

# Chemical Constituents and Biological Functions of Different Extracts of *Milletia speciosa* Leaves

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**Abstract** *Milletia speciosa*, a traditional Chinese folk medicine, used as immunity enhancer and anti-bronchitis. The study was proposed to investigate phytochemicals and pharmacological activities (antityrosinase, sunscreen and anticancer) of the different fractions of *M. speciosa* leaves. The sample was extracted with solvents of increasing polarity i.e. petroleum ether (PE), ethyl acetate (EtOAc), methanol (MeOH) and the total flavonoid content for each fraction was determined. The GC-MS analysis revealed various important bioactive compounds. The identified major compounds included beta sitosterol (12.039%), (Z)-9-octadecenamide (6.299%) and gamma sitosterol (4.910%). The highest flavonoid content was recorded for the PE extract i.e.  $47.50 \pm 0.40\%$ . The PE extract showed significant antityrosinase activity ( $70.30 \pm 0.90\%$ ) with  $IC_{50}$  0.035 mg/ml followed by the MeOH extract i.e.  $66.50 \pm 1.20\%$  with  $IC_{50}$  0.039 mg/ml. The highest sunscreen activity was noted for the EtOAc extract by observing maximum absorbance value ( $1.518 \pm 0.49$ ) in UVA region followed by  $1.214 \pm 0.46$  in UVB zone. The highest cytotoxicity ( $29.45 \pm 1.17\%$  viability) towards MCF-7 breast cancer cells line was noted for the PE extract with  $IC_{50}$   $6.73 \mu\text{g/ml}$ . Overall, our study revealed the presence of potential bioactive compounds for each extract that exhibited significant therapeutic activities and therefore validate the importance of *M. speciosa* leaves as a source of natural medicine.

**Keywords:** *Milletia speciosa*, GC-MS analysis, bioactivities, flavonoid, sunscreen, antityrosinase, anticancer

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## 1. Introduction

Medicinal plants serve as an important source of new drug leads due to their diverse phytochemical composition. Many studies have confirmed that medicinal plants contain a number of biologically important compounds like phenols, alkaloids and other compounds soluble in water and organic solvents [1]. While, other works have characterized the medicinal values of plants which may include anticancer, antidiabetic and antioxidant properties, etc. Different extracts of different plants, can show a wide range of medicinal effects such as hepatoprotection, antimalarial activity, antibacterial and many others [2]. About 80% people living in developing world use herbal medicines as a primary and traditional healthcare source. During the past decade, a remarkable public acceptance and interest for natural therapies has been witnessed both

in developing and developed countries [3]. Extracts obtained from various parts (stems, leaves, roots, flowers etc.) are widely used for *in vitro* and *in vivo* scientific studies. To date, approximately 500, 000 plant species have been reported around the globe, and only 1% has been investigated for phytochemicals. This situation creates a huge potential to search for unexploited plants and discover new therapeutic compounds [4].

*M. speciosa* is a tropical plant of Leguminosae family, which is widely distributed in China, tropical and sub-tropical regions of the world. The plant has been reported to be used as immunity enhancer and anti-bronchitis [5]. However, no detail research studies on GC-MS analysis and pharmacological activities of *M. speciosa* leaves have been reported so far. In present study, we report phytochemical composition, tyrosinase inhibition, sunscreen and anticancer activities of petroleum ether (PE), ethyl acetate (EtOAc) and methanolic (MeOH) extracts of *M. speciosa* leaves.

## 2. Material and Methods

### 2.1. Collection of Plant Material

*Millettia speciosa* was collected from Wanning city, Hainan province in May 2018, and was identified by Professor Zhong Qiongxin, College of Life Sciences of Hainan Normal University, Haikou, China. The sample was stored in Hainan Key Laboratory of Tropical Medicinal Plant Chemistry, College of Chemistry and Chemical Engineering, Hainan Normal University having sample number (20110511MS).

The leaves of *M. speciosa* were selected for the study. The collected sample was washed with tap water to remove dust. The sample was dried in shade for several days till complete dryness. The dried sample was grounded into powder with lab-crusher HS-400Y and kept in an air tight container for future use.

### 2.2. Extraction

Extraction of dried powder was carried out by maceration using different organic solvents of increasing polarity (petroleum ether, ethyl acetate and methanol). A 300 gm of each sample was separately and sequentially extracted. The samples were immersed in 5 L petroleum ether for 3 days and filtered through fresh cotton plug and finally with Whatman filter paper. The filtrate was concentrated using rotary evaporator. The residue from first step was immersed back in 5 L ethyl acetate for 3 days. Same procedure was followed to get raw extract of ethyl acetate. The residue from second immersion was extracted with methanol, resulted in raw methanolic extract. The raw extracts were kept in an air tight container at 4°C till further study [6].

### 2.3. GC-MS analysis

The procedure for GC-MS analysis of crude extracts of *M. speciosa* leaves was followed as described by Mustapha et al. [7]. Briefly, analysis were carried out on equipment model Agilent 7890A/5975C using capillary column operating in EI mode. An HP-5 MS column (30 m x 250 µm x 0.25 µm) was used. Helium was used as a carrier gas at a constant flow rate of 1.2ml/min. The percent composition of chemical constituents in crude extracts was expressed as percentage by peak area. The identification and characterization of compounds was based on their retention indices. Mass spectra were obtained by EI at 70 eV over the scan range 35-650amu. The mass spectra were matched with standards available in NIST 08.L Mass Spectrum libraries.

### 2.4. Determination of Total Flavonoids

The total flavonoids content was determined by using spectrometry method [1,8] with slight modifications. Rutin was used as Standard to prepare stock solution. A series of different dilution was made to establish a linear equation based on a calibration curve.  $y=10.818x-0.0217$ ,  $R^2=0.9975$ . Where “y” is the absorbance and “x” is the flavonoids content in mg/ml.

