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Original Article

STEROLS BIOACTIVITY OF *RUTA GRAVEOLENS* L. AND *MURRAYA PANICULATA* L.

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ABSTRACT

Objective: *Ruta graveolens* L. (*R. graveolens*) and *Murraya paniculata* L. (*M. paniculata*) are medicinal plants belonging to Rutaceae family have many uses in traditional medicine. The aim of the present study was to investigate sterols bioactivity of the two Rutaceae plant leaves.

Methods: Sterols of the two Rutaceae plant leaves were identified using GC/MS. The antioxidant activities of the sterols of these herbs were evaluated by three different methods; free radical scavenging using 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and total antioxidant activity. The anticancer activity of the sterols was determined by MTT assay against colorectal cancer HCT116, breast cancer MCF7, liver cancer HepG2 and lung cancer A549 cell lines. Anti-inflammatory activity was evaluated using albumin denaturation assay and antiviral activities against H5N1 virus were carried out using plaque reduction assay.

Results: GC/MS assay showed β-Sitosterol (36%) as the most abundant sterols of *R. graveolens* followed by stigmasterol (18%), while stigmasterol (25.2%) was the most abundant one of *M. paniculata* steroids. The anti-inflammatory potential of *R. graveolens* steroids was significantly higher than that of diclofenac sodium (standard drug). *M. paniculata* sterols have higher antiviral activity (IC_{50}= 0.15 of µg/ml) than *R. graveolens* sterols (IC_{50}= 7.8 of µg/ml). The sterols of *R. graveolens* showed anticancer activity against MCF7 and A549 cells with inhibition 84.3 and 81%, at 100 µg/ml respectively. While *M. paniculata* sterols showed 77.3% inhibition against A549 cells.

Conclusion: The current study suggests that the sterols of *M. paniculata* have more anti-viral activity than *R. graveolens* sterols which showed more anticancer and anti-inflammatory activities.

Keywords: *Ruta graveolens* L., *Murraya paniculata* L., phytosterols, bioactivity

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INTRODUCTION

Therapeutic plants are broadly utilised as a part of the treatment of many diseases. So, there are increasing interests to use plants in traditional medicine. They show more natural movement than manufactured medications without side effects, easily available, safer and cheaper. In the treatment of oxidative diseases such as inflammation, cancer and aging, antioxidant therapy has earned an importance. Reactive oxygen species (ROS) increase inflammation by the release of tumor necrosis factors, which stimulate neutrophil and macrophages. Thus, ROS are important mediators that stimulate inflammatory processes and consequently, their neutralisation by radical scavengers can reduce inflammation [1].

Rutaceae family are known for their high diversity of secondary metabolites including flavonoids, alkal oids, terpenes, limonoids and coumarins; these bioactive groups show remarkable medicinal effects [2]. *R. graveolens* and *M. paniculata* considered the most important traditional medicine in Rutaceae family [3]. They have antidiabetic, antifungal, antiandrogenic, anti-rheumatic pain, contraceptive, anti-hangover, antidote and insecticide effects [4-12]. Rutaceace popular with its secondary metabolites such as; alkaloids (graveoline, dictamnine, γ-fagarine, skimmianine, rutalinium and furacridone); coumarins (bergaptene); flavonoids (rutin); essential oil (2-undecyl acetate, 2-nonylacetate and 2-nonanone) [13].

Phytosterols are cholesterol-like molecules found in herbs and plants, the most common phytosterols are stigmasterol, β-sitosterol, and campesterol. They have been shown to reduce cancer, oxidation, inflammation, viral infection and blood cholesterol. On the basis of the uses of these compounds in traditional medicine, the present work was focused on *R. graveolens* and *M. paniculata* sterols to study their antioxidant, anti-inflammatory, antiviral and anticancer activities.

MATERIALS AND METHODS

Plant material

Plants were adopted from horticulture Dept. Agriculture Research Centre. They were identified by phytochemistry Dept. National Research Centre. The plants were air-dried at room temperature.

Determination of total lipid content

10 g of air-dried fine powder of each Rutaceae plant leaves were extracted by petroleum ether 40-60 °C using Soxhlet apparatus till complete extraction. The solvent was evaporated by rotary evaporator at 40 °C till dryness then the residue was kept in vacuum desiccators till constant weight and the concentration of total lipids were calculated [14].

