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In vitro cytotoxic, antioxidant, antimicrobial activity and volatile constituents of *Coccoloba peltata* Schott cultivated in Egypt

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Abstract

Coccoloba peltata Schott (*C. peltata*) is an evergreen, small tree mainly growing in Brazil. Since there is no relevant report for its biological activities or phytochemical data, this study aimed to assess the volatile components and bioactivity potential of leaves extract and fractions of *C. peltata* Schott cultivated in Egypt. Gas chromatography mass spectrometry (GC-MS) analysis allowed the tentative identification of thirty secondary metabolites including α-tocospiro A (17.20%), α-tocospiro B (12.02%), squalene (7.78%), 13-epi-manoyl oxide (6.80%), nonacosane (5.12%), pentatriacontane (4.17%) and hexadecanoic acid methyl ester (3.78%) as major compounds. The crude extract was subjected to *in vitro* bioassays revealing a promising results against the studied cancer cell lines, HepG-2, MCF-7, HCT-116, PC3 and HeLa (IC_{50} 7.56-14.26 µg/mL) in comparison to doxorubicin while the corresponding fractions exihibited lower activity. The antioxidant assay based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid as a reference, indicated the crude extract as the most active (IC_{50} 32.86 µg/mL) followed by chloroform fraction (IC_{50} 46.05 µg/mL), n-hexane fraction (IC_{50} 57.42 µg/mL) and then butanol fraction (IC_{50} 50.06 µg/mL). Finally, the total extract exihibited potent antimicrobial activity against *E. coli B. subtilis* and *C. albicans* with activity indices of 46.1, 73.9, 81.5%, respectively, as compared to ciprofloxacin and clotrimazole. Taken together, the crude extract from *Coccoloba peltata* displayed higher responses to the various assays, it has been suggested as a synergism among the compounds therefore.

Keywords: *Coccoloba peltata*; volatile constituents; cytotoxicity; antioxidant; antimicrobial; *in vitro* activity.

1. Introduction

Coccoloba resembles a genus of trees or shrubs which includes 120-150 species belongs to the family Polygonaceae [1]. *Coccoloba* is indigenous to tropical and subtropical zones of America, South America, in Central America and Caribbean, with two more species spreading to Florida [2]. Plants of genus *Coccoloba* are commonly identified by their simple and alternate leaves and characteristic ochrea [3]. *Coccoloba* species have been used traditionally in different countries as anti-inflammatory, astringent, antipyretic, to treat menstrual problems, gonorrhea, hemorrhoids, hemorrhages, asthma, insomnia, vision impairment, sexual impotence, skin diseases, intestinal parasites and warts cures [4-6]. Recently, interest in *Coccoloba* plants has considerably increased as many studies have confirmed their therapeutic properties. The biological properties of different *Coccoloba* species, phytoterapic preparations and secondary metabolites have been investigated using numerous *in-vivo* and *in-vitro* experimental models [7]. Most of the biological activities attributed to *Coccoloba* species are mainly due to the presence of phenolic compounds. Characterization of a few members of *Coccoloba* shows a high diversity of metabolites as flavonoids and tannins [8-10], terpenoids and sterols [11-13], anthraquinones [14] and volatile oils [15].

Coccoloba peltata Schott is a small tree that is native mainly to Brazil. The scarce literature concerning *C. peltata* with only one previous study [16] encouraged us to carry out further investigation of biological activities of *C. peltata as well as studying its*

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phytochemical constituents. Therefore, this study was planned to investigate the leave extract of *C. peltata* cultivated in Egypt for possible cytotoxic, antioxidant and antimicrobial activity as well as profiling of its volatile constituents using GC-MS which offers systematic evidence for commercial uses of the plant. Also studying for individual compounds may offer important drug leads but this step still under investigation, which will be reported in a fellow research work after further scrutiny.

2. Experimental

2.1. Chemicals

All the chemicals used in the extraction and fractionation procedures were of analytical grade, obtained from Sigma-Aldrich (St. Louis, MO, USA), and used as received. HPLC-grade solvent was used for the GC-MS analysis.

