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Antioxidant and Cytotoxic Activities of *Gmelina arborea* ROXB. Leaves

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HAS, LAR and MAG designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors MAM and AMS performed the statistical analysis. Author HMFM managed the analyses of the study. Author MAG managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The leaves of *Gmelina arborea* (ROXB.) (Family Verbenaceae) are widely used in the folklore to treat various types of diseases. In this study, the antioxidant and cytotoxic activities of different methanolic extracts and the derived subfractions of 90% methanolic extract of this plant were evaluated.
Methodology: The antioxidant activity was carried out via three different quantitative assays as well as qualitative one. Total phenolic was determined via Folin-Ciocalteu and total flavonoid via $AlCl_3$ assays. The cytotoxic activity was carried out via brine shrimp test and toward human cancer cell line; HepG2 using Sulphorhodamine-B assay. The 90% methanolic extract was fractionated using pet. ether then the 90% defatted methanol undergoes fractionation using ($CHCl_3$, EtOAc and n-BuOH).
Results: The antioxidant results showed that the; DPPH antioxidant activity was (19.20, 14.10 and 28.94 µg/ml); total antioxidant capacity was (412.69, 518.45 and 390.41; mg AAE /g extract); reducing power was (0.649, 0.715 and 0.396; 200 mg/ml) and total phenolic was (330.22, 400.66 and 244.76; mg GAE/g extract), respectively for 90% methanol, n-BuOH and EtOAc. The cytotoxic results showed that the; mortality of brine shrimp larvae (LC_{50}) against different dosages of defatted 90% methanol, n-BuOH and

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EtOAc respectively was (158.48, 39.81 and 199.52; µg/ml) and the results of HepG2 assay showed that n-BuOH fractions have cytotoxic activity with IC_{50} ≤ 20 µg/ml (IC_{50} = 17.3 µg/ml) which falls within the American Cancer Institute criteria followed by 90% methanol and EtOAc (IC_{50} = 22.1 µg/ml).
Conclusion: It was concluded that *Gmelina arborea* extracts possess a powerful antioxidant and cytotoxic activities.

Keywords: Gmelina arborea; antioxidant; anticancer; total phenolic content.

1. INTRODUCTION

Human bodies are exposed to exogenous oxidizing agents such as pollutants, different chemicals and smoking, also are exposed to endogenous ones produced via metabolic processes. Chemical substrates that act as oxidizing agents contain reactive oxygen species (ROS) like superoxide anion ($O2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), and peroxyl ($ROO^{\bullet}$) radicals as well as reactive nitrogen species (RNS) like peroxynitrite anion ($ONOO^{-}$) and nitric oxide ($NO^{\bullet}$) radical; overall harmful results occurred when such species attack human cell and tissues leading to cancer [1]. Cancer is the second leading cause of death in the world, so scientists over the entire world do their best to discover safe cancer therapy. Cancer is considered as a major public health problem either in the developed and developing countries over the world. It was estimated that 12.7 million recent cancer cases and about 7.6 million cancer deaths take place in the year 2008, which reflected the harmful effect of cancer on human by its various types [2]. In fact, most of the artificial agents currently being used in cancer therapy are toxic and produce damage to normal cells [3]. Therefore, chemoprevention or chemotherapy via nontoxic agents could be one solution for decreasing the harmful effects of cancer. Previous studies of tumor cells have led to an emphasis of the cytotoxic effect of dietary polyphenols; raising the possibility that; these compounds could contribute to the prevention and treatment of cancer [3]. Furthermore, many plants have been investigated in order to identify new and effective safe antioxidant and anticancer compounds, as well as to elucidate the mode of action of cancer inhibition [4]. Verbenaceae is a family of mainly tropical flowering plants; it includes some 35 genera and 1200 species. The phenolic compounds [5] and iridoids [6] were isolated from different members of the family. *Gmelina arborea* (ROXB.) family verbenaceae is locally known as 'Gambhari'. In English it is known as the 'Candahar tree' or 'White teak', is a fast growing deciduous tree occurring naturally throughout greater part of India [7]. Pervious phytochemical studies on gmelina genus showed the presence of several compounds such as phenolic compounds [8-9] and iridoids [10]. Pervious phytochemical studies on different parts of *Gmelina arborea* showed that; different classes of secondary metabolites were identified like flavonoids [9,11], phenolics [8] and iridoids [12-13]. It is reported to contain alkaloid, glycoside, lignann derivatives and sesquiterpenoid; furthermore, phytochemical screening analysis reveals the presence of carbohydrates, saponins, tannins, anthraquinones and cardiac glycosides [11]. The root, leaf and bark part of *Gmelina arborea* has possesses anti-inflammatory action [14], antimicrobial [15-16], antioxidant [16-19], anthelmintic [20], cytotoxic activity [21], anti-ulcer activity [22], diuretic effect [17,23], anti diabetic activity [24-25] and cardioprotective [18]. Furthermore, the aqueous extract of *Gmelina arborea* bark showed remarkable antihyperglycemic activity against STZ induced diabetes in rats [26]. I was reported that the hexane extract of *Gmelina arborea* leaves exhibited vasorelaxant properties [19]. Also, previous toxicological study revealed that the acute and repeated dose toxicity of the methanol extract of the *Gmelina arborea* stem bark was evaluated in tested mice and rats and the effects on body weight,

food and water consumption, organ weight, hematology, clinical chemistry as well as histology were studied. The results showed that the administration of methanol extract from the *Gmelina arborea* bark at 300–5000 mg/kg did not produce mortality or significant changes in the clinical signs and the no-observed adverse effect level was 5000 mg/kg. There were no significant differences in the general condition, growth, organ weights, hematological parameters, clinical chemistry values, or gross and microscopic appearance of the organs from the treatment groups as compared to the control group [27-28]. On the other side, the immunomodulatory effects of roots of *Gmelina arborea* were investigated and the methanolic extract and its ethyl acetate fraction were used for evaluating the pharmacological activity. The modulating effect was evaluated on humoral and cell-mediated immune response using animal models like cyclophosphamide-induced myelosuppression, delayed-type hypersensitivity (DTH) response, and humoral antibody (HA) titre. The results showed that both test extracts produced significant increase in HA titre, DTH response, and levels of total white blood cell count. It was concluded that such extracts were found to be a potential immunostimulant [29]. Accordingly, in this work, the antioxidant and cytotoxic activities of 90% MeOH extract of *G. arborea* as well as its derived fractions were done via different assays.