The extracts were dissolved with dimethyl sulfoxide. In a 10 ml test tube, 2ml of extracts, 3.4 ml of 30% methanol, 0.15 ml of NaNO<sub>2</sub> (0.5 M) and 0.15 ml of AlCl<sub>3</sub>.6H<sub>2</sub>O (0.3 M) was mixed. After 5 min, 1 ml of NaOH (1 M) was added. The solution was mixed well and the absorbance was measured against the reagent blank at 510 nm. Samples were run in triplicate and mean values were calculated as mg rutin per g dry weight (% w/w). The total content of flavonoid was calculated using the following formula;

$$\text{Total flavonoid content} = RE * V / m$$

Where, RE is the concentration of rutin solution obtained from the calibration curve; V, is the volume of extract (ml) and m, is the weight of pure leaf extract (g).

### 2.5. Antityrosinase Activity

The tyrosinase inhibitory activity of all extracts was determined using previously described method [9] with some modifications. In short, 1 ml of potato tyrosinase was added to phosphate buffer (pH= 6.8) and 2 ml of L-tyrosinase and 2 ml of different concentration of leaf extracts. The experimental reaction time was 30 min at 37°C. After incubation, absorbance of the mixture was measured at 490 nm using UV-spectrophotometer. Arbutin (0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 mg/ml) and Vitamin C (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4 mg/ml) was used as positive control. The percent inhibition of tyrosinase was calculated using the following formula;

% Inhibition

$$= 100 - \left( \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \right) \times 100.$$

### 2.6. Sunscreen Activity

The spectrophotometric method was used to determine sunscreen activity as described earlier but with some modifications [10,11]. The test solutions were prepared by taking 100-200 µg/ml concentration. The absorbance was measured in UVC (200-280nm), UVB (280-320nm) and UVA (320-400nm) region using dual beam ultraviolet-visible spectrophotometer (TU-1901, Beijing General Analysis Instruments). Rutin and 4-methylbenzylidene camphor (sunscreen) was used as positive control.

### 2.7. Anticancer Activity

#### 2.7.1. Cell Culture

MCF-7 cells (human breast cancer cell line) were procured from Kunming Cell Bank, Chinese Academy of Sciences, China. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics penicillin (5000 units/mL), which were purchased from Gibco, Invitrogen, CA, USA. Cells were maintained at 37°C in 5% CO<sub>2</sub> humidified incubator. Cells were subcultured at 80% confluency, and fresh media was applied every 2-3 days interval [12,13,14].

### 2.7.2. Evaluation of Cellular Cytotoxicity

The cytotoxicity was evaluated by the crystal violet staining (CVS) method with some modification [15,16]. In short, the cells were seeded in a 96-well tissue culture microplate, at a cell concentration of  $4 \times 10^3$  cells per well in 100  $\mu$ l of growth medium. Fresh medium containing different concentrations of each fraction (i.e. PE, EtOAc and MeOH) was added after 24 hrs of seeding at 37°C. Serial two-fold dilutions (200  $\mu$ g/ml, 100  $\mu$ g/ml, 50  $\mu$ g/ml, 25  $\mu$ g/ml and 12.50  $\mu$ g/ml) of the extracts were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates using a multichannel pipette. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub> for a period of 72 hrs. Three wells were used for each concentration of the test sample i.e. the samples were tested in triplication. Control cells (MCF-7 cells with medium) were also incubated without test samples.

At the end of the incubation period, media was aspirated and the crystal violet solution (100  $\mu$ l of 1%) was added along with fetal bovine serum (1:4.5) to each well for at least one and half h. The stain was removed and the plates were rinsed using distilled water. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly. The viable cells yield was determined by a colorimetric method. The percentage of viable cells was calculated by using formula; % Viability= absorbance of treated cells/absorbance of control cells \*100 [13]. The quantitative analysis (colorimetric evaluation of fixed cells) was performed by absorbance measurements in an automatic Microplate reader (Thermo Scientific Multiskan Go) at 590 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. The effect on cells growth was calculated as the difference in absorbance

percentage in presence and absence of the test extracts. The results were illustrated in a dose-response manner. The IC<sub>50</sub> (concentration at which 50% growth inhibition of cancer cells occurs) was calculated. Doxorubicin was used as the standard anticancer drug.

## 3. Results

### 3.1. GC-MS Analysis

GC-MS chromatograms were obtained for extracts of solvents with increasing polarity (petroleum ether, ethyl acetate and methanol) are given in Figure 1 - Figure 3, respectively. GC-MS chromatogram of the PE extract depicted different peaks resulting the presence of 54 compounds (Table 1). The most abundant compounds identified were gamma sitosterol (4.910%), followed by n-hexadecanoic acid (4.745%), stigmast-4-en-3-one (2.939%) and gamma tocopherol (2.845%).

The peaks in the chromatogram of the EtOAc extract resulted in 14 compounds identified according to their retention time (Rt) on capillary column (Table 2). Out of these compounds, beta sitosterol (12.039%) was found a major chemical constituent. This was followed by (Z)-13 docosenamide (4.270%), campesterol (2.705%), 2-pentadecanone, 6, 10, 14-trimethyl (2.046%) and phytol (1.787%).

The GC-MS analysis of the MeOH extract of *M. speciosa* leaves led to the identification of 12 chemical compounds (Table 3) with higher similarity index. Among these compounds, beta sitosterol (9.860%) was characterized and identified as the most significant phytochemical. Other major compounds observed were (Z)-9-octadecenamide (6.299%), stigmasterol (6.071%) and n-hexadecanoic acid (4.316%).

