Phytoconstituents and bioactivities of the bark of *Pleiogynium timorense* (DC.) Leenh (Anacardiaceae)

Gehan Fawzy Abdel Raoof 1*, Ataa Abdelhaleem Said1, Khaled Younes Mohamed2, Hesham A. Gomaa3,4

1Pharmacognosy Department, National Research Centre, Dokki, Giza, Egypt
2Internal Medicine Department, Medical Division, National Research Centre, Giza, Egypt
3Biochemistry Department, Faculty of Pharmacy, Nahda University, Beni-Suef, Egypt
4Pharmacology Department, College of Pharmacy, Jouf University, Sakakah, Saudi Arabia

**Introduction:**

The purpose of this study was to evaluate the phytoconstituents and various bioactivities of *Pleiogynium timorense* bark as a step towards the production of a new drug from natural origin to overcome the complications of the synthetic drugs.

**Methods:**

The phenolic compounds were isolated and identified by chromatographic and spectroscopic methods as ultra violet (UV) and nuclear magnetic resonance (NMR) spectra. The isolated compounds, as well as 70% methanol extract of *P. timorense* bark were tested for cytotoxicity against human colon carcinoma (HCT 116), human hepatocellular liver carcinoma (HepG2), normal melanocytes (HFB-4) and human breast carcinoma (MCF-7) cell lines. In addition, the methanol extract was evaluated for renal protective, hepatoprotective, antioxidant and antihyperglycaemic activities.

**Results:**

Seven phenolic compounds were isolated from the bark of the plant for the first time which were identified as; pyrogallol, catechin, gallic acid, kaempferol, quercetin, rutin and quercitrin. Moreover, the methanol extract of the bark showed a promising cytotoxic effect against HepG2 cell line more than that of the isolated compounds comparing with doxorubicin (a positive control), where catechin and gallic acid showed moderate effects. In addition, the methanol extract showed potent antioxidant, hepatorenal protective and antihyperglycaemic effects.

**Conclusion:**

*Pleiogynium timorense* extract possesses a potent cytotoxic effect against HepG2 cell line and significant antioxidant, hepatorenal protective and antihyperglycaemic effects.

**Implication for health policy/practice/research/medical education:**

The results of this study suggest that the methanol extract of *Pleiogynium timorense* bark, as well as catechin and gallic acid can be used as promising cytotoxic agents against HepG2 cell line. Moreover, the methanol extract can be used as a potent antioxidant, hepatoprotective and antihyperglycaemic agent upon further clinical studies.
the leaves of the plant (6). Cyanidin-3-glucoside was isolated from *Pleiogynium* fruits which showed a potent antioxidant activity (7). In our previous work, rutin, catechin, quercetin and quercetin were isolated from the pericarp of the plant, as well as the methanol extracts of the pericarp and seeds showed anti-inflammatory, antioxidant, analgesic, hepatorenal protective effects (8). Moreover, GC/MS analysis of the lipoidal matter of the seeds showed that 1-heptene was the major compound in unsaponifiable matter, while linoleic acid was the major fatty acid (9). Furthermore, dichloromethane extract of the bark showed a significant activity against the A2780 human ovarian cancer cell line due to the presence of three new trihydroxy alkylcyclohexenones which were isolated from this extract (10). In our recent work the constituents of *P. timorense* pericarp and seeds were identified by using high-performance liquid chromatography (HPLC) with electrospray ionization mass spectrometry (11). Moreover, the volatile constituents of *P. timorense* fruits were identified which showed a moderate cytotoxic effect on human hepatoma cells and a powerful cytotoxic effect against laryngeal carcinoma and breast adenocarcinoma human tumor cell lines (12). The present study aims to the methanol extract of the bark of this plant as antioxidant, antihyperglycaemic, cytotoxic and hepatorenal protective agent with the aim of producing a natural drug.

**Materials and Methods**

**Equipments, materials and chemicals**

Solvent mixtures; $S_1$: BAW (n-butanol: acetic acid: water (4:1:5, v/v/v) upper phase), $S_2$: (water: glacial acetic acid (85:15, v/v)) and $S_3$: (methanol: chloroform (30:70)). Thin layer chromatography (TLC) was done on TLC silica gel F$_{254}$ plates (200 mm layer thickness) (Merck; Darmstadt, Germany), column liquid chromatography was done on Silica gel (0.063–0.200 mm) and Sephadex LH-20 (Pharmacia Fine Chemicals). Nuclear magnetic resonance (NMR) spectra were measured on JEOL EX-500 spectrometer (Tokyo, Japan) with 500 MHz for $^1$H-NMR and 125 MHz for $^{13}$C-NMR. MS was done using Finnigan MAT SSQ 7000, 70 eV. Ultraviolet spectra were recorded by using UV/VIS: Shimadzu UV; chromatograms were visualized under visible recording spectrophotometer model-UV 240 (NRC, Egypt).

