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^{1*}Sami A. Al-Robai , ^{1,2}Abdelazim A. Ahmed , ^{3,4}Aiman A.E. Ahmed , ⁵Sami A. Zabin , ^{1,6}Haidar A. Mohamed  and ¹Abdullah A. A. Alghamdi 

¹Department of Biology, Faculty of Science, Albaha University, Al-Baha, Saudi Arabia.

^{2,3}Department of Botany, Faculty of Science, University of Khartoum, Sudan.

³Pharmacology Department, Faculty of Medicine, Albaha University, Al-Baha, Saudi Arabia.

⁴Pharmacology Department, Faculty of Pharmacy, Omdurman Islamic University, Khartoum, Sudan

⁵Department of Chemistry, Faculty of Science, Albaha University, Al-Baha, Saudi Arabia.

⁶Medicinal and Aromatic Plants Research Institute, National Center for research, Sudan.

*Correspondence: E-mail: dr.alrobai@gmail.com.

Phenols, Antioxidant and Anticancer properties, of Three Medicinal Plants: In Vitro and In Silico Evaluation

Abstract. Recently, antioxidant and anticancer properties of phenolic compounds have been an area of growing interest. This work aimed at assessing the phenolic content and selected bioactivity of three medicinal plants. Standard spectrophotometric, chromatographic and biological methods were adopted. In silico screening for the phenolic acids as cytotoxic agents for cancer was performed. *Euphorbia granulata* showed significantly-high amount of polyphenols. The highest level of flavonoid and tannin contents was detected in *Galinsoga parviflora*. Ten phenolic acids were identified and quantified via GC-MS in all extracts. p-Hydroxybenzoic was the most dominant acid in *Tagetes minuta* while gallic was the predominant acid in *E. granulata*. Studied extracts showed higher reactive oxygen and nitrogen species scavenging activities and exhibited lower hydrogen peroxide inhibition value. *T. minuta* exhibited strong activity on A2780 cell line. The cytotoxicity of *G. parviflora* was highly significant against all cancer cells. *E. granulata* proved to be most active towards MCF7 and A2780 cell lines. In silico data revealed that caffeic acid had a high cytotoxicity. Studied crude extracts showed promising antioxidant and anticancer activities which could be due to the different phenolic contents in the studied plants.

Introduction

Nowadays, medicinal plants and their derived bioactive components have gained much attention in non-clinical and clinical researches [1]. The health benefit and biological activity of natural phenols from plants have been reported by several works [2,3]. In Saudi Arabia, medicinal plants have been used in traditional medicine since ancient times and local people still use them to cure many diseases [4]. According to Alqahtani et al. [5] and Abdel-Sattar et al. [6] more than half of the flowering plants in the Kingdom are used in folk medicine or have healing properties. Aati et al. [7] have reported that there are 471 out of 2253 plant species in the flora of Saudi Arabia are traditionally used for medicinal purposes. Al Baha province, southwestern of Saudi Arabia is characterized by a variety of habitats (mountains, escarpments valleys, coastal plain, sand dunes). This geographic diversity has provided an ideal atmosphere for the growth of various medicinal herbs in this region.

In recent years, *T. minuta* is gaining importance in scientific research because of its richness in chemical components that have bioactive and therapeutic properties towards plant, animal and human pathogens [8]. In previous works, many flavonoid compounds (quercetagetin, quercetagetin-7-arabinosyl-galactoside, quercetagetin-3-arabinosyl-galactoside, quercetagetin-7-glucoside, patuletin, patuletin-7-glucoside and isorhamnetin) were detected in leaves extract of *T. minuta*. Quercetagetin-7-arabinosyl-galactoside, which is the dominant compound in the leaves extract, had

antimicrobial activity towards some pathogenic microbes [9].^[60] Moreover, the essential oil obtained from leaves showed high antifungal activity towards *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* [10]. The potentiality of *T. minuta* was reported as a good source of antioxidant [11-13]^[36] and anticancer [14-16] agents.

E. granulata has many ethnobotanicals uses such as controlling human immunodeficiency virus (HIV-1) [17], expelling intestinal worms and having diuretic activity [18] as well as antimicrobial properties [19]. Ghauri et al. [20] have recently demonstrated the analgesic and pyretic activities of the aqueous methanolic extract of *E. granulata* in animal model.

Crude extracts and the pure isolated compounds from *G. parviflora* have been reported as potent cytotoxic, antimicrobial, and antioxidant agents [21]. The chemical constituents of *G. parviflora* are useful for treating snakebites, wounds, eczemas, lichens, flu and colds [22]. The cytotoxic activity and protective property of different extracts from *G. parviflora* on human fibroblast model have been reported by Bazylko, et al. [23].

The targeted plants *T. minuta*, *E. granulata*, and *G. parviflora* are growing in different places in Al Baha region, KSA and are commonly used by the local inhabitants in folk medicine. The available studies of antioxidant and anticancer properties as well as phenolic constituents of these plants are poorly described. This work aimed at evaluating the phenolic contents, antioxidant and anticancer properties of the methanolic extracts obtained from these plants in order to give scientific evidence for their uses in traditional medicine.

^[23] Materials and Methods

Plant materials and extraction. Three wild medicinal plants, *T. minuta* L. (Asteraceae), *G. parviflora* Cav. (Asteraceae) and *E. granulata* Forssk. (Euphorbiaceae), were collected from different localities of Al Baha region. Each plant material (both aerial and root parts) was washed by distilled water, dried under shade for 22 days, then gently ground to coarse powder using an electric mill. Plant powdered materials were defatted with petroleum ether (40 – 60 °C) for 24 h, then filtered and dried. Afterwards, each plant sample (10g) was macerated in 100 mL methanol and kept at room temperature for 72 h with shaking using an orbital shaker (150 rpm).^[62] The mixture was filtered and the obtained extract was dried using a rotary evaporator (Buchi, USA).^[62] The dried extracts were stored at 4 °C prior to analysis.

