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Original Article

CHEMICAL COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL AND EXTRACT OF *ALPINIA MALACCENSIS* ROSCOE (ZINGIBERACEAE)

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ABSTRACT

Objective: The present study was conducted to examine the chemical composition, *in vitro* antioxidant and antimicrobial activity of both the essential oil and methanolic extract of *Alpinia malaccensis* leaves.

Methods: The essential oils obtained from the leaves of *Alpinia malaccensis* were analyzed by gas chromatography/mass spectrometry to determine chemical compositions. Antioxidant activity of both oil and extract were determined using DPPH and ABTS assay whereas the antimicrobial effects were tested by inhibition zone diameter and minimum inhibitory concentration.

Results: The GC–MS analysis of the essential oil identified 10 components comprising 92.7% of the oil. The major constituents of the oil are α-phellandrene (43.9%), β-cymene (31.7%), β-pinene (4.6%). Total phenolic content of the leaf extract of *A. malaccensis* was found to be 76.25 mg GAE/g of the extract. Essential oil and methanolic extract displayed significant antioxidant activities with IC_{50} values of 18.26µg/ml and 22.5µg/ml in DPPH and 20µg/ml and 26.23µg/ml in ABTS respectively. Oil and extract showed very good activity against all four microbial strains.

Conclusion: The result showed that the essential oil has better activity than extract. Thus it could be served as potential source of natural antioxidant, natural antimicrobial as well as natural preservative ingredient in cosmetics, food and pharmaceutical industry.

Keywords: *Alpinia malaccensis*, Antioxidant, Antimicrobial, Essential oil, α-phellandrene.

INTRODUCTION

In this modern age of chemotherapeutics, the herbal medicinal system has drawn the attention of both academician and researchers for the development of novel drugs, use in cosmetic industries, food preservation etc. Among the plant products essential oils and extracts have been used for many thousands of years in food preservation, pharmaceuticals, alternative medicine, and natural therapies as well as in perfumes, cosmetics, aromatherapy, phototherapy, spices and nutrition [1]. Zingiberaceae has a rich source of compounds of phytomedicinal interest. The genus *Alpinia* is the largest, most widespread and most taxonomically complex genus in the Zingiberaceae with 230 species occurring throughout tropical and subtropical Asia [2]. *Alpinia malaccensis* is a perennial medicinal plant belonging to the family Zingiberaceae, growing widely in the subtropical and tropical regions and native of Indonesia and Malaysia. It is called "Kha Pa" in Thailand and "Ring Malacca" in Vietnam. *A. malaccensis* is cultivated as an ornamental in Bangladesh, Bhutan, India, Indonesia, Malaysia, Myanmar and Thailand. This beautiful tropical plant is becoming a popular house plant as well as a landscape plant. *A. malaccensis* is used to cure wounds and sores (pounded rhizome), it is chewed together with betel nut to make the voice strong and clear (rhizome), it is applied on gastralgia with tympanites (decoction of fruit or the crushed seed) and it is used for bathing feverish people [2-4]. However the scientific evidence about *Alpinia malaccensis* remains limited.

Antioxidants act as radical scavengers, inhibit lipid peroxidation and other free radical-mediated processes and are able to protect the human body as well as processed foods from oxidative damage attributed to the reaction of free radicals. The use of synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ), in foods is discouraged due to their perceived carcinogenic potential and safety concerns [5]. Food-borne diseases are a severe health problem in the world, even in well-developed nations [6]. The consumption of food contaminated with food-borne microorganisms can pose a serious threat to human health. The existence of microorganisms causes spoilage and results in reduction of the quality and quantity of processed foods [7]. Essential oils, extracts and bioactive constituents of several spices and herbs are well known to exert antioxidant and antimicrobial activities [8].