Separation of unsaponifiable matter and fatty acids

One ml oil of the two species was saponified with methanolic KOH (20 ml, 10%) at 80˚C for 3 h under reflux. The unsaponifiable matter of each oil was extracted with ether (4 x 10 ml), washed several times with distilled water, dried over anhydrous sodium sulphate. Then the solvent was evaporated and the unsaponifiable matter was quantified (g) and kept for further analysis.

Identification of the unsaponifiable matter

The unsaponifiable matters of the two Rutaceae plant leaves were identified using GC/MS, with the following conditions:

Hewlett Packard HP 6890 apparatus equipped with HP-1 methyl siloxane capillary column (0.25 mm x 30 m), using flame ionisation detector (FID), and nitrogen was used, as a carrier gas. Nitrogen, hydrogen and air gases were set at flow rates 30, 30 and 300 ml/min, respectively. Oven temperature was programmed from 70-280˚C at rate 8˚C/min. Temperatures of detector and injectors were 300 and 250˚C, respectively. The hydrocarbon and sterol compounds

were identified by comparing the relative retention times of the separated components with those of available standard materials injected under the same conditions. The quantitative estimation of each compound was based on the area of the recorded peak area.

Antioxidant activity

The antioxidant activity was determined using the following methods:-

ABTS free radical scavenging assay

The ABTS free radical scavenging assay was carried out according to Arnao *et al.* [15]. Potassium persulfate (2.6 mmol) was added to 7.4 mmol of $ABTS^{+}$ and kept for 12-16 h at room temperature in the dark. The $ABTS^{+}$solution (1 ml) was diluted with 60 ml methanol to an absorbance of 1.1±0.02 at 734 nm before analysis. $ABTS^{+}$solution (2.80 ml) was added to sample fractions (0.150 ml, 50–150 µg/ml). After incubation for 2h in the dark, the absorbance was measured at 734 nm. Ascorbic acid was used as the positive control. The $ABTS^{+}$ free radical-scavenging capacity (%) was calculated using the following equation:

$$\text{ABTS scavenging activity \%} = [1 - \frac{A_1 - A_2}{A_0}] \times 100$$

Where, A_0 was the absorbance of the control (without sample), A_1 was the absorbance in the presence of the sample and A_2 was the absorbance without $ABTS^{+}$).

DPPH free radical scavenging assay

The ability of different sterols from the two Rutaceae plant to scavenge DPPH free radical were determined according to Ye *et al.* [16] method. Briefly, 0.1 mmol of sterols DPPH solution was prepared to give the initial absorbance value of 0.993 at 517 nm. The different concentration of samples (in 0.1 ml) of each sample (with appropriate dilution if necessary) was added to 3.0 ml of ethanolic DPPH solution. After incubation for 30 min in the dark, the absorbance was measured at 517 nm. The DPPH scavenging activity was calculated using the following formula:

$$\text{DPPH scavenging activity \%} = [1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}}] \times 100$$

Total antioxidant capacity

The total antioxidant assay was carried out according to Prieto *et al.* [17]. One ml of different extracts from the sterols of the two Rutaceae sp. (100 to 400 µg/ml) were mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mmol sodium phosphate and 4 mmol ammonium molybdate). The tubes were capped and incubated at 95 °C for 90 min. After cooling, the absorbance of each sample was measured at 695 nm. Standard series concentrations of ascorbic acid were treated as the sample.

Anti-inflammatory activity

Anti-inflammatory of the sterols of the two Rutaceae sp. was tested using the method of Rahman *et al.* [18]. The different concentrations of both plant sterols and standard drug; diclofenac sodium (50, 100, 150,200 ug/ml) were mixed with 0.45 ml bovine albumin serum. The sample extracts were incubated at 37 °C for 20 min and then heated to 57 °C for 3 min after cooling the samples was added 2.5 ml phosphate buffer pH 6.4. The absorbance was measured using UV-visible spectrophotometer at 255 nm.

