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Cytotoxicity of *Enterolobium timbouva* Plant Extract and Its Isolated Pure Compounds

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The present study aimed to evaluate the cytotoxic activities of the aqueous alcohol extract of *Enterolobium timbouva* leaves as well as its isolated pure compounds.
Place and Duration of Study: Department of Pharmacognosy, Faculty of pharmacy, Ain Shams University, between March 2010 and May 2012.
Methodology: *In vitro* Cytotoxic study was conducted for *the* aqueous methanol extract and the isolated pure single compounds to determine the IC_{50} by sulphorhodamine B (SRB) assay.
Results: Phytochemical investigation of the extract resulted in the isolation and structural determination of ten phenolic compounds isolated for the first time from entitled genus viz; 3,4-Dihydroxy-Cinnamic acid (Caffeic acid) (1); Quercetin-3-*O*-*β*-D-glucopyranoside (Isoquercitrin) (2); Quercetin-3-*O*-*β*-D-galacto-pyranoside (Hyperin) (3); Kaempferol-3-*O*-*β*-D–glucopyranoside (Astragalin) (4); Hesperetin-7-*O*-rutinoside (Hesperidin) (5); Quercetin 3-*O*-rutinoside (Rutin) (6); Quercetin (7); Kaempferol (8); 7-methoxycoumarin (Herniarin) (9); and Chrysin (10). The aqueous alcohol extract exhibited potent cytotoxic activity against diffferent cancer cell lines with IC_{50} values of 2.67 µg/mL against MCF-7

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cell line, 3.89 µg/ml against HCT_{116} cells, 4 µg/mL against HEp_2 cells, 4.5 µg/mL against HeLa cells, 1.7 µg/mL against PC-3 cells, and 5.7 µg/mL against Huh-7 cells. *In vitro* cytotoxic assay of the isolated pure compounds against Huh-7 cell Line showed that compounds 1, 9 and 10 are the only tested compounds exhibiting potent cytotoxic activity with IC_{50} of 3 µg/mL, 0.76 µg/mL, and 18.51 µg/mL respectively. The rest of tested compounds exhibited IC_{50} exceeding 1000 µg/mL which reflects their safety.
Conclusion: The current study indicated that the phenolic compounds isolated from *Enterolobium timbouva leaves* are promising molecules with potentially useful cytotoxic activity profiles. This confirms that this terrestrial plant has great value as a source of lead compounds with pharmaceutical applications.

Keywords: Enterolobium timbouva; fabaceae; phenolics; in vitro cytotoxic assessment.

1. INTRODUCTION

In the last few years, the identification and development of phenolic compounds or extracts from different plants have become a major area of health- and medical-related research**.** Fabaceae occupies a distinguishable situation among the famous plant families which include genera embracing phenolic rich species. Fabaceae is particularly rich in flavonoids and related compounds; about 28% of all flavonoid and 95% of all isoflavonoid aglycone structures known from the plant kingdom are produced by legumes [1].

Enterolobium timbouva Mart. *(*synonym: *Enterolobium contortisiliquum*), a member of the tree family Fabaceae, sub-family Mimosoideae, commonly known in English as the earpod tree, is a tree more than 20 m high which is characterized with its black ear-shaped fruits. It is native to tropical South America, southern Brazil, and temperate South America. But, it is widely populated and cultivated in Egypt .Since nothing could be traced in literature concerning the phenolic content of the various parts of *Enterolobium timbouva* and as part of an ongoing study to discover potential bioactive phenolics from terrestrial plant sources [2,3], the present study was directed to investigate the phenolics present in the aqueous alcohol extract obtained from the leaves of *Enterolobium timbouva* and to investigate their *in vitro* cytotoxic activities.

2. EXPERIMENTAL

2.1 Plant Material

Fresh leaves of *Enterolobium timbouva* (Fabaceae) were collected from plants grown in El-Orman botanical garden, Ministry of Agriculture, Giza, on March (2010).They were kindly authenticated by Mrs. Tereize Labib, agricultural engineer, El-Orman botanical garden, Giza, Egypt. A voucher specimen of the authenticated plant (ETF-5001) was deposited at the department of Pharmacognosy, Faculty of pharmacy, Ain Shams University, Cairo, Egypt.