**Plant identification and collection**

The bark of *P. timorense* was prepared from Zoo garden, Giza, Egypt. The plant was identified by Dr. M. El-Gebaly, the taxonomist at the Department of Botany, National Research Centre (NRC), Giza, Egypt. A voucher specimen (possessing number 2001) was kept in NRC.

**Phytochemical screening**

The constituents of the methanolic extract of the bark were identified by standard procedures as previously described (13,14).

**HPLC determination of phenolics and flavonoids**

The identification and quantification of flavonoids and phenolics in 70% methanol extract of *P. timorense* bark were performed by HPLC according to the previously described methods (15,16).

**Extraction and isolation**

The air dried powdered bark of *P. timorense* (1 kg) was extracted with petroleum ether (60-80°C) for defatting. The defatted powder was extracted with 70% methanol by percolation, then the concentrated extract (70 g) was phytochemically screened using the standard procedures which were previously described (13) to identify its constituents. The extract (40 g) was subjected to polyamide CC and eluted with gradient water: methanol (10:0) to (0:10) to give five fractions. Fraction 1, one compound which was eluted with water: methanol (90:10), was purified on Sephadex LH-20 CC and was eluted with methanol to give compound 1. Fraction 2, two compounds which were eluted with water: methanol (80:20), was applied on Sephadex LH-20 C using methanol as eluent to give compounds 2 and 3. Fraction 3, two compounds which were eluted with water: methanol (70:30), was applied on Sephadex LH-20 C to give compounds 4 and 5. Fraction 4, one compound which was eluted with water: methanol (60:40), was purified on Sephadex LH-20 C to give compound 6. Fraction 5, one compound which was eluted with water: methanol (50:50), was purified on Sephadex LH-20 C to give compound 7.

**Cytotoxicity assay procedures**

**Human tumor cell lines**

Human breast carcinoma (MCF-7), normal melanocytes (HFB-4), human hepatocellular liver carcinoma (HepG2) and human colon carcinoma (HCT116) cell lines were obtained from the American Type Culture Collection and were maintained by serial sub-culturing in the National Cancer Institute, Cairo, Egypt.

**Culture media**

The cells were suspended in RPMI 1640 medium (SIGMA ALORICH) supplemented with 10% fetal calf serum (SIGMA, USA) in presence to 1% antibiotic antimycotic mixture (10.000 U/mL K-penicillin, 10.000 μg/mL streptomycin sulphate and 25 μg/mL amphotericin B) and 1% L-glutamine (all purchased from Lonza, Belgium).

**Assay method for cytotoxic activity**

The cytotoxicity against HCT116, Hep-G2, HFB4 and MCF-7 cells were tested in the National Cancer Institute, according to the SRB (Sulforhodamine B) assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method using Adriamycin® (Doxorubicin) 10
mg vials (Pharmacia, Sweden) as the reference drug, the method was previously described by (17).

**Animals**

Sprague Dawley rats (130-150 g) were selected and kept in the controlled environmental conditions with free access to water and diet. The handleings with animals were complied with the ethical guidelines of the Medical Ethical Committee of the National Research Centre in Egypt and in accordance with the guidelines of the International Association for the Study of Pain Committee for Research and Ethical Issues.

**Acute toxicity study**

The LD$_{50}$ of methanol (70%) extract of *P. timorense* bark was determined using Karber method that was previously described (18).

**Antioxidant, hepatoprotective, and renal-function protective effects**

Rats were classified into normal and damaged liver (received carbon tetra chloride (CCl$_4$)) groups. Each group was classified into 4 different groups (4 rats in each); group 1 (control) received distilled water; group 2 received silymarin (50 mg/kg), while groups 3 and 4 received 150 and 300 mg/kg tested extract of *P. timorense*, respectively, for 15 days. At the end of experimental period, the rats were anaesthetized as previously described (19). Blood samples were collected, centrifuged at 3000 rpm for 15 minutes and stored at - 20°C before they were analyzed.

**Analytical methods**

Serum total antioxidant capacity (TAC) level was determined as previously described (20). Serum alanine amino transferase (ALT) and aspartate amino transferase (AST) activities were determined colorimetrically (21). The level of uric acid was determined according to Bartles and Bohmer method (22), while creatinine was measured using Barham and Sugar method was previously described by (17).

**Anti hyperglycaemic effect**

The methanol extract of *P. timorense* bark was evaluated for the anti hyperglycaemic activity using the method that was described previously (24).

**Statistical analysis**

One-way ANOVA was used to analyze the data. The result was considered statistically significant at *P* value < 0.05.

**Results**

**Phytochemical screening**

The results of phytochemical analysis of the bark of *P. timorense* revealed the presence of carbohydrate, terpenoids, coumarins, saponins flavonoids and tannins, while alkaloids were absent (Table 1).