2. MATERIALS AND METHODS

2.1 Plant Material and Chemicals

The leaves of the plant under investigation were collected from Zoo Garden, Giza, Egypt in July 2011. The identity of the plant was established by Prof. Dr. Wafaa Amer, Professor of Plant Taxonomy, Faculty of Science, Cairo University, Giza, Egypt. Voucher specimen (given number GA) was kept in the Department of Medicinal Chemistry, Theodor Bilharz Research Institute (TBRI). The plant material was air-dried in shade place at room temperature and then powdered by electric mill, finally kept in tightly closed container in a dark place till the extraction process. All solvents and reagents used were of analytical grade. 1,1'-diphenyl-2-picrylhydrazyl (DPPH) free radical and Folin–Ciocalteu's reagent (FCR) were purchased from (Sigma-Aldrich Co.). Trichloroacetic acid (TCA), pot. ferricyanide, ferric chloride, aluminum trichloride, sodium carbonate, disodium phosphate, ammonium molybdate, rutin, ascorbic acid and gallic acid were purchased from (Merck Chemical Co.), all solvents and acids (methanol, petroleum ether, chloroform, ethyl acetate, n-butanol), were purchased from (Sigma-Aldrich Co.). The absorbance measurements for antioxidant activity were recorded using the UV-Vis spectrophotometer Spectronic 601 (Milton Roy, USA).

2.2 Extraction and Fractionation

Small scale extraction process was carried out via taking five samples from dry powder of fresh leaves of the plant (20 g), then extracted separately with different solvents (100 ml); 100% methanol and methanol-water mixtures (90, 85, 70 and 50%) in room temperature with shaking day by day followed by filtration and again extraction for four times. Then each extract was filtered using Whatmann filter paper No.1 and concentrated by using a rotatory evaporator (Buchi, Switzerland) at (28 ± 2ºC) affording known weight of each crude methanol extract. The crude extracts were collected and stored at room temperature in the dark for the further process. Large scale extraction was carried out via taking plant powder (200 g), soaking it in (2000 ml) of 90% methanol using the last mentioned extraction procedures. The 90% methanolic crude extract (30 g) was defatted by washing several times

with petroleum ether (60-80°C). The defatted crude methanol extract was ready for bioassays. Twenty gram of the defatted methanol extract was undergoes fractionation process by using different organic solvents; $CHCl_3$; EtOAc and n-BuOH (4 x 150 ml solvent).

2.3 Determination of Total Phenolic Content

The total phenolic content was determined using Folin- Ciocalteu's reagent according to the method described by Kumar et al., 2008. In this method, the reaction mixture was composed of (100 µl) of plant extract (100 µg/ml), 500 µl of the Folin-Ciocalteu's reagent and 1.5 ml of sodium carbonate (20%). The mixture was shaken and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 h, and then the absorbance was measured at 765 nm; gallic acid was used as standard. All determinations were carried out in triplicate. The total phenolic content was expressed as mg gallic acid equivalent (GAE) per g extract [30].

2.4 Determination of Total Flavonoid Content

The total flavonoid content was determined according to the reported procedures by Kumeran and Karunakaran, 2006; using rutin as a standard. Briefly, 100 µl of plant extract in methanol (100 µg/ml) was mixed with 100 µl of aluminium trichloride ($AlCl_3$) in methanol (20 mg/ml) and then diluted with methanol to 500 µl. The absorption at 415 nm was read after 40 min against the blank. The blank consists of all reagents and solvents without $AlCl_3$. All determinations were carried out in triplicate. The total flavonoid in plant extracts was determined as mg rutin equivalents (RE)/g extracts [31].

2.5 Antioxidant Activity

2.5.1 Rapid screening of antioxidant by dot-blot and DPPH staining

Each diluted sample of the G. arborea extract/fraction was carefully loaded onto a 20 cm × 20 cm TLC layer (silica gel 60 F254; Merck) and allowed to dry (3 min). Drops of each sample were loaded, in order of decreasing concentration (2, 1, 0.5, 0.25 and 0.125 mg/ml), along the row. The staining of the silica plate was based on the reported procedure [32]. The sheet bearing the dry spots was placed upside down for 10 s in a 0.4 mM DPPH solution. Then the excess of solution was removed with a tissue paper and the layer was dried with a hair-dryer blowing cold air. Stained silica layer revealed a purple background with white spots at the location where radical-scavenger capacity presented. The intensity of the white color depends upon the amount and nature of radical scavenger present in the sample [33].

2.5.2 DPPH radical scavenging activity

The scavenging activity of the stable 1,1'-diphenyl-2-picrylhydrazyl free radical was determined by the method described by Marwah et al., 2007. Briefly, the reaction medium contained 2 ml of 100 µM DPPH purple solution in methanol and 2 ml of plant extract, ascorbic acid was used as standard. The reaction mixture was incubated in the dark for 20 min and the absorbance was recorded at 517 nm. The assay was carried out in triplicate. The DPPH radical scavenging activity was calculated according to the equation:

$$\% \text{ DPPH radical scavenging activity} = 1 - [A_{sample}/A_{control}] \times 100$$

Where $A_{control}$ and A_{sample} are the absorbencies of control and sample after 20 min, respectively. The SC_{50} (concentration of sample required to scavenge 50% of DPPH radicals) values were determined. The decrease of absorbance of DPPH solution indicates an increase of the DPPH radical scavenging activity [34].

