ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 5, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

NITRIC OXIDE PRODUCTION AND ANTIOXIDANT ACTIVITY OF DRIED FRUIT EXTRACTS OF *TERMINALIA CHEBULA*

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Received: 18 December 2017, Revised and Accepted: 09 January 2018

ABSTRACT

Objective: The dried ripe fruits of *Terminalia chebula* have traditionally been used to treat various ailments as it has a wide spectrum of pharmacological activities. Hence, in the present study, we aimed to explore the antioxidant activity, nitric oxide production, cytotoxicity, and phytocompounds present in the aqueous and methanol extract of *T. chebula.*

Methods: The dry fruits of *T. chebula* were extracted using water and methanol, and the extracts were concentrated by lyophilization method. Phytochemical analysis was done by gas chromatography and mass spectrometry and Fourier-transform infrared spectroscopy. The free radical scavenging activity of *T. chebula* was estimated by 1,1diphenyl 2, picrylhydrazyl method. RAW 264.7 cells were stimulated with aqueous and methanol extracts, and the production of nitric oxide was estimated by spectrophotometric method using Griess reagent. Cytotoxicity assay was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method and percentage of cell viability was calculated.

Results: Aqueous and methanolic extracts of the dry fruit of *T. chebula* showed non-toxic to RAW 264.7 cells at the concentration of 2 mg and 1.5 mg, respectively. These concentrations showed high free radical scavenging activity and production of optimum concentration of nitric oxide in RAW 264.7 cells.

Conclusion: Fruit extracts of *T. chebula* possess properties of nitric oxide production and high free radical scavenging activity; these properties could be useful in the development of immunomodulatory drugs as well as protection against various human diseases associated with oxidative stress.

Keywords: *Terminalia chebula*, Antioxidant, Nitric oxide, Cytotoxicity, Gas chromatography and mass spectrometry, Fourier-transform infrared, RAW264.7.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i5.24316

INTRODUCTION

The use of medicinal plants for treating various types of human illnesses goes back to the early man, as evidenced from ancient and North African civilizations [1]. According to the World Health Organization, 80% of the world's rural population depends on medicinal plants for their primary health-care need. At present, 25% of the prescribed drugs are active and synthetic compounds derived from medicinal plants [2]. India is one of the few countries in the world which has a unique wealth of medicinal plants, vast traditional knowledge, and use of herbal medicines for the cure of various diseases [3].

Terminalia chebula belongs to the family of Combretaceae and native of India and Asia. Dried ripe fruits of *T. chebula* have traditionally been used to treat various ailments in Asia due to its wide spectrum of pharmacological activities associated with the biologically active chemicals present in this plant [4]. Fruits of *T. chebula* are known to possess antimicrobial, anticancer, anti-diabetic, immunomodulatory, and hepatoprotective properties [5]. The major bioactive constituents of the fruit include tannins, anthraquinones, chebulinic acid, chebulagic acid, chebulic acid, ellagic acid, and gallic acid [6].

Reactive oxygen species (ROS) are produced during aerobic respiration, infection, exercise, exposure to pollutants, ultraviolet (UV) light, and ionizing radiation [7,8]. In a normal cell, there is a balance between formation and removal of ROS. Oxidative stress occurs if this balance is affected and causes various diseases [9]. Antioxidants from plant materials scavenge the free radicals thereby protecting the body from stress-related diseases [10]. In biological systems, nitric oxide (NO) is a gaseous free radical synthesized by a family of enzymes called nitric oxide synthases [11], mainly involved in regulation of vascular tone, neurotransmission, and host defense mechanisms [12]. Over the past decade, NO has gained considerable attention by the researchers due to its activity against different types of bacteria, viruses, parasites, and tumors [13]. NO production activity of medicinal plants accounts for their reported health-care benefits [14]. Hence, in the present study, we made an attempt to evaluate the antioxidant and nitric oxide production potentials of *T. chebula* fruit extracts.

METHODS

Preparation of extract

Dried fruits of *T. chebula* were procured from Tamil Nadu Medical Plant Farms and Herbal Medicine Corporation Ltd., (TAMCOL), Chennai, and powdered with mechanical blender. 50 g of powder was mixed with 500 ml of sterile double distilled water for aqueous extraction and methanol for solvent extraction for 48 h. The extracts were then filtered, freeze-dried and stored at 4°C until further analysis [15].

