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ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF *TECTONA GRANDIS* LINN. LEAVES

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ABSTRACT

Cancer prevention and treatment have attracted increasing interest. Leaves of *Tectona grandis* Linn. (Verbenaceae) are widely used in the folklore to treat various types of diseases. This study characterizes the antioxidant activity, total phenolic content (TPC) and cytotoxic activity of different methanolic extracts as well as the derived subfractions (Pet. ether; $CHCl_3$, EtOAc and n-BuOH) from the 90% methanolic extract of *T. grandis*. The antioxidant activity was evaluated via three assays; 1,1'-diphenyl-2-picraylhydrazyl free radical (DPPH), phosphomolybdenum method (total antioxidant capacity; TAC and reducing power antioxidant assay (RPAA). Due to the high antioxidant activity of the tested fractions, their cytotoxic activity was evaluated via using preliminary brine shrimp lethality test and toward liver cancer cell line; HepG2 using Sulphorhodamine-B assay. The DPPH activity was ranged from (15.78, 10.63 and 19.58 µg/ml); TAC was ranged from (477.58, 589.50 and 400.30; mg AAE /g extract); RPAA (OD value) was ranged from (0.699, 0.783 and 0.423; 200 mg/ml) and TPC was ranged from (208.32, 400.56 and 295.67; mg GAE/g extract) all respectively for defatted 90% methanol, n-BuOH and EtOAc fractions. A positive linear relationship existed between antioxidant activity and TPC (all R^2 values > 0.91). The mortality of brine shrimp larvae (LC_{50}) against different dosage of defatted 90% methanol, n-BuOH and EtOAc respectively was (100, 15.84 and 125.89). The HepG2 results showed that defatted 90% MeOH and n-BuOH fractions have cytotoxic activity with $IC_{50} \leq 20$µg/ml which falls within the American Cancer Institute criteria. It was concluded that *T. grandis* extracts possess a powerful antioxidant and cytotoxic activities.

Key words: *Tectona grandis*, Antioxidant activity, Cytotoxic activity, Total phenolic content.

INTRODUCTION

Free radicals are highly reactive unstable chemical compounds, containing an unpaired electron; the presence of unpaired electrons usually confers a considerable degree of reactivity cause damage to other molecules by extracting electrons from them in order to reach a stable form (Lee *et al.*, 2003; Valko *et al.*, 2007). These reactive species can be classified into; reactive oxygen species (ROS) namely (oxygen-centered radicals) such as superoxide anion ($O2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals or reactive nitrogen species (RNS which include agents like peroxynitrite anion ($ONOO^-$) and nitric oxide ($NO^{\bullet}$) radical, among other; in addition, there are non-free radical species (oxygen-centered nonradical derivatives) such as hydrogen peroxide (H_2O_2), singlet oxygen $^1O_2$ (O-O:), nitric oxide (NO) and hypochlorous acid (HClO) (Golden *et al.*, 2002). The continuous production of such species in human body due to exposure to exogenous oxidizing agents from environmental pollutants (cigarette smoke, pesticides and UV radiation), life style and to endogenous ones produced by metabolism, lead to harmful destructive changes in structures of all human cells and tissues including; cellular membranes, nucleotides in DNA and critical sulfhydryl bonds in proteins, these all aspects are consider as an introduction to the up normal cell growth and finally leading to cancer (Machlin and Bendich,

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1987). Cancer is the second leading cause of death in the worldwide, and is considered as one of the most fearsome causes of morbidity and mortality in all over the world. Cancer is only preceded by cardiovascular, infectious and parasitic disease. Although, the disease is often been regarded principally as a problem of the developed world, more than half of all cancers occur in the developing countries (Mathers *et al*., 2001). Antioxidants are substances that may protect the human cells and tissues from the damage occurred via the destructive action of chemically unstable molecules known as reactive species either oxygen or nitrogen. The protective mechanism of the antioxidant compounds based up on the neutralization of these reactive species via pairing the highly energetic reactive odd electrons which responsible for the oxidation process, so the antioxidant molecule is capable of slowing or preventing the oxidation of other molecules (Sies, 1997). A great number of natural medicinal herbs and plants have been tested as naturally occurring antioxidant agents and results have shown that the raw extracts or isolated pure compounds from such plants were more effective antioxidants *in vitro* than commonly known synthetic one like BHT or vitamin E (Gordon and Weng, 1992; Gu and Weng, 2001; Pyo *et al*., 2004). A number of natural product extracts have been studied for anticancer activity leading to the development of numerous clinically promising anticancer compounds (Da Rocha *et al*., 2001). Hence, natural products are now considered of exceptional value in the development of effective anticancer drugs with minimum host cell toxicity. The present study investigates *Tectona grandis* (Verbenaceae). Verbenaceae is a family of mainly tropical flowering plants, that contains trees, shrubs and herbs notable for heads, spikes or clusters of small flowers, many of which have an aromatic smell. Verbenaceae family includes some 35 genera and 1200 species. The essential oils, phenolic compounds, iridoids and triterpenes were isolated from different members of the family (Zhang and Cheng, 2000; Siddiqui *et al*., 2007; Lui *et al*., 2009; Meshkatalsadat *et al*., 2010). Teak is the common name for the tropical hardwood tree species *T. grandis*; *T. grandis* is native to South and Southeast Asia, mainly India, Indonesia, Malaysia and Myanmar. The plant used in treatment of urinary discharge, bronchitis, laxative, sedative, diuretic, and in scabies (Nayeem and Karverkar, 2010). Literature survey has revealed that *Tectona genus* contain phenolic compounds and sterols (Nayeem and Karvekkar, 2010; Macias *et al*., 2010; Singh *et al*., 2010). It also revealed presence of monoterpenes and apocarotenoids (Macias *et al*., 2008).

MATERIALS AND METHODS

Plant material

The leaves of the plant under investigation were collected from Zoo Garden, Giza, Egypt in June 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 TG) 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 and kept in tightly closed container in dark places until subjected to the extraction process.

Materials and chemicals

All solvents and reagents used were of analytical grade. 1,1'-diphenyl-2-picraylhydrazyl (DPPH) free radical and Folin–Ciocalteu's reagent (FCR) was purchased from (Sigma-Aldrich Co.). Trichloroacetic acid (TCA), Pot. ferricyanide, ferric chloride, aluminum chloride, sodium carbonate, sodium phosphate, ammonium molybdate, rutin, ascorbic acid and gallic acid were purchased from (Merck Chemical Co.), all solvents and acids [petroleum ether, chloroform, ethyl acetate, n-butanol, acetic acid, and sulphuric acid), dimethylsulphoxide (DMSO)] were purchased from (Sigma-Aldrich Co.). Paper chromatography (PC) was done on Whatmann No. 1 (57 x 46 cm) while thin layer chromatography (TLC) was performed over pre-coated silica plates (GF254, Merck). The absorbance measurements for antioxidant activity were recorded using the UV-Vis spectrophotometer Spectronic 601 (Milton Roy, USA).

Extraction and fractionation

Small scale extraction process was carried out via taking five samples from the dry powder of the 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 ($28 \pm 2^{\circ}C$) 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 the plant powder (200 g), was soaked in (2000 ml) of 90% methanol for one week at room temperature with shaking day by day followed by filtration and again extraction for four times. The organic solvent was removed in vacuo using rotatory evaporator. The 90% methanolic crude extract (34.5 g) was defatted by washing several times with petroleum ether (60-80°C). The defatted crude methanol extracts were ready for bioassay. Twenty grams 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). Each fraction was filtered and then concentrated by removing the solvent by using rotary evaporator. The yield of each fraction was determined and kept in dark for further analysis.

Phytochemical and biological studies

Different methanolic extracts (100%, 90%, 85%, 70% and 50%), and the successive fractions obtained from 90% methanolic extract of the plant under investigation were *in vitro* tested for their phytochemical analysis, total phenolic content, total flavonoid content, antioxidant and cytotoxic activities according the following procedures.

