Journal of Medicinally Active Plants

Volume 1 | Issue 3

January 2012

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DOI: https://doi.org/10.7275/R5Q23X59
https://scholarworks.umass.edu/jmap/vol1/iss3/6

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Essential Oil Constituents and Biological Activity of *Aegle marmelos* (L.) Corr. Serr. from Nepal

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Date received: June 10, 2012.

Keywords: Antimicrobial activity, brine shrimp lethality, cytotoxic activity, essential oil composition, insecticidal activity, mosquito larvicidal activity, nematocidal activity.

ABSTRACT

Aegle marmelos, a deciduous fruit tree that grows in Nepal, has numerous uses in traditional medicine, being used as an anti-inflammatory, anti-diarrheal, heart disease treatment, and in birth control. In this study, leaves were collected to obtain, analyze, and examine the bioactivity of the essential oil of *A. marmelos,* which is often referred to as bael tree in Nepal. The essential oil from leaves was obtained by hydrodistillation. The chemical composition, determined by GC-MS, revealed 82 compounds, with 81 components being identified. The major component was limonene (64.1%), with the other two abundant components being (*E*)-β-ocimene (9.7%) and germacrene B (4.7%). Bioassay screening of the essential oil indicated marginal toxicity against MCF-7 human breast adenocarcinoma cells (LC_{50} = 98.2 μg mL^{-1}), but good larvicidal activity against mosquitoes (*Culex pipiens*) (LC_{50} = 2.15 μg mL^{-1}), nematocidal activity against *Caenorhabditis elegans*, (LC_{50} = 113 μg mL^{-}) and insecticidal activity against termites (*Reticulitermes virginicus*), fruit flies (*Drosophila melanogaster*), and fire ants (*Solenopsis invica* × *richteri*). The essential oil of *A. marmelos* showed remarkable brine shrimp lethality. Antimicrobial and antifungal activity of the leaf oil was negligible. Most of the observed biological activity was apparently due to the relatively high level of limonene (64.1%) in the essential of the leaf essential oil from *A. marmelos*.

INTRODUCTION

Aegle marmelos (L.) Corr. Serr. (Rutaceae) is one of only three species in genus *Aegle* (Sharma et al., 2011), is a subtropical fruit-bearing, deciduous tree that grows throughout hills and plains of the sub-Himalayan countries of Nepal, India, Sri Lanka, and Bangladesh (Sekar et al., 2011). The species, which is normally characterized by a thick trunk with spiny branches and a soft, flaky bark (Sekar et al., 2011), grows to a height of 7 to 8 m and has pale-green leaves with 3-5 leaflets that release an aroma when bruised. The plant has greenish-white flowers that bloom in late spring and produce an oval-shaped, sweet-tasting, soft fruit covered in a woody shell that turns yelow when the fruit ripens (Dhankhar et al., 2011; Sekar et al., 2011).

A. marmelos has several common names, and in Nepal, is referred to as the bael tree. The plant is often seen growing in temple gardens of Nepal where the leaves are used in prayers to Lord Shiva and an important fertility ritual, Bel biha (Sekar et al., 2011). Considered one of the most important medicinal plants in southern and southeastern Asia, the entire plant is traditionally used to treat an array of ailments.

Various tissues of *A. marmelos* have demonstrated positive effects on heart diseases (root bark) (Kakiuchi et al., 1991), dysentery and inflammation (leaves) (Arul et al., 2005), diabetes (leaf extract) (Kumar et al., 2009), and ulcers (Udupa et al., 1994).

Fruit from *A. marmelos* is used as an antioxidant and a form of birth control (Gheisari et al., 2011).

Earlier antimicrobial studies using essential oils from *A. marmelos* leaves have demonstrated that terpenoid constituents in the oil are *in-vivo* antifungal components (Rana et al., 1997; Balakumar et al., 2011). Tannins, eugenol, and cuminaldehyde contribute to the antibacterial activity of the fruit extract, particularly against *Escherichia coli* (Gheisari et al., 2011; Duke, 1992). *A. marmelos* bark extract was cytotoxic to tumor cell lines and lethal to brine shrimp (Costa-Lotufo et al., 2005). An extract from the leaves has demonstrated activity against breast cancer cells, thought to be attributable to the presence of skimmianine (Lambertini et al., 2004; Jagetia et al., 2004).

