Volume 9, Issue 2, 2020, 1111 - 1116 ISSN 2284-6808

Letters in Applied NanoBioScience

https://nanobioletters.com/

https://doi.org/10.33263/LIANBS92.11111116

Original Research Article Open Access Journal

Received: 02.03.2020 / Revised: 05.05.2020 / Accepted: 10.05.2020 / Published on-line: 18.05.2020

Phytochemical composition and biological investigation of *Trichilia emetica Vahl*. seed extracts

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ABSTRACT

The current study investigated the phytochemical analysis, chemical composition, antioxidant, cytotoxic and antifungal activity of Trichilia emetica seed extracts. Preliminary phytochemical screening and quantification were realized by GC-MS. Consecutive extractions (methanol, ethanol, ethyl acetate, hexane, chloroform and distilled water) were carried out and phytochemical tests performed to represent the variety of phytochemicals. Seed extracts variably displayed alkaloids, cardiac glycosides, phenols, sterols, terpenoids and flavonoids but there was no indication of saponins and tannins in all extracts. The biological investigation of the methanolic extract showed significant biological activities. The results obtained revealed that methanol extract has presented significant IC50 =5.94 µg/mL radical scavenging activity. Minimum inhibitory concentration (MIC) of hexane and chloroform fractions displayed antifungal activity (40.95-100 and 76.27-100.11, respectively) against all three fungal pathogens, whilst the methanol extract inhibited *C. krusei* and *C. parapsilosis* and the ethyl acetate inhibited *C. parapsilosis* only. The results obtained revealed the medicinal importance of the plant and methanol extract of *T. emetica* would aid researchers to exploit as potential antifungal and antioxidant agent.

Keywords: *Anti-oxidant; antifungal; GC MS; methanol extract; Trichilia.*

1. INTRODUCTION

Medicinal plants play an important role in human health care. It is estimated that about 20-30% of all drugs evaluated as therapeutic agents are derived from natural products [1-4]. In South Africa, 60-80% of the population depend on the traditional use of plants as a source of medical relief from both human and animal ailments [5]. Approximately 10% of the documented South African tree species are threatened [6, 7].

This is of great concern as some indigenous tree species may become extinct before their potential curative value has been investigated.

T. emetica is an evergreen tree, native to Africa [8]. The surfeit of applications established for T. emetica in traditional medicine has engaged the curiosity of scientists, thus encouraging screening for a broad range of biological and pharmacological activities, before incorporation into the country's official health care system. Different plant material of *T. emetica* is used in traditional medicine for controlling various sicknesses [9]. Powder obtained from roots is used to treat abdominal pains, dysmenorrhoea and hepatic disorders [10]. The stem bark is employed in the treatment of bronchial inflammation and fever [10]. A study conducted on leaf extracts of *T. emetica* displayed good antioxidant activity [11]. Leaves [2] and root extracts of *T. emetica* exhibited promising antibacterial activity [12, 13] whilst fruit extracts showed inhibition of fungal growth [14]. Root extracts of *T. emetica* also exhibited proliferation inhibition of MCF-7 and murine sarcoma (S180) cells [15].

Additionally, T. *emetica* also exhibited anti-inflammatory [16], antischistosomal [17], antiplasmodial [18], anticonvulsant [19], antitrypanosomal [20], antitussive [21], antimutagenic [22] and hepatoprotective properties [12].

The only known traditional use of *T. emetica* seeds are for cosmetic purposes [23]. This plant seeds has not been profoundly screened for antimicrobial activity.

Keeping in view the untapped area and pharmacological significance, *T. emetica* was selected to investigate its phytochemical constituents, in vitro antioxidant, and antifungal activity of crude seed extracts.

2. MATERIALS AND METHODS

2.1. Chemicals.

Chemicals and growth media used in this study were of analytical grade and purchased from Merck (Pty) Ltd, South Africa unless otherwise stated. Solvents used for extraction were of high performance liquid chromatography (HPLC) grade and were purchased from Sigma-Aldrich (USA).

2.2. Seed material.

Mature seeds were collected from *T. emetica* trees growing in St. Lucia, KwaZulu-Natal, South Africa (13 53 N, 60 58 W). The plant was authenticated by the WARD Herbarium within the School of Life Sciences, University of KwaZulu-Natal, Durban, South Africa. The aril was removed, seeds were air-dried at room temperature for several days and crushed to a fine powder and

stored in airtight containers for subsequent use in solvent extractions.

