BIOLOGICAL PROPERTIES AND CHEMICAL INVESTIGATION OF *MURRAYA KOENIGII* L. SPRENG LEAVES (PYINDAW-THEIN)

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Abstract

In the present work, locally grown *M. koenigii* leaves were selected to investigate the chemical constituents and some bioactivities. The main aim of this research was to study the chemical constituents of essential oil and some biological activities such as antioxidant, antimicrobial, and antiproliferative activities of leaves. The preliminary phytochemical tests indicated that various types of compounds, such as alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, and terpenoids were present in the selected leaves samples, except for flavonoids, tannins, and starch. The essential oil (2.44%) was extracted from the leaves of *M. koenigii* by steam distillation method. The total phenolic content was found to be 2.73 ± 0.004 µg GAE/mg in the ethanol extract and 2.56 ± 0.003 µg GAE/mg in the watery extract by the Folin-Ciocalteu assay. The antioxidant activity of an ethanol extract (IC_{50} = 34.84 µg/mL) was found to be more effective than watery extracts (IC_{50} = 42.92 µg/mL) by the DPPH assay. The antimicrobial activity of the leaves was screened by using the agar well diffusion method. The PE, EtOAc, and EtOH extracts showed antimicrobial activity against all tested microorganisms (16 to 18 mm). *In vitro* antiproliferative activity of ethanol and watery extracts was tested on five cancer cell lines by the CCK-8 assay. The ethanol extract inhibited the growth of MCF7 (IC_{50} 1 µg/mL), A549 (IC_{50} 1 µg/mL), Kato III (IC_{50} 15.3 µg/mL), GSU (IC_{50} 1 µg/mL), and Hela (human cervical cancer) (IC_{50} 1 µg/mL). The watery extract, was tested against MCF7 (IC_{50} > 100 µg/mL), A549 (IC_{50} 62.1 µg/mL), Kato III (IC_{50} > 100 µg/mL), GSU (IC_{50} 9.6 µg/mL) and Hela (IC_{50} > 100 µg/mL). GC-MS analysis of essential oil from *M. koenigii* leaves showed the presence of *p*-xylene, α-methyl styrene, azulene, 3-carene, methyl hexadecanoate, 9,12- octadecadienoate, 9-octadecadienoate, methyl stearate and methyl eicosanoate respectively.

Keywords: essential oil, antioxidant activity, antimicrobial, antiproliferative activity

Introduction

Plants have a significant role in maintaining human health and improving the quality of human life. The world health organization (WHO, 1999) estimated that 80 % of people rely on traditional medicine. *M. koenigii* leaf is an important leafy vegetable with many uses. It is belonging to the family Rutaceae based on semi-evergreen aromatic trees found throughout Malaysia, India, Bangladesh, Nepal, Sri Lanka, and Myanmar. It is commonly known as curry leaves. *M. koenigii* is widely used in Indian cookery for centuries and has a versatile role to play in traditional medicine. *M. koenigii* has various notable pharmacological activities in the plant such as activity on the heart, antidiabetic and cholesterol-reducing properties, antimicrobial activity, antiulcer activity, antioxidative property, cytotoxic activity, antidiarrhea activity, and phagocytic activity. The chemical composition of the fresh leaves of *M. koenigii* consists of volatile oil. *M. koenigii* is most popular on account of its diverse medicinal properties and its use as a flavorings agent in different curries and foods since ancient times. The leaves of the plant are used traditionally in the Indian Ayurveda system to treat diabetes (Pehlivan *et al*., 2012).

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Materials and Methods

Plant materials

The selected sample used in this study was the curry leaves of *M. koenigii*. The sample was collected in Kamayut Township and identified at the Department of Botany, University of Yangon. The collected sample was washed with water and dried in the air. The dried pieces were made into powder by using a grinding machine. The powder sample was stored in an airtight container to prevent moisture change and other contamination.

Chemicals

Chemicals were procured from the BDH and E. Merck.

Instruments

Autoclave, incubator, distillation set, vacuum rotatory evaporator

Preliminary Phytochemical Investigation

Phytochemical tests for the leaves of *M. koenigii* were carried out according to the reported methods to investigate the presence and absence of phytoconstituents such as alkaloids, α-amino acid, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins, and terpenoids (M-Tin Wa, 1972 Marini-Bettolo *et al.*, 1981).

