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

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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 its 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 JEDOL 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 1,1-diphenyl 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 2×10⁶ 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% naphthylethylenediamine 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, 2×10⁶/well of RAW 264.7 cells were plated in 24-well tissue culture plates and incubated for 24 h at 37°C with 5% CO₂. 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*](image1)

![Fig. 2: Nitric oxide production by RAW 264.7 cell line upon stimulation of aqueous and methanol extract of an extract of *Terminalia chebula*](image2)

![Fig. 3: Cytotoxicity of aqueous and methanol extracts of *Terminalia chebula*](image3)
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-Methylenecholestane-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
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* extract suppressed tumor growth through NO production through nuclear factor-κB signaling pathway.

### 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     | Carboxylic compounds (such as aldehydes, ketones, acids, esters, and lactones) |
| 3.    | 1614 (s)                        | N-H deformation    | Aromatic nitro compounds                      |
| 4.    | 1536 (s)                        | N=O stretching     | Aromatic hydrocarbons                         |
| 5.    | 1448 (m)                        | C=N stretching     | Primary amine                                 |
| 6.    | 1340 (s)                        | C-0 stretching     | Phenols                                       |
| 7.    | 1209 (s)                        | C-0 stretching     | Carboxylic compounds (aldehydes, ketones, acids, and esters), primary alcohols |
| 8.    | 1032 (s)                        | C-0 stretching     | Meta disubstituted aromatic compounds         |
| 9.    | 834.761 (m)                     | C-H deformation    | Meta disubstituted aromatic compounds         |
| 10.   | 700-600 (s)                     | 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               | C₁₁H₁₈O₂          | ![Structure](https://example.com/structure1.png) |
| Pyrano[4,3-b] benzopyran-1,9-dione, 5a-methoxy-9a-methyl-3-(1-propenyl) perhydro- | 16.60 | 7.70   | 308.162              | C₁₇H₂₄O₅          | ![Structure](https://example.com/structure2.png) |
| Methyl(4-iodophenyl) propanoate | 17.28 | 38.20  | 289.98               | C₁₁H₁₁I O₂        | ![Structure](https://example.com/structure3.png) |
| Dasycarpidan-1-methanol, acetate (ester) | 17.98 | 29.00  | 326.19               | C₁₇H₂₁N₂O₂        | ![Structure](https://example.com/structure4.png) |
| Oxacyclotetradecane-2,11-dione, 13-methyl | 18.97 | 45.40  | 240.17               | C₁₄H₂₁O₃          | ![Structure](https://example.com/structure5.png) |
| 2-methylenecholestan-3-ol | 19.80 | 39.60  | 400.68               | C₁₈H₃₀O          | ![Structure](https://example.com/structure6.png) |

GC-MS: Gas chromatography and mass spectrometry, *T. chebula*: *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.
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-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: Extraction preparation, manuscript editing.

CONFLICTING OF INTEREST

None.

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