Total polyphenol content. The total polyphenol content in plant extract samples was evaluated spectrophotometrically according to Cliffe et al. [24] method with slight modifications.^[10] Plant aliquot of the extract solution (0.1 mL; 0.5 mg /mL) was mixed well with Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL) and the mixture was left at 25 °C for 10 min. Then, sodium carbonate solution (1.5 mL; 25%) was added to the reaction mixture and incubated at 40 °C for 30 min. The absorbance was read at 765 nm on a spectrophotometer (PD-303UV, Apel, Saitama, Japan) against a blank contained 0.1 mL of methanol instead of 0.1 mL of plant sample in the reaction mixture. Gallic acid (0.1 – 0.5 mg/mL)^[10] was used as a reference standard and the results were expressed as milligram gallic acid equivalents per gram dry weight (mg GAE/g DW).

Total flavonoid content. For estimating the amount of total flavonoid in each extract, Dewanto et al. [25] assay was followed with some modifications. A mixture of plant's extract sample (0.5 mL; 0.5 mg/mL), distilled water (1 mL) and sodium nitrite solution (0.2 mL; 5%) was prepared and left to stand for 10 min at 25 °C. To the prepared mixture,^[5] 0.4 mL of aluminum chloride (10% w/v in methanol) was added and the mixture was incubated at 25 °C for 5 min. Sodium hydroxide solution (1 mL; 1M) was added, and the volume of the reaction mixture was completed to 6 mL with distilled water. The optical density was recorded at 510 nm against a blank solution^[10] (composed of 0.5 mL methanol instead of plant sample in the reaction mixture). Quercetin (0.01 – 0.05 mg/mL) was used as a reference standard and the results of the total flavonoid content were expressed as mg quercetin equivalent per g dry weight (mg QE/g DW).

Total tannin content. Total tannin content in each plant extract was quantified according to the method described by Bhat et al. [26]^[19] with some modifications. One mL (0.5 mg/mL) of each extract

or tannic acid solution (0.1 – 0.5 mg/mL) was mixed with 3 mL of vanillin/methanol solution (4%) then concentrated hydrochloric acid (1.5 mL) was added to the mixture. After left for 30 min at room temperature, the absorbance was measured at 500 nm against methanol as a blank. The amount of tannin content was expressed as mg equivalent of tannic acid per gram dry weight of the plant sample (mg TAE/g DW).

In vitro antioxidant assays. The antioxidant activity of methanolic extracts from *T. minuta*, *E. granulata* and *G. parviflora* was determined using four different complementary assay methods as described below. Ascorbic acid as natural antioxidant and butylated hydroxytoluene (BHT) as synthetic antioxidant were used as reference standards.

DPPH radical scavenging activity. The radical scavenging effect of the plant extract samples against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was performed following Koh et al. [27] assaying method. Every plant extract (1mL) with a concentration of (0.5 mg/mL) or standard was added to 2.7 mL of (6×10^{-5} mol/L) methanolic solution of DPPH and mixed well. After incubation at room temperature in the dark for 60 min, the absorbance was read at 517 nm. DPPH solution without plant sample or standard was used as a control and methanol as a blank. The radical scavenging activity was estimated using the % of DPPH inhibition as follow:

$$\% \text{ DPPH radical scavenging} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c = Control absorbance and A_s = Sample absorbance.

Experiment was repeated thrice and results are presented as mean with standard deviation.

ABTS radical scavenging assay. The radical scavenging activity of the extracts together with the standard solutions were measured by ABTS (2,2'-azino-bis (3- ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay [28]. Ten mL of potassium persulfate (2.4 mM) was mixed with 10 mL of ABTS solution (7 mM) then the mixture was left to react in the dark for 12 h at room temperature. This ABTS solution was diluted with methanol to obtain an absorbance of 0.700 at 734 nm prior to assay. Plant samples or standard solutions (1 mL., 0.5 mg/mL) were added to 2.5 mL of the diluted ABTS then the reaction mixture was allowed to react for 7 min at room temperature in the dark before recording the absorbance at 734 nm. The ABTS scavenging activity of the targeted plant extracts was expressed as % of ABTS+ similar to DPPH scavenging method as follow:

$$\% \text{ ABTS radical scavenging} = \frac{A_c - A_s}{A_c} \times 100$$

Experiment was repeated thrice and results are presented as mean with standard deviation.

Nitric oxide radical scavenging activity (NO). The assay method reported by Ebrahimzadeh et al. [29] was adopted for measuring the percentage of nitric oxide radical scavenging activity in each suspension. A solution containing a mixture of 0.5 mL of plant extract or positive standard (0.5 mg/mL), 2 mL of sodium nitroprusside (10 mM) and 0.5 mL of phosphate buffer saline (50 mM, pH 7.4) was prepared then left for 2.5 h at 25 °C. Griess reagent was prepared by mixing 1.0 mL of sulfonic acid (33%) in glacial acetic acid (20%). The above prepared mixture (0.5 mL) was mixed with the Griess reagent (0.5 mL) and 1.0 mL of naphthylene diamine dichloride (0.1% w/v). After incubation at room temperature for 30 min, the absorbance was recorded at 540 nm.

$$\% \text{ nitric oxide radical scavenging} = \frac{A_c - A_s}{A_c} \times 100$$

Experiment was repeated three times and results are presented as mean with standard deviation.

Hydrogen peroxide scavenging activity. Ruch et al. [30] method was adopted for measuring the capacity of each extract to scavenge the hydrogen peroxide radical in the reaction medium. A mixed solution of 40 mM H₂O₂ and 50 mM phosphate buffer saline (pH 7.4) was prepared and stored in a dark brown bottle. One mL of each sample (0.5 mg/mL) was added to 0.6 mL of the above mixed solution. After 15 min at room temperature, the absorbance was recorded at 230 nm. A blank was prepared by mixing plant samples with the buffer and without hydrogen peroxide.

$$\% \text{H}_2\text{O}_2 \text{ radical scavenging} = \frac{A_c - A_s}{A_c} \times 100$$

Experiment was repeated three times and results are presented as mean with standard deviation.

