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RESEARCH ARTICLE

CYTOTOXIC EFFECT, ANTIOXIDANT POTENTIAL, AND PHYTOCHEMICAL STUDY OF THE ETHYL ACETATE EXTRACT OF *PLEIOGYNIUM TIMORENSE* SEEDS

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Abstract

Objective: The aim of the current research was to evaluate the cytotoxicity of *Pleiogynium timorense* seeds against different human cancer cell lines, its antioxidant activity and to investigate its phytoconstituents.
Methods: Ethyl acetate extract of *Pleiogynium timorense* seeds was assayed for the cytotoxic effect against liver cancer cell line (HepG2), ovarian cancer cell line (SKOV-3) and prostate cancer cell line (PC-3) using SRB (Sulforhodamine B) assay. The antioxidant activity was evaluated by the DPPH radical scavenging assay using Trolox as a standard. The phytochemical components of the plant extract were examined using various phytochemical screening methods. The polyphenolic contents of the extract were analyzed using high performance liquid chromatography (HPLC).
Results: The result revealed that the seed extract exhibited very potent effect against HepG-2 cancer cell line with IC_{50}=1.62 µg/mL, and against SKOV-3 cancer cell line with IC_{50}=6.37 µg/mL, while a moderate effect against PC-3 cancer cell line with IC_{50}=46 µg/mL, by comparing with that of Doxorubicin. Moreover, IC_{50} values of Trolox and the seed extract were 24.42±0.87 and 90.4±0.32 µg/ml, respectively. The results revealed the presence of the flavonoids, tannins and triterpenes and/or sterols in the seed extract. While, it revealed the absence of coumarins, alkaloids, saponins and carbohydrate and/or glycosides from the extract.
Conclusion: In conclusion, the current study highlights the effect of ethyl acetate extract of *Pleiogynium timorense* seeds as antioxidant and a potent cytotoxic agent against different human cell lines aiming to be the first step towards the discovery of safe natural anticancer drug.
Keywords: Antioxidant; cytotoxic; ethyl acetate extract; phytoconstituents; *Pleiogynium timorense* seeds.

INTRODUCTION

Anticancer medications are regarded as the first choice for the treatment of many kinds of cancer[1]. There are numerous anticancer medications with various sources and modes of action; nevertheless, majority of these medications have detrimental side effects. Thus, it is necessary to create new anticancer medications with less adverse effects[2]. Nowadays, medicinal plants are used to cure serious diseases like cancer. These plants contain several phytochemicals such as tannins, triterpenes, alkaloids and flavonoids which showed promising biological activities[3].

Pleiogynium timorense (DC.) Leenh. From the family of Anacardiaceae, is commonly known as Gambozia. The plant is a rich source of biologically active components that are crucial to the bioactivities of plants such as polyphenolic compounds[3-5]. Gallic acid and catechin isolated from *P. timorense* bark exhibited a potential activity against HepG2 cancer cell line[4]. In addition, trihydroxy alkylcyclo hexenones isolated from *P. timorense* bark dichloromethane extract exhibited cytotoxic activity against the A2780 cancer cell line[6]. The methanol extract of *P. timorense* seeds, pericarp and fruits exhibited a strong antioxidant activity as a result of polyphenolic compounds[7,8].

The volatile constituents of the fruits exhibited a promising cytotoxic effect against different human cell lines[9]. In addition, *P. timorense* seeds methanol extract exhibited antihyperlipidemic and antihyperglycaemic activities[10]. The leaves of the plant exhibited promising biological activities such as; antimicrobial, anti-inflammatory, and hypoglycaemic, activities due to the presence of polyphenolic compounds[11,12]. Recently, *P. timorense* leaves ethyl acetate extract exhibited a potent cytotoxic activity against (SKOV-3), a reasonable activity against (HepG-2) with lower activity against (PC- 3)[3].

Nothing could be found in the literature that is currently available that discusses phytoconstituents or bioactivities of *P. timorense* seeds ethyl acetate extract. Thus, the aim of this study is to evaluate antioxidant and anticancer potentials of *P. timorense* seeds ethyl acetate extract against different human cancer cell lines and to investigate its phytoconstituents.