**HPLC analysis of flavonoids and phenolic compounds in 70% methanol extract of *Pleogynium timorense* bark**

Fourteen flavonoidal compounds were identified representing 36.97 mg/g of the total content, the major flavonoid was quercetin (5.31 mg/g) followed by naringenin (5.12 mg/g). On the other hand, 16 phenolic compounds were identified representing 25.85 mg/g of the total content. The major phenolic compound was catechin (4.56 mg/g) followed by $\rho$-hydroxy benzoic acid (3.26 mg/g) (Table 2).

**Characterizations of compounds**

**Compound 1**

Compound 1, (10 mg), was isolated as yellow powder with $R_f$ = 0.31 in n-butanol: acetic acid: water (4:1.5, v/v/v) (S1) and 0.43 in water: glacial acetic acid (85:15, v/v) (S2). Under UV, it gave purple color and upon exposure to ammonia and with AlCl$_3$, the color changed to yellow. UV spectral data: $\lambda_{max}$ nm in methanol; 257, 358, with sodium methoxide: 271, 411, with AlCl$_3$: 273, 390, and with NaOAc/H$_3$BO$_3$: 260, 389.

$^1$H-NMR spectral data in DMSO-d$_6$: 6.6 (d, J = 8.3 Hz, 5′), 6.3 (d, J = 2.2, H-8), 7.47 (dd, J = 2.1, 8.3 Hz, H-6′), 6.1 (d, J = 2.2 Hz, H-6), 7.4 (d, J = 2.2 Hz, H-2′), 5.3 (d, J = 7.1 Hz, H-1), 1.2 (d, J = 6.7 Hz, Me) and 4.2 (d, J = 1.4 Hz, H-1′).

**Compound 2**

Compound 2, (7 mg), was isolated as yellow amorphous powder with $R_f$ = 0.55 in S1 and 0.64 in S2. Under UV, it gave purple color which turned by ammonia to yellow. Acid hydrolysis gave quercetin (as aglycone) and rhamnose (as sugar). UV spectral data: $\lambda_{max}$ nm in methanol; 257, 352, with NaOAc: 273, 393, with AlCl$_3$/HCl: 270, 402, with NaOAc: 273, 390, and with NaOAc/H$_3$BO$_3$: 260, 389.

$^1$H-NMR spectral data in DMSO-d$_6$: 6.6 (d, J = 8.3 Hz, 5′), 6.3 (d, J = 2.2, H-8), 7.47 (dd, J = 2.1, 8.3 Hz, H-6′), 6.1 (d, J = 2.2 Hz, H-6), 7.4 (d, J = 2.2 Hz, H-2′), 5.3 (d, J = 7.1 Hz, H-1), 1.2 (d, J = 6.7 Hz, Me) and 4.2 (d, J = 1.4 Hz, H-1′).

**Compound 3**

Compound 3, (9 mg), was isolated as yellow powder with $R_f$ = 0.93 in S1 and 0.15 in S2. It gave yellow color under UV light which was turned to yellow with ammonia vapor. UV spectral data: $\lambda_{max}$ nm in methanol; 261, 367, with ammonia and with AlCl$_3$: 270, 402, with NaOAc: 273, 390, and with NaOAc/H$_3$BO$_3$: 260, 389.

$^1$H-NMR spectral data in DMSO-d$_6$: 6.3 (d, J = 2.4, H-8), 6.13 (d, J = 2.4, H-6), 6.84 (d, J = 8.9 Hz, 5′), 7.25 (m, H-2′ and H-6′), 0.77 (d, J = 6 Hz, Me) and 5.3 (d, J = 2.1 Hz, H-1′).

---

Table 1. Results of phytochemical screening of the bark methanol extract of *Pleogynium timorense*

| Chemical constituents         | Present (+) or absent (-) |
|-------------------------------|--------------------------|
| 1. Carbohydrate and/or glycosides | +                        |
| 2. Coumarins                  | +                        |
| 3. Saponins                   | +                        |
| 4. Flavonoids                 | +                        |
| 5. Sterols and/or triterpenes  | +                        |
| 6. Alkaloids and/or nitrogenous bases | -                  |
| 7. Tannins                    | +                        |
Table 2. HPLC analysis of flavonoids and phenolic compounds in 70% methanol extract of Pleiogynium timorense bark