Preparation of Crude Extracts

100 g of dried powdered sample was percolated in 500 mL of petroleum ether (PE, 60–80 °C) for one week and filtered. This procedure was repeated three times. Then the filtrate was concentrated by a vacuum rotatory evaporator to get the respective PE extract. Similarly, ethyl acetate and 70% EtOH extracts of the dried powdered sample were prepared according to the above procedure. The crude extract was then dried and stored in a desiccator after each solvent was removed using a rotary evaporator. In the preparation of watery extract 100 g of dried powdered sample was soaked in 500 mL of distilled water in a conical flask. These flasks were boiled in a water bath for 6 h and filtered. This process was carried out three times. The combined filtrates were dried to dryness over a water bath at 100 °C to get the corresponding watery extract.

Extraction of Essential Oil by Steam Distillation Method

The essential oil was extracted from the leaves of *M. koenigii* by the steam distillation method (Srivastava *et al.*, 2003). The sample of the fresh leaves of curry (100 g) was placed in the insert of a glass jacket. The glass jacket was filled with distilled water. The glass jacket was fitted to the set which was joined to the water condenser. When the glass jacket was heated, the condensed oil was collected in the receiver flask. The oil extracted with n-hexane was evaporated at 60-70 ˚C to get essential oil. Organic constituents in essential oil were detected by GC-MS spectroscopic method at Research and Innovation, National Analytical Laboratory.

Determination of Total Phenol Content

The total phenol content (TPC) in each crude extract was determined by the Folin-Ciocalteu reagent method (Song *et al.*, 2010). First, 0.5 mL of each extract solution was mixed with 5 mL of FCR reagent (1:10) and incubated for 30 min. 4 mL of 1 M sodium carbonate

solution was added to each tube and the tubes were kept at room temperature for 2 h. The UV absorbance reaction mixture was read at λ_{max} 765 nm. The blank solution was prepared by using distilled water instead of the sample solution. Total phenol contents were estimated as microgram gallic acid equivalents per milligram of different extracts (µg GAE / mg).

Determination of Antioxidant Activity by DPPH Assay

The DPPH radical scavenging activity of 70% ethanol and watery extracts was determined by a UV-visible spectrophotometer. The control solution was prepared by mixing 1.5 mL of ethanol and 1.5 mL of 0.002% DPPH solution in the brown bottle. The sample solution was also prepared by mixing 1.5 mL of 0.002 % DPPH solution and 1.5 mL of tested sample solution. These bottles were shaken on a shaker for 30 min and incubated at room temperature. After incubation, these solutions were measured at 517 nm and the percentage of radical scavenging activity was calculated. The antioxidant power of IC_{50} (50% inhibition concentration) values was calculated by the linear-regressive Excel program (Marinova & Batchvarob, 2011).

Antimicrobial Screening by Agar Well Diffusion Method

The antimicrobial screening of the polar and nonpolar extracts was determined by the agar well diffusion method. *Agrobacterium tumefaciens, Bacillus pumilus, Bacillus subtilis, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens,* and *Staphylococcus aureus* were used as tested organisms. Four small wells of 8 mm in diameter each were cut out of the inoculated agar to place the samples to be tested. The volume of each sample placed in each well was 0.1 mL. The Petri dishes were then incubated at 37 ºC for 48 h. The diameters of the clear inhibition zones around the well were measured (Finegold & Martin, 1982).

Determination of Antiproliferative Activity

The anticancer or antiproliferative activity of ethanol and watery extracts of *M. koenigii* leaves samples was determined against five cancer cell lines such as Hela (Cervix cancer), MCF 7 (Breast cancer), Kato III (Stomach cancer), GSU (gastric cancer), and A 549 (lung cancer) by CCK-8 assay (Fatma *et al*., 2015). The tests were done at the Department of Natural Products Chemistry, Institute of Natural Medicine, and University of Toyama, Japan.

Determination of Organic Compounds in Essential Oil from the Leaves of *M. koenigii* by GC-MS Method

Gas chromatography-mass spectrometry (GC-MS) is a method that combines gas chromatography and mass spectrometry to identify different substances with a test sample (Robert and Webster, 1998). The application of GC-MS includes the identification and quantitation of volatile and semi-volatile organic compounds in complex mixtures (James and Martin, 1952). Organic constituents in essential oil from the leaves of *M. koenigii* were detected by GC-MS spectroscopic method at Research and Innovation, National Analytical Laboratory.

