ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Evaluation of Total Phenol, Anticancer and Antioxidant Properties by Different Extracts of *Terminalia Belerica* Roxb. Leaves: An *In Vitro* Analysis.

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ABSTRACT

The present study was aimed to compare *in vitro* anticancer and antioxidant effects as well as total phenols contents of five different exclusive extracts namely; methanol, aqueous methanol, ethyl acetate, chloroform and petroleum ether extracts of *Terminalia belerica* Roxb. leaves. The obtained results reveal that there is a moderate correlation between the total phenols content and the antioxidant activities of these extracts. In addition, the antioxidant activity and the total phenols contents increased with increasing the solvent polarity as both methanol extract and the aqueous methanol extract showed the highest activities compared to the other extracts. Furthermore, petroleum ether extract showed the most potent anticancer activities followed by chloroform against all the fourteen cell lines from ten different human cancer types, namely: ovarian carcinoma, liver carcinoma, breast carcinoma, HeLa contaminant, cervical carcinoma, CNS-human glioblastoma, non-small lung cancer, colon adenocarcinoma, fibrosarcoma, leukemia and melanoma. The other extracts showed potent anticancer activities only against leukemia and melanoma. Based on these results, it can be postulated that, especially petroleum ether extract represents a highly promising extract for anticancer candidate drugs. However, further fractionation of the petroleum ether extract is needed to purify and identify the active metabolites, which are responsible for these activities.

Keywords: *Terminalia belerica* Roxb. leaves; Anticancer; Antioxidant.

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ISSN: 0975-8585

INTRODUCTION

About 80% of the population in the third world countries relies on traditional plant based medicines for their primary health care needs [1]. Natural products and related drugs are used to treat 87% of different human diseases [2]. About 25% of the prescribed drugs in the world are prepared from a variety of plant materials as leaves, stems, roots, bark etc. [3,4]. However, the majority of these plants have not yet undergone chemical, pharmacological and toxicological studies to investigate their bioactive compound(s) as well as their mode of action [5]. The plant *Terminalia bellerica* Roxb. is a member of the genus Combreta of the family Combretaceae. This plant has been introduced by the Arabs from India and has been reported to have many medicinal properties and used as popular folk medicine in Asian and African countries [6]. *Terminalia bellerica*, is one of the most important medicinal plants with wide pharmacological applications. It is widely used in Ayurveda, Siddha, Chinese medicine and Unani. Furthermore, It has been found to show antimicrobial, antioxidant, antidiarrhoeal, antidiabetic, analgesic, immunomodulatory, antihypertensive, antisalmonella, hepatoprotective, antispasmodic, bronchodilatory activities and treatment of gastric ulcer, constipation and general debility. Hence, this plant provides a significant role in the prevention and treatment of several diseases. Further evaluation needs to be carried out in order to explore the concealed areas and their practical clinical applications, which can be used for the welfare of the mankind [7].

Only limited research has been performed on the leaves of *Terminalia bellerica* [8]. Therefore, in this study, we report for the first time on the cytotoxic activity of five different leave extracts of *Terminalia bellerica* to evaluate their anticancer potential as well as their corresponding total phenol content and antioxidant activities.

MATERIALS AND METHODS

Materials

Sulforhodamine B (SRB), Roswell Park Memorial Institute (RPMI) 1640 medium, gallic acid, rutin, 1,1-diphenyl-2-picryl hydrazyl (DPPH), fluorouracil® ≥99% (HPLC), cytarabine® ≥90% (HPLC), gemcitabine® ≥98% (HPLC), aldesleukin® ≥98% (HPLC), tamoxifen® ≥99% and doxorubicin® 98.0-102.0% (HPLC) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Capecitabine® supplied by Roche, Basel, Switzerland. The Folin-Ciocalteu's phenol reagent was from Fluka Chemie AG, Buchs, Switzerland. Fetal bovine serum (FBS) was purchased from Gibco, UK. Dimethyl sulfoxide (DMSO) and methanol were of HPLC grade and all other reagents and chemicals were of analytical reagent grade.