2.3. Extraction of plant material.

The dried seed powder (100 mg) of *T. emetica* was extracted by cold percolation method [24] using several organic solvents, viz. methanol, ethanol, ethyl acetate, hexane, chloroform and distilled water. Briefly, 1 mL of distilled water and 1 mL of the respective solvent was added to 100 mg of dried powdered seed material, vortexed for 1 min and allowed to stand at room temperature. After 24 h, the extract was centrifuged at 5000 rpm for 10 min, the supernatant was collected and the solvents were evaporated. The dry extract was stored at 4oC for further use.

2.4. Preliminary qualitative phytochemical screening.

The seed extracts were assessed for phytochemicals analysis by using the following standard methods as described by [24].

2.4.1. Test for alkaloids

a. Dragendorff's reagent test. Two mL of Dragendorff's reagent (potassium bismuth iodide solution) and 2 mL of diluted hydrochloric acid were added to 1 mL of seed extract. A reddish brown precipitate was considered to be indicative of the presence of alkaloids.

2.4.2. Test for flavonoids

a. NaOH test. One mL of 1N NaOH solution was added to 1 mL of seed extract. Formation of a yellow colour demonstrated a positive presence of flavonoids.

2.4.3. Test for cardiac glycosides

a. Keller-Killani test. One mL of glacial acetic acid was carefully added to 2 mL of seed extract and mixed well. Thereafter, 2 drops of 5% ferric chloride (FeCl3) solution was added after cooling to room temperature. This solution was transferred carefully to a test tube containing 2 mL of concentrated sulphuric acid (H2SO4). The formation of a reddish brown ring at the junction of two liquid layers was considered to be indicative of the presence of glycosides.

2.4.4. Test for terpenoids

a. Salkowski test. Five mL of seed extract was added to 2 mL of chloroform. Thereafter, 3 mL of concentrated H2SO4 was slowly added to form a layer at the interface. A reddish brown colour at the interface was considered indicative of the presence of terpenoids.

2.4.5. Test for steroids

a. Lieberman-Buchard test. Two mL of acetic anhydride was added to 5 mL of seed extract. Thereafter, 1 mL of H2SO4 was carefully added. The formation of a blue-green colour indicated the presence of steroids.

2.4.6. Test for saponins

a. Foam test. Five mL of seed extract was shaken vigorously in 20 mL distilled water. Formation of a stable honeycomb-like foam was an indication of the presence of saponins.

2.4.6. Test for phenols

a. Phenol test. On the addition of 0.5 mL $FeCl_3.6H_2O$ (w/v) solution to 2 mL of extract, the formation of an intense dark green colour was considered to be indicative of the presence of phenols.

2.4.7. Test for tannins

a. Gelatin test. The test solution was evaporated to dryness and the resultant residue was dissolved in 1% (w/v) liquefied gelatin; to this was added 10% (w/v) sodium chloride (NaCl) solution. A white precipitate was considered to be indicative of the presence of tannins.

2.5. Gas Chromatography/Mass Spectroscopy (GC/MS) analysis.

Analysis of all extracts by GC–MS was performed using PerkinEmler® Gas Chromatography (Clarus® 580) equipped with MSD mass spectrometer (Clarus® SQ8S) instrument with built–in autosampler. An aliquot of extract (1 mL) was then injected into apparatus. The samples were analysed on an Elite-5MS (30 m x 0.25 mm id x 0.25 µm) column. The oven temperature was programmed to proceed from 37-320°C at a rate of 18-25°C/min and held for 0.5 and 1.85 min at 18 and 320°C, respectively. The injector temperature was 250°C, MS Ion Source temperature was 280°C, with a full scan, and solvent delay of 0-2.30 min. MS Scan Range was m/z 35-500 in 0.10 sec. One µL of each sample was injected in helium carrier gas at a split flow rate of 20 mL/min.