Results and Discussion

Phytochemical Constituents in the Leaves of *M. koenigii*

The phytochemical constituents present in the leaves of *M. koenigii* were investigated by the test tube method. The phytochemical tests revealed that alkaloids, α-amino acid carbohydrates, cyanogenic glycosides, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, and terpenoids were present in the sample. However, flavonoids, starch, and tannins were not detected in the leaves of *M. koenigii*.

Total Phenol Content of Crude Extracts of *M. koenigii* Leaves

The presence of phenolic compounds in medicinal plants is responsible for their antioxidant and anti-inflammatory activities. The total phenol contents of ethanol and watery crude extracts of leaves of *M. koenigii* were evaluated with the spectrophotometric method using the Folin-Ciocalteu reagent. The principle of this method is the reduced ability of the phenol functional group. Gallic acid (3, 4, 5- trihydroxy benzoic acid) was used to construct a standard calibration curve at λ_{max} 765 nm. Total phenol content was expressed as micrograms of gallic acid equivalent per milligram (µg GAE/mg) and was detected in ethanol extract (2.73 ± 0.004 µg GAE/mg) rather than watery extract (2.56 ± 0.003 µg GAE/mg). The phenolic compounds were found to be more soluble in ethanol (Table 1).

Table 1. Total Phenol Content of Ethanol and Watery Extract from the Leaves of *M. koenigii*

No.	Extracts	TPC (µg GAE/mg ± SD)
1	Ethanol	2.73 ± 0.004
2	Watery	2.56 ± 0.003

GAE = Gallic acid equivalent

Antioxidant Activity of Crude Extracts of *M. koenigii* Leaves

The antioxidant activity of watery and 95% ethanol extracts of *M. koenigii* leaves was determined by using the DPPH assay method. The antioxidant activity was expressed as a 50% oxidative inhibitory concentration (IC_{50}). In this experiment, five different concentrations of each crude extract in 95 percent ethanol solvent were used: 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL. Butylated hydroxytoluene was used as a standard, and ethanol without crude extract was employed as a control. Determination of absorbance was carried out at a wavelength of 517 nm using a UV-visible spectrophotometer. The IC_{50} values were found to be 34.84 µg/mL for 95% ethanol extract and 42.92 µg/mL for watery extract. Butylated hydroxytoluene was used as a standard and the lower IC_{50} value of 95% ethanol extract was found to be more effective than the watery extract in free radical scavenging activity (Table 2).

Table 2. Antioxidant Activity (% RSA) of Ethanol and Watery Extracts from the Leaves of *M. koenigii*

Sample	% RSA±SD of different concentrations (µg/mL)						IC_{50} (µg/mL)
	31.25	62.5	125	250	500	1000	
Ethanol	47.27 ± 0.007	71.01 ± 0.002	92.22 ± 0.003	96.63 ± 0.0014	93.90 ± 0.015	87.39 ± 0.017	34.84
Water	31.93 ± 0.007	80.25 ± 0.004	85.08 ± 0.004	86.13 ± 0.000	86.4 ± 0.005	76.49 ± 0.039	42.92
Standard butylated hydroxytoluene	17.01 ± 0.009	31.16 ± 0.002	59.06 ± 0.023	77.09 ± 0.008	81.47 ± 0.002	87.28 ± 0.000	22.26

Antimicrobial Activity of Crude Extracts of *M. koenigii* Leaves

In the present work, the antimicrobial activity of extracts was tested on seven strains of bacteria: *Agrobacterium tumefaciens, Bacillus pumilus, Bacillus subtilis, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens,* and *Staphylococcus aureus*, and one strain of fungus, *Candida albicans.*The measurable zone diameter, including the agar well diameter (8 mm), shows the degree of antimicrobial activity. The greater the agar well diameter, the higher the antimicrobial activity. From the experimental results, it was found that PE, EtOAc, and EtOH extracts of leaves showed antimicrobial activity (16 -19 mm) against all tested microorganisms. This shows these extracts have good antimicrobial activity (15 -19 mm). However, the watery extract inhibited *E. coli, M. luteus, P. fluorescens,* and *C. albicans* (11 -14 mm). It indicates that watery extract has low antimicrobial activity (10 - 14 mm) (Figure 1 and Table 3).

