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Volatile Constituents and Cytotoxic Activity of the Fruits of *Pleiogynium timorense* (Dc.) Leenh.

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Abstract

The volatile constituents which obtained from fresh fruits of *Pleiogynium timorense*(Dc.) Leenh (Anacardiaceae) by hydro-distillation method were investigated by Gas chromatography and gas chromatography–mass spectrometry. Twenty seven compounds were identified representing 98.6% of the total oils. D-Limonene was the major oil constituent (64.51 %), followed by γ-Terpinene (5.60%), while, α-Copaene and trans-Caryophyllene showed the same percentage (4.74 %). In addition, phytochemical screening, proximate analysis, total phenolic and total flavonoid contents of the fruits were determined. Furthermore, the volatile constituents showed a cytotoxic effect against breast adenocarcinoma MCF7 and laryngeal carcinoma HEp2 human tumor cell lines and a moderate cytotoxic effect on human hepatoma HepG2 cells.

1. Introduction

Aiming to avoid the complications accompanied chemical drugs; many studies were carried out to produce drugs from plant origin, as edible plants possess relatively safer constituents. Gambozia is the common name of *Pleiogynium timorense* (DC.) Leenh. which belongs to family (Anacardiaceae) that contains many edible plants [1-3]. *Pleiogynium timorense* (DC.) Leenh. is characterized by its edible fruits that were used in jellies and jams [4]. The leaves of gambozia showed hypoglycaemic, anti-inflammatory, antioxidant, and antimicrobial activities. Moreover, phenolic contents as flavonoids were isolated and identified from gambozia leaves [5, 6]. Regarding the fruits, similar to the leaves, they have antioxidant properties with cyaniding-3-glucoside as one of the constituents [7].

The seeds and pericarp of gambozia showed hepato-renal protective, analgesic, antioxidant and anti-inflammatory activities, the study showed the isolation and identification of phenolic contents such as; quercetin, quercetrin ,catechin and rutin from the pericarp of the plant [8]. Lipoidal matter of the seeds was analyzed by the GC/MS analysis, 5, 24 (28)-cholestadien-24-methylen-3b-ol and α–amyrin were isolated and identified from gambozia seeds [9]. Furthermore, three new bioactive trihydroxy alkylcyclohexenones were isolated and identified from dichloromethane extract of the bark which showed activity against the A2780 human ovarian cancer cell line [10]. Recently, identification of the constituents from both seeds and pericarp of *Pleiogynium timorense* were identified using High-Performance Liquid chromatography with Electrospray Ionization Mass Spectrometry [11].

In this study, the volatile constituents of the fruits of *Pleiogynium timorense* (Dc.) Leenh were identified for the first time. In addition, phytochemical screening, proximate analysis, total phenolic and total flavonoid contents of the fruits were determined. Furthermore, we tested the possible cytotoxic effect of the isolated volatile oil against several human tumor cell lines. The obtained results demonstrated the beneficial effects of the fruits of *Pleiogynium timorense* (DC.) Leenh. It may participate as a new flavouring agent in different industries

including food and pharmaceutical products. In addition, it might provide a new candidate plant in the treatment of different human cancers.

2. Materials and methods

2.1. Plant material

The fresh fruits of *Pleiogynium timorense* (DC.)Leenh. (Anacardiaceae) plant were collected from Zoo garden, Giza, Egypt in April 2015. The plant was identified by Dr Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC), Egypt and by Mrs. Tereeza Labib consultant of plant taxonomy at the ministry of agriculture and director of Orman botanical garden, Giza, Egypt. Voucher specimen was kept in our laboratory in NRC, possessing number 2001.

2.2. Phytochemical screening

Chemical tests were carried out on the 70% methanolic extract using standard procedure to identify the constituents as described by [12, 13].