2.6. Minimum Inhibitory Concentration (MIC) for antifungal determination.

The MIC of seed extracts against Candida albicans (ATCC 90028), Candida krusei (ATCC 6258) and Candida parapsilosis (ATCC 22019) were determined using the broth microdilution assay [25] with slight modifications. Briefly, seed extracts were diluted with 5% (v/v) DMSO to yield a range of concentrations: 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL. Forty µL of each concentration of the extract was added to a 96-well microtiter plate. Yeasts were harvested and suspended in 1% sterile saline solution and the turbidity of the supernatants measured spectrophotometrically at 625 nm with an absorbance of 0.08–0.1 equivalents to the No. 0.5 McFarland standard following the NCCLS M27-A2 guidelines. Once the desired absorbance was achieved using saline, the working suspension was diluted 1:20 in a mixture containing RPMI (Roswell Park Memorial Institute) 1640 medium (BioWhittakerTM, Lonza) with 0.165 M morpholinepropanesulfonic acid (MOPS) (BioWhittakerTM, Lonza) buffered to pH 7.0. The working suspension was further diluted with RPMI (1:50) to obtain a final test inoculum of $1\text{-}5x10^3$ CFU/mL. Then 160 µL of the working inoculum suspension was dispensed into each well containing the extract. Amphotericin B made up using sterile distilled water at 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL, were used as a positive control whilst the different solvents used to prepare the seed extracts and 5% DMSO were used as negative control agents. Plates were then incubated in an aerobic environment at 35°C for 24 h. After incubation, 20 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-18sulfophenyl)-2H-terazolium salt) was added directly to each well, incubated at 37°C for 4 h and the absorbance recorded at 490 nm on a microtitre plate reader (BioTek Synergy HT, Germany). Cell growth was determined using Equation 1.

Cell viability (%) = (Mean OD- Control OD) × 100 (Equation 1)

IC_{50} values were determined via linear regression analyses. Each extract at their various concentrations was assayed in triplicate and expressed as the mean percentage of viable cells.

2.7. DPPH Free Radical Scavenging Assay.

The DPPH assay was employed to determine the free radical scavenging activity of all seed extracts, according to a modified method [26-27]. DPPH solution was prepared by adding 3.7 mg of DPPH to 15 mL of methanol. To each well of a microtitre plate, 150 µL of DPPH solution and 50 µL of methanol extract at varying

concentrations (200 μg/ml, 100 μg/ml, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, and 6.25 μg/mL) were added to give a final volume of 200 μL. The plate was then left to incubate in the dark for 30 min, after which the absorbance was read using a microtitre plate reader (BioTek Synergy HT, Germany) at 517 nm. Ascorbic acid served as a positive control whilst methanol was used as a negative control. The different concentrations of each extract were assayed in triplicate and the experiment was repeated twice. Free radical activity was calculated using the equation 2.

IC_{50} of seed extract and ascorbic acid required to scavenge the DPPH free radical by 50% was calculated using linear regression analysis.

2.8. Statistical analyses.

Statistical analyses were performed on SPSS software, Version 22. Percentage data obtained were arcsine transformed, analysed for normality and thereafter subjected to a One Way Analysis of Variance (ANOVA). Results were considered significantly different if p values were less than 0.05.

$$\text{Free radical scavenging activity (\%)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \qquad \text{(Equation 2)}$$

Where

A_{sample} = methanol extract of seeds
A_{blank} = methanol
A_{control} = DPPH-methanol

3. RESULTS

3.1. Preliminary phytochemical analysis.

The preliminary qualitative phytochemical screening for different extracts of *T emetica* showed alkaloids, cardiac glycosides, phenols, sterols, terpenoids and flavonoids but there was no indication of saponins and tannins in all extracts (Table 1). The chloroform extract resulted in the highest diversity of secondary metabolites while distilled water resulted in the least. The most commonly occurring secondary metabolites (across the various extracts) were sterols and glycosides, whilst terpenoids were only found in the methanol extract.

