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Research Article

PROFILING OF BIOACTIVE COMPONENTS PRESENT IN *Ziziphus mauritiana* Lam FOR *IN-VITRO* ANTIOXIDANT AND *IN-VIVO* ANTI-INFLAMMATORY ACTIVITIES

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ABSTRACT

The aim of the present study is to analyze the methanol extract of *Ziziphus mauritiana* Lam leaves for its chemical composition using gas chromatography – mass spectroscopy (GC- MS) analysis correlating *in-vitro* anti-oxidant, *in vivo*-anti-inflammatory activity and to determine the total phenolic & flavonoid contents. The processed plant material was extracted using methanol. Phytochemical profiling of the extract revealed presence of flavonoids, terpenoids, alkaloids and glycosides. The antioxidant activity of the extracts was determined using 2,2'-Diphenyl-1-picrylhydrazylhydrate (DPPH), 2,2′-azino bis-3-ethylbenzothiazoline-6 sulfonic acid (ABTS) and reducing power assay. Anti-inflammatory activity was evaluated by cotton pellet granuloma in wistar rat model. Twenty three phytoconstituents identified using GC-MS analysis out of that nine were familiar with earlier reports on its pharmacological background. The IC_{50} values for the DPPH activity was 7.64µg, for ABTS activity 43.79 µg and for reducing power assay 26.35µg. The methanol extract of Z. *mauritiana* leaves showed a significant reduction ($p < 0.001$) in the cotton pellet granuloma in a dose-dependent with a maximum that reaches at 500 mg/kg. Hence, flavonoids and phenolic compounds were associated with the antioxidant and anti-inflammatory activities observed for *Z. mauritiana*. The effect of the extract might be due to the presence of active phytomolecules such as n – hexadecanoic acid and L-(+)-ascorbic acid 2, 6-dihexadecanoate. From these results it was clearly evident that the phenolic and flavonoid compounds from Z. *mauritiana* leaves are responsible for anti-inflammatory and antioxidant activities.

Keywords: *Ziziphus. mauritiana*, anti-inflammatory, terpenoids, alkaloids glycosides & flavonoids.

INTRODUCTION

Drugs from natural origin play a significant role in the public health care system of the nation [1]. Plants are regarded as molecular factory, as they have capacity to synthesize enormous diversity of metabolites termed as bioactive compounds [2]. Plants contain a variety of phyto pharmaceuticals, which are found to possess important applications in the field of agriculture, human and veterinary medicine. Population raise, demand for drugs, increase in the cost of treatment, side effects of drugs and resistance formation of drugs are the main reasons that leads to pay more attention for plant based herbal medicines for a variety of human disorders [3].It is quite interesting to notice that the developed countries primarily used modern medicines, but as a result of side effects of these drugs people turned their minds towards phyto medicines and medicinal plants in the recent era. Currently, traditional herbal medicine has been widely used not only in the developing countries but also in the industrialized world as an alternative [4]. Many scientists and researchers have compensated attention to the crude extracts and active pure compounds isolated from plants species used in herbal and traditional remedies [5]. Nearly about 80% of the world's populations still depend upon traditional remedies together with folklore system mainly based on phytotherapy. Its efficacy, safety, lesser cost-factor and almost nil adverse effects have attracted the attention of economically developed and developing countries. The ethno botanical use of medicinal plants was transmitted from generation to generation. Intake of edible medicinal plants as a source of diet has been increased in the recent era due to the presence of important health components [6].

Ziziphus mauritiana Lam. is an evergreen shrub or trees distributed throughout low latitudes of warm, tropical and subtropical regions including Asia, Africa and Australia. The genus *Ziziphus* (Rhamnaceae) comprises of approximately 170 species and is importantly used in the treatment of various diseases. The ripe fruits of *Z. mauritiana* are mostly consumed raw and the leaves are cooked and eaten in Indonesia [7]. Hence, the present study was aimed to standardize and to identify the phyto-constituents present in the methanol extract of Z. *mauritiana* Lam leaves by GC-MS and to perform *in-vitro* antioxidant and *in-vivo* anti-inflammatory activities.

MATERIALS AND METHODS

Chemicals

Gallic acid was purchased from SD fine Chemicals, India. 2, 2'-Diphenyl-1-picrylhydrazylhydrate (DPPH) was purchased from Hi media. Folin-ciocalteu reagent, trichloroacetic acid, was purchased from Qualigens fine chemicals, India. 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), N-(1-Naphthyl) ethylenediamine dihydrochloride and 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphoric acid and potassium ferricyanide were from Merck, USA. All chemicals and solvents used in this study were of analytical grade.

Collection of plant materials

The fresh leaves of the *Ziziphus mauritiana* Lam was collected from wild source in the month of August 2013. The plant was identified and authenticated by Dr. G. V. S. Murthy, Join Director, Botanical Survey of India, TNAU campus, Coimbatore. A voucher specimen was preserved in our institution for further references.

Extraction of plant materials

The coarsely powdered leaves were extracted with methanol to obtain the crude methanol extract of *Z. mauritiana* leaves. The methanol extract was concentrated to dryness under reduced pressure in a rotary vacuum yielding a dry residue (yield 9.47% w/v). The extracted residue was subjected to preliminary phytochemical screening for the profiling of various plant constituents using standard methods.

Phytochemical screening of leaves extract

The freshly prepared crude methanol extract of leaves of *Z. mauritiana* was subjected to qualitative chemical tests to identify various classes of bioactive chemical constituents present in the leaves using standard procedures for the presence of alkaloids, tannins, glycosides, flavonoids, phenols and saponins (Trease and Evanns 2002).

GC-MS analysis

The methanol extract from the leaves of *Z. mauritiana* were analyzed using a Perkin Elmer GC–MS equipped with a fused silica capillary column (30 m × 0.25 i.d, film thickness 0.25m) coupled with a Perkin Elmer Clarus 600C MS. An electron ionization system with ionization energy 70 eV was used for the detection of compounds. Inert gas helium was used as a carrier gas at constant flow rate of 1 ml/min. Mass transfer line and injector temperatures were set at 220 and 300°C, respectively. The oven temperature was programmed started from 50 to 150°C at 3°C/min, then held for 10 min and finally rose to 300°C at 10°C/min. The crude samples were diluted with appropriate solvent (1/100, v/v) and filtered. The particle-free diluted crude extract was injected with split mode. The split ratio was of 1:120.

Identification of components

Interpretation on mass spectrum GC-MS was conducted using data base of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of unknown components was compared with the spectrum of known compounds stored in NIST library. The name, molecular weight and structure of the components of the test materials were tabulated.

Determination of total phenol content

Total phenol content in the plant extract was determined by Folin-Ciocalteu method [8]. Briefly, 0.1 ml of plant extract was mixed with 0.5 ml of distilled water followed by the addition of 0.25 ml of Folin - Ciocalteu phenol reagent and allowed to stand for 6 min. Then, 0.75 ml of 20% of sodium carbonate solution was added and the final volume was made up to 3.5 ml with distilled water and the absorbance was read at 765 nm. Total phenol content of plant extract was expressed as gallic acid equivalents (mg of GAE /g of plant extract).

Determination of total flavonoid content

Total flavonoid content was analyzed using modified calorimetric method [9]. Briefly, 0.5 ml of plant extract was mixed with 0.9 ml of distilled water and 1 ml of aluminum chloride solution. The reaction mixture was allowed to stand for 1 h at room temperature and the formation of yellow color indicates the presence of flavonoid and read at 420 nm. Total flavonoid content of plant extract was calculated as rutin equivalents (mg of RE /g of plant extract).

***In-vitro* antioxidant activity**
DPPH radical scavenging assay

DPPH radical scavenging activity was estimated by method described by Liana-Pathirana and Shahidi [10]. 1ml of DPPH solution (0.135 mM) was mixed with different concentrations of plant extract and kept in dark at room temperature for 30 min. Finally, the absorbance was measured at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation.

$$\text{Percentage of inhibition} = [(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}/\text{Standard})] / (\text{Abs}_{\text{Control}})] \times 100$$

The final result was expressed as an IC_{50} value (the concentration of sample producing 50% inhibition of the DPPH free radicals; µg/ml).

ABTS radical scavenging assay

ABTS radical scavenging assay was performed as described by Re et al [11]. ABTS stock solution (7 mM ABTS and 2.4 mM potassium persulphate) was prepared. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12h at room temperature in the dark. The plant extract were allowed to react with 1 ml of ABTS working solution for 7 min and absorbance was measured at 734 nm. The percent of ABTS free radical scavenging inhibition capacity of the extract was calculated from the following equation:

$$\text{Percentage of inhibition} = [(\text{Abs control} - \text{Abs sample}/\text{standard})] / (\text{Abs control})] \times 100$$

The final result was expressed as an IC_{50} value (the concentration of sample producing 50% inhibition of the ABTS free radicals; µg/ml).

Ferric reducing power assay

Ferric reducing power of the sample was measured according to the method of Oyaizu [12]. The sample with different concentrations was added with 2.5 ml of sodium phosphate buffer followed by addition of 2.5 ml of 1% potassium ferricyanide. The reaction mixture was vortexed well and incubated at 50°C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. To 5 ml of the supernatant, 5 ml of deionized water was added with 1 ml of 1% ferric chloride and incubated at 35°C for 10 min. The absorbance was read at 700 nm.

***In-vivo* anti-inflammatory activity**

The method was adopted with slight modification described by Winter and Porter [13]. Four groups of five animals in each group were taken and anaesthetized with ketamine and xylazine cocktail. The extracts and the standard drug were administered

orally 30min prior to the cotton pellet implantation. After anesthesia, small incision was made subcutaneously and sterile cotton pellets weighing 50mg was implanted into the dorsal region of the rats. After implantation, test drug (aspirin 100mg/kg), methanol extract of *Z. mauritiana* leaves 250 and 500mg/kg, were administered orally for 7 consecutive days. On 8th day rats were euthanized and the cotton pellet (along with granular tissue) was removed. Blood was collected by retro-orbital plexus for biochemical estimation. Serum was separated for SGOT, SGPT and ALP analysis using standard kits.

RESULTS

The results of the preliminary phytochemical analysis confirmed presence of triterpenoids, steroids, flavonoids, tannins, phytosterols and carbohydrate in the methanol extract of *Z mauritiana* leaves (Table 1). This study showed that total phenolics content of leaf as 2.78 ± 0.018 mg of GAE/g, total flavonoid content of leaf extract was found to be 62.38 ± 0.003 mg of RE/g (Table 2).

In-vitro antioxidant activity of methanol extract of *Z. mauritiana* leaves was represented in figure 1. The DPPH scavenging activity of methanol extract of *Z. mauritiana* leaves exhibited significant radical scavenging activity with increasing concentration of extracts. The calculated IC_{50} values for all the methods were tabulated (Table 2). The calculated IC_{50} was found to be 38.07µg/ml for DPPH method. However ascorbic acid was used as standard and its radical scavenging activity was found to be 7.64µg/ml. Similarly, in ABTS method the methanol extract of *Z. mauritiana* leaves was found to be effective in scavenging radicals with increase was concentration of the extracts. At the dose of 50µg/ml it showed the maximum protection of 99.11%. The calculated IC_{50} was found to be 19.8µg/ml for ABTS scavenging activity. The higher absorbance shows greater reducing power of the plant. Methanol extract of *Z. mauritiana* leaves showed concentration-dependent reducing power. The maximum protection of 57.81% was observed in 100µg/ml. The calculated IC_{50} was found to be 90.70µg/ml. GC-MS chromatogram of compounds identified from the *Z. mauritiana* Lam leaves was illustrated in figure 2. The active principles were confirmed with their retention time (RT), molecular formula and the molecular weight (MW) with reference from previous literatures (Table 3). Major compounds identified in methanol extract were trehalose, tetradecanoic acid, n – hexa decanoic acid (Palmitic acid), Z-2-Dodecenol, L-(+)-ascorbic acid 2, 6-dihexadecanoate, vitamin E and squalene. The results of the cotton pettet granuloma in rats were illustrated in figure 3. These results revealed that the methanol extract of *Z. mauritiana* leaves shows dose dependent inhibition of weight of cotton pellets. The extract significantly ($p < 0.001$) decreases the weight of cotton pellets in rats which received 500mg/kg. The extract at the dose of 500mg/kg produced 31.1% protection to the inflammation compared to the 16.9% in 250mg/kg. The standard drug produced`44.9% protection to the inflammation. Similarly, there was significant ($p < 0.001$) decrease in the levels of SGPT, SGOT and ALP was observed in the treated groups compared to the control group in cotton pellet granuloma model.

Table 1: Preliminary phytochemical analysis of methanol extract of *Ziziphus mauritiana* lam leaves

Test	Methanol Extract
Carbohydrates	+
Proteins & amino acids	-
Glycosides	-
Alkaloids	-
Phytosterols	+
Flavonoids	+
Saponins	+
Tannins & phenolic compounds	+

Table 2: IC_{50} for i*n-vitro* antioxidant assay of methanol extract of *Ziziphus mauritiana* lam leaves

S. No	Method	IC_{50} Value
1	DPPH method	38.07µg/ml
2	ABTS method	19.8µg/ml
3	Reducing power assay	90.70 µg/ml

Table 3: GC-MS Spectral analysis of methanol extract of *Ziziphus mauritiana* lam leaves

S. No.	RT min	Name of the compound	Molecular formula	Molecular weight
1.	4.02	1H-Azonine	$C_8H_{17}N$	127
2.	4.80	3-Acetylthymine	$C_7H_8N_2O_3$	168
3.	5.40	4-Methylpiperidine-1-carboxylic acid, phenyl ester	$C_{13}H_{17}NO_2$	219
4.	5.51	2-Aziridinone,1-ert-butyl-3-(1-methylcyclohexyl)-	$C_{13}H_{23}NO$	209
5.	5.92	Oxirane,[(dodecyloxy)methyl]-	$C_{15}H_{30}O_2$	242
6.	8.68	2-Pentyne,5-methoxy-	$C_6H_{10}O$	98
7.	12.75	Dodecanoic acid	$C_{12}H_{24}O_2$	200
8.	13.31	Z-2-Dodecenol	$C_{12}H_{24}O$	184
9.	15.95	Phthalic acid, butyl undecyl ester	$C_{23}H_{36}O_4$	376
10.	13.81	Octadecen-1-ol	$C_{18}H_{36}O$	268
11.	18.73	α-D-Glucopyranoside,O-α-D-glucopyranosyl-	$C_{18}H_{32}O_{16}$	504
12.	19.65	Heptadecanoic acid	$C_{17}H_{34}O_2$	270
13.	23.13	Cyclododecanol	$C_{12}H_{24}O$	184
14.	20.89	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270
15.	27.14	L-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652
16.	28.73	Tetradecanoic acid	$C_{14}H_{28}O_2$	228
17.	29.36	7-Hexadecenoic acid, methyl eser,(Z)-	$C_{17}H_{32}O_2$	268
18.	30.80	Oleic acid	$C_{18}H_{34}O_2$	282
19.	31.85	Vitamin E	$C_{29}H_{50}O_2$	430
20.	32.87	Squalene	$C_{30}H_{50}$	410
21.	34.59	E-2-Tetradecen-1-ol	$C_{14}H_{28}O$	212
22.	36.30	n- Hexadecanoic acid	$C_{16}H_{30}O_4$	286
23.	36.81	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{30}H_{50}O_4$	474

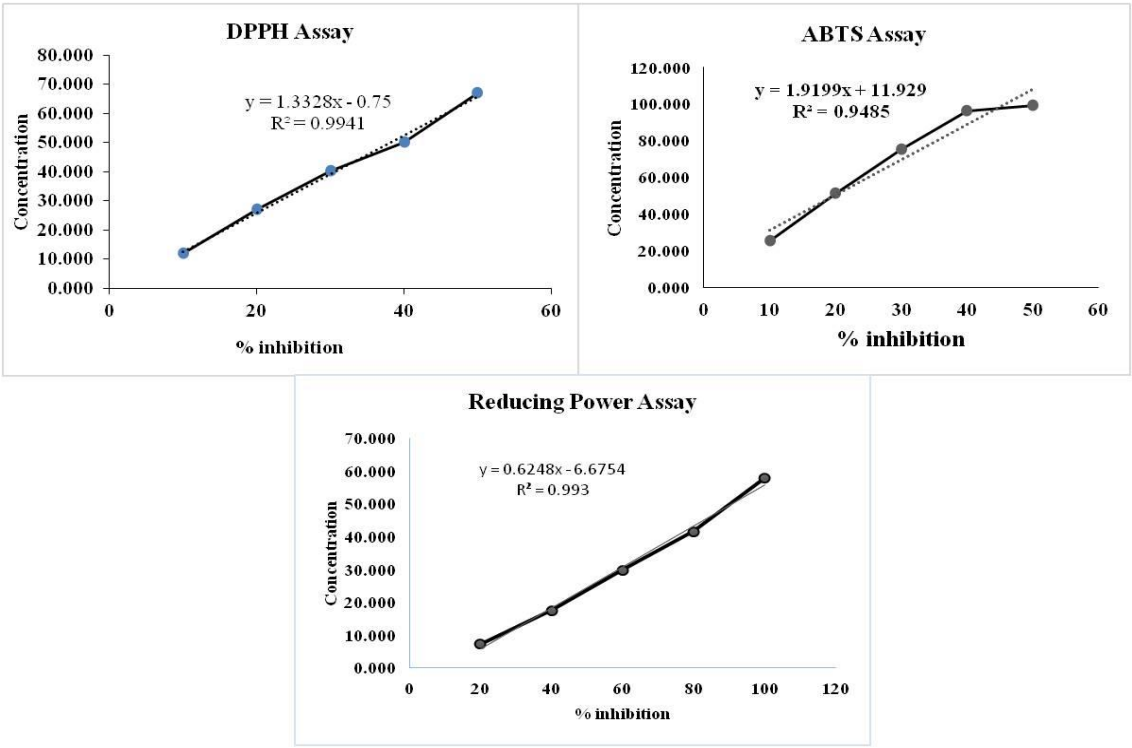

Figure 1: *In-vitro* antioxidant activity of methanol extract of *Ziziphus mauritiana* lam leaves

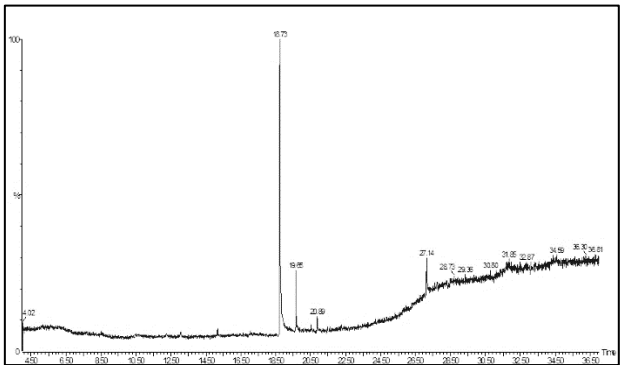

Figure 2: GC-MS chromatogram of methanol extract of *Ziziphus mauritiana* lam leaves

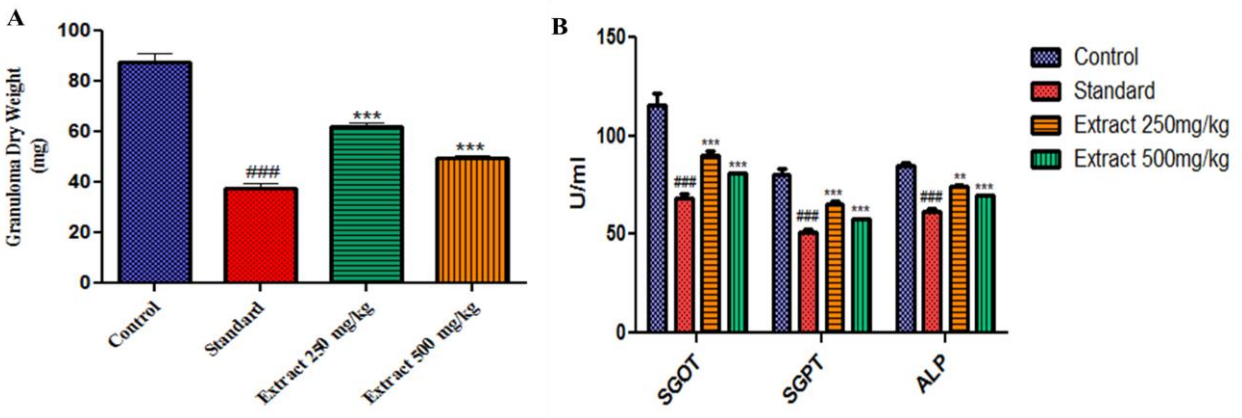

Figure 3: A) Effect of of methanol extract of *Ziziphus mauritiana* lam leaves cotton-pellet induced granuloma in rats B) Effect of methanol extract of *Ziziphus mauritiana* lam leaves on various biochemical changes in cotton-pellet induced granuloma in rats

DISCUSSION

Natural products are a primary source for bioactive compounds which have potential to develop novel therapeutic agents [14]. This preliminary phytochemical screening may be suitable for the proper identification of bioactive principles from plants which may lead to the drug discovery and development [15]. The results of the preliminary phytochemical analysis confirmed the various phytochemicals such as triterpenoids, steroids, flavonoids, tannins, phytosterols and carbohydrate in the methanol extract of *Z mauritiana* leaves. Phenolic compounds like flavonoids, phenolic acids and tannins are the major contributors of the antioxidant capacity of plants. Diverse biological activities such as anti-inflammatory, anti-atherosclerotic and anti-carcinogenic activities are documented by these plant derived compounds [16]. Earlier reports suggested that tannins have general antimicrobial and antioxidant activities. Similarly, saponins were found to possess antifungal properties. It is used as anti-hypercholesterolemia, anti-hyperglycemia, antioxidant, anticancer, anti-inflammatory and weight loss [17]. Therefore, the chemical constituents present in *Z. mauritiana* leaves can be used as a lead compound for the treatment of various diseases.

Pants enriched with phenols, flavonoids and carotenoids exhibited high antioxidant capacity due to their redox properties [18]. The methanol extract of *Z. mauritiana* leaves exhibit strong antioxidant activity in all the assays investigated. Flavonoids effectively scavenge most of oxidizing agents and other free radicals involved in several diseases. Total phenols and flavonoids possess diverse chemical and biological activities including radical scavenging properties due to cleaving complex chemical structures. Many disorders like neurodegeneration, cancer and AIDS attributed through free radicals [19]. Antioxidants are useful for the management of those diseases due to their scavenging activity. DPPH assay is a sensitive way to analyze the antioxidant activity of plant extracts. The excess electrons in DPPH radicals are paired by suitable reducing agents to form subsequent hydrazine [20]. Super oxide anion is one of the biologically important ROS radical as it forms singlet oxygen and hydroxyl radical. Elevation of super oxide anion radical in biological system leads to redox imbalance and associated with destructive physiological functions [21]. Similarly, nitric oxide was synthesized by endothelial cells, macrophages, neurons act as a potent oxidant in many physiological functions. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite radicals. Excess nitric oxide levels were observed in various disorders like AIDS, cancer, alzheimer's and arthritis [22]. According to our study, the high phenolic content in *Z. mauritiana* can explain its high free radical scavenging activity.

Gas Chromatography-Mass Spectrometry (GC-MS) is one of the valuable tools for identifying the phyto compounds. The initial step in investigating the metabolites of any medicinal plant is phytochemical screening which gives a broad idea on the nature of chemical constituent's presents [23]. In the present study nine major compounds were identified from the methanol extract of leaves of *Z. mauritiana* by Gas chromatography– Mass spectrometry (GC-MS) analysis. Earlier research showed that trehalose a disaccharide (α - D-Glucopyranoside, O-α – D glucopyranosyl) has protective effect on osteoporosis. Moreover, trehalose was also found to acquire suppressive effect on the development of osteoporosis [24]. n-hexadecanoic acid revealed cytotoxicity to human leukemic cells, MOLT-4 and also showed *in-vivo* antitumor activity in mice [25]. Furthermore, it also has a vital role in antioxidant, hypocholesterolemic, anti-inflammatory, nematicide, pesticide, lubricant, anti-androgenic and flavoring agent [26]. Natural ascorbic acid helps in the growth and repair of tissues in all parts of the body and helps the body to absorb iron from non heme sources. It is required for connective metabolism especially in the tissue, bones and teeth [27]. L-(+)-ascorbic acid 2, 6-dihexadecanoate is scientifically reported as wound healer, antinociceptive, anti-inflammatory, antioxidant and hyaluronidase inhibitors [28]. The presence of squalene is an antioxidant agent and possesses chemo preventive activity against colon carcinogenesis. It is also used as anti-bacterial, antitumor, cancer preventive, immunostimulant, chemo preventive, lipoxygenase-inhibitor and pesticide.

Cotton pellet granuloma method was one of the sub-acute models to screen inflammation. The transudative, exudative and proliferation phases of inflammation phases were analyzed using the cotton pellet granuloma method [29]. The inflammation mechanism initiates from the proliferation of fibroblasts generation of small blood vessels surrounding the granuloma. Kinine, an important mediator of granuloma produced both vasodilation and increase vascular permeability which indicates early signs of inflammation. The ability of the extract to inhibit the formation of fibroblasts surrounding the granuloma was considered to have effective anti- inflammatory property [30]. Since the methanol extract of *Z. mauritiana* leaves inhibits the fibroblast formation in a dose dependent manner, which reduced the number of new fibroblasts and inhibits the collagen, mucopolysaccharide formation in the granuloma, was considered the potent anti-inflammatory property. Free radicals may provoke inflammation which sequestered by antioxidants to attenuate inflammation. The plant is supposed have the phytoconstituents like L-(+)-ascorbic acid 2, 6-dihexadecanoate, n-hexadecanoic acid, which inhibits cyclooxygenase enzyme for producing inflammation peripherally to produce anti-inflammatory effect. Thus, the result of the present study supports the traditional use of *Z. mauritiana* leaves in the treatment of inflammation and related diseases. However, further studies are needed on the isolation and identification of individual phytochemical constituents from the plant extract related to pharmacological and toxicity aspects will give fruitful social benefit to promote healthy living.

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