Determination of individual phenolic acids (GC-MS analysis).^[32] Five mL of methanol was added to 0.5 g of each plant extract sample, then vortex for 1 min. Extraction was done by sonication in ultrasonic bath for 30 min. Extracts were concentrated to 1 mL under a gentle stream of high purity nitrogen (N₂). For derivatization, 20 µL concentrated sample was transferred to 2 mL vial and dried under a gentle stream of high purity nitrogen then 30 µL of N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) was added. After incubation at 70 °C for 3 h, samples were redried under gentle stream of N₂ to remove excess BSTFA then redissolved in 300 µL of hexane. Derivatized samples were injected in gas chromatography mass spectrometry (GC-MS) on an Agilent (Palo Alto, CA) 6890N gas chromatograph equipped with an Agilent HP-5MS column (30 m×0.25mm ×0.25µm film thickness) and 5973N mass selective detector. Samples were analyzed by selected-ion-monitoring (SIM) mode using electron impact and negative chemical ionization mass spectrometry. Samples were scanned for individual phenolic acids and quantized against calibration curves made from 10 individual phenolic standards.

Determination of cytotoxicity (Cell culture).^[14] Different cell lines were brought from the ATCC, USA: MCF-7 (human breast adenocarcinoma), A2780 (human ovary adenocarcinoma), HT-29 (human colon adenocarcinoma); and MRC-5 (Normal human foetal lung fibroblast). Cells were sub-cultured at 37°C, 5% CO₂, 95% air and 100% relative humidity. RPMI-1640 medium [10% fetal bovine serum (FBS), L glutamine and 1% antibiotic-antimycotic] was used for all cells except MRC-5 cell, which was maintained in Eagle's minimum essential medium (EMEM, 10% FBS).^[8]

Cytotoxicity assay. The cytotoxicity of the prepared plant samples was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay [31]. The tested cell lines (100 µL) were placed in 69-well (3×10³ cells/well) plates then incubated at 37 °C overnight. Final concentrations of each extract were: 0, 0.05, 0.5, 5, 25, 50 µg/mL in media (DMSO, 0.1%). Doxorubicin (0.01 – 10 µg/mL) was used in this experiment as a reference drug. Each concentration was tested in triplicates and the experiment was repeated twice. The plates were incubated for 72 h then MTT solution (10 µL; 5 mg/mL) was added to each well. Optical density of the purple formazan A570 produced by treated cells is compared with the amount of formazan produced by untreated control cells, the strength of the drug in causing growth inhibition can be determined, thus compound concentration causing 50% inhibition (IC₅₀) compared to control cell growth (100%) were determined. GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA was used for analysis.

In silico molecular docking screening (Protein source as suggested targets). The crystallographic structure of the five selected protein targets were retrieved from protein data bank as PDB format; Recombinant human placental annexin v complexed with K-201 as a calcium channel activity inhibitor [PDB code: 1HAK], with a resolution of 3.00 Å [32]. Estrogen receptor alpha ligand binding domain in complex with Lsz102 [PDB code: 6B0F], with a resolution of 2.86 Å [33]. Structure, Thermodynamics, and Kinetics of Plinabulin Binding to two Tubulin Isotypes [PDB code: 6S8L], with a resolution of 1.80 Å [34]. Crystal structure of a double-stranded DNA containing the major adduct of the anticancer drug Cisplatin [PDB code: 1AIO], with a resolution of 2.60 Å [35] and Human topoisomerase II alpha bound to DNA [PDB code: 4FM9], with a resolution: Doxorubicin of 2.90 Å [36]. They were downloaded as PDB structure.

Ligand sources. The selected ligands (standard drugs and the detected individual phenolic acids) were sketched using built in services in the zinc database in SDF format. The two standards Doxorubicin and Tamoxifen were collected from ZINC database with ID ZINC3918087 [37] in SDF format. Then converted to PDBQT format with three-dimensional structure using Open babel.

Binding pocket identification and preparation. AutoDock Tools 1.5.6 [38] were used to prepare and convert the protein to PDBQT format. All five selected protein targets were checked for missing atoms, chain breaks and water molecules. Unnecessary residues were also removed from

the protein structures, only the active binding site was kept, hydrogens were added, partial charges were calculated.

Ligands preparation. The ligands were converted to their corresponding three-dimension structures using chemical file format converter openbabel 2.4.1 [39] (by adding "--gen3d" argument). Auto Dock Tools 1.5.6^[16] was used to prepare and convert the molecules to PDBQT format, rotatable bonds were detected, polar hydrogens were added, non-polar hydrogens were deleted and their charges were merged with carbon atoms, hydrogen bond donors and hydrogen bond acceptors were defined for each ligand, partial charges were calculated.

Geometry optimization and pre-docking procedure. In order to prepare the selected compounds for docking, hydrogens and Gasteiger charges were added and all the hetero-atoms and water molecules were removed from protein structure^[2]. The protonated protein initially optimized in order to remove all the bad steric clashes using AMBER 99 force field [40], while MMFF94s force field parameters [41,42]^[2] were performed for small molecules. Ligands were optimized by minimizing energy to minimum RMS Gradient of 0.010 kcal/mol using Chem3D.

Molecular docking approach. AutoDock Vina 1.1.2 [43] was used to perform molecular docking simulations of the standard ligands and tested compounds that previously prepared in PDBQT format were carried out against the three-dimensional structure of all listed five proteins and binding energy was recorded as described here:

(1) The co-crystallized protein with its inhibitor compound was identified; therefore, the binding site was identified with its residues. (2) The inhibitor compound is then removed, optimized the active site pocket and re-docked again into the active site pocket to gain the same results. (3) A search space (grid box) that covers the entire binding site for each target was set; other Vina configurations were left as default, such as following:

1HAK: (16.5992, 18.4811, 14.6966) centered (22.7988, 16.0331, -11.475); 6B0F: (15.3105, 18.2803, 20.7096) centered (-38.8628, 5.27725, -28.2858); 6S8L: (17.8539, 20.5174, 23.1907) centered (17.2629, -4.42538, 26.6303); 1DDP: (10.4729, 10.3155, 10.1) centered (-0.495224, -3.74989, 0.609371); 4FM9: (77.1944, 111.975, 70.9791) centered (38.0454, 46.9917, 16.8529).

(4) Ligand interactions were computed for the X-ray co-crystallized protein to reveal the different types of interactions as a validation for the coming docking procedure.^[2] (5) A cluster of 10 conformers of the ligand were retained with highest and best score by default.