Phytochemical analysis of *T. chebula* by gas chromatography and mass spectrometry (GC-MS)

Phytochemical analysis was performed at the sophisticated analytical instruments facility, IIT-Madras by JEOL GC MATE II GC-MS data system with high resolution. Aqueous and methanol extracts of *T. chebula* were subjected for compound identification and major compounds were identified by comparing with the National Institute of Standards and Technology database.

Fourier-transform Infrared (FTIR) spectroscopy analysis

Functional groups were identified by the Perkin Elmer system one FTIR/attenuated total reflection using KBr sampling technique with a scan range of MIR-400-4000/cm and resolution of 1/cm.

Evaluation of antioxidant properties of *T. chebula*

Antioxidant properties of *T. chebula* extracts were estimated using 1,1diphenyl 2, picrylhydrazyl (DPPH) as described by Khalaf *et al.* [16]. Briefly, 800 µl of Tris (100 mM pH 7.4) was mixed with 200 µl of both aqueous and methanol extracts ranging from 2 mg to 7.81 µg (test), with ascorbic acid as a positive control and distilled water as negative control. To this mixture equal volume of DPPH (100 µM in ethanol) was added and incubated in the dark at room temperature for 20 min with intermittent shaking. After incubation, the optical density was read in UV-spectrophotometer (UV-1800, Shimadzu, Japan) at 517 nm. The percentage scavenging activities were calculated using the following formula:

% DPPH Scavenging=OD value of control–OD value of test/OD value of control×100.

Cell culture and stimulation of RAW 264.7 cells with *T. chebula*

RAW 264.7 cells were obtained from the National Center for Cell Science Pune, India. Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin). Cells were plated in 24 well tissue culture plates at the concentration of 2×10^6 cells/well. After 24 h, cells were stimulated with different concentrations of aqueous and methanol extracts of *T. chebula* ranging from 2 mg to 7.81 mg with lipopolysaccharide (LPS) as a positive control. Supernatants were collected at 24 and 48 h poststimulation and tested for nitric oxide production [17].

Nitric oxide assay

100 µl of cell culture supernatant was mixed with 100 µl of Griess reagent (1% sulfanilamide in 5% of phosphoric acid and 0.1% naphthlethylenediamine dihydrochloride) and incubated for 10 min at room temperature. After incubation, optical density was measured at 540 nm in Microplate Spectrophotometer (BioTek USA). Nitrite concentration was determined using dilution of sodium nitrite as a standard [18].

Cytotoxicity assay

Cytotoxicity assay was carried out as described by Mosmann *et al.* [19]. Briefly, 2×10^6/well of RAW 264.7 cells were plated in 24-well tissue culture plates and incubated for 24 h at 37°C with 5% CO_2. After 24 h, complete medium was removed, and cells were treated with different concentrations of aqueous and methanol extracts in serum-free medium and incubated for 24 h. After incubation, 250 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated at 37°C for 4 h. After incubation, MTT was completely removed, and 200 µl dimethyl sulfoxide (DMSO) was added, plates were gently shaken and read at 570 nm. The percentage cell viability was calculated using the formula:

% Cell viability=OD value of the sample/OD value of the control×100.

Statistical analysis

Statistical analysis was done by ANOVA two factor test using Microsoft Excel 2013.

RESULTS

Antioxidant properties of aqueous and methanol extracts of *T. chebula* were estimated by DPPH method. The dose response assay was performed with concentrations ranging from 2 mg to 7.81 µg; the results were expressed as percentage scavenging. Aqueous and methanol extracts of *T. chebula* showed significant free radical scavenging activity in dose-dependent manner ($p<0.0001$) which is 92.18–85.93% and 92.50–88.12%, respectively, it was comparable to ascorbic acid, showing 93.43% scavenging activity (Fig. 1). Between the two extracts, methanol showed significant free radical scavenging activity ($p<0.001$).

