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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(4): 3846-3853. **OPEN ACCESS**

Isolation and characterization of polyphenols from the fruits of the bioactive traditional plant *Terminalia arjuna* (Roxb.) Wight & Arn.

Manal H. Shabana

Phytochemistry and Plant Systematics Department, National Research Center, Dokki,12622 **Egypt**.
***Correspondence: shabanamhs61@hotmail.com** **Received: 24-10-2019, Revised: 21-11-2019, Accepted: 29-11-2019 e-Published: 15-12-2019**

The aqueous methanol extracts of *Terminalia arjuna* W & A fruits, passed through series of separations on column and paper chromatography to afford eight pure polyphenolic compounds. A new gallate ester of rhamnose: 1, 2, 3, 4-tetra-O-galloyl -∝ - L- rhamnopyranose (1) was isolated and identified. Hydrolysable tannins, ellagic glycosides, ellagic and gallic acid were also obtained. U.V, ^{1}H and ^{13}C NMR spectral analysis, ESI-MS/MS and chemical analysis were used for the identification and structure illustration of the pure polyphenols. The physical and chemical analysis indicated that the remaining isolated compounds were: 4- O - (∝- L- rhamnopyranosyl) ellagic acid (2); 4- O -(3",4"-di-O-galloyl-∝- L- rhamnopyranosyl) ellagic acid (3); 3'-O- methyl-4- O - (∝- L- rhamnopyranosyl) ellagic acid (4); 3'-O-methyl 4- O - (4"-O-galloyl-∝- L- rhamnopyranosyl) ellagic acid (5); 3'-O-methyl- 4- O -(3",4"-di-O-galloyl-∝- L- rhamnopyranosyl) ellagic acid (6), ellagic acid (7), and gallic acid (8). The study showed that the fruits of *T. arjuna* contained substantial amount of polyphenols, which supply antioxidant effect thus authenticating its extensive use in folk medicine.

Keywords: *Terminalia arjuna;* polyphenols; 1, 2, 3, 4- tetra-O- galloyl -∝ - L- rhamnopyranose.

INTRODUCTION

Terminalia arjuna (*T. arjuna*) family: Combretaceae is a large tree about 20-30 m height. The family consists of 200 species spread around the world. The tree grows mainly along the ponds and the riversides. *T. arjuna* fruits are ovoid, 5 cm in length, they are woody with 5 wings curved upwards.

T. arjuna is considered as one of the most beneficial traditional plant that has been used in folk medicine for preventing and curing diseases (Amalraj and Gopi 2017; Mahajan and Chaudhari 2012; Dhiman and Kumar 2006; Gosh 2003). According to Ayurvedic literature, the bark of *T. arjuna* is reputed for its health benefits on the heart (Dhiman and Kumar 2006 ; Sharma et al., 2005). It was traditionally used for cardiovascular disorders (Khaliq et al., 2013; Maulik and Talwar 2012; Dwivedi 2007).It provides hypo cholesterolaemic, anti-dyslipidaemic, anti-hyperlipidemic and antioxidant properties (Subramanian et al., 2011; Aquil et al., 2006; Raghavan and Kunari 2006; Chander et al 2004; Gupta et al., 2001). Many phytoconstituents present in the bark were isolated. The bark contained triterpenes, saponins, flavonoids, tannins, polyphenols and sterols (Singh et al., 2002 a and 2002 b; Wang et al., 2010, Honda et al., 1976; Sharma et al 1982; Saha et al., 2012; KuO et al., 2005; Rastogi and Mahrota 1993). The polyphenols amount in *T.arjuna* bark is higher than polyphenols in common sources of polyphenols like tea and grapes (Callemien et al., 2008). Reported studies have focused on the chemical composition and the bioactivity of the bark. However, till date no report exists on the

constituents of the fruit. Therefore this study was planned to carry out for the first time a detailed analysis of the polyphenols in *T.arjuna* fruits. The identification of the polyphenols was performed by chemical and physical means: U.V, NMR analysis and ESI/ MS/MS. It is important to characterize these polyphenols, as they are effective antioxidants and provide insights to interpret the benefits *T.arjuna* in Ayurvedic literature. The evaluation of new medicines from plants opened a wide area for research and made a transition state, from traditional herbal medicine to modern medicine (Liu 2011).