Table 1. GC-MS analysis of petroleum ether extract of *Millettia speciosa* leaves

NO.	Rt (min)	Compound Name	Similarity	MW*	Rc <sup>o</sup> (%)
1	4.011	Dimethyl sulfone	91	94.009	0.261
2	6.957	Cyclopentasiloxane, decamethyl-	91	370.094	0.048
3	7.722	Ethanone, 1-(3-methylphenyl)-	93	134.073	0.034
4	8.291	Cyclotetrasiloxane, octamethyl-	91	296.075	0.079
5	9.091	Tridecane	90	184.219	0.068
6	9.355	Cyclohexasiloxane, dodecamethyl-	93	444.113	0.096
7	10.043	Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-	95	174.141	0.021
8	11.008	3-Buten-2-one, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-, (E)-	92	192.151	0.028
9	11.328	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	95	194.167	0.068
10	11.502	Dimethyl phthalate	97	194.058	0.053
11	11.849	Cycloheptasiloxane, tetradecamethyl-	93	518.132	0.553
12	12.579	Phenol, 2,4-bis(1,1-dimethylethyl)-	97	206.167	0.340
13	13.677	Dodecanoic acid	95	200.178	0.135
14	13.843	1-Hexadecene	99	224.25	0.321
15	13.989	Hexadecane	99	226.266	0.073
16	14.379	Cyclohexadecane	97	224.25	0.031
17	16.477	Heptadecane	98	240.282	0.079
18	18.506	Cyclooctane, methyl-	94	126.141	0.036
19	18.61	5-Octadecene, (E)-	90	252.282	0.047
20	18.93	Tetradecanoic acid	96	228.209	0.080
21	19.263	1-Octadecene	99	252.282	0.436
22	20.591	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	96	280.313	0.084
23	21.056	2-Pentadecanone, 6,10,14-trimethyl-	98	268.277	0.735
24	23.912	Hexadecanoic acid, methyl ester	98	270.256	0.282

NO.	Rt (min)	Compound Name	Similarity	MW*	Rc* (%)
25	24.586	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	94	292.204	0.239
26	25.399	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	95	278.152	1.332
27	26.504	n-Hexadecanoic acid	94	256.24	4.745
28	28.971	Benzenamine, N-[4-(1-methylethyl)benzylidene]-4-(1-pyrrolidylsulfonyl)-	91	356.156	1.180
29	31.166	Phytol	91	296.308	1.823
30	32.931	Linoleic acid ethyl ester	99	308.272	0.775
31	33.188	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	95	306.256	1.321
32	34.314	Heptadecanoic acid, 15-methyl-, ethyl ester	92	312.303	0.848
33	40.394	4,8,12,16-Tetramethylheptadecan-4-olide	97	324.303	0.426
34	41.033	1-Nonadecene	93	266.297	0.126
35	41.958	1-Docosene	99	308.344	0.202
36	44.814	9,12-Octadecadienoic acid (Z,Z)-	95	280.24	0.059
37	45.911	Pentacosane	96	352.407	0.070
38	49.573	Hexacosane	96	366.423	0.105
39	50.581	Octacosane	98	394.454	0.810
40	51.582	Stigmasterol	99	412.371	0.771
41	52.436	Ergost-22-en-3-ol, (3. Beta.,5. alpha.,22E,24R)-	91	400.371	0.610
42	53.777	Pentadec-7-ene, 7-bromomethyl-	93	302.161	0.498
43	56.091	trans-13-Docosenamide	90	337.334	0.744
44	57.05	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-	93	281.078	0.232
45	57.342	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	99	410.391	0.621
46	58.669	Gamma.-Sitosterol	99	414.386	4.910
47	60.017	1-Hexacosene	99	364.407	1.766
48	61.351	Tritriacontane	98	464.532	0.892
49	61.622	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-, [2R-[2R*(4R*,8R*)]]-	98	402.35	2.286
50	63.811	4,22-Stigmastadiene-3-one	99	410.355	1.661
51	65.187	Beta.-Tocopherol	94	416.365	0.757
52	65.868	Gamma.-Tocopherol	95	416.365	2.845
53	68.237	Heptacosane, 1-chloro-	97	414.399	1.598
54	69.69	Stigmast-4-en-3-one	91	412.371	2.939

Table 2. GC-MS analysis of ethyl acetate extract of *Milletia speciosa* leaves

No.	Rt (min)	Compound Name	Similarity	MW	Rc* (%)
1	15.81	Cycloheptasiloxane, tetradecamethyl-	90	518.132	0.155
2	19	1-Nonadecene	91	266.297	0.200
3	25.893	Octadecane	94	254.297	0.497
4	26.998	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	91	280.313	0.114
5	27.442	2-Pentadecanone, 6,10,14-trimethyl-	98	268.277	2.046
6	28.276	Phthalic acid, isobutyl octyl ester	90	334.214	0.376
7	32.265	Eicosane	97	282.329	1.234
8	35.795	Phytol	91	296.308	1.787
9	38.199	Docosane	97	310.36	0.609
10	41.798	Oxirane, hexadecyl-	93	268.277	0.237
11	43.681	Tetracosane	98	338.391	0.844
12	51.686	Campesterol	95	400.371	2.705
13	53.25	13-Docosenamide, (Z)-	95	337.334	4.270
14	57.989	Beta.-Sitosterol	95	414.386	12.039

Table 3. GC-MS analysis of methanolic extract of *Milletia speciosa* leaves

No.	Rt (min)	Compound Name	Similarity	MW	Rc* (%)
1	30.055	Hexadecanoic acid, methyl ester	99	270.256	2.233
2	31.257	Dibutyl phthalate	96	278.152	1.851
3	31.758	n-Hexadecanoic acid	99	256.24	4.316
4	35.176	9,15-Octadecadienoic acid, methyl ester, (Z,Z)-	95	294.256	0.440
5	35.371	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	95	264.245	0.844
6	35.788	Phytol	90	296.308	1.527
7	36.177	Octadecanoic acid, methyl ester	99	298.287	0.944
8	37.004	2(1H)-Naphthalenone, octahydro-4a-methyl-7-(1-methylethyl)-, (4a.alpha., 7.beta.,8a.beta.)-	95	208.183	1.108
9	37.685	Octadecanoic acid	96	284.272	0.531
10	53.236	9-Octadecenamide, (Z)-	97	281.272	6.299
11	53.778	Stigmasterol	91	412.371	6.071
12	57.864	Beta.-Sitosterol	99	414.386	9.860

\*MW= Molecular weight, Rc= Relative content.