RAW 264.7 cells were treated with different concentrations of aqueous and methanol extracts of *T. chebula* for 24 and 48 h. 24 h poststimulation with aqueous extract showed the significant nitric oxide production ($p<0.01$). The concentration of 23.39 µM and 2.81 µM nitric oxide was produced at 2 mg and 7.81 µg of aqueous extract, respectively (Fig. 2). Cells treated with LPS produced 176 µM.

The cytotoxicity concentration 50 was 2 mg for the aqueous extract and 1.5 mg in the case of methanol extract. Aqueous extract showed significant cell viability ($p<0.01$) compared with methanol extract (Fig. 3).

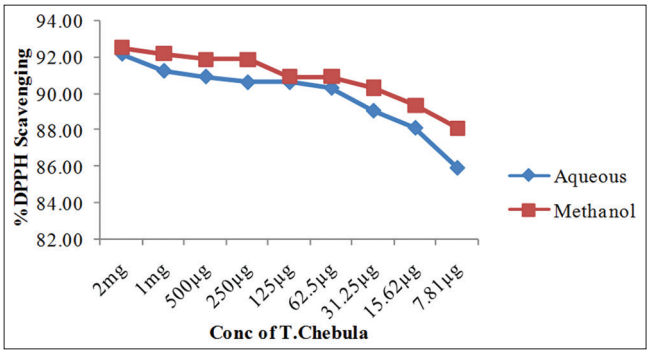


Fig. 1: 1,1diphenyl 2, picrylhydrazyl scavenging activity of aqueous and methanol extract of *Terminalia chebula*

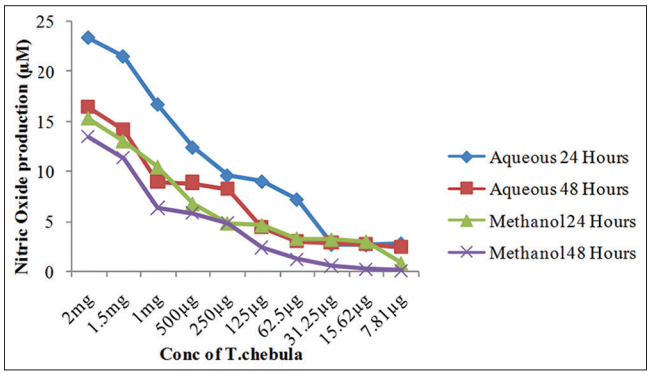


Fig. 2: Nitric oxide production by RAW 264.7 cell line upon stimulation of aqueous and methanol extract of an extract of *Terminalia chebula*

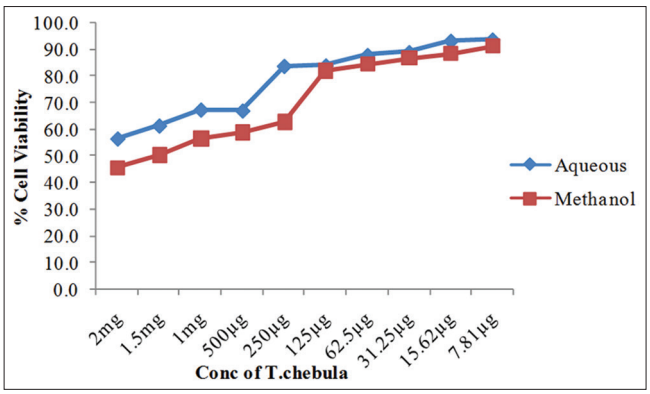


Fig. 3: Cytotoxicity of aqueous and methanol extracts of *Terminalia chebula*

FTIR peaks revealed the presence of high concentration of phenols, primary amines, aromatic hydrocarbons, medium to strong bonded groups, saturated carboxylic acids, broad peaks of ammonium ions, amino acids (zwitterions), N–O nitro-compounds, aromatic meta- and mono-disubstituted-benzene, conjugated aromatic groups, bromoalkanes, aliphatic, and aromatic amines (saturated or unsaturated) (Figs. 4 and 5, Table 1).

Major compounds present in the aqueous and methanol extract of *T. chebula* was identified by GC-MS analysis. Figs. 6 and 7 showing the chromatogram of compounds present in the aqueous and methanol extract. Major compounds identified were listed in Tables 2 and 3.