MATERIALS AND METHODS

Plant material

T.arjuna fruits were collected from *T.arjuna* tree in El –Orman botanical garden, the fruits were authenticated by Dr. I.El-Garf, professor of Taxonomy, Faculty of Science, Cairo University. A voucher specimen has been deposited in the herbarium of the flora department, El- Orman garden, Giza, Egypt

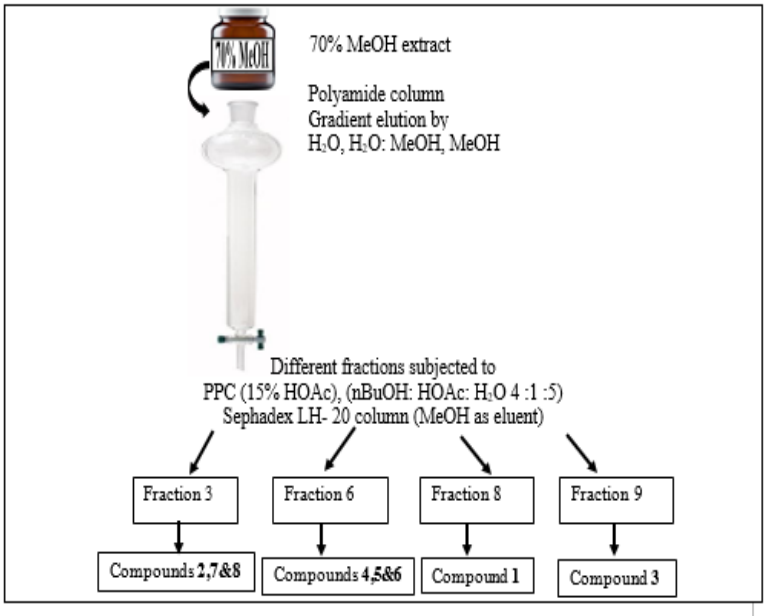
Extract preparation

The collected fruits of *T.arjuna* were dried and pulverized into powder. The aqueous methanol extract was prepared as described by Chaudhari and Mahajan (Chaudhari and Mahajan, 2015). The solvent was evaporated on rotary apparatus at40 °C leaving a dry residue. The residue was subjected to a polyamide column, eluted by water followed by H_2O/ MeOH with increasing MeOH amounts till reaching 100% MeOH. According to paper chromatography profile of the column fractions, the fractions were gathered together in ten fractions. The solvent used for P.C were: (1) HOAc-H_2O (3: 17); (2) n BuOH-OHAc-H_2O (4: 1:5, upper layer); (3) C_6H_6-nBuOH-H_2O-Pyridine (1:5:3:3). For sugar analysis we used solvents (2 and 3). Fractions purification were done by a combination of preparative paper chromatography (PPC) on Whatman No3 MM using solvent (1)and (2), followed by immobilization on Sephadex LH-20 column using MeOH as eluent. Eight compounds were obtained in pure form.

Methods:

The ^{1}HNMR spectrum (CD_3OD) was registered on Varian-Mercury 400 VX NMR at 400 MHz and assigned on the basis of coupling constants. ^{13} C was registered at 100MHz. Mass spectrometry was performed in the negative mode using Waters 3100 USA equipped with ESI source and ion trap mass analyzer. UV spectra were measured with a Shimadzu UVPC 240UV-Vis recording spectrometer.

Scheme for chromatographic fractionation of 70% MeOH extract of *Terminalia arjuna* fruit



RESULTS

Polyphenols characterization

A total of eight compounds were isolated in pure form and were identified in the aqueous methanol extract of *T.arjuna* fruits (fig.1) .They were hydrolysable tannins including gallotannins, ellagitannins, ellagic and gallic acid. The new compound (1), a gallate ester of rhamnose, was identified based on the products of its acid hydrolysis, its UV absorption, ^{1}HNMR, ^{13}CNMR spectral analysis and confirmation of the established structure through ESI-MS/MS analysis.

Compound (2) and its di-O-galloyl derivatives (compound 3); compound (4) and its mono and di -O-galloyl derivatives (compound 5 and 6) were identified by chemical and spectral analysis and comparison with the published literature data. For compounds 2, 3, 4, 5 and 6, the rhamnoside linkage was determined at position 4- ellagic acid, on the basis of previous NMR analysis of *T.arjuna* ellagic glycosides (Saha et al., 2012). The position of the galloyl residue was determined in compounds 1, 3, 5 & 6 on the basis of NMR analysis. Compounds 7 and 8 were identified by CoPC with authentic samples and by spectral analysis. The sequence of compounds from 1-8 was done to facilitate their comparison but it was not their order of elution from the column.