PRELIMINARY PHYTOCHEMICAL SCREENING

Thin layer and paper chromatography

For active fractions phytochemical screening was performed for testing the presence of secondary metabolites by TLC analyses (Pre-coated aluminum silica gel plates, GF254, Merck) using different eluting systems which cover non-, medium- and high polar components. The solvent systems are ($CHCl_3$: EtOAc: MeOH; 2:2:1), ($CHCl_3$: MeOH, 9:1 and 8.5:1.5), ($CHCl_3$: MeOH: H_2O; 7:3:0.5), (n-BuOH: MeOH: H_2O, 4:1:0.5), (n-hexane: Diethyl ether, 32:1) (AcOH: H_2O: HCl, 30:10:3). The following spraying reagents were used in order to develop the spots: 1% ferric chloride (tannins), 2% aluminium chloride in ethanol (flavonoids), 40% sulphuric acid/methanol (saponins), and Dragendorff reagent (alkaloids). Also, one dimensional paper chromatography was performed on Whatmann No. 1 (57 x 46 cm) using BAW solvent systems (n-BuOH: AcOH: H_2O, 4:1:5 organic layer) and 15% AcOH/H_2O. After drying the change of spots colour on the chromatograms was detected by exposing to ammonia vapor or spraying with 1% methanol $AlCl_3$ or $FeCl_3$ (Mabry *et al*., 1970).

Phytochemical tests

Conventional standard protocols for detecting the presence of different chemical constituents in the plant extracts were employed. The tests for the secondary metabolites viz. alkaloids, tannins, sterols, saponins, glycosides, sterols/terpenes, reducing sugars, non-reducing sugars, flavonoids and phenols were carried out with the methanolic extracts of leaves of *T.grandis* using preliminary phytochemical screening (Trease and Evans, 1983; 1989; Harborne, 1993; Sofowora, 1993; Edeoga *et al*., 2005).

BIOLOGICAL INVESTIGATIONS

Determination of total phenolic content

The total phenolic content of each plant extract was determined using Folin - Ciocalteu's reagent. Gallic acid was used as standard. In this method, the reaction mixture was composed of (100 µl) of plant extract (100 µg/ml) and 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. Then the absorbance was measured at 765 nm. All determinations were carried out in triplicate. The total phenolic content was expressed as mg gallic acid equivalent (GAE) per g extract (Kumar *et al*., 2008).

Determination of total flavonoid content

The content of flavonoids of each extract was determined according to the reported procedures, 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 solvent without $AlCl_3$. All determinations were carried out in triplicate. The total flavonoid in plant extracts was determined as mg rutin equivalents (RE)/g extract (Kumaran and Karunakaran, 2006).

ANTIOXIDANT ACTIVITY

Rapid screening of antioxidant by dot-blot and DPPH staining

Each diluted sample of the *T. grandis* extracts/fractions 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 procedure (Soler-Rivas *et al*., 2000; El-Sayed *et al*., 2011). 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 (Dong-Jiann Huang *et al*., 2005; El-Sayed *et al*., 2011).

DPPH radical scavenging activity

The scavenging activity of the stable 1,1'-diphenyl-2-picraylhydrazyl (DPPH) 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 decrease in absorbance on

addition of test samples was used to calculate the antiradical activity, as expressed by the inhibition percentage (%IP) of DPPH radical, following the equation: %IP = [Ac - As]/ Ac X 100; where Ac and As are the absorbencies of the control and of the test sample after 20 min, respectively (Marwah *et al*., 2007).

Determination of total antioxidant capacity

The antioxidant activity of each 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 sodium 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) (Prieto *et al*., 1999; El-Sayed *et al*., 2011).

Reducing power antioxidant assay (RPAA)

A Spectrophotometric method described by Ferreira *et al*., 2007; was used for the measurement of reducing power. For this 2.5 ml of each of the extracts 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 used as standard. Three replicates were made for each test sample (Ferreira *et al*., 2007; El-Sayed *et al*., 2011).

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 extract of tested plant were carried out using the correlation and regression by Microsoft Excel program (Annegowda *et al*., 2010).

CYTOTOXICITY ACTIVITY

Brine shrimp lethality bioassay (BSLT)

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 of 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 calculated according to Reed-Muench method**.** Potassium dichromate was used as standard (Ipsen and Feigi, 1970; Miya *et al*., 1973).

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 (Ipsen and Feigi, 1970; Miya *et al*., 1973).

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 (10^4cells/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 (Skehan *et al*., 1996).

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Table 1. Phytochemical constituent of different extracts of *T. grandis* using TLC and PC analyses

No.	Constituents	Elution System	TLC and/or PC	Spraying reagent	Inference
1	Flavonoids	n-BuOH: AcOH: H_2O; 4: 1: 5 AcOH: H_2O; 15:85	TLC/PC; Long/Short UV light	$AlCl_3$ and/ or NH_3 and/ or $FeCl_3$	Dark Purple /Yellow spots
2	Phenolic acids	n-BuOH: AcOH: H_2O; 4: 1: 5 AcOH: H_2O; 15:85	TLC/ PC; Long/Short UV light	$AlCl_3$ and/ or NH_3 and/ or $FeCl_3$	Blue/Violet spots
3	Saponins	($CHCl_3$: MeOH: H_2O; 7: 3: 0.5) $CHCl_3$: EtOAc: MeOH; 2:2:1) ($CHCl_3$: MeOH, 9:1 and 8.5:1.5) (n-BuOH: MeOH: H_2O; 4:1:0.5)	TLC	MeOH/H_2SO_4 (60: 40 v/v)	Rosy Pink spots
4	Sterols	n-hexane: Diethyl ether; 32: 1	TLC	MeOH/H_2SO_4 (60: 40 v/v)	Dark Pink spots
5	Tannins	AcOH: H_2O: HCl; 30: 10: 3	PC; Short UV light	$FeCl_3$	Deep Violet/ Dark Greenish spots

Table 2. Results of phytochemical screening of (90%) methanolic extract of leaves of *T. grandis* as well as its derived fractions

Secondary Metabolite	Chemical Test	Sample (Extract or Fraction)					
		90%M[b]	DM	PE	C	E	B
Tannins	i. $FeCl_3$	+[a]	+	-	-	+	+
Flavonoids	i. Shinoda	++	+++	+	+	+++	+++
	ii. $AlCl_3$	++	+++	+	+	+++	+++
Saponins	i. Frothing	+++	++	+	++	+++	+++
Terpenoids & Steroids	i. Sslkowski	+	++	+	++	++	+++
	ii. Libarman Burchard's	+	++	+	++	++	+++
Anthraquinones	i. Borntrager's	+	+	-	-	+	++
Alkaloids	i. Dragendorff's	+	+	-	-	-	+
	ii. Meyer's	+	+	-	-	-	+
Carbohydrates	i. Molisch's	+++	+++	-	-	+++	+++
	ii. Barfoed's	++	++	-	-	+++	+++
Coumarins	i. NaOH	+	+	-	-	-	+

[a] +: Low; ++: Moderate; +++: High; -: Absent
[b] 90% M: 90% MeOH; DM: Defatted MeOH; PE: Petroleum ether; E: Ethyl acetate; B: n-butanol.

Table 3. Total extractable content (TEC), total phenolic content (TPC), total flavonoid (TFC), free radical scavenging potential (DPPH) and total antioxidant capacity of the different methanolic extracts of *T. grandis* (L.)