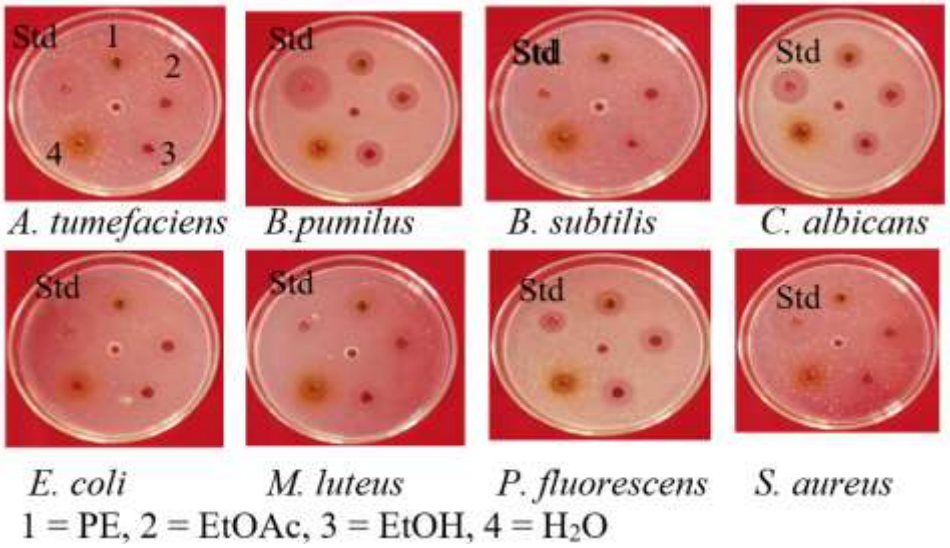

Figure 1. Antimicrobial screening of crude extracts of leaves of *M. Koenigii*

Table 3. Antimicrobial Screening of Leaves of *M. koenigii*

Microorganisms	Inhibition zone diameters of different crude extracts (mm)				
	PE	EtOAc	EtOH	Water	Standard chloramphenicol
A. tumefaciens	18(++)	18(++)	16(++)	-	29(+++)
B. pumilus	17(++)	19(++)	16(++)	-	27(+++)
B. subtilis	17(++)	17(++)	17(++)	-	29(+++)
E. coli	16(++)	17(++)	16(++)	13(+)	31(+++)
M. luteus	19(++)	18(++)	17(++)	13(+)	19(++)
P. fluorescens	17(++)	17(++)	16(++)	11(+)	15(++)
S. aureus	17(++)	18(++)	17(++)	-	29(+++)
C. albicans	17(++)	17(++)	16(++)	14(+)	22(+++)

Agar well diameter = 8 mm, No activity = (-)
10 mm ~ 14 mm = (+), 15 mm ~ 19 mm = (++), 20 mm and above = (+++)

Antiproliferative Activity of Crude Extracts of *M. koenigii* Leaves

The antiproliferative activity of ethanol and watery extracts of leaves against MCF7 (human breast cancer), A549 (human lung cancer), Kato III (human stomach cancer), GSU (human gastric cancer), and Hela (human cervix cancer) was evaluated by using the CCK-8 Assay. The anticancer effect was expressed as IC_{50} values (50% inhibitory concentration). The antiproliferative activity of ethanol and water extracts for MCF7, A549, Kato III, Hela, and GSU cell lines was evaluated as the percent cell viability of the sample at different concentrations and IC_{50} values. Ethanol extract from the leaves was found to possess the highest antiproliferative activity against MCF7, A549, GSU, and Hela cancer cell lines because of its very low IC_{50} value of less than 10 µg/mL. The water extract of the leaves exhibited low activity compared with ethanol extract because of their high IC_{50} value (> 100 µg/mL) (Table 4).

Table 4. Antiproliferative Activity of Ethanol and Watery Extracts of Leaves of *M. Koenigii* against Cancer Cell Lines

Extracts	IC_{50} µg/mL				
	MCF7	**A549**	**Kato III**	**GSU**	**Hela**
EtOH	<1	<1	15.3	<1	<1
Water	>100	62.1	>100	9.6	>100
***5FU	16.4	16.9	3.4	1.62	17.6

*5FU = Standard drug

Organic Compounds in Essential Oil by GC-MS Method

Gas chromatography-mass spectrometry (GC-MS) is the single most important tool for the separation and identification of unknown organic compounds by matching them with reference spectral. The GC-MS chromatograms of essential oil from the leaves of *M.koenigii* are shown in figures 2 to 10. The interpretation of the mass spectral was compared with their peak distributions against the database of the National Institute Standard and Technology (NIST MS 14.0, Gaithersburg, MD, USA) and those from the literature (Table 5).