Ligand efficacy estimation. A set of standard ligands and the detected phenolic compounds were explored in AutoDock Tool to calculate different parameters and saved in CSV format and then evaluated regarding their efficiencies in order to see the drug candidate properties. The ΔG values for our ligands were calculated by AutoDock 1.1.4. However, the ligand efficiency (LE) value was introduced to normalize the free binding energy values [44]. Ligand efficiency indices ($\Delta G/NHA$) were calculated based on the number of heavy atoms for our ligands using equation to calculate the LE from in vitro data: $LE_v = -p(EC_{50})/NHA$.

Statistical analysis. Initially the AutoDock Tool was used to calculate the binding free energy (ΔG) for our ligands clusters in silico, while the GraphPad prism software was used to manipulate and compare the data using One-way ANOVA with Dunnett's PostHoc Test and Tukey's Multiple Comparison Test.^[15] Results were considered statistically significant at p -value ≤ 0.05 .

Results and discussion

Total polyphenol, flavonoid and tannin contents. In this investigation, the amounts of total phenol, flavonoid and tannin contents were varied among the studied plants (Fig.1). The quantity of the flavonoid compounds was significantly ($p < 0.05$) higher in *G. parviflora* extract than the other two plant extracts. The highest tannin content was observed in *G. parviflora* extract (6.07 ± 0.33 mg/g) and the least (1.98 ± 0.24 mg/g) was in the extract of *E. granulate*. The amount of polyphenols (33.88 ± 0.72 mg/g) in the crude methanolic extract of *E. granulata* was significantly ($p < 0.05$) higher than that in *T. minuta* (19.71 ± 0.51 mg/g) and *G. parviflora* (19.62 ± 1.15 mg/g).

No significant differences between *T. minuta* and *G. parviflora* in their polyphenol contents were observed.

On comparing the observed results reported by Rezaei et al. [45] the polyphenol ($34.17 \pm 0.6 \text{ mg g}^{-1}$) and flavonoid ($14.86 \pm 0.4 \text{ mg g}^{-1}$) concentrations in *T. minuta* were higher than our obtained values. Moreover, the total phenolic content in *T. minuta* ($67 \pm 7 \text{ mg g}^{-1}$) reported by Ranilla et al. [46] was higher than the results of this study for the same plant. The presence of flavonoids and tannins as secondary metabolites in *E. granulata* has been reported by Ali et al. [15]. The richness of the genus *Euphorbia* in phenolic [47] and tannin [48] compounds has been revealed in previous studies. The amount of flavonoids in *G. parviflora* for this study was inconsistent with the result reported by Bazylko et al. [23] for ethanol extract ($685.4 \pm 69.6 \text{ mg g}^{-1}$) and water extract ($41.4 \pm 3.5 \text{ mg g}^{-1}$). The results reported by Studzińska-Sroka et al. [49] for total polyphenol ($98.30 \pm 0.14 \text{ mg g}^{-1}$) and flavonoid ($6.15 \pm 0.41 \text{ mg g}^{-1}$) contents in *G. parviflora* were disagreed with this result. Moreover, flavonoids and other natural polyphenols have been reported in *G. parviflora* by several researchers [21,23,50].

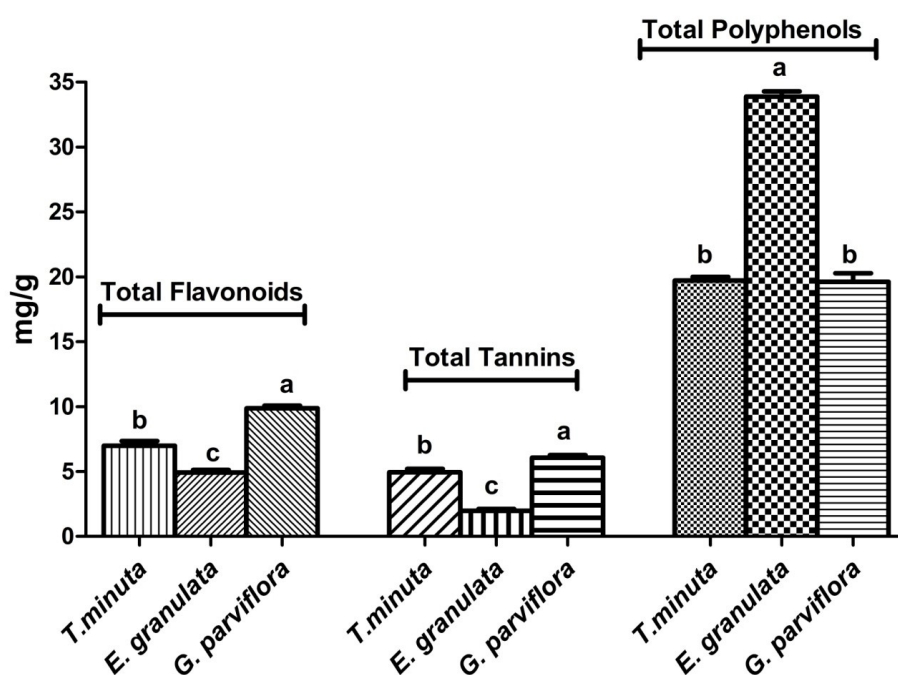


Figure 1 – Total polyphenol, flavonoid and tannin contents in the studied plants.

Different letters in the same test are significantly different (Tukey's Multiple Comparison Test, $p \leq 0.05$).