2.2 Instruments and Material for Chemical Investigation

^{1}H-NMR spectra were measured by a Jeol ECA 500 MHz NMR spectrometer (JEOL, Tokyo, Japan) at 500 MHz. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C-NMR chemical shifts to dimethyl sulfoxide (DMSO)-*d6* and converted to the tetramethylsilane (TMS) scale by adding 39.5. Typical conditions: spectral width = 8 kHz for ^{1}H and 30 kHz for

^{13}C, 64 K data points, and a flip angle of 45. UV recording was done on a Shimadzu UV Visible-1601 spectrophotometer (Shimadzu, Kyoto, Japan). For column chromatographic analysis the following adsorbents were used: 1) Polyamide powder, polyamide S_6 for CC, Riedel-De Haen AG, seelze Hannover, Germany. 2) Sephadex LH-20, GE Healthcare Bio-Sciences, Uppsala, Sweden). Paper chromatographic analysis was carried out on Whatman No. 1 paper (Whatman, Kent, UK) using solvent systems: 1) H_2O; 2) 6% AcOH; and 3) BAW (*n*-BuOH/AcOH/H_2O, 4:1:5, v/v, upper layer). Solvents 2 and 3 were also used for preparative paper chromatography (PPC).

2.3 Extraction, Isolation, and Purification of Phenolics from *Enterolobium timbouva*

The intact air dried plant material 2 kg were homogenized in MeOH-H_2O (3:1) mixture (three extractions each with 5 L). The dried filtrate (50 g) of the homogenate was applied on a polyamide 6s column (500 g, 120 x 5 cm) and eluted with H_2O followed by H_2O-MeOH mixtures of decreasing polarities to yield 36 individual fractions (2L each) that were examined separately under UV light. Each fraction was investigated by two-dimensional paper chromatographic investigation (2D-PC). Similar fractions were pooled to yield seven main fractions (I-VII) that were separately dried in a vacuum and subjected to 2D-PC.

Compound 1 (90 mg) was isolated pure from fraction II (eluted with 20% MeOH) by column fractionation (CF) (30 cm L x 4.5 cm D) of 2.00 g material over 30 g Sephadex LH-20 using 3 L of H_2O for elution. Compounds 2 (120 mg), 3 (105 mg), and 4 (150 mg) were individually separated from fraction III (eluted with 40% MeOH) by repeated PPC, using BAW as solvent, while compounds 5 (250 mg)| and 6 (170 mg) were obtained from 3 g of fraction IV (eluted with 60% MeOH) by fractionation on Sephadex LH-20column (30 cm L x 4.5 cm D), using 5 L of a mixture of H_2O/MeOH (1:2) for elution. Compounds 7 (170 mg) and 8 (140 mg) were obtained from 1.5 g of fraction V (eluted with 80% MeOH) by fractionation on a polyamide 6s (20 g) column (30 cm L x 4.5 cm D) and elution with *n*- butanol saturated with water. Compounds 9 (70 mg) and 10 (100 mg) were individually isolated pure through CF of 2.50 g of fraction VI (eluted with MeOH) on a polyamide 6s (20 g) column (30 cm L x 4.5 cm D) and elution with a mixture of MeOH-C_6H_6-H_2O (60:38:2).

2.4 Cell Culture

Human breast (MCF-7), colon (HCT_{116}), larynx (HEp_2), cervica (HeLa), prostate (PC -3), and liver (Huh-7) carcinoma cell lines were purchased frozen in liquid nitrogen (-180 ^{0}C) from Cell culture unit, VACSERA, Agouza, Giza, Egypt (The Holding Company for Biological Products & Vaccines). Cells were grown in Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal bovine serum (10%), penicillin G (100 IU/mL), and streptomycin (100µg/mL). Cells were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity. Cell culture reagents were obtained from Gibco Invitrogen (Carlsbad, CA, USA).