2.5.3 Determination of total antioxidant capacity

The antioxidant activity of the plant extract was determined according to phosphomolybdenum method, using ascorbic acid as standard. In this method, 0.5 ml of each extract (200 µg /ml) in methanol was combined in dried vials with 5 ml of reagent solution (0.6 M sulfuric acid, 28 mM disodium phosphate and 4 mM ammonium molybdate). The vials containing the reaction mixture were capped and incubated in a thermal block at 95 °C for 90 min. After the samples had cooled at room temperature, the absorbance was measured at 695 nm against a blank. The blank consisted of all reagents and solvents without the sample and it was incubated under the same conditions. All experiments were carried out in triplicate. The antioxidant activity of the extracts was expressed as the number of equivalents of ascorbic acid (AAE) [35].

2.5.4 Reducing power antioxidant assay

A Spectrophotometric method described by Ferreira *et al.*, 2007; was used for the measurement of reducing power. For this, 2.5 ml of each extract was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide (10 mg/ml). The mixture was incubated at 50 °C for 20 min, then rapidly cooled, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (2.5 ml) of the supernatant was diluted with distilled water (2.5 ml), and then ferric chloride (0.5 ml, 0.1%) was added and allowed to stand for 10 min. The absorbance was read spectrophotometrically at 700 nm, ascorbic acid was used as standard. Three replicates were made for each test sample [36].

2.5.5 Statistical Analysis

All data were presented as mean ± SD using SPSS 13.0 program. Correlation analysis of the antioxidant activity and free radical scavenging activity versus the total phenolic content of the different extracts of tested plant were carried out using the correlation and regression by Microsoft Excel program [37].

2.6 Cytotoxicity Activity

2.6.1 Brine shrimp lethality bioassay test

A solution of instant ocean sea salt (Aquarium System, Ohio) was made by dissolving 2.86 g in distilled water (75ml). 50 mg of *Artemia salina* Leach eggs (Artemia, Inc., California) was added in a hatching chamber. The hatching chamber was kept under an inflorescent bulb for 48 h for eggs to hatch into shrimp larvae. 20 mg of the tested extract was dissolved in 2 ml methanol or solvent in which it was soluble and from this, 500, 400, 300, 200, 100, 50, 5 µl of each solution was transferred into vials corresponding to 1000, 800, 600, 400, 200,100, and 10 µg/ml, respectively. Each dose was tested in triplicate. The vials and the control containing 500 µl of solvent were allowed to evaporate to dryness in about 48h at room temperature. 4.5 ml of instant ocean sea solution were added to each vial and 10 larvae of

Artemia salina (taken 8-72 h after the initiation of hatching) were added to each vial. The final volume of solution in each vial was adjusted to 5 ml with sea salt solution immediately after adding the shrimp. 24 h later the number of surviving shrimp at each dosage was counted and recorded. LC_{50} values were determined with 95% confidence intervals by analyzing the data. The data were analyzed and LC_{50} values were calculated and carried according to Reed-Muench method. Potassium dichromate was used as standard [38-39].

2.6.2 Statistical Analysis

The Reed-Muench method assumes that an animal that survived a given dose would also have survived any lower dose, and conversely, that an animal that died with a certain dose would have also died at any other higher dose. Thus, the information from any one group can be added to that of the other groups in the range of dose tested [38-39].

2.6.3 Liver carcinoma cell line (HepG2)

Potential cytotoxicity of the extracts, fractions and certain isolated pure compounds of the two plants were tested using method of Skehan *et al.,* 1996, using cell line HEPG2. Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the compounds or extract to allow attachment of cell to the wall of the plate. Different concentrations of the compounds or extract under test (0, 1, 2.5, 5, and 10 µg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37 °C and atmosphere of 5 % CO_2. After 48 h, cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is platted to get the survival curve of each tumor cell line after the specified compound [40].

3. RESULTS AND DISCUSSION

3.1 Total Flavonoid Content

The total flavonoid content of the different methanolic extracts of *G. arborea* plant using aluminum chloride colorimetric assay was showed in (Table 1), which ranged from 84.72 to 5.55 (mg RE / g ext.), the values showed decreasing in the order; 90% (84.72) > 100% (61.99) > 85% (40.27) > 70% (17.83 > 50% (5.55) MeOH (mg RE / g ext.) [24]. All derived fractions from the 90% MeOH extract contained a considerable amount of flavonoid content ranged from 95.36 to 6.78 mg RE/g extract. The n-BuOH fraction has possesses the highest total flavonoid content (95.36) (mg RE / g ext.), while pet .ether fraction comprised of the lowest total flavonoid content (6.78) (mg RE / g ext.) (Table 2) [9].

3.2 Total Phenolic Content

This assay based on the reduction of hexavalent Mo (VI) to pentavalent Mo (V) via the donation with single electron by the antioxidant substance. Further more, under alkaline conditions, Folin-Ciocalteu's (FC) phenol reagent (yellow colour) reacts with phenolic compounds leading to the formation of a phenolate anion via dissociation of a phenolic hydrogen atom. The overall reaction involving the reversible one- or two-electron reduction

leading to the formation of blue-coloured chromophores between phenolate and the FC reagent [41]. The total phenolic content of the different methanolic extracts of *G. arborea* ranged from 190.41 to 38.77 mg gallic acid equivalents/g extract (Table 1). It has appeared that the 90% MeOH extract of has the highest content of phenolic compounds (190.41) and 50% MeOH extract has the lowest one (38.77) (mg of GAE/g). As shown in (Table 2), the total phenolic content in BuOH fraction derived from 90% MeOH showed the highest amount (400.66), followed by defatted 90% methanolic extract (330.22), EtOAc fraction (244.76), $CHCl_3$ fraction (25.45) and pet. ether fraction (16.43) (mg of GAE/g), which may be responsible for antioxidant activity [42]. Plant phenolics constitute one of the major groups of compounds acting as primary antioxidant or free radical terminators. Phenolic compounds such as flavonoids, phenolic acids and tannins are considered to be the major contributor to the antioxidant activity of medicinal plants, so as the phenolic content increased as much as possible in the tested samples (high phenolic content), the number of free hydroxyl groups will increased which are mainly responsible for scavenging of reactive species (ROS or RNS) [42-44]. Many studies have revealed that the phenolic contents in plants are related to their antioxidant activities, and the antioxidant activities of phenolic compounds are probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [45]. Also, phenolic compounds were found to have excellent antioxidant properties against some free radical species and were found to have excellent antioxidant activity in the inhibition of LDL (low-density lipoproteins) oxidation [41]. The bioactivity of these compounds may be related to their ability to chelate metals and inhibit lipoxygenase [45]. In conclusion, high ability of phenolics to neutralize radicals results from their chemical structure; the higher the number of hydroxyl groups (especially those bonded to the B-ring) and double bonds in the molecule, the greater the ability to scavenge radicals. Phenolic compounds contribute significantly to antioxidant properties of plant extracts, which is often demonstrated by high correlation between the level of phenolics in the tissue and antiradical activity of the extract [46].