Nowadays, the interests in natural products are looking into sources of alternative, more natural and environment friendly antimicrobials, antioxidants, antibiotics and other bioactivities. There are only a few reports available for *Alpinia malaccensis* worldwide which prompted us to study chemical composition, antioxidant and antimicrobial activities of oil and extract of *Alpinia malaccensis* [9]. To the best of our knowledge, no literature data is available on the antioxidant and antimicrobial activities of the essential oil from the leaves of *A. malaccensis.*

MATERIALS AND METHODS

Plant material

The rhizomes of *Alpinia malaccensis* were collected from Phulbani, Odisha in the month of July, 2010 and the specimen was authenticated by Dr. P.C. Panda, Senior Scientist, Taxonomy and conservation division, Regional Plant Resource Centre, Bhubaneswar. The collected rhizomes were planted in the greenhouse of Centre of Biotechnology and after one and half years of complete growth the leaves were collected from these plants.

Microorganism and media

The test organisms used in this study were *Staphylococcus aureus* (MTCC-3160), *Pseudomonas aeruginosa* (MTCC-424), *Candida albicans* (MTCC-183) *and Aspergillus niger* (MTCC-281) obtained from the Microbial Type Culture Collection, Chandigarh, India. All the strains maintained in recommended media were purchased from Hi-Media India private Ltd., Mumbai.

Extraction and quantification of essential oil

Essential oil was extracted by hydro-distillation of fresh leaves of field grown *Alpinia malaccensis* plants in a clevenger's apparatus following the method of Guenther [10]. The fresh leaves was washed to remove soil, peeled and sliced. Sliced leaves (100 g) were mixed with distilled water. A flask containing the sliced leaves was heated for 3-4 hours and the condensed vapor was separated throughout an

auto-oil/water separator. The oil present at the upper most layers was collected in the ependroff tube. Each essential oil extraction was run in triplicate. Yield percentage was recorded as fresh weight basis. The distilled leaf essential oil was dried over anhydrous sodium sulphate and stored at -4°c in air tight container for further uses.

Preparation of extract

The fresh leaves were taken, washed and chopped into pieces and dried under shade for 15-20 days. The shade dried leaves were grounded to coarse powder. For extraction, extra pure methanol (AR grade 99.8%) was added to100g of powdered leaves and placed in soxhlet apparatus for 24 h. The methanol extract was filtered with Whatman 40 filter paper and then concentrated by using rotary evaporator to yield a semisolid mass (15.96% w/w). The residue obtained was stored in refrigerator for further study. Each methanolic extraction was run in triplicate.

Determination of TPC

TPC of methanol extract of *A. malaccensis* was determined by Folin-Ciocalteu method with little modifications, using Gallic acid as a standard phenolic compound [11-12]. The extracts were diluted with distilled water to a known concentration in order to obtain the readings within the standard curve range of 0.0 to 600 µg of Gallic acid/ml. 250 µl of diluted extract or Gallic acid solution was mixed with 1 ml of distilled water in a test tube followed by the addition of 250 µl of Folin-Ciocalteu reagent. The samples were mixed well and then allowed to stand for 5 min at room temperature in order to allow complete reaction with the Folin-Ciocalteu reagent. Then, 2.5 ml of 7% sodium carbonate aqueous solution was added and the final volume was made up to 6 ml with distilled water. The absorbance of the resulting blue colour solution was measured at 760 nm using spectrophotometer after incubating the samples for 90 min. The result was expressed as mg of Gallic acid equivalents (GAE) / g of extract by using an equation that was obtained from standard Gallic acid graph. All the experiment was conducted in three replicates.

GC-MS Analysis and identification of Compound

GC-MS analysis of the oil was carried out on a 6890 series instrument (Agilent Technologies, Palo Alto, CA, USA), equipped with MS detector and a HP-5 fused silica capillary column (30 m x 0.25 mm internal diameter), (film thickness 0.25 µm). The temperature was programmed from 50°C-240°C at 4°C/ min; from 240°C to 270°C at 15°C/ min; held isothermal at 50°C for 1 min and at 270°C for 15 min. The temperatures of both injector and detector were kept at 280°C; sample injection volume, 1 µl; split ration was 100:1. The carrier gas was helium at a flow rate of 1.2 ml/ min. GC-MS (70eV) data were measured on the same gas chromatograph coupled with MSD 5973. MS source temperature at 230°C; MS quadrapole temperature at 150°C; interface temperature at 290°C; mass scan, 20-600 amu. The retention indices were calculated using a homologous series of n-alkanes. Compounds were identified by comparison of their retention indices the data given in the literature and using NIST Library (Table-1) [13-14].