Antiviral activity

Cytotoxicity assay

The cytotoxic effect of each sterol was tested individually in Madin-Darby Canine kidney cells (MDCK) (friendly obtained from St. Jude Children's Research Hospital) using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Lonza) with minor modification as previously described [19]. Briefly, MDCK cells were seeded in 96 well culture plates and incubated for 24 h at 37 °C in 5% CO_2. After 24 h post culture, the cells were treated with desired concentrations of the tested compounds in triplicates. After further 24 h, the medium was aspirated and cell monolayer was washed with phosphate buffer saline (PBS) 3 times then MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for 4 h. A volume of 200 µl of DMSO was added to each well to dissolve formazan crystals. The absorbance of formazan solutions was measured at λmax 540 nm. The percentage of cytotoxicity of each sterol was determined with the following equation:

$$\text{Cytotoxicity \%} = \frac{\text{Absorbance of cell without treatment-Absorbance of cell with treatment}}{\text{Absorbance of cell without treatment}} \times 10C$$

The plot of cytotoxicity % versus concentration of tested sterols was used to calculate the concentration which exhibited 50% cytotoxicity (TC_{50}).

Plaque reduction assay

Antiviral activities of tested sterols were carried out using plaque reduction assay as previously described [20]. Briefly, MDCK cells were seeded in 6 well culture plates (10^5cells/ml) and incubated for 24 h at 37 °C in 5% CO_2. Previously titrated A/duck/ Egypt/ Q5569D/2012(H5N1) virus was diluted to optimal virus dilution that gave countable plaques and mixed with the safe concentration of each tested sterols then incubated for 1hr at 37 °C before being added to the cells. Growth medium was removed from the 6 well cell culture plates and virus-compound mixtures were inoculated in duplicates, After 1hr contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose, 1% Antibiotic-antimycotic mixture and 4% bovine serum albumin (BSA, Sigma) was added onto the cell monolayer, plates were left to solidify and incubated at 37 °C till formation of viral plaques (3 d). 10% Formalin was added to each well for 1 h then over layer was removed. Fixed cells were stained with 0.1% crystal violet in distilled water. Control untreated virus was included in each plate. Finally, plaques were counted and the percentage of reduction in virus count was recorded as following:

$$\text{\% inhibition} = \frac{\text{Viral count (untreated)-viral count (treated)}}{\text{Viral count (untreated)}} \times 100$$

Cytotoxic effect on human cell lines

Cell viability was assessed by the mitochondrial-dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan [21].

Procedure: All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in RPMI 1640 medium for (HepG2, MCF-7 and HCT116) and DMEM for A549, 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Ampho-tericin B) and 1% L-glutamine at 37 °C under 5% CO_2.

Cells were batch cultured for 10 d, then seeded at a concentration of $10x10^3$ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO_2 using a water-jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of the sample to give a final concentration of (100-50-25-12.5-6.25-3.125-0.78 and 1.56 µg/ml). After 48 h of incubation, the medium was aspirated, 40 µl MTT salt (2.5µg/ml) were added to each well and incubated for further four hours at 37 °C under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200µl of 10% Sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37 °C. A positive control which composed of 100µg/ml was used as a known cytotoxic natural agent which gives 100% lethality under the same conditions [22, 23]. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula:

$$\% \text{ of viability change} = [\frac{\text{Reading of extract}}{\text{Reading of negative control}} - 1] \times 100$$

A probit analysis was carried for IC_{50} and IC_{90} determination using SPSS 11 program.

Statistical analysis

Results are expressed as mean value of three replicate. Data were statistically analysed through analysis of variance (ANOVA) and Duncan's test at P>0.01 using CoStat-Statistics Software.

RESULTS AND DISCUSSION

The data obtained from GC/MS analysis of the unsaponifiable matter of the sterols extracted from *R. graveolens* L. and *M. paniculata* L. are presented in table (1). The highest content of the sterols identified are β-sitosterol (36%) and stigmasterol (18%) followed by campesterol (8.99%) for *R. graveolens*. While the major phytosterol in *M. paniculata* are stigmasterol (25.2%) followed by eicosane (10.9%) and octadecane (10.5%).