3.2. Chemical composition of methanolic extract.

The greatest diversity of phytocompounds was obtained when methanol was used as the extracting solvent (Fig 1) when compared to ethanol, ethyl acetate, hexane and water. After, mass spectrometric analysis for each peak of methane extract from T. emetica seed showed the presence of 16 different phytocompounds (Table 2) belonging to different chemical families with pentanoic acid, 5-hydroxy-, 2,4-di-T-butylphenyl esters (10.85%) and p-xylene (9.63%) occurring in greatest abundance. The presence of several biologically active compounds suggests that the crude seed extracts of *T. emetica* possess pharmacological value, thus corroborating its use in herbal medicine. In a similar study carried out by Nana et al., 2013 using methanol, hexane and ethyl acetate fractions from stem bark of T. emetica yielded similar phytocompounds. Flavonoids, phenols and terpenoids were found to be major phytoconstituents, with no indication of the presence of saponins and sugars [25]. With regard to secondary metabolites of *T. emetica* obtained from other parts of the tree, numerous limonoids have been isolated and limonoids occurrence is restricted to the Meliaceae and Rutaceae families of the plant kingdom [28, 29]. Further investigation involving fractionation and characterization of bioactives present in the extracts of *T. emetica* is needed to ascertain the presence of limonoids. This could potentially be a new source of limonoids to explore.

3.3. Antifungal activity.

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of extract that is responsible for almost complete inhibition of microbial growth in a broth culture [30, 31]. The reference drug Amphotericin-B was used as a positive control in this study (Table 3). The methane, hexane and chloroform fractions exhibited good activity (37.46-75.45, 40.95-100 and 76.27-100.11, respectively) against all three fungal pathogens, whilst the ethyl acetate inhibited *C. parapsilosis* growth only. In contrast, the ethanol and aqueous extracts displayed no antifungal activity. These data suggest that the extracts were effective against *C. albicans* and most effective against *C. parapsilosis* (particularly, in terms of the methanol, ethyl acetate and hexane extracts). The activity of some of the plant extracts on different organisms explains their broad spectrum nature while most of the plant extracts found to have effect on one organism may be due to their narrow spectrum of activity. This difference of activity appears to be directly related to the qualitative and/or quantitative diversity of the compounds that are being accumulated by the plants investigated [14]. Though the MIC of the plant extracts are no match to those of the standard antibiotics, it is hoped that they might produce comparable effect on further purifications and/or isolation of the active constituents.

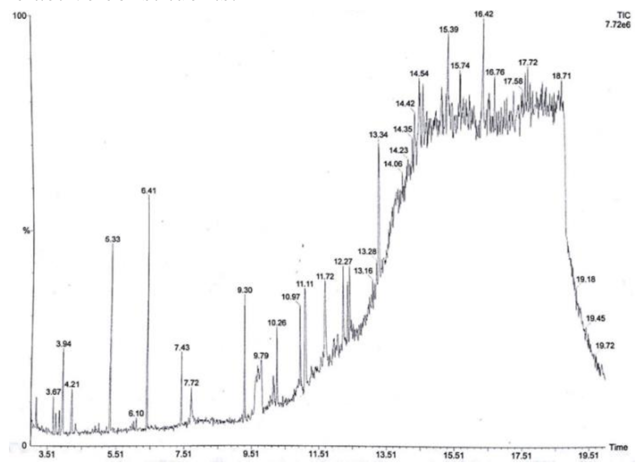

Figure 1. Gas chromatogram of methanol crude seed extract of *T. emetic.*

Fungal infections of the skin, nails and hair affect a large proportions of the world,, accounting for about 20% of new out patient referrals in the tropical countries within Africa where the damp and humid climatic conditions coupled with the advent of HIV/AIDS infection tend to aggravate skin disorders of fungal

origin [32]. The situation is further compounded by the lack of patient compliance to antibiotic regimen and by the exorbitant costs of the antibiotics. The preliminary results of the present study, therefore, not only confirm the justifiable use of seed extracts against these microorganisms in the traditional health care system but also reflects the hope for development of effective chemotherapeutic agents in the future from the same or similar extracts.

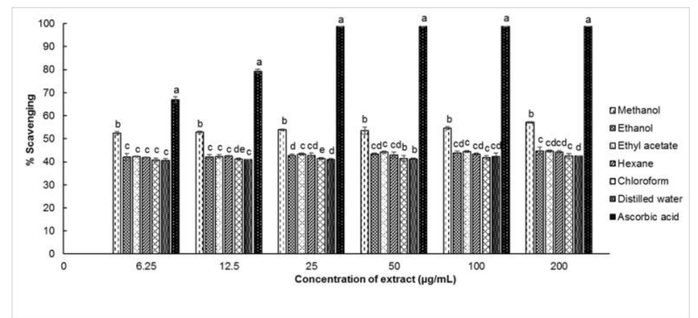

Figure 2. Free Radical scavenging activity of the crude seed extracts of *T. emetica.*

Bars labelled with different letters are significantly different when compared within extract type, across concentrations (ANOVA; p<0.05). Values represent mean ± SD of 3 trials of 3 replicates each.