DPPH radical scavenging activity

Radical scavenging activity of the leaf essential oils, major compound of oil α-phellandrene from sigma Aldrich (W285609 Aldrich) and methanolic leaf extract of *Alpinia malaccensis* were determined by a spectrophotometric method based on the reduction of a methanol solution of DPPH using the method of Blois with little modification [15]. 1 ml of various concentrations of oil and extract in methanol was added to 2 ml of 0.1 mM methanol solution of DPPH. The mixture was shaken vigorously and left to stand at room temperature for 30 min in dark. Then the colorimetric changes (from deep violet to light yellow), when DPPH is reduced, were measured at 517 nm on a uv/visible spectrophotometer. Absolute methanol was used to zero the spectrophotometer. The DPPH solution was freshly prepared daily, stored in a flask covered with aluminium foil and kept in the dark. Tests were carried out in triplicate. Vitamin C (Ascorbic Acid), a standard antioxidant was used as positive control. Absorbance of the DPPH radical without antioxidant, the control, was measured. Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution.

Radical scavenging activity was expressed as percentage inhibition of DPPH radical and was calculated by following equation-

$$\% \text{ Inhibition} = (A_{control} - A_{test}) \times 100 / A_{control}$$

Where, $A_{control}$ is the absorbance of the control [solution without extract] and A_{test} is the absorbance of samples (extract/oil and ascorbic acid).

The antioxidant activity of each sample was expressed in terms of IC_{50} (micro molar concentration required to inhibit DPPH radical formation by 50%), calculated from the graph after plotting inhibition percentage against extract concentration.

ABTS radical scavenging assay

To determine ABTS radical scavenging assay, the method of Re *et al.* was adopted [16]. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The resulting solution was then diluted by mixing 1 ml of freshly prepared ABTS solution to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 2.5 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as

$$\text{ABTS radical scavenging activity (\%)} = (A_{control} - A_{test}) \times 100 / A_{control}$$

Where $A_{control}$ is the absorbance of ABTS radical + methanol; A_{test} is the absorbance of ABTS radical + sample extract /standard.

Antimicrobial activity

Initial screening: For initial screening the disc diffusion method as described previously by was followed with slight modifications [17]. Briefly, Nutrient Agar NA] and Potato Dextrose Aagar (PDA) plates were swabbed with freshly grown cultures of the test pathogens by the help of a pre-sterilized cotton swab. Sterile filter paper discs (5 mm diameter) were kept on the above plates at equidistance. Varying volumes (2, 5 and 10 µl) of oil and extract were loaded over the sterile filter paper discs. The plates were incubated at 37°C 18-24 hours for bacteria, 48 hours for fungi and observed for a zone of clearance around the discs which indicated positive microbicidal activity of the oil and extract. All the experiments were carried out in triplicate.

Determination of MIC [minimum inhibitory concentration]

Minimum inhibitory concentration of oil and extract were determined by the serial dilution method [18]. The oil was diluted with NBT & PDBT (Nutrient and potato dextrose broth supplemented with 0.75% of Tween-20) to give sample concentration of 5 µg/ml to 100 µg/ml. 50 µl of (fresh culture) of the test organisms was inoculated into 1ml of NBT and PDT containing various concentrations of both the samples. The tubes were incubated at 37°C, for 18-24 h, (48 h for fungi) and the lowest concentration inhibiting bacterial & fungal growth (no turbidity) was noted as MIC.