Plant Material

Terminalia belerica leaves were collected from Giza Zoo, Cario-Egypt in the summer of 2013. A voucher specimen identified by Dr. T. Labeb, Herbarium of Orman garden, was deposited in the Herbarium of the National Research Centre, Cairo, Egypt. The leaves were cleaned, air-dried in the shade, and then powdered by laboratory mill to 24 meshes. Powdered materials were maintained in an airtight container at room temperature (28 ± 2ºC), and protected from light until use [8].

Plant Extraction

The dried plant leaves were powdered and extracted (25g) exclusively with 100 ml (1 x 3) each of petroleum ether, chloroform, ethyl acetate, methanol and 70% aqueous methanol in a soxhlet extractor for 4 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40-50°C). The five extracts yielded greenish brown residues, weighing 0.45 g (0.018 w/w), 0.39 g (0.0156 w/w) and 0.41 g (0.0164 w/w), 0.70 g (0.028 w/w) and 0.66 g (0.0264 w/w) respectively. All the extracts were preserved in a refrigerator until further use [9].

Biochemical Screening

Determination of Total Polyphenol Content (TPC)

The total phenolic content in each extract was determined according to the Folin-Ciocalteu method [10] with some modification to minimize the volume of the reactants used to microlitres [9]. 10 µL of each extract (1 mg/mL) was mixed with 50 µL of Folin–Ciocalteu phenol reagent (10 x dilutions) and allowed to react for 5 min. Then 40 µL of 20% saturated Na_2CO_3 solution was added and allowed to stand for 1 h in the dark before the absorbance of the reaction mixture was read at 725 nm using SpectraMax® Paradigm® Multi-Mode microplate reader (Molecular Devices). A gallic acid standard curve was obtained for the calculation of phenolic content. The total polyphenol content (TPC) of each extract was expressed as mg gallic acid equivalents per gram of plant extract on a dry-weight basis.

DPPH Radical Scavenging Assay

The antioxidant activity of each extract and standard were assessed based on their radical scavenging effect of the stable DPPH free radical [11]. Different concentrations of each extract were dissolved in distilled DMSO and used. 10 µL of each extract or standard (from 0.0 to 300 µg/mL) was added to 90 µL of a 100 µM methanolic solution of DPPH in a 96-well microtitre plate (Sigma-Aldrich Co., St. Louis, MO, US). After incubation in the dark at 37°C for 30 min, the decrease in absorbance of each solution was measured at 520 nm using SpectraMax® Paradigm® Multi-Mode microplate reader. Absorbance of blank samples containing the same amount of either water or DMSO and DPPH solution was also prepared and measured. The scavenging potential was compared with a solvent control (0% radical scavenging) and ascorbic acid. The percentage of $DPPH^{\bullet}$ bleaching was utilized to calculate the IC50 (half-maximal inhibition concentration).

Radical scavenging activity was calculated by the following formula:

$$\% \text{ Reduction of absorbance} = [(A_B - A_A) / A_B] \times 100,$$

where: A_B – absorbance of blank sample and A_A – absorbance of tested extract solution (t = 30 min) [10,12].

In vitro Anticancer Screening

Cell Culture

Fourteen human cell lines from ten different cancer types, namely: SK OV-3 (human ovarian carcinoma cell line), Hep-G2 (liver carcinoma cell line), MCF-7 (breast carcinoma cell line), KB (human-HeLa contaminant; cervical carcinoma), HeLa (cervical carcinoma), SF-268 (CNS-human glioblastoma cell line), NCIH460 (non-small lung cancer), RKOP27 (colon adenocarcinoma), HT-1080 (fibrosarcoma), HL60; U937; K561 (leukemia) and G361; SK-MEL-28 (melanoma) were purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100U/ml penicillin and 100U/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Cytotoxic Activity (Sulforhodamine B Assay)