2.3. Total phenolic assay

The total phenolic content (TP) was determined by applying the Folin–Ciocalteu colorimetric method using gallic acid as a standard [14]. It was expressed as milligrams of gallic acid equivalents (GAE)/g of the dry plant materials.

2.4. Total flavonoid assay

Total flavonoid content (TFC) was measured by using an aluminum chloride colorimetric assay [15]. A calibration curve was established using quercetin as a standard. TFC was expressed as mg quercetin equivalent (QE)/g of the dry plant materials.

2.5. Proximate analysis

Percentages of moisture content, total ash, water soluble ash, acid-insoluble ash, and crude fibre values were assessed according to the official methods [16].

2.6. Essential oil extraction

The fresh fruits of *Pleiogynium timorense* (500g) were cut into small pieces and subjected to hydro-distillation for 3 hours at 50^{o}C in Clevenger-type apparatus according to the Egyptian Pharmacopoeia [16]. The resulted essential oil was dehydrated with anhydrous sodium sulphate to yield 0.022 % (w/w) and kept in the freezer at (-20^{o}C) until GC-MS analyses.

2.7. GC-MS analysis

The GC/MS analysis of the essential oil was carried out by using gas chromatography-mass spectrometry instrument stands at the Department of Medicinal and Aromatic Plants Research, National Research Centre with the following specifications. Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TG-5MS column (30 m x 0.25 mm i.d., 0.25 µm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 ml/min and a split ratio of 1:10 using the following temperature program: 60 ^{o}C for 1 min; rising at 3.0 ^{o}C /min to 240 ^{o}C and held for 1 min. The injector and detector were held at 240 ^{o}C. Diluted samples (1:10 hexane, v/v) of 0.2 µL of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV using a spectral range of m/z 40-450.

2.8. Identification of components

Compounds were identified by comparing their spectral data and RI, Retention Index relative to standard n-alkanes, with Wiley spectral library collection and NIST Mass Spectral Library. In addition, retention times and mass spectra were also compared with those of authentic pure samples.

2.9. Cell culture

Human hepatoma HepG2, human laryngeal carcinoma HEp2, human breast adenocarcinoma MCF7 cell lines were obtained from VACSERA, Giza, Egypt. Cells were maintained in Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient Mixture (Gibco™, USA), supplemented with 10% heat-inactivated fetal bovine serum (PAA laboratories, Canada), 2 mM l-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco™, USA). Cells were grown in 75-cm^2 tissue culture flasks at 37°C in a 5% CO2 humidified incubator.

2.10. Determination of cytotoxicity of the total volatile constituents

The effect of total volatile constituents of Gambozia fruits on HepG2, MCF7, and HEp2 cell viability was determined by measuring the capacity of reducing enzymes present in viable cells to convert [3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazoliumbromide] (MTT, Sigma, USA) to formazan crystals as described previously [17]. Briefly, cells were incubated with increasing concentrations of Gamboziatotal volatile constituents dissolved in DMSO in serum free media onto a 96-well cell culture plate at 37°C under a 5% CO_2 humidified incubator for 24 h, the media were removed and 100 µL of serum-free media containing 1.2 mM of MTT dissolved in phosphate-buffered-saline (PBS), pH 7.4, was added to each well. After 2 h incubation in a CO_2 incubator at 37°C, the media were then decanted off by inverting the plate, and a 100 µL of isopropyl alcohol was added to each well, with shaking for 1 h to dissolve the formazan crystals. The color intensity of the blue formazan solution formed in each well was measured at wavelength of 570/690 nm using Biochrom Anthos Zenyth 200 microplate reader (Bio-chrom LTD, USA). The percentage of cell viability was calculated relative to control wells (DMSO-treated cells) designated as 100% viable cells. Doxorubicin HCl (Adricin®, EUP, Egypt) was used as a positive control that showed more than 85% cytotoxicity for all tested cell lines.