Major active compounds identified in aqueous extract of *T. chebula*

Oxacyclotetradecane-2,11-dione, 13-methyl.
2-Methylenecholestan-3-ol.
Methyl (4-iodophenyl) propanoate.

DISCUSSION

In humans, the major system of defense against oxidative damage is by the production of antioxidants. Antioxidants can reduce oxidative stress and consequently ameliorate the progress of stress-related diseases. Recently, the research focus has been shifted to natural sources of antioxidants; especially from medicinal plants due to the adverse side effects of synthetic antioxidants [20]. Gupta *et al.* [21], reported that *Terminalia bellerica* fruit extract showed 31.66–84.16% of free radical scavenging activity at the concentrations ranging from 50 to 200 µg/ml. In the present study, we observed aqueous, and methanol extracts of *T. chebula* showed antioxidant property in a dose-dependent manner. Both the extracts showed 92% of free radical scavenging activity and whereas standard ascorbic acid showed 93.43%.

Nitric oxide is a multi-functional paracrine and autocrine signal molecule which involved in many physiological and pathological

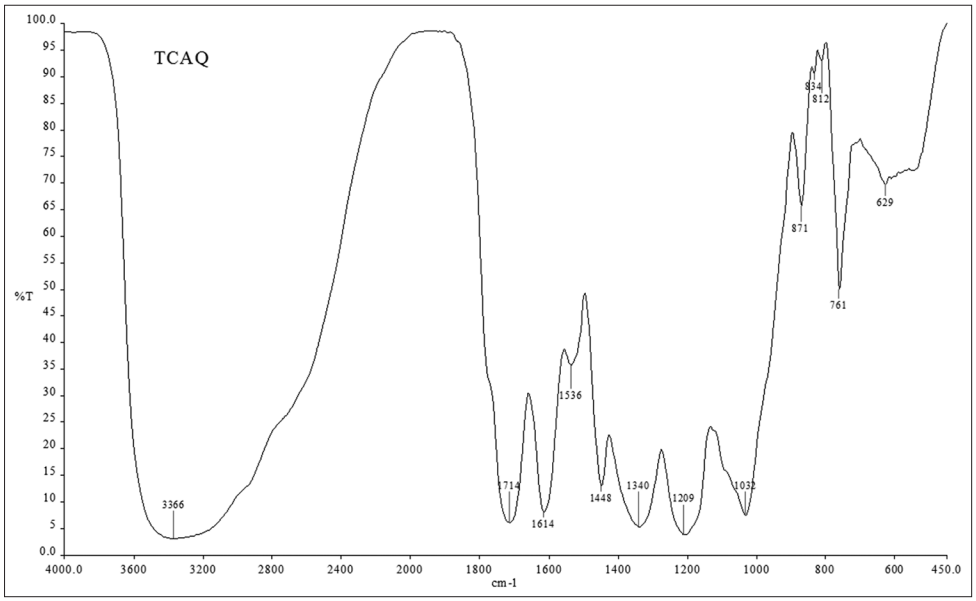


Fig. 4: Fourier-transform infrared analysis - aqueous extract of *Terminalia chebula*