Much of the previous work (Arul, et al., 2005; Kumar, et al., 2009; Dhankhan, et al., 2011) on *A. marmelos* as a medicinal plant has focused on the plant fruit. The current study was initiated to explore the composition of the essential oil in leaves of *A. marmelos* plants growing in Nepal, and to broaden the biological activity profile of this understudied taxon.

MATERIALS AND METHODS

Plant material. Leaves of *Aegle marmelos* Corr. Serr. (Rutacease) were randomly collected from one individual tree growing in the city of Biratnagar (26°28′ N, 87°16′ E, 72 m asl) in the Morang district of the Koshi Zone of Nepal on May 17, 2011, for use in this study. The plant was identified by Tilak Gautam, and a voucher specimen (1701) has been deposited in the herbarium of the located in the Botany Department on the Post-Graduate Campus of Tribhuvan University in Biratnagar, Nepal. The fresh leaf samples (100 g) were crushed and hydrodistilled using a Clevenger type apparatus for 4 h and yielded a clear, pale yellow, essential oil (1.5 g), which was stored at 4°C until analysis.

Oil analysis. The essential oil from the *A. marmelos* leaves was analyzed by GC-MS using an Agilent 6890 GC (Santa Clara, CA) with Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV), scan range = 45-400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a (5% phenyl)-polymethyl-siloxane stationary phase a film thickness of 0.25 μm, a length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 48.7 kPa and a flow rate of 1.0 mL min^{-1}. Injector temperature was 200°C and the detector temperature was 280°C. The GC oven temperature program was programed for: 40°C initial temperature, hold for 10 min; increase in temperature 3°C/min to 200°C; increase in temperature 2°/min to 220°C. A 1% w/v solution of the sample in CH_2Cl_2 was prepared and 1 μL was injected using a splitless injection technique.

Identification of the oil components was based on the retention indices determined by reference to a homologous series of *n*-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature (Adams, 2007), stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.080)]. The percentages of each component are reported as raw percentages based on total ion current without standardization (Table 1).

Antimicrobial screening. The essential oil and the major component, limonene, were screened for antimicrobial activity against Gram-positive bacteria [*Bacillus cereus* (ATCC No. 14579) and *Staphylococcus aureus* (ATCC No. 29213)] and Gram-negative bacteria [*Pseudomonas aeruginosa* (ATCC No. 27853) and *Escherichia coli* (ATCC No. 10798)]. Minimum inhibitory concentrations (MICs) were determined using the microbroth dilution technique (Sahm and Washington, 1991).

Dilutions of the samples were prepared in cation-adjusted Mueller Hinton broth (CAMHB), beginning with 50 μL of 1% w/w solutions of samples in DMSO plus 50 μL CAMHB. The sample solutions were serially diluted (1:1) in CAMHB in 96-well plates to give concentrations of 2500, 1250, 625, 313, 156, 78, 39, and 20 μg mL^{-1}. Organisms at a concentration of approximately 1.5×10^8 colony-forming units (CFU) mL^{-1} were added to each well, and the plates were incubated at 37°C for 24 h. The minimum inhibitory concentration was determined as the lowest concentration of oil or limonene without

turbidity. Gentamicin was used as a positive antibiotic control and DMSO was used as a negative control.

Antifungal activity was determined, as described above for bacteria, using *Candida albicans* (ATCC No.10231) in a yeast-nitrogen base growth medium (Difco Laboratories, Inc., Detroit, MI) with approximately 7.5×10^7 CFU mL^{-1}. Amphotericin B was used as the positive control. An additional test for antifungal activity against *Aspergillus niger* (ATCC No. 16888) was determined as above using YM broth inoculated with *A. niger* hyphal culture diluted to a McFarland turbidity of 1.0. Amphotericin B was the positive control.

Brine shrimp lethality assay. The brine shrimp (*Artemia salina*) lethality test was done using a modification of the procedure of McLaughlin (1990). *Artemia salina* eggs were hatched in a sea salt solution (Instant Ocean®, Spectrum Brands, Inc. Madison, WI) (38 g L^{-1}) with an incandescent light bulb as the heat source. After 48 h, the newly hatched nauplii were counted using a micropipette and transferred to 20 mL vials.

