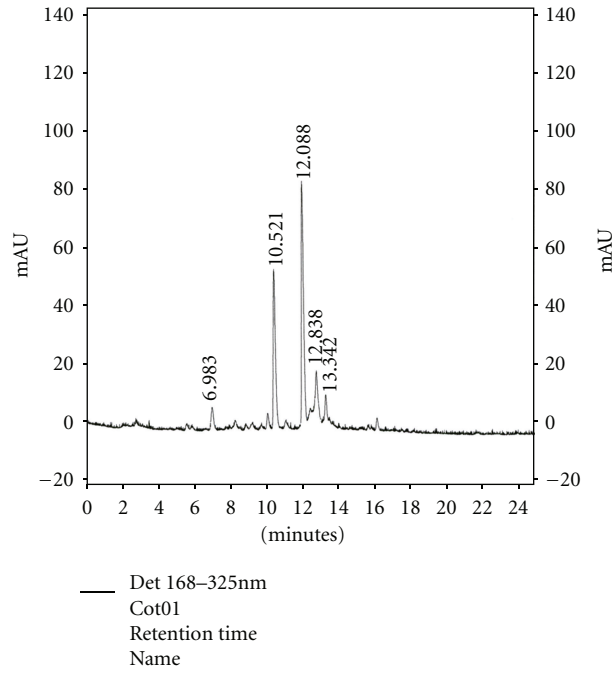

Figure 1: HPLC fingerprint of leaf methanol extract of *Cotyledon orbiculata.*

Table 2: Effect of leaf methanol extract of *Cotyledon orbiculata* on hot-plate-induced nociception in mice.

Treatment groups	Dose (mg/kg)	Response time (s)				
		0 min	15 min	30 min	45 min	60 min
PS	0.25 mL	4.13 ± 0.13	6.63 ± 0.60	4.38 ± 0.78	3.38 ± 0.68	2.75 ± 0.31
C. orbiculata	100	6.63 ± 0.92	10.25 ± 1.46	11.75^{**} ± 1.07	7.13 ± 1.27	5.13 ± 0.08
	200	6.63 ± 0.85	7.38 ± 1.08	12.38^{+} ± 1.43	6.88 ± 1.57	6.25 ± 1.45
	400	5.25 ± 0.47	19.13^{*} ± 5.01	26.63^{++} ± 3.35	22.63^{+} ± 3.41	24.5^{++} ± 2.55
Morphine	10	3.38 ± 0.64	26.63^{++} ± 4.83	36.50^{++} ± 6.55	22.88^{++} ± 2.93	16.63^{++} ± 2.07
DMSO	0.25 mL	5.00 ± 0.76	6.13 ± 0.99	3.88 ± 0.38	4.75 ± 0.86	4.50 ± 0.91

$^{*}P < 0.05$, $^{**}P < 0.025$, $^{+}P < 0.02$, $^{++}P < 0.001$ versus physiological saline control, ANOVA ($n = 8$). The response time in seconds was expressed as Mean ± SEM.
PS: physiological saline.
DMSO: dimethylsulfoxide.

Table 3: Effect of leaf methanol extract of Cotyledon orbiculata on carrageenan-induced oedema in the right hind paw of rat.