3.3 Antioxidant Activity

Polyphenols as source of antioxidants play vital role to the prevention of cardiovascular diseases, cancers, osteoporosis, neurodegenerative diseases and diabetes mellitus [47]. Antioxidants benefits in the diet are very promising as cancer inhibitors because of their low toxicity, safety and general acceptance [48-49]. Cyanidin as an example of naturally occurring antioxidant is anthocyanin pigment found in many berries (grapes, blackberry, blueberry, cherry, cranberry, raspberry etc.), apples, plums and red cabbage, exerts several biological activities like; antioxidant, anti-inflammatory and anticancer [50]. Also, flavan-3-ols from tea, cocoa, chocolate, fruits, vegetables and wine, are highly potent antioxidant compounds able to educe incidence of stroke, heart failure and diabetes and cancer [48]. Furthermore, antioxidants are used in food products to delay or inhibit the oxidation process maximizing product shelf life and quality. Antioxidants are primarily added to foods in combination with synergists like ascorbic, tartaric and phosphoric acids to increase efficiency [51]. Previous studies on the evaluation of the antioxidant activity reported that there are a large number of *in vitro* methods have been developed to evaluate the activity of natural antioxidants either in the form of pure compounds or as extracts. Scientists have been classified the in *vitro* methods into two major groups; hydrogen atom transfer reactions like; oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant potential (TRAP) and β carotene bleaching and electron transfer reactions like trolox equivalent antioxidant capacity (TEAC), Ferric reducing antioxidant power (FRAP), 1, 1'-diphenyl-2-picryl-hydrazyl assay (DPPH), superoxide anion assay, hydroxyl assay and nitric oxide

assay [41]. These methods are popular due to their high speed, accuracy and sensitivity. However, we need to use more than one method to evaluate antioxidant capacity of plant materials because of the complex nature of phytochemicals [52]. Therefore, in the present study three different assays were employed in order to determine and compare the antioxidant properties of *G. arborea* extracts.

3.3.1 Rapid screening of antioxidant by dot-blot and DPPH staining

The antioxidant potential activity of the defatted 90% methanol extract of *G. arborea* as well as its derived fractions was determined via eye-detected semi-quantitatively via a rapid DPPH staining-TLC technique. Each diluted sample was applied as a TLC layer that was stained with DPPH solution. This method was depend up on the inhibition of the accumulated of oxidized products and the generation of free radicals was inhibited via the addition of antioxidant and masking of the free radicals [32, 53]. Initial faint spots appeared and weak spots could be observed in sample row, and the appearance of white spots has potential value for the evaluation of the different tested fractions [54]. These white spots with strong intensity appeared quickly at the concentration of 0.50 mg/ml of each extract [32], ascorbic acid was used as a positive control. Results in (Fig. 1) revealed that, all the tested extracts showed promising activity but the n-BuOH fraction showed the potent activity followed by the EtOAc. These results revealed that all the tested extracts react positively with DPPH and these reactions based on the ability of these extracts/fractions as free radical scavenging compounds. The wider diameter as well as high color intensity of the resulting dots (spots) indicates the high radical masking activity of the tested fractions [33,53].

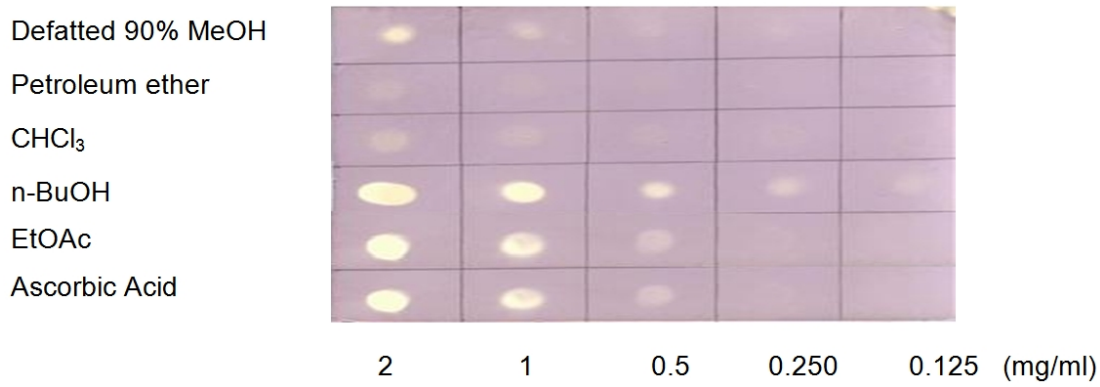

Fig. 1. Dot-blot qualitative antioxidant assay of different fractions of *G. arborea* on silica sheet stained with DPPH solution in methanol.

