Research Article

GCMS analysis, antioxidant and antibacterial activities of ethanol extract of *Anisomeles malabarica* (L.) R.Br. ex. Sims leaves

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Received: 1 October 2018 Revised: 24 October 2018 Accepted: 7 November 2018

Abstract

Objective: The leaves of *Anisomeles malabarica* belonging to the family Lamiaceae was evaluated for antioxidant and antimicrobial activities. **Materials and methods:** The ethanol extract of leaves of *Anisomeles malabarica* revealed the presence of alkaloids, phenols, steroids, glycosides, etc. The total phenolic content and total flavonoid content were carried out by Folin-ciocalteau reagent and $AlCl_3$ reagent methods. The antioxidant activities of ethanol extract of *Anisomeles malabarica* were carried out by $DPPH^{\cdot}$ radical, Superoxide radical (O_2^{-}) and $ABTS^{+}$ radical cation scavenging assays, Phosphomolybdenum reduction and Fe^{3+} reduction assays. **Results:** The IC_{50} of $DPPH^{\cdot}$ radical, Superoxide radical (O_2^{-}) and $ABTS^{+}$ radical cation scavenging assays were 94.18, 65.31 and 40.84 µg/mL concentration respectively. Also, the RC_{50} of phosphomolybdenum reduction and Fe^{3+} reduction were 19.16 and 34.98 µg/mL concentration respectively. The antibacterial activity for ethanol extract of leaves of *Anisomeles malabarica* was carried out against bacterial pathogens such as *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris*. The major findings of GCMS analysis were Phenol, 2, 4-bis (1, 1-dimethylethyl)-, Oleic acid, Coumarine, 3-[2-(1-methyl-2-imidazolylthio)-1-oxoethyl]- exhibiting significant therapeutic applications. **Conclusion:** The results provide justification for the uses of ethanol extract of *Anisomeles malabarica* to treat various infectious diseases. Further the ethanol extract could be turned as an effective drug for cancerous cells.

Keywords: Free radicals, $ABTS^{\cdot+}$ radical cation, superoxide radical (O_2^{-}), RC_{50}, agar well diffusion, GCMS

Introduction

Anisomeles malabarica R.Br. Ex Sims is an aromatic, densely pubescent, perennial herb, 1.2–2.0 m in height belonging to the family Lamiaceae. It is commonly found in Western Ghats from Maharashtra to Karnataka, Andhra Pradesh, Kerala and Tamil Nadu. The plant is reported to possess anti-periodic, diaphoretic, emmenagogue properties. Ethno botanically, the leaves of the plant are used against convulsions, dyspepsia in intermittent fevers, colic, boils and tetanus. The herb is also reported to be useful in inflammation, cough, cold, stomachache, itches and uterine affections. *Anisomeles malabarica* R.Br. Ex Sims is also known to possess antifertility, antispasmodic, anticancer, diuretic, antimicrobial and anticonvulsant activities (Yuan et al., 2016).

Traditional medicines have great importance with the usage of available natural products such as Ayurveda, unani, traditional chinese and Korean medicine. They have emerged in orderly-regulated systems of medicine. In their various forms, they may have certain defects, but they are still a valuable repository of human knowledge (Fabricant and Farnsworth, 2001; Alves and Rosa, 2007). According to an estimate, only 5-15 % of terrestrial plant species have been investigated pharmacologically. 10,000-15,000 of world's plant species have been reported as medicinal plants and about 150-200 species have been integrated into western medicine. About 25% of all medicines today have plants origins (Gurnani et al., 2014).

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Materials and methods

Collection of plant material and preparation of extracts

Leaves of *Anisomeles malabarica* were collected from Thiruvanamalai regions, Tamilnadu. Extraction of leaves in

DOI: https://doi.org/10.31024/ajpp.2019.5.1.26

Figure 1. Habitat of *Anisomeles malabarica*

ethanol was performed by maceration method (Trease and Evans, 1983). In this method, coarse powdered leaves were soaked in ethanol for 72 hours. The supernatant was filtered using filter paper and the above soaking process was repeated further two times in the same leaves material but with fresh ethanol. All supernatants collected together, condensed by rotary evaporator at 50ºC, which yields dark gummy mass and weighed. The extracted residues were weighed and re-dissolved in suitable solvents to yield 1mg/mL solutions ready for further analysis.