Dealing with sterols, the unsaponifiable matter of most Ruteceae species contained campesterol, stigmasterol and β-sitosterol [24]. Phytosterol has been demonstrated to relive hypercholesterolemia and control cardiovascular diseases, oxidative stress, inhibit inflammation and inhibit cancer. Many studies demonstrated that there are a relation between intake of phytosterol and a decrease the risk of some dangerous disease [25, 26]. Many studies have embroiled oxidative cell damage emerging from an imbalance between free radicals and oxidative defence as the essential driver of dangerous diseases [27].

Antioxidant activity

The present study demonstrated powerful antioxidant activity in a dose-dependent relationship as shown in the table (2). Table (2) demonstrated that all samples possesses ABTS radical scavenging activity less than 60% at 150 µg/ml and less than ascorbic acid as standard antioxidant (122.3%). The scavenging activity at 150 µg/ml can be arranged in the following order: ascorbic acid>*R. graveolens*>*M. paniculata*. There are significant differences due to the scavenging activity by ABTS.

Table 1: GC/MS analysis of unsaponifiable matter of the sterols fraction of *R. graveolens* and *M. paniculata*

Compounds	Relative percentage	
	R. graveolens	*M. paniculata*
Hexadecane	-	7.8
Octadecane	8.99	10.5
Nonadecane	2.84	1.8
Eicosane	0.2	10.9
Docosane	8.95	7.7
Tricosane	4.16	5.8
Tetracosane	1.68	5.5
Pentacosane	2.77	1.7
Hexacosane	1.37	5.6
Heptacosane	2.83	1.3
Nonacosane	0.92	--
Triocontane	2.3	0.8
Campasterol	8.99	5.5
Stigmasterol	18	25.2
β-Sitosterol	36	9.9

Table 2: Antioxidant activity (%) of the sterols fraction of *R. graveolens* and *M. paniculata* against ABTS, DPPH radical and their total antioxidant activity

Samples	ABTS			DPPH			Total antioxidant capacity			
	Sample concentration (µg/ml)									
	50	100	150	50	100	150	50	100	150	200
R. graveolens	44.06±3.19 cd	55.08±2.11 cd	57.24±10.14 c	32.85±0.70 c	36.34±2.04 c	43.635±6.68 c	119.96±6.70 h	135.11±2.60 fg	142.85±4.50 f	186.04±2.02 d
M. paniculata	58.89±11.49 c	54.99±10.75 cd	56.61±8.27 cd	47.98±11.45 c	39.50±9.67 c	43.13±11.63 c	144.61±3.91 f	160.84±3.90 e	173.55±4.01 de	201.72±1.98 c
Ascorbic acid	40.76±0.37 d	81.53±0.75 b	122.3±1.13 a	45.86±4.64 c	84.2±4.39 b	113.87±10.83 a	127.97±8.93 gh	147.37±12.15 f	230.16±6.20 b	243.46±3.56 a
LSD 0.01	9.84			10.98			6.63			

Data are represented as mean±SD, Statistical analysis is carried out by two-way analysis of variance using COSTAT program, un-shared letters between brackets were a significant value between groups.

DPPH results revealed that the antioxidant activity of ascorbic acid was significantly higher than *R. graveolens*, *M. paniculata* sterols. No significant different between different sterols. From table (2), the data clearly showed that, all samples exhibited high scavenging activity were concentration dependent.

The results also revealed that the antioxidant capacity of the extracts can be arranged according to the total antioxidant activity in the following order: ascorbic acid, *M. paniculata* and *R. graveolens*. The results of antioxidant activity are agreed with the results of Valentina and Neelamegam [28] which revealed the high antioxidant potential of plant sterols. Several studies revealed the highest antioxidant activity of *M. paniculata* extracts [29], essential oil [30] and phenolic content [31] compared with vitamin E [32], BHT and BHA [30]. In the same time, Haraguchi [33] demonstrated that the potency of *R. graveolens* extract was nearly the same as BHT. Both *M. paniculata* and *R. graveolens* sterols demonstrated the acceptable effect in free radical scavenging activity.

Anti-inflammatory activity

Results in the table (3) showed the anti-inflammatory activity of the sterols of the Rutaceae plants under investigation, significant high percentages of an anti-inflammatory inhibition was recorded for *R.*

graveolens at the different concentrations in a dose-dependent manner followed by diclofenac sodium and *M. paniculata*.