2.5 *In vitro* Assay for Cytotoxic Activity

Enterolobium timbouva aqueous methanol extract and its isolated compounds were screened for their cytotoxicity to determine the IC_{50} (50% growth inhibition) by sulphorhodamine B (SRB) assay according to the method of Skehan [4]. Cytotoxicity of the aqueous methanol extract of *Enterolobium timbouva* was evaluated on human breast (MCF-7), colon (HCT_{116}), larynx (HEp_2), cervica (HeLa), prostate (PC -3), and liver (Huh-7)

carcinoma cell lines. The cytotoxic activities of the isolated compounds caffeic acid (1), isoquercitrin (2), hesperidin (5), rutin (6), quercetin (7), herniarin (9) and chrysin (10) were evaluated against Huh-7 cell line. Doxorubicin was used as positive control.Exponentially growing cells were collected using 0.25% Trypsin-EDTA. Viability was determined by trypan blue exclusion using the inverted microscope. Cells were seeded in 96-well plates at 4000 cells/well in Dulbecco's Modified Eagle's Medium (DMEM) supplemented medium. After 24 hours, cells were incubated with the appropriate concentration ranges of drugs, completed to total of 200 µl volume/well using fresh medium and incubation was continued for 72 hours. Control cells were treated with vehicle alone. For each drug concentration, 4 wells were used. Following 72 h treatment, the cells were fixed with 10% trichloroacetic acid for 1 h at 4 ºC. Wells were stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The plates were air dried for 24 h and the dye was solubilized with Tris-HCl (10 mM, pH 7.4) for 5 minutes on a shaker at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 564 nm with the ELIZA microplate reader.The percentage of cell survival was calculated as follows:

Survival fraction = O.D. (treated cells)/ O.D. (control cells)

The IC_{50} values were calculated according to the equation for Boltzman sigmoidal concentration response curve using the nonlinear regression fitting models (Graph Pad, Prism Version 5). The experiment was repeated 3 times for each cell line.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Study of the Aqueous Alcohol Extract

As a result of intensive phytochemical study of *Enterolobium timbouva* aqueous alcohol leaf extract, 10 phenolic constituents were individually isolated and identified for the first time from this genus. These compounds are: 3,4-Dihydroxy-Cinnamic acid (Caffeic acid) (1); Quercetin-3-*O*-*β*-D-glucopyranoside (Isoquercitrin) (2); Quercetin-3-*O*-*β*-D-galacto-pyranoside (Hyperin) (3); Kaempferol-3-*O*-*β*-D–glucopyranoside (Astragalin) (4); Hesperetin-7-*O*-rutinoside (Hesperidin) (5); Quercetin 3-*O*-rutinoside (Rutin) (6); Quercetin (7); Kaempferol (8); 7-methoxycoumarin (Herniarin) (9); and Chrysin (10) (Fig. 1). The structures of these compounds were unambiguously determined by their chromatographic behaviors as well as spectroscopic analysis via UV, ^{1}H-NMR and ^{13}C–NMR (if necessary).

3.1.1 Caffeic acid; 3, 4-Dihydroxy-Cinnamic acid (1):

Amorphous buffy yellow powder; UV λ_{max} (nm) in MeOH: 240, 280, 350, sh; ^{1}H-NMR (δ ppm): 6.13 (1H, *d*, *J*= 12.2 Hz, H-8), 6.93 (1H, *dd*, *J*= 8.4, 1.5 Hz, H-6), 6.72 (1H, *d*, *J*= 8.4 Hz, H-5), 6.99 (1H, *d*, *J*= 1.5 Hz, H-2), 7.37 (1H, *d*, *J*= 12.2 Hz, H-7); ^{13}C- NMR (δ ppm): 125.42 (C-1), 114.86 (C-2), 145.21 (C-3), 148.35 (C-4), 115.75 (C-5), 121.35 (C-6), 141.41 (C-7), 127.50 (C-8), 174.65 (C-9), [5]**.**