Individual phenolic acids. As shown from Table 1, the GC-MS analysis revealed the presence of ten individual phenolic acids in all extracts. The quantity of vanillic acid and 3,4 dihydroxybenzoic acid was very low in all plant extracts. The highest level of the total individual phenolic acids ($397 \mu\text{g/g}$) was detected in *T. minuta* extract followed by *E. granulata* ($300 \mu\text{g/g}$) and *G. parviflora* ($130 \mu\text{g/g}$). *p*-Hydroxybenzoic acid ($70 \mu\text{g/g}$) was the dominant phenolic acid in *T. minuta* extract. *p*-Coumaric acid was the second dominant phenolic acid in *T. minuta* extract. The amount of sinapic acid observed in *T. minuta* extracts ($42 \mu\text{g/g}$) was twice that of *G. parviflora* ($21 \mu\text{g/g}$). *T. minuta* and *E. granulata* extracts contained equal amount ($25 \mu\text{g/g}$) of trans-cinnamic acid. *E. granulata* was rich in both gallic acids and ferulic acids as compared with that of *G. parviflora* and *T. minuta*. The concentration of gallic and caffeic acids in *E. granulata* represented 45% of the total content of the individual phenolic acids detected in this plant. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) was the second predominant phenolic acid ($62 \mu\text{g/g}$) in the

extract of *E. granulata*. The amount gallic and caffeic acids present in *E. granulata* was nearly equal to one-third of the total individual phenolic acid content. *E. granulata* and *G. parviflora* contained same amounts (8 µg/g) of 3,4-dihydroxybenzoic acid and syringic acids. The studied plant extracts showed variation in their vanillic acid content.

However, phenolic extracts which contained hydroxybenzoic acid has the ability to protect Caco-2 cells against pro-oxidant induced toxicity [51]. The study carried out by Farhoosh et al. [52] has revealed the anti-DPPH radical activity of the derived compounds from p-hydroxybenzoic acid. Ester p-hydroxybenzoic acid has been widely used as an ingredient in cosmetic, food and drug products [53]. p-Coumaric acid has various pharmacological effects such as antioxidant [54,55] anticancer [56] neuroprotective [57]. Recently, the potentiality of this acid to control the inflammatory diseases through different molecular mechanisms has been stated by Ferreira et al. [58] Sinapic acid has various pharmacological and therapeutic applications such as antioxidant, anticancer and anti-inflammatory [59]. However, most of the phenolic compounds in plant tissues are derived from t-cinnamic acid [60]. Gallic acid which was the dominant phenolic acid in *E. granulata* extract possesses various industrial and biological properties [61]. It has a high antioxidant property as well as protective activity in healthy people against apoptosis [62]. The potent antioxidant activity of the gallic acid is attributed to its tendency to donate hydrogen [63]. Gallic acid has been reported as an effective compound against various diseases such as alzheimer, Parkinson [64] and cancer [65]. Ferulic acid plays an essential role for treatment of several chronic disorders such as cancer disease and has cytoprotective potential to inhibit the free radicals in living cells [66]. Ferulic acid which is found in many plants [67] has the ability to scavenging both the reactive oxygen species and nitrogen radicals [68] and has an inhibition effect on the cytotoxic enzymes [69]. Caffeic acid which was found at different concentrations in the studied plants has been reported as antioxidant ingredient [55]. The therapeutic potential of vanillic acid has been reviewed by Brimson et al. [70] who indicated that vanillic acid is a probable anti-inflammatory and anticancer candidate and also inhibit nitric oxide (NO) production. In general, the health benefits of phenolic acids and their uses in food and medicine have been recently confirmed by Yang et al. [71].

Table 1 – Individual phenolic acids (µg/g) in the studied herb plants as analysed by GC-MS

Compound	<i>T. minuta</i>	<i>E. granulate</i>	<i>G. parviflora</i>
t-Cinnamic acid	25	25	9
p-Hydroxybenzoic acid	70	43	15
Vanillic acid	20	8	10
3,4-Dihydroxybenzoic acid	19	8	8
Syringic acid	35	8	8
p-Coumaric acid	57	24	13
Gallic acid	44	73	12
Ferulic acid	40	62	14
Caffeic acid	45	25	20
Sinapic acid	42	24	21
Total	397	300	130

Antioxidant activity. The potential of the methanolic extracts of the investigated plants to scavenge the free radicals in the reaction medium was carried out using four different in vitro methods. Ascorbic acid and butylated hydroxytoluene (BHT) were used as reference compounds. Results, summarized in figures 2 and 3, showed that the values of antioxidant activities of the studied extracts exhibited good antioxidant activities although none of the crude extracts in this investigation had a higher activity than the used controls in each assay. It was observed that the DPPH, ABTS and NO radical scavenging activities of all crude extracts were higher than 80% and slightly less than the activity of the tested controls. The three tested plant extracts showed potent antioxidant activity when using DPPH% and ABTS% methods (Fig. 2). The data of DPPH assay indicated that there were significant differences ($p \leq 0.05$) between the scavenging activity of the

plant extracts and that of the controls. The anti-radical activities of the plant samples were significantly different when compared with ascorbic acid and BTH as controls. The tested extracts of the three plants showed statistically significant difference in their ability to scavenge the free nitric oxide radicals in the reaction medium (Fig. 3). They displayed good ability to scavenge the nitric oxide radicals in the prepared solutions. The antioxidant potential (NO) of *E. granulata* extract ($82.77 \pm 0.30\%$) was nearly equal that of BHT ($83.14 \pm 0.28\%$) and lower than ascorbic acid ($87.76 \pm 1.10\%$) as controls. The potentiality of the three crude extracts to inhibit or quench the hydrogen peroxide radicals in the prepared suspensions was very weak ($1.73 \pm 0.27 - 6.62 \pm 0.44\%$) as compared with the control ($16.27 \pm 0.60\%$). *E. granulata* extract exhibited more DPPH ($87.70 \pm 0.75\%$) and ABTS ($87.65 \pm 0.49\%$) inhibition as compared with the *T. minuta* ($85.41 \pm 1.55\%$) and *G. parviflora* ($83.22 \pm 0.34\%$) extracts.

