Global Journal of Pharmacology 7 (4): 448-456, 2013
ISSN 1992-0075

DOI: 10.5829/idosi.gjp.2013.7.4.76103

Chemical Investigation and Antioxidant Activity of Phenolic Acids from the Leaves of *Terminalia arjuna*

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Abstract: *Terminalia arjuna*, a well-known medicinal plant has extensively been used in Ayurvedic medicines to treat various diseases. The present study was investigated the phenolic content and antioxidant activity of 70% acetone extract of *Terminalia arjuna* and to isolate and identify its certain phenolic constituents as well as assessment the antioxidant potential of purified compounds via two qualitative *in vitro* antioxidant assays (1,1'-diphenyl-2-picrylhydrazyl) free radical scavenging (DPPH) and phosphomolybdenum as wall as total phenolic content was determined via Folin-Ciocalteu assay. Owing to the high antioxidant of the 70% acetone extract it undergoes successive fractionation via; diethyl ether, petroleum ether, CH_2Cl_2, EtOAc and n-BuOH. n-BuOH sub-fraction undergoes chromatographic separation upon polyamide CC. to afforded three compounds (1-3), their structures were established via UV, IR, ^1H and ^{13}C-NMR analyses and identified as; gallic acid (1), methyl gallate (2) and ellagic acid (3). Also, EtOAc sub-fraction undergoes chromatographic separation upon silica gel CC. to afforded one compound, identified as; vanillic acid (4). The DPPH results for all tested fractions was ranged from 9.06 to 132.54 µg/ml and for the four isolated compounds showed DPPH scavenging activity with SC_{50} values of 6.0, 7.06, 6.38 and 7.87 µg/ml, respectively for compounds 1, 2, 3 and 4 and total antioxidant capacity of all tested fractions was ranged from 84.81 to 439.23 (mg AAE /g extract); and for compounds (1-4) was; 307.10, 215.55, 274.0 and 178.19 (mg AAE /g compound), respectively for compounds 1, 2, 3 and 4. It was concluded that, the isolated phenolic compounds of *Terminalia arjuna* leaves exhibited a potent antioxidant activity.

Key words: *Terminalia arjuna* · Chromatographic Isolation, Phenolics · Antioxidant Activity

INTRODUCTION

Free radicals can be classified into oxygen, nitrogen, carbon and sulphur based molecules and include hydroxyl radical (OH^-), superoxide anion ($O2^-$), singlet oxygen ($^1O2$) and hydrogen peroxide (H_2O_2). The hydroxyl radical is the most reactive of the oxygen centered radicals and will react with the first available bio-molecule they encounter. Other free radicals include the 2 gaseous radicals nitric oxide (NO^-) and nitrogen dioxide (NO^{2-}) and the carbon centered radicals (R^-) of organic compounds; alkoxyl (RO^-) and peroxyl (ROO.) formed during the peroxidation of lipids. While the sulphur based radicals (RS.) and ($RS\text{-}SR^-$) are involved in cellular function [1]. Reactive oxygen species (ROS), including superoxide anion radical ($O2^-$), Hydroxyl radical (OH^-), nitric oxide (NO^-) radical and hydrogen peroxide (H_2O_2) are physiological metabolites. Small amounts of ROS indicate that exposure to ROS leads to deleterious changes of cell function by a number of alterations, such as lipid peroxidation, enzyme inactivation and oxidative DNA damage [2]. Also, the accumulation of ROS has been postulated to be implicated in the aging process [3]. Therefore, excessive generation of ROS induced various stimuli, which exceeds the antioxidant capacity of the organism, leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity and cancer [4]. The approach to develop new drugs through natural products might be the