Test for bactericidal and fungicidal effect

In order to evaluate the effect (microbicidal /microbiostatic) of the oil and extract, one loop from the MIC tube was sub cultured on to the NA & PDA plates which were then incubated at 37°C over night to check whether the oil and extract merely had bactericidal or fungicidal activity i.e. no growth on sub-culturing.

Determination of MKT (Minimum killing time)

This experiment was designed to determine the time required to kill the bacteria and fungus *in-vitro* by the oil and extract. One ml of NB, supplemented with 0.75% of DMSO and one ml of PDB

supplemented with 0.75% of DMSO at MIC level of both samples were prepared and inoculated with 0.1 ml of freshly grown test organisms and incubated at 37°C. One loop of the sample from the above test tubes were sub cultured onto NA plates at 0, 5,10,15, 30,45, 60, 90, 120, 180 min. intervals and incubated overnight. Two sets of tubes were incubated for each test organisms from which sub-culturing were carried out alternatively (to avoid time lapse during subculture).

Statistical analyses

Each experiment was carried out in triplicate. The data were statistically analyzed using SPSS 10.0. A least significant difference (LSD0.05) was used to test the effects of oil and extract through a general linear model. The test was statistically significant at $p<0.05$.

RESULT AND DISCUSSION

Chemical composition of essential oil

The fresh leaves of *Alpinia malaccensis* yielded 0.2% of essential oil.

The analysis of oil was performed using gas chromatography-mass spectrometry (GC-MS). In total, ten volatile constituents, representing 92.7% of the oil, were identified in the leaf oil (Table 1). The most abundant components found in the leaf oil were α-phellandrene (43.9%) followed by β-cymene (31.7%), β-pinene (4.6%) (Fig-1). The major compound (α-phellandrene) obtained in this study was also found to be the major compound of the essential oil obtained from leaves of *Alpinia malaccensis* growing in Bangladesh [4].

According to Bhuiyan, *A.malaccensis* leaf essential oil contained α-phellandrene (31.8%), eucalyptol (13.7%), O-cymene (11.4%), β-pinene (11.3%), limonene (6.4%), β-myrcene (5.6%) and α-pinene (5.5%) as major components. Nor Azah, *et al.* (2005) revealed that the compounds of essential oils of *Alpinia malaccensis* var. *nobilis* from Terengganu extracted from leaves, rhizomes and stems [19]. The most abundant compound in the leaf oil was (*E*)-methyl cinnamate with 88.0 %. The other components present in the leaf oil were 1, 8- cineole (1.8 %) and *p*-cymene (1.5%). *β*-pinene (1.6%), *α*-phellandrene (1.9 %).