Human cancer cell lines were grown in RPMI 1640 medium (37°C, 5% CO_2) to assess the growth inhibition by a colorimetric assay, which estimates cell number indirectly by staining total cellular protein with Sulforhodamine B (SRB) dye [13,14]. Logarithmically growing cells were seeded at a density of 10^4 cells/well into 96-well plates, and allowed to adhere for 24 h at 37°C. After 24 h, two plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of each extract addition. In the rest of the plates, the supernatant was replaced by 100 µL culture medium supplemented with each extract in DMSO at different concentrations and incubated at 37°C for 48 h. The final concentration of DMSO in the solution in each well was 0.5%. Treatment with DMSO only was always used as a control. At the end of the treatment, the supernatant from each well was discarded and cells were fixed by layering 100 µL ice-cold 10% trichloroacetic acid (TCA) on top of the growth medium and then incubated at 4°C for 1 h. The plates were then washed five times with cold water, the excess water was drained off, and the plates were air-

dried. SRB stain (100 µL; 0.4 (w/v) in 1% acetic acid) was added to each well and left in contact with the cells for 1 h. Subsequently, the cells were washed with 1% acetic acid and rinsed four times. The plates were dried, and 100 µL of 10 mM Tris base was added to each well to dissolve the dye. The plates were shaken gently for 20 min, and absorbance (OD) of each well was read at 540 nm. Cell survival was measured as the percentage of absorbance compared to the control. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 mL of 80% TCA (final concentration, 16% TCA). The $\log_{10}GI_{50}$ parameter, which represents the 50% growth inhibition concentration (GI_{50}) at which the percentage growth (PG) is 50, was calculated for each cell line [15-17].

Statistical Analysis

All the experiments were repeated at least three times in three different days, and each experiment was conducted in triplicate and error bars represent standard deviations (SD). All the values were represented as mean ± SD. The IC_{50} values were determined by probit analysis using SPSS software program (SPSS Inc., Chicago, IL). Correlation coefficients (R) to determine the relationship between two variables (radical scavenging test and content of total phenolic compounds) were calculated using MS Excel software (CORREL statistical function).

RESULTS AND DISCUSSION

The extensive literature survey revealed that plant drugs are considered less toxic and free from side effects than synthetic drugs. *Terminalia bellerica* is a large deciduous tree which represent an important medicinal plant with diverse pharmacological spectrum [18]. Different parts of *Terminalia bellerica* exhibited many pharmacological activities [19-22]. However, only limited research has been performed on its leaves, [8, 23]. In this study we report, for the first time, investigation of the anticancer and antioxidant activities of different *Terminalia bellerica* leaf exclusive extracts. Therefore, in this study, a series of five exclusive extracts from *Terminalia belerica* Roxb. leaves, differing with regard to solvent polarity, namely; methanol, 70% aqueous methanol, ethyl acetate, chloroform and petroleum ether extracts, have been prepared and investigated for their antioxidant activities, total phenol contents as well as a preliminary anticancer screening for the first time.

DPPH Radical Scavenging Activity

The stable radical DPPH has been used widely for the determination of primary antioxidant activity of pure antioxidant compounds, extracts and food materials. The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 520 nm. Organic solvent extracts of *Terminalia belerica* Roxb. were assayed for their radical scavenging activity using the DPPH colorimetric test. Figure **1** shows the antioxidant activities of the five extracts compared to that of ascorbic acid and rutin. The antioxidant activities of these extracts ranged from 3.6 µg/mL to 68.6µg/mL as shown in Table **1**. The highest DPPH radical scavenging effect was detected in the methanol extract with an IC_{50} of 3.6 µg/mL followed by the aqueous methanol extract that showed an IC_{50} of 4.2µg/mL compared to the antioxidant activity of ascorbic acid (IC_{50} 7.6 µg/mL) and that of rutin (IC_{50} 17.2 µg/mL) which are often used as positive controls because of their high antioxidant activities. The ethyl acetate extract showed an IC_{50} of 45.5 µg/mL, while the chloroform extract showed an IC_{50} of 49.9 µg/mL. The petroleum ether extract showed the least antioxidant activity with IC_{50} of 68.6µg/mL. In examining radical scavenging capacity, it can be observed that the more polar protic solvents are more effective at extracting the antioxidant components in the *Terminalia belerica* Roxb. extract. These results are in agreement with the previously reported data [10,12].