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single most successful strategy [5, 6]. Natural products like; plant extract, can provide unlimited opportunities for new drug discoveries because of their unmatched chemical diversity. Medicinal plants provide important ingredients in modern medicines, nutraceutical, food supplements, folk medicines and lead compounds for synthetic drugs [7, 8]. Screening medicinal plants for novel bioactive compounds are the sole remedy since, plant based drugs are biodegradable, safe and have fewer side effects [9]. Thus, the therapeutic potential of the herbs opens up new vista in the future pharmacological research of herbal drug development. Besides, in the last few years, interest in the antioxidant activity of the plant extracts has increased tremendously which is very important due to the fact that free radicals can be responsible for various diseases like; heart diseases, strokes, arteriosclerosis, cancer as well as for ageing processes. Potent sources of natural antioxidants and antimicrobials can reduce the use of synthetic forms such as Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT). Since, many antioxidants are of plant origin, they can react rapidly with these free radicals thereby causing retardation in the extent of oxidative deterioration [10]. Moreover, consumption of antioxidant or addition of antioxidant to food materials could protect the body against these harmful effects of free radicals [11]. In modern days, the antioxidants and antimicrobial activities of plant extracts have formed the basis of many applications in pharmaceuticals, alternative medicines and natural therapy [12]. Promising free radical scavenging activity of some plant extracts against food spoilage have also been well reported [13]. In recent years plant derived medicines have received great deal of attention compared to synthetic ones, due to their potent antioxidant activities, very less side effects and economic viability. Numerous naturally occurring phenolic antioxidants that readily deal with oxidation and have potential to minimize the effects of autoxidation, have been identified in many plant sources [14]. *Terminalia arjuna* Wight & Arn. (family: Combretaceae) popularly known as 'Arjuna' is a large deciduous tree. The plants of genus Terminalia comprising of 250 species are widely distributed in tropical areas of the world and warm especially in Africa [15]. It is traditionally used in the management of cardiovascular diseases, myocardial infarction, amyloidosis, acute pancreatitis, arthritis, atherosclerosis, inflammatory bowel disease, diabetes, senile dementia and retinal degeneration [16]. *In vitro* antioxidant of *T. arjuna* has been well investigated [17]. The genus Terminalia is known as a rich source of pentacyclic triterpenes and their glycoside derivatives, flavonoids, tannins and other aromatic compounds [18]. Therefore, the present investigation was undertaken to isolate and elucidate certain phenolic compounds from the plant under investigation as well as evaluation their total antioxidant capacity and their DPPH free radical scavenging activity.

MATERIALS AND METHODS

Plant Materials: Leaves of *Terminalia arjuna* (Combretaceae) were collected from Zoological Garden, Giza, Egypt in August-September, 2010. Authentication and identification of the plant was kindly established by Mrs. Teresa Labib, General Manager and head of plant Taxonomy in El-Orman Botanical Garden, Giza, Egypt. Voucher specimen given (TA_1) was deposited at Laboratory of Medicinal Chemistry, Theodor Bilharz Research Institute. The fresh plant leaves were completely dried in shade place at room temperature and then powdered by electric mill. The dried powder was kept until subjected to the further process.

Experimental Procedures: ^{1}H-NMR (500 MHZ, DMSO-d6) and ^{13}C-NMR (125 MHZ, DMSO-d6) spectra were recorded on JEOL-GX-spectrometer (NRC). The chemical shifts were expressed in δ (ppm) with reference TMS and coupling constant (J) in Hertz. UV spectra were determined in methanol (Micro Analytical Center, Faculty of Science, Cairo). Melting points were determined on an electrothermal apparatus. Silica gel 60 GF254 (Fluka) were used for analytical TLC. Sephadex LH-20 (25-100 μm, Sigma) or silica gel (70-230 mesh, Merck) were used for column chromatography. Paper chromatography was carried out on Whatmann No. 1 or No. 3 paper sheets (Whatmann, England). Spots were visualized by absorption of UV radiation and spraying with methanolic 1% $FeCl_3$ or/and 5% $AlCl_3$ or 40% H_2SO_4 followed by thermal activation.

Material and Chemicals: All solvents and reagents used were of analytical grade. 1,1'-diphenyl-2-picraylhydrazyl ($DPPH^{\bullet}$) free radical was purchased from (Sigma-Aldrich Co.). Aluminum chloride, sodium phosphate, ammonium molybdate, ascorbic acid and gallic acid were purchased from (Merck Chemical Co.), all solvents and acids [petroleum ether, chloroform, methylene chloride, ethyl acetate, n-butanol, acetic acid and sulphuric acid), were purchased from (Sigma-Aldrich Co.). The absorbance measurements for antioxidant activity were recorded using the UV-Vis spectrophotometer Spectronic 601

(Milton Roy, USA).

Extraction and Chromatographic Isolation: The dry powder of *Terminalia Arjuna* leaves (1.5 Kg) was soaked in 70 % acetone (7 L x 3) at room temperature, for 6 weeks. After concentration in vacuo it gives (420 g) crude 70% acetone extract. The crude acetone extract was successively extracted with diethyl ether, petroleum ether, methylene chloride and ethyl acetate to give (18 g, 11.6 g, 3.04 g and 8.8 g), respectively for; ether extract, petroleum ether extract, methylene chloride and ethyl acetate extracts. The residue was partitioned between water and n.butanol to give (34 g) butanol extract and the remaining water extract was desalted by dissolving the extract with water then precipitation with methanol. The filtrate evaporated till dry to give (51.6) g water extract. Preliminary phytochemical screening and the two dimensional paper chromatography (TDPC) of each of ethyl acetate and n-BuOH extracts using two common solvent systems 15% AcOH (AcOH: H_2O; 15:85) and BAW (n-BuOH: AcOH: H_2O; 4:1:5 upper layer) revealed the presence of promising variety of phenolic compounds. Therefore these two extracts were submitted to chromatographic isolation.

Determination of Total Phenolic Content: The total phenolic compounds of plant extracts were determined by the Folin-Ciocalteau method [19]. Briefly, 100 µl of extract solution (containing 100 µg extract in 1.0 ml methanol), 0.5 ml of Folin-Ciocalteau reagent (Sigma-Aldrich Chemie, Steinheim, Germany) was added and mixed thoroughly. Three minutes later 1.5 ml of 20 % sodium carbonate was added and the mixture was diluted with 7.9 ml of distilled water. And the mixture was incubated at ambient temperature (25-27°C) for 90 min with intermittent shaking. The absorbance of the blue color that developed was read at 760 nm using spectrophotometer (UV-VIS spectrophotometer, Milton Roy 601). The concentration of total phenols was expressed as mg/g of dry extract. The concentration of total phenolic compounds in the extract was expressed as mg gallic acid equivalents (GAE) per g of extract (mg GAE/g).

Antioxidant Activity

DPPH Radical Scavenging Activity: DPPH free radical scavenging assay was measured using DPPH free radical test [20]. DPPH is one of the powerful free radical which is used to evaluate the electron donating capacity of antioxidants. The different concentrations of each of the extracts and pure compounds were prepared in methanol and were added to 3ml of 0.1mM methanolic solution of DPPH. Control was prepared containing the same solvents and reagents without any extract. The tubes were shaken vigorously and allowed to stand for 30 min at room temperature in the dark. Changes in absorbance of samples were measured at 517 nm. A control reading was obtained using methanol instead of the extract. Ascorbic acid served as the standard. Free radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula:

$$\% \text{ inhibition} = ((A_0 - A_t) / A_0 \text{ X } 100$$

where, A_0 is the absorbance of the control, A_t is the absorbance of test samples. All the tests were performed in triplicates and the results are reported as SC_{50}, which is the amount of antioxidant necessary to decrease the initial $DPPH^{\bullet}$ concentration by 50%. The lower SC_{50} value corresponds to a higher scavenging activity (higher antioxidant activity) of plant extract [21].

Determination of Total Antioxidant Capacity: Total antioxidant activity (TAA) of each sequential extract and pure compound was determined according to the method of Prieto *et al*. [22] Briefly 0.3 ml of sample was mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95°C for 90 min under water bath. Reading was taken at 695 nm after cooled to room temperature against blank, the blank consisted of all reagents and solvents without the sample and it was incubated under the same conditions. All the tests were performed in triplicate and the total antioxidant activity is expressed as the number of equivalents of ascorbic acid (AAE).

Chromatographic Isolation of n-butanol and Ethyl Acetate Extracts of *T. arjuna:* Butanol extract (30 g) was subjected to polyamide column (5 x 125 cm) chromatography using elution system water, water: methanol with different gradients, finally pure methanol. Fractions (500 ml each) were collected; tested using paper chromatography (PC), developed with solvent systems 15 % Acetic acid and BAW (n-BuOH: AcOH: H_2O; 4:1:5 upper layer). Similar fractions were collected together to give 13 major fractions which subjected separately for further purification according to the result of PC chromatogram. Finally three pure compounds (1-3) were obtained. The ethyl acetate soluble part from the acetone

extract (8 g) was subjected to open column chromatography (70 x 3 cm) packed with silica gel 60 (70-230 mesh, Merk) adsorbent as stationary phase. Elution was started with petroleum ether (60-80) followed by gradient mixtures of petroleum: EtOAc till reached (100%) ethyl acetate then followed by gradient mixtures of ethyl acetate: MeOH and till reached 100% methanol at the end. Fractions of 100 ml were collected and combined together according to their PC behavior after screening of their spots over paper chromatography in two common eluents (15%) acetic acid and BAW and over thin layer chromatography (TLC) using different solvent system according to their nature. The combined fractions eluted with 70% petroleum ether-EtOAc were passed through a Sephadex LH-20 column and eluted with $CHCl_3$: MeOH (2:1) mixture and then subjected to preparative TLC to give compound (4).

- Compound 1 was obtained as beige to off-white powder, m.p. (251-252 °C) and R*f* = 0.24 (hexane: ethyl acetate: acetic acid, 2:1:0.3 v/v/v), on silica gel TLC and 0.52 (15% AcOH), 0.78 (BAW) on PC. It gave a positive test with $FeCl_3$. IR v_{max} (KBr): 3373.83, 3288.04, 1704.76, 1618.95, 1537.95, 1450.21, 1248.66 and 770.42 Cm^{-1}. UV λ_{max} (nm): (MeOH) 211, 267. ^{1}H-NMR (500 MHz, DMSO-d6), spectra showed peaks at: δ 6.91 (2H, s, H-2, H-6). ^{13}C-NMR (125 MHz, DMSO-d6): δ 120.9 (C-1), 109.7 (C-2, C-6), 145.9 (C-4, C-6), 138.51 (C-5) and 168.04 (C-7).
- Compound 2 was isolated as white solid; m.p. (201-203 °C) and R*f* = 0.69 (15% AcOH) and 0.61 (BAW) on PC, it appear as violet in UV light and gave a positive test with $FeCl_3$.

IR v_{max} (KBr): 3362.40, 2919.71, 1693.91, 1418.58, 1259.68, 1162.48 and 738.99 Cm^{-1}. UV λ_{max} (nm): (MeOH) 225, 273. ^{1}H NMR (500 MHz, DMSO-d_6), spectra showed peaks at: 7.08(2H, s, H-3, 7) and 3.78 (3H, s, -OCH3). ^{13}C NMR (125 MHz, DMSO-d_6): 166.87 (C-7), 146.14 (C-4, C-6), 138.98 (C- 5), 119.80 (C-3), 109.05 (C-2, C-6) and 52.12 (OCH_3).

- Compound 3 was isolated as cream to light yellow powder; m.p. (162-165 °C) and R*f* = 0.69 (15% AcOH) and 0.61 (BAW) on PC, it show faint yellow in visible light, blue in UV. Spraying with $AlCl_3$ gave yellow while gave dark blue color with $FeCl_3$ reagent. IR v_{max} (KBr): 3440, 1730, 1560, 1520 Cm^{-1}. UV λ_{max} (nm): (MeOH) 280, 310. ^{1}H NMR (500 MHz, DMSO-d_6), spectra showed peaks at: 7.63 (2H: H-5, H-5`), 8.3 (2H, OH-4`, OH-4), 12.10 (2H, OH-3`, OH-3). $^{13}$C NMR (125 MHz, DMSO-$d_6$): 113.60 (C-1, C-1`), 136.80 (C-2, C-2`), 140.38 (C-3, C-3`), 148.74 (C-4, C-4`), 111.54 (C-5, C-5`), 108.80 (C-6, C-6`) and 161.28 (C-1``, C-2``).
- Compound 4 was isolated as white to light yellow powder; m.p. (210-213 °C) and R*f* = 0.68 (BAW) and 0.84 (15% AcOH) on PC and 0.98 (ethyl acetate: formic acid: acetic acid: water; 100:11:11:26) on TLC, it show blue in visible light, faint blue in UV. UV λ_{max} (nm): (MeOH) 259, 292 nm. ^{1}H NMR (500 MHz, DMSO-d_6), revealed the presence of the following signals at: 6.43 (1H, d, H-5), 7.17 (1H, d, H-2), 7.25 (1H, dd, H-6), 9.18 (1H, s, 4-OH) and 3.58 (3H, s, 8-OCH_3). ^{13}C NMR (125 MHz, DMSO-d_6): 189.99 (C-7; C=O), 160.36 (C-3), 149.97 (C-4), 128.40 (C-6), 125.12 (C-1), 116.48 (C-5), 109.91 (C-2) and 55.67 (C-8).

Statistical Analysis: All data were presented as mean ± SD in triplicate 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 [23].

RESULTS AND DISCUSSION

Determination of Total Phenolic Contents: Polyphenolic compounds including variety of secondary metabolites (flavonoids, tannins, phenolic acids and among others). Polyphenolics are very vital constituents in medicinal plants due to their potential scavenging and powerful chain breaking antioxidant abilities as well as their ability to stabilized lipid peroxidation, which return to their hydroxyl pattern [24]. The total phenolic content (TPC) of 70% acetone extract of *T. arjuna* was 96.04 (mg GAE / g ext.) and its derived subfractions showed different values of TPC which decreasing in the order; n-BuOH (996.52), CH_2Cl_2(388.64), EtOAc (242.98), diethyl ether (166.43) and pet. ether (109.16) (mg GAE / g ext.). Due to the high phenolic contents in n-BuOH and EtOAc subfractions, these fractions were subjected to chromatographic isolation to identify its phenolic constituents. Plant polyphenols, a diverse group of phenolic compounds (flavanols, flavonols, anthocyanins, phenolic acids, etc.) possess an ideal structural chemistry for free radical scavenging activity [25, 26].

Antioxidant Activity

Determination of Free Radical Scavenging Activity

(DPPH): The antioxidant activities in the plant extracts were focused on phenolic compounds and this assay used to assess their antioxidant activities is based on radical scavenging mechanism. DPPH free radical assay is a method used to evaluate the antioxidant activities in a relatively short time compared with other methods. This method is not affected by side reactions such as metal chelation and enzyme inhibition which is a major disadvantage associated with laboratory generated free radicals. The different extracts of *T. arjuna* showed effective free radical scavenging activity in DPPH radical assay. It exhibited remarkable antioxidant effect even at very low concentration. Free radical scavenging activity of 70% acetone extract of *T. arjuna* as well as its derived subfractions and purified compounds (1-4) was evaluated on DPPH-free radical (Tables 2, 3). Inhibition of DPPH radicals above 50 % is considered to be significant for antioxidant properties of any compound [22]. The DPPH results for all tested fractions were ranged from 9.06 to 132.54 µg/ml. This results were supported by previous studies which revealed that the *T. arjuna* bark extract have shown antioxidant effect on N-nitrosodiethylamine (DEN) induced hepatocellular carcinoma in rats [27]. The four isolated compound showed DPPH scavenging activity with SC_{50} values of (6.0, 7.06, 6.38 and 7.87 µg/ml), respectively for compounds 1, 2, 3 and 4 with respect to the standard ascorbic acid 8.65 µg/ml The antioxidant activity of phenolic acids (cinnamic acid or benzoic acid derivatives) and their esters based upon the number and arrangement of free hydroxyl groups attached to the phenolic acid nucleus (hydroxylation pattern), in which this activity would be enhanced via steric hindrance [28, 29].

Determination of Total Antioxidant Capacity: Free radical scavenging of phenolic compounds is an important property 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 [22]. The total antioxidant capacity of the 70% acetone extract of *T. arjuna* as well as the derived fractions of was 317.08, 84.81, 359.27, 121.21, 370.70 and 439.23 (mg AAE /g ext.), respectively for; 70% acetone, diethyl ether, pet. ether, CH_2Cl_2, EtOAc and n-BuOH extracts. This activity may be due to presences of high amounts of phenolic compounds like tannins [30]. On other side, the total antioxidant capacity of the four purified phenolic compounds (1-4), was in the order; 1 (307.10) > 3 (274.0) > 2 (215.55) > 4 (178.19) (mg AAE /g compound). Compound 1 (gallic acid; 3,4,5-tihydroxy benzoic acid) showed total antioxidant capacity (307.10), equivalent to its three hydroxyl groups, while on its ester form compound 2 (Methyl gallate) the esterification decrease its activity to (215.55), on other hand the displacement of 3-hydroxyl group with methoxy group and absence of 5-hydroxy group in compound 4 (vanillic acid), lead to reducing the activity to (178.19), when compared with three free hydroxyl groups in compound 1 [28, 29]. In case of compound 3 (ellagic acid), the comparison between its antioxidant activity and its chemical structure as a nucleus of condensed tannin, the increase of galloyl group and ortho-hydroxyl structure significantly enhanced its activity. Previous study revealed that the antioxidant activity of both condensed and hydrolyzable tannins increased with the increase of their molecular weight in the following order: monomer < dimer < trimer <dimer-di gallate. The larger the tannin molecules were, they possessed more galloyl groups and ortho-hydroxyl groups and their activities were stronger [29].

Identification of the Isolated Phenolic Compounds (1-4): The ethyl acetate and n-BuOH subfractions were subjected to chromatographic isolation and their chemical structures were determined via certain spectroscopic analysis (Table 1 and Figure 1).

UV spectral data λ_{max} (nm) of compound 1 in methanol showed two characteristic peaks at 211 nm (conjugation of hydroxyl groups with benzene ring) and 267 nm (conjugation of carbonyl group with benzene ring) [31, 32]. The IR spectrum of compound 1 showed absorption peaks at 3373, 1704.7 and 1618.95 cm^{-1} in indicated the presence of hydroxyl (Ar-O-H), carbonyl groups (carboxylic O-H) and aromatic carbon (Ar. C=C), respectively [33]. The ^{1}H-NMR spectrum of compound 1 revealed the presence of only one peak characteristic for aromatic protons H-2 and H-6 at 6.91 (2H, s, H-2,6) indicating the substitution of benzene ring in four positions (tetra-substituted). ^{13}C-NMR spectra showed peaks at 120.9 (C-2), 109.7 (C-3 & C-7), 145.9 (C-4 & C-6), 138.51 (C-5) and 168.04 (C-1), indicating that it contains seven carbon atoms and the signal at δ 168.04 indicate the presence of carboxylic group which was confirmed by UV and IR spectra. Accordingly, compound 1 was identified as gallic acid (3, 4, 5-trihydroxy benzoic acid) from this data and via comparison with authentic sample (Co-PC, Co-TLC and Co-m.p.) [34, 35].

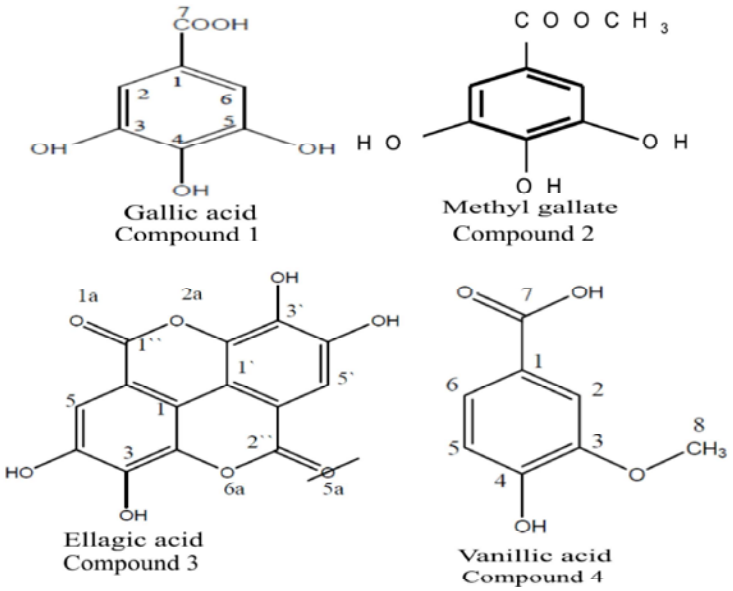

Fig. 1: Chemical structures of phenolic compounds 1-4 isolated from T. arjuna leaves.

Table 1: ^{1}H-^{13}C-NMR (DMSO-d6) spectral data for the isolated phenolic compounds (1-4).

	1		2		3		4	
Carbons	δ_c	δ_H (ppm)	δ_c	δ_H (ppm)	δ_c	δ_H (ppm)	δ_c	δ_H (ppm)
1	120.9		119.8		133.6		125.12	
2	109.7	6.91 (2H, s)	109.05	7.08 (1H, s)	136.8		109.91	7.17 (1H, d, J=2)
3	145.9		146.14		140.38		160.36	
4	138.51		138.98		148.74	12.10	149.97	3.94
5	145.9		146.14		111.54	8.30	116.48	6.43 (1H, d, J=2)
6	109.7	6.91 (2H, s)	109.05	7.08 (1H, s)	108.80	7.63	128.40	7.25 (1H, dd, J=8,2)
7	168.04	9.17 (1H, s)	166.87		--------		189.99	9.18 (1H, s)
8			52.12	3.78 (3H, s)	--------		55.67	3.58 (3H, s)
1’					133.6			
2’					136.8			
3’					140.38	12.10		
4’					148.74	8.30		
5’					111.54	7.63		
6’					108.8			
1’’					161.28			
2’’					161.28			

Table 2: Total phenolic content (TPC), free radical scavenging potential (DPPH) and total antioxidant capacity of the 70% acetone extract of *T. arjuna* (L.) as well as its derived subfractions.

Sample	TPC[a] (mg GAE / g ext.)	DPPH (SC_{50})[b] [µg/ml]	Total antioxidant capacity (mg AAE /g ext.)[c]
70 % Me_2CO	96.04 ± 3.19378	27.42± 0.044	317.08 ± 2.53
Diethyl ether	166.43 ± 0.02	19.80 ± 11.50	84.81 ±2.56
Pet. ether	109.16 ± 2.47	80.49 ± 0.01	359.27 ± 2.99
CH_2Cl_2	388.64 ± 7.05	13.99 ± 0.13	121.21 ± 6.06
EtOAc	242.98 ± 6.42	132.54 ± 0.04	370.70 ± 7.06
n-BuOH	996.52 ± 5.75	9.06 ± 1.12	439.23 ± 8.85
Ascorbic acid	……	8.25 ± 0.95	……

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

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

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

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

Table 3: Free radical scavenging potential (DPPH) and total antioxidant capacity of the isolated phenolic compounds (1-4) from *T. arjuna* (L.).

Compound	DPPH (SC_{50}) [µg/ml]	Total antioxidant capacity (mg AAE /g ext.)
1	6.0 ± 0.12	307.10 ± 4.12
2	7.06 ± 1.56	215.55 ± 5.44
3	6.38 ± 2.14	274.00 ± 2.05
4	7.87 ± 0.84	178.19 ± 1.12
Ascorbic acid	8.65 ± 1.30	……