In previous studies, antioxidant activity was revealed in extracts from some species belong to the genus *Euphorbia* such as *E. hirta*, *E. retusa* and *E. gaditana* [72]. Mostafa et al. [73] had reported that at concentration of 150 mg/ml, the ethyl acetate fraction from *G. parviflora* demonstrated strong antioxidant activity. The ability of extracts from *G. parviflora* to scavenging DPPH free radicals was reported by Bazytko et al. [74]. A previous study showed that both aqueous and ethanol extracts from *G. parviflora* have the ability to scavenge the generated reactive oxygen species (superoxide anion and hydrogen peroxide) in cell-free systems [23]. The antioxidant property of essential oils obtained from *T. minuta* was reported by Osée Muyima et al. [11] and de Oliveira et al. [13]. The studies carried out by Shirazi et al. [16] and Igwaran et al. [12] revealed that *T. minuta* could be a useful source of antioxidant agents. Thiophene and flavonoid compounds which were isolated from the methanolic extract of *T. minuta* had showed potent antioxidant activity when compared with propyl gallate as a standard [75]. The obtained results revealed that the investigated plants were rich in polyphenols, flavonoids and tannins molecules, which had anti-radical activity. The presences of phenolic, flavonoid and tannin compounds in the tested plants could be linked with the antioxidant properties of these extracts. This is consistent with the several recent studies, which suggested that the antioxidant properties could be referred to phenolic compounds present in plants [72,76]. Among the reported secondary metabolites in plants, phenolic components had been recognized as excellent substances for reducing the oxidative damage caused by the free radical in living cells [77]. However, the direct relationship between the phenolic and flavonoid contents in different medicinal plants and antioxidant activity has been revealed by El-Zayat et al. [78]. The potentiality of the medicinal plants as a source of therapeutic agents is often related to their antioxidant capacity, that depend on to the amount of the phenol and flavonoid components present in these plants [79].

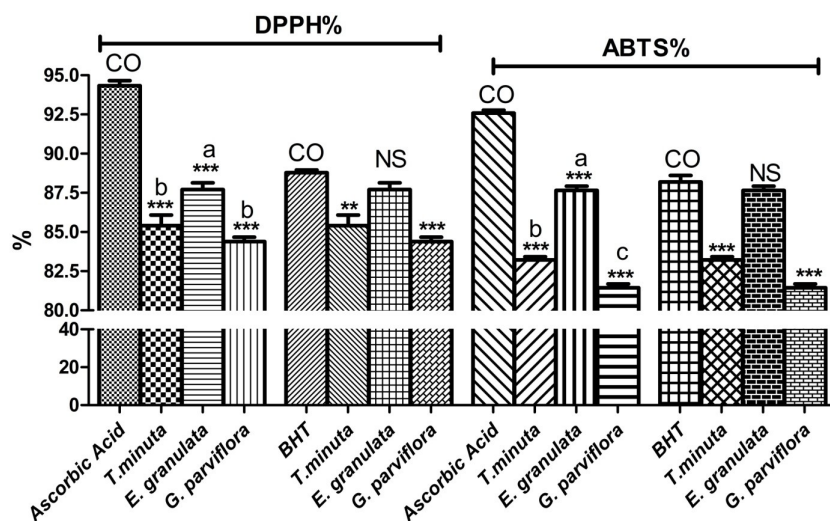


Figure 2 – Antioxidant activity of plant extracts evaluated by DPPH and ABTS assays

The results are presented as mean with standard deviation (\pm); $n = 3$, sample values were compared with the control value using one way ANOVA+ Post Hoc test, where $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), Not significant (NS), Control (CO). Different letters in the same lot are significantly different (Tukey's Multiple Comparison Test, $p \leq 0.05$).

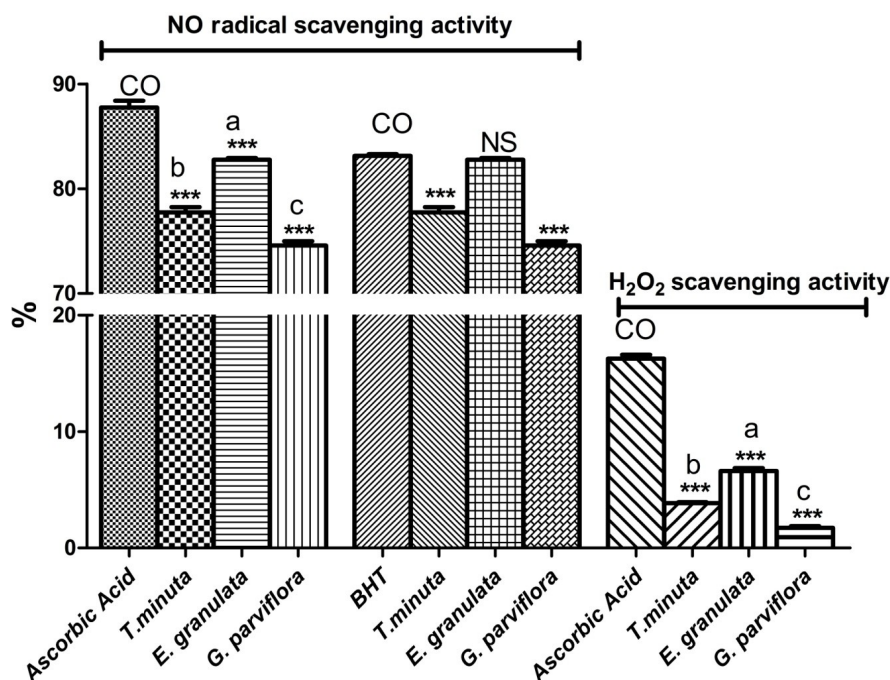


Figure 3 – Antioxidant activity of plant extracts evaluated by nitric oxide (NO) and hydrogen peroxide assays

The results are presented as mean with standard deviation (\pm); $n = 3$. sample values were compared with the control value using one way ANOVA+ Post Hoc test, where $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), Not significant (NS), Control (CO). Different letters in the same lot are significantly different (Tukey's Multiple Comparison Test, $p \leq 0.05$).

Anticancer activity. ^[14] The cytotoxic activity results of the tested extracts on MCF7 (human breast adenocarcinoma), A2780 (human ovary adenocarcinoma), HT29 (human colon adenocarcinoma) and MRC5 (normal human fetal lung fibroblast) cell lines are summarized in Table 2. A clear variation was observed among the three plant extracts regarding their toxicity against the tested cell lines. The recorded IC_{50} values of *G. parviflora* extract for the three tested cell lines were significantly ($p \leq 0.001$) high when compared with IC_{50} value of the control. The IC_{50} values of the three extracts were significantly different ($p \leq 0.05$) for A2780 and MCF7 cell lines. No significant difference was observed between *T. minuta* and *G. parviflora* in their inhibition effect towards HT29 cell line. Extracts of *E. granulata* and *G. parviflora* showed same cytotoxic activity on MCF7 cell lines and A2780 cell line, respectively. Both MCF7 and HT29 cell lines were weakly responded to extracts of *T. minuta* and *E. granulata* as compared with the normal cell. The highest anticancer activity ($8.92 \pm 2.32 \mu\text{g mL}^{-1}$) was presented by *T. minuta* extract on A2780 cell line. The lowest cytotoxic activity was demonstrated for *T. minuta* extract ($35.01 \pm 3.57 \mu\text{g mL}^{-1}$) against MCF7 cell line and *E. granulata* extract ($41.89 \pm 0.07 \mu\text{g mL}^{-1}$) towards HT29 cell line.