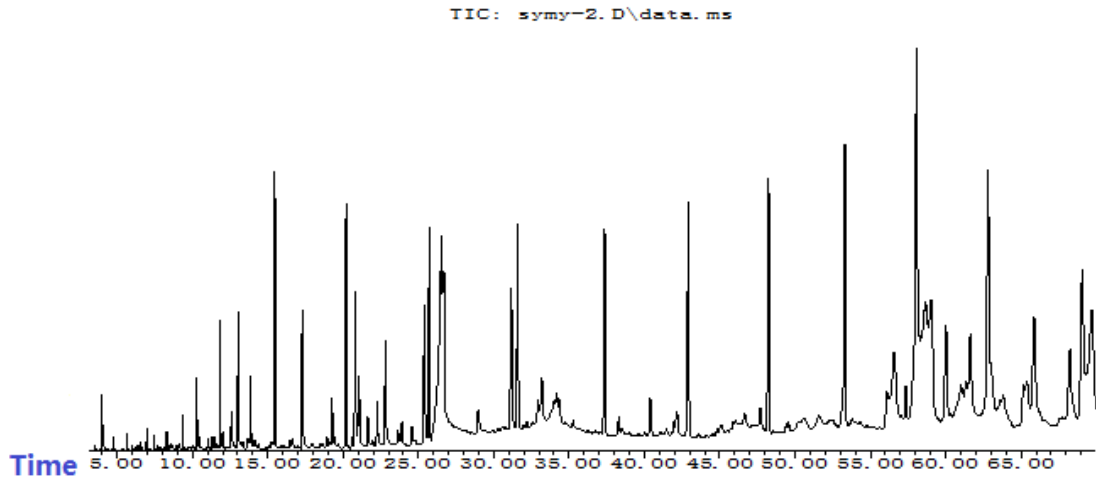


Figure 1. Total ion chromatogram for GC-MS analysis of petroleum ether extract of leaves

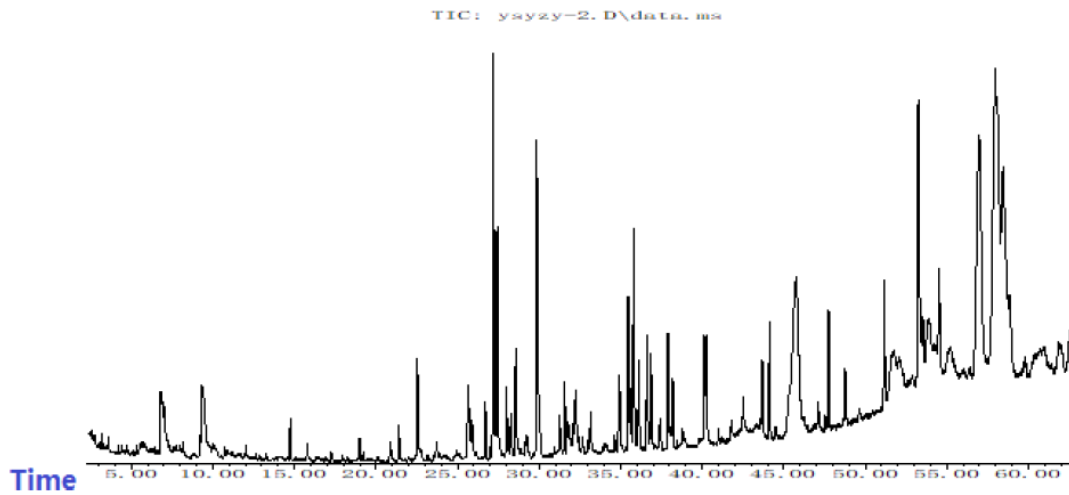


Figure 2. Total ion chromatogram for GC-MS analysis of ethyl acetate extract of leaves

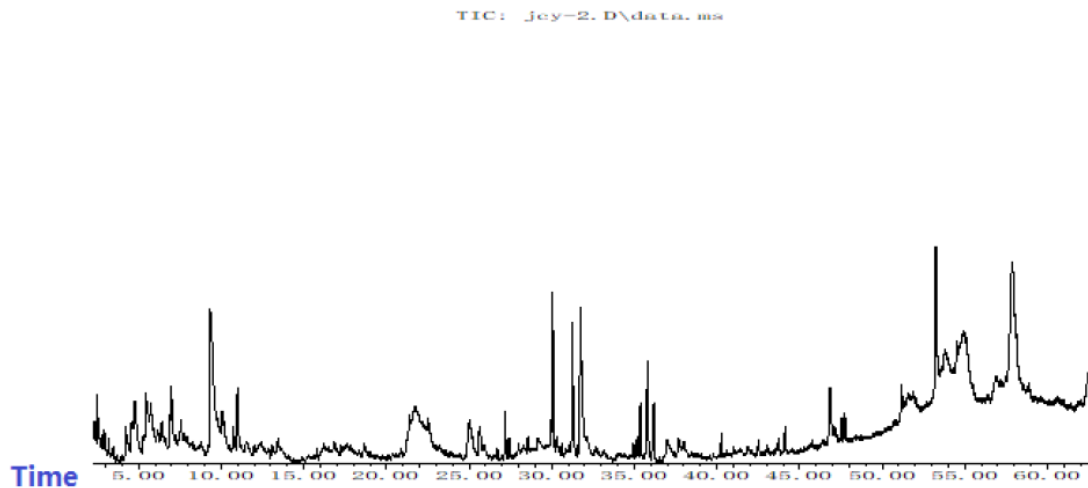


Figure 3. Total ion chromatogram for GC-MS analysis of methanol extract of leaves