Compound 1: 1, 2, 3, 4-tetra-O-galloyl -∝- L-rhamnopyranose

A new compound isolated as yellow powder, R_f in solvent (1) = 0.22, dark band on Sephadex LH-20 and under U.V light. Upon acid hydrolysis (HCl, 2hrs, 100°C), it yielded gallic acid and rhamnose (CoPC). UV λ_{max} MeOH: 217, 276. ESI-MS/MS negative mode: 771([M-H]$^-$), 619([M-H]$^-$ - [galloyl unit]), 467([M-H]$^-$ - [2 galloyl units]), 169([gallic acid-H]$^-$). ^{1}HNMR: four gallic residues appeared at δ ppm 7.11, 7.08, 7.06, 7.00 (each of them d, J=2Hz). Rhamnose moiety appeared at δ ppm 6.20(br. s, H-1rh), 5.48(m, H- 3rh), 5. 00(m, H-2&4rh), 3.75 (H-5rh), 1.14(d, J=6Hz, CH_3 of rh).^{13}CNMR and ^{1}HNMR were illustrated in table1.

Compound 2: 4- O – (∝- L- rhamnopyranosyl) ellagic acid

It had a buff color under long U.V light and yellow band on Sephadex L.H 20. U. V λ_{max} MeOH: 217, 263, 367. R_f in solvent (1) = 0.40. Upon normal acid hydrolysis, it gave ellagic acid & rhamnose (CoPC). The molecular ion peak was at m/z 447([M-H]$^-$) corresponding to a molecular weight 448. The major fragments were 447, 301([M-H]$^-$ -146). ^{1}HNMR: protons of ellagic acid at δ ppm 7.50 (s), 7.38 (s), protons of rhamnose at 5.00 (s, anomeric-H-1''rh), 3.6-3.8 (the rest of rhamnose protons), 1.15 (d, J=6Hz, CH_3 of rhamnose).

Compound 3: 4- O - (3'', 4''-di-O-galloyl-∝-L-rhamnopyranosyl) ellagic acid

Dark spot under U.V, R_f in solvent (1) = 0.24. Upon acid hydrolysis, it hydrolyzed to ellagic, gallic acid and rhamnose (CoPC). U. V λ_{max} MeOH: 219, 273, 362. The molecular ion peak appeared at m/z: 751 ([M-H]$^-$) corresponding to a molecular weight of 752. Other fragments were 599 ([M-H]$^-$ - [galloyl unit]), 447([M-H]$^-$ - [2galloyl units]), 301,169 ([gallic acid-H]$^-$).^{1}HNMR: protons of ellagic acid at δ ppm 7.40 (s), 7.28 (s). Protons of two galloyl moieties δ ppm 7.13 (d, J=2Hz), 7.09 (d, J=2Hz). Protons of rhamnose at 5.00 (br. s, anomeric H-1''rh), 4.58 (d, J=9Hz, H-4'' rh), 4.52 (m, H-3''rh), 3.6- 3.80 (m, the rest of rhamnose protons), 1.15 (d, J= 6Hz, CH_3 of rhamnose).

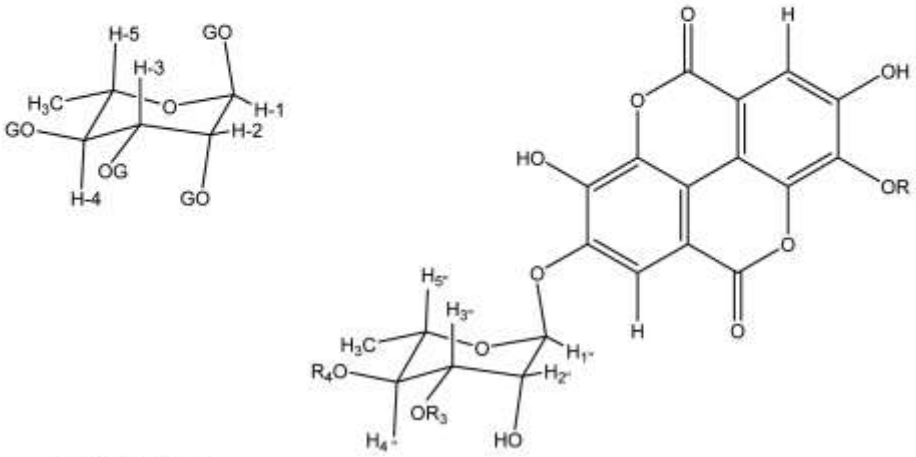
Compound 4: 3'-O- methyl-4- O - (α- L-rhamnopyranosyl) ellagic acid