Sample	TEC %[a]	TPC[b] (mg GAE / g ext.)	TFC[c] (mg RE / g ext.)	DPPH (SC_{50})[d] [µg/ml]	Total antioxidant capacity (mg AAE /g ext.)[e]
MeOH 100%	15.05	188.90 ± 2.65	73.61 ± 2.37	28.67 ±1.49	384.69 ± 1.89
MeOH 90 %	17.22	208.32 ± 2.32	108.32 ± 7.21	15.78 ± 2.33	477.58 ± 2.65
MeOH 85 %	18.82	160.96 ± 4.12	52.11 ± 1.40	34.22 ± 2.51	348.46 ± 1.32
MeOH 70 %	21.44	111.82 ± 3.76	30.55 ± 3.40	43.90 ± 0.23	324.58 ± 2.53
MeOH 50 %	9.31	46.87 ± 1.75	9.72 ± 1.13	90.20 ± 1.47	123.91 ±2.56
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 4. Total extractable content (TEC), total phenolic content (TPC), total flavonoid (TFC), free radical scavenging potential (DPPH) and total antioxidant capacity of the defatted 90% methanol extract of *T. grandis* as well as its derived fractions

Sample	TEC %	TPC (mg GAE /g ext.)	TF (mg RE / g ext.)	DPPH SC_{50} [μg/ml]	Total antioxidant capacity (mg AAE /g ext.)
Defatted 90% MeOH	12.0	385.67 ± 2.23	80.02 ± 2.42	14.45 ± 0.54	490.73 ± 1.23
Pet. ether	1.97	30.20 ± 1.95	4.73 ± 1.68	134.04 ± 0.15	96.41 ± 2.52
$CHCl_3$	0.71	46.59 ± 0.47	9.90 ± 0.93	125.89 ± 0.31	201.89 ± 0.95
EtOAc	0.27	295.67 ± 3.56	55.22 ± 1.54	19.58 ± 0.88	400.30 ± 1.65

Table 5. Mortality of brine shrimp larvae after 24 h of exposure to different concentrations 90 % methanol, defatted 90% methanol, ethyl acetate and n-butanol extracts of *T. grandis*

Dose ppm	% Mortality of brine shrimp larvae			
	90% MeOH	Defatted 90% MeOH	EtOAc	n-BuOH
1000	95.68	97.41	92.36	99.45
800	87.90	91.91	83.47	98.71
600	78.57	85.0	72.32	98.42
400	66.66	75.90	59.04	92.45
200	50.0	61.53	43.56	84.52
100	34.41	43.90	27.72	71.87
10	16.30	22.36	13.59	43.75

Table 6. Cytotoxic activity of 90% methanol, defatted 90% methanol, EtOAc and n-BuOH extracts of *T. grandis*

Plant/extract	($LC_{50} \pm$ SE)[a]	(CL)[b]
90% methanol	100.0 ± 8.67	(117.34 – 82.66)
Defatted 90% methanol	79.43 ± 7.99	(95.41 – 63.45)
EtOAc	125.89 ± 11.47	(148.83 – 102.95)
n-BuOH	15.84 ± 6.98	(29.8 – 1.88)

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

Table 7. Cytotoxicity of 90% defatted methanol, EtOAc and n-BuOH extracts of *T. grandis*

Conc. μg/ml	SF (HEPG2)[a]		
	Defatted 90% MeOH	EtOAc	n-BuOH
0.000	1.000	1.000	1.000
5.000	0.745	0.841	0.643
12.500	0.598	0.652	0.483
25.000	0.426	0.457	0.449
50.000	0.446	0.464	0.428
IC_{50}	19.7 μg/ml	22.1 μg/ml	11.6 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%.

RESULTS AND DISCUSSION

Thin layer and paper chromatography

According to the TLC and PC analyses performed with the extracts, flavonoids glycosides, saponins, phenolic acids and alkaloids were detected. Flavonoids and phenolic acids appear to be present in most of the extracts. On the other hand, spots with no fluorescence under UV 254 or 365 nm could be seen in these extracts and that may indicate the presence of saponins or terpenes which characteristic orange or yellow colours were found in most of the extracts. $AlCl_3$ and/ or NH_3 and/ or $FeCl_3$ reagent indicates the presence of flavonoids if spots become yellow, orange or green when analyzed under UV 365 nm (Table 1) (Mabry *et al.*, 1970; Wagner and Bladt, 2009).

Fig 1. Percentage of yield of constituents in different extraction of *T. grandis*

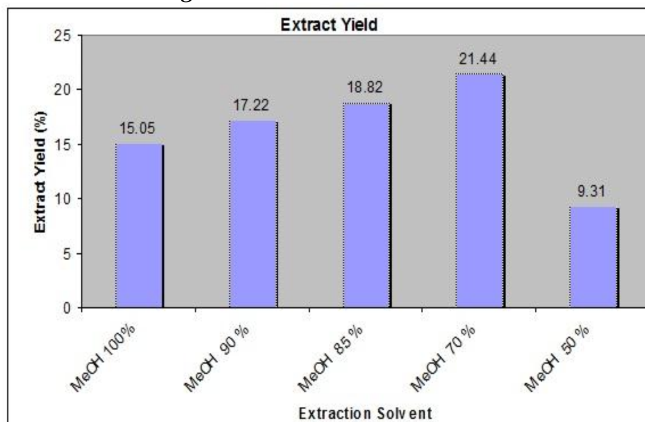

Fig 2. Dot blot assay of different fractions of *T. grandis* on silica sheet stained with DPPH solution in methanol

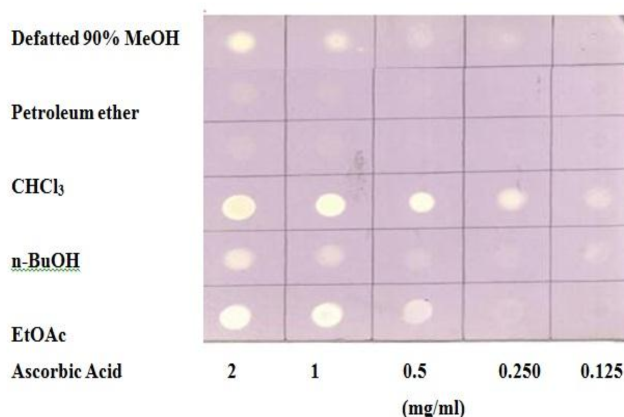

Fig 3. Correlation between DPPH free radical scavenging activities of the different methanolic extracts of *T. grandis* and their total phenolic contents (TPC)

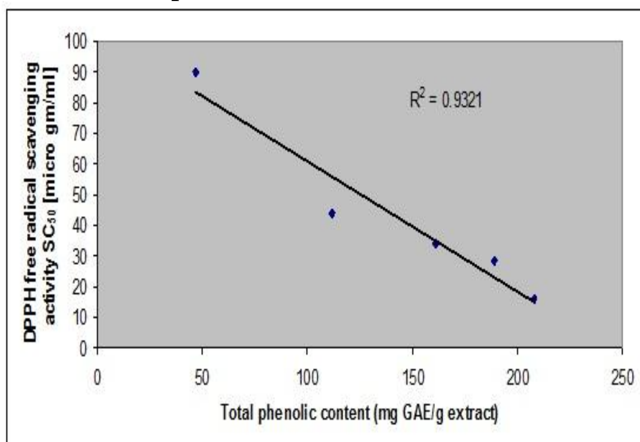

Fig 4. Correlation between the total antioxidant capacity of different methanolic extracts of *T. grandis* and their total phenolic contents (TPC)

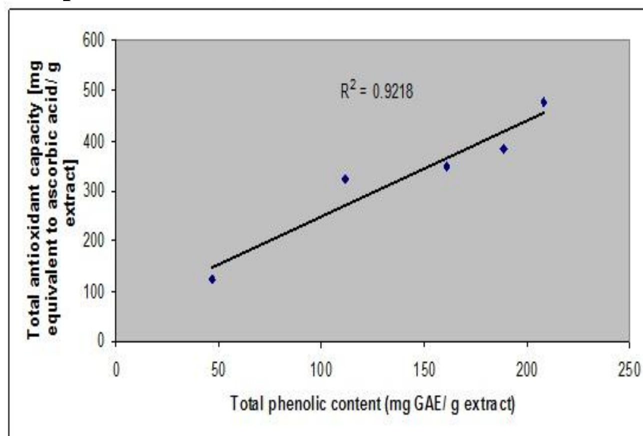

Fig 5. Correlation between DPPH free radical scavenging activities and total phenolic contents (TPC) of the defatted 90% methanolic extract of *T. grandis* as well as its derived extracts