According to the Us National Cancer Institute (NCI) a crude extract is considered to be active if it has an IC_{50} value of $< 20 \mu\text{g/mL}$ [80]. Based on this criterion, the IC_{50} values of *T. minuta* and *G. parviflora* extracts on A2780 cell line as well as *E. granulata* extract on MCF7 cell line were within the set limit of NCI guidelines for crude extracts at 50% inhibition. ^[13] In this study the anticancer activity of *T. minuta* crude extract on MCF7 cell line ($35.01 \pm 3.57 \mu\text{g mL}^{-1}$) was high when compared with the result ($54.7 \pm 6.2 \mu\text{g mL}^{-1}$) reported by Ali et al. [15]; and its low as compared with the activity ($4.68 \mu\text{molL}^{-1}$) of the pure isolated tagetone (A) compound from *T. minuta* flowers [81]. The activity of *T. minuta* extracts on other cancer cells such as Lewis lung

carcinoma has been reported by Ickesh et al. [14]. In addition, the reproductive activity of the essential oils from *T. minuta* against human tumor cell lines (liver hepatocellular carcinoma (HepG2) and nasopharyngeal cell lines) has been presented by Shirazi et al. [16]. The anticancer result of *E. granulata* extract on MCF7 cell ($16.23 \pm 4.50 \mu\text{g mL}^{-1}$) obtained in this study was inconsistent with the result reported by Ghauri et al. [20] who stated that the aqueous methanolic extract of *E. granulata* had no anti-proliferative activity towards the breast cancer cell line (MCF7). The weak cytotoxic activity of *G. parviflora* extract on MCF7 cell lines which was reported by Mostafa et al. [73] disagreed with the result obtained in this study. On the other hand, Pan et al. [82] have found that chloroform and ethyl acetate extracts from *G. parviflora* have good cytotoxic activity on HL60 (human promyelocytic leukemia cell).

Table 2 – Inhibition concentration of plant extracts and doxorubicin as a reference drug against three cancer cell lines and one normal fibroblast (MTT 72 h, $\text{IC}_{50} \pm \text{SD} \mu\text{g mL}^{-1}$)

Sample	MRC5 (Control)	A2780	HT29	MCF7
<i>T. minuta</i>	$37.10 \pm 1.61\text{b}$	$8.92 \pm 2.32^{***}, \text{a}$	$21.22 \pm 0.57^{***}, \text{a}$	$35.01 \pm 3.57\text{NS}, \text{c}$
<i>E. granulata</i>	$40.22 \pm 9.48\text{b}$	$22.80 \pm 1.55^{**}, \text{c}$	$41.89 \pm 0.07 \text{NS}, \text{b}$	$16.23 \pm 4.50^{***}, \text{a}$
<i>G. parviflora</i>	$57.40 \pm 1.96\text{a}$	$16.53 \pm 0.92^{***}, \text{b}$	$22.39 \pm 1.73^{***}, \text{a}$	$27.83 \pm 0.13^{***}, \text{b}$
Doxorubicin	0.74 ± 0.06	0.02 ± 0.01	0.38 ± 0.03	0.01 ± 00

Results are presented as mean with standard deviation (\pm); $n = 4$. IC_{50} values were compared with the IC_{50} value of MRC5 normal cells in the same row using one way ANOVA+ Dunnett's Multiple Comparison Test, where $p \leq 0.01$ (**), $p \leq 0.001$ (***), Not significant (NS). Means followed by different letters in the same column are significantly different according to Tukey's means test at $p \leq 0.05$.

In silico molecular docking results. The in silico molecular docking screening results showed that caffeic acid had a least (ΔG_{bind} , kcal/mol) values against the five different selected target receptors, particularly α -estrogen receptor (6b0f) – 6.24 ± 0.08 kcal/mol] and two tubulin isotypes (6s8l) [$- 6.17 \pm 0.07$ kcal/mol] compared to the relevant standard ligands tamoxifen [$- 8.40 \pm 0.46$ kcal/mol], cisplatin-Zn/plinabulin [$- 5.57 \pm 0.92$ kcal/mol] (Table 3). In addition to, caffeic acid exhibited a ratio of 72 – 95% when it was correlated with both in vitro activity against the tested cell-lines and in silico virtual efficiency against the five different selected target receptors as estimated from the numbers of heavy atoms (Table 4). On other hand, similar activity to the standards tamoxifen and cisplatin/ plinabulin on estrogen receptors ($\text{ER}\alpha$ and/or $\text{ER}\beta$) and two tubulin isotypes was observed by caffeic acid (Table 3 and 4).

Lesser the binding energy, is an indication of high activity and better is the binding of the ligand and receptor [83,84]. Referring to this hypothesis the in silico molecular docking results proved that caffeic acid had a least (ΔG_{bind} , kcal/mol) value among the ten screened compounds against the different selected targets. This indicated that this compound possessed a high affinity and thus expected to have a greatest cytotoxic activity.

In addition to, caffeic acid exhibited a ratio of (72 – 95%) regarding the correlated in vitro activity and in silico efficiency; these findings confirmed the pre-determined cytotoxic activity of caffeic acid and can be taken as evidence to confirm the cytotoxic activity against the selected cancer cell-lines. This finding was in line with the recent trend that scientists usually combining both in silico and in vitro approaches to identify and validate the potential biological activity of lead compound(s) at pre-clinical testing level [85-87].