Gave a reddish buff band on Sephadex LH-20 column, R_f in solvent (1)= 0.20, by acid hydrolysis, it gave rise to 3'-O-methyl ellagic acid and rhamnose (CoPC), U.V λ_{max} MeOH:220, 267, 366, m/z at 461 ([M-H]$^-$) .The major fragments ions appeared at 461, 315([M-H]$^-$ - 146), 301.^{1}HNMR δ ppm 7.50 (s, H-5 of ellagic acid), 7.38 (s, H-5' of ellagic acid), 4.95 (s, anomeric H-1'' rh), 3.85 (OCH_3 at 3' of ellagic acid), 3.60-3.80 (m, the rest of rhamnose protons) 1.15 (d, J= 6Hz, CH_3 of rhamnose).

Compound 5: 3'-O-methyl-4-O- (4''- O- galloyl-α-L- rhamnopyranosyl) ellagic acid

Gave dark spot under short U.V and an orange band on Sephadex LH.20 column, R_f in solvent (2) =0.48. Upon normal acid hydrolysis, it hydrolyzed to 3'-O-methyl-ellagic acid, gallic acid (U.V and CoPC) and rhamnose (CoPC). U.V λ_{max} MeOH: 210, 278, 360.The molecular ion peak m/z was 613 ([M-H]$^-$). The daughter fragment ions m/z 315 ([M-H]$^-$ - [galloyl+ 146]), 301, 169 ([gallic acid -H]$^-$). ^{1}HNMR : δ ppm 7.50 (s, H-5 of ellagic), 7.38 (s, H-5' of ellagic), 7.1 (d, J =2Hz, H-2and H-6 of gallic acid), 4.95 (br. s. anomeric proton of rhamnose), 4.50 (d, J=9, H-4'' rh), 3.85(s, OCH_3 at 3' of ellagic), 3.60 -3.80(m, the rest of rhamnose protons), 1.15 (d, J= 6Hz, CH_3 of rhamnose).

1:G=galloyl



2: R=R_3=R_4=H

3: R=H, R_3=R_4=galloyl

4: R=OCH_3, R_3=R_4=H

5:R=OCH_3, R_3= H, R_4= galloyl

6: R=OCH_3, R_3=R_4=galloyl

7 **8**

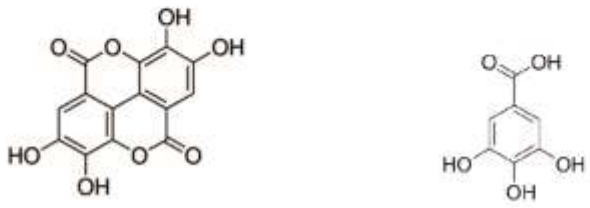


Fig.1 Structuresof the polyphenols from the fruits of *T. arjuna*

Table-^{1}HNMR (400MHz) and ^{13}C (100MHz, solvent CD_3OD) spectral data of compound (1)

Position	^{1}H shift	J coupling in HZ	^{13}C shift
α-L-Rha			
1	6.20	2.0 (1,2)	101.4
2	5.00	3.2 (2,3)	72.7
3	5.48	9.5 (3,4)	73.5
4	5.00	9.5 (4,5)	72.4
5	3.75	6.1 (5,6)	69.5
6-Me	1.14	6	20
1-O-Galloyl			
1'			121.30
2', 6'	7.08		110.16
3' , 5 '			146.11
4'			140.01
7'			168.31
2-O-Galloyl			
1'			121.48
2', 6 '	7.06		169.92
3' , 5 '			146.46
4'			139.82
7'			168.26
3-O-Galloyl			
1'			120.74
2', 6'	7.10		109.98
3' , 5 '			145.13
4'			139.37
7'			168.68
4-O-Galloyl			
1'			120.06
2', 6'	7.00		109.94
3' , 5 '			145.92
4'			140.15
7'			167.76

Compound 6: 3'-O-methyl-4-O- (3'', 4''-di-O-galloyl-α-L- rhamnopyranosyl) ellagic acid.