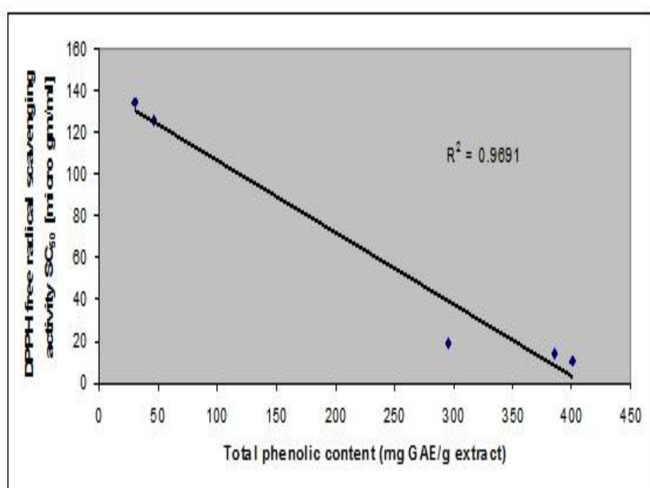

Fig 6. Correlation between the total antioxidant capacity and total phenolic contents (TPC) of the defatted 90% methanolic extract of *T. grandis* as well as its derived extracts

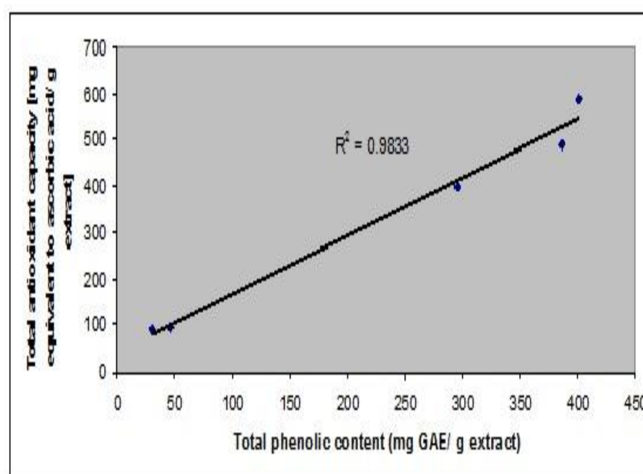

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Fig 7. Reducing power assay of the different methanolic extracts of *T. grandis* at concentration 200 µg/ml

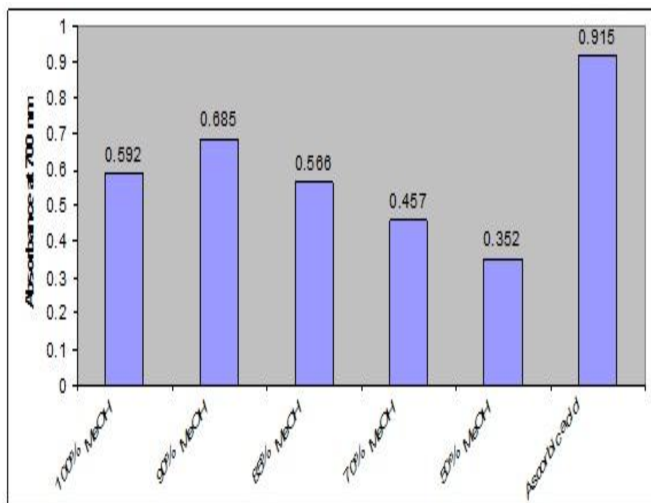


Fig 8. Reducing power assay of the defatted 90% methanolic extract of *T. grandis* as well as its derived fractions at concentration 200 µg/ml

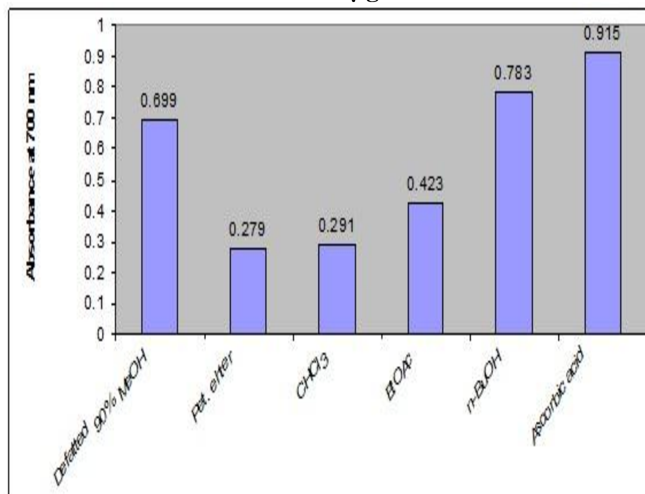


Fig 9. Estimation of LC_{50} by plot of percent mortality of brine shrimp larvae against different dosage of different extracts of *T. grandis*

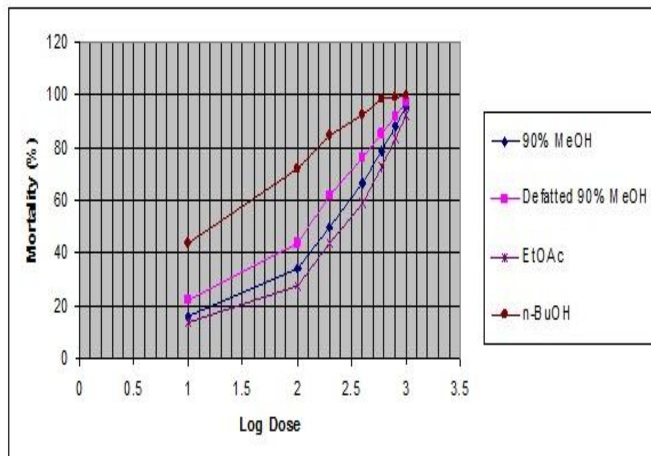


Fig 10. Effect of 90% defatted methanol of *T. grandis* against liver tumor cell line

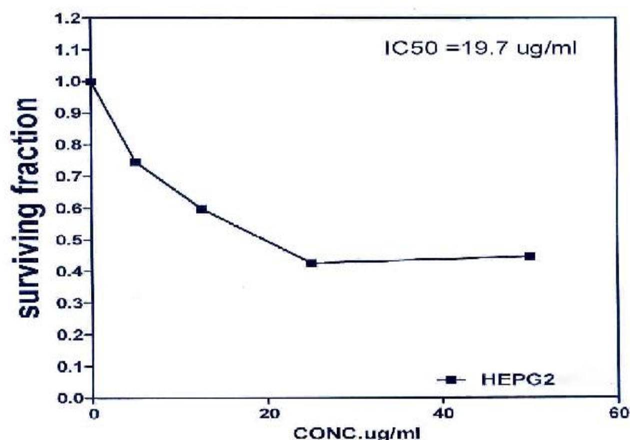


Fig 11. Effect of ethyl acetate extract of *T. grandis* against liver tumor cell line

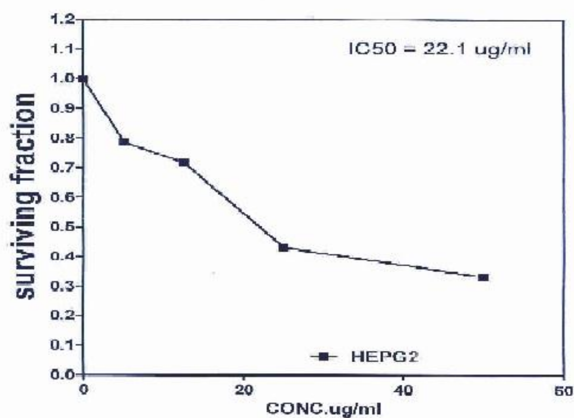
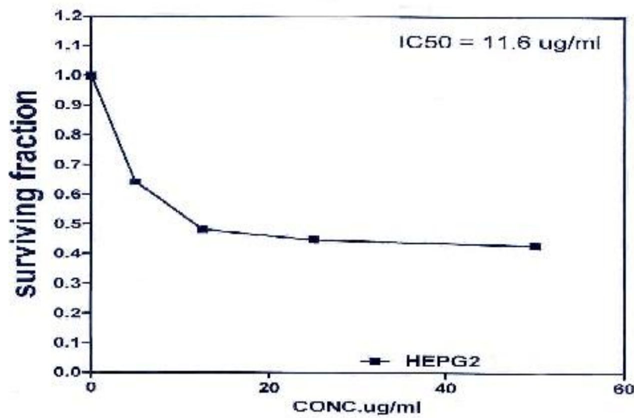