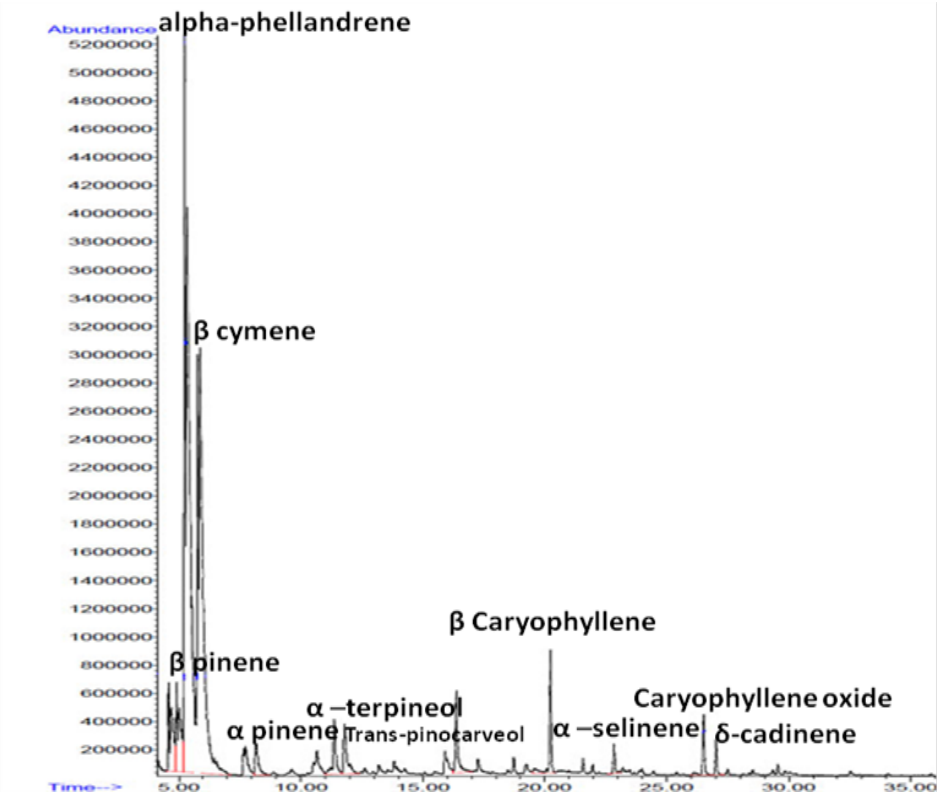

Fig. 1: GC Chromatogram of leaf oil of *Alpinia malaccensis.*

Table 1: Chemical composition of essential oil of *Alpinia malaccensis* leaf

S. No.	Compound	Area percent (%)	(RI)[a]	(RI)[b]	References
1	β-Pinene	4.6 ± 0.2	934	933	Jordan *et* al 2002 [13]
2	α-Phellandrene	43.9 ± 3.5	1005	1004	Adams 1995 [14]
3	β-cymene	31.7 ± 2.5	1082	1081	Adams 1995 [14]
4	α –pinene	1.5 ±0.15	982	983	Jordan *et* al 2002 [13]
5	α –terpineol	2.2 ±0.15	1198	1197	Jordan *et* al 2002 [13]
6	Trans-pinocarveol	2.2 ±0.16	1139	1137	Adams 1995 [14]
7	β Caryophyllene	3.3 ±0.25	1418	1416	Adams 1995 [14]
8	α –selinene	0.7 ±0.25	1494	1495	Adams 1995 [14]
9	Caryophyllene oxide	1.7±0.15	1581	1580	Adams 1995 [14]
10	δ-cadinene	0.8 ±0.23	1524	1522	Adams 1995 [14]

RI[a]: Retention Indices from literature., RI[b]: Experimental value, Average value (area %) of four replicate samples. Standard deviation (±) is given besides area percent in bracket.

DPPH radical scavenging activity

The antioxidant activity of the leaf essential oil, major compound α-phellandrene from sigma Aldrich (W285609 Aldrich) and extract obtained from *A. malaccensis* were evaluated using DPPH radical assay. In the present study, the extract showed significant DPPH radical inhibiting activity at a concentration of 500 µg/ml. (Fig -2) showed the dose response curve of DPPH radical scavenging activity of *A. malaccensis* compared with standard ascorbic acid. It was observed that the extract had DPPH scavenging activity with IC_{50} value of 22.5 µg/ml while the IC_{50} value of the standard antioxidant ascorbic acid was 6.58 µg/ml. As can be seen from the graph, activity was increased with the increasing concentration of the samples. The free radical scavenging activity of leaf essential oil of *A. malaccensis* revealed percentage inhibitions of 21.77, 31.53, 52.26 and 81.70% at concentrations of 5, 10, 20 and 50 µg/ml respectively.

Assessed essential oil was able to reduce the stable violet DPPH radical to the yellow DPPH-H, reaching 50% of reduction with IC_{50} value 18.26 µg/ml. In addition, the major component of the oil i.e. α-phellandrene has shown better activity having IC_{50} value of 12.5µg/ml. We assume that the higher antioxidant activity of the leaf oil may be due to its major compound. Lower IC_{50} value indicates higher antioxidant activity. The result showed that, the inhibition on DPPH radical scavenging assay of the essential oils was higher as compared to the methanolic leaf extract of the of *A.malaccensis*.