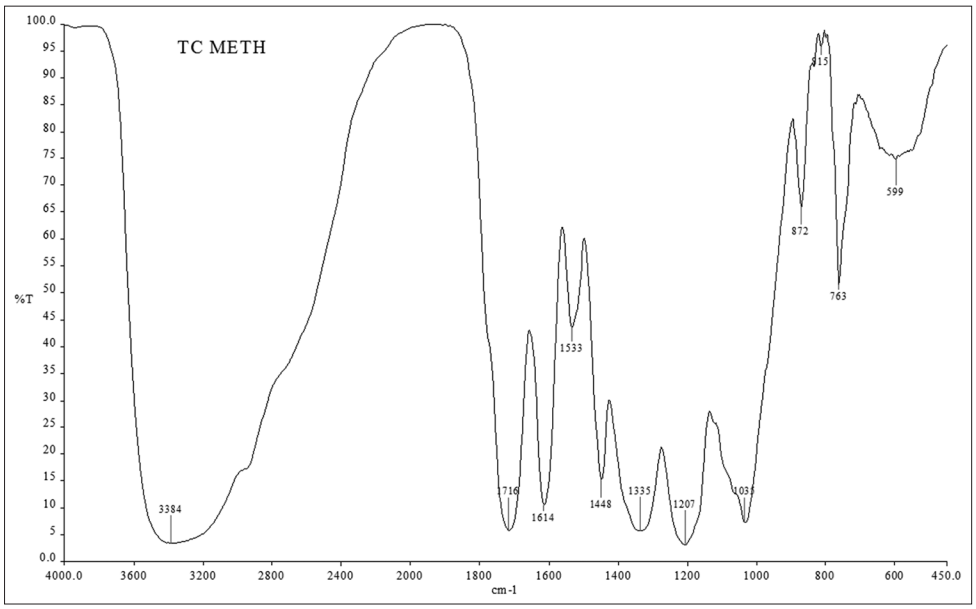


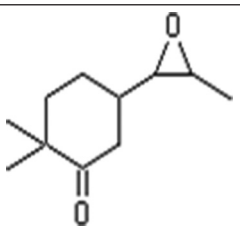
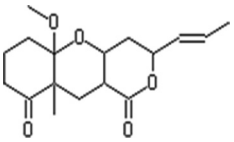
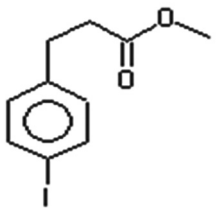
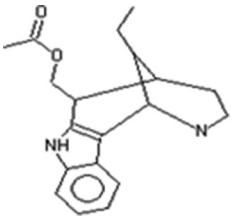
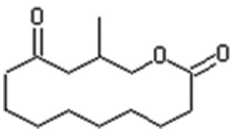
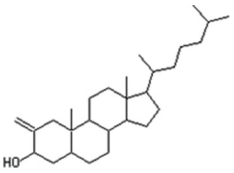
Fig. 5: Fourier-transform infrared analysis - methanol extract of *Terminalia chebula*

Table 1: Bioactive functional groups identified in *T. chebula* by FTIR analysis

S. No	Frequency in cm^{-1} and intensity	Types of vibration	Functional group present in *T. chebula* extract
1.	3366 (s)	O-H stretching	Alcohols and phenols (polymeric association)
2.	1715 (s)	C=O stretching	Carbonyl compounds (such as aldehydes, ketones, acids, esters, and lactones)
3.	1614 (s)	N-H deformation	Amides
4.	1536 (s)	N=O stretching	Aromatic nitro compounds
5.	1448 (m)	C=C stretching	Aromatic hydrocarbons
6.	1340 (s)	C-N stretching	Primary amine
7.	1209 (s)	C-O stretching	Phenols
8.	1032 (s)	C-O stretching	Carbonyl compounds (aldehydes, ketones, acids, and esters), primary alcohols
9.	834, 761 (m)	C-H deformation	Meta disubstituted aromatic compounds
10.	700-600 (s)	C-H deformation, C-Cl stretching	Alkene, halogen compounds

FTIR: Fourier-transform infrared, *T. chebula: Terminalia chebula,* s: Strong, m: Medium

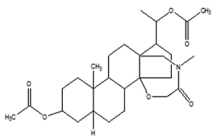
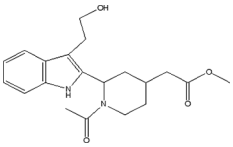
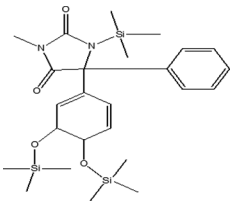
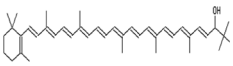
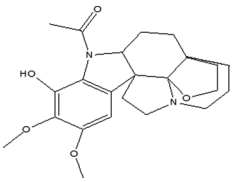
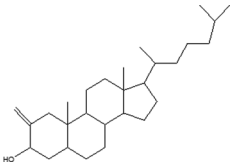
Table 2: GC-MS analysis of *T. chebula*