Treatment group	Dose (mg/kg)	Paw volume (mL) (Mean ± SEM)								
		0	30	60	90	120	150	180	210	240 (min)
UR	—	0.11 ± 0.01	0.12 ± 0.08	0.10 ± 0.03	0.09 ± 0.01	0.11 ± 0.04	0.11 ± 0.05	0.09 ± 0.07	0.10 ± 0.03	0.09 ± 0.05
PS	0.25 mL	0.09 ± 0.04	0.35 ± 0.05	0.48 ± 0.03	0.52 ± 0.02	0.61 ± 0.04	0.68 ± 0.02	0.72 ± 0.01	0.69 ± 0.05	0.69 ± 0.03
C. orbiculata	50	0.09 ± 0.01	0.29 ± 0.01	0.34^{*} ± 0.01	0.41^{*} ± 0.07	0.50^{*} ± 0.02	0.53^{*} ± 0.01	0.61^{*} ± 0.04	0.59^{*} ± 0.05	0.58^{*} ± 0.03
	100	0.08 ± 0.05	0.26^{*} ± 0.01	0.31^{**} ± 0.04	0.37^{**} ± 0.01	0.38^{+} ± 0.02	0.36^{+} ± 0.03	0.36^{+} ± 0.02	0.33^{+} ± 0.01	0.36^{+} ± 0.04
	200	0.11 ± 0.03	0.22^{*} ± 0.02	0.26^{+} ± 0.01	0.27^{+} ± 0.01	0.28^{+} ± 0.04	0.31^{+} ± 0.02	0.30 ± 0.02	0.20^{+} ± 0.01	0.30^{+} ± 0.01
	400	0.10 ± 0.04	0.19^{*} ± 0.02	0.22^{+} ± 0.01	0.21^{+} ± 0.03	0.21^{+} ± 0.02	0.20^{+} ± 0.04	0.22^{+} ± 0.01	0.19^{+} ± 0.01	0.19^{+} ± 0.02
Indomethacin	10	0.11 ± 0.02	0.14^{+} ± 0.02	0.18^{+} ± 0.04	0.20^{+} ± 0.06	0.19^{+} ± 0.03	0.18^{+} ± 0.01	0.17^{+} ± 0.02	0.16^{+} ± 0.03	0.15^{+} ± 0.04
DMSO	0.25 mL	0.11 ± 0.03	0.36 ± 0.06	0.44 ± 0.04	0.53 ± 0.07	0.64 ± 0.03	0.66 ± 0.04	0.70 ± 0.02	0.69 ± 0.03	0.67 ± 0.01

$^{*}P < 0.05$, $^{**}P < 0.025$, $^{+}P < 0.001$ versus physiological saline control, ANOVA ($n = 8$).
UR: untreated rats.
PS: physiological saline.
DMSO: dimethylsuloxide.

drug [3], significantly attenuated the thermal stimulation or nociception produced by the hot plate. *C. orbiculata* also significantly attenuated the nociception produced by hot plate. It is probable that the plant species may be acting via certain central pain receptors to attenuate the nociception produced by hot plate in this study. According to Koster et al. [9], Williamson et al. [10] and Eddy and Leimback [11], acetic acid writhing and hot plate tests are used to evaluate peripherally and centrally acting analgesic drugs respectively. In this study, *C. orbiculata* attenuated both the acetic acid-induced writhing and the nociception produced by hot plate which may suggest that the plant species may have both peripheral and central antinociceptive effect.

Swingle [17] has shown that prostaglandins, histamine, serotonin, and bradykinin are mediators of different phases of carrageenan-induced oedema. Di Rosa et al. [18], Capasso et al. [19], and Salvemini et al. [20] have also reported the involvement of histamine, 5-hydroxytrptamine, bradykinin, prostaglandin, and nitric oxide in carrageenan-induced paw oedema. Nag-Chaudhuri et al. [21] in their report on their work on the anti-inflammatory and related actions of *Syzygium cuminii* seed extract suggested that prostaglandin E_1, histamine, serotonin, and bradykinin mediate carrageenan-induced rat paw oedema. Indomethacin has been shown to produce its anti-inflammatory effect by inhibiting the enzyme, cyclooxygenase, thus inhibiting prostaglandin synthesis [22]. It has also been shown that the nonsteroidal anti-inflammatory drugs may antagonize mediators such as serotonin, bradykinin, and capsaicin [23] some of which have been implicated in carrageenan-induced paw oedema. It is not surprising that in this study, indomethacin attenuated carrageenan-induced rat right hind paw oedema. *C. orbiculata* also attenuated the carrageenan-induced rat right hind paw oedema which may suggest that probably, the plant species may be affecting a host of mediators to produce its anti-inflammatory effect.

Amabeoku et al. [8] have shown that the leaves of *C. orbiculata* contain tannins, saponins, triterpene steroid, reducing sugar, and cardiac glycosides. Bruneton [24] reported that saponins have both analgesic and anti-inflammatory properties. It is possible, therefore, that saponins may also be contributing to the antinociceptive and anti-inflammatory activities of *C. orbiculata* in this study. The HPLC fingerprint of the plant species obtained revealed major characteristic peaks at the following retention times

(minutes): 6.983, 10.521, 12.088, 12.838, and13.342. The acute toxicity test carried out showed that the LD_{50} value obtained for *C. orbiculata* could be greater than 4000 mg/kg (p.o.).