A total of nine vials, each containing 10 *A. salina* nauplii in 10 mL of sea salt solution (the same as the hatching solution) were prepared. Of these vials, three were labeled as controls with one vial containing no DMSO, a second vial containing 10 µL of DMSO, and the third vial containing 100 µL DMSO. A second set of three replicate vials contained 10 µL of 1% essential oil solution in DMSO, and the remaining three vials were prepared by adding 100 µL of 1% essential oil solution in DMSO. After 24 h, surviving *A. salina* were counted in each vial and LC_{50} values (Table 2) were determined using the Reed-Muench method (Reed and Muench, 1938) .

Cytotoxicity screening. Human MCF-7 breast adenocarcinoma cells (ATCC No. HTB-22) (Soule et al., 1973) were grown in a 3% CO_2 environment at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100,000 units penicillin, and 10.0 mgL^{-1} streptomycin, 15 mM of HEPES, and buffered with 26.7 mM $NaHCO_3$, pH 7.35. Cells were plated into 96-well cell culture plates at 2.5×10^4 cells per 100 µL well. After 48 h, the supernatant fluid was removed by suction and replaced with 100 µL of growth medium containing 1.0 µL of DMSO solution containing an essential oil (1% w/w in DMSO) for a final oil concentration of 100 µg mL^{-1} in each well.

Solutions were added to wells in four replicates. Medium and DMSO controls (10 µL DMSO mL^{-1}) were used as negative controls and tingenone (Setzer et al., 1998) was used as a positive control. After the addition of the test compounds, the plates were incubated for 48 h at 37°C in 5% CO_2. Following incubation, the medium was removed by suction, and 100 µL of fresh medium was added to each well. To establish kill rates, the MTT assay for cell viability was done (Ferrari et al., 1990). Colorimetric readings were recorded, using a Spectra MAX Plus microplate reader at 570 nm (Molecular Devices, LLC, Sunnyvale, CA). Absorbency averages, standard deviations, and percent kill ratios ($\%kill_{cmpd}/\%kill_{DMSO}$) were calculated.

Termiticidal activity. Termiticidal activity was determined using worker termites (*Reticulitermes virginicus*) (Item number 143736) purchased from Carolina Biological Supply (Burlington, NC). Assays of activity were done using a six-well culture plate in which each well was fitted with a filter paper disc. The essential oil and limonene solutions were prepared in 1% aqueous Tween® 80 solutions at 500, 1000, and 2000 µg mL^{-1}. Sample solutions (200 µL) of each concentration were sprayed into three wells. Water and 1% aqueous Tween® 80 solution were used as controls in the remaining wells. In each well, six termites were placed and termiticidal activity was determined 24 h later.

Mosquito larvicidal activity. Larvae of *Culex pipiens* (Item number 144478) were obtained from Carolina Biological Supply (Burlington, NC). For the bioassay, 10 mL of sterile water was placed into each of five 20-mL vials. Into each vial 10 mosquito larvae were transferred using soft brush. Of the vials, three were labeled as control with one containing 10 µL DMSO, a second one containing 100 µL DMSO and a third one containing sterile water. Into the remaining two vials, 10 µL and 100 µL of a 1% solution of essential oil in DMSO were added, respectively, for final concentrations of 10 and 100 µg mL^{-1}). Surviving mosquito larvae were counted after 24 h. The experiments were done at 23 ± 2ºC.

Nematocidal assay. A nematocidal assay using *Caenorhabditis elegans* was done using a modification of the procedure of Park and co-workers (Park, et al., 2007). Briefly, a 1% solution of *A. marmelos* leaf oil in DMSO was used to make dilutions for the sample solutions. The sample solutions were prepared in sterile water beginning with 50 µL of the 1% essential oil solution mixed in 50 µL sterile water. This sample solution was serially diluted (1:1) with sterile water in a 96-well plate. Into each well, 10-30 *C. elegans* (mixtures of juvenile and adult nematodes, male:female:juvenile ~1:1:2) per 50 µL of sample solution. Sterile water and serially diluted DMSO were used as controls. The dead and living nematodes were counted after 24 h using a microscope. Dead nematodes were identified by their immobility and straight body, even after transfer to clean water. LC_{50} values were determined using the method of Reed and Muench (1938).