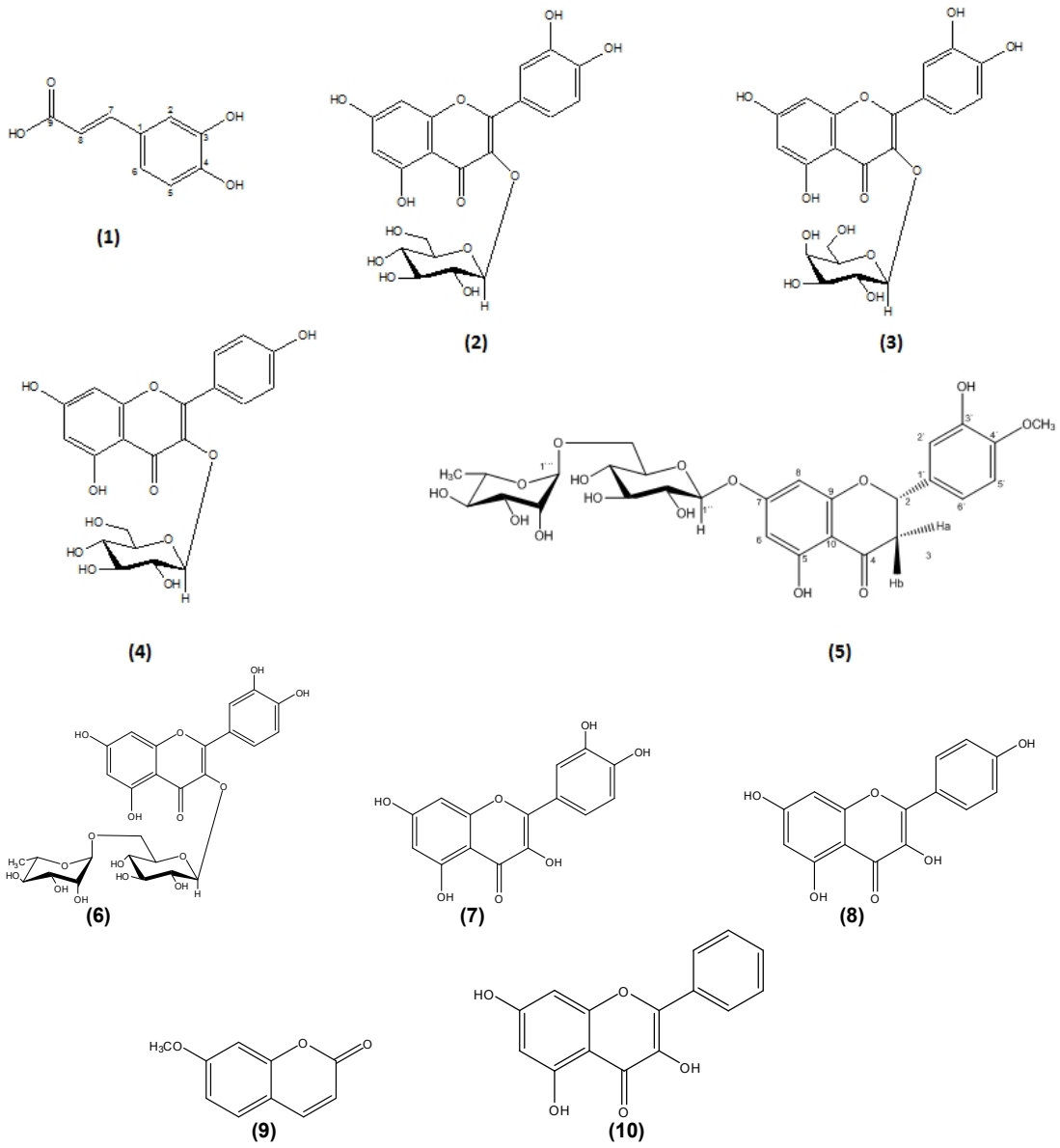

Fig. 1. Chemical compounds isolated from fractionation of the aqueous methanol leaf extract:(1) Caffeic acid; (2) Isoquercitrin; (3) Hyperin; (4) Astragalin; (5) Hesperidin; (6) Rutin; (7) Quercetin; (8) Kaempferol; (9) Herniarin; and (10) Chrysin.

3.1.2 Quercetin 3-*O*-*β*-D-glucopyranoside; Isoquercitrin (2):

Amorphous yellow powder; UV λ_{max} (nm) in MeOH: 257, 358; ^{1}H-NMR (δ ppm): 6.17 (1H, *d*, *J* = 1.5, H-6), 6.42 (1H, *d*, *J*= 1.5, H-8), 6.88 (1H, *d*, *J* = 8, H-5`), 7.61 (1H, *dd*, *J* = 8 & 1.5, H-6`), 7.68 (1H, *d*, *J* = 1.5, H-2`), 5.03 (1H, *d*, *J* = 7 Hz, H-1``), 3.30-3.80 (m, remaining sugar protons), [6].