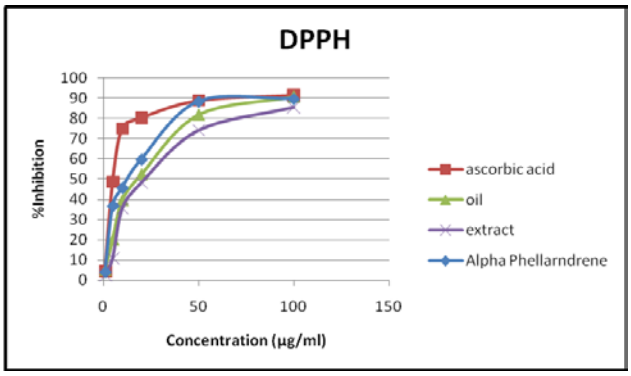

Fig. 2: The DPPH radical scavenging activity of essential oil and methanolic extract of *Alpinia malaccensis.*

ABTS radical scavenging activity

The leaf oils and extract of *A. malaccensis* were fast and effective scavengers of the ABTS radical (Fig-3). The oil, α-phellandrene and extract exhibited potent scavenging effects against ABTS with an IC_{50} value of 20 µg/ml, 20 µg/ml and 26.23 µg/ml respectively which is almost equivalent to that of standard BHT (IC_{50} value 19.47 µg/ml).

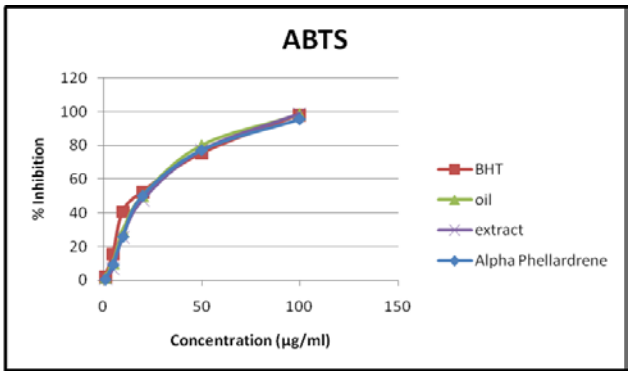

Fig. 3: The ABTS radical scavenging activity of essential oil and methanolic extract of *Alpinia malaccensis.*

The plant extracts showed higher inhibitory activity in removing ABTS radicals from the reaction system at100 µg/ml. The ABTS assay is based on the inhibition of the absorbance of radical cation, $ABTS^{+}$, which has a characteristic wavelength at 734 nm, by antioxidants. The principle behind the technique involves the reaction between ABTS and potassium per sulphate to produce the ABTS radical cation ($ABTS^{+}$) which is a blue green chromogen. In the presence of antioxidant reluctant, the coloured radical is converted back to colourless ABTS [20]. ABTS de-colorization is a system from potassium persulfate and ABTS. Thus, any active compound in *A. malaccensis* must be act with ABTS radical directly. In this study the oil (with the major compound α-phellandrene) was found to have better activity than methanolic extract of leaf.

There have been reports that the antioxidant activities from the Zingiberaceae family are from less polar constituents isolated such as curcuminoid, kava pyrones and gingerols [22-23]. Recently, Chan reported the antioxidant activity from methanol extracts of the leaves of five *Alpinia* species [24]. *Alpinia zerumbet, A. purpurata, A. zerumbet 'Variegata', A. malaccensis* and *A. galanga* displayed low to high radical scavenging activity ranging from 90 to 2180 mg AA/100 g. Among these *Alpinia* species, leaf of *Alpinia zerumbet* showed high radical scavenging activity with value of 2180 mg AA/100 g.

In general, antioxidant properties of extracts and essential oils depend on their chemical components, their structural features, concentration, temperature, light, type of substrate and physical state of the system, as well as on micro components acting as a pro-oxidant or synergists [25]. It has been described that the kinetic behaviour of some compounds can affect radical scavenging abilities [26]. For slow reacting compounds, the influence was attributed to the complex reacting mechanism.