Qualitative phytochemical analysis of *Anisomeles malabarica*

Screening of phytochemicals for *Anisomeles malabarica* (ethanol extract) was carried out comparatively using standardized methods (Harborne, 1978; Raaman, 2006). Understanding the presence of active phytoconstituents would be a definite pathway for evaluating the therapeutic purpose.

Quantitative estimations of total phenols and flavonoids

Determination of total Phenols

Folin-Ciocalteau reagent method was used to determine the total phenolic compounds with slight modifications (Spanos and Wrosltad, 1990). One hundred µL of ethanol extract of *Anisomeles malabarica* (1mg/mL) was mixed with 900 µL of distilled water and 1 mL of Folin-Ciocalteau reagent (1:10 diluted with distilled water). After 5 mins, 1 mL of Na_2CO_3 (20% w/v) solution was added. The mixture was then allowed to stand for 30 mins incubation in dark at room temperature. The absorbance was measured by UV-vis spectrophotometer at 765 nm. The total phenolic content was expressed in terms of gallic acid equivalent (µg/mg of extract), which is a common reference compound.

Determination of total flavonoids

The total flavonoid content of ethanol extract of *Anisomeles malabarica* was determined using aluminium chloride reagent method with slight modifications (Liu et al., 2007). Five hundred µL of extract (1mg/mL) was mixed with 0.5 mL of methanol and 0.5 mL of (5% w/v) sodium nitrite solution. Then, 0.5 mL (10% w/v) aluminium chloride solution was added followed by 1 mL of 1M NaOH. The mixture was incubated for 30 minutes at room temperature and the absorbance was measured at 510 nm. The result was expressed as (µg/mg of extract) quercetin equivalent.

In vitro antioxidant activities of *Anisomeles malabarica*

(a) Free radical Scavenging Activity

The antioxidant activity was determined by DPPH scavenging assay in which various concentrations of ethanol extract of *Anisomeles malabarica* was been pipetted out in clean test tubes (Blois, 1958). Freshly prepared DPPH (1, 1-Diphenyl-2-picryl hydrazyl) solution (1mL) was added to each tube and the samples were incubated in dark at 37°C for 20 mins and read at 517 nm. The data were expressed as the percent decrease in the absorbance compared to the control. Ascorbic acid was used as reference compound. The percentage inhibition of radical scavenging activity was calculated.

$$\%\ \text{of DPPH}^{\cdot}\ \text{radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

(b) Superoxide radical (O_2^-) scavenging activity

Superoxide radical (O_2^-) scavenging activity was carried out and the reaction mixture contains 1 mL of different concentrations of ethanol extract of *Anisomeles malabarica* with 50 mM of phosphate buffer (pH 7.4), 200 µL of 1.5 mM of riboflavin, 200 µL 12 mM of EDTA and 100 µL 50 mM of NBT, added in that sequence (Lokesh Deb et al., 2009). The reaction was started by illuminating the reaction mixture for 15 min in UV lamp. After illumination, the absorbance was measured at 590 nm and the IC_{50} was calculated. Ascorbic acid was used as positive control.

$$\%\ \text{of Superoxide radical } (O2^{\bullet -})\ \text{inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

(c) $ABTS^+$ (2, 2-azinobis (3-ethylbenzo thiazoline-6-sulfonic acid) radical cation scavenging activity

The ethanol extract of *Anisomeles malabarica* from the

stock solution was taken in various concentrations and this assay was performed according to the method (Delgado-Andrade et al., 2005). The stock solutions included 7.4 mM ABTS solution and 2.6 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 hours at room temperature in the dark. Fresh ABTS solution was prepared for each assay. Plant extract of varying concentration were allowed to react with 500 µL of the ABTS solution for 15 minutes in dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. The $ABTS^{+}$ radical cation scavenging activity was calculated as:

$$\%\ \text{of}\ ABTS^{\bullet+}\ \text{radical cation inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