2.2. Plant collection, extraction and fractionation

The plant material was collected in March 2019 from Orman Botanic Garden, Giza region, Egypt, and identified by Ms. Therese Labib, Consultant at Orman Botanical Garden and El Qubba Botanical Garden, Giza region, Egypt. A voucher specimen (no. CP2019) of the plant has been deposited at the Herbarium of the Orman Botanical Garden, Giza region, Egypt. Two-week dried leaves was ground into fine powder (100 g) and soaked in dichloromethane:methanol (1:1) for three days at room temperature (whole process of extraction was done thrice). Organic extract was filtered and concentrated *in vacuo* by rotary evaporator to obtain a dark green crude extract (CP-LE, 12 g). The crude extract was suspended in water (1 L) and fractionated using n-hexane (CP-H, 3.1 g), chloroform (CP-C, 4.3 g) and butanol (CP-B, 3.5 g).

2.3. Gas chromatography-mass spectrometry analysis

The volatile constituents of *C. peltata* was analysed using Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) equipped with a capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). Temperature of the column oven was firstly held at 50°C and then gradually increased by 5°C /min to 250 °C and hold for 2 min and then increased to 300 °C by 30 °C /min and hold for 2 min. Temperature of the injector and MS transfer line was kept at 270, 260°C respectively; Helium was employed as a carrier gas with a constant flow rate of 1 mL/min. The delay of solvent was 4 min and automatic injection of diluted samples of 1 µl was performed using Autosampler AS1300 coupled with GC in the split mode. EI mass spectra were obtained at 70 *eV* ionization voltages within the range of *m/z* 50–650 in the full scan mode. Temperature of the ion source was set at 200 °C. Identification of the components was achieved by comparison of the retention times and mass spectra with those reported WILEY 09 and NIST 14 mass spectral database [17].

2.4. Cytotoxicity assay

2.4.1. Cell lines

Five human tumor cell lines namely Hepatocellular carcinoma (HePG-2), Epitheliod Carcinoma (Hela), Human prostate cancer (PC3), Mammary gland (MCF-7) and Colorectal carcinoma (HCT-116). The cell lines were purchased from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt.

2.4.2. MTT assay

The above mentioned cells were used to determine the inhibitory effects on cell growth using the MTT assay. This is a colorimetric assay based on the change of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Cell lines were cultivated in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics were added in concentrations as follow: 100 µg/mL streptomycin and 100 units/mL penicillin at 37 ºC in a 5% CO_2 incubator. The seeding procedure of cell lines was performed in a 96-well plate at a density of $1.0x10^4$ cells/well at 37 ºC for 48 h under 5% CO_2. After incubation, different concentration of crude extract/fractions were added to the cells and incubated for 24 h. After 24 h of drug treatment, 20 µl of MTT solution at 5mg/mL was added and incubated for additional 4 h. Dimethyl sulfoxide (DMSO) in volume of 100 µl is added to each well to dissolve the formed purple formazan. This assay is measured and recorded at an absorbance of 570 nm by a plate reader (EXL 800, USA). The percentage of relative cell viability was calculated as (A570 of treated samples/A570 of untreated sample) X 100 [18,19]. Doxorubicin was used as a reference anticancer drug. The reagents RPMI-1640 medium, MTT and DMSO are purchased from sigma company (St. Louis, USA) while Fetal Bovine serum is from (GIBCO, UK).

2.5. DPPH free radical scavenging activity

The compounds' ability to donate hydrogen atom or electron was evaluated by the decolorizing of the purple methanolic solution of diphenylpicrylhydrazyl (DPPH). This spectrophotometric analysis uses the

stable radical (DPPH) as a reagent. The samples were dissolved in methanol to obtain a final concentration of 1 mg/mL. 200 µL of each sample were added to 0.4 mL of 0.1 mM DPPH in methanol. After 30 min of incubation in the dark, the absorbance was recorded against a blank at 517 nm [20]. Ascorbic acid was used as standard antioxidant (positive control). Blank sample was run without DPPH and using methanol instead of sample. Negative control sample was run with methanol instead of tested compound. The radical scavenging activity was calculated from the following equation:

% Inhibition = (A_{blank} – A_{sample})/(A_{blank}) x 100

2.6. Antimicrobial activities

The anti-bacterial activity of the extract/fractions was tested against gram positive bacteria (*Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*) while the anti-fungal activity was tested against (*Candida albicans*) by the agar diffusion technique [21,22]. Crude extract as well as fractions were dissolved in DMSO and solutions of the concentration 1 mg/mL were prepared separately on autoclaved paper discs of Whatman filter paper with standard size (5 mm). The paper discs were soaked in the desired concentration and placed aseptically in the petri dishes having nutrient agar media (agar 20g + beef extract 3g + peptone 5g) seeded with *B. subtilis* , *E. coli* and *C. albicans.* The petri dishes were incubated at 36 ºC and the inhibition zones were read after 24 h of incubation. Each experiment was done in triplicates. Antibacterial and antifungal activity of ciprofloxacin and clotrimazole were recorded under the same procedure, concentration and solvents as a standard reference compounds. The % activity index for the complex was calculated by the following formula:

%Activity Index = Zone of inhibition by test compound (diameter)/ Zone of inhibition by standard (diameter) x 100

3. Results and discussion

3.1. GC/MS analyses of C. peltata leaves extract

The chemical constituents of *C. peltata* were tentatively identified by the comparison of their mass spectra with related counterparts reported by NIST, Wiley9, Mainlib, Replib libraries and/or authentic spectra [17]. Analysis of *C. peltata* by GC-MS revealed the presence of thirty compounds (Figures 1,2 and Table 1), accounting to 75.12 % of the total extract composition. α- Tocospiro A (17.20%), α-tocospiro B (12.02%), squalene (7.78%), 13-epi-manoyl oxide (6.80%), nonacosane (5.12%), pentatriacontane (4.17%) and hexadecanoic acid methyl ester (3.78%) were the main components in the leaves extract.

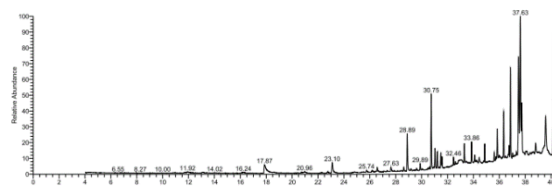

Figure 1: GC-MS chromatogram of the *C. peltata* leaves extract.

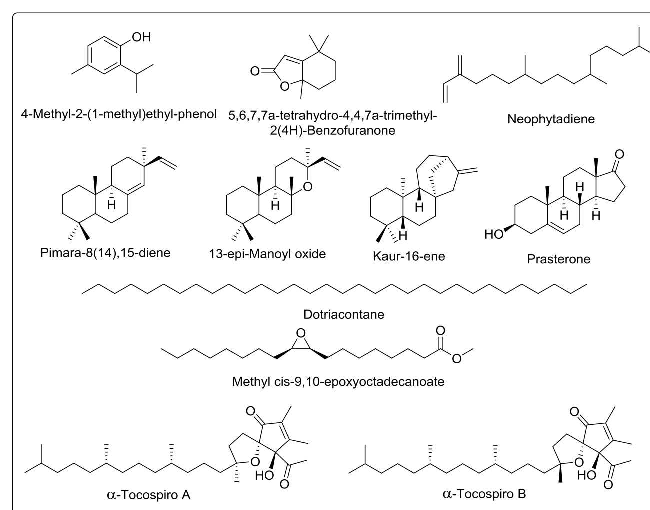

Figure 2: chemical structures of major compounds depicted from the GC-MS.

3.2. Cell viability test (Determination of IC_{50})

In this study, the cytotoxic effect of *C. peltata* leave extract (CP-LE) and its subsequent fractions (CP-H, CP-C and CP-B) was tested by MTT assay against five cancer cell lines namely; HepG-2, MCF-7, HCT-116, PC3 and HeLa cancer cells [23]. The results presented in Table 2 showed that leaves extract had the highest potency to inhibit the viability of all tested cell lines followed by chloroform fraction then butanol fraction, while n-hexane fraction exhibited the least inhibition of cell viability. Noticeably, HeLa cell line was the most sensitive to leave extract followed by chloroform, butanol and n-hexane fractions with an IC_{50} value of 7.56, 17.18, 25.37, 39.92 µg/mL respectively. On the other hand, HCT-116 and PC3 cell lines were the most resistant to both crude leave extract and its fractions (Table 2), while HepG-2 and MCF-7 cancer cells were moderately affected.