3. Results and discussion

3.1. Phytochemical screening

Phytochemical analysis of the medicinal plants is very important in the evaluation of biologically active compounds. Table 1 showed that flavonoids, tannins, saponins, terpenoids, and coumarins were present, while alkaloids were absent in the fruits of *Pleiogynium timorense*.

Table 1: Results of phytochemical screening of *Pleiogynium timorense* pericarp and seeds

Constituents	***Pleiogynium timorense* Fruits**
1.Carbohydrates and/or Glycosides	+ ve
2. Tannins	++ ve
3. Alkaloids and/or nitrogenous bases	-ve
4. Flavonoids	++ ve
5. Sterols and/or triterpenes	+ ve
6. Saponins	±ve
7. Coumarins	+ ve

+ ve denotes the presence of the constituents, - ve denotes the absence of the constituents
±ve denotes the presence of the constituents in minute amounts

3.2. Total phenolic and flavonoids contents

The phenolic content was expressed as mg / g gallic acid equivalent (GAE).

The results showed that the total phenolic content of the fruits of *Pleiogynium timorense* was 15.6 mg gallic acid equivalent / g of the dry plant materials, while the total flavonoid was 12.3 mg quercetin equivalent (QE)/g of the dry plant materials. These results indicate that the fruits of gamposia contain high percentage of the phenolic content. Moreover, the diet that rich of flavonoids could protect the human from many diseases [18-20].

3.3. Proximate analysis

Table 2 showed the percentages of total ash, acid-insoluble ash, water soluble ash, crude fiber, and moisture content of the fruits of *Pleiogynium timorense*. From these results, it could be concluded that, these constants could be used as criteria for the purity of the fruits of *Pleiogynium timorense*.

Table 2: Percentages of certain pharmacopoeial constants of the fruits of *Pleiogynium timorense*

Pharmacopoeial constants	**Fruits of *Pleiogynium timorense***
Moisture (%)	8.76
Total Ash (%)	3.56
Water-soluble ash (%)	2.34
Acid-insoluble ash (%)	0.98
Crude fibre (%)	9.98

3.4. Volatile Constituents and Cytotoxic Activity

The pharmacological and medicinal activities of essential oils from plant origin such as anti-inflammatory, antioxidant and antimicrobial activities resulted in using these oils in many potential applications as in pharmaceuticals and in perfumes [21]. Our study showed the identification of the volatile constituents from the fruits of *Pleiogynium timorense*(Dc.) Leenh (Anacardiaceae) for the first time. Results of composition of volatile oils from fresh fruits of *Pleiogynium timorense* (Dc.) Leenh are shown in table 3. The relative area of each compound of the oils was measured by calculating the peak area of the compound relative to total peak area. According to the order of the elution, the constituents are listed where twenty seven compounds were identified representing 98.6% of the total oils. D-Limonene, a monocyclic monoterpene compound, was the major oil constituent (64.51 %), followed by γ-Terpinene (5.60 %) while, α-Copaene and trans-Caryophyllene showed the same percentage (4.74 %) (Table 3). Results of cytotoxic properties of volatile constituents of the fruit of Gambozia are shown in Figure (1).

Table 3. Composition of volatile oils from fresh fruits of *Pleiogynium timorense*(Dc.) Leenh