Name of the compound	RT	Area %	Molecular mass g/mol	Chemical formula	Structure of the compound
2,2-dimethyl-5 (3-methyloxiranyl) cyclohexanone	16.33	34.50	182.25	$C_{11}H_{18}O_2$	
Pyrano[4,3-b] benzopyran-1,9-dione, 5a-methoxy-9a-methyl-3-(1-propenyl) perhydro-	16.60	7.70	308.162	$C_{17}H_{24}O_5$	
Methyl (4-iodophenyl) propanoate	17.28	38.20	289.98	$C_{10}H_{11}IO_2$	
Dasycarpidan-1-methanol, acetate (ester)	17.98	29.00	326.19	$C_{20}H_{26}N_2O_2$	
Oxacyclotetradecane-2,11-dione, 13-methyl	18.97	45.40	240.17	$C_{14}H_{24}O_3$	
2-methylenecholestan-3-ol	19.80	39.60	400.68	$C_{28}H_{48}O$	

GC-MS: Gas chromatography and mass spectrometry, *T. chebula: Terminalia chebula*

processes such as regulation of blood pressure, neurotransmission, signal transduction, antimicrobial defense, cellular redox regulation, apoptosis [22], and immunomodulation [23]. Tomimori *et al*. [24], reported that *Crassocephalum crepidioides* extract suppressed tumor growth through NO production through nuclear factor-κB signaling pathway. Ugusman *et al*. [25], demonstrated that *Piper sarmentosum*

Table 3: Major active compounds identified in methanol extract of *T. chebula* by GC-MS analysis

Name of the compound	RT	Area %	Molecular mass g/mol	Chemical formula	Structure of the compound
5a-pregnan3,20a-diol, 14a, 18a-(4-methyl-3-oxa (1-oxa-4-azabutane-1,4-diyl) -diacetate	12.22	21.50	489.64	$C_{28}H_{43}O_6N$	
4-Piperidineacetic acid 1-acetyl-5-ethyl-2[3-(2-hydroxyethyl)-1H-indol-2-yl] -a-methyl-methyl ester	14.3	12.90	400.48	$C_{23}H_{32}O_4N_2$	
2,4-Imidazolidinedione, 5-[3,4-bis[(trimethylsilyl) oxy] phenyl]-3-methyl-5-phenyl-1-[trimethylsilyl	16.08	28.08	516.84	$C_{25}H_{40}O_4Si_3N_2$	
Psi-Carotene, 3',4'-didehydro-1',2'-dihydro-1',2'-dihydroxy-[2R]	19.17	12.80	568.86	$C_{40}H_{56}O_2$	
Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy	21.1	48.20	414.49	$C_{23}H_{30}O_5N_2$	
2-Methylenecholestan-3-ol	21.80	22.40	400.680	$C_{28}H_{48}O$	

GC-MS: Gas chromatography and mass spectrometry, *T. chebula: Terminalia chebula*