In conclusion, the data obtained show that *C. orbiculata* has both antinociceptive and anti-inflammatory activities which may be produced by the plant species inhibiting various chemical mediators including prostaglandins and bradykinin. The relatively high LD_{50} value of 4000 mg/kg (p.o.) obtained for the plant species shows that it may be safe in or nontoxic to mice. The result obtained justifies the use of the plant species by traditional medicine practitioners in South Africa for the treatment of painful conditions such as headache, earache, toothache, and inflammation. However, more studies are needed to further elucidate the mechanism of the antinociceptive and anti-inflammatory actions of *C. orbiculata*.

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Research Article

Anti-Inflammatory Activity of *Delonix regia* (Boj. Ex. Hook)

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The present work was to evaluate the anti-inflammatory activity of *Delonix regia* leaves (Family: Caesalpiniaceae). The powder of *Delonix regia* leaves was subjected to extraction with ethanol in soxhlet extractor. The ethanol extract after preliminary phytochemical investigation showed the presence of sterols, triterpenoids, phenolic compounds and flavonoids. The anti-inflammatory activity was studied using carrageenan-induced rat paw edema and cotton pellet granuloma at a three different doses (100, 200, and 400 mg/kg b.w. p.o.) of ethanol extract. The ethanol extract of *Delonix regia* leaves was exhibited significant anti-inflammatory activity at the dose of 400 mg/kg in both models when compared with control group. Indomethacin (10 mg/kg b.w. p.o) was also shown significant anti-inflammatory activity in both models.

1. Introduction

Inflammation is a series of pathological changes associated with local vascular reaction and cellular response, the living tissue, an injury insufficient to kill the tissue. This is distinguished from the wider problem of generalized reactions of the body. However, it is related to infection caused by microorganisms, and various pathological changes are associated with it [1]. Traditional medicines play an important role in health services around the globe. About three-quarters of the world population relies on plants and plant extracts for healthcare. The rational design of novel drugs from traditional medicine offers new prospects in modern healthcare. *Delonix regia* (Boj. Ex. Hook) (Family: Caesalpiniaceae) is a medium-sized tree found in greater parts of India. The decoction of the leaves is traditionally used in treating gastric problems, body pain, and rheumatic pains of joints [2, 3]. Ethanolic extracts of flower and bark were investigated to anti-inflammatory activity in rats [4]. The leaves are reported to antibacterial [5] and antimalarial [6]. *Delonix regia* contains proteins, flavonoids, tannins, phenolic compounds, glycosides, sterols, and triterpenoids. However, no data were found regarding the pharmacological and phytochemical evaluation of the leaves of the plant. The aim of the present study was to investigate the anti-inflammatory activity of the ethanol extract of the leaves of *Delonix regia* (Boj. Ex. Hook).

2. Materials and Methods

2.1. Plant Material. *Delonix regia* leaves were procured from the local market in Jalgaon, Maharashtra, India, identified, and authenticated. A voucher specimen (voucher specimen number-Vaishu 9200) was deposited in the herbarium of the Department of Botany, Rashtrasant Tukdoji Maharaj, Nagpur University, Nagpur.

2.2. Extraction of Plant Material. The leaves were dried under a shade and pulverized. The coarse powder (1000 g) was extracted with ethanol using a soxhlet apparatus. The extract was dried using a rotary vacuum evaporator and stored in

a desiccator until further use. The percentage yield of ethanol extract of *Delonix regia* (EEDR) was 25.0% w/w.

2.3. Phytochemical Screening. A preliminary phytochemical screening of *Delonix regia* was carried out [7]. The presence of alkaloid (Dragendorff reagent and Mayer's reagent), flavonoids (Shinoda test), steroids (Liberman Burchard test), and terpenes (Vanillin sulfuric acid reagent) were analyzed.

2.4. Drugs and Chemicals. Indomethacin was purchased from Themis Pharmaceutical Ltd. India. Carrageenan was purchased from Sigma Aldrich, USA. Tween 80 and other reagents of analytical grade were purchased from S. D. Fine Chem. Ltd, India.

2.5. Animals. Wistar albino rats (150–200 g) and mice (20–25 g) of either sex were purchased from Calcutta Fish Aquarium, Indore, India and were housed under standard conditions of temperature and light. Animals had free access to food (Amrut Feeds, Pune, India) and water. The Institutional Animal Ethics Committee approved the protocol of the study.