3.1.3 Quercetin 3-*O*- *β*-D-galactopyranoside; Hyperin (3)

Amorphous yellow powder; UV λ_{max} (nm) in MeOH: 258, 268sh, 300sh, 362; ^{1}H-NMR (δ ppm): 6.15 (1H, *d*, *J* = 1.5 Hz, H-6), 6.35 (1H, *d*, *J* = 1.5 Hz, H-8), 6.78 (1H, *d*, J= 8.0 Hz, H-5`), 7.54 (1H, *d*, *J* = 1.5 Hz, H-2`), 7.59 (1H, *dd, J* = 1.5 & 8.0 Hz, H-6`), 5.32 (1H, *d*, *J* = 8.0 Hz, H-1``), 3.45-3.85 (m, remaining sugar protons); $^{13}$C-NMR (δ ppm): 178.00 (C-4), 164.65 (C-7), 161.68( C-5), 156.76 (C-2, C-9), 147.59 (C-4`), 145.19 (C-3`), 133.98 (C-3), 123.54 (C-6`), 121.98 (C-1`), 116.36 (C-5`), 115.71 (C-2`), 104.09 (C-10), 102.28 (C-1``), 99.98 (C-6), 98.98 (C-8), 76.21 (C-5``), 73.68(C-3``), 71.78 (C-2``), 68.07 (C-4``), 60.65 (C-6``), [7].

3.1.4 Kaempferol-3-*O*- *β*-D -glucopyranoside; Astragalin (4)

Amorphous yellow powder; UV λ_{max} (nm) in MeOH: 266, 300 sh, 325; ^{1}H-NMR (δ ppm): 6.16 (1H, *d*, *J* = 1.5, H-6), 6.40 (1H, *d*, *J* = 1.5, H-8), 6.89 (2H, *d*, *J* = 7.6, H-3`& H-5`), 8.02 (2H, *d*, *J* = 7.6, H-2`& H-6`), 5.01 (*d*, 1H, *J* = 7.6 Hz, H-1``), 3.27–3.67 (m, remaining sugar protons), [8].

3.1.5 Hesperetin-7-*O*-rutinoside; Hesperidin (5)

Amorphous white powder; UV λ_{max} (nm) in MeOH: 214, 226sh, 284, 328 sh;
^{1}H-NMR (δ ppm): 2.73 (1H, *dd, J*=17.0 & 3.0 Hz, H-3b), 3.51 (1H, *dd, J*=17.0 & 12.0 Hz, H-3a) ,3.74 (3H, *s*, 4′-OCH_3), 5.46 (1H, *m*, H-2), 6.08 (1H, *d*, *J* = 2.0 Hz, H-6), 6.11 (1H, *d*, *J* = 2.0 Hz, H-8), 6.86 (1H, *d*, *J* = 8.0 Hz, H-5`), 6.90 (2H, *m,* H-2`&H-6`), 4.94 (1H, *d*, *J* = 5.4 Hz, H-1``), 4.48 (1H, *d, J* = 1.5 Hz, H-1```), 1.04 (3H, *d*, *J*= 6.5 Hz, Me-6```); 3.05-3.41 (m, remaining rutinosyl protons), [9].

3.1.6 Quercetin 3-*O*-rutinoside; Rutin (6)

Amorphous yellow powder; UV λ_{max} (nm) in MeOH: 256, 266sh, 300sh, 360; ^{1}H-NMR (δ ppm): 6.10 (1H, *d*, *J* = 1.5 Hz, H-6), 6.30 (1H, *d*, *J* = 1.5 Hz, H-8), 7.32 (1H, *d*, *J* = 7.6 Hz, H-5`), 7.51 (2H, m, H-2` and H-6`), 5.35 (1H, *d*, *J* = 7.6 Hz, H-1``), 4.35 (1H, *d, J* = 1.9 Hz, H-1```), 1.11 (3H, *d*, *J*= 4.5 Hz, Me-6```); 3.15-3.41 (m, remaining rutinosyl protons), [10].

3.1.7 Quercetin (7)

Amorphous yellow powder; UV λ_{max} (nm) in MeOH:, 269 sh, 301sh, 370; ^{1}H-NMR (δ ppm): 6.19 (1H, *d*, *J* = 1.5 Hz, H-6), 6.45 (1H, *d*, *J* = 1.5 Hz, H-8), 7.59 (1H, *d*, *J* = 8.0 Hz, H-5`), 7.61 (1H, *dd*, *J* = 8.0 & 1.5 Hz, H-6`), 7.71 (1H, *d*, *J* = 1.5 Hz, H-2`), [11].