MATERIALS AND METHODS

Materials for phytochemical study

Plant material

Fruits of *P. timorense* plant were collected from Zoo Garden, Giza, Egypt, and identified by Dr Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC), Egypt. Voucher specimen was deposited in the Herbarium of NRC, with the possessing number of 2001.The seeds were separated from fruits, dried, powdered, and were kept in dark well-closed containers.

Phytochemical analysis

Preparation of plant extract

The air-dried powder (1.5 kg) of *P. timorense* seeds was extracted by maceration with ethyl acetate till exhaustion. The extract was filtered and concentrated to dryness at 40°C by using rotatory evaporator.

Phytochemical screening

Using the previously mentioned standard techniques, the components of the plant extract were determined[13,14].

HPLC analysis

An Agilent 1260 series was used for the HPLC analysis. The Kromasil C18 column (4.6 mm x 250 mm i.d., 5 µm) was used for the separation. Water (A) and 0.05 percent trifluoroacetic acid in acetonitrile (B) are the components of the mobile phase, and their flow rate is 1 ml/min. The linear gradient was sequentially programmed into the mobile phase as follows: 0 minutes (82 percent A), 0 to 5 minutes (80 percent A), 6 to 8 minutes (60 percent A), 12 to 15 minutes (85 percent A), and 16 to 18 minutes (82 percent A). At 280 nm, the multi-wavelength detector was observed. For each of the sample solutions, a volume of 10 µL was injected. The column was kept at a constant 35 °C temperature. The identification was based on matching UV spectra and retention times to those of the standards[15].

In vitro cytotoxic activity

Cancer cell lines

The cytotoxicity assay was carried out against human cancer cell lines such as HepG2 (liver cancer cell line), PC-3 (Prostate cancer cell line) and SKOV-3 (Ovarian cancer cell line). From Nawah Scientific Inc., these cell lines were purchased (Mokatam, Cairo, Egypt).

Cell culture

Cells were kept at 37°C in RPMI medium provided with penicillin (100 units/ml), streptomycin (100 mg/ml), and 10% of heat-inactivated fetal bovine serum in humidified atmosphere with CO_2 (5% (v/v)[16,17].

SRB assay

SRB assay was used to measure cell viability. The 96-well plates were seeded with aliquots of the 100 µL cell suspension (5x10^3 cells), which were then cultured completely for 24 hours. Total 100 µL of another aliquot media with the tested drugs of different concentrations (0.01, 0.1, 1, 10, 100 µg/mL) was used to treat the cells. Cells were fixed by changing medium with 10% TCA (150 µL) and incubating at 4°C for 1 hour after 72 hours of drug exposure. After the TCA solution was withdrawn, distilled water was used to wash the cells five times. 70 µL of SRB solution was added with concentration 0.4%w/v, and the mixture was then incubated for 10 minutes at room temperature and in a dark area. The acetic acid solution (1%) was used for washing the plates three times, and they were then left overnight to air dry. The protein-bound SRB dye was then dissolved in 150 µL of 10 mM TRIS. A BMG LABTECH®- FLUO star Omega microplate reader (Ortenberg, Germany) was used to measure the absorbance at 540 nm[16,17].

In vitro antioxidant activity

Sample preparation

Initial screening step:

Solution of the seed extract was prepared in DMSO with two concentrations (1000 and 100 µg/mL).

IC_{50} determination:

Five concentrations were produced by serially dilution of extracts that exhibited an inhibition more than 50% in any of the concentrations used in the initial screening stage.

Trolox stock solution:

100 µM of trolox in methanol was prepared in various concentrations (5, 10, 15, 20, 30, 40, and 50 µM).

DPPH Assay

According to the procedure reported by Boly *et al.*, the free radical assay of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) was performed[18]. Briefly A 96-well plate containing 100 µL of the sample was introduced to 100 µL of DPPH reagent freshly prepared with percentage 0.1% in methanol (n=3). The reaction was allowed to sit in the dark for 30 min at room temperature. The decrease in colour intensity of DPPH at 540 nm was assessed at the end of incubation time.