Table 5. Chemical Composition of Essential Oil from the Leaves of *M. koenigii*

No.	Compound	Molecular weight	Molecular formula
1	*p*-xylene	106	C_8H_{10}
2	α-methyl styrene	118	C_9H_{10}
3	Azulene	128	$C_{10}H_8$
4	3-carene	136	$C_{10}H_{16}$
5	Methyl hexadecanoate	270	$C_{17}H_{34}O_2$
6	Methyl 9,12-octadecadienoate	294	$C_{19}H_{34}O_2$
7	Methyl 9-octadecenoate	296	$C_{19}H_{36}O_2$
8	Methyl stearate	298	$C_{19}H_{38}O_2$
9	Methyl eicosanoate	326	$C_{21}H_{42}O_2$

According to the GC-MS chromatogram (Figure 2), the peak appears at the retention time of 2.53 min, indicating the molecular weight of a compound to be 106 with the molecular formula C_8H_{10}. Therefore, it can be referred that the compound as *p*-xylene. At the retention time of 3.547 min, the GC-MS spectrum (Figure 3) shows the molecular ion peak at m/z 118, indicating the molecular formula C_9H_{10}. Therefore, the compound is α-methyl styrene. At the retention time of 6.197 min, the GC-MS spectrum (Figure 4) shows the molecular ion peak at m/z 128, indicating the molecular formula $C_{10}H_8$. Therefore, the compound is azulene.

At the retention time of 3.079 min, the GC-MS spectrum (Figure 5) shows the molecular ion peak at m/z 136, indicating the compound 3-carene with the molecular formula $C_{10}H_{16}$. At the retention time of 24.67 min, the GC-MS spectrum (Figure 6) shows the molecular ion peak at m/z 270, indicating the compound, methyl hexadecanoate with the molecular formula $C_{17}H_{34}O_2$. At the retention time of 27.77 min, the GC-MS spectrum (Figure 7) shows the molecular ion peak at m/z 294, indicating the compound is methyl 9,12-octadecadienoate, $C_{19}H_{34}O_2$. At the retention time of 28.00 min, the GC-MS spectrum (Figure 8) shows the molecular ion peak at m/z 296, indicating the molecular weight of a compound methyl 9-octadecenoate to be 296 with the molecular formula $C_{19}H_{36}O_2$. At the retention time of 28.42 min, the GC-MS spectrum (Figure 9) shows the molecular ion peak at m/z 298 which indicates the compound methyl stearate, $C_{19}H_{38}O_2$. At the retention time of 32.5 min, the GC-MS spectrum (Figure 10) shows the molecular ion peak at m/z 326 which indicates the molecular weight of a compound methyl eicosanoate with the molecular formula $C_{21}H_{42}O_2$.

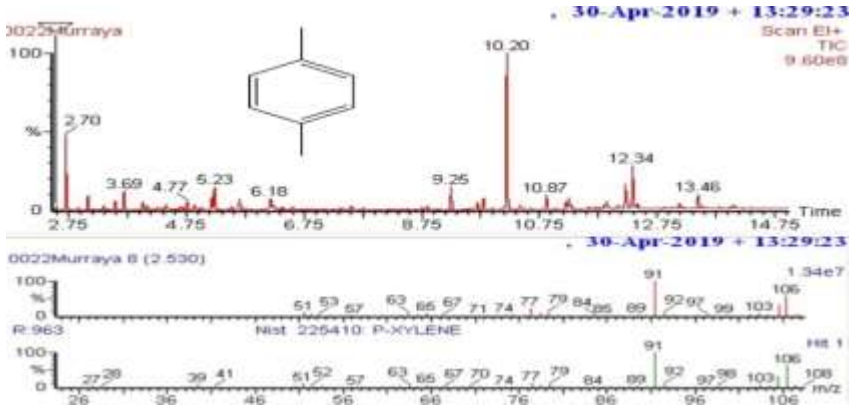

Figure 2. GC-MS chromatograms of *p*-xylene at retention time (2.530 min)