| Flavonoids                          | Concentration (mg/100 g) | Phenolics                          | Concentration (mg/100 g) |
|-------------------------------------|--------------------------|------------------------------------|--------------------------|
| Apigenin                            | 241.32                   | Caffeine                           | 73.23                    |
| Rhamentin                           | 351.66                   | Shikimic acid                      | 14.33                    |
| Naringenin                          | 168.06                   | Galloylquinic acid                 | 96.98                    |
| Kaempferol                          | 223.42                   | Gallic acid                        | 315.32                   |
| Hesperitin                          |                          | Pyrogallol                         | 284.89                   |
| Quercetin                           | 531.42                   | Methyl gallate                     | 199.75                   |
| Apigenin -7-glucose                 | 045.32                   | Protocatchuic acid                 | 73.56                    |
| Quercetin                           | 452.16                   | Ellagic acid                       | 88.92                    |
| Rutin                               | 413.23                   | Vanilllic acid                     | 17.09                    |
| Hesperidin                          | 091.46                   | Coumaric acid                      | 69.89                    |
| Naringenin                          | 512.66                   | Caffeic acid                       | 69.53                    |
| Apigenin-7-glucose                  | 210.42                   | Catechlin                          | 456.84                   |
| Apigenin-6-rhamnoside-8-glucose     | 125.87                   | Cinnamnic acid                     | 19.73                    |
| Apigenin-6-arabinose-8-galactose    | 198.31                   | Benzoi acid                        | -                       |
| Acacetin neo rutinoside             |                          | Ferulic acid                       | -                       |
| Acacetin-7-neo hesperides           |                          | p-Hydroxy benzoic acid             | 326.34                   |
| Kaempferol-3,7-dirhamnoside         | 132.15                   | Catechol                           | 285.24                   |
|                                    |                          | Chlorogenic acid                   | 193.87                   |

NaOMe: 273, 404, with AlCl3: 273, 445, with AlCl3/HCl: 265, 432, with NaOAc: 273, 405 and with NaOAc/H3BO3: 262, 386. 1H-NMR spectral data in MeOD: 6.5 (d, J = 2.1, H-8), 6.93 (d, J=H7, 5), 6.17 (d, J = 2.1, H-6), 7.54 (d, J = 2.2, 8.5 Hz, H-6) and 7.73 (d, J = 2.1 Hz, H-2).

**Compound 4**

Compound 4, (8 mg), was isolated as yellow powder with Rf = 0.66 in S1 and 0.9 in S2. It gave dull yellow color under UV, with ammonia vapor and with AlCl3. UV spectral data in methanol: 269, 365 with NaOMe: 277, 415, with AlCl3: 269,423, with NaOAc: 274, 385 and with NaOAc/H3BO3: 266, 370.

**Compound 5**

Compound 5, (13 mg), was isolated as white crystals with Rf = 0.6 in methanol: chloroform (30:70) S 3. With vanillin sulphuric acid reagent, it gave strong pink color. 1H-NMR spectral data: 6.76 (H-5’, d, J(H-5’, H-6’) 8.04 Hz), 6.87 (H-2’, d, J(H-2’, H-6’) 1.93 Hz), 6.03 (H-8, d, J(H-8, H-6) 2.5 Hz), 5.88 (H-6, d, J(H-6, H-8) 2.5 Hz), 2.92 (H-4e, dd, J(H-4e, H-3a) 5.53 Hz, J(H-4e, H-4a) 16.12 Hz), 2.56 (H-4a, dd, J(H-4a, H-3a) 8.52 Hz, J(H-4a, H-4e) 16.13 Hz), 4.02 (H-3, m), δ 4.54 (H-2, d, J(H-2, H-3a) 7.5 Hz), 6.75 (H-6’, dd, J(H-6’, H-2) 1.96 Hz, J(H-6’, H-5) 8.17 Hz) and 8.03. 13C-NMR spectral data: 114.7 (C-2), 80.7 (C-2), 95.3 (C-8), 93.6 (C-6), δ 66.1 (C-3), 27.4 (C-4), Peaks at δ of 99.3, 130.8, 144.3, 144.5, 155.2, 156.3, and 156.6 for other aromatic carbons.

**Compound 6**

Compound 6, (15 mg), was isolated as white amorphous powder with Rf = 0.69 in S1 and 0.55 in S2, it gave violet color under short UV light. 1H-NMR spectral data of compound 6: δ 6.70 (s, H-2, 6). 13C-NMR spectrum of compound 6: δ 169.18(C=O), 144.96 for (C 3, 5), δ 138.17 (C-4), δ 120.76 (C-1) and δ 108.7 (C-2, 6).

**Compound 7**

Compound 7, (15 mg), was isolated as brown needles with Rf = 0.85 in S1 and 0.6 in S2, its melting point was 132-135°C and it gave violet color under short UV light, Rf values = 0.85 in S1 and 0.6 in S2. EI-MS m/z: 126 (100, M+). 1H-NMR spectral data in DMSO-d6: δ 6.43 (t, H-5) and 6.26 (d, J = 8.1, 2H, H-4 and 6), 13C-NMR spectral data in DMSO-d6: δ 119.5 (C-5), 107.6 (C-4, C-6), 133.5 (C-2) and 147.07 (C-1, 3).