Data analysis:

Microsoft Excel® was used to analyse the data, and GraphPad Prism 5® was used to determine the IC_{50} value using logarithmic value of the concentration and applying the following equation (log (inhibitor)/ normalised response- equation of variable slope)[19]. The antioxidant effect was assessed using IC_{50} that express the concentration at which 50% of the DPPH molecules are reduced.

RESULTS AND DISCUSSION

Phytochemical study

Phytochemical screening

The results of the phytochemical screening of ethyl acetate extract of *P. timorense* seeds revealed the presence of the flavonoids tannins and triterpenes and/or sterols in the plant extract. While it revealed the absence of coumarins, alkaloids, saponins and carbohydrate and/or glycosides from the plant extract.

Table 1: Polyphenolic compounds identified in ethyl acetate extract of *P. timorense* seeds.

S.N.	Polyphenols	Area %
1	Gallic acid	7.3
2	Chlorogenic acid	24.7
3	Catechin	17.2
4	Methyl gallate	5.5
5	Caffeic acid	3.9
6	Coumaric acid	7.4
7	Ellagic acid	4.5
8	Vanillin	6.3
9	Naringenin	5.9
10	Taxifolin	4.5
Total identified compounds		87.2%

These results confirmed that the seed extract is a rich source with the phytochemical constituents that contribute significantly in the plant bioactivities. This result was in agreement with what were reported by previous studies which found that flavonoids, terpenoids, saponins, carbohydrate, tannins and coumarins, were detected in the leaves, bark and fruits of *P. timorense* with absence of alkaloids[3,4,10].

HPLC Analysis

HPLC analysis of polyphenolic compounds revealed the identification of ten polyphenolic compounds in ethyl acetate extract of *P. timorense* seeds representing 87.2% of the total area, where chlorogenic acid (24.7 %), catechin (17.2%), Coumaric acid (7.4%), and gallic acid (7.3 %) represent the major compounds (Table 1 and Figure 1). The result was in agreement with previous studies which reported that *P. timorense* is a rich source with polyphenolic compounds[3-5].

Said *et al.*, reported that the methanol extracts of pericarp and seeds of *P. timorense* were analyzed by HPLC–ESI-MS/MS, the result revealed the presence of diversity of polyphenolic compounds in each extract[5]. Abdel Raoof *et al.*, stated that the polyphenolic compounds of *P. timorense* bark were analyzed by HPLC, the result revealed the identification of 16 phenolic compounds and 14 flavonoidal compounds[4]. Recently, *P. timorense* leaves ethyl acetate extract was analyzed by HPLC analysis and the study identified 11 polyphenolic compounds in which chlorogenic acid, gallic acid, catechin and taxifolin were the major compounds[3].

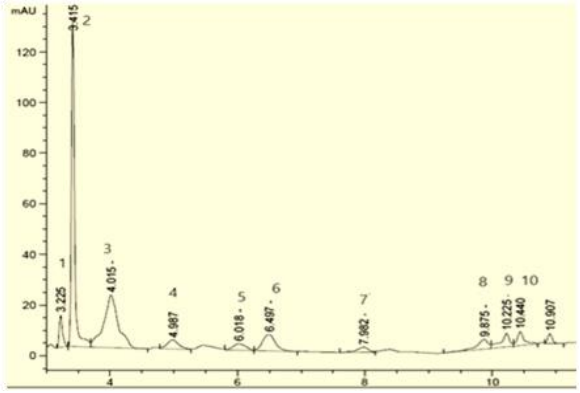

Figure 1: HPLC analysis of polyphenolic compounds in ethyl acetate extract of *P. timorense* seeds.