Table 1: Chemical constituents of *C. peltata* identified by GC-MS:

No.	RT (min)	Tentative indentification	Area%	Mol. formula	Mol. mass	Cpd. class
1	17.88	4-Methyl-2-(1-methyl)ethyl-phenol	1.81	$C_{10}H_{14}O$	150	Monoterpenoid
2	25.75	1-Docosanol	0.36	$C_{22}H_{46}O$	326	Fatty alcohol
3	26.18	5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4*H*)-Benzofuranone	0.34	$C_{11}H_{16}O_2$	180	Terpenoid
4	26.57	Neophytadiene	0.64	$C_{20}H_{38}$	278	Hydrocarbone
5	27.64	6,10,14-trimethyl-2-Pentadecanone	0.61	$C_{18}H_{36}O$	268	Ketone
6	28.63	2-methyl-1-Hexadecanol	0.47	$C_{17}H_{36}O$	256	Fatty alcohol
7	28.89	Hexadecanoic acid methyl ester	3.78	$C_{17}H_{34}O_2$	270	Fatty acid ester
8	29.17	(*Z*)-Methylhexadec-11-enote	0.23	$C_{17}H_{32}O_2$	268	Fatty acid ester
9	29.61	*E*-8-methyl-9-tetradecene-1-ol acetate	0.27	$C_{17}H_{32}O_2$	268	Fatty acid ester
10	29.89	Pimara-8(14),15-diene	0.62	$C_{20}H_{32}$	272	Diterpenoid
11	30.04	Hexadecanoic acid-14-methyl methyl ester	0.24	$C_{18}H_{36}O_2$	284	Fatty acid ester
12	30.63	3-Ethyl-5-(2-ethyl butyl)-octadecane	0.19	$C_{26}H_{54}$	366	Hydrocarbone
13	30.75	13-epi-Manoyl oxide	6.80	$C_{20}H_{34}O$	290	Diterpenoid
14	31.22	Kaur-16-ene	1.66	$C_{20}H_{32}$	272	Diterpenoid
15	31.49	(Z, Z, Z)-9,12,15-Octadecatrienoic acid methyl ester	1.41	$C_{19}H_{32}O_2$	292	Fatty acid ester
16	31.58	Pentacosane	0.99	$C_{25}H_{52}$	352	Hydrocarbone
17	32.61	Ergosterol	0.25	$C_{28}H_{44}O$	396	Steroid
18	33.31	Docosane	1.56	$C_{22}H_{46}$	310	Hydrocarbone
19	33.86	4,8,12,16-Tetramethylheptadecan-4-olid	1.88	$C_{21}H_{40}O_2$	324	Lactone
20	34.10	Dotriacontane	0.83	$C_{32}H_{66}$	450	Hydrocarbone
21	34.45	Prasterone	0.73	$C_{19}H_{28}O_2$	288	Steroid
22	34.87	2,2,4,15,17,17-hexamethyl-7,1,2-bis(3,5,5-trimethylhexyl)-octadecane	1.72	$C_{42}H_{86}$	590	Hydrocarbone
23	35.99	Stigmast-5-en-3-ol	0.88	$C_{29}H_{50}O$	414	Steroid
24	36.34	Pentatriacontane	4.17	$C_{35}H_{72}$	492	Hydrocarbone
25	36.47	Methyl cis-9,10-epoxyoctadecanoate	0.20	$C_{18}H_{34}O_3$	298	Fatty acid ester
26	36.87	Squalene	7.78	$C_{30}H_{50}$	410	Hydrocarbone
27	37.32	9-Hexadecenoic acid-9-octadecenyl ester	0.36	$C_{34}H_{64}O_2$	504	Fatty acid ester
28	37.49	α-Tocospiro B	12.02	$C_{29}H_{50}O_4$	462	Sesterterpenoid
29	37.63	α- Tocospiro A	17.20	$C_{29}H_{50}O_4$	462	Sesterterpenoid
30	37.74	Nonacosane	5.12	$C_{29}H_{60}$	408	Hydrocarbone
Total %			75.12			

Table 2: Influence of the extract/fractions of *C. peltata* on the viability of different cancer cell lines:

Extract/Fractions	*In vitro* Cytotoxicity IC_{50} (µg/ml) [a]				
	HepG-2	MCF-7	HCT-116	PC3	HeLa
CP-LE	9.39±0.8	8.52±0.7	11.48±1.0	14.26±1.1	7.56±0.5
CP-H	42.54±2.8	36.81±2.4	54.02±3.2	59.10±3.5	39.92±2.5
CP-C	24.68±1.9	19.28±1.8	32.79±2.4	35.07±2.5	17.18±1.5
CP-B	31.72±2.3	22.59±2.0	44.18±2.9	41.88±2.6	25.37±2.1
Doxorubicin	4.50±0.2	4.17±0.2	5.23±0.3	8.87±0.6	5.57±0.4

[a] **IC_{50} (µg/mL)**: 1 – 10 (very strong). 11 – 20 (strong). 21 – 50 (moderate). 51 – 100 (weak) and above 100 (non-cytotoxic)