The structures of the isolated compounds are illustrated in Figure 1.

The cytotoxic activity

The cytotoxic activity of 70% methanol extract of P. timorense bark was assessed in vitro against HFB-4, MCF7, HepG2 and HCT116 cell lines in comparison with doxorubicin (Table 3). The results showed cytotoxic activity of the extract with IC50 of 42.89, 23.45, 4.39 and 10.78 (µg/mL), respectively, by comparing these results with that of doxorubicin IC50 (3.52, 3.12, 3.86 and 4.49 µg/mL, respectively). It was found that the methanol extract possessed a potent cytotoxicity against HepG2 (IC50 = 4.39 µg/mL).

On the other hand, Table 4 shows in vitro cytotoxic activity of the isolated compounds against HepG2 cell line in comparison with doxorubicin. Rutin, quercetin, quercetin, kaempferol, catechin, gallic acid and pyrogallol had cytotoxic activities with IC50 = 15.7, 18.8, 25.5, 29.3, 6.4, 9.6 and 12.3, respectively.

Acute toxicity study

LD50 was found to be 4.8 g/kg body weight, this relatively
carbon tetrachloride treatment alone. Liver enzymes were significantly increased in CCl₄-toxicated groups. The two doses of the bark extract showed a significant decrease in the elevated level of liver enzymes that was caused by CCl₄. Uric acid and creatinine were significantly elevated by CCl₄ treatment. The bark extract in the two dose levels showed a significant decrease in creatinine and uric acid values compared with both control and silymarin groups (Table 6).

**Antihyperglycaemic test**
Serum glucose level of the rats was significantly decreased by treating with the two doses of the bark extract in a dose-dependent manner (Table 7).

**Discussion**
Phytochemical screening of the bark of *P. timorense* revealed the presence of various phytoconstituents which are responsible for the bioactivities of the plant. So. the current study attempted to discover the relation between the phytoconstituents of *P. timorense* bark and the tested bioactivities. HPLC analysis confirmed the presence of extensive number of phenolic compounds with reasonable amounts. Recently, many studies have examined the cytotoxicity, antioxidant and antihyperglycemic activities of phenolic compounds (25-27). Chromatographic examination of the 70% methanol extract of *P. timorense* bark resulted in the isolation and identification of seven compounds for the first time from the bark.

UV spectrum of compound 1 gave a bathochromic shift in band I in addition of NaOMe with an increase in intensity which indicated the presence of the free hydroxyl group at 4'. Band II showed bathochromic shift on addition of sodium acetate which indicated the presence of free hydroxyl group at position 7. The hypsochromic shift with HCl after addition of AlCl₃ confirmed the presence of orthodihydroxy pattern in ring B. Acid hydrolysis showed that the quercetin was aglycone, while sugars were rhamnose and glucose. ¹H-NMR spectrum of compound 1 showed a doublet at δ 6.6 (J = 8.3Hz) for H-5'.
of doublet at $\delta$ 7.47 ($J = 2.1, 8.3$ Hz) for H-6'. It showed a doublet at $\delta$ 7.4 ($J = 2.2$ Hz) for H-2', while H-6 and H-8 appeared at $\delta$ 6.1 and $\delta$ 6.3, respectively as meta coupling protons. The anomeric glucose proton H-1' showed doublet at $\delta$ 5.3 with $\beta$- linkage, while those at $\delta$ 4.2 and at 6.1 for rhamnose with $\alpha$- linkage. These data were confirmed with the data which were reported for rutin (28).

UV spectrum of compound 2 gave a bathochromic shift in band I in addition of NaOMe with a marked increase in the intensity which confirmed free hydroxyl group at 4'. On addition of sodium acetate, band II showed a bathochromic shift with a marked increase in intensity which indicated the presence of free hydroxyl group at 4'. On addition of sodium acetate, band II showed a bathochromic shift that confirmed the presence of free hydroxyl group at position 7 was free. The presence of free hydroxyl group at position 5 was indicated after addition of AlCl$_3$/HCl due to bathochromic shift in band Ia when it was compared with that in methanol. The presence of ortho-dihydroxy pattern at ring–B was indicated after addition of sodium acetate with boric acid, as band I showed a bathochromic shift when it was compared with that in methanol. $^1$H-NMR spectrum of compound 3 showed doublet at $\delta$ 7.73 for H-2' with $J = 2.1$ Hz for meta-coupling with proton at H-6' that showed signal at $\delta$ 7.54. For H-5', it showed a doublet at $\delta$ 6.93 with $J = 8.1$ Hz that showed ortho-coupling with H6'. The two meta-coupling protons for H-8 and H-6 appeared at $\delta$ 6.5 and $\delta$ 6.1. The two meta-coupling protons for H-8 and H-6 appeared at $\delta$ 6.5 and $\delta$ 6.1, respectively as meta coupling protons. The anomeric proton of the rhamnose showed signal at $\delta$ 5.3 with $\beta$- linkage, while those at $\delta$ 4.2 and at 6.1 for rhamnose with $\alpha$- linkage. These data were confirmed with the data which were reported for quercetin (28).