It appeared dark spot under short U.V and on Sephadex LH.20 column, R_f in solvent (2) =0.52. It hydrolyzed to 3'-O-methyl-ellagic acid, gallic acid (U.V and CoPC) and rhamnose (CoPC) by acid hydrolysis. U.V λ_{max} MeOH: 219, 273, 365.The molecular ion peak m/z was 765 ($[M-H]^-$). The daughter fragment ions appeared at m/z 613($[M-H]^-$ - galloyl), 315($[M-H]^-$ - [galloyl+ 146]), 301, 169 ([gallic acid -H]$^-$). ^{1}HNMR : δ ppm 7.50 (s, H-5 of ellagic), 7.38 (s, H-5' of ellagic), 7.03 (d, j =2Hz, H-2 and H- 6 of first gallic), 6.88 (d, J=2Hz, H-2 and 6 of second gallic), 4.95 (br. s. anomeric proton of rhamnose , 4.50 (d, J=9 Hz, H-4'' rh), 4.38 (m, H-3'' rh), 3.85 (s, OCH_3 at 3' of ellagic acid), 3.60 -3.80(m, the remaining rhamnose protons), 1.15 (d, J= 6Hz, CH_3 of rhamnose).

Compound 7: ellagic acid

It was obtained as off-white powder and appeared as buff spot under short U.V, R_f in solvent (1) = 0.15, R_f in solvent (2) = 0.45. U. V λ_{max} MeOH: 260, 362. ^{1}HNMR: δ ppm 7.50 (s, H-5 of ellagic), 7.38 (s, H-5' of ellagic). ESI-MS: 301 ($[M-H]^-$).

Compound 8: gallic acid

It was obtained as buff powder, appeared as dark spot under short U.V. R_f in solvent (1)=0.52, R_f in solvent (2)= = 0.65, U.V λ_{max} MeOH: 270nm.^{1}HNMR : δ ppm 7.00(d, J=2Hz, H-2&6). ESI-MS: 169 ($[M-H]^-$).

DISCUSSION:

Compound 1: was a new compound, it showed in ^{1}HNMR (table1) four doublets (J=2Hz) at δ ppm 7.00, 7.06, 7.08, 7.10 each integrated to two protons, attributed to 4 galloyl moieties. In the

sugar regions, there was a broad singlet at δ 6.20 attributed to α -configurated anomeric proton of rhamnose. The attachment of four galloyl residues to the positions C1, C2, C3 and C4 of rhamnose follows from the strong downfield shifts of the signals of H1, H2, H3 and H4. These findings were in accordance with ^{13}CNMR of (**1**) table 1, The ESI/MS/MS negative mode, proved that compound (1) underwent sequential losses of dehydrated galloyl moieties (152amu) and a hexose moiety (146 amu) from its parent ion at 771 $(M-H)^-$, giving rise to a daughter ion at m/z 169 (gallic acid –H). Consequently, compound (1) is 1, 2, 3, 4-tetra-O-gallyol-α-L- rhamnopyranose, a new gallate ester of rhamnose which has not yet been isolated before from nature.

Compound 2: was an ellagic glycoside which hydrolyzed to ellagic acid and rhamnose. The mass fragmentation gave daughter ions of 301 (ellagic acid –H) while parent peak at m/z 447$(M-H)^-$. ^{1}HNMR showed two signals at δ 7.50 & 7.38 referred to H-5 & H-5' of ellagic acid. The signals of anomeric proton of rhamnose appeared at 4.95. The glycosidic linkage is assumed to be at position 4 of ellagic acid , based on previous NMR analysis of *Terminalia* species(Amalraj and Gopi 2017), compound 2 was therefore 4- O -(∝- L- rhamnopyranosyl) ellagic acid. It had been previously identified as Eschwerleilenol C and was isolated from *Eschweilera coriacea* and from longan seeds (Yang et al., 1998, Zheng et al., 2009).