Fig 12. Effect of n-BuOH extract of *T. grandis* against liver tumor cell line



Phytochemical screening tests

Thousands of diverse natural products are produced by plants and many of these are involved in plant defense. The phytochemical diversity of antioxidant and anticancer compounds include terpenoids, saponins, phenolics, phenyl propanoids and alkaloids (Cooper *et al*., 2006). In this study, the phytochemical analysis of the defatted 90% methanol extract of the *T. grandis* as well as its derived fractions showed the presence of different groups of secondary metabolites such as alkaloids, flavonoids, saponins, terpenoids, steroid, tannins, anthraquinones, coumarins and sterols which are of medicinal importance (Table 2). The preliminary qualitative tests performed by Pooja *et al*., 2010; indicated that the leaves of *T. grandis* plant contain flavonoids, phenols and tannins (Pooja *et al*., 2010). A previous study the of *T. grandis* stem methanolic extract showed presence of alkaloids and absence of flavonoids and terpenoids (Gupta and Singh, 2004).

Extraction yield

Methanol and methanol-water mixtures solvents are used for extraction of the chemical constituents of the *T. grandis*. The amount of the extractable substances of each extract was expressed as a percentage by weight of dried leaves of plant. The results in (Tables 3, 4 and Fig. 1) showed that the methanol and methanol-water mixtures have different abilities for extraction of different percents of extractable substances. The highest amount of the total extractable content (TEC) from the plant was obtained by 70% methanol (21.44 %) followed by 85 % (18.82 %), 90 % (17.22 %), 100 % (15.05 %) and 50% (9.31) methanol. No significant association could be found neither between the extraction yields and total phenols nor between the extraction yields and the results from the different antioxidant assays. Our results showed that the yield of the defatted 90% methanol extract of *T.grandis* and its derived fractions was (12.0 %, 1.97 %, 0.71 %, 0.27% and 4.81 %) respectively for defatted 90% methanol extract, pet. ether, $CHCl_3$, EtOAc and n-BuOH. Variation in the percent yield of extracts might be due to chemical composition of plant, nature of the soil and agro-climatic condition. The other factors could be the effectiveness of the extracting solvent to dissolve endogenous compounds (Anwar *et al*., 2006; Hsu *et al*., 2006; Sultana *et al*., 2007; Shabbir *et al*., 2011; Mohammad Asif, 2011).

Total phenolics and flavonoids contents (TPC)

The total phenolics and flavonoids assays are considered simple, sensitive and precise bioassays (Huang *et al*., 2005; Prior *et al*., 2005). The total phenolic and flavonoid contents of different crude extracts as well as the derived fractions of 90% methanolic extract of *T. grandis* was determined and expressed in terms of gallic acid and rutin equivalents respectively (Tables 3, 4). There was a wide range of phenol concentrations in tested extracts. The content of phenolics and flavonoids of different metanolic extracts of *T.grandis* decreased in the order (TPC/TFC): 90% MeOH (208.32/ 108.32); 100% MeOH (188.90/ 73.61); 85% MeOH (160.96/ 52.11); 70% MeOH (111.82/ 30.55) and 50% MeOH (46.87/ 9.72). On the light these results the 90% MeOH extract was promising to undergo successive fractionation. Furthermore, the content of phenolics and flavonoids of derived fractions of *T. grandis* decreased in the order (TPC/TFC): n-BuOH > defatted 90% MeOH > EtOAc > $CHCl_3$> pet. ether. The content of total phenols varied from 30.20 to 400.56 (mg GAE / g ext.); 400.56 385.67, 295.67, 46.59 and 30.20 (mg GAE / g ext.), respectively for the n-BuOH, defatted 90% MeOH, EtOAc, $CHCl_3$ and pet. ether (Table 3). The content of total flavonoids varied from 4.73 to 80.13 (mg RE / g ext.); 80.13, 80.02, 55.22, 9.90 and 4.73 (mg RE / g ext.), respectively for the n-BuOH, 90% MeOH, EtOAc, $CHCl_3$ and pet. ether. These results are in agreement with those available in the literature (Rao *et al*., 2011).The high phenolic content in n-BuOH as well as EtOAc extracts contributes to their increased antioxidant potential in comparison to the other extracts. The total phenolics assay actually measures the reducing capacity of a sample. The antioxidant and cytotoxic activities of many plants have been attributed to their phenolic contents (Rodney *et al*., 2012). These findings should encourage the development of new antioxidant and anticancer drugs in the future. More studies are required to find out more specific biochemical, pharmacological and molecular aspects of the targeted molecules. Further works on the isolation of bioactive phytoconstituents of *T. grandis* should be carried out to provide the exact mechanism of activity.

ANTIOXIDANT ACTIVITY

Rapid screening of antioxidant by dot-blot and DPPH staining

The antioxidant potential activity of the defatted 90% methanol extract of *T. grandis* 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 (Soler-Rivas, 2000; El-Sayed *et al*., 2011). Initial faint spots appeared and weak spots could be observed in sample row, and the appearance of white spots has potential value for the indirect evaluation of the different tested fractions (Chang *et al*., 2002). These white spots with strong intensity appeared quickly at the concentration of 0.50 mg/ml of each extract, ascorbic acid was used as a positive control. Results in (Fig. 2) revealed

that, all the tested extracts showed promising activity but the n-BuOH extract 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 (Dong-Jiann Huang *et al*., 2005; El-Sayed *et al*., 2011).

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

The free radical-scavenging activity of different extracts/fractions was determined by the DPPH$^{\bullet}$ method and the results are shown in (Tables 3, 4). The DPPH$^{\bullet}$ radical is one of the most widely used and stable chromogen compound used to evaluate the antioxidant activity of the tested compounds. The characteristic chemical structure of DPPH$^{\bullet}$ refer that; It is a stable nitrogen-centered free radical, the colour of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to undergo such reaction can be considered as antioxidants and therefore radical scavengers compounds (Brand-Williams *et al*., 1995; Zheng and Wang, 2010). A low SC_{50} value indicates that the mechanism of antioxidant action of extracts was as a hydrogen donor and it could reach the termination process via the transforming the free radicals to another chemically stable forms. For the different methanolic extracts, the values of SC_{50} varied from 15.78 to 90.20 [µg/mL]; this showed DPPH scavenging activity in the order, 90% MeOH (15.78) > 100% MeOH (28.67) > 85% MeOH (34.22) > 70% MeOH (43.900 > 50% MeOH (90.20) [µg/ml] (Table 3). These results showed the potent antioxidant activity of 90% MeOH extract when compared with the other extracts, so it promising to undergoes further fractionations to determine the actual fraction/s which responsible for this activity. The DPPH radical scavenging activities of water extracts were the lowest compared to those of other solvents. This suggests that the use of water for extraction of radical scavenging compounds from selected medicinal plants was not effective, which is in accordance with previous research that demonstrated that the lowest antioxidant and radical scavenging activities were in water extracts (Majhenič *et al*., 2007; Arabshahi-Delouee and Urooj, 2007). Also, the different derived fractions of 90% MeOH extract showed DPPH scavenging activity which in the order, n-BuOH (10.63), defatted 90% MeOH (14.45), EtOAc (19.58), $CHCl_3$ (125.89) and pet. ether (134.04) [µg/ml]) and that of the standard ascorbic acid was 8.25 (µg/ml) (Table 4). Therefore, n-BuOH activity was the highest in the five tested samples and pet. ether activity was the lowest one. The total phenolic content of all tested extracts of *T.grandis* exhibited the strongest correlation with the DPPH antioxidant activity. Thus, the antioxidant efficiency of the tested extracts appears to be largely based upon the total phenolic level. These results are in agreement with other reports in the literature (Rapisarda *et al*., 1999; Roginsky and Lissi, 2005; Prior *et al*., 2005; Park *et al*., 2006). From these results there is a linear correlation between the content of phenolics and antioxidant potential of the active extracts under investigation. This point of correlation was accepted by many authors who established a linear correlation between the total contents of phenolics and the antioxidant capacity (Cai *et al*., 2004; Park *et al*., 2006). Whereas few authors reported that there is no correlation (Yu *et al*., 2002). The significant correlation between total phenolic content and the DPPH antioxidant activity of the defatted 90% methanolic extract as well as its derived fractions was confirmed through the high value of correlation coefficient respectively (R^2 = 0.93 and 0.96) (Fig. 3, 5). On the light of such correlation it was concluded that the phenolic compounds containing free hydrogen are largely responsible for antioxidant activity (Evans *et al*., 1996; Evans *et al*., 1997). Thus the phenolic compounds of *T. grandis* can be referred to be responsible for the antioxidant activity.