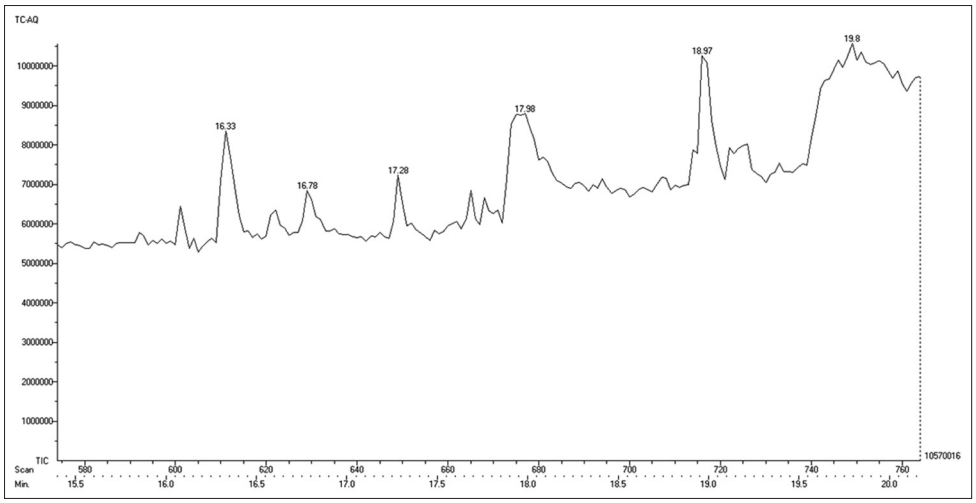


Fig. 6: Gas chromatogram of an aqueous extract of *Terminalia chebula*

increased NO production and could protect human umbilical vein endothelial cells from oxidative stress and also reduce the risk of atherosclerosis. Karupiah *et al*. [26], correlated NO production in macrophage and antiviral activity of interferon gamma. In the present study, we observed 23.24 µM and 15.30 µM NO production in RAW264.7 cells after 24 h poststimulation with aqueous and methanol extracts, respectively. Aqueous extract had shown increased nitric oxide production on comparison with the methanol extract which may be due to the higher free radical scavenging activity of the methanol extract. 2 mg and 1.5 mg of aqueous and methanol extracts were found to be nontoxic and exhibited above 50% cell viability, respectively.

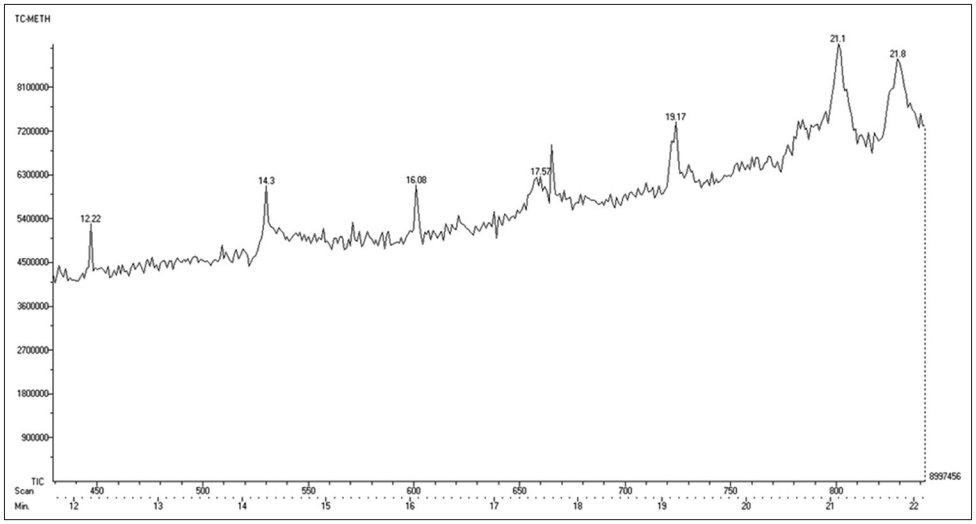


Fig. 7: Gas chromatogram of methanol extract of *Terminalia chebula*

The major bioactive compounds present in aqueous extract were oxacyclotetradecane-2,11-dione, 13-methyl, 2-methylenecholestan-3-ol and Methyl (4-iodophenyl) propanoate. In methanol extract aspidospermidin-17-ol,1-acetyl-19,21-epoxy-15,16-dimethoxy and 2,4-imidazolidinedione,5-[3,4-bis[(trimethylsilyl)oxy]phenyl]-3-methyl-5-phenyl-1 [trimethylsilyl.

CONCLUSION

In the present study, we observed that methanol extract of *T. chebula* has higher free radical scavenging activity whereas the aqueous extract exhibited higher nitric oxide production and both the aqueous and methanol extracts were non-toxic at higher concentrations. The major active compounds identified in the aqueous extract were Oxacyclotetradecane-2, 11-dione, 13-methyl, in methanolic extract Aspidospermidin-17-ol, 1-acetyl-19, 21-epoxy-15, 16-dimethoxy which might be responsible for the observed biological activities. Further studies are required to investigate the molecular mechanism behind the antioxidant and NO production activities of *T. chebula*. Our study could be useful in the development of immunomodulatory drugs as well as protection against various human diseases associated with oxidative stress.

AUTHOR'S CONTRIBUTION

DHANASEZHIAN ARIDASS: Concepts, design, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing. Seetharaman Srivani: Concepts, design, data analysis, manuscript editing, manuscript review. Marimuthu Ragavan Rameshkumar: Extract preparation, manuscript editing.

CONFLICTING OF INTEREST

None.

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