Fruit fly lethality test. Wild type *Drosophila melanogaster* were obtained from a breeding colony sourced and maintained using a *Drosophila* culture kit (Carolina Biological Supply, Burlington, NC). The *Drosophila* medium (2 mL) was placed into each of five 20-mL glass vials. Of the vials, three vials were labeled as control, the first containing only *Drosophila* medium, the second with 20 µL DMSO, and the third with 150 µL of DMSO. Of the remaining two vials, one contained 20 µL of 1% essential oil solution in DMSO and the second one contained 150 µL of 1% essential oil solution in DMSO. Individual fruit flies were transferred into each vial (10 flies $vial^{-1}$). Each test was done in triplicate. Surviving fruit flies were counted at 24 h after initiation of the treatments.

Fire ant lethality test. Worker red imported fire ants, most probably *Solenopsis invicta* × *richteri* hybrid (Chen et al., 2012), were collected from the University of Alabama campus, Huntsville, AL. Sample solutions of 250 µg mL^{-1}, 500 µg mL^{-1} and 1000 µg mL^{-1} were prepared in 1% aqueous Tween-80® solution. The control was 1% Tween solution. Each assay was done using a 400 mL beaker, fitted with a filter paper disk on the bottom. The filter paper was sprayed with 600 µL of sample solution and 20 fire ant workers were transferred to the beaker. The beaker was sealed at the top with Parafilm® (Pechiney Plastic Packaging Co., Chicago, IL) and the mortality of the fire ants was recorded after 24 h. The bioassay was done in triplicate at room temperature. LC_{50} values were calculated using the method of Reed and Muench (1938).

RESULTS

The analysis of the essential oil (1.5% yield) obtained from the leaves of *A. marmelos* revealed a total of 82 components of which 81 were identified representing 99.8% of the essential oil composition (Table 1). The major components of oil from Nepal were limonene (64.1%), (*E*)-β-ocimene (9.7%), and germacrene B (4.7%), with smaller amounts of (*E*)-caryophyllene (2.4%), myrcene (2.0%), (*Z*)-β-ocimene (1.9%), (2*Z*,6*E*)-farnesol (1.9%), linalool (1.8%), and γ-curcumene (1.7%).

The leaf essential oil from was not appreciably antimicrobial, having MIC values ≥ 625 µg mL^{-1} against the tested organisms (Table 2). The major component of the essential oil, limonene is not considered antimicrobial and was generally less active than the oil *per se*. The slightly higher activity of the essential oil as compared with limonene may be due to synergistic effects of minor components in the oil rather than to limonene alone. In this study, bacteria concentrations of approximately 1.5×10^8 CFU mL^{-1} per test were used. Had lower bacterial concentrations been used, lower MIC values may have been achieved.

A. marmelos leaf oil demonstrated *in-vitro* cytotoxic activity on MCF-7 human breast adenocarcinoma cells with 51.4 ± 14.7% of the cells killed at an oil treatment rate of 100 µg mL^{-1}. The cytotoxic activity can be attributed to the major component, limonene, which was also was cytotoxic on MCF-7 cells with a 90.8 ± 9.6% kill rate at 100 µg mL^{-1}. Consistent with the measured cytotoxic activity on MCF-7 human breast cells, *A. marmelos* leaf oil was also notably active in the brine shrimp lethality assay (Anderson, et al., 1991).

Table 1. Chemical composition of the leaf essential oil of *Aegle marmelos* from Nepal.