(d) Phosphomolybdenum reduction assay

Total antioxidant capacity can be calculated in which various concentrations of ethanol extract of *Anisomeles malabarica* from the prepared sample (1mg/mL) was been pipetted out and 1mL of the reagent solution was added, followed by incubation in boiling water bath at 95°C for 90mins (Prieto et al., 1999). After cooling the sample to room temperature, the absorbance of the solution was measured at 695 nm in UV spectrophotometer. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions. Ascorbic acid served as standard.

$$\%\ \text{of Phosphomolybdenum Reduction} = \frac{\text{Sample} - \text{Control}}{\text{Sample}} \times 100$$

(e) Ferric (Fe^{3+}) reducing power assay

The ethanol extract of *Anisomeles malabarica* was taken in various concentrations and was mixed with 1mL of phosphate buffer (0.2M, pH-6.6) and 1mL of potassium ferricyanide (1% w/v), and incubated in water bath at 50°C for 30 mins. Then, 0.5mL of trichloroacetic acid (10% w/v), 0.5mL $FeCl_3$ (0.01% w/v) was added to the mixture and then centrifuged at 3000 rpm for 10 mins and the absorbance was measured at 700 nm (Oyaizu, 1986). Ascorbic acid served as standard.

$$\%\ \text{of}\ Fe^{3+}\ \text{Reduction} = \frac{\text{Sample} - \text{Control}}{\text{Sample}} \times 100$$

Thin layer chromatography analysis

Thin layer chromatography (TLC) analysis was carried out for on silica gel aluminium sheet ethanol extract of *Anisomeles malabarica* (Merck Silica gel 60 F254) (Stahl, 2005). Each extract was spotted at 0.5 mm above from the bottom of the TLC plate. The spotted TLC plate was placed in a 100mL beaker containing solvent mixture. The chromatogram was developed and the spots were visualized under UV light at 254 nm as well as in iodine vapour. The ratio in which distinct coloured bands appeared was optimized and R_f values were calculated.

R_f = Distance travelled by the solute/ Distance travelled by the solvent

Screening of crude extract for antibacterial activity

Agar well diffusion assay

Nutrient agar was prepared and poured in the sterile petri dishes and allowed to solidify. 24 hours grown bacterial pathogens were swabbed on nutrient agar plates (Eloff, 1998). Then, the stock crude of ethanol extract of *Anisomeles malabarica* individually (10mg/mL) was prepared in sterile test tubes. Varying concentration (250µg, 350µg, 450µg) of ethanol extract was loaded in the wells made using sterile Cork borer. The preferred solvent (ethanol) was used as control. Tetracycline was used as standard. The plates were then incubated at 37ºC for 24hours. After incubation the inhibition diameter was measured using zone scale.

Identification of bioactive compounds by Gas chromatography-Mass spectrometry analysis

The presence of active compounds were been confirmed by thin layer chromatography and the compounds were identified using gas chromatography and mass spectrometry (GC-MS) method, (TSQ QUANTUM XLS). The name of the instrument is Gas Chromatography-Mass Spectrometry and the instrument made is of Thermo scientific. The software required for analytical studies is XCALIBUR (ver-2.2). The column size is of TG-5MS (30mX0.25mmX0.25um). The injector temperature and interface temperature (°C) was at 280°C.

Statistical analysis

The experiments were conducted in duplicates and the data entered in tables were average of two replicates. The data mentioned were reported as the mean ± standard deviation of two replicates.

Results and discussion

In vitro* antioxidant activities of *Anisomeles malabarica

(a) Free radical scavenging activity

The antioxidant activity was carried out by DPPH assay according to the method of (Blois, 1958). Antioxidant molecules can quench DPPH free radicals (i.e by providing hydrogen atoms or by electron donation, via a free radical

Table 1. DPPH$^{\cdot}$ radical and Superoxide radical (O_2^{-}) scavenging activity of ethanol extract of leaves of *Anisomeles malabarica*

Concentration (µg/mL)	% of inhibition	
	DPPH$^{\cdot}$ radical	Superoxide radical ($O_2^{\bullet-}$)
20	19.71±0.43	28.16±0.32
40	27.42±0.36	32.86±0.38
60	38.59±0.25	45.93±0.21
80	42.47±0.28	55.47±0.45
100	47.86±0.10	69.05±0.17
120	51.95±0.18	74.58±0.29

attack on the DPPH molecule) and convert them to colourless. The percentage of DPPH scavenging activity was found to be higher as 51.95±0.18 at 120µg/mL for ethanol extract of *Anisomeles malabarica* (Table 1). The IC_{50} value for ethanol extract was found to be 94.18µg/mL concentration (Figure 2) and was compared with standard (Ascorbic acid, IC_{50} value as 12.83µg/mL concentration).