Kuntz et al. [88] mentioned that molecules that achieve a given potency with fewer heavy atoms are more efficient. Accordingly, caffeic acid which possess 14-NHA can be consider as an efficient cytotoxic compound. Strong correlation between the in vitro activity and in silico virtual efficiency prediction of caffeic acid as cytotoxic agent was observed. Caffeic acid showed closely related values; 0.32, 0.33 with MCF7 and HT29 cell lines in vitro and 0.44, 0.44 with 6b0f and 6s8l as target receptors in silico. This indicates that docking calculations can be used to determine which compound had the best drug candidate tendency or feature(s) to serve as a future new drug as

mentioned by Defant et al. [89]. The obtained results suggested that caffeic acid may act either as estrogen receptors blocker [90] or as tubulin polymerization inhibitor that disrupt the microtubules and induce cell cycle arrest in G 2-M phase and formation of abnormal mitotic spindles [91] or may act by both mechanisms. further confirmatory tests were required to proof or rule out these hypotheses.

Table 3 – In silico molecular docking: affinity for the different standards and tested ligands

Ligand/Target	1hak : Annexin V (C25dH32dN2dO2dS)	6b0f: α -Estrogen Receptor (C25dH17dF3dO4dS)	6s8l: Plinabulin + Two Tubulin Isotypes	1AIO: DNA Containing Cisplatin	4FM9: α -topoisomerase II
Standard (Control)	- 6.87 \pm 0.07C	-8.40 \pm 0.46C	-5.57 \pm 0.92C	-2.54 \pm 0.09C	- 6.21 \pm 0.15C
Vanillic acid	- 4.51 \pm 0.11***	-5.42 \pm 0.15***	-5.45 \pm 0.045 NS	-5.19 \pm 0.07***	-5.89 \pm 0.09*
3,4 Dihydroxy Benzoic acid	-4.58 \pm 0.085***	-5.53 \pm 0.18***	-5.62 \pm 0.09 NS	-5.36 \pm 0.07***	-5.93 \pm 0.06*
Gallic acid	-4.93 \pm 0.091***	-5.52 \pm 0.16***	-5.68 \pm 0.11 NS	-5.72 \pm 0.05***	-6.51 \pm 0.17*
p-Coumaric acid	-5.06 \pm 0.12***	-6.11 \pm 0.09***	-5.98 \pm 0.04 NS	-4.82 \pm 0.11***	-6.14 \pm 0.15*
Caffeic acid	-5.10 \pm 0.15***	-6.24 \pm 0.08***	-6.17 \pm 0.07 NS	-5.36 \pm 0.05***	-6.21 \pm 0.15*
Ferulic acid	-5.33 \pm 0.09***	-6.24 \pm 0.11***	-6.07 \pm 0.06 NS	-5.20 \pm 0.04***	-6.16 \pm 0.13*
Sinnapic acid	-5.33 \pm 0.12***	-5.93 \pm 0.17***	-5.79 \pm 0.05 NS	-5.33 \pm 0.06***	-6.04 \pm 0.08*
Syringic acid	-4.54 \pm 0.08***	-5.51 \pm 0.07***	-5.44 \pm 0.08 NS	-5.59 \pm 0.05***	-6.23 \pm 0.06*
p-Hydroxybenzoic acid	-4.46 \pm 0.09***	-5.44 \pm 0.15***	-5.34 \pm 0.09 NS	-4.80 \pm 0.08***	-5.86 \pm 0.09*
t-Cinamic acid	-4.70 \pm 0.16***	-5.90 \pm 0.11***	-5.79 \pm 0.03 NS	-4.68 \pm 0.07***	-5.63 \pm 0.15*

Standard ligands: K-201, Tamoxifen, Cisplatin-Zn and Doxorubicin. Where $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), Not significant (NS) and control (C)

Table 4 – Comparison of calculated LE based on NHA, in vitro and in silico for the most promising tested compound; caffeic acid from the T. minuta plant

Targets	STD Ligands	Cell line	In vitro activity		In silico efficiency		Ratio
			IC ₅₀	LEv	Δ Gbind, kcal/mol	LEs	
1hak : Annexin V (C25dH32dN2dO2dS)	Cisplatin/ K-201	HT29	21.22 \pm 0.57	0.33	-5.10 \pm 0.15	-0.36	91.7%
6b0f: α -Estrogen Receptor (C25dH17dF3dO4dS)	Tamoxifen/ Lsz102	MCF7	35.01 \pm 3.57	0.32	-6.24 \pm 0.08	-0.44	72.7%
6s8l: Two Tubulin Isotypes	Cisplatin/ Plinabulin	HT29	21.22 \pm 0.57	0.33	-6.17 \pm 0.07	-0.44	75%
1AIO: DNA Containing Cisplatin	Cisplatin/ Plinabulin	A2780/MRC5	8.92 \pm 2.32	0.36	-5.36 \pm 0.05	-0.38	94.7%
			37.10 \pm 1.61	0.32			84.2%
4FM9: α -topoisomerase II	Doxorubicin	MCF7	35.01 \pm 3.57	0.32	-6.21 \pm 0.15	-0.44	72.7%

LEv = p (IC₅₀)/NHA, LEs = Δ Gbind /NHA.

Conclusion

The results obtained showed that the amount of polyphenol content in the studied extracts was in the following order E. granulata T. minuta G. parviflora. Both flavonoids and tannins were higher in G. parviflora compared to the other two plant extracts. Moreover, ten different phenolic acids were quantified and identified in the studied plants. p-Hydroxybenzoic acid was the most

dominant of all phenolic acids in *T. minuta* extract while gallic acid was the major acid in *E. granulata* extract. Vanillic acid and 3,4-dihydroxybenzoic acid were found in a few amounts in the investigated plants. Most of the extracts showed high DPPH, ABTS and NO radical scavenging activity as compared with the control. All extracts displayed various cytotoxic activities towards the tested cell lines. *T. minuta* extract exhibited highest cytotoxicity against A2780 cell line and was the lowest against MCF7 cell line. In silico screening revealed that phenolic acids have a noticeable cytotoxic activity and caffeic acid was the most promising compound that possessed high affinity and ligand efficiency against the selected virtual targets. The cytotoxic activity of caffeic acid as estrogen receptor blockers or tubulin polymerization inhibitor required more in vitro and in vivo confirmatory studies. Further research is required to complete the pharmacological and toxicological profile of the investigated components in the studied plants.

^[20] Acknowledgments

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