The IR spectrum of compound 2 confirmed the presence of OH at (3362.40 cm^{-1} broad), C-H stretching at (2920 cm^{-1}) and revealed the presence of a carbon-carbon double bond at (1693 cm^{-1}). The UV spectrum of compound 2 in methanol showed two characteristic bands at 225 and 273 nm indicating its phenolic nature as hydroxy benzoic acid derivatives [36]. The ^{1}HNMR spectrum of compound 2 showed two characteristic peaks at 7.08 (2H, s, H-2, H-6) and 3.78 (3H, s, -OCH3), respectively for two aromatic protons H-2 and H-6 of tetra-substituted benzene ring as well as three methoxy group protons [37]. The ^{13}C-NMR data of compound 2 showed the presence of ester carbonyl at δ 166.87 (C-7), one methoxy carbon at δ 52.12 (OCH3), three hydroxyl carbons at δ 146.14 (C-3, C-5), 138.98 (C- 4), two unsubstituted carbons at δ 109.05 (C-2, C-6) and 119.80 (C-1). Via comparison it's chromatographic and spectral data with previous reported data in the literature [36, 37], compound 2 identified as methyl gallate (methyl 3, 4, 5-trihydroxybenzoate).

The IR spectrum of compound 3 exhibited the presence of hydroxyl group at (3440 cm^{-1}), α, β-unsaturated carbonyl of lactones at (1730 cm^{-1}) and aromatic ring carbons peaks at (1560 and 1520 cm^{-1}). The UV spectrum of compound 3 in methanol showed two characteristic bands at 280, 310 nm indicating its characteristic hydroxylation pattern and extended conjugation up on aromatic rings [38]. The ^{1}HNMR spectrum of compound 3 showed three characteristic sets of peaks at 7.63 (2H; H-5, H-5`), for two aromatic protons H-5, H-5` as well as peaks at 8.3 (2H; OH-4`, OH-4) and 12.10 (2H; OH-3`, OH-3), for hydroxyl protons at OH-4`, OH-4, OH-3` and OH-3 respectively [39]. The ^{13}C-NMR data of compound 3 showed the presence of characteristic peak at 161.28 (C1``, C2``), for lactonic carbonyl carbons and other sets of peaks at 113.60 (C1, C1`), 136.80 (C2, C2`), 140.38 (C3, C3`), 148.74 (C4, C4`), 111.54 (C5, C5`), 108.80 (C6, C6`), assigned for hydroxy-substituted and unsubstituted carbons. Therefore, according to the comparison of it's chromatographic and spectral data with previous reported data in the literature [38, 39], compound 3 identified as 2, 3, 7, 8-tetrahydroxy-chromeno [5,4,3-cde]chromene-5,10-dione] (ellagic acid).

The UV spectrum of compound 4 in methanol showed two characteristic bands at 259 (conjugation of hydroxyl groups with benzene ring) and 292 nm (conjugation of carbonyl group with benzene ring) [31, 32]. The ^{1}HNMR spectrum of compound 4 revealed the presence of the following signals at; 6.43 (1H, d, H-5), 7.17 (1H, d, H-2), 7.25 (1H, dd, H-6), respectively for three aromatic protons H-5,H-2 and H-6 and peaks at 9.18 (1H, s, 4-OH) and 3.58 (3H, s, 8-OCH3), assigned for hydroxyl and methoxy protons, indicating its tri-substituted skeleton [40]. The ^{13}C-NMR data of compound 4 showed the presence of characteristic peak at 189.99 (C-7) assigned to carbonyl carbon and other sets of hydroxy-substituted and unsubstituted carbons at 160.36 (C-3), 149.97 (C-4), 128.40 (C-6), 125.12 (C-1), 116.48 (C-5), 109.91 (C-2) as well as methoxy carbon at 55.67 (C-8) [41]. Therefore, according to the comparison of its chromatographic and spectral data with previous reported data in the literature, compound 4 identified as 4-hydroxy-3-methoxybenzoic acid (ellagic acid) [42, 43].

CONCLUSION

This study indicated that the 70% acetone extract of *T. arjuna* showed high extent of phenolic contents which related to its own antioxidant activity. Therefore, the 70% acetone extract of the plant under investigation can be recommended as a natural source of antioxidant agents.

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