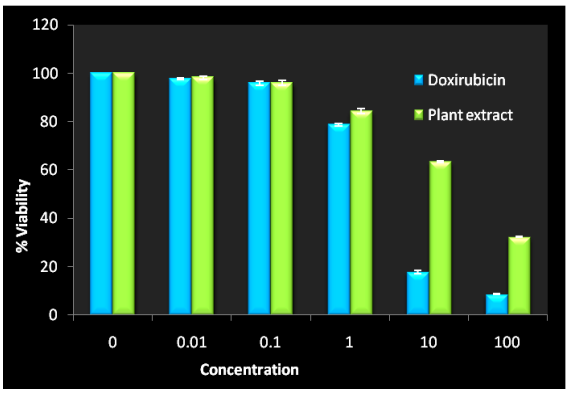

Figure 2: Cytotoxic activity of ethyl acetate extract of *P. timorense* seeds against PC-3 human cell line *in vitro*.

Cytotoxicity activity

Ethyl acetate extract of *P. timorense* seeds was evaluated for its *in vitro* cytotoxic activity against different cancer cell lines using Doxorubicin as a reference anticancer agent. The results revealed that the plant extract exhibited a potent cytotoxic activity in dose dependent manner. The percentage of the tested cancer cell lines was markedly decreased by the plant extract (Figure 2-Figure 4). Moreover, the result revealed that the plant extract exhibited a very potent effect against HepG-2 cancer cell line with IC_{50}=1.62 µg/mL, a potent cytotoxic effect against SKOV-3 cancer cell line with IC_{50}=6.37 µg/mL, while it showed a moderate effect against PC-3 cancer cell line with IC_{50}=46 µg/mL, by comparing with that of Doxorubicin (Table 2). Previous studies confirmed the cytotoxic activity of *P. timorense* on different human cancer cell lines[4,6,9]. *P. timorense* bark exhibited a potent cytotoxic effect against the A2780 human ovarian cancer cell line[6], and liver cancer cell line (HepG-2)[4]. In addition, the volatile constituents of *P. timorense* fruits exhibited potent cytotoxic effect against breast (MCF7) and laryngeal (HEp2) human cancer cell lines[9]. Recent research reported that *P. timorense* leaves ethyl acetate extract exhibited a powerful cytotoxic activity against (SKOV-3), a reasonable activity against (HEPG-2) with lower activity against (PC-3)[3]. The phytoconstituents of *P. timorense* play a vital role as cytotoxic agent[4,6]. Where, trihydroxy alkylcyclo-hexenones isolated from *P. timorense* bark dichloro-methane extract, exhibited cytotoxic activity against the A2780[6].

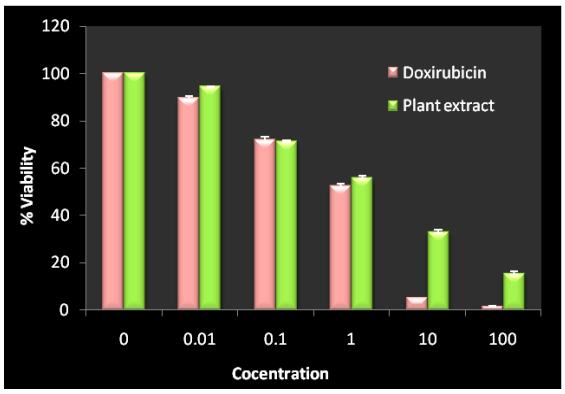

Figure 3: Cytotoxic activity of ethyl acetate extract of *P. timorense* seeds against HEPG-2 human cell line *in vitro*.

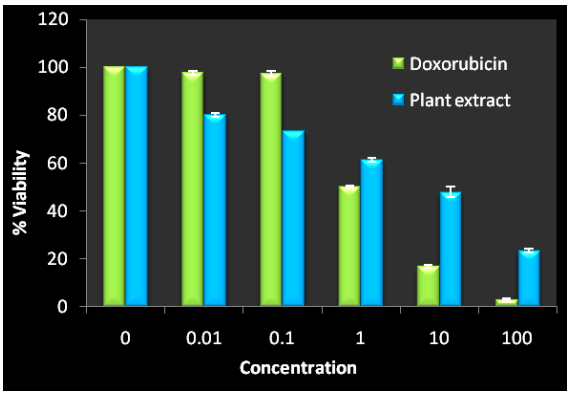

Figure 4: Cytotoxic activity of ethyl acetate extract of *P. timorense* seeds against SKOV-3 human cell line *in vitro*.