Inflammation is a complex process and various mediators eg, prostaglandin, leukotrienes, etc, have been reported to be involved in the development of inflammatory diseases. The results of the present study go parallel with results obtained by Harsha and Latha [34], they found that Methanol extract of *R. graveolens* showed significant anti-inflammatory activity and also provide evidence for the leaves *R. graveolens* in folk treatment as an anti-inflammatory agent. The results of the current study revealed the strong anti-inflammatory activity of *R. graveolens* and are in accordance with that of the findings of Ratheesh *et al.* [3]. They found that the alcoholic extract of *R. graveolens* indicated most extreme (90.9%) restraint on carrageenan incited rodent paw edema. The impact was fundamentally ($P<0.05$) higher than that of the Voveran (standard medication 72.72%). The highest activity of *R. graveolens* sterols may be due to β-sitosterol as the predominant sterol that accounted for 36% of the total amount of sterols. This compound was found to possess potent anti-inflammatory activity [35] similar to hydrocortisone and oxyphenbutazone when administered intra-peritoneally. This compound was also orally effective against carrageenin-induced odema. Prieto *et al.* [36] revealed that β-sitosterol can modulate a cell-mediated edema. In another study carried out by Laidlaw [37] showed that plant sterols were found to reduce IL-6 levels which associated with inflammation possibly.

Table 3: Anti-inflammatory activity of sterols fraction of *R. graveolens* and *M. paniculata*

	Sample concentration (µg/ml)			
	50	**100**	**150**	**200**
Diclofenac sodium	74.05±0.37^{e}	81.74±0.63^{d}	85.5±0.20^{cd}	88.48±0.30^{c}
R. graveolens	66.86±4.68^{f}	86.74±1.54^{c}	101.07±1.57^{b}	112.98±2.03^{a}
M. paniculata	52.80±0.42^{h}	55.37±0.54^{gh}	57.80±2.59^{g}	58.21±1.01^{g}
LSD 0.01	2.08			

Data are represented as mean±SD, Statistical analysis is carried out by two-way analysis of variance using COSTAT program, Unshared letters between brackets were a significant value between groups.

Antiviral activity

The antiviral and cytotoxic activities of the described sterols were tested for antiviral activity against the avian influenzaH5N1 virus, and the results were illustrated in table (4) and fig. (1). The results in table (4) and fig. (1) Showed that, *M. paniculata* sterols have high inhibition (IC_{50}= 0.15 of µg/ml) than *R. graveolens* sterols (IC_{50}= 7.8of µg/ml) and this may be due to that *R. graveolens* more cytotoxic than *M. paniculata*.

The causative agents of the most fatal diseases are viruses, such as herpes simplex, AIDS and cancer [38]. Available medications for infectious diseases have always been limited. The results of the present study give conclusive evidence that *M. paniculata* sterols and *R. graveolens* sterols have antiviral activity against avian influenza H5N1 virus and this may be due to high phytosterol content.

These results concord with the results by Malini and Vanithakumari [39] and Khan *et al.* [40] who found that plant sterols are helping to combat the chronic viral infections. Other studies by Wachsman *et al.* [41, 42] found that the brassinosteroid and its derivatives inhibited the *in vitro* replication of herpes simplex type 1 (HSV-1) thymidine kinase $(TK)^{+}$ and TK-strains.

Table 4: TC_{50} and antiviral activity (IC_{50}) of the tested compounds against H5N1 virus

Extract	TC_{50} Conc. (mg/ml)	IC_{50} Conc. (µg/ml)
M. paniculata	0.54	0.15
R. graveolens	0.12	7.80

Where, TC_{50}: the concentration which exhibited 50 % cytotoxicity; IC_{50}: the half maximal inhibitory concentration.