### 3.2. Total Flavonoids

The results for total flavonoids of different extracts of *M. speciosa* leaves are given in Table 4. The highest amount ( $P < 0.05$ ) of flavonoids were recorded for the PE extract ( $47.50 \pm 0.40\%$ ), followed by the MeOH extract i.e.  $45.00 \pm 0.30\%$ . Whereas, the least amount ( $26.70 \pm 0.30\%$ ) was calculated for the EtOAc extract.

Table 4. Total flavonoid content of various *Millettia speciosa* leaves extracts

Extract	Total flavonoid content (%)
Petroleum ether	$47.50 \pm 0.40$
Ethyl acetate	$26.70 \pm 0.30$
Methanol	$45.00 \pm 0.30$

### 3.3. Antityrosinase Activity

As tyrosinase is an important enzyme for melanin synthesis, the plant material which has inhibitory activity for tyrosinase is thought to be a best candidate for skin whitening and protection against sun-related injuries. Hence, we were interested to investigate the antityrosinase activity of different *M. speciosa* leaves extracts, arbutin and vitamin C at various concentrations (Table 5, Figure 4). The results indicated highest tyrosinase inhibitory activity (70.30 ± 0.90%) for the PE extract at 0.090 mg/ml concentration with IC<sub>50</sub> value 0.035 mg/ml. This was followed by higher value (66.50 ± 1.20 %) noted for the MeOH extract at 0.090 mg/ml concentration with IC<sub>50</sub> value 0.039 mg/ml. While, the lowest inhibition (33.33 ± 0.86%) was calculated for the MeOH extract at 0.015 mg/ml concentration.

### 3.4. Sunscreen Activity

The absorbance values for sunscreen effect of *M. speciosa* leaves were measured and given in Table 6 and Figure 5. The EtOAc extract exhibited maximum absorbance (1.518 ± 0.49) in UVA region (320~400 nm) at concentration of 200 µg/ml as compared to rutin (1.014 ± 0.28) and 4-methylbenzylidene camphor (0.512 ± 0.19) at 40 µg/ml concentration. Similarly, maximum absorbance (1.214 ± 0.46) in UVB region was also observed for the EtOAc extract but less than 4-methylbenzylidene camphor (3.350 ± 0.52) at given concentrations. While in UVC region, maximum absorbance (0.863 ± 0.38) was recorded for the PE extract but less than rutin (1.908 ± 0.61) and 4-methylbenzylidene camphor (3.485 ± 0.46). The least absorbance (0.459 ± 0.10) was measured for MeOH extract at 320~400 nm.

Table 5. Antityrosinase activity and IC<sub>50</sub> (mg/ml) of various *Millettia speciosa* leaves extracts

Compound	Concen. (mg/ml)	Petroleum ether		Ethyl acetate			Methanol		IC <sub>50</sub> (mg/ml)
		Inhibition (%) ± SD	IC <sub>50</sub> (mg/ml)	Inhibition (%) ± SD	IC <sub>50</sub> (mg/ml)	Inhibition (%) ± SD	IC <sub>50</sub> (mg/ml)		
Extract	0.015	34.43 ± 0.88	0.035	34.09 ± 0.91	0.051	33.33 ± 0.86	0.039	0.039	
	0.030	50.00 ± 0.73		40.21 ± 1.11		50.00 ± 0.45			
	0.045	54.21 ± 1.11		46.13 ± 1.03		52.10 ± 0.84			
	0.060	60.13 ± 0.83		51.50 ± 0.90		57.13 ± 0.63			
	0.075	64.50 ± 0.90		54.70 ± 0.90		60.21 ± 0.71			
	0.090	70.30 ± 0.90		59.31 ± 0.90		66.50 ± 1.20			
Positive Control	Concen. (mg/ml)								IC <sub>50</sub> (mg/ml)
Arbutin	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.64
	Inhibition (%) ± SD								
	71.43 ± 0.24	87.50 ± 0.70	37.50 ± 0.62	25.00 ± 1.00	25.00 ± 0.65	20.00 ± 1.05	20.00 ± 0.81	10.00 ± 1.05	
Vitamin C	Concen. (mg/ml)								
	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.58
	Inhibition (%) ± SD								
25.00 ± 0.50	25.00 ± 0.70	62.00 ± 0.50	40.30 ± 0.70	25.00 ± 0.60	25.00 ± 0.80	22.00 ± 0.30	18.00 ± 0.40		