3.1.8 Kaempferol (8)

Amorphous yellow powder; UV λ_{max} (nm) in MeOH: 266, 294 sh, 322sh, 367; ^{1}H-NMR (δ ppm): 6.19 (1H, *d*, *J*=2.0 Hz, H-6), 6.39 (1H, *d*, *J* = 2.0 Hz, H-8), 6.89 (1H, *d, J*=8.0 Hz, H-3`& H-5`), 8.04 (1H, *d*, *J* =8.0 Hz, H-2`,H-6`), [12].

3.1.9 7-methoxycoumarin; Ayapanin; Herniarin (9)

Amorphous yellowish white powder ; UV λ_{max} (nm) in MeOH: 252sh, 290sh, 322; ^{1}H- NMR (δ ppm): 6.24 (1H, *d*, *J* = 10 Hz, H-3), 6.89 (1H, *dd, J* = 8.4 & 1.5 Hz, H-6), 6.93 (1H, *d, J* = 1.5 Hz, H-8), 7.57 (1H, *d*, *J* = 8.4 Hz, H-5), 7.94 (1H, *d*, *J* = 10 Hz, H-4), 3.81 (3H, *s*, Me-7); [13].

3.1.10 5, 7-Dihydroxyflavone; Chrysin (10)

Amorphous yellow powder; UV λ_{max} (nm) in MeOH: 270,314; ^{1}H-NMR (δ ppm): 6.18 (1H, *d, J* = 2.0 Hz, H-6), 6.48 (1H, *d*, *J* = 2.0 Hz, H-8), 6.93 (1H, *s* , H-3), 7.57 (3H, *m* , H-3`, H-4`& H-5`), 8.02 (2H, *d, J* = 7.0 Hz, H-2` & H-6`) ; [14].

3.2 *In vitro* Assay for Cytotoxic Activity

In vitro cytotoxic assay of the aqueous alcohol extract of *Enterolobium timbouva* showed potential cytotoxic activity against different cancer cell lines with IC_{50} values of 2.67 µg/mL against MCF-7 cell line, 3.89 µg/mL against HCT_{116} cells, 4 µg/ml against HEp_2 cells, 4.5 µg/mL against HeLa cells, 1.7 µg/ mL against PC-3 cells, and 5.7 µg/mL against Huh-7 cells (Fig. 2).

In vitro cytotoxic assay of the isolated compounds showed potent cytotoxic activity against Huh-7 cell Line with IC_{50} of 3 µg/mL , 0.76 µg/mL and 18.51 for µg/mL caffeic acid (1), herniarin (9) and chrysin (10) respectively. On the other side, isoquercitrin (2), hesperidin (5), rutin (6), and quercetin (7) did not show promising cytotoxic effects. They all exhibited IC_{50} exceeding 1000 µg/mL which reflects their safety (Fig. 3).

According to the American National Cancer Institute, the IC_{50} limit to consider a crude extract active against cancer cells should be lower than 20 µg/mL [15]. It can be concluded that the aqueous alcohol extract of *Enterolobium timbouva* possess potential cytotoxic activity against breast, colon, larynx, cervical, prostate, and liver cancer cells and most potent against human prostate cancer.

Results also suggest that caffeic acid (1), herniarin (9) and chrysin (10) are probably the compounds responsible for this potent cytotoxic activity of the aqueous methanol leaf extract. The inhibitory activity of caffeic acid on cell viability and cell proliferation was in accordance with the previous report by Chung et al. [16] who confirmed that the anti-metastatic and anti-tumor effects of caffeic acid are mediated through the selective suppression of matrix metalloproteinase (MMP)-9 enzyme activity and transcriptional down-regulation by the dual inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) as well as MMP-9 catalytic activity. Also the cytotoxicity of herniarin (9) in the present study matched that obtained by Hadi [17] who confirmed that herniarin induces its cytotoxic effect through apoptosis induction and chrysin cytotoxicity was in accordance with the previous report by Choo et al. [18] who confirmed that chrysin inhibits proliferation and induces apoptosis via caspase activation and inactivation of the Akt signaling. These findings consequently merit further exploration of the extract in subsequent *in-vivo* studies and later in controlled clinical trials.