In our study, antioxidative capacities of the essential oil were attributed to α-phellandrene and β-cymene, both presented in the oil at relatively high amount. Also activity in methanolic extracts of leaf was positively correlated with the higher phenolics content. Most likely, the major constituent from methanolic extracts of leaf of *A. malaccensis* is involved in one or more secondary reactions, which resulted in the slower reduction of DPPH solutions.

Antimicrobial activity

From the preliminary screening studies by disc diffusion method, it was observed that the test pathogens were susceptible to both oil and extract. However a difference in the zone sizes were observed with different pathogens (Table-2).Oil showed better activity against all organisms than extract. The MIC of the oil and extract were ranged between 1-7 µl/ml and 2-10 µl/ml respectively (Table-2).Though a variance was observed in the zones of inhibition and the MIC values, the three test pathogens except *S. aureus* were killed at same time i.e. took more than 24h while *S. aureus* was killed after 15 min with oil (Fig 4-7). All the test pathogens were sensitive to all the antibiotics tested.

The results of this study suggest that the antimicrobial activity of the essential oil of *A. malaccensis* is bactericidal against *S. aureus*. The oil was significantly effective [$p<0.05$] against the test pathogens. Bacterial and fungal susceptibility towards the oil and extracts were observed at 2.5 µl per disc but higher concentrations showed larger zones of inhibition, when tested by agar plate technique.

The results were highly significant for all the treatments, determining MICs, MKTs and even when the activities compared with standard antibiotic. In general, there seemed to be overall agreement between the size of inhibition zones obtained by the disc diffusion method (DDM) and the minimum inhibitory concentration (MIC) values, i.e. larger zones of inhibition correlated with lower MIC values [27-28]. Since all the test pathogens were sensitive to the specific antibiotic studied; the microbicidal activity of the oil and extract could be attributed as multidimensional. The antimicrobial activity of some essential oils and extracts against food-borne pathogens, including mycotoxin producing fungi, has also been tested [29-30]. However in our investigation, for the first time we have documented the antimicrobial activity of leaf oil and extracts of *A. malaccensis*. Of course, other studies are highly necessary to study the toxicity of these oils and extracts in order to set an appropriate formulation for novel drugs.