Total Polyphenol Contents (TPC)

The total amount of phenolic compounds in different exclusive extracts were determined using the Folin-Ciocalteu method. The Folin–Ciocalteu phenol reagent is used to obtain an estimate of phenolic compounds present in an extract. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the reagent. However, the assay has been shown not specific to polyphenols in that other oxidizable components can react with the Folin reagent [10,24,25]. In addition, phenolic compounds, depending on the number of phenolic groups they have, respond differently to the Folin–Ciocalteu reagent [23]. The total phenol content of these extracts ranged from 72.1 mgGAE/g extract

ISSN: 0975-8585

to 131.1 mgGAE/g extract as shown in Table **1**. The methanol extract exhibited the highest level of polyphenols content (131.1mgGAE/g extract). The aqueous methanol extract exhibited the second highest level of polyphenols (126.7 mgGAE/g extract). The ethyl acetate extract exhibited the third level of polyphenols content (124.7 mgGAE/g extract) followed by the chloroform extract, which exhibited the fourth level of polyphenols content (121.3 mgGAE/g extract). The petroleum ether extract showed the least level of polyphenolic content (72.1 mgGAE/g extract) among all the five investigated extracts. From the above results, we can estimate that methanol and aqueous methanol extracts, which showed the highest antioxidant activities, also had the highest amount of polyphenols. Ethyl acetate and chloroform extracts, which showed moderate antioxidant activities, had moderate TPC as well. Petroleum ether extract showed the lowest TPC and the lowest antioxidant activity.

Figure 1: The antioxidant activities of five different extracts of *Terminalia belerica* Roxb. leaves using DPPH scavenging method.