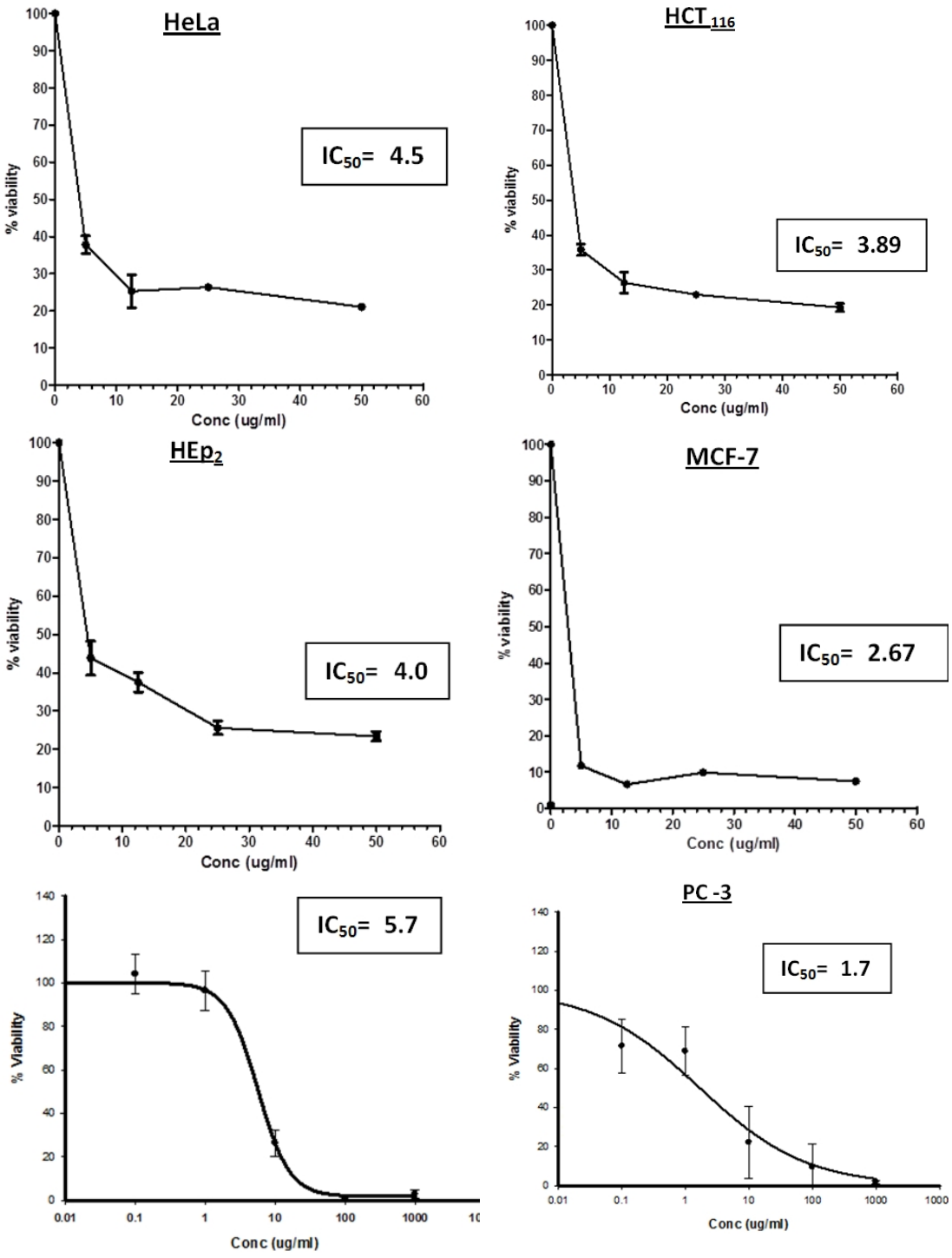

Fig. 2. Concentration response plots of *Enterolobium timbouva* extract on different cancer cell lines. Error bars are the standard deviations from three experiments