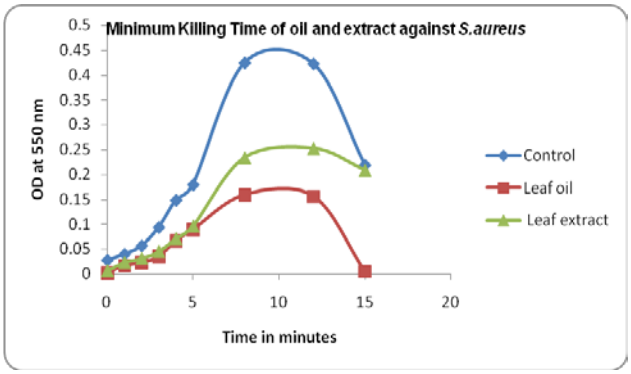

Fig. 4: Minimum Killing time of oil and extract against *S. aureus*

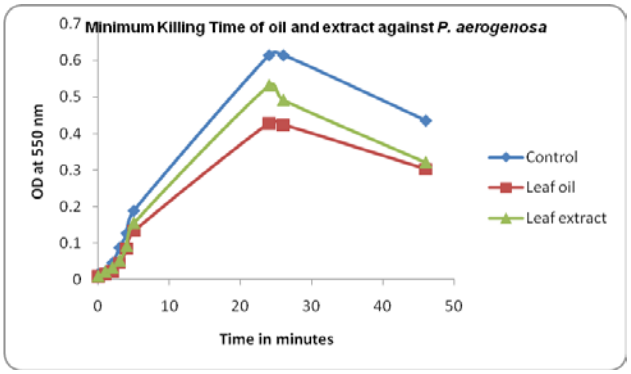

Fig. 5: Minimum Killing time of oil and extract against *P. aerogenosa*

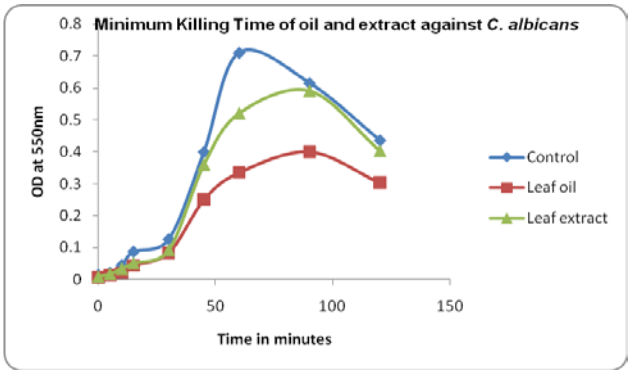

Fig. 6: Minimum Killing time of oil and extract against *C. albicans*

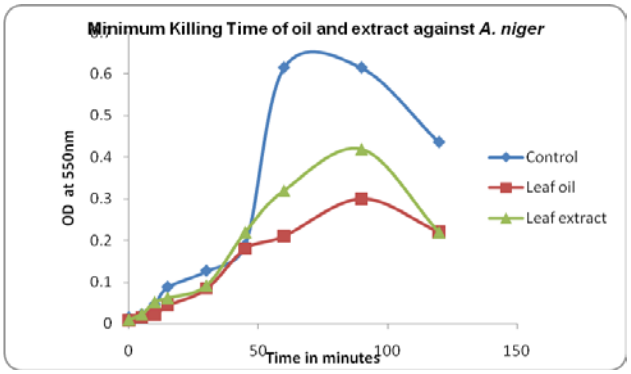

Fig. 7: Minimum Killing time of oil and extract against *A.niger*

Table 2: Antimicrobial activity of leaf oil and extract by Disc Diffusion Method (DDM) and Minimum inhibitory concentration (MIC) assay

Micro-Organism	Mean zone sizes in mm by DDM* using oil at			Mean zone sizes in mm by DDM* using extract at 5 µl			Inhibition zone diameter of gentamycin in mm	MIC of oil (µl/ml)	MIC of extract (µl/ml)
	2 µl	5 µl	10µl	2 µl	5 µl	10µl			
S. aureus	26.12±0.22	35.33 ± 0.57	42.25±0.57	10±1	28 ± 1	34.4±0.2	17±1	1.95	2.5
P. aeruginosa	8.24±0.03	12.33 ± 0.57	17.75±0.57	4±1	10 ± 1	18.67±0.57	25.67±0.57	7.81	10
C. albicans	13.63±1	24.67 ± 0.57	30.26±0.57	10.14±0.57	19.67 ± 0.57	24.93±0.34	27.67±0.57	5.5	8.5
A. niger	6.98±0.57	16.33 ± 0.57	21.24±0.57	5.28±0.57	12.33 ± 0.57	18±1	25±1	6.7	9.5

* Mean ± standard deviation (SD) where n=3 and data is significant at P<0.05.

CONCLUSION

GC-MS analysis of *A. malaccensis* leaf essential oil showed major compounds as α-phellandrene, β-cymene, β-pinene, caryophyllene. There were many other compounds in minor amounts. The essential oil and methanolic extracts of leaves possess good antioxidant activity. In addition, the oil and extract exhibited a broad spectrum of antimicrobial activities against four microbes. With high antioxidant activity and antimicrobial activity, leaves of these *A. malaccensis* species can be developed into skin-lightening products and natural preservatives to inhibit food spoilage. The leaf oil and extract of *Alpinia malaccensis* exhibited strong antioxidant and antimicrobial activity thereby implying its potential in providing protection against oxidative diseases and its use as a natural antioxidant and antimicrobial agent in food and confectionary industries.

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CONFLICT OF INTEREST

We do not have any conflict of interest

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