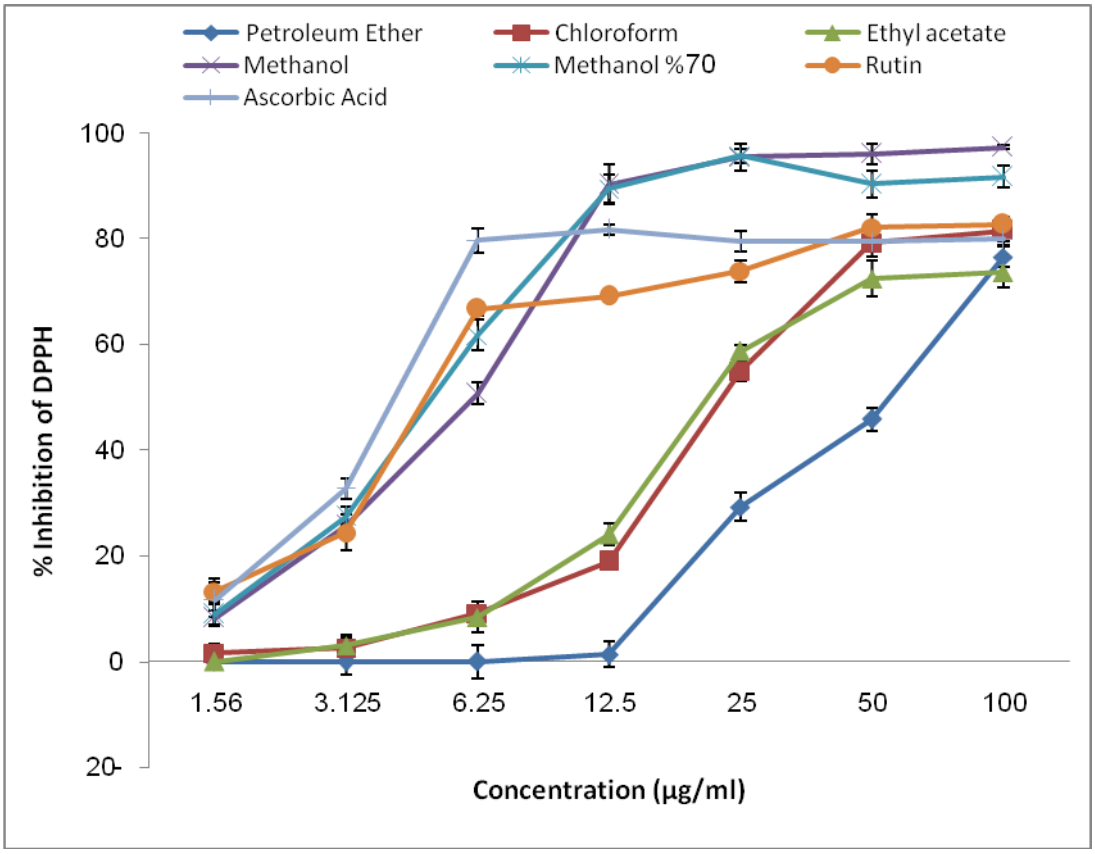

Table 1: Antioxidant activities and total phenol contents of five different exclusive extracts of *Terminalia belerica* Roxb. leaves.

Extract	DPPH-IC_{50} (µg/ml) Mean ± SD (n = 3)	TPC mgGAE/g plant extract Mean ± SD (n = 3)
70% Methanol	4.2 ± 1.23	126.7 ± 2.26
Methanol	3.6 ± 1.98	131.1 ± 3.40
Ethyl acetate	45.5 ± 2.09	124.7 ± 2.92
Chloroform	49.9 ± 3.12	121.3 ± 1.59
Petroleum Ether	68.6 ± 2.74	72.1 ± 2.46
Ascorbic acid	7.6 ± 2.18	-
Rutin	17.2 ± 1.83	-

ISSN: 0975-8585

Table 2: The $log_{10}GI_{50}$ of the *in vitro* anticancer activities of the five extracts of *Terminalia belerica* Roxb. leaves against fourteen cell lines from ten different human cancer types.

Cells	70%Methanol	Methanol	Ethyl acetate	Chloroform	Petroleum Ether	Fluorouracil	Doxorubicin	Cytarabine	Gemcitabine	Capecitabine	Aldesleukin	Tamoxifen
KB	NA	NA	NA	NA	4.64×10^{-17}	4.46×10^{-3}						
SK OV-3	NA	NA	NA	NA	8.86×10^{-14}		4.16×10^{-3}					
SF-268	NA	NA	NA	NA	8.44×10^{-14}			7.68×10^{-3}				
NCI H 460	NA	NA	NA	NA	5.55×10^{-17}				2.13×10^{-3}			
RKOP27	NA	NA	NA	NA	5.97×10^{-17}					4.33×10^{-3}		
HL60	6.45×10^{-14}	8.55×10^{-17}	5.67×10^{-17}	4.56×10^{-17}	3.45×10^{-17}		1.13×10^{-3}					
U937	6.89×10^{-14}	$5,78 \times 10^{-17}$	5.32×10^{-14}	4.56×10^{-13}	7.89×10^{-17}		4.45×10^{-3}					
K561	5.89×10^{-14}	5.43×10^{-17}	5.67×10^{-17}	5.78×10^{-13}	7.89×10^{-17}		6.66×10^{-3}					
G361	6.59×10^{-17}	4.58×10^{-17}	5.68×10^{-17}	4.57×10^{-13}	7.69×10^{-14}						6.66×10^{-3}	
SK-MEL-28	6.79×10^{-13}	7.69×10^{-17}	5.69×10^{-17}	4.69×10^{-13}	9.87×10^{-17}						3.45×10^{-3}	
HeLa	NA	NA	NA	5.69×10^{-17}	6.59×10^{-17}							0.11×10^{-3}
MCF-7	NA	NA	NA	5.66×10^{-17}	5.69×10^{-17}		1.98×10^{-3}					
HT-1080	NA	NA	NA	8.79×10^{-13}	8.79×10^{-13}							1.16×10^{-3}
HepG2	NA	NA	NA	6.77×10^{-17}	7.89×10^{-17}							1.31×10^{-3}