3.4. *In vitro* free radical (DPPH) scavenging activity.

The DPPH free radical assay has been used extensively as a model scheme to evaluate the scavenging activity of antioxidants in vitro [32]. From the results shown in Fig. 2 it can be observed that except for the methanol extract all the seed extracts of *T. emetica* demonstrated radical scavenging ability in the concentration range 6.25-200 µg/mL. The methanol seed extract exhibited good radical scavenging activity with an IC50 (50% inhibitory concentrations) value of 5.94 µg/mL. However, it must be noted (that despite this low IC50 value, the radical scavenging ability of the methanol extract was not dose-dependent: Free radical scavenging of 52.37% and 57.13% was noted at concentrations of 6.25 µg/mL and 200 µg/mL, respectively (Fig. 2). The free radical scavenging ability of the standard, ascorbic acid, increased in a dose-dependent fashion with an IC50 value of 4.67 µg/mL.

It has been established that aromatic amines (p-aminophenol, p-phenylene diamine, etc.), ascorbic acid, flavonoids, glutathione, tannins and tocopherol have the ability to reduce and decolourise DPPH by their hydrogen donating ability [33, 34] resulting in potent antioxidant activity. All crude seed extracts, with the exception of methanol, exhibited poor antioxidant activity (Table 4) showing no IC50 in the concentration range tested. The methanol seed extract of T. *emetica* displayed good radical scavenging activity with an IC50 value of 5.94 µg/mL. It was reported in an earlier study, that seeds of Trichilia contain 40-60% fats that are made up of linoleic, oleic and palmitic acids [8]. This antiradical activity of the methanol crude seed extract could be attributed to the presence of phenols in the fatty acids of the seeds. Good radical scavenging activity was also reported for the methanol leaf extract *of T. emetica* by Frum and Viljoen [11] where an IC50 value of 17.9 µg/mL was noted. The phytocompounds identified in this study may be responsible for the antioxidant potential of the methanol seed extract; however, their precise mode of action needs further investigation.

Table 1 Comparison of preliminary phytochemical qualitative screening of various fractions of crude seed extracts of *T. emetica*

Secondary metabolites	Methanol extract	Ethanol extract	Ethyl acetate extract	Hexane extract	Chloroform extract	Distilled water extract
Alkaloids	-	-	+	+	+	-
Cardiac glycosides	+	+	+	-	+	-
Phenols	+	-	+	+	+	-
Sterols	-	+	+	+	+	-
Flavonoids	+	+	-	-	+	+
Saponins	-	-	-	-	-	-
Terpenoids	+	-	-	-	-	-
Tannins	-	-	-	-	-	-

Notes: (+) indicate presence of phytocompound and (-) absence of phytocompound.

Table 2 Phytocompounds of methanol seed extract of *T. emetica* acquired via GC/MS

RT (minutes)	Compounds	Molecular formula	Molecular weight	Peak area %
3.67	1,1-dimethyl-1-silacyclobutane	$C_5H_{12}Si$	100	4.89
3.94	p-xylene	C_8H_{10}	106	9.63
5.33	Hentriacontane	$C_{31}H_{64}$	436	8.87
6.10	Nonadecane, 2,6,10,14-tetramethyl	$C_{23}H_{48}$	324	3.58
6.41	Dodecane, 1-fluoro	$C_{12}H_{25}F_2$	188	8.62
7.43	Sydnone, 3-(3,3-dimethylbutyl)-	$C_8H_{14}O_2N_2$	170	6.97
7.72	Benzaldehyde, 2,5-dimethyl-	$C_9H_{10}O$	135	5.72
9.79	Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	518	4.16
10.26	Pentanoic acid, 5-hydroxy-,2,4,-di-T-butylphenyl esters	$C_{19}H_{30}O_3$	306	10.85
11.11	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	592	4.29
11.72	Disulphide, di-tert-dodecyl	$C_{24}H_5OS_2$	402	3.48
14.35	Trimethyl[-4-(1,1,3,3-tetramