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The Essential Oil Constituents of *Zornia diphylla* (L.) Pers, and Anti-Inflammatory and Antimicrobial Activities of the Oil

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Abstract: The essential oil obtained from hydrodistillation of *Zornia diphylla* (L.) Pers (whole plant) was analyzed by gas chromatographic techniques. Thirty seven compounds comprising 96.3% of the oil were identified by GC-MS. Monoterpenoids predominated the oil (83.9%) and the major constituent was sabinene (43.1%) followed by terpinene 4-ol (13.2%). The oil was screened for antibacterial, antifungal, anti-inflammatory and cytotoxic activities. Sabinene was tested for its anti-microbial property. The essential oil showed significant *in vitro* antibacterial activity against *Salmonella typhi*, one of the causative organisms of food poisoning. Sabinene exhibited promising anti-bacterial and anti-fungal activities. The essential oil showed anti-inflammatory activity against carageennan-induced paw edema in rats. The oil (50 µg/mL) was devoid of any significant *in vitro* cytotoxicity to thymocytes, macrophages and Dalton's lympohoma ascitis cells. This is the first report on the essential oil constituents of *Zornia diphylla* (L.) Pers.

Keywords: Essential oil; *Zornia diphylla*; anti-inflammatory; antimicrobial; cytotoxicity.

1. Introduction

Essential oil has been in use since ancient times as food and drink flavors, perfumes, deodorants, disinfectants and medicines. Essential oils with a wide range of biological use have been reported such as anti-malarial, insect repellent, anti-cancer, anti-oxidant, anti-inflammatory and cytotoxic [1-6]. In recent years, food safety researchers, food regulatory authorities and food processors are increasingly concerned with food-borne disease outbreaks caused by microbes [7, 8]. The most common type of food poisoning is of bacterial origin. Numerous studies have been published on the antimicrobial activities of essential oil against many different types of microbes, including food-borne pathogens [9, 10]. The main constituents of essential oils are responsible, to a large extent, for the fragrance and anti-microbial activities of aromatic medicinal plants. Due to these properties, spices and herbs are added to food, not only as flavoring agents but also as preservatives [11]. The use of less toxic natural preservatives and flavoring agents must be promoted for safer and healthy food. Many plants used in folk and tribal medicine in Kerala (India) to prevent as well as treat various infections, are not known to the main stream medical practitioners, one such plant is *Zornia diphylla* (L.) Pers belonging to the family Fabaceae. The traditional use of this plant has been mentioned in the ancient repository of Malabar medicinal plants, Hortus Malabaricus, for the treatment of dysentery and venereal diseases [12-14]. Recently the biological activities of *n*-hexane, alcohol and water extracts were evaluated in our laboratory. The *n*-hexane extract showed anti-fungal

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activity and a promising anti-fungal fraction was isolated from the hexane extract [15]. The n-hexane extract also showed promising anti-cancer activity; a steroid positive anti-cancer compound was isolated from the anti-cancer fraction (which is different from the anti-fungal fraction isolated from the *n*-hexane extract) [16]. The anti-cancer fraction induced apoptosis to DLA cells and protected mice challenged with lethal dose of DLA cells [16]. The essential oil constituents of this plant have not been studied and the plant possesses a characteristic smell that prompted us to study the essential oil of this plant. An attempt was made in the present study to identify the constituents of essential oil and evaluate its antibacterial, antifungal, anti-inflammatory and cytotoxic activities. The anti-microbial properties of the major constituent of the oil, sabinene are also reported here.

2. Materials and Methods

2.1. Plant material

Fresh whole plant was collected during May - June, 2009 from the coastal area of Thiruvananthapuram District, Kerala (India) and was identified by Dr. T. Sabu and Prof. N. Ravi, the taxonomists of Tropical Botanic Garden and Research Institute (TBGRI). A voucher specimen (No. 50976) has been deposited in the herbarium of TBGRI.

2.2. Chemicals and reagents

RPMI-1640 medium, phosphate buffered saline (PBS), trypan blue, streptomycin, fluconazole and ketoconazole were purchased from Himedia, India Limited. All other chemicals and reagents used were analytical grade and purchased from E. Merck India Ltd.

2.3. Essential oil

The essential oil was isolated from fresh whole plant (500 g) by hydrodistillation for 4 h on a Clevenger-type apparatus according to the method recommended in the current European Pharmacopoeia [17]. The oil was dried over anhydrous sodium sulphate and stored under nitrogen at 4^{o}C. The isolation was done 10 times (500 g X 10) to get about 0.4 mL oil from 5 kg plant material.

2.4. GC/FID analysis

The analysis was carried out by splitless injection of 0.1 µL of isolated volatile oil into a Varian CP-3800 (Varian Inc., USA), gas chromatograph equipped with a flame ionization detector (FID) and a CP Sil 8CB fused silica capillary column (30 m, 0.32 mm i.d., 0.25 µm film thickness). Nitrogen was used as the carrier gas at a flow rate of 1 mL/min. Oven programme: injector temperature 220^{o}C, oven temperature 60-246^{o}C at 3^{o}C/min, detector temperature 250^{o}C. Relative percentages of individual components of the oil were obtained from the GC-FID peak area-percent report.

2.5. GC/MS analysis

The analysis was done on a Hewlett Packard 6890 gas chromatograph fitted with a cross-linked 5% PH ME siloxane HP-5 MS capillary column (30 m x 0.32 mm, 0.25 µm film thickness) coupled with a 5973 series mass selective detector with splitless injection of 1.0 µL of essential oil, helium as the carrier gas at 1.4 mL/ min constant flow mode. The temperature program for the analysis was, injector temperature 220^{o}C and oven temperature 60^{o}C to 246^{o}C (3^{o}C/ min). The mass spectrum was electron impact (EI^{+}) mode at 70eV and ion source temperature 250^{o}C. The essential oil components were identified by MS library search (Wiley 2.75), retention times, relative retention indices (RRI) [18] calculated using homologues of *n*-alkanes as standards and by literature references [19, 20].

2.6. Isolation of sabinene from the essential oil

Separation of sabinene from essential oil isolated from *Z. diphylla* was done using preparative chromatography. Briefly 10 µL of essential oil was spotted in a readymade silica gel 60 F_{254} (Merck) plate and developed with pure *n*-hexane as solvent system and individual component bands were derivatized with anisaldehyde-sulphuric acid spray reagent. The essential oil was subjected to preparative silica gel-G (20 x 20 cm plates) thin layer chromatography (TLC) using pure *n*-hexane as solvent system. After separation, individual bands in TLC plate were visualized by spraying with

anysaldehyde-sulphuric acid spray reagent masking major portion of preparative TLC plate followed by heating at 80 ^{0}C. The silica gel corresponded to sabinene was carefully separated and eluted with chloroform, filtered and dried free of solvent using a rotary evaporator [21]. The sabinene thus obtained was tested for its antifungal activity.

2.7. Pharmacological screening

2.7.1. Animals

Inbred Swiss albino mice (25 to 30 g weight) reared at TBGRI animal house were used. Animals were caged in uniform hygienic conditions and fed with standard pellet diet (Lipton, India Laboratories, Bangalore) and water *ad libitum.* Animals were maintained under standard laboratory conditions as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Animal experiments were approved by the Institute Animal Ethics Committee (IAEC).

2.7.2. Determination of antimicrobial activity

2.7.2.1. Bacterial and fungal strains

Gram-positive bacteria, *Staphylococcus aureus* (MTCC 96), *Bacillus cereus* (MTCC 430), *Bacillus subtilis* (MTCC 441), Gram-negative bacteria, *Serratia marcescens* (MTCC 97), *Pseudomonas aeruginosa* (MTCC 741), *Klebsiella pneumoniae* (MTCC 109), *Proteus vulgaris* (MTCC 426), *Escherichia coli* (MTCC 443), *Salmonella typhi* (MTCC733) and the fungi, *Candida albicans* (MTCC 227), *Fusarium oxysporum* (MTCC 284), *Aspergillus fumigatus* (MTCC 343), *Aspergillus niger* (MTCC 1344) and *Trychophyton rubrum* (MTCC 296) were obtained as the Microbial Type Culture Collection (MTCC) from the Institute of Microbial Technology, Chandigarh, India.

2.7.2.2. Antimicrobial screening

The antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) and the minimum microbicidal concentration (MMC) using the broth dilution method [22]. In order to facilitate the dispersion in the aqueous nutrient medium, the oil was diluted (1:1) with 5% Tween 80 in broth and sonicated to confirm uniform mixing. Each strain was tested with sample that was serially diluted in broth to obtain concentrations ranging from 2 to 100 µg/mL. The sample, previously sterilized with Millipore filter of 0.20 µm, was stirred and inoculated with 50 µL of suspension of the tested microorganisms, containing 5 X 10^6 CFU/ mL for bacteria and 5 x 10^6 CFU/ml spore for fungal strains, and incubated for 24 h at 37^{0}C for bacteria, and 48 h at 30^{0}C for fungi. The MIC value was determined as the lowest concentration of the sample at which the tested microorganisms did not show any visible growth after incubation. Controls contained only 5 % Tween 80 instead of the essential oil. Streptomycin and fluconazole were used as standards. The MMC is defined as the lowest concentration of the essential oil at which inoculated microorganisms were completely killed [23-25].

2.7.3. Anti inflammatory activity

Anti-inflammatory activity of the essential oil isolated from *Z. diphylla* was evaluated by carrageenan-induced paw edema in mice [26]. Briefly male mice were divided into eight groups of six animals each. Inflammation of the hind paw was induced by injecting 0.1 mL of 1% (w/v) fresh carrageenan into the sub-plantar surface of the right hind paw. Animals in the p.o. group were pretreated with drugs 30 min before injection of carrageenan, while animals in external drug application group received the drug externally immediately after carrageenan injection. The essential oil was diluted with purified coconut oil for p.o. and external route of administration. Among the two control group animals, one group was treated with coconut oil (0.5 mL/animal), p.o. and the other group of control animals were treated with coconut oil (50 µL/paw) externally. Among the two positive control groups of animals, one group of animals was treated with indomethacin at a dose of 10 mg/kg, p.o. and the other group was treated with 1% w/w diclofenac sodium gel externally. Animals in the two p.o. test groups were treated with essential oil at a dose of 5 % (0.5 mL/animal), and 10 % (0.5 ml/animal), p.o. and for the other two test groups, 5 % v/v (50 µL/paw) and 10 % v/v (50 µL/paw)

essential oil in coconut oil was applied externally. Measurement of foot volumes were accomplished by displacement technique using a plethysmometer (Ugo Basile plethysmometer, Italy), immediately before and at every hour interval. Inhibition percentage of the inflammatory reaction was determined for each animal by comparison with controls and calculated using the data taken at 3 h after carrageenan injection. It is calculated with the formula,

$$\text{inhibition (\% of control)} = \left[\frac{(dc-dt)}{dc}\right] X\ 100$$

where dt is the difference in paw volume in the drug-treated group and dc is the difference in paw volume in the control group

2.7.4. Determination of cytotoxicity

2.7.4.1. Cell Lines

Dalton's Lymphoma ascitis (DLA) cells, originally obtained from Amala Cancer Research Centre, Trissur, India were maintained as transplantable tumors in the peritoneal cavity of mice.

2.7.4.2. Collection of thymocytes

Mice were sacrificed by cervical dislocation and thymus glands were carefully separated without adjoining lymph nodes. The separated thymus glands were transferred to RPMI-1640 medium and single cell suspension of thymocytes was prepared. Viability was assessed by Trypan blue exclusion method using a Neubauer counting chamber.

2.7.4.3. Collection of peritoneal macrophages

Mice were sacrificed by cervical dislocation and immediately injected with 5 mL chilled RPMI-1640 medium to the peritoneal cavity and peritoneal exudates cells (PEC) were collected. The glass adherent cell population (macrophages) was separated and viability was assessed by Trypan blue exclusion method using a Neubauer counting chamber [27].

2.7.4.4. In vitro cell viability assay: Trypan blue exclusion method

Effect of different concentrations of essential oil on short term cell viability was assessed by incubating 1 X 10^6 DLA cells or peritoneal macrophages or thymocytes in 1 mL phosphate buffered saline (PBS) containing vehicle (0.1% DMSO) or different concentrations of essential oil (25 and 50 µg/ml) or curcumin (50 µg/mL) for 3 h in a CO_2 incubator at 37^0 C, 5% CO_2, 95% air and 95 % relative humidity. The cell viability was assessed by Trypan blue exclusion method [27].

2.7.5. Statistical analysis

The data were expressed as mean ± standard deviation, statistical analysis was performed by one way ANOVA followed by Tukey-Kramer multiple comparison test, p values <0.05 were considered as significant.

3. Results and Discussion

3.1. Chemical composition of the essential oil

The light greenish yellow volatile oil of *Zornia diphylla* was obtained at a yield of 0.08% (v/w) (fresh weight basis) by hydrodistillation method. The oil obtained was clear with a good smell. A total of 37 constituents comprising 96.3% were characterized from the essential oil. In Table 1, the retention indices, amount in percentage and identification methods are given. The components are listed in order of elution on a HP 5MS column. Monoterpenoids predominated the oil (83.9%) with 67.0% hydrogenated monoterpenes and 16.9% oxygenated monoterpenoids. Sabinene (43.1%) and terpinene 4-ol (13.2%) were the major compounds.

Table 1. Chemical composition of *Z. diphylla* essential oil

RRI[a]	RRI[b]	Component	Identification[c]	%
Monoterpene hydrocarbons				
917	921	Tricyclene	1:2	0.2
919	924	α- Thujene	1:2	0.5
928	932	α- Pinene	1:2	1.5
962	969	Sabinene	1:2:3	43.1
970	974	β- Pinene	1:2:3	4.7
995	1001	δ-2-Carene	1:2	3.7
1011	1014	α- Terpinene	1:2	0.8
1020	1025	β- Phellandrene	1:2	1.4
1040	1044	*trans*-β-ocimene	1:2	2.2
1049	1054	γ- Terpinene	1:2:3	7.3
1081	1086	Terpinolene	1:2	1.6
Oxygenated monoterpenes				
1093	1098	*trans*-Sabinene hydrate	1:2	1.2
1113	1118	*cis-p*-Menth 2-en-1-ol	1:2	0.9
1169	1174	Terpinene-4-ol	1:2:3	13.2
1182	1186	α-Terpineol	1:2	0.5
1190	1195	*cis*-Piperitol	1:2	0.4
1205	1207	*trans*-Piperitol	1:2	0.4
1218	1218	*endo*-Fenchyl acetate	1:2	0.1
1237	1235	*trans*-Chrysanthenyl acetate	1:2	0.2
Sesquiterpene hydrocarbons				
1391	1387	β-Bourbonene	1:2	0.2
1404	1400	β-Longipinene	1:2	1.3
1419	1417	β-Caryophyllene	1:2	2.6
1458	1452	α-Humulene	1:2	0.4
1484	1478	γ-Muurolene	1:2	1.3
1511	1505	(*E,E*)-α-Farnesene	1:2	0.3
Oxygenated sesquiterpenes				
1569	1561	(*E*)-Nerolidol	1:2	0.2
1590	1582	Caryophyllene oxide	1:2	0.3
1602	1594	Carotol	1:2	0.3
1642	1630	Muurola-4,10(14)-dien-1-β-ol	1:2	0.3
1650	1639	Caryophylla-4(12),8(13)-dien-5-ol	1:2	0.2
1656	1644	α-Muurolol	1:2	0.3
1664	1652	α-Cadinol	1:2	0.3
1668	1652	α-Eudesmol	1:2	0.2
1698	1685	Germacra-4(15),5,10(14)-trien-1-α-ol	1:2	0.4
Phenyl propanoids				
1574	1565	3-(*Z*)-Hexenyl benzoate	1:2	0.5
1774	1759	Benzyl benzoate	1:2	0.2
Diterpenoids				
2114	2105	Phytol	1:2	3.1

[a] - Relative retention index on a HP 5MS column. [b] - Relative retention index from literature.
[c] - 1 = linear retention index; 2 = mass spectrum; 3 = coinjection with authentic compound

present in the oil. Sesquiterpenoids were present to the extent of 8.6% with β-caryophyllene being the major one (2.6%). Phenyl propanoids and diterpenoids were present in minute quantities. The component band with an Rf value of 0.71 on TLC was found to be the major compound, sabinene which was isolated in pure form (liquid, refractive index: 1.469).

3.2. Antimicrobial activity

The *in vitro* antimicrobial activity of the essential oil of *Z. diphylla* against nine species of bacteria and five fungal species are given in Table 2. Among the six gram negative bacteria tested, only *Salmonella typhi.* (MIC - 5.3 µg/mL) was found to be the most susceptible to the oil. The essential oil exhibited mild to moderate antibacterial activity against gram negative bacterial species

Serratia marcescens, Proteus vulgaris, Klebsiella pneumonia, *Pseudomonas aeruginosa* and *Escherichia coli.* Among the three gram positive bacterial species tested, the essential oil showed moderate activity against *Bacillus subtilis* which often contaminates foods. The oil exhibited mild antifungal activity against *Candida albicans, Trychophyton rubrum* and *Aspergillus fumigatus.*

Evaluation of the anti-microbial activity of sabinene resulted in the discovery of potent anti-microbial property of this compound for the first time (Table 2A). It was active against streptomycin resistant *S. typi* (MIC: 3.12 µg/mL) and showed activity comparable to that of

Table 2. Antimicrobial activity of *Zornia diphylla* essential oil in mice [a]

Microorganism (MTCC)	Essential oil (MIC, µg/mL)	Streptomycin (MIC, µg/mL)	Fluconazole (MIC, µg/mL)
Gram positive bacteria			
Bacillus cereus (430)	>100	8.7 ± 1.2	NT
B. subtilis (441)	23.3 ± 2.9	10.0 ± 2.0	NT
Staphylococcus aureus (96)	>100	6.7 ± 1.2	NT
Gram negative bacteria			
Serratia marcescens (97)	25.0 ± 0.0	7.3 ± 1.2	NT
Pseudomonas aeruginosa (741)	13.3 ± 2.9	10.7 ± 1.2	NT
Proteus vulgaris(426)	21.7 ± 2.9	8.0 ± 0.0	NT
Salmonella typhi (733)	5.3 ± 1.2	R	NT
Escherichia coli (443)	>100	6.0 ± 0.0	NT
Klebsiella pneumoniae (109)	21.7 ± 2.9	7.3 ± 1.2	NT
Fungi			
Candida albicans (227)	100.0 ± 0.0	NT	9.3 ± 1.2
Fusarium oxysporum (284)	R	NT	7.3 ± 1.2
Aspergillus niger (1344)	R	NT	10.0 ± 0.0
A. fumigatus (343)	50.0 ± 0.0	NT	8.7 ± 1.2
Trychophyton rubrum (296)	100.0 ± 0.0	NT	6.7 ± 1.2

NT – not tested; R: resistant

[a] The values shown represent the average of three determinations ± standard deviations

MIC- Minimum Inhibition Concentration

streptomycin in the case of *P. aeruginosa*. It exhibited very good anti-fungal activity also (Table 2A). The anti-microbial activity of sabinene was substantially more than that of the oil. The anti-microbial activity of the oil could be solely, or to a large extent, due to this compound. Previously, the extract of *Tridax procumbens* containing α-peneine, β-pinene, phellandrene and sabinene as major compounds, has been reported to have anti-fungal activity [28]. The present study shows that the anti-fungal activity of the extract could be, at least to a large extent, due to sabinene.

3.3. Anti-inflammatory activity

The essential oil isolated from *Z. diphylla* was evaluated for anti-inflammatory activity by carrageenan induced paw edema method. The difference of paw volume at various time intervals were measured and given in Table 3. The oil showed significant anti-inflammatory activity when administered orally along with coconut oil. The essential oil showed only moderate anti-inflammatory activity when applied topically on the paw. In this connection, it should be noted that the 2 major components of the oil, sabinene (43.1 %) and terpinene-4-ol (13.2 %) are reported to have anti-inflammatory properties under cell culture systems. Sabinene isolated from *Oenanthe crocata* shows anti-inflammatory activity by inhibiting nitric oxide production in lipopolysaccharide (LPS) triggered macrophages [29]. Terpinen-4-ol present in the essential oil of *Melaleuca alternifolia* (tea tree oil) is reported to suppress the production of inflammatory mediators (TNF-α, Il-1, IL-8 and PGE_2) by LPS stimulated human monocytes [30]. Further, *in vivo* studies have shown that lens protein-induced inflammation in rabbit's eye is inhibited by the instillation of 1 % sabinene [31]. In view of these, the *in vivo* anti-inflammatory activity of *Z. diphylla* essential oil observed in the present study in mice could be due to these 2 major compounds present in the oil.

Table 2A. Antimicrobial activity of sabinene isolated from *Zornia diphylla* essential oil [a]

Microorganisms	MIC (µg/mL)	Streptomycin (µg/mL)	Fluconazole (µg/mL)
Gram positive bacteria			
Bacillus subtilis	12.50	6.25	NT
Gram negative bacteria			
Pseudomonas aeruginosa	6.25	6.25	NT
Salmonella typhi	3.13	R	NT
Escherichia coli	25.00	6.13	NT
Fungi			
Candida albicans	25.00	NT	8.13
Aspergillus fumigatus	12.50	NT	6.25
Trychophyton rubrum	12.50	NT	6.25

NT, not tested; R, resistant; MIC, Minimum Inhibition Concentration
[a] Values are mean of three determinations

Table 3. Anti-inflammatory activity of essential oil from *Z. diphylla* (carrageenan-induced paw edema in mice)

Test groups	Inflammation (mL)		
	1[a]	2[b]	3[c]
Control, coconut oil (0.5 mL/ animal), p.o.	0.104 ± 0.005	0.103 ± 0.005	0.103 ± 0.006
Control, coconut oil (50 µL/paw), topical	0.096 ± 0.003	0.095 ± 0.003	0.095 ± 0.004
Test group 1 (0.5 mL of 5 % essential oil in coconut oil, p.o.)	0.070 ± 0.005	0.058 ± 0.006	0.052 ± 0.008*(50)
Test group 2 (0.5 mL of 10 % essential oil in coconut oil, p.o)	0.053 ± 0.007	0.035 ± 0.004	0.031 ±0.003** (70)
Indomethacin (10 mg/kg, p.o.)	0.035 ± 0.006	0.024 ± 0.005	0.019 ±0.004* * (82)
5 % oil in coconut oil, topical (50 µL/paw)	0.084 ± 0.004	0.075 ± 0.003	0.071 ± 0.004* (25)
10 % oil in coconut oil, topical (50 µL/paw)	0.061 ± 0.006	0.049 ± 0.005	0.044 ± 0.005** (54)
1% diclofenac sodium gel, topical (50 mg /paw)	0.045 ± 0.005	0.034 ± 0.005	0.032 ± 0.003** (66)

[a] The difference in paw volume after 1 h; [b] The difference in paw volume after 2 h;
[c] The difference in paw volume after 3 h;
Data are mean ± standard deviation; n=6.
* $p < 0.05$, ** $p < 0.001$ compared with the respective control group (one-way ANOVA).
Values in parentheses represent % inhibition in inflammation

3.4 In vitro cytotoxicity

The essential oil of *Z. diphylla* was tested for cytotoxicity using Trypan blue exclusion method. The cell viability assay was done using Dalton's lymphoma ascitis cells, normal peritoneal

macrophages and thymocytes. At 50 µg/mL level the essential oil showed only marginal cytotoxicity to macrophages and thymocytes, but curcumin, a food additive, showed considerable cytotoxicity to these cells (Table 4). DLA cells were more susceptible compared to normal cells.

Table 4. *In vitro* cytotoxicity (Ttrypan blue exclusion method)

Test material	Concentration (µg/mL)	% cell viability [a]		
		DLA cells	Macrophages	Thymocytes
Control	0.1% DMSO	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Essential oil	25	98.7 ± 1.2*	100.0 ± 0.0	100.0 ± 0.0
Essential oil	50	94.3 ± 2.1*	98.3 ± 0.6*	97.7 ± 0.6*
Curcumin	50	34.2 ± 3.2*	36.3 ± 2.0*	18.3 ± 2.2*

[a] – The values shown represent the average of three determinations ± standard deviation
* $p < 0.05$ compared with the control group (one-way ANOVA).

4. Conclusion

This is the first report on the constituents and pharmacological activities of *Z. diphylla* essential oil. The major constituents of the essential oil were sabinene and terpinene-4-ol. The essential oil showed potent anti-*S. typhi* activity. Further, it showed considerable anti-inflammatory activity against carageenan-induced paw edema in mice. Sabinene showed promising anti-bacterial and anti-fungal activates.

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