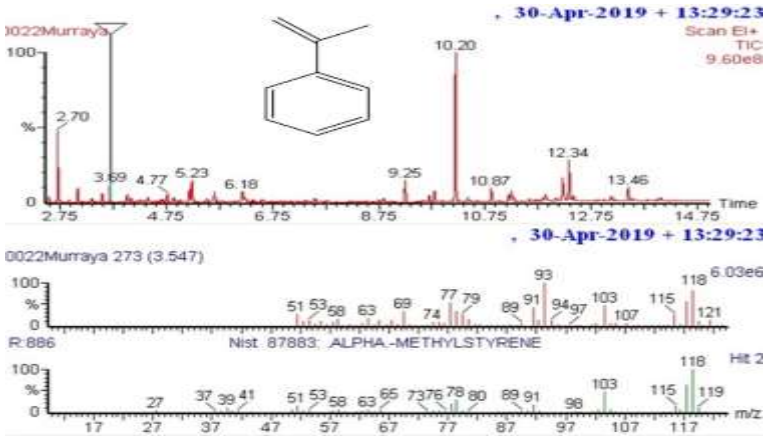

Figure 3. GC-MS chromatograms of α-methyl styrene at retention time (3.547 min)

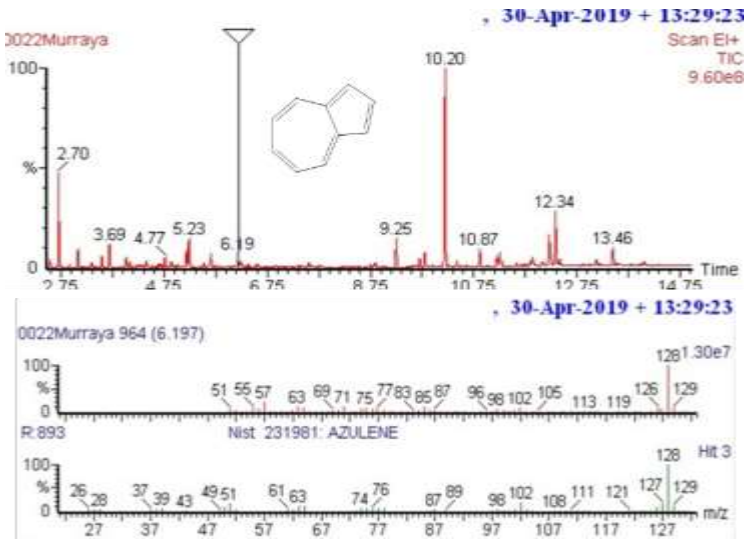

Figure 4. GC-MS chromatograms of azulene at retention time (6.197 min)

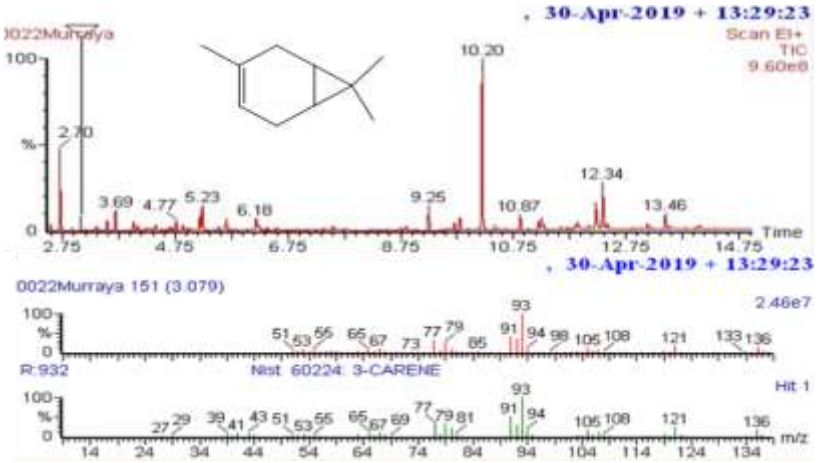

Figure 5. GC-MS chromatograms of 3-carene at retention time (3.079 min)