Phosphomolybdenum antioxidant assay

Free radical scavenging ability of phenolic compounds is an important phenomenon underlying their various biological and pharmacological activities. The phosphomolybdenum assay is based on the reduction of Mo^{VI} to Mo^{V} by antioxidant compounds and a formation of a green phosphate/Mo^{V} complex with a maximal absorption at 765 nm (El-Sayed *et al*., 2011). The assay has been successful in the quantification of vitamin E antioxidant activity (Prieto *et al*., 1999). In addition, it was efficient to extend its application to the phenolic compounds extracted and isolated from the medicinal plants (Lu and Foo, 1995). The total antioxidant capacity of the different methanolic extracts of *T. grandis* was 477.58, 384.69, 348.46, 324.58 and 123.91 (mg AAE /g ext.), respectively for 90%, 100%, 85%, 70% and 50% MeOH extracts. These results also, supported the results obtained from the DPPH assay that concluded that the 90% MeOH extract showed high total antioxidant capacity when compared with the other extracts of *T. grandis*, which may be due to presences of high percentages of phenolic compounds (Rajuri *et al*., 2010). The total antioxidant capacity values for the derived fractions of 90% MeOH extract decreased in the order: n-BuOH (589.50) > defatted 90% MeOH (490.73) > EtOAc (400.30) > $CHCl_3$ (201.89) > pet. ether (196.41) (mg AAE /g ext.) (Table 4). The total phenolic compounds contents were also highly correlated with the antioxidant

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activity of different methanolic extracts (R^2= 0.92) and (R^2= 0.98) for derived fractions of 90% MeOH extract (Fig. 4,6). A good correlation found between polyphenol content and antioxidant activities in all screened samples showing that antioxidants activities present in the extracts were essentially due to the existence of the polyphenols (Rice-Evans *et al*., 1996; Mohammed *et al*., 2010; Rao *et al*., 2011; El-Sayed *et al*., 2011).

Reducing power antioxidant assay (RPAA)

Reducing power assay measures the electron-donating capacity of an antioxidant substance. The reducing properties are generally associated with the presence of reductones, which have been shown to exhibit antioxidant ability via breaking the chain reactions and donating a hydrogen atom to the reactive species. Furthermore, reductones are also reported to react with desired precursors of peroxide, thus preventing peroxide formation (Li and Lin, 2010). Being good electron donors, phenolic compounds show the reducing power and have ability to transform the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) via donating single electron (Shon *et al*., 2004). Increasing absorbance at 700 nm indicates an increase in reductive ability, and the reducing power of the tested compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al*., 1995; El-Sayed *et al*., 2011). In this study, we investigated the Fe^{3+}-Fe^{2+} transition to measure the *T. grandis* reducing capacity. The reducing power of different metanolic extracts of *T. grandis* was shown in (Fig. 7), at absorbance 700 nm and concentration (200 µg/ml), with ascorbic acid as a positive control; which there absorbance was decrease in the order: 90% (0.685), 100% (0.592), 85% (0.566), 70% (0.457) and 50% (0.352) MeOH extracts, while ascorbic acid absorbance was 0.915. The increasing absorbance of the tested sample at 700 nm indicates an increase in reductive ability; accordingly the 90% MeOH showed the highest reducing power activity in comparison with the other tested methanolic extracts near the value of ascorbic acid. The reducing power of different fractions of *T.grandis* was shown in (Fig. 8), with ascorbic acid as a positive control. The reducing power of defatted 90% methanolic extract (0.699), pet. ether (0.279), $CHCl_3$ (0.291), EtOAc (0.423) and n-BuOH (0.783) was compared with ascorbic acid (0.915). The n-BuOH has the highest reducing power at 200 µg/ml concentration. However, pet. ether fraction showed lowest reducing power. The reducing power may be based upon the hydrogen donation from phenolic compounds (Shimada *et al*., 1992; Nayeem and Karvekar, 2010; El-Sayed *et al*., 2011). Besides, phenolic compounds and flavonoids are also widely distributed in plants, which have been reported to exert numerous biological applications, involving antioxidant, free radical scavenging abilities and anticarcinogenic (Miller, 1996; Sahaa *et al*., 2008).

CYTOTOXIC ACTIVITY

Brine shrimp lethality test (BSLT)

Brine shrimp cytotoxicity assay has been considered as primary screening bio-assay for anticancer activity. Brine shrimp assay is suggested to be a convenient probe for the pharmacological activities in medicinal plant extracts (Mayerhof *et al*., 1991). BST bioassay can be successfully used as a cheaper, reliable and quicker tool for isolating the biologically active fractions especially anticancer agents from the natural sources (Pathak *et al*., 1988). In the present study, extracts of *T. grandis* plant were evaluated by the brine shrimp lethality bioassay using the procedure (Meyer *et al*., 1982). The brine shrimp toxicity data are summarized in (Tables 5, 6). The n-BuOH extract of showed the most potent toxic effect at LC_{50} = 15.84 µg/mL, followed by defatted 90% MeOH extract which showed cytotoxicity at LC_{50} = 79.43 µg/ mL, 90% MeOH and ethyl acetate extracts showed a significant cytotoxic effect at LC_{50} = 100.0 and 125.89 µg/ mL respectively (Fig. 9). Several classes of plant secondary metabolite are responsible for the observed cytotoxic activity, but the most important and diverse bio-potencies have been observed in phenolic acids, flavonoids and tannins, so the observed activity may be due to the presence of secondary metabolites in *T.grandis* fractions (Pooja *et al*., 2010). This indicates that this plant contain potential bioactive compounds, which if properly and extensively studied, could provide many chemically interesting and biologically active drug candidates, including some with potential antitumor properties. A thorough chemical study is required to isolate the molecules that are responsible for the activities.

Liver carcinoma cell line (HepG2)

Cancer or malignant disease is one of the major causes of death in humans 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/AIDS (Mathers *et al*., 2001). Cancer is considered as a major health problem in both developed and developing countries over the world. It was estimated 12.7 million recent cancer cases and about 7.6 million cancer deaths take place in the year 2008 (Ferlay *et al*., 2010; Abdel-Hameed *et al*., 2012). The environmental, chemical, metabolic and genetic parameters play an important role in the generation of all cancer types. The weak success chances of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treatment of cancer, was monitored by the high mortality rates, indicates that there is an urgent requirement of new cancer dealing actions (Dai and Mumper, 2010; Abdel-Hameed *et al*., 2012). Drug discovery from medicinal plants has played a vital role in

the treatment of cancer (Balunas and Kinghorn, 2005; Abdel-Hameed *et al*., 2012). Thus, it is urgent to find more and safer new active constituents that attack and kill cancer cells. (Table 7) showed the cytotoxic effects of the defated 90% methanolic extract, ethyl acetate and n-butanol fractions of the leaves part of *T. grandis* against HepG2 cell line using the sulforhodamine B (SRB) method (Skehan *et al*., 1990). The n-BuOH fraction showed high cytotoxic activity toward the HepG2 cell line with IC_{50} = 11.6 µg/ mL, followed by the 90% defatted methanol with IC_{50} = 19.7 µg/ mL and ethyl acetate fraction with IC_{50} =22.1 µg/ mL comparing with the reference standard doxorubicin with IC_{50} = 4 µg/ mL.