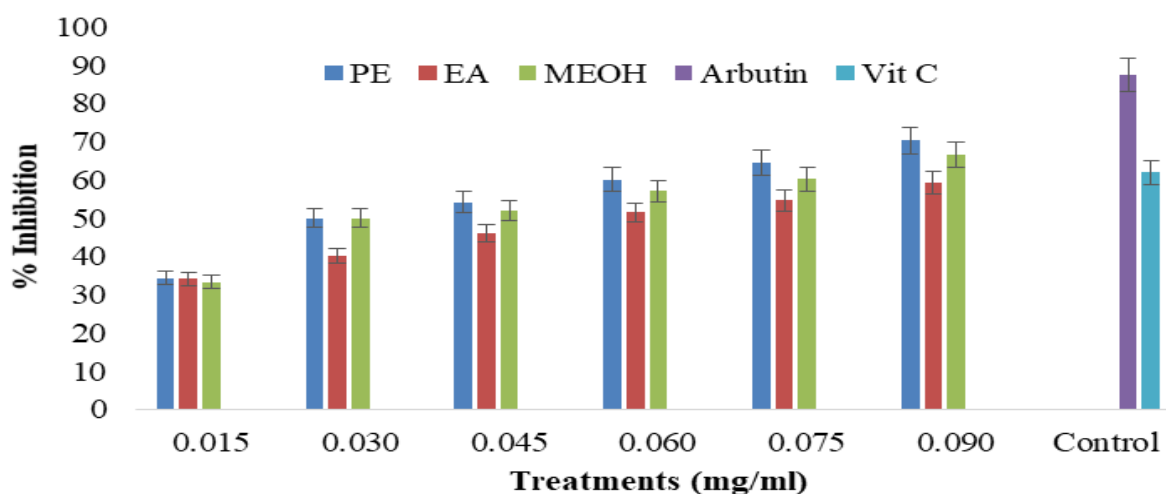


Figure 4. Antityrosinase activity of *Millettia speciosa* leaves extracts, Arbutin and Vit C. Values are expressed as means ± SD (n=3)

Table 6. Sunscreen activity of various extracts of *Millettia speciosa* leaves

Compound	Concentration (µg/ml)	UVA Zone (320~400nm) Abs.	UVB Zone (280~320nm) Abs.	UVC Zone (200~280nm) Abs.
Petroleum ether extract	200	0.818 ± 0.18	0.704 ± 0.23	0.863 ± 0.38
Ethyl acetate extract	200	1.518 ± 0.49	1.214 ± 0.46	0.710 ± 0.33
methanol extract	200	0.459 ± 0.10	0.664 ± 0.30	0.470 ± 0.25
Rutin*	40	1.014 ± 0.28	0.663 ± 0.32	1.908 ± 0.61
4-methylbenzylidene camphor*	40	0.512 ± 0.19	3.350 ± 0.52	± 0.46

\*Commercial sunscreen.

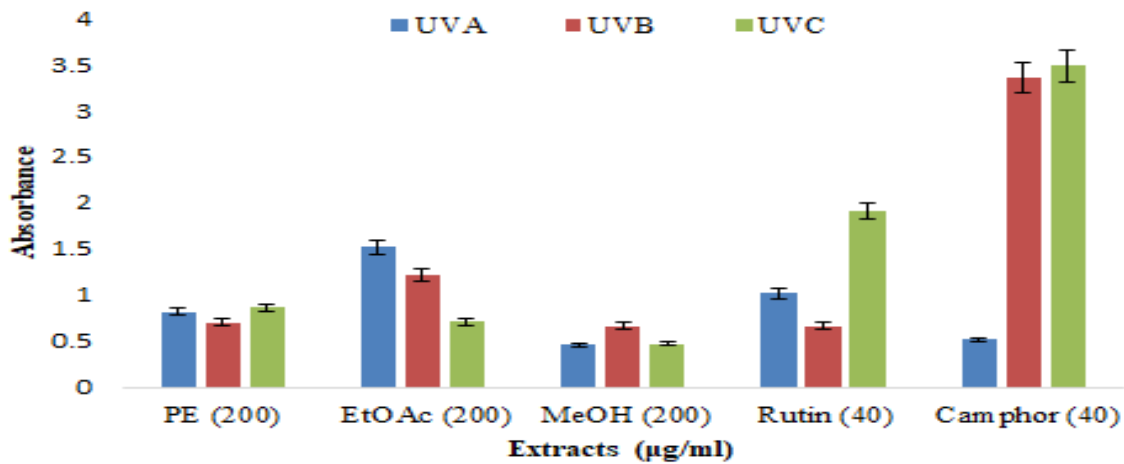


Figure 5. Sunscreen activity of *Millettia speciosa* leaves extracts, rutin and camphor. Values are expressed as means ± SD (n=3)

### 3.7. Anticancer Activity

During the last decade, medicinal plants with significant anticancer potential has immensely attracted scientist to develop new therapeutics. In present study, the

anticancer effect of different extracts of *M. speciosa* leaves against MCF-7 cells was investigated. A dose dependent effect was noted as given in Table 7. The experiment was run in triplicate for different concentrations ranged from 12.50-200 µg/ml.

Table 7. Cells Viability (%) and IC<sub>50</sub> (µg/ml) for various *Millettia speciosa* leaves extracts

Compound	Concen. (µg/ml)	Petroleum ether		Ethyl acetate		Methanol	
		Viability (%) ± SD	IC <sub>50</sub> (µg/ml)	Viability (%) ± SD	IC <sub>50</sub> (µg/ml)	Viability (%) ± SD	IC <sub>50</sub> (µg/ml)
Extract	12.5	95.62 ± 1.22	6.73	91.66 ± 1.05	4.14	95.13 ± 0.93	2.55
	25	80.31 ± 1.12		88.27 ± 1.06		90.63 ± 1.06	
	50	71.54 ± 1.23		76.35 ± 1.45		80.95 ± 1.10	
	100	47.91 ± 1.022		66.71 ± 1.31		79.51 ± 1.33	
	200	29.45 ± 1.17		43.51 ± 1.25		53.44 ± 1.11	
Positive Control	Concen. (µg/ml)						IC <sub>50</sub> (µg/ml)
Compound	1.25	2.50	5.00	10.00	20.00	15.19	
	Viability (%) ± SD						
Doxorubicin	37.52 ± 1.21	36.41 ± 1.15	35.72 ± 1.18	34.58 ± 1.34	33.93 ± 0.98		