3.3.2 1,1'-diphenyl-2-picraylhydrazyl free radical scavenging assay (DPPH)

$DPPH^{\bullet}$ (1,1'-diphenyl-1-picrylhydrazyl radical) is a stable radical and is often used in assessment of the antioxidant activity. The free radical DPPH possesses a characteristic absorption at 517 nm (purple in color), which decreases significantly when exposed to radical-scavengers (due to hydrogen atoms transfer from antioxidant to DPPH). A lower absorbance at 517 nm indicates a higher radical scavenging activity of tested sample [41]. In this assay, the ability of the investigated *G. arborea* fractions to act as donors of hydrogen atoms or electrons in transformation of DPPH radical into its yellow reduced form DPPH-H was investigated [55]. The DPPH radical scavenging activity of the different methanolic

extracts was evaluated, the activity of different extracts (expressed by SC_{50} value) was decreased in the order; 90% MeOH (20.45) > 100% (30.45) > 85% (36.21) > 70% (49.66) > 50% (105.52) (µg/ml) (Table 1). DPPH radical scavenging activity of the defatted 90% methanolic extract and their derived fractions (pet. ether, $CHCl_3$, EtOAc and n-BuOH) was also investigated. As shown in (Table 2), the highest activity was observed in the BuOH fraction, followed by defatted 90% methanolic extract, while EtOAc fraction also showed good inhibitory effects. The DPPH radical inhibition of derived fractions decreased in the following order according to SC_{50} [µg/ml]; BuOH (14.10) > defatted 90% MeOH (19.20) > EtOAc (28.94) > $CHCl_3$ (138.57) > pet. ether (160.89). There are highly positive correlation between the antioxidant scavenging activity of the tested fractions and their total phenolic content, which obviously appeared from the high value of the correlation coefficient (R^2 =0.93) (Fig. 2), this strong evident for the highly polyphenols constituents exist in these extracts [8,56]. Phenolic compounds are known to be powerful chain breaking and free radicals terminators antioxidants due to their characteristic nature as a vital phytoconstituents of medicinal plants. Accordingly, phenolic compounds may contribute directly to the potential antioxidant activity. The results of our study showed that most of the tested fractions exhibits significant free radical scavenging actions which exist in direct positive correlation with phenolic content, these finding was supported by the previous study of Patil et al., 2009 [42].The variation on the antioxidant activity return to the fact that; several methods have been developed to quantify the antioxidant compounds individually. It would be desirable to establish and standardize methods that can measure the total antioxidant capacity level directly from medicinal plant extracts containing phenolics. The techniques are different in terms of mechanism of reaction, effectiveness, sensitivity, reagents, substrates, experimental condition, reaction medium and standard analytical evaluation methods [57]. It is known that, the activity of antioxidant extracts or compounds are strongly affected by many factors such as antioxidant concentration, temperature, pH and storage [58]. For example, temperature is one of the most important factors affecting antioxidant activity. Generally, heating causes an acceleration of the initiation reactions. It was reported that the antioxidant activity of phenolic acid compounds decreased with increasing temperature [59]. Also, it can be said that the polarity of the solvent had significant impact on the extraction of phytochemicals such as antioxidants [60]. Another study showed that the antioxidant activity of the tested plant samples based on the plant part (tissue) but it is not seasonal. In previous study carried out by Fernandes *et al.*, 2007, the antioxidant potential of different parts of turnip plants, involving (leaf, flower, stem and root) was evaluated. Flowers were found to be the most active part followed by leaf, stem and root parts [61]. Also, solvent extraction process is commonly used for the isolation of antioxidants from medicinal plant material. Antioxidant activities of the developed extracts are strongly dependent on the type of solvent [62].

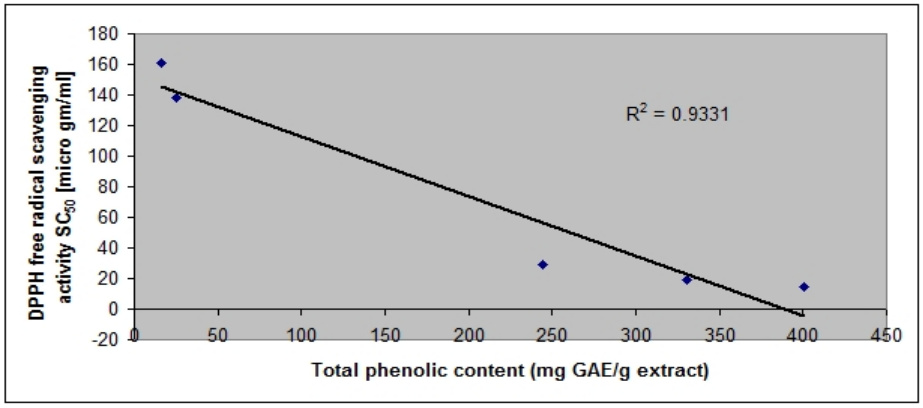

Fig. 2. Correlation between DPPH free radical scavenging activity and total phenolic content of the defatted 90% methanolic extract of *G. arborea* as well as its derived extracts.

3.3.3 Phosphomolybdenum antioxidant assay

The basic principle to assess the antioxidant capacity through phosphomolybdenum assay includes the reduction of Mo (VI) to Mo (V) by the sample analyte followed by formation of green phosphate/Mo (V) complex with a maximum absorption at 695 nm [35]. In the present study, the total antioxidant capacity values of the different methanolic extracts of *G. arborea* were evaluated which decreased in the order; 90% MeOH (425.13) > 100% MeOH (358.90) > 85% MeOH (330.04) > 70% MeOH (300.54) > 50% MeOH (108.30) (mg AAE /g ext.) (Table 1). Also, the total antioxidant capacity values of the derived fractions decreased in the order; n-BuOH (518.45) > defatted 90% MeOH (412.69) > EtOAc (390.41) > $CHCl_3$ (89.20) > pet. ether (79.20) (mg AAE /g ext.) (Table 2). The antioxidant capacity of different fractions observed in this study may be due to the presence of high phenolic compounds in such fractions [8, 63]. The high total polyphenol content increases the antioxidant activity and proves a linear positive correlation between phenolic content and total antioxidant activity of the tested fractions (R^2 = 0.98) (Fig. 3). The presence of these bioactive compounds might contribute to diverse scavenging effects of *G. arborea* [56,64]

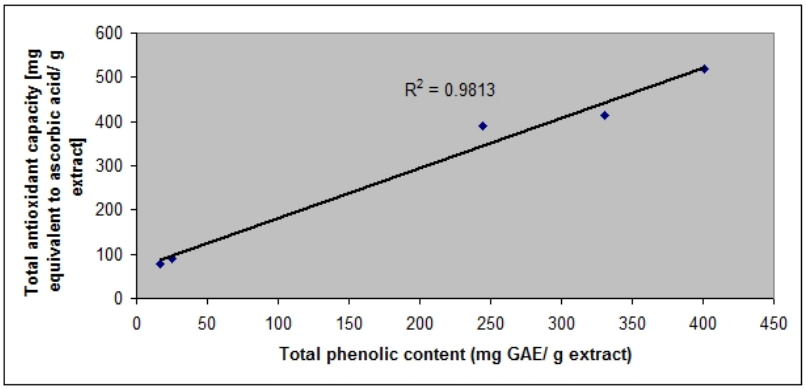

Fig. 3. Correlation between the total antioxidant capacity and total phenolic content of the defatted 90% methanolic extract of *G. arborea* as well as its derived extracts.