(b) Superoxide radical (O_2^{-}) scavenging activity

The superoxide anion radical-scavenging activity of the extract may be due to the presence of phenolic compounds. Generation of super oxide radical is by auto oxidation of riboflavin in presence of light and thereby reduces Nitro Blue Tetrazolium (NBT). The percentage of superoxide radical (O_2^{-}) scavenging activity was found to be higher as 74.58±0.29 at 120µg/mL for ethanol extract of *Anisomeles malabarica* (Table 1). The IC_{50} value for ethanol extract was found to be 65.31µg/mL concentration (Figure 2) and was compared with standard (Ascorbic acid, IC_{50} value as 9.15µg/mL concentration).

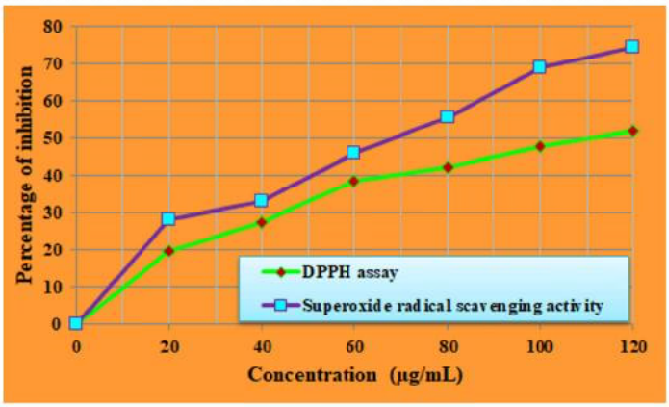


Figure 2. DPPH$^{\cdot}$ radical and Superoxide radical (O_2^{-}) scavenging activity of ethanol extract of leaves of *Anisomeles malabarica*

(c) ABTS^{+} radical cation scavenging activity

ABTS^{+} (2,2 – azinobis (3-ethylbenzo thiazoline-6-sulfonic acid) assay measures the relative ability of antioxidant to scavenge the ABTS generated in aqueous phase (Table 2). ABTS is generated by reacting with a strong oxidizing agent (Potassium per sulfate) with ABTS salt. Reduction of blue green ABTS-radical coloured reaction by hydrogen-donating antioxidant is measured at 734 nm (Figure 3). The maximum ABTS^{+} radical cation scavenging activity of ethanol extract of *Anisomeles malabarica* was found to be 59.28±0.11 at 60µg/mL concentration. The IC_{50} value for ethanol extract of *Anisomeles malabarica* was found to be 40.84µg/mL concentration and was compared with standard Ascorbic acid (IC_{50} value as 6.43µg/mL concentration).

Table 2. ABTS^{+} radical cation scavenging activity of ethanol extract of leaves of *Anisomeles malabarica*

Concentration (µg/mL)	% of inhibition
	ABTS$^{\bullet+}$ radical cation
10	28.05±0.35
20	37.19±0.26
30	42.36±0.20
40	48.97±0.45
50	55.42±0.50
60	59.28±0.11

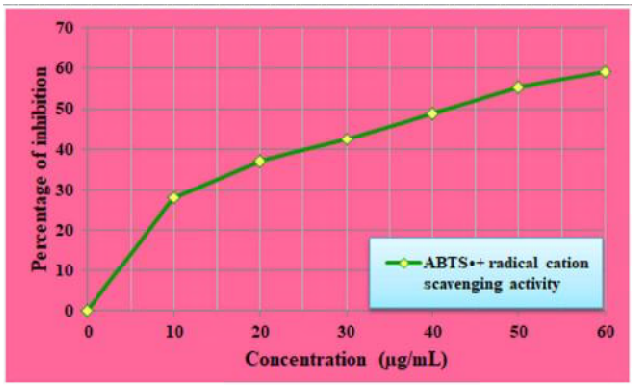


Figure 3. ABTS^{+} radical cation scavenging activity of ethanol extract of leaves of *Anisomeles malabarica*