Moreover, gallic acid and catechin isolated from *P. timorense* bark exhibited a potent effect against HepG2 cell line[4]. In addition, *P. timorense* was nontoxic up to 5g/kg that indicated that the plant was safe and nontoxic for the normal cells[7].

Table 2: IC_{50} values (µg/ml) of ethyl acetate extract of *P. timorense* seeds *in vitro* on different human cell lines.

Type of cell line	IC_{50} µg/ml	
	Ethyl acetate extract of *P. timorense* seeds	Doxorubicin
SKOV-3	6.37	0.96
PC-3	46.00	5.38
HEPG-2	1.62	0.66

Antioxidant activity

The antioxidant activity of ethyl acetate extract of *P. timorense* seeds was evaluated by the DPPH radical scavenging test using Trolox as a reference. IC_{50} values of Trolox and the extract were 24.42±0.87 and 90.4± 0.32 µg/ml, respectively (Figure 5 and Figure 6).

This result revealed the antioxidant activity of the seed extract that is correlated with the the presence of polyphenolic compounds. The result of HPLC analysis revealed that chlorogenic acid and catechin were the major compounds in the seed extract which were also the major contributors to the antioxidant activity of the extract. It has been widely stated that chlorogenic acid and catechin are well-known antioxidants via affecting the balance of reactive oxygen species (ROS) regulation[20,21]. That was in agreement with previous studies which stated that the methanol extract of *P. timorense* seeds, pericarp and fruits exhibited a potent antioxidant activity due to the presence of polyphenolic compounds[7,8].

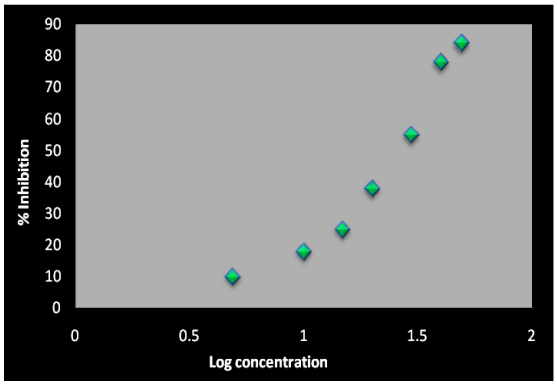

Figure 5: DPPH radical scavenging effect of Trolox.

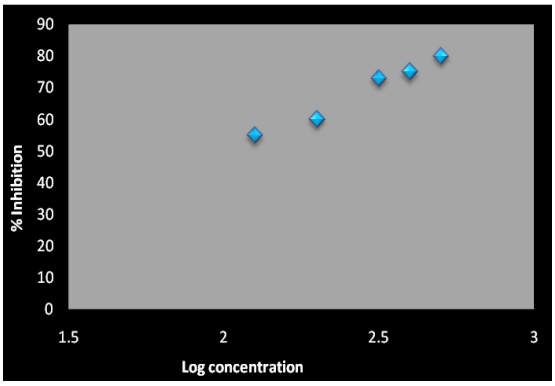

Figure 6: DPPH radical scavenging effect of *P. timorense* seeds extract.

CONCLUSIONS

P. timorense ethyl acetate extract of the seeds exhibited a promising cytotoxic activity against different human cancer cell lines and a potent antioxidant activity. The seed extract is a rich source for polyphenolic components that are essential for the bioactivities of the plant. Further contributions by researchers are needed to isolate and identify the biologically active compounds of the plant extract, and to perform clinical trials to enter the field of drug discovery.

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AUTHOR'S CONTRIBUTION

Raoof GFA: writing original draft, phytochemical study. **Said AAH:** investigation, data interpretation. **Mohamed KY:** methodology, biological study. **El**

Kareem AMZA: literature survey, formal analysis, review. **Mahmoud TH:** critical review, supervision. The final manuscript was read and approved by all authors.

DATA AVAILABILITY

The data supporting the findings of this study are not currently available in a public repository but can be made available upon request to the corresponding author.

CONFLICTS OF INTEREST

None to declare.

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