RI[1]	Compound	%[2]		RI[1]	Compound	%[2]
787	3-Methyl-2-butenal	0.1		1437	α-Zingiberene	0.2
856	(3*Z*)-Hexenol	0.3		1445	β-Funebrene	0.2
941	α-Pinene	0.2		1454	α-Humulene	0.8
963	Benzaldehyde	0.1		1459	(*E*)-β-Farnesene	0.3
976	Sabinene	0.2		1474	*trans*-Cadina-1(6),4-diene	tr
994	Myrcene	2.0		1483	γ-Curcumene	1.7
1030	Limonene	64.1		1484	*ar*-Curcumene	0.3
1047	(*Z*)-β-Ocimene	1.9		1493	*trans*-Muurola-4(14),5-diene	tr
1053	(*E*)-β-Ocimene	9.7		1496	*epi*-Cubebol	0.1
1063	γ-Terpinene	tr		1497	α-Farnesene	0.1
1075	*cis*-Linalool oxide (furanoid)	tr		1506	Germacrene A	0.1
1088	α-Terpinolene + trans-Linalool oxide (furanoid)	0.1		1511	(*E*,*E*)-α-Farnesene	0.1
1104	Linalool	1.8		1514	α-Cedrene	0.2
1121	*cis*-*p*-Menth-2,8-dien-1-ol	0.1		1518	Cubebol	0.5
1130	Alloocimene	tr		1525	δ-Cadinene	0.3
1134	*cis*-Limonene oxide	tr		1534	*trans*-Cadina-1,4-diene	tr
1135	*trans*-*p*-Menth-2,8-dien-1-ol	0.1		1535	Italicene ether	tr
1138	*trans*-Limonene oxide	tr		1548	*cis*-Sesquisabinene hydrate	0.8
1188	(3*Z*)-Hexenyl butanoate	0.2		1553	Elemol	0.2
1190	α-Terpineol	0.1		1562	Germacrene B	4.7
1200	*cis*-Piperitol	0.4		1566	(*E*)-Nerolidol	0.2
1208	*trans*-Carveol	0.1		1570	(3*Z*)-Hexenyl benzoate	tr
1218	γ-Isogeraniol	0.2		1576	Germacrene D-4-ol	tr
1226	Nerol	tr		1581	*trans*-Sesquisabinene hydrate	0.2
1228	*cis*-Carveol	tr		1584	Caryophyllene oxide	0.1
1239	Neral	tr		1628	1-*epi*-Cubenol	0.1
1252	*p*-Anisaldehyde	tr		1632	Eremoligenol	0.1
1252	Geraniol	0.2		1635	Caryophylla-4(12),8(13)-dien-5-ol	tr
1270	Geranial	tr		1643	τ-Muurolol	0.1
1337	δ-Elemene	0.1		1646	α-Muurolol (= Torreyol)	tr
1349	α-Cubebene	0.1		1651	β-Eudesmol	tr
1375	α-Copaene	0.3		1655	α-Cadinol	0.2
1385	(3*Z*)-Hexenyl hexanoate	tr		1671	*epi*-β-Bisabolol	0.1
1391	7-*epi*-Sesquithujene	0.3		1682	*epi*-α-Bisabolol	tr
1392	β-Elemene	0.2		1686	Germacra-4(15),5,10(14)-trien-1α-ol	tr
1396	Methyl perillate	0.1		1690	Shyobunol	0.1
1399	(*Z*)-Jasmone	0.1		1699	(2*Z*,6*Z*)-Farnesol	0.1
1408	Methyl *N*-methylanthranilate	tr		1728	(2*Z*,6*E*)Farnesol	1.9
1421	(*E*)-Caryophyllene	2.4		1742	(2*E*,6*E*)-Farnesal	tr
1434	γ-Elemene	0.4		2109	(*E*)-Phytol	0.3
					Total Identified	99.8

[1]RI = Retention Index determined in reference to a series of *n*-alkanes on an HP-5 ms column; compounds are listed in order of elution (increasing RI).
[2]Percent of total oil.

The leaf essential oil of *A. marmelos* was toxic to the nematode *C. elegans* with an LC_{50} of 113 µg mL^{-1}. The major constituent of the oil, limonene, was also toxic to *C. elegans* (LC_{50} = 85 µg mL^{-1}), suggesting noted activity was due to the concentration of limonene in the leaf tissue oil. The notable larvicidal activity of the essential oil against *C. pipiens* and the insecticidal activity against termites (*R. virginicus*), fruit flies (*D. melanogaster*), and red imported fire ants (*S. invicta* × *richteri*) is probably also due to the high concentration of limonene in the leaf.