3.3. DPPH free radical scavenging assay

The DPPH is an assay used to evaluate the antioxidant potential of different chemicals. It is considered as an effective method to estimate the radical-scavenging action of a compound by a chain-breaking mechanism [24,25]. This method depends on the ability of an antioxidant to decolorize DPPH. The results of the DPPH free radical scavenging activity listed in Table 3. The leaves extract was the most active in this assay (IC_{50} 32.86 µg/mL) with comparable activity to that of ascorbic acid (IC_{50} 29.70 µg/mL). The three fractions showed lower radical scavenger activity with IC_{50} 46.05, 50.06 and 57.42 µg/mL for chloroform, hexane and butanol respectively.

Table 3: *In vitro* DPPH free radical scavenging assay of the extract/fractions of *C. peltata*:

Extract/Fractions	Conc. (µg/mL)						
	10	20	30	40	50	60	IC_{50}
CP-LE	14.2	26.1	45.2	59.3	66.2	79.1	32.86±0.18
CP-H	10.8	24.6	30.2	38.7	42.5	54.9	57.42±0.25
CP-C	13.5	23.1	31.2	42.5	51.7	64.5	46.05±0.21
CP-B	9.7	25.2	33.6	40.8	46.8	59.6	50.06±0.24
Ascorbic acid	19.7	33.9	48.1	57.6	69.3	81.5	29.70±0.13

3.4. Antimicrobial activity evaluation

The antimicrobial potential of the crude leave extract/fractions was evaluated against gram-negative bacteria *E. coli*, gram-positive bacteria *B. subtilis* and the pathogenic yeast *C. albicans*. A standard agar diffusion technique was employed [26] and the diameters [mm] of inhibition zones are summarized in Table 4. Obviously, the leave extract was the most active showing the highest activity indices 46.1, 73.9 and 81.5% against *E. coli*, *B. subtilis* and *C. albicans* respectively in comparison to ciprofloxacin (antibacterial) and clotrimazole (antifungal) as a reference drugs. Chloroform fraction came in the second place after the leave extract with 30.8, 56.5 and 59.2% activity index against *E. coli*, *B. subtilis* and *C. albicans* respectively. The third place was occupied by butanol while hexane fraction was the least active fraction. It is also clear that the fungal strain *C. albicans* was the most sensitive showing the highest inhibition by *C. peltata* leaves extract/fractions while *B. subtilis* was moderately inhibited and the highest resistance was shown by the pathogenic gram-negative bacteria *E. coli*.

Table 4: Antimicrobial activities of the extract/fractions of *C. peltata* using agar diffusion assay:

Extract/Fractions	Diameter inhibition zone in mm (% activity index) [a]		
	E. coli	***B. subtilis***	***C. albicans***
CP-LE	12 (46.1)	17 (73.9)	22 (81.5)
CP-H	4 (15.4)	7 (30.4)	10 (37.0)
CP-C	8 (30.8)	13 (56.5)	16 (59.2)
CP-B	6 (23.1)	10 (43.5)	13 (48.1)
Ciprofloxacin	26 (100)	23 (100)	----
Clotrimazole	----	----	27 (100)

[a] Diameters (mm) of zones of inhibition are provided. In each case, 5 mm disks with 1 mg/mL of the test compounds were incubated. Ciprofloxacin and clotrimazole were used as the positive control (1 mg/mL).

4. Conclusion

C. peltata was investigated for its volatile secondary metabolites composition using GC-MS and was also evaluated for possible cytotoxic, antioxidant and antimicrobial activity. *C. peltata* showed a promising potential in all biological assays. These results assumed that *C. peltata* can be considered as a potential source for bioactive compounds, suggesting further investigation and isolation of chemical constituents to be carried out broadly, which may afford potential drug leads against different diseases.

5. list of abbreviations

Abbreviation	Title
C. peltata	*Coccoloba peltata*
GC-MS	Gas chromatography mass spectrometry
HePG-2	Hepatocellular carcinoma
Hela	Epitheliod Carcinoma
MCF-7	Mammary gland
PC3	Human prostate cancer
HCT-116	Colorectal carcinoma
DPPH	2,2-diphenyl -1-picrylhydrazyl
CP-LE	*C. peltata* leaves extract
CP-H	*C. peltata* hexane extract
CP-C	*C. peltata* chloroform extract
CP-B	*C. peltata* butanol extract

6. Disclosure statement

There are no conflicts to declare.

7. References

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