Table 1. Total extractable content, total phenolic content, total flavonoid content, free radical scavenging potential and total antioxidant capacity of the different methanolic extracts of *G. arborea*.

Sample	TEC (%)[a]	TPC[b]	TFC[c]	DPPH (SC_{50})[d]	Total antioxidant capacity[e]
MeOH 100%	14.56	166.45 ± 2.15	61.99 ± 4.16	30.45 ± 2.77	358.90 ± 2.49
MeOH 90 %	19.02	190.41 ± 4.03	84.72 ± 2.40	20.45 ± 2.34	425.13 ± 3.78
MeOH 85 %	20	152.29 ± 2.34	40.27 ± 2.01	36.21 ± 1.59	330.04 ± 1.61
MeOH 70 %	22.42	100.03 ± 1.89	17.83 ± 2.61	49.66 ± 3.19	300.54 ± 2.11
MeOH 50 %	7.5	38.77 ± 3.20	5.55 ± 3.09	105.52 ± 2.97	108.30 ± 3.75
Ascorbic acid	…….	…….	……	8.25 ± 0 .95	……

Results are expressed as mean values ± standard deviation (n = 3).

[a] TEC (total extractable content).

[b] TPC (total phenolic content) values are expressed as mg gallic acid equivalent/g extract (mg GAE/g ext.).

[c] TFC (total flavonoid content) values are expressed as mg rutin/g extract (mg RE/ g ext.).

[d] DPPH values are expressed as µg dry extract/ml (µg/ml).

[e] Total antioxidant capacity values are expressed as mg ascorbic acid equivalent/g extract (mg AAE/g ext.).

Table 2. Total extractable content, total phenolic content, total flavonoid content, free radical scavenging activity and total antioxidant capacity of the defatted 90% methanol extract of *G. arborea* as well as its derived fractions.

Sample	TEC (%)[a]	TPC[b]	TFC[c]	DPPH (SC_{50})[d]	Total antioxidant capacity[e]
Defatted 90% MeOH	13.46	330.22± 0.63	74.12 ± 1.59	19.20± 1.74	412.69 ± 2.34
Petroleum ether	1.53	16.43± 3.50	6.78 ± 3.69	160.89± 2.41	79.20 ± 0.50
$CHCl_3$	0.53	25.45± 0.33	12.67 ± 1.09	138.57± 1.32	89.20 ± 1.42
EtOAc	0.36	244.76± 1.78	40.89 ± 4.21	28.94 ± 0.73	390.41 ± 0.94
n-BuOH	4.30	400.66± 4.60	95.36 ± 0.52	14.10 ± 1.68	518.45 ± 1.35

3.3.4 Reducing power antioxidant assay

The reducing power of the tested compounds can be used as a significant indicator of its potential antioxidant activity. In this assay, the yellow color of the test solution changes to green depending on the reducing power of tested substances. The presence of reductants in the reaction medium leads to the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by the measurement of the absorbance (OD value) at 700 nm [65]. Compounds with reducing power acting as electron donors and can reduce the oxidized intermediates of lipid peroxidation, accordingly they act as primary and secondary antioxidants [66]. As shown in (Fig. 4), the reducing power of the different methanolic extracts *G. arborea* decreased in the order (OD value); 90% MeOH (0.625) > 100% MeOH (0.575) > 85% MeOH (0.535) > 70% MeOH (0.445) > 50% MeOH (0.295) at 200 µg/ml. Also, the reducing power of the defatted 90% methanolic extract as well as its derived fractions at 200 µg/ml is in the order; BuOH fraction (OD value = 0.715) > defatted 90% MeOH extract (OD value = 0.649) > EtOAc fraction (OD value = 0.386) > $CHCl_3$ fraction (OD value = 0.257) > pet. ether fraction (OD value = 0.215) and ascorbic acid used as a positive control with (OD value = 0.915) (Fig. 5). The antioxidant principles present in the

fractions of *G. arborea* caused the reduction of Fe^{3+}/ ferricyanide complex to the ferrous form, and thus proved the reducing power ability. The antioxidant activity of *G. arborea* extracts is well known [16, 42] and is to be expected due to a high content of phenolic compounds [67]. Reducing power is associated with antioxidant activity and serves as a significant reflection of the antioxidant activity and measures the electron-donating capacity of an antioxidant samples [65]. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, via converting them to more stable products and consequently, terminate radical chain reactions, so that they can act as primary and secondary antioxidants [66]. The reducing properties are generally associated with the presence of reductones, which have been shown to exhibit antioxidant action by breaking the chain reactions and donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation [68]. Being good electron donors, phenolic compounds show the reducing power and have ability to convert the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by donating an electron [69]. These results are in full agreement with the previous studies which reported that the reducing power of plant extracts are correlated with the phenolic content [53]. The antioxidant activities of polyphenols were attributed to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, as well as their metal chelating abilities [70-71]. Higher absorbance indicates a higher reducing power and higher antioxidant activity. All the investigated extracts have reductive capabilities (reducing power) and potential antioxidant activity (absorbance) increases in concentration-dependent manner. Hence, the plant extracts were found to contain high amounts of reductones, which could react with radicals to stabilize and terminate radical chain reactions [53]. The reducing power of the tested fractions was very strong and the tested plant extracts able to reduce the most Fe^{3+} ions, which had a lesser reductive activity than the standard of ascorbic acid. Furthermore, absorbance increasing (OD values) indicated the increasing in reducing power of the samples. On the basis of the results obtained in this study, it is concluded that the 90% methanolic extract of *G. arborea* as well as its subfractions contains large extent of phenolic compounds and exhibits high reducing power capabilities, also these finding was supported by the previous study of Patil et al., 2009 [42].