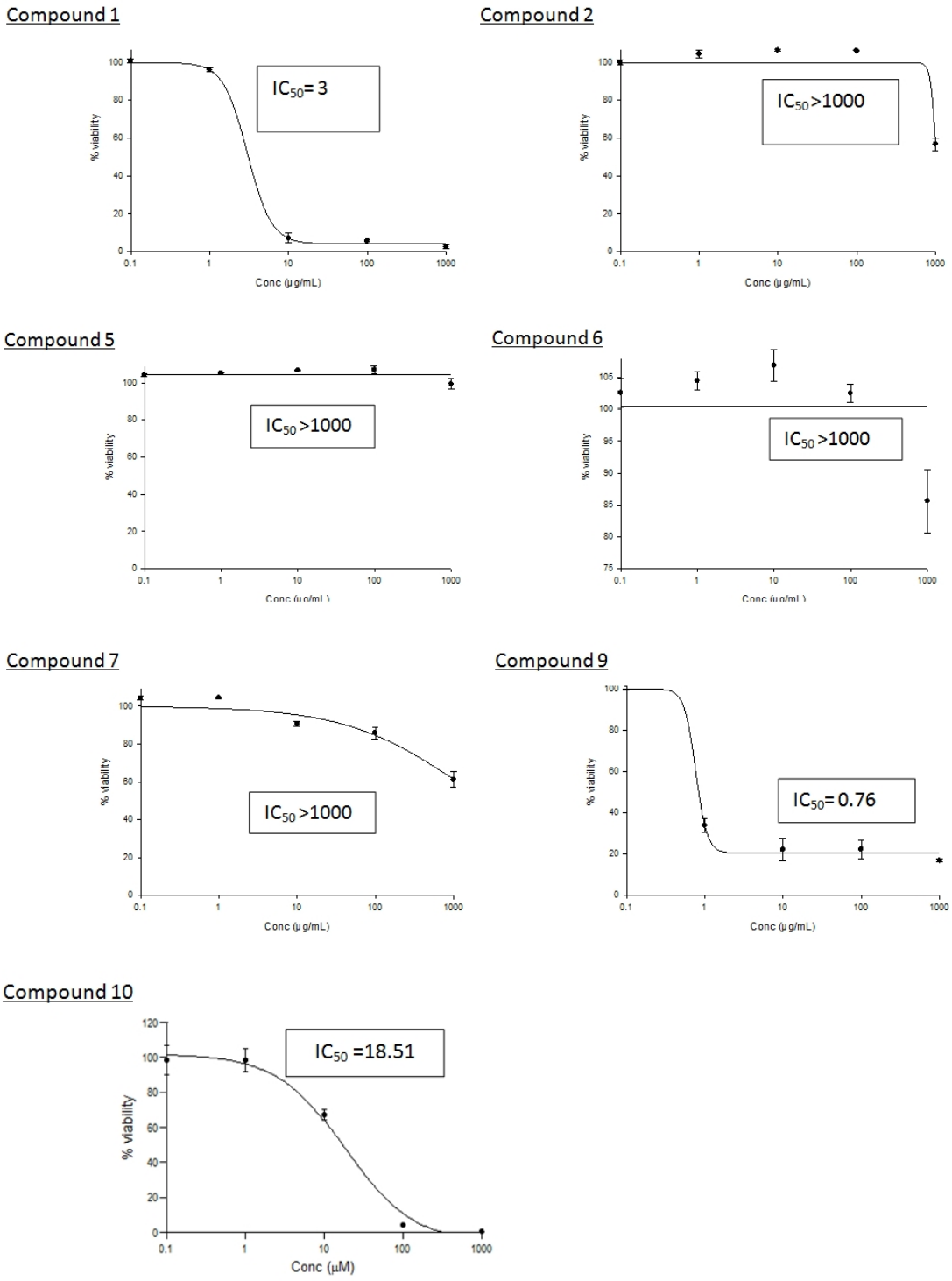

Fig. 3. Concentration response plots of the isolated compounds 1, 2, 5, 6, 7, and 9 on Huh-7 cell line. Error bars are the standard deviations from three experiments

4. CONCLUSIONS

Intensive phytochemical investigation of *Enterolobium timbouva* leaf extract confirmed that it is capable of synthesizing and accumulating appreciable amounts of several phenolics, thus leading to the isolation and characterization of ten of these constituents. The current study indicated that the phenolic compounds isolated from *Enterolobium timbouva leaves* are promising molecules with potentially useful cytotoxic activity profiles. This confirms that this terrestrial plant has great value as a source of lead compounds with pharmaceutical applications.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

The authors declare that no competing interests exist.

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