For compound 3, on addition of sodium methoxide, band I showed a bathochromic shift with a marked increase in intensity which indicated the presence of free hydroxyl group at 4'. On addition of sodium acetate, band II showed a bathochromic shift that confirmed that hydroxyl group at position 7 was free. The presence of free hydroxyl group at position 5 was indicated after addition of AlCl$_3$/HCl due to bathochromic shift in band Ia when it was compared with that in methanol. The presence of ortho-dihydroxy pattern at ring–B was indicated after addition of sodium acetate with boric acid, as band I showed a bathochromic shift when it was compared with that in methanol. $^1$H-NMR spectrum of compound 3 showed doublet at $\delta$ 7.73 for H-2' with $J = 2.1$ Hz for meta-coupling with proton at H-6' that showed signal at $\delta$ 7.54. For H-5', it showed a doublet at $\delta$ 6.93 with $J = 8.1$ Hz that showed ortho-coupling with H6'. The two meta-coupling protons for H-8 and H-6 appeared at $\delta$ 6.5 and $\delta$ 6.1 with $J = 2.1$ Hz. These data were confirmed with the data which were reported for quercetin (28).

The UV spectrum of compound 4 showed a bathochromic shift in band I on addition of NaOMe and the intensity was increased which proved that hydroxyl group at position 4' was free. Band II showed a bathochromic shift on addition of sodium acetate that confirmed the
presence of free hydroxyl group at position 7. The absence of orthodihydroxy pattern in ring B was confirmed by the absence of hypsochromic shift with AlCl3/HCl. These data were confirmed with the data which were reported for kaempferol (28).

1H-NMR spectrum of compound 5 gave doublet of doublet peak at δ 6.75 for proton H-6 with ortho-coupling with H-5′ and meta-coupling with H-2′, doublet peak at δ 6.88 for proton H-6 which formed with H-8 meta coupling, doublet peak at δ 6.79 for proton H-5 with ortho-coupling with H6′, doublet peak at δ 6.87 for proton H-2′ with meta-coupling with H-6. H-8 showed peak at δ 6.03 as doublet with meta coupling with H-6, while proton H-4e gave peak at δ 2.92, proton H-4a showed peak at δ 2.56, multiple at δ 4.02 for H-3, doublet peak at δ 6.54 as for H-2 and multiple peaks at δ 8.03 for phenolic protons. 13C-NMR spectrum of compound 5 gave peak at δ 93.6 for carbon (C-6), (C-8) showed peak at δ 95.3, peak at δ 80.7 for carbon (C-2), peak at δ 66.1 for carbon (C-3), peak at δ 27.4 for carbon (C-4) and showed peaks at δ 156.6, 156.3, 155.2, 130.8, 144.85, 144.3 and 99.3 for other aromatic carbons. By comparing these data with the previously published data (29), compound 5 was identified as catechin.

1H-NMR spectrum of compound 6 gave a sharp singlet peak at δ 7.09 ppm for two protons H-2, 6. 13C-NMR spectrum of compound 6 showed the carbon of carbonyl of carboxylic group at δ 169.18, δ 144.96 for C-3,5, δ 138.17 for C-4, δ 120.76 for C-1 and δ 108.7 for C-2,6. So, compound 6 was identified as gallic acid.

1H-NMR spectrum of compound 7 showed a triplet peak at δ: 6.43 for H-5 and a doublet peak at 6.26 for two protons H-4, 6. 13C-NMR spectrum of compound 7 gave a peak at δ: 107.6 for C-4 & C-6, peak at 119.5 for C-5, peak at 133.5 for C-2 and peak at 147.07 for C-1,3. By comparing with the previously published data (30), compound 7 was identified as pyrogallol.

As the antioxidant activity of the isolated compounds were previously reported (31,32), so they played an important role in appearing the bioactivities of the extract. Recently, dichloromethane extract of the bark showed a significant activity against the A2780 human ovarian cancer cell line due to the presence of three new trihydroxy alkyloxyhexones which were isolated from this extract (10). This reported research is the first study regarding the bark of P. timorense. Therefore, we aimed to continue the study of the cytotoxic effect of the methanol extracts and the isolated compounds against different cell lines. The methanol extract showed a promising cytotoxic activity against HepG2 cell line (IC50 = 6.4, & 9.6 µg/mL, respectively, by comparing with Doxorubicin (IC50 = 3.86 µg/mL). We can conclude that the methanol extract of P. timorense bark has more potent in vitro cytotoxicity against HepG2 cell line (IC50 = 4.39 µg/mL) than that of the isolated compounds. This might be due to the synergistic effect of the phenolic contents in the methanol extract which play a vital role in the bioactivities.