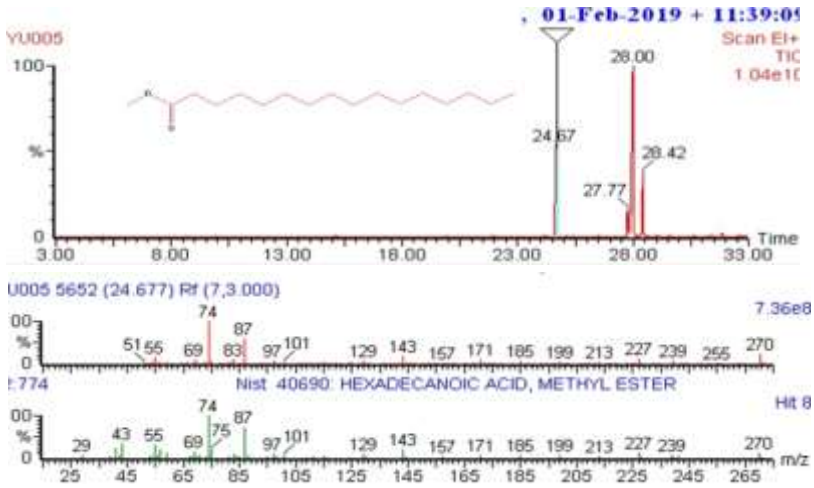

Figure 6. GC-MS chromatograms of methyl hexadecanoate at retention time (24.67 min)

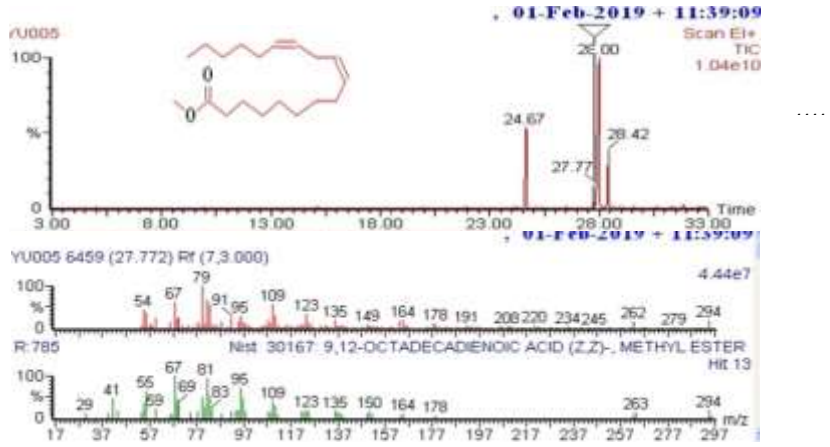

Figure 7. GC-MS chromatograms of methyl 9,12-octadecadienoate at retention time (27.77 min)

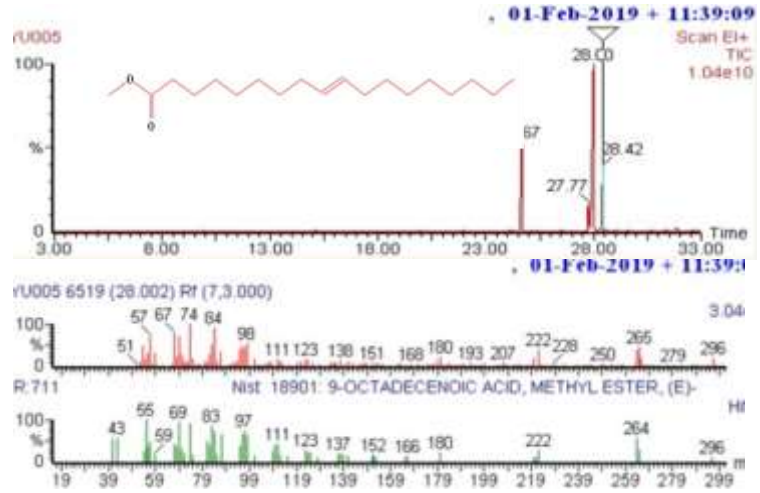

Figure 8. GC-MS chromatograms of methyl 9-octadecenoate at retention time (28.00 min)

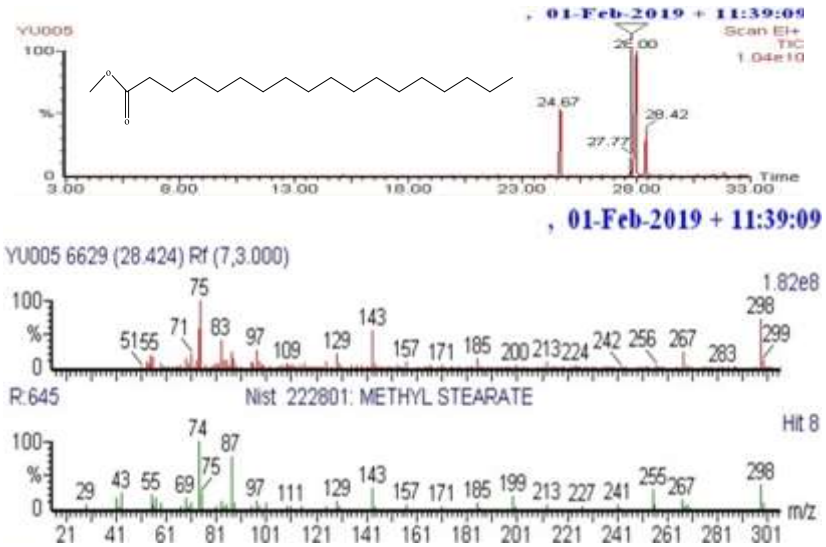

Figure 9. GC-MS chromatograms of methyl stearate at retention time (28.42 min)