(d) Phosphomolybdenum assay

The total antioxidant activity of ethanol extract of *Anisomeles malabarica* was measured spectrophotometrically by phosphomolybdenum reduction method which is based on the reduction of Mo (VI) to Mo (V) by the formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption at 695 nm. The maximum

reducing ability for ethanol extract of *Anisomeles malabarica* was 86.08±0.37 at 120µg/mL concentration (Figure 4). The experiment demonstrated higher antioxidant activity the RC_{50} of 19.16µg/mL concentration for ethanol extract of *Anisomeles malabarica* (Table 3) and was compared with standard Ascorbic acid (RC_{50} value as 15.37µg/mL concentration).

(e) Ferric (Fe^{3+}) reducing power assay

The antioxidant activity of ethanol extract of *Anisomeles malabarica* was calculated according to Oyaizu, 1986. The inhibition in reducing power assay denotes the yellow color of the test solution changes to various shades of green and blue depends upon reducing power of each compound. The maximum reducing ability for ethanol extract of *Anisomeles malabarica* was 79.42±0.11 at 120µg/mL concentration (Figure 4). The RC_{50} value for ethanol extract of *Anisomeles malabarica* was found to be 34.98µg/mL concentration (Table 3) and was compared with the standard (27.46µg/mL concentration) Ascorbic acid.

Qualitative phytochemical analysis of *Anisomeles malabarica*

The results of phytochemical analysis for ethanol extract of *Anisomeles malabarica* showed the presence of phenols, terpenoids, tannins, steroids (Table 4) and was quantified.

Table 3. Phosphomolybdenum and Fe^{3+} reduction of ethanol extract of *Anisomeles malabarica*

Concentration (µg/mL)	% of reduction	
	Phosphomolybdenum reduction	Fe^{3+} reduction
20	52.19±0.38	40.77±0.35
40	67.92±0.29	57.17±0.26
60	69.50±0.46	64.26±0.20
80	77.46±0.31	74.19±0.45
100	82.49±0.23	77.74±0.50
120	86.08±0.37	79.42±0.11

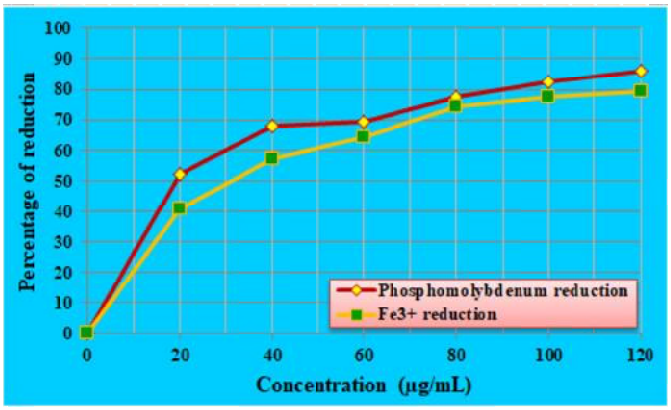


Figure 4. Phosphomolybdenum and Fe^{3+} reduction of ethanol extract of *Anisomeles malabarica*

Quantitative estimations of total phenols and flavonoids

Total phenolic content was found to be 278.6±0.42 µg/mg of GAE for ethanol extract of *Anisomeles malabarica*. Total flavonoid content was 61.78±0.36 µg/mg of QE for ethanol extract of *Anisomeles malabarica*. From the results, it is significant that due to presence of higher phenolic content and flavonoid content, antioxidant and antibacterial activities were found to be higher for ethanol extract of *Anisomeles malabarica.*

Table 4. Qualitative analysis of ethanol extract of leaves of *Anisomeles malabarica*