The two doses of the 70% methanol extract of P. timorense bark (150 & 300 mg/kg) showed significant increase in TAC and significant decrease in the liver enzymes (ALT& AST), urea and creatinine levels compared with control and silymarin groups in both normal and hepatotoxic groups. Moreover, the two doses of the 70% methanol extract significantly decreased the blood glucose level. These results revealed that the 70% methanol extract of P. timorense bark had promising antioxidant, hepatorenal protective and antihyperglycemic effects, and the phytoconstituents in the methanol extract were responsible for these activities.

Conclusion
The present study revealed the positive effects of methanol extract of the bark of P. timorense (DC.) Leenh for use as antioxidant, hepatorenal protective, antihyperglycemic and anticancer. Detailed information on the phytoconstituents and the above mentioned biological activities were discussed in this research.

Acknowledgments
We acknowledge to the National Research Centre and Faculty of Pharmacy Nahda University, Egypt and College of Pharmacy, Jouf University, Sakakah, Saudi Arabia for using laboratory instruments in doing research.

Authors’ contribution
All authors conceived, designed and performed the experiment and analyzed the data; GFAR wrote and revised the paper; all authors read and confirmed publication of the paper.

Conflict of interests
The authors have no conflict of interests to declare.

Ethical considerations
Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors. The handling with animals were complied with the ethical guidelines of the Medical Ethical Committee of the National Research Centre in Egypt and were in accordance with the guidelines of the International Association for the Study of Pain Committee for Research and Ethical Issues.
Phytoconstituents and bioactivities of *Pleiogynium timorense* Bark

Funding/Support
This research had no financial support.