According to the American Cancer Institute (ACI), 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 (Boik, 2001; Abdel-Hameed *et al*., 2012). Two tested fractions (defatted 90% MeOH and n-BuOH) showed IC_{50} values exist under the ACI criteria, accordingly these fractions are considered as promising cytotoxic agents.

CONCLUSION

In the present study, the antioxidant activity from different extracts of *T. grandis* was evaluated as well as the cytotoxic activity. *T. grandis* showed strong antioxidant activity by DPPH, phosphomolybdenum and reducing power activities when compared with standard L-ascorbic acid. Also, most of these extracts showed a significant cytotoxic effect. In addition, the *T. grandis* was found to contain a noticeable amount of total phenols, which play major role in controlling oxidation. The results of this study show that the *T. grandis* can be used as easily accessible source of natural antioxidant. The anti-oxidant and cytotoxic activities of *T. grandis* may be due to the presence of different phyto-constituents such as tannins, phenolic acids and flavonoids. Therefore, it is suggested that further investigation to determine antioxidant activity by *in vivo* methods of *T. grandis* could be considered.

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REFERENCES

Abdel-Hameed ES, Salih AB, Mohamed MS, Mortada ME, Eman AE. Phytochemical studies and evaluation of antioxidant, anticancer and antimicrobial properties of *Conocarpus erectus* L. growing in Taif, Saudi Arabia. *European Journal of Medicinal Plants*, 2(2), 2012, 93-112.

Anwar F, Jamil A, Iqbal S, Sheikh MA. Antioxidant activity of various plant extracts under ambient and accelerated storage of sunflower oil. *Grasas Aceites Sevilla*, 57, 2006, 189-197.

Annegowda HV, Nee CW, Mordi MN, Ramanathan S, Mansor SM. Evaluation of phenolic content and antioxidant property of hydrolysed extracts of *Terminalia catappa* (L.) leaf. *Asian Journal Plant Sciences*, 9, 2010, 479-485.

Arabshahi-Delouee S, Urooj A. Antioxidant properties of various solvent extracts of mulberry (*Morus indica* L) leaves. Food Chemistry, 102, 2007, 1233-1240.

Brand-Williams W, Cuvelier M, Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology*, 28, 1995, 25-30.

Balunas MJ, Kinghorn AD. Drug discovery from medicinal plants. *Life Sciences*, 78, 2005, 431-441.

Boik J. Natural compounds in cancer therapy. Oregon Medical Press, Minnesota, USA, 2001.

Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 Chinese medicinal plants associated with anticancer. *Life Sciences*, 74, 2004, 2157-2184.

Chang WC, Kim SC, Hwang SS, Choi BK, Ahn HJ, Lee MY, Park SH, Kim SK. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science*, 163, 2002, 1161-1168.

Cooper J, Niggli U, Leifert C. Handbook of organic food safety and quality. Abington Hall, England, 2006.

Da Rocha AB, Lopes RM, Schwartsmann G. Natural Products in Anticancer Therapy. *Current Opinion in Pharmacology* 1, 2001, 364-369.

Dai J, Mumper RJ. Plant Phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules* 15, 2010. 7313-7352.

Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology*, 4, 2005, 685-688.

El-Sayed MM, Salah AA, Hanan AE, Mahfouz MA, Maher MA, El-Sayed SA, Waffa SA, Ezzat EA. Evaluation of Antioxidant and Antimicrobial Activities of Certain *Cassia* Species. *Australian Journal of Basic and Applied Sciences*, 5(9), 2011, 344-352.

Evans RCA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, 20, 1996, 933.

Evans RCA, Miller NJ, Paganga G. Antioxidant properties of phenolic compounds. *Trends in plant science*, 2, 1997, 152.

Ferreira ICFR, Baptista M, Vilas-Boas BL. Free radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal; Individual cap and stipe activity. *Food Chemistry*, 100, 2007, 1511-1516.

Ferlay J, Shin H, Bray F, Forman D, Mathers C, Parkin D. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer*, 127, 2010, 2893-2917.

Golden TR, Hinerfeld DA, Melov S. Oxidative stress and aging: beyond correlation. *Aging Cell*, 1, 2002, 117-123.

Gordon MH, Weng XC. Antioxidant properties of extracts from Tanshen (*Salvia miltiorrhiza* Bunge). *Food Chemistry*, 44, 1992, 119-122.

Gu LW, Weng XC. Antioxidant activity and components of *Salvia plebeia* R.Br. a Chinese Herb. *Food Chemistry*, 73, 2001, 299-305.

Gupta PK, Singh P. A napthoquinone derivative from *Tectona grandis* (Linn.). *Journal of Asian Natural Products Research*, 6, 2004, 237-240.

Harborne JB. Phytochemistry. Academic Press, London, 1993, 89-131.

Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, 53, 2005, 1841-1856.

Huang DJ, Lin CD, Chen HJ, Lin YH. Antioxidant and antiproliferative activities of sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57') constituents. *Botanical Bulletin of Academia Sinica*, 45, 2005, 179-186.

Hsu B, Coupar IM, Ng K. Antioxidant activity of hot water extract from the fruit of the Doum palm, *Hyphaene thebaica*. *Food Chemistry*, 98, 2006, 317-328.

Ipsen J, Feigi P. Bancroft's Introduction to Biostatistics, 2nd ed., Harper & Row. New York, 1970, 15.

Kumar KS, Ganesan K, Rao PV. Antioxidant potential of solvent extracts of *Kappaphycus alve*rezii (Doty). Edible seaweed. *Food Chemistry*, 107, 2008, 289-295.

Kumaran A, Karunakaran RJ. *In vitro* antioxidant activities of methanol extracts of fine *Phyllanthus* species from India. *LWT-Food Science and Technology*, 40, 2006, 344-352.

Lee K, Kim Y, Lee H, Lee C. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *Journal of Agricultural and Food Chemistry*, 51(25), 2003, 7292-7295.

Li C, Lin E. Antiradical capacity and reducing power of different extraction method of *Areca catechu* seed. *African Journal of Biotechnology*, 9, 2010, 7831-7836.

Lu F, Foo LY. Phenolic antioxidant component of evening primrose, in Nutrition, Lipids, Health and Diseases, Ed. By Ong ASH, Niki E and Packer L. Champaign: American Oil Chemists Society Press, 1995, 86-95.

Lui S, Zhou T, Zhang S, Xuan L. Chemical constituents from *Clerodendron bungei* and their cytotoxic activities. *Helvetica Chimica Acta*, 9, 2009, 1070-1079.

Mabry TT, Markham KR, Thomas MB. The systematic identification of flavonoids. New York: Springer-Verlag, 1970.

Machlin LJ, Bendich A. Free radical tissue damage: protective role of antioxidant nutrients. *Federation of American for Experimental Biology Journal*, 1, 1987, 441-445.

Macias FA, Lacret R, Varela RM, Nogueiras C, Molimillo JMG. Isolation and phytotoxicity of terpenes from *Tectona grandis*. *Journal of Chemical Ecology*, 36, 2010, 396-404.

Macias FA, Lacret R, Varela RM, Nogueiras C, Molinillo JM. Bioactive apocarotenoids from *Tectona grandis*. *Phytochemistry*, 69, 2008, 2708-2715.

Majhenič L, Škerget M, Knez Z. (2007) Antioxidant and microbial activity of guarana seed extracts. *Food Chemistry*, 104, 2007, 1258-1268.

Marwah RG, Fatope MO, Mahrooqi RA, Varma GB, Abadi HA, Al-Burtamani SKS. (2007) Antioxidant capacity of some edible and wound healing plants in Oman. *Food Chemistry*, 101, 2007, 465-470.

Mathers CD, Boschi-Pinto C, Lopez AD, Murray, CJL. Cancer incidence, mortality and survival by site for 14 regions of the world. *World Health Organization*, 2001, 3.