Phytochemicals	Tests	Results
Alkaloids	Mayer's test	+
	Hager's test	+
Phenols	Ferric chloride (5%) test	+
Tannins	Ferric chloride (0.1%) test	+
Flavonoids	Sodium hydroxide test	+
Glycosides	Legal's test	+
Steroids	Libermann-Burchard test	+
Terpenoids	Salkowski test	+
Saponins	Foam test	+

Thin layer chromatography analysis

Thin layer chromatography analysis was carried out in the solvent system of Toluene (0.2mL): Chloroform (1.8mL) (Figure 5). The separated active compounds were visualized in UV light and iodine balls. The R_f values of the separated compounds were found to be 0.83 and 0.72.

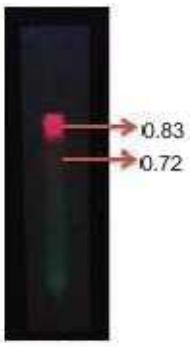


Figure 5. Thin Layer Chromatography of ethanol extract of *Anisomeles malabarica*

Screening of crude extract for antibacterial activity

Agar well diffusion assay

After 24hours of incubation, the inhibition diameter was measured using zone scale. The maximum inhibition for ethanol extract of *Anisomeles malabarica* was against

Escherichia coli (16mm), *Shigella flexneri* (15mm), *Staphylococcus aureus* (14mm) (Table 5 and Figure 6). From the above results it's proven that ethanol extract of *Anisomeles malabarica* could be used as broad spectrum of antibiotics to treat bacterial infections.

Identification of bioactive compounds by Gas chromatography-Mass spectrometry analysis

Table 5. Antibacterial activity of ethanol extract of *Anisomeles malabarica*

Bacterial pathogens	Control (Ethanol)	Zone of inhibition (mm)			
		Standard (Tetracycline)	250 µg/mL	350 µg/mL	450 µg/mL
S. aureus	-	14	13	13	14
B. subtilis	-	16	13	13	14
S. flexneri	-	13	14	14	15
P. vulgaris	-	12	11	12	12
E. coli	-	13	15	16	16

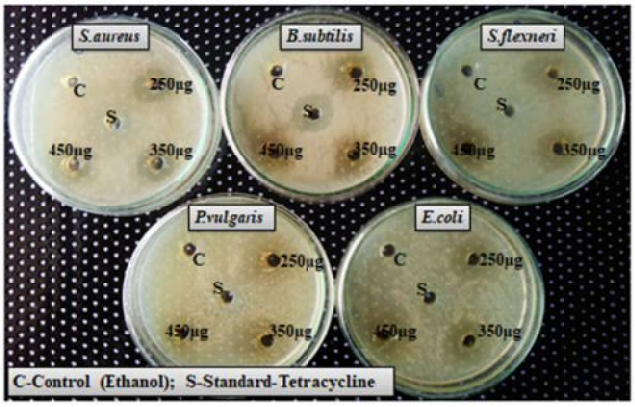
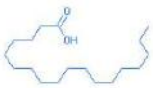
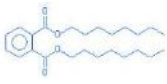
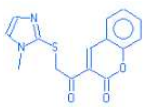


Figure 6. Antibacterial activity of ethanol extract of *Anisomeles malabarica*

The GCMS analysis for ethanol extract of *Anisomeles malabarica* revealed the presence of phytoconstituents

Table 6. GC-MS analysis of ethanol extract of *Anisomeles malabarica*

S. No	Compounds Name	RT	Compounds Structure	Molecular Weight	Molecular Formula (g/mol)
1	Phenol,2,4-bis(1,1-dimethylethyl)-	12.03		206	$C_{15}H_{24}O$
2	1H-Imidazo(4,5-c)pyridine,2-(3,4-dimethoxyphenyl)-	15.7		255	$C_{15}H_{14}N_2O_2$
3	3,7-Dimethyl-6-nonen-1-ol acetate	16		212	$C_{13}H_{24}O_2$
4	Hexadecanoic acid, methyl ester	16.82		270	$C_{17}H_{34}O_2$
5	Pentadecanoic acid, methyl ester	17.48		256	$C_{16}H_{32}O_2$
6	Oleic acid	18.38		282	$C_{18}H_{34}O_2$
7	Octadecanoic acid	19.15		284	$C_{18}H_{36}O_2$
8	Di-n-octyl phthalate	22.35		390	$C_{24}H_{38}O_4$
9	Phenol,2,6-bis(1,1-dimethylethyl)-4-[(4-hydroxy-3,5-dimethylphenyl)methyl]-	24		340	$C_{29}H_{44}O_2$
10	2H-Naphtalen-1-one, 3,4-dihydro-6-methoxy-2-(4-methoxybenzylideno)-	19.78		293	$C_{19}H_{18}O_3$
11	Coumarine,3-[2-(1-methyl-2-imidazolylthio)-1-oxoethyl]-	20.98		300	$C_{14}H_{12}N_2O_3S$