References
1. Quattrocchi U. CRC World Dictionary of Plant Names, Vol M-Q. New York, Washington DC: CRC Press; 1999: 2107.
2. Rozefelds A, Dettmann M, Clifford T, Hocknull S, Newman N, Godthelp H, et al. Traditional and computed tomographic (CT) techniques link modern and Cenozoic fruits of *Pleioquium* (Anacardiaceae) from Australia. Alcheringa: An Australasian Journal of Palaeontology. 2015;39(1):24-39. doi: 10.1080/03115518.2014.951916.
3. Cavalcanti SBT, Teles HL, Silva DHS, Furlan M, Young MCM, Bolzani VS. New tetra-acetylated oligosaccharide diterpene from *Cupania vernalis*. J Braz Chem Soc. 2001;12(3):413-6. doi: 10.1590/S0103-50532001000300014.
4. Everett TH. The New York Botanical Garden Illustrated Encyclopedia of Horticulture. Vol. 8. New York, London: Garland; 1981. p. 2721.
5. El-Fiki NM, Ahmed FI. Phytochemical study of *Pleioquium solandri* (Benth.) Engl J Pharm Sci. 1999;24:38-50.
6. Said A, Aboutabl EA, Abdel Raoof GF, El-Gendy MAM, Abdel Raoof GF, Abd N, Singab AE, et al. Chemical composition and bioactivity of *Pleioquium timorense* (Anacardiaceae). Nat Prod Commun. 2010;5(4):545-50.
7. Netzel M, Netzel G, Tian Q, Schwartz S, Konczak I. Native Australian fruits—a novel source of antioxidants for food. Innov Food Sci Emerg Technol. 2007;8(3):339-46. doi: 10.1016/j.ifset.2007.03.007.
8. Said A, Aboutabl EA, Abdel Raof GF, Hufner A, Nada SA. Phenolic contents and bioactivities of periperc and seeds of *Pleioquium solandri* (Benth.) Engl. (Anacardiaceae). Iran J Basic Med Sci. 2015;18(2):164-71.
9. Said A, Aboutabl EA, Hussein AA, Abdel Raof GF. The composition of the lipoidal matter of the seeds of *Pleioquium timorense* (DC.) Leenh. Egypt Pharm J. 2015;14(1):65-8. doi: 10.4103/1687-4315.154725.
10. Eaton AL, Rakotondraibe LH, Brodie PJ, Goetz M, Kingston J, et al. Phytochemical and biological study of *Pleioquium solandri* (Benth.) Engl J Pharm Sci. 1999;24:38-50.
11. Said A, Aboutabl EA, Abdel Raof GF. Identification of Constituents from *Pleioquium timorense* (DC.) Leenh Pericarp and Seeds Using High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometry. AASCIT J Chem. 2017;3(4):30-36.
12. Said A, Omer EA, El Gendy MAM, Abdel Raof GF, Abd EL-Kader AE, Fouad R. Volatile constituents and cytotoxic activity of the fruits of *Pleioquium timorense* (DC.) Leenh. J Mater Environ Sci. 2018;9(8):2274-9.
13. Harbone JB. Phytochemical methods: a guide to modern techniques of plant analysis. London: Chapman & Hall; 1973. p. 49.
14. Sowofora A. Medicinal plants and traditional medicine in Africa. Ibadan, Nigeria: Spectrum Books Ltd; 1993. p. 289.
15. Mattila P, Astola J, Kumpulainen J. Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. J Agric Food Chem. 2000;48(12):5834-41. doi: 10.1021/jf000661f.
16. Guopy P, Hugues M, Boivin P, Amiot MJ. Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds. J Sci Food Agric. 1999;79(12):1625-34. doi: 10.1002/(sici)1097-0010(199909)79:12<1625::aid-jfsa411>3.0.co;2-8.
17. Skehan P, Storeg R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst. 1990;82(13):1107-12. doi: 10.1093/jnci/82.13.1107.
18. Chinedu E, Arome D, Ameh FS. A new method for determining acute toxicity in animal models. Toxicol Int. 2013;20(3):224-6. doi: 10.4103/0971-6580.121674.
19. Coccheto DM, Bjornsson TD. Methods for vascular access and collection of body fluids from the laboratory rat. J Pharm Sci. 1983;72(5):465-92. doi: 10.1002/jps.2600720503.
20. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. Method for the measurement of antioxidant activity in human fluids. J Clin Pathol. 2001;54(5):356-61. doi: 10.1136/jcp.54.5.356.
21. Huang B, Ban X, He J, Tong J, Tian J, Wang Y. Hepatoprotective and antioxidant activity of ethanolic extracts of edible lotus (*Nelumbo nucifera* Gaertn.) leaves. Food Chem. 2010;120(3):873-8. doi: 10.1016/j.foodchem.2009.11.020.
22. Barham D, Trinder P. An improved colour reagent for the determination of blood glucose by the oxidase system. Analyst. 1972;97(151):142-5. doi: 10.1039/an9729700142.
23. Bartels H, Bohmer M, Heierli C. [Serum creatinine determination without protein precipitation]. Chim Clin Acta. 1972;37:193-7. doi: 10.1016/0009-8981(72)90432-9.
24. Yaro AH, Aliyu M, Borodo SB, Nazifi AB. Anthopyrgeal and antihyperlipidemic activities of ethanol leaf extract of *Eleusine corocana* (Linn.) Gaertn. in alloxan-induced hyperglycaemic rats. J Appl Pharm Sci. 2018;8(7):28-32. doi: 10.7324/JAPS.2018.8705.
25. Nandi S, Vraco M, Bagchi MC. Anticancer activity of selected phenolic compounds: QSAR studies using ridge regression and neural networks. Chem Biol Drug Des. 2007;70(5):424-36. doi: 10.1111/j.1747-0285.2007.00575.x.
26. Bendaiif H, Melhaoui A, Bouyazesi A, Hambouzi B, El Ouadi Y. The study of the aqueous extract of leaves of *Pancratium foetidium* Pom as: characterization of polyphenols, flavonoids, antioxidant activities and ecofriendly corrosion inhibitor. J Mater Environ Sci. 2017;8(12):4475-86.
27. El-Sherif MM, Ragheb AT, Mohosraraa SA, Marzouk MM, Kassem MES, Saleh NAM. *Peyrygotta alata* (Roxb.) R. Br.: chemical constituents, anti-hyperglycemic effect and anti-oxidative stress in alloxan-induced diabetic rats. J Mater Environ Sci. 2018;9(1):245-55. doi: 10.26872/jmes.2018.9.1.28.
28. Mabry TJ, Markham KR, Thomas MB. The systematic identification of flavonoids. New York: Springer-Verlag; 1970. p. 3371-8.
29. Hye A, Taher MA, Ali MY, Ali MU, Zaman S. Isolation of (+)-catechin from *Acacia catechu* (Cutch Tree) by a convenient method, J Sci Res. 2009;1(2):300-5. doi: 10.3329/jcr.v1i2.1635.
30. Lu CH, Li YY, Li LJ, Liang LY, Shen YM. Anti-inflammatory activities of fractions from *Geranium nepalense* and related polyphenols. Drug Discov Ther. 2012;6(4):194-7.
31. Sang S, Tian S, Wang H, Stark RE, Rosen RT, Yang CS, et al. Chemical studies of the antioxidant mechanism of tea catechins: radical reaction products of epicatechin with peroxyl radicals. Bioorg Med Chem. 2003;11(16):3371-8. doi: 10.1016/s0968-0896(03)00367-5.
32. Sano M, Yoshida R, Degawa M, Miyase T, Yoshino K. Determination of peroxyl radical scavenging activity of flavonoids and plant extracts using an automatic potentiometric titrator. J Agric Food Chem. 2003;51(10):2912-6. doi: 10.1021/jf0211276.