Mayerhof ER, Koncz-Kalman RZ, Nawrath C, Bakkeren G, Crameri A, Angelis K, Redei GP, Schell JB, Hohn KJ. T-DNA integration: a mode of illegitimate recombination in plants. *The EMBO Journal*, 10, 1991, 697-704.

Meir S, Kanner J, Akiri B, Hadas SP. Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. *Journal of Agricultural and Food Chemistry*, 43, 1995, 1813-1817.

Meshkatalsadat MH, Papzan A, Abdollahi A. Determination of bioactive volatile organic components of *Lippa citriodora* using Ultrasonic assisted with headspace solid phase micro extraction coupled with GC-MS. *Digest Journal of Nonmaterials and Biostructures*, 6, 2010, 319-323.

Meyer BN, Ferrrigni NR, Putnam JE, Jacobsen LB, Nichols E, Mclaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica*, 45, 1982, 31- 34.

Miller AL. Antioxidant flavonoids: structure, function and clinical usage. *Alternative Medicine Review*, 1, 1996, 103.

Miya TS, Holck HGO, Yim GKW, Mennear JH, Spratto R. Laboratory Guidein In: Pharmacology, 4th Ed, Burgess Publishing, Minneapolis, 1973, 1237.

Mohammad A. *In vivo* analgesic and antiinflammatory effects of *Tectona grandis* (Linn.) stem bark extracts. *Malaysian Journal of Pharmaceutical Sciences*, 9, 2011, 1-11.

Mohammed FAK, Nagendra P, Kong KW, Amin I. Flavonoid, hesperidine, total phenolic contents and antioxidant activities from Citrus species. *African Journal of Biotechnology*, 9, 2010, 326-330.

Nayeem N, Karvekkar MD. Isolation of phenolic compounds from the methanolic extract of *Tectona grandis*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 1, 2010, 221-225.

Nayeem N, Karverkar MD. Analgesic and anti-inflammatory activity of the methanolic extract of the frontal leaves of *Tectona grandis*. *Internet Journal of Pharmacology*, 8, 2010, 1531-2976

Nitha B, Meera CR, Janardhanan KK. Antitumor activity of ethanolic extract of *Lentinus dicholamellatus*. *Amala Research Bulletin*, 25, 2005, 165-168.

Park Y, Jung S, Kang S, Delgado-Licon E, Ayala A, Tapia MS, Martin-Belloso O, Trakhtenberg S, Gorinstein S. Drying of persimmons (*Diospyros kaki* L.) and following changes in the studied bioactive compounds and the total radical scavenging activities. *LWT-Food Science and Technology*, 39, 2006, 748-755.

Pathak NKR, Neogi P, Biswas M, Tripathi YC, Pandey VB. Betulin aldehyde, an antitumour agent from the bark of *Tectona grandis*. Indian *Journal of Pharmaceutical Sciences*, 50, 1988, 124-125.

Pooja, Kartick CS, Sukhbir LK, Priyanka SVS, Vikas G. Free radical scavenging activity of *Tectona grandis* roots. *International Journal of Pharmaceutical Sciences and Research*, 1, 2010, 159-163.

Prieto P, Pineda M, Aguilar M. Spectrophotometric quantation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Analytical Biochemistry*, 269, 1999, 337- 341.

Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53, 2005, 4290-4302.

Pyo YH, Lee TC, Logendrac L, Rosen RT. Antioxidant activity and phenolic compounds of Swiss chard (Beta vulgaris subspecies cycla) extracts. *Food Chemistry*, 85, 2004, 19-26.

Rajuri A, Rao KNV, David B, Chaithanya RK. A Review on *Tectona grandis* (Linn.): Chemistry and medicinal uses (Family: Verbenaceae). *Herbal Tech Industry*, 2010, 6-9.

Rao KNV, Aradhana R, David B, Chaitanya R, Anil KA. *In vitro* antioxidant and free radical scavenging activity of various extracts of *Tectona grandis* Linn leaves. *Journal of Pharmacy Research*, 4, 2011, 440-442.

Rapisarda P, Tomaino A, Lo Cascio R, Bonina F, De Pasquale A, Saija A. Antioxidant effectiveness as influenced by phenolic content of fresh orange juices. *Journal of Agricultural and Food Chemistry*, 47, 1999, 4718-4723.

Rice-Evans C, Miller N, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, 20, 1996, 933-956.

Rodney L, Rosa MV, Jose´ MG, Molinillo CN, Francisco AM. Tectonoelins, new norlignans from a bioactive extract of *Tectona grandis*. *Phytochemistry Letters*, 5, 2012, 382-386.

Roginsky V, Lissi EA. Review of methods to determine chain-breaking antioxidant activity in food. *Food Chemistry*, 92, 2005, 235-254.

Sahaa MR, Hasana SMR, Aktera R, Hossain MM, Alam MS, Alam MA, Mazumder ME H. *In vitro* free radical scavenging activity of methanol extract of the leaves of *Mimusops elengi* Linn. *Bangladesh Journal of Veterinary Medicine* , 6, 2008, 197-202.

Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autoxidation of soyabean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry*, 40, 1992, 945-948.

Shon MY, Choi SD, Kahng GG, Nam SH, Sung NJ. Antimutagenic, antioxidant and free radical scavenging activity of ethyl acetate extracts from white, yellow and red onions. *Food and Chemical Toxicology*, 42, 2004, 659-666.

Siddiqui BS, Ahmed F, Sattar FA, Begum S. Chemical constituents from the aerial parts of *Lippa nidiflora* (Linn.). *Archives of Pharmacal Research*, 30, 2007, 1507-1510.

Sies H. Oxidative stress: oxidants and antioxidants. *Experimental Physiology*, 82, 1997, 291-295.

Singh N, Shukla N, Singh P, Sharma R, Rajendran SM, Maurya R, Palit G. Verbascoside isolated from *Tectona grandis* mediates gastric protection in rats via inhibiting protop pump activity. *Fitoterapia*, 81, 2010, 755-761.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. New colorimetric cytotoxicity assay for anticancer-drug screening. *Food and Chemical Toxicology*, 34, 1996, 449- 456.

Shabbir G, Anwar F, Sultana B, Khalid ZM, Afzal M, Khan MQ, Ashrafuzzaman M. Antioxidant and antimicrobial attributes and phenolics of different solvent extracts from leaves, flowers and bark of Gold Mohar [*Delonix regia* (Bojer ex Hook.) Raf.]. *Molecules*, 16, 2011, 7302-7319.

Sultana B, Anwar F, Przybylski R. Antioxidant activity of phenolic components present in barks of barks of *Azadirachta indica, Terminalia arjuna, Acacia nilotica, and Eugenia jambolana* (Lam.) trees. *Food Chemistry*, 104, 2007, 1106-1114.

Sofowora A. Medicinal plants and Traditional medicine in Africa. Spectrum Books Ltd, Ibadan, Nigeria, 1993, 289-300.

Soler-Rivas C, Espin JC, Wichers HJ. An easy and fast test to compare total free radical scavenger capacity of foodstuffs. *Phytochemical Analysis*, 11, 2000, 330-338.

Trease GE, Evans WC. Pharmacognosy, 12th Ed. Bailliere Tindall, London, 1983, 21-22.

Trease GE, Evans WC. Pharmacognosy, 13th Ed. Bailliere Tindall, London, 1989, 176-180.

Valko M, Leibfritz D, Moncol J. Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal* of *Biochemistry* & *Cell Biology*, 39, 2007, 44-84.

Wagner H, Bladt S. Plant drug Analysis, A thin Layer Chromatography. Springer, Heidelberg, Germany, 2009.

Yu L, Haley S, Perret J, Harris M, Wilson J, Qian M. Free radical scavenging properties of wheat extracts. *Journal of Agricultural and Food Chemistry*, 50, 2002, 1619-1624.

Zhang YH, Cheng DL. Two new iridoid glycosides from *Caryopteris mongholica*. *Chinese Chemical Letters*, 11, 2000, 319-322.

Zheng W, Wang S. Antioxidant activity and phenolic composition in selected herbs. *Journal of Agricultural and Food Chemistry*, 49, 2010, 5165-5170.