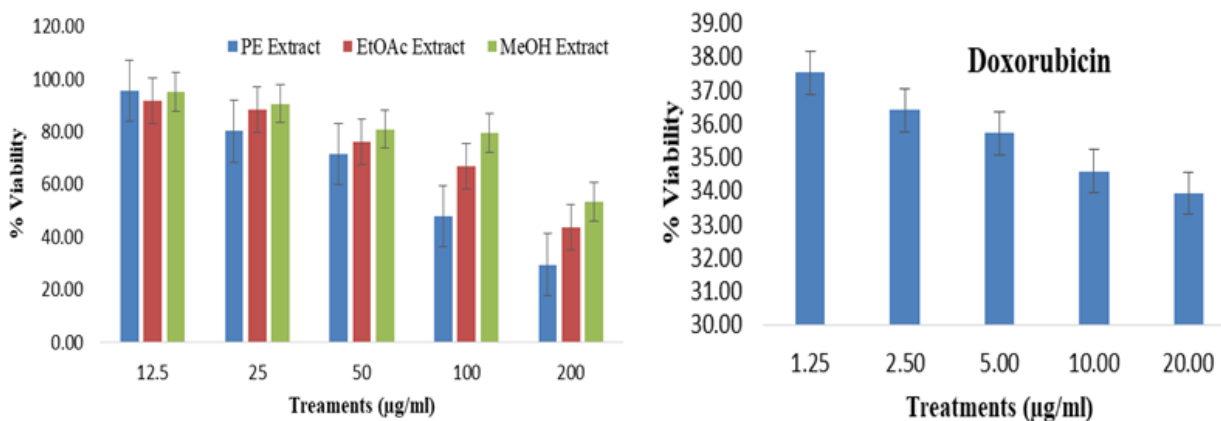


Figure 6. Anticancer activity of *Millettia speciosa* leaves extracts and Doxorubicin. Values are expressed as means ± SD (n=3).

The most significant effect ( $29.45 \pm 1.17\%$ ) was shown by the PE extract at 200  $\mu\text{g/ml}$  with an  $\text{IC}_{50}$  value of 6.73  $\mu\text{g/ml}$ . This was followed by the EtOAc extract ( $43.51 \pm 1.25\%$ ) at the same concentration level with an  $\text{IC}_{50}$  value of 4.14  $\mu\text{g/ml}$ . While, at highest concentration, the cell viability was reduced to  $53.44 \pm 1.11\%$  which revealed the least effect of the MeOH extract at  $\text{IC}_{50}$  2.55  $\mu\text{g/ml}$ . The % viability of MCF-7 cells at different strength of *M. speciosa* leaves extract is given in Figure 6.

## 4. Discussion

### 4.1. GC-MS Analysis

To know the medicinal importance of any plant, the initial study is to investigate phytochemical constituents. It gives broad information about nature of phytoconstituents belonging to various classes to develop health promoting novel drugs. The presence of different classes of bioactive compounds e.g. alkaloids, steroids, phenolics, steroids etc. in plant extract have been reported to demonstrate important therapeutic activities [17].

For the first time, various extracts of the leaves of *M. speciosa* were investigated for bioactive compounds. In the present study, GC-MS analysis of the PE extract revealed 54 compounds. According to the results, gamma sitosterol was found with maximum relative content value. The compound has an important role to induce G2/M cell cycle arrest and apoptosis in MCF-7 and A549 cells [18]. Other major compounds included; n-hexadecanoic acid, stigmast-4-en-3-one and gamma tocopherol. n-hexadecanoic acid has been found responsible for anticancer cytotoxic potential [19]. Stigmast-4-en-3-one is a sterol that exhibits antitumor, antidiabetic and hypoglycemic activities [20]. While anti-inflammatory and antioxidant properties have been reported for gamma tocopherol [21].

Likewise, GC-MS analysis of the EtOAc extract predicted beta sitosterol being a main component. The compound has various pharmacological activities like; anti-oxidant, anticancer, immunomodulatory etc. [22]. While, (Z)-13-docosenamide and phytol are reported to have antinociceptive and anti-inflammatory properties [23]. Similarly, campesterol is responsible for anticancer, antioxidant, cholesterol lowering and antidepressant activities. The compound 2-Pentadecanone, 6,10,14-trimethyl- has anti-inflammatory, cardio-protective, antioxidant and antibacterial capabilities [24].

The compound beta sitosterol and n-hexadecanoic acid was also noted for the MeOH extract. The reports suggested strong antioxidant and antimicrobial properties for (Z)-9-Octadecenamide [25]. While stigmasterol was reported to have anticancer, anti-inflammatory, antioxidant, diuretic and antimicrobial properties [26]. This is the first study to investigate various extracts of *Millettia speciosa* leaves for bioactive compounds through GC-MS analysis. The presence of various therapeutic compounds in *M. speciosa* leaves with significant biological activities suggests its pharmaceutical importance and further detailed study for new drug development.

### 4.2. Total Flavonoid Content

Flavonoids are plant secondary metabolites that exhibit different biological activities due to free hydroxyl groups [27]. The total flavonoid content of different extracts of *M. speciosa* leaves was colorimetrically determined. Each extract showed different amount of flavonoid content. Our experiment recorded highest amount for the PE extract, while the least for EtOAc extract. The flavonoid content of *M. speciosa* leaves extracted by PE, EtOAc and MeOH cannot be compared with other results due to unavailability of suitable literature about selected plant and part. However, several reports are available in literature about other medicinal plants. Previously, Sushant et al. revealed  $37.86 \pm 0.53$  mg QE/g in the MeOH extract of *Cassia tora* [27]. Similarly,  $23.25 \pm 0.11$   $\mu\text{g Qu/g}$ ,  $4.04 \pm 0.23$   $\mu\text{g Qu/g}$  and  $20.44 \pm 0.26$   $\mu\text{g Qu/g}$  was recorded for aerial parts of *Hyoscyamus gallergheri* extracted with hexane, EtOAc and MeOH, respectively [28]. It is clear from the literature that the total flavonoid content of medicinal plants is significantly affected by genetic, biological and environmental variations [29].