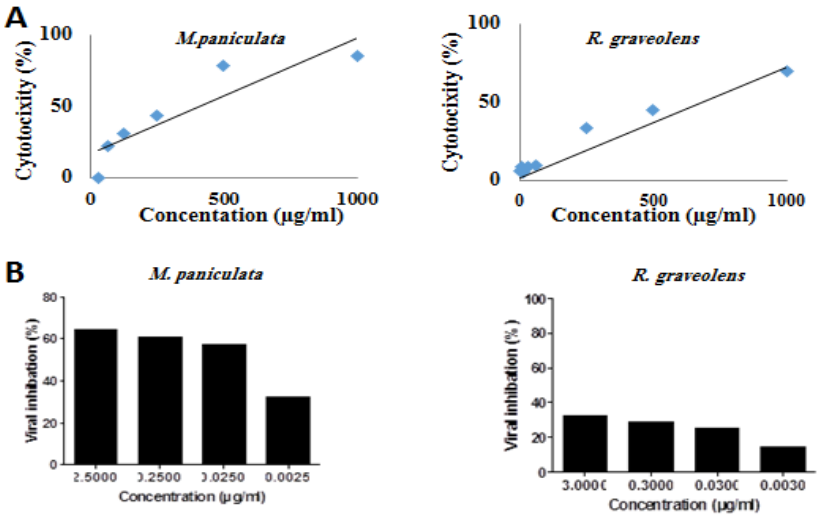

Fig. 1: Cytotoxicity (A) and antiviral (B) activities of M. paniculata and R. graveolens sterols

Anti-tumor activity

The anticancer properties of two Ruteace sterols using different cancer cell lines were studied. The results presented in table (5) showed that *R. graveolens* extract reduced the viability of treated cancer cells in a dose-dependent manner. Based on the cell viability measured after only 24 h, the approximate 50% inhibitory concentration (IC_{50}) of *R. graveolens* sterols was

approximately 32.3 µg/ml (A549 cells) with inhibition 81% at 100 µg/ml, 58.3 µg/ml (MCF7) with inhibition 84.3% at 100 µg/ml. While, *M. paniculata* sterols showed 77.3% inhibition against A549 cells.

Table 5: Antitumor activity of sterol fraction of *R. graveolens* and *M. paniculata* against cancer cell lines (HCT116, MCF7, HepG2 and A549)

Cancer cell lines	Sterols fraction of *M. paniculata* (µg/ml)				Sterols fraction of *R. graveolens* (µg/ml)			
	HCT116	MCF7	HePG2	A549	HCT116	MCF7	HepG2	A549
Inhibition%100µg/ml	0	36.5	16.8	77.3	47.6	84.3	34.8	81
IC_{50} µg/ml	0	-	-	65.1	-	58.3	-	32.3
IC_{90} µg/ml	0	-	-	106.8	-	94.7	-	56.8

Where, IC_{90}

The present results confirmed the antitumour activity of *R. graveolens* sterols which in agreement with Fadlalla *et al.* [43] who found that *R. graveolens* extract contain biologically active compounds which have potent anti-cancer activity, exhibited through strong anti-survival effects on cancer cells. While *M. paniculata* came in the second rank and give IC_{50} 65.1 µg/ml (A549 cells) with inhibition 77.3% at 100µg/ml. In these regards, Choi *et al.* [44] and Lopes *et al.* [45] found that phytosterols have anti-cancer effects, against stomach cancer [46], lung cancer [47], ovary [48] and breast cancer [49] by enhancing stimulation of X-receptor and switch of sphingomyelin, suppression of cell division and induce apoptosis by activation of caspase [50]. It has been speculated that phytosterols inhibit cancer-cell growth, metastasis, invasion, and induce apoptosis [51]. Some epidemiological results suggest a correlation between sterol intake and a reduction in cancer risk. It has been estimated that phytosterols intake may be related to a reduction in cancer risk upwards of 20% [52]. Chai *et al.* [53] found that β-sitosterol could inhibit the MCF-7 cells proliferation, also showed apoptosis activities in human leukemic U937 cells and human breast cancer MDA-MB-231 cells by activating of caspase-3 and Bax/Bcl-2 ratio [54, 55].

CONCLUSION

R. graveolens and *M. paniculata* sterols contain bioactive compounds that open door for the treatment of many diseases such as inflammation, cancer, oxidation and viral infection which can be further explored for their potent use in pharma industries.

CONFLICT OF INTERESTS

Declared none

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