Table 2. Biological activity of *A. marmelos* essential oil and limonene.

Test organism	Antimicrobial activity (MIC mg mL^{-1})	
	A. marmelos oil	Limonene
B. cereus	625	1250
S. aureus	625	1250
E. coli	625	1250
P. aeruginosa	1250	1250
C. albicans	1250	313
A. niger	625	156
	Toxicity assays (LC_{50} µg mL^{-1})	
MCF-7 cells	98.2±14.9	74.7±4.1
A. salina	4.18±0.82	6.33±3.35
C. elegans	113[1]	85.1[1]
C. pipiens	2.15[1]	nt[2]
R. virginicus	500[1]	158[1]
D. melanogaster	238±24	249±62
Solenopsis	413±65	1290±80

[1]Due to shortage of test organisms, only one assay was done.
[2]nt = not tested.

DISCUSSION

The relatively high level of limonene in the leaf oil of *A. marmelos* sourced in Nepal is consistent with the plant material being limonene chemotype, comparable to samples from India (Kaur et al., 2006; Kumar et al., 2007; Garg et al., 2006). The chemical composition of the Nepal sample was markedly different from *A. marmelos* samples from Thailand, which are primarily composed of sylvestrene (Kamkaen et al., 2008), and phellandrene (Jarikasem et al., 2004). Additional chemotypes of *A. marmelos* that have been reported include a phellandrene chemotype from India (Baslas and Deshpandey, 1951; Bhandari and Gupta, 1972), a myrcene chemotype from India (Garg et al., 1995), a 1,8-cineole chemotype from India (Singh et al., 1993), and a δ-cadinene chemotype from Cuba (Pino et al., 2005). Such differences in composition could be attributed to differences in genotype or geographical location, climate conditions, and time of harvest.

Previous studies have revealed *A. marmelos* leaf oil to be antibacterial to *Aeromonas*, *E. coli*, *Pseudomonas solanacearum*, and *Xanthomonas vesicatoria* (Pandey et al., 1981). Additionally, root extracts of *A. marmelos* have demonstrated anti-bacterial activity against *Vibrio cholerae, Salmonella typhimurium*, *Klebsiella pneumoniae*, *E. coli*, *P. aeruginosa*, *Bacillus subtilis* and *S. aureus* (Pitre and Srivastava, 1987). Antifungal activity has also been previously reported for the leaf oil (Rana et al., 1997) as well as fruit extracts Gheisari et al., 2011).

The toxicity of the leaf essential oil to the nematode *C. elegans* is comparable to *Cymbopogon martinii* essential oil (Kumaran et al., 2003). The oil constituent, limonene, which was also toxic to *C. elegans*, was not as active as the phenolic monoterpenoids thymol and carvacrol (Lei et al., 2010). Limonene has been shown to be relatively inactive against the root-knot nematode, *Meloidogyne incognita* (Ntalli et al., 2010). Many essential oils have relatively high LC_{50} values against this nematode (Ntalli et al., 2010, 2011).

The activity of the essential oil against *C. pipiens* is consistent with earlier studies with *Citrus* oils containing high concentrations of limonene (Michaelakis et al., 2009). Limonene has shown mosquito larvicidal activities against *Aedes aegypti* (Cheng et al., 2009; Santos et al., 2011), while limonene-rich essential oils were also active against *A. aegypti* (Pitasawat et al., 2007). Crude leaf extracts of *A. marmelos* had previously shown larvicidal activity against *A. aegypti* (Patil et al., 2010). The broad insecticidal activity against termites, fruit flies, and fire ants undoubtedly also results from the limonene, a compound known to be toxic to insects, including termites (Raina et al., 2007) and weevils (Papachristos et al., 2004).

In summary, the leaf oil of *A. marmelos* from Nepal is a limonene chemotype. The oil has shown remarkable toxicity to *A. salina* nauplii, *C. elegans*, and *C. pipiens* larvae, as well as insecticidal activity toward termites, fruit flies, and fire ants. The oil was

only marginally cytotoxic on MCF-7 cells, and virtually devoid of antimicrobial activity. This medicinal plant may also serve as an alternative to synthetic pesticides.

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