Compound 3: was a (galloyl-∝- L-rhamnopyranosyl) ellagic acid as seen by the products of hydrolysis: gallic and ellagic acid plus rhamnose. MS fragmentation gave the parent peak at m/z 751 $(M-H)^-$, a daughter peak at 301 ([ellagic acid -H]) $^-$ and a daughter peak at 169 ([gallic acid –H] $^-$). From ^{1}HNMR, ellagic acid protons appeared at 7.40 & 7.25. Two galloyl residues were deduced at 7.13 & 7.09, each of them was a doublet with coupling constant 2Hz.While the anomeric proton of rhamnose appeared as a broad singlet at 5.00.The attachment of the two galloyl to C-3''& C-4'' of the rhamnose followed from the downfield shift of the signals of 4'' H and 3''H. From the above data, compound 3 was identified as 4- O- (3'', 4''-di-O-galloyl-α- L- rhamnopyranosyl) ellagic acid. It was isolated before, from *Terminalia chebula* and *T. horrida* by (Pfundstein et al., 2010).

Compound 4: which is the methyl ellagic acid analog of compound 2 based on ^{1}HNMR. A methoxy signal appeared at 3.85 while the remaining signals of compounds 4 and 2 were similar. The ESI-MS/MS analysis revealed a molecular ion peak m/z 461($[M-H]^-$), daughter fragments at m/z 301, 315 corresponding to ellagic acid and methyl derivative of ellagic acid respectively. These data were in accordance with published spectral data for 3'-O- methyl-4- O-(∝- L- rhamnopyranosyl) ellagic acid (Saha et al., 2012, Kim et al., 2001).

Compound 5: had a signal at 7.10 (d, j=2Hz) in ^{1}HNMR, assigned to one galloyl moiety. The attachment of the galloyl residue to the position C-4'' of rhamnose came from the downfield shift of H-4'' (4.50ppm). This downfield shift is probably due to galloylation at the geminal OH-4'' of this proton. The rest of the spectrum was identical to compound 4. Therefore compound 5 was identified as 3'-O-methyl 4- O- (4''-O-galloyl-α- L-rhamnopyranosyl) ellagic acid. MS data confirmed this finding. It was previously isolated from longan seeds (Chen et al., 2015).

Compound 6: the methyl ellagic acid analog of compound 3, was structure illucidated on the basis of ^{1}HNMR and MS data and comparing with published literature. It showed ^{1}HNMR spectrum similar to that of compound 3, with an extra singlet at δ 3.85 ppm, corresponding to methoxy group at C-3' of ellagic acid. In the MS, there were fragments at 765 ($[M-H]^-$), 613 ($[M-H]^-$ -[galloyl unit]), 315 ([methoxy ellagic-H]$^-$), 169 ([gallic acid -H]$^-$) , compound 6 was identified as 3'-O-methyl 4-O- (3'', 4''- di -O-galloyl-α- L- rhamnopyranosyl) ellagic acid. It was identified before in *T.chebula* fruits by, only, MS spectrometry (Pfundstein et al 2010). In this paper ^{1}HNMR and U.V for compound 6 were done and reported for the first time.

Compound 7: had chromatographic data, ^{1}HNMR, MS and U.V typical to ellagic acid which was previously isolated from many plants (Srivastava et al., 2007).

Compound 8: was characterized as gallic acid based on comparison of its ^{1}HNMR, MS and U.V with the previous literature reports (Al-Zahrani 2012).The ellagic acid derivatives isolated from the fruits of *T.arjuna* were known to exert antioxidant effect (Zaffrila et al., 2001).We may attribute the heart health benefits effect to these compounds. Many phytochemical substances, that are present in the medicinal plants, exhibited definite physiological effect on the human body. These bioactive substances include terpenoids, tannins, polyphenols, flavonoids, steroids and carbohydrates (Kim and Song 2013, Sharma et al., 2012).It also may be these polyphenols and tannins that supply antioxidant activity as well as

vascular amplification effect, in this way authenticating the therapeutic potential of *T.arjuna* in cardiovascular disorders.

CONCLUSION

Based on the literature evidences, *T.arjuna* acts as potent antioxidant and is used for the treatment of cardiovascular diseases. The present study showed that the fruits of *T.arjuna* contained substantial amount of polyphenols. Tannins and the related compounds, isolated from the fruits, were structure elucidated with the chemical and the spectral analysis. These results merit controlled clinical trials, with a standardized product to develop herbal drug, to combat against heart diseases.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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