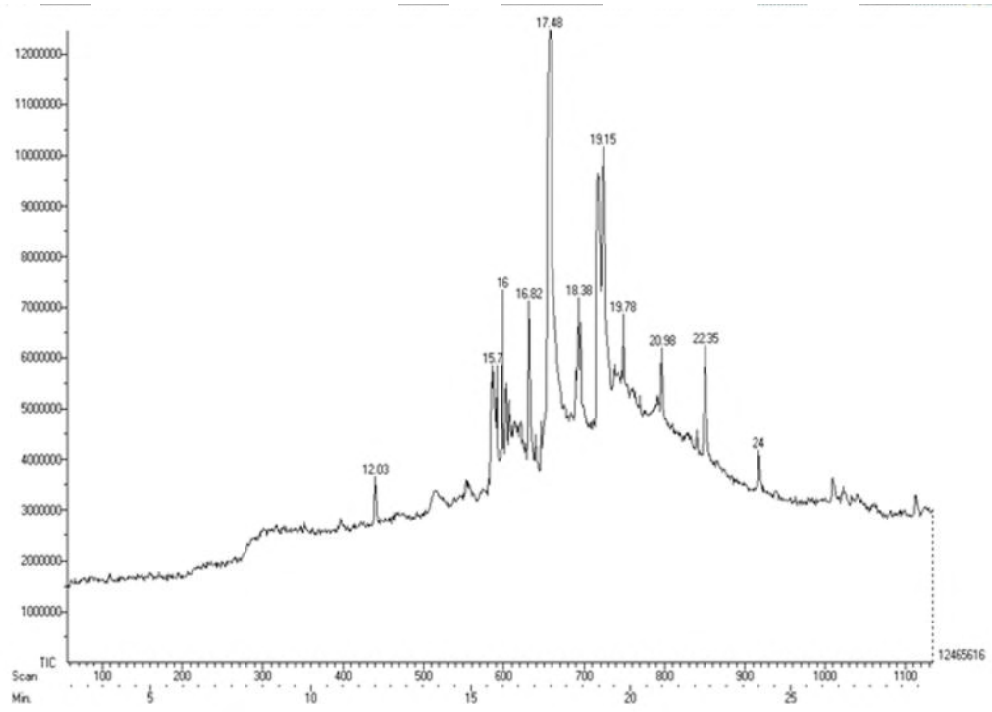


Figure 7. GCMS Chromatogram of ethanol extract of *Anisomeles malabarica*

(Table 6 and Figure 7) such as Phenol, 2,4-bis(1,1-dimethylethyl)-, Hexadecanoic acid, methyl ester, Oleic acid, Coumarine,3-[2-(1-methyl-2-imidazolylthio)-1-oxoethyl]- exhibiting biological activities (Table 7).

Conclusion

From the present research study, plant derived secondary metabolites possessed potent antioxidant and antimicrobial property. Naturally available phytomolecules have been widely used both invitro and invivo purpose. Usage of natural phytoconstituents for cancer therapy and treatment has been a promising and curable approach. In future, the pharmacokinetic and pharmacodynamic interactions, metabolic activity, toxicity response, clinical trials could be performed for the pure compound from fraction isolated from column chromatography with validated structure of the active drug.

Acknowledgement

The authors wish to thank Armats Biotek Training and Research Institute and SAIF, IIT, Madras for providing necessary facilities needed for the research.

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