2.6. Acute Toxicity Studies. Healthy adult Swiss albino mice of either sex weighing between 20 and 25 g were subjected to acute toxicity studies as per guidelines (AOT no. 425) suggested by the Organization for Economic Cooperation and Development [8]. Groups of six mice each were administered orally graded doses ranging from 0.1 to 5 g/kg. The mice were observed continuously for 2 h for behavioral, neurological, and autonomic profiles for any lethality or death for the next 48 h.

2.7. Anti-Inflammatory Activity

2.7.1. Carrageenan-Induced Paw Edema. The anti-inflammatory activity of the extract was carried out using Wistar albino rats (150–200 g) of either sex [9, 10]. The rats were divided into five groups of six rats each. The control group received 1% (v/v) Tween 80 in water, p.o. at a dose of 10 mL/kg. The positive control group was treated orally with the standard drug, indomethacin (10 mg/kg). Ethanol extract was administered orally to the other groups in doses of 100, 200, and 400 mg/kg as shown in Table 1. All the suspensions were administered 30 min before the induction of oedema by administering 0.1 mL of 1% w/v carrageenan in saline [11, 12]. The degree of paw oedema of all the groups was measured using a plethysmometer at 0, 1, 3, 5, and 7 h after the administration of carrageenan to each group.

2.8. Cotton Pellet Granuloma. Two autoclaved cotton pellets weighing 10 ± 1 mg were implanted subcutaneously into both sides of the groin region of each rat. The rats were divided into five groups of six rats each. The control group received 1% (v/v) Tween 80 in water, p.o. at a dose of 10 mL/kg. The positive control group was treated orally with the standard drug, indomethacin (10 mg/kg). Ethanol extract was administered to the other groups in doses of 100, 200, and 400 mg/kg orally for 7 days. After 7 days, the animals were sacrificed, and the pellets together with the granuloma tissues were carefully removed, dried in an oven at 60°C, weighed, and compared with control [13].

2.9. Statistical Analysis. All values are expressed as Mean ± SEM. Statistical analysis was performed by one-way analysis of Variance (ANOVA), and individual comparisons of the group mean values were done using Dunnet's *t*-test, with the help of Graph Pad prism 4.0 software. The value of P lower than 0.05 was considered as significant (P is probability) [14, 15].

3. Results

3.1. Phytochemical Studies. The preliminary phytochemical studies of ethanol extract of *Delonix regia* was indicated the presence of sterols, triterpenoids, phenolic compounds, and flavonoids.

3.2. Acute Toxicity Studies. In acute oral toxicity study, mice given graded doses ranging from 0.1 to 5 g/kg appeared normal. *Delonix regia* was safe up to a dose level of 5000 mg/kg of body weight. No lethality or any toxic reactions were found up to the end of the study period.

3.3. Anti-inflammatory Activity

3.3.1. Carrageenan-Induced Paw Edema. The ethanol extract (400 mg/kg) significantly inhibited carrageenan-induced paw oedema. The ethanol extract produced a dose-dependent inhibition of carrageenan oedema which was comparable with known anti-inflammatory drugs. The ethanol extract of *Delonix regia* produced significant ($P < 0.01$) anti-inflammatory activity. Significant reduction of paw oedema was observed at 3 h after carrageenan injection. The reduction in carrageenan-induced paw oedema by 400 mg/kg of ethanol extract after 3 h was 48.1%, while oedema reduction by the standard drug, indomethacin (10 mg/kg), was 65.8% (Table 1).

3.3.2. Cotton Pellet Granuloma. The ethanol extract significantly inhibited cotton pellet granuloma. The percent inhibition of ethanol extract was 42.4% at dose of 400 mg/kg, and this inhibition was less than that produced by indomethacin (61.6%) (Table 2).

4. Discussion

Carrageenan-induced oedema of rat foot is used widely as a working model of inflammation in the search for new anti-inflammatory agents [16] and appeared to be the basis for the discovery of indomethacin, the anti-inflammatory drug [17]. The oedema which develops in rat paw after carrageenan injection is a biphasic event. The initial phase is attributed to the release of histamine and serotonin, the oedema maintained between the first and second phase to kinin, and

Table 1: Effect of ethanol extract of *Delonix regia* (100, 200, and 400 mg/kg) on paw volume in carrageenan-induced paw edema rats.