No.	R_t (min)	RI	Area %	Compounds
1	6.30	969	0.67	β-Myrcene
2	7.21	997	0.32	α-Terpinene
3	7.64	1010	64.51	D-Limonene
4	8.19	1027	0.33	β Ocimene Y
5	8.65	1040	5.60	γ-Terpinene
6	9.62	1066	0.70	α- Terpinolen
7	10.30	1082	0.38	L-Linalool
8	10.55	1088	3.83	Nonanal
9	13.66	1163	0.60	4-Terpineol
10	13.99	1170	1.38	Naphthalene
11	14.37	1178	0.34	α-Terpineol
12	14.51	1181	0.38	Estragole
13	15.31	1197	0.28	α-Cyclocitral
14	15.64	1205	2.96	Thymyl Methyl Ether
15	21.22	1330	0.35	Cyclosativene
16	21.53	1337	4.74	α-Copaene
17	23.37	1378	4.74	trans-Caryophyllene
18	23.92	1389	0.33	α-Bergamotene
19	24.15	1394	0.36	Dihydro-β-ionone
20	24.90	1411	0.49	α-Caryophyllene
21	26.07	1439	0.45	β Ionone
22	26.96	1460	0.79	Butyl Hydroxy Toluene
23	27.47	1472	2.23	δ-Cadinene
24	32.05	1581	0.44	γ-Eudesmol
25	32.98	1602	0.58	β-Eudesmol
26	47.70	2004	0.50	Nonadecane
27	48.10	2016	0.32	Phytol

R_t= Retention Time

RI= Retention Index relative to standard n-alkanes.

The obtained results showed that the total volatile constituents is cytotoxic to human breast cancer MCF7 cells (IC_{50}= 0.021 µL/mL) and human laryngeal cancer HEp2 cells (IC_{50} = 0.0263 µL/mL). However, lower effect was exhibited with human hepatoma HepG2 cells (IC_{50} = 0.172 µL/mL), suggesting a promising use of the fruits and their oil as an antitumor agents. Although there is no data regarding the cytotoxicity of the Gambozia fruit, it was demonstrated that the bark possesses three trihydroxy alkyl cyclohexenones when tested against the A2780 human ovarian cancer cell line [10].In agreement with our results, the main active ingredient identified, D-limonene, was found to enhance cytotoxicity of docetaxel against human prostate cancer cells [22].

Figure 1

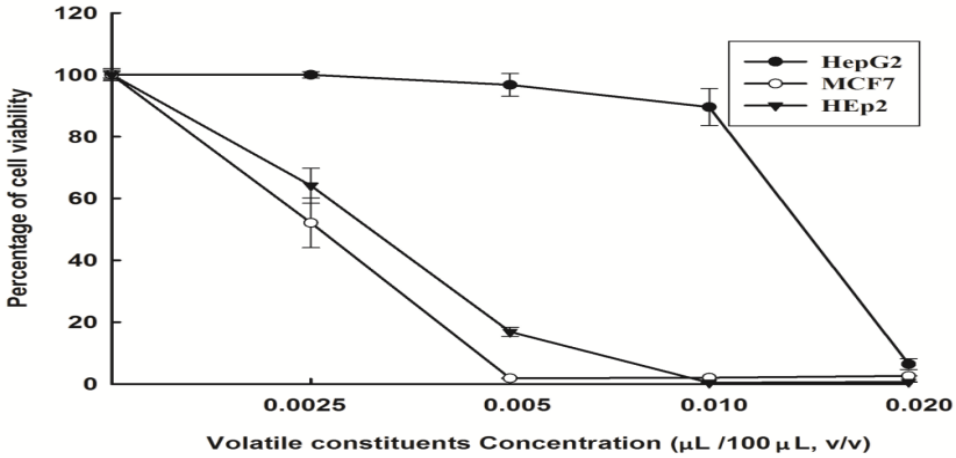

Figure (1). Cytotoxic properties of volatile constituents of the fruit of Gambozia

Conclusion

The volatile constituents of the fruits of *Pleiogynium timorense* (Dc.) Leenh were identified for the first time in the present study. Furthermore, the volatile constituents possess a cytotoxic activity against breast (MCF7) and laryngeal (HEp2) human cancer cell lines and a lower effect on human hepatoma HepG2 cells. The obtained results demonstrate the beneficial effects of the fruits of *Pleiogynium timorense* (DC.) Leenh and its volatile constituents that may participate as a new flavouring agent in different industries including food and pharmaceutical products. In addition, it might provide a new candidate plant in the treatment of different human cancers.

Conflicts of interest There are no conflicts of interest.

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