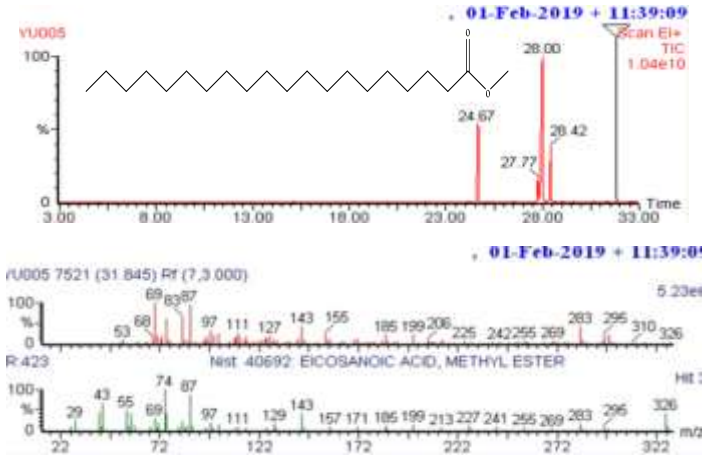

Figure 10. GC-MS chromatograms of methyl eicosanoate at retention time (31.845 min)

Conclusion

From the overall assessment of the chemical and biological investigation of curry leaves (*M. koenigii*), the following inferences could be deduced. The phytochemical tests revealed that alkaloids, α-amino acid, carbohydrates, cyanogenic glycosides, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, and terpenoids were present in the sample. However, flavonoids, starch, and tannins were not detected in the leaves of *M. koenigii*. The total phenol content evaluated by the Folin-Ciocalteu reagent in terms of Gallic acid equivalent (µg/mL) in ethanol extract (2.73 ± 0.0030 µg/mL) was higher than that of watery extract (2.56± 0.0046 µg/mL). For the investigation of the antioxidant potential of ethanol and the watery extract by the DPPH assay, the IC_{50} values of ethanol and watery extracts were observed at 34.24 µg/mL and 42.92 µg/mL respectively. Therefore, the antioxidant activity of the ethanol extract is more potent than watery extract but weaker than standard BHT (IC_{50} = 22.26 µg/mL). The antimicrobial activity of the polar and nonpolar extracts was screened by using the Agar Well Diffusion method on eight microorganisms. PE, EtOAc, and EtOH extracts (16 mm - 19 mm) showed antimicrobial activity against all tested microorganisms. However, the water extract inhibited only (11mm-14 mm) against *P. aeruginosa, M. luteus, C. albicans, and E. coli*. The antiproliferative activity of ethanol and watery extracts from the leaves of *M.koenigii* were tested on five cancer cell lines by the CCK-8 assay method. It was observed that the ethanol extract was found to be more antiproliferative activity against human breast cancer) (IC_{50} < 1 µg/mL), human lung cancer (IC_{50} < 1 µg/mL), human stomach cancer (IC_{50} < 15.3 µg/mL), human gastric cancer (IC_{50} < 1 µg/mL) and human cervical cancer (IC_{50} < 1 µg/mL).In the case of water extracts, against human breast cancer (IC_{50} > 100 µg/mL), human lung cancer (IC_{50} 62.1 µg/mL), human stomach cancer (IC_{50} > 100 µg/mL), GSU human gastric cancer (IC_{50} 9.6 µg/mL) and human cervical cancer (IC_{50} > 100 µg/mL). The chemical constituents of essential oil from the leaves of *M. koenigii* were identified by the gas chromatography-mass spectrometry method. The nine organic compounds (terpenoids, esters) were found in the essential oil of leaves of *M. koenigii*. The results of the present study indicate that the leaves of *M. koenigii* L. can be used as raw material for the production of insecticides, medicines, and flavoring ingredients.

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