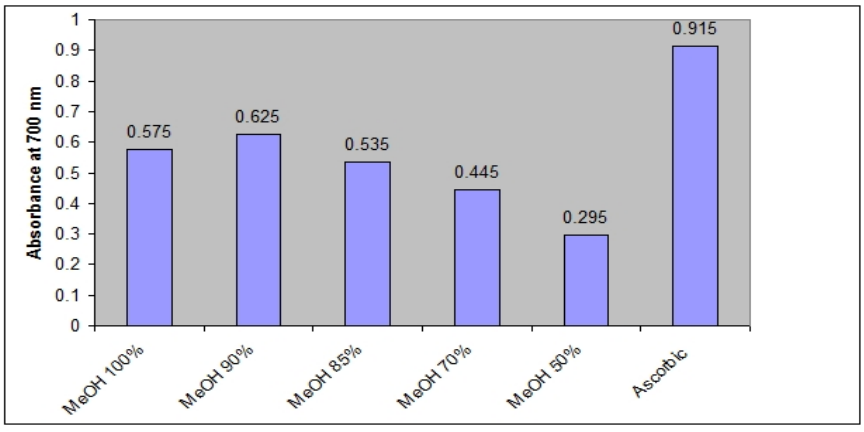

Fig. 4. Reducing power activity of the different methanolic extracts of *G. arborea* at concentration 200 µg/ml.

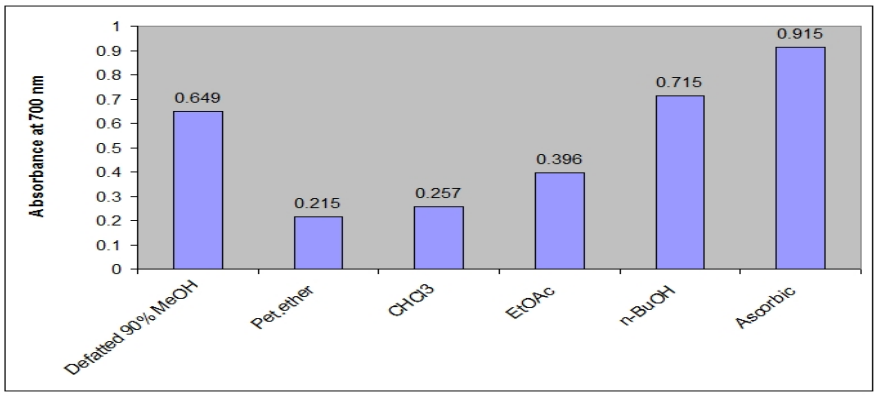

Fig. 5. Reducing power activity of the defatted 90% methanolic extract of *G. arborea* as well as its derived fractions at concentration 200 µg/ml.

3.4 Cytotoxic Activity

Actually, *in vitro* cytotoxicity assays are widely used to chemicals including cancer chemotherapeutics, pharmaceuticals, biomaterials, natural toxins, antimicrobial agents and industrial chemicals because they are rapid and economical. These cytotoxicity tests measure the concentration of the substance that damages components, structures or cellular biochemical pathways, and they also allow direct extrapolation of quantitative data to similar *in vitro* situations [72].

3.4.1 Brine shrimp test

Brine shrimp (*Artemia salina* L.) bioassay considered as a preliminary screening for the presence of antitumor compounds and used to determine the toxicity of plant extracts [73]. Using brine shrimp larvae, pharmacognosists and natural product chemists were able to detect and isolate plant constituents as well as active compounds with a variety of pharmaceutical activities [74]. Brine shrimp considered rapid, inexpensive, in-house bioassay for screening and fractionation monitoring of physiologically active plant extracts [75]. According to Meyer *et al*. (1982) several extracts derived from natural products which have $LC_{50} \leq 1000$ µg/ml using brine shrimp bioassay were known to contain physiological active principles [73]. The 90% methanolic extract of *G. arborea* plant as well as its derived fractions were tested for their toxicity using brine shrimp test. Criterion of brine shrimp toxicity for fraction, compound or plant extract was established according to as LC_{50} values above 1000 µg/ml are non toxic, between 500 & 1000 µg/ml are weak toxic, and that below 500 µg/ml are toxic [76]. The n-BuOH extract of showed the most potent toxic effect at LC_{50} = 39.81 µg/ml followed by defatted 90% MeOH at LC_{50} = 125.89 µg/ml, 90% MeOH and ethyl acetate extracts showed a remarkable cytotoxic effect at LC_{50} = 158.48 and 199.52 µg/ml respectively (Table 3, Fig. 6). All tested extracts showed promising cytotoxic activity according to criteria of NCI [76]. The results could serve for further pharmacological and phytochemical research, which clearly indicate to the toxic effects of extracts prepared from leaves of *G. arborea*. The presence of phenolic acids and flavonoids may be responsible for the observed brine shrimp lethality activity [56].

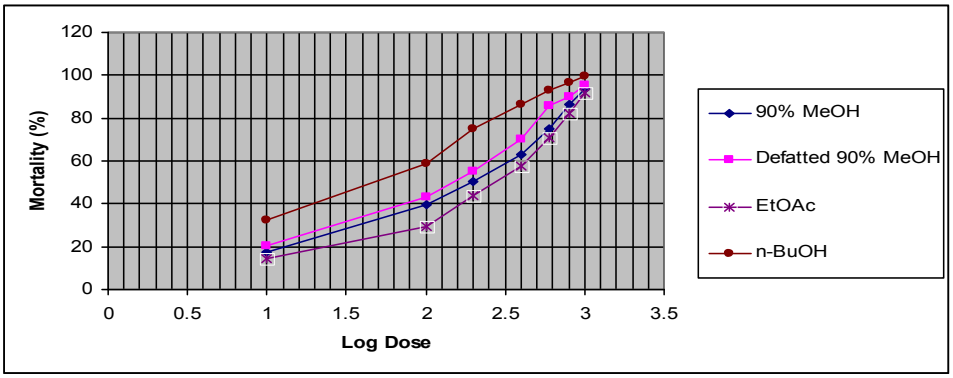

Fig. 6. Estimation of LC_{50} by plot of percent mortality of brine shrimp larvae against different dosage of different extracts of *G. arborea*.