NA= No Activity

Statistical Analysis of TPC against the Antioxidant Activity

With reference to the above mentioned results, it is obvious that, the correlations of TPC against the antioxidant activity based on the DPPH assay involving all five extracts were moderately correlated reflected by the moderate correlation coefficient (≤ 0.7), confirming that, not only the phenolic compounds contribute to the radical scavenging activity of these extracts [10] but there may be other compounds than phenolics are responsible for these high antioxidant activities. This may explain our data which showed that only minor differences are found in total phenol content, but major differences in antioxidant activity: E.g., by comparing the 70% methanol extract against the chloroform extract, a more than ten-fold difference in DPPH-IC_{50} between the 70% methanol extract (4.2 ± 1.23) and the chloroform extract (49.9 ± 3.12) could not be based on a less than 5% difference in TPC (126.7 ± 2.26 vs. 121.3 ± 1.59).

Anticancer Activity

The five extracts were screened for their *in vitro* anticancer activities using SRB assay. Each extract was tested at seven different concentrations against fourteen cell lines of ten types of human cancers, namely, liver, cervical, leukemia, lung, colon, CNS, melanoma, ovarian, breast and fibrosarcoma cancer. Results are expressed as $\log_{10}GI_{50}$, which is the $\log_{10}$ extract concentration (µg) that caused a 50% reduction in the proliferation for each cell line compared to the control cells during the drug incubation. Their activities were compared as well with the cytotoxicity of different anticancer drugs namely, fluorouracil®, cytarabine®, gemcitabine®, capecitabine®, aldesleukin®, tamoxifen® and doxorubicin®; positive controls. The data were expressed as average values obtained from five wells. Table **2** represents the anticancer activities of each extract. These results reveal that, all the five extracts show very high anticancer activities toward leukemia (HL60; U937; K561) and melanoma (G361; SK-MEL-28) cell lines compared to the positive controls, doxorubicin® and aldesleukin® respectively. In addition, chloroform and petroleum ether extracts showed very high anticancer activities against HeLa (cervical carcinoma), MCF-7 (breast carcinoma cell line), HT-1080 (fibrosarcoma) and Hep-G2 (liver carcinoma cell line) compared to tamoxifen® and doxorubicin®. However, the methanol, 70% methanol and ethyl acetate extracts did not show any significant anticancer activities against those four human cancer cell lines. Furthermore, petroleum ether extract showed very high anticancer activities against KB (human-HeLa contaminant; cervical carcinoma), SK OV-3 (human ovarian carcinoma cell line), SF-268 (CNS-human glioblastoma cell line), NCIH460 (non-small lung cancer) and RKOP27 (colon adenocarcinoma) compared with the positive controls; fluorouracil®, doxorubicin®, cytarabine®, gemcitabine® and capecitabine® respectively. The other four extracts did not show any anticancer activity against those five human cancer cell lines.

CONCLUSION

Based on the results of this study, it can be postulated that, *Terminalia belerica* Roxb. leaves are a potential source of phenols, natural antioxidants as well as anticancer candidates. Phenolic content, the antioxidant activities and the anticancer potency of the extracts depend on the solvents used for extraction. Especially petroleum ether extract represents a highly promising extract for anticancer candidate drugs. However, further fractionation of the petroleum ether extract is needed to purify and identify the active metabolites, which are responsible for these activities. To identify the metabolites responsible for the anticancer and antioxidant activities observed in these different extracts, a large-scale phytochemical analysis of *Terminalia belerica* Roxb. leaves is now being performed at our laboratory and the results that will be obtained will be published elsewhere.

ACKNOWLEDGEMENTS

This work was supported financially by: Cross-Border Cooperation within the European Neighborhood and Partnership Instrument (ENPI), Mediterranean Sea Basin Programme 2007-2013, Ref No: I-B/4.1/257 (contract no.12/2337); BRAMA (Botanicals Risk Assessment training in the Mediterranean Area).

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