Treatment	Dose mg kg^{-1}	Carrageenan-induced rat paw edema Mean ± SEM*			
		+1 h	+3 h	+5 h	+7 h
Control	—	0.36 ± 0.06	0.79 ± 0.05	0.64 ± 0.04	0.68 ± 0.01
Indomethacin	10	0.18 ± 0.03[a]	0.27 ± 0.04 (65.82)[b]	0.29 ± 0.03 (54.68)[b]	0.33 ± 0.06 (51.47)[b]
EEDR	100	0.28 ± 0.02 (22.22)[NS]	0.46 ± 0.03 (41.77)[b]	0.39 ± 0.03 (39.06)[b]	0.41 ± 0.03 (35.93)[b]
EEDR	200	0.25 ± 0.01 (30.55)[NS]	0.43 ± 0.01 (45.56)[b]	0.38 ± 0.03 (40.62)[b]	0.39 ± 0.03 (42.64)[b]
EEDR	400	0.21 ± 0.02 (41.66)[NS]	0.41 ± 0.02 (48.10)[b]	0.37 ± 0.01 (42.18)[b]	0.4 ± 0.01 (41.17)[b]

*The number of animal was 6 in each group. Figure in parenthesis indicates percent inhibition in paw volume. The probability values were calculated using one way ANOVA followed by Dunnet's *t*-test: a < 0.05, b < 0.01, NS: not significant.

Table 2: Effect of ethanol extract of *Delonix regia* (100, 200 and 400 mg/kg) on cotton pellet granuloma in rats.

Treatment	Dose mg kg^{-1}	Cotton pellet granuloma Weight of pellets Mean ± SEM
Control	0.3 mL	51.73 ± 2.35
Indomethacin	10	19.82 ± 2.52 (61.68)[b]
EEDR	100	37.24 ± 2.00 (27.99)[NS]
EEDR	200	30.85 ± 3.72 (40.36)[b]
EEDR	400	29.77 ± 3.76 (42.44)[a]

*The number of animal was 6 in each group. Figure in parenthesis indicate percent inhibition in cotton pellet granuloma. The probability values were calculated using one way ANOVA followed by Dunnet's *t*-test: a < 0.05, b < 0.01, NS: not significant.

the second phase to prostaglandin [18]. All the mediators appear to be dependent upon an intact complement system for their activation and release [19]. It has been shown that, in the early phase of the oedema, the dominant cells are polymorphonuclears whereas in advanced stages mononuclears predominate. In this study, indomethacin (nonsteroidal) anti-inflammatory drug was tested on carrageenan oedema [20]. Phytochemical studies on *Delonix regia* revealed that it contains β-sitosterol, tannin, lupeol, and flavonoids [21]. Hentriacontane, hentriacontanol and it's D-glucoside, and campesterol were identified as constituents of *Delonix regia* [22].

Most of the anti-inflammatory triterpenes isolated have lupane, oleanane, ursane, and taraxastane. Lupeol and ursolic acid showed significant anti-inflammatory activity in various models [23, 24]. Lupeol has been reported to possess dose-dependent suppression of PGE_2 without any effect of LTC_4 release. Thus, ursolic acid and lupeol [25] were able to prevent the production of some inflammatory mediators which likely contributed to anti-inflammatory effect of *Delonix regia*. Jiang and Dusting have reported that phenolic compounds have potential role in inflammatory conditions [26]. Satya Prasad et al. have further shown that phenolics inhibit polymorphonuclear lipoxygenase, an enzyme involved in inflammatory conditions [27]. The cotton pellet granuloma method has been widely employed to assess the transductive, exudative, and proliferative components of chronic inflammation and is a typical feature of established chronic inflammatory reaction. The fluid absorbed by the pellet greatly influences the wet weight of the granuloma, and dry weight correlates well with the granuloma of the granulomatous tissue formed [28, 29]. Administration of ethanol extract at the doses of 400 mg/kg significantly reduced the granulomatous tissue formation when compared to control.

5. Conclusion

Preliminary phytochemical analysis indicated that the ethanol extract of *Delonix regia* contains sterols, triterpenoids, flavonoids, and phenolic compounds. The anti-inflammatory activity of ethanol extract of *Delonix regia* may probably be due to the presence of several bioactive anti-inflammatory principals. However, it needs isolation, structural elucidation, and screening of any of the above-mentioned active principle/s to pin point activity of drug. It is thus apparent that ethanol extract of *Delonix regia* possesses anti-inflammatory activity.

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