Table 3. Cytotoxic activity of 90% methanol, defatted 90% methanol, EtOAc and n-BuOH extracts of *G. arborea*.

Extract/fraction	(LC_{50} ± SE)[a]	(CL)[b]
90% methanol	158.48 ± 10.30	(179.08 – 137.88)
Defatted 90% methanol	125.89± 7.96	(141.81 – 109.97)
EtOAc	199.52 ± 11.70	(222.93 – 176.11)
n-BuOH	39.81 ± 6.35	(52.51 – 27.11)

[a] *Results are expressed as mean values ± standard error (n = 3).*
[b] *95 % confidence limits (CL= LC_{50} ± 2 SE LC_{50}) in parentheses.*

3.4.2 Liver carcinoma cell line (HepG2)

Cancer or malignant disease is one of the major causes of death in humans. It was reported that malignant neoplasm is the third (12.4%) leading cause of death worldwide, the first (30%) being cardiovascular disease and the second (18.8%) being infectious diseases which include HIV and AIDS [77]. It is well known that cancer is second only to cardiovascular disease as a natural cause of death, with an incidence of over 6 million cases reported annually across the globe [78]. Previous report by WHO stated that during the year 2003, of the 56 million fatalities that occurred worldwide during the year 2000, cancer was responsible for 12% of these, with 5.3 million males and 4.7 million females developing neoplasms, which claimed the lives of 6.2 million of these individuals [79]. Thus, it is urgent to find more and safer new active constituents that attack and kill cancer cells. (Table 4 and Fig. 7) showed the cytotoxic effects of the 90% methanolic extract, ethyl acetate and n-butanol fractions of the leaves part of *G. arborea* against HepG2 cell line using the sulforhodamine B (SRB) method respectively [40]. The SRB method, which was developed in 1990, remains one of the most widely used methods for *in vitro* cytotoxicity screening. It has been widely used for drug-toxicity testing against different types of cancerous and non-cancerous cell lines [80]. The n-BuOH fraction showed high cytotoxic activity toward the HepG2 cell line with IC_{50} = 17.3 µg/ml, followed by the 90% defatted methanol with IC_{50} = 22.1 µg/ml and ethyl acetate fraction with IC_{50} =22.1 µg/ml (Fig. 7). According to the National Cancer Institute (NCI), the criteria and the conditions of cytotoxic activity for the crude extract is an IC_{50} values ≤ 20 µg/ml, is considered to be potentially cytotoxic [81-82]. The n-BuOH fraction is considered active against liver tumor and the remaining tested fractions

showed IC_{50} exist under the NCI criteria, thus these fractions are considered as promising cytotoxic agent.

Table 4. Potential cytotoxicity of 90% defatted methanol extract as well as its ethyl acetate and n-BuOH sub fractions of *G. arborea* against liver tumor cell line.

Conc. µg/ml	SF (HEPG2)[a]		
	Defatted 90% MeOH	EtOAc	n-BuOH
0.000	1.000	1.000	1.000
5.000	0.787	0.557	0.643
12.500	0.718	0.516	0.483
25.000	0.432	0.517	0.449
50.000	0.333	0.524	0.428
IC^{b}_{50}	22.1 µg/ml	22.1 µg/ml	17.3 µg/ml
Reference standard	Doxorubicin IC_{50} = 4 µg/ml		

[a] SF = Surviving fraction; [b] IC_{50}= Dose of the extract which reduces survival to 50%.

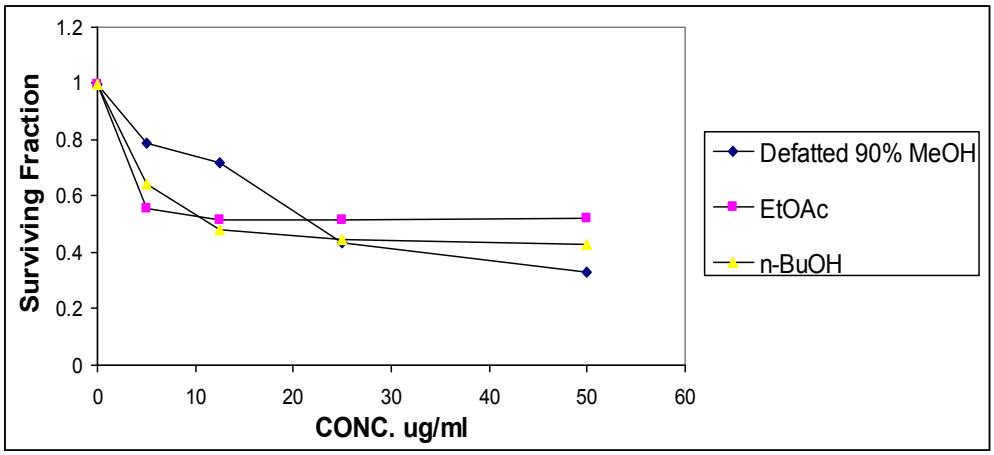

Fig. 7. Effect of 90% defatted methanol, EtOAc and n-BuOH extracts of *G. arborea* against liver tumor cell line.

4. CONCLUSION

The present study demonstrates that the plant *G. arborea* has antioxidant and cytotoxic activities and these activities are due to presence of certain bioactive secondary metabolites compounds in each extract, also these activities seem to depend on the content of phenolic constituents in each extract. On the basis of the results obtained in the present study, it is concluded that most tested extracts of *G. arborea* leaves, exhibit high free radical scavenging activities; it also chelates iron and has reducing power as well as cytotoxic activity. These *in vitro* assays indicate that the plant extracts are a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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