### 4.3. Antityrosinase Activity

Tyrosinase is a copper containing glycoprotein, which plays an important role in melanogenesis. The enzyme catalyzes hydroxylation of monophenols to diphenols and oxidation of *o*-diphenols to *o*-quinones to finally form melanin resulting various skin disorders like melasma, age spots etc. [30,31]. Thus tyrosinase inhibitors from natural source are considered attractive targets in cosmetics. In our study, different extracts of *M. speciosa* leaves were investigated for tyrosinase inhibition. The tyrosinase inhibition was noted in a dose-dependent manner and highest inhibition ( $70.30 \pm 0.90\%$ ) was recorded for the PE extract at 0.090 mg/ml concentration, followed by MeOH extract ( $66.50 \pm 1.20$ ) at the same dose. The inhibition could be due to copper chelating agents present in plant extracts, which prevents copper ions to bind with oxygen, thereby leading to deactivation of tyrosinase enzyme [32]. It is also assumed that tyrosinase inhibition may be due to the presence of flavonoids. Other authors consider inhibition of tyrosinase due to other chemical constituents [33]. The study suggests the leaves of *M. speciosa* an alternative to commercially available Arbutin and Vitamin C as the extracts showed significant effect at comparatively lower concentrations. This is the first study to report anti-tyrosinase activity of various extracts of *M. speciosa* leaves, suggesting its possible use in cosmetics for skin whitening and treatment of hyperpigmentation.

### 4.4. Sunscreen Activity

During the last few years, human activities has damaged the stratosphere and resulted in a significant ultraviolet (UV) radiations reaching the earth. As a result, various diseases and disorders have been increased alarmingly due to constant exposure to solar UV radiations [34]. The UV radiations have been categorized into three regions i.e. UV-C (200-280nm), UV-B (280-320nm) and UV-A



(320-400nm). Among these, UV-C can cause severe damage to skin and eyes for even a short exposure. However, these radiations are completely filtered and absorbed by atmosphere. UV-A and UV-B radiations are not completely absorbed and responsible for sunburn and other skin related disorders [35]. So to avoid such problems, sunscreen agents are used for protection against harmful radiations. Although, synthetic sunscreens are available in the market but with realization of their side effects, herbal based photoprotective agents are more likely to be preferred. In this study, we investigated various extracts of *M. speciosa* leaves for sunscreen activities in different regions. All the extracts exhibited sunscreen activities almost in all zones of radiations. However, the EtOAc extract showed maximum absorbance in UV-A and UV-B zone as compared to other two extracts. While moderate activities were noted for the PE and MeOH extracts. The sunscreen activity of plant based products is thought to be due to the presence of flavonoids, polyphenols and alkaloids [34,36]. Further study is suggested to screen these classes of compounds to identify the sunscreen compounds, hence it will be a cheaper and safe alternative to harmful synthetic chemicals used in sun-protection cosmetics.

#### 4.5. Anticancer Activity

Breast cancer is the most common life threatening cancer amongst women. According to International Agency for Research on Cancer (WHO) report 2018, 1 in 4 new cancer cases diagnosed in women globally are breast cancer [37]. During the last few years, researchers have started venture into natural bioactive compounds that could possibly lead to comparatively efficient and safe therapeutics to synthetic drugs. Hence, many plants were investigated and reported to show significant potential against breast cancer cells [38]. Different classes of compounds in plant extracts, such as polyphenols, alkaloids and terpenoids have been reported to demonstrate anticancer activities [39].

For the first time, various extracts of *M. speciosa* leaves were investigated for anticancer properties. From the studies conducted, it may be concluded that the PE extract contained phytochemicals that are responsible for stronger cytotoxic effect against breast cancer cell lines. The results were in agreement to Gunji et al. who reported toxic effects of the PE extract of *Cynodon dactylon* against MCF-7 cells [40]. The EtOAc extract exhibited moderate cytotoxicity leading to death of cancer cells by almost 57%. These results may be supported by studies conducted by Mardina et al. who reported anticancer activities of the EtOAc extract of *Sphagneticola trilobata* (L.) J.F. Pruski on MCF-7 cells line [41]. The *in vitro* studies of the MeOH extract also showed potential for anticancer activity which was in favor of Kamalanathan and Natarajan who reported the anticancer potential of the MeOH extract of leaf and leaf-derived callus of *Aerva javanica* [42].

These preliminary results suggest the promising anticancer potential of the PE, EtOAc and MeOH extracts of *M. speciosa* leaves that might be an ideal choice to develop novel plant-based anticancer drugs.

#### 4.6. Statistical Analysis

All the experiments for bioactivities were run in triplicate. The results were expressed as means  $\pm$  SD (standard deviation). The data was analyzed using one-way analysis of variance (ANOVA) at  $\alpha = 0.05$  with SPSS 22.0 software.

### 5. Conclusion

The screening performed on extracts of the leaves of *M. speciosa* for phytochemical composition, antityrosinase, sunscreen and anticancer properties reveals that they are endowed with potentially exploitable natural bioactive compounds and may therefore be suggested as a source of natural medicines. Further, isolation and purification of the active compounds, and *in vivo* evaluation of bioactivities, along with toxicity studies of the extracts from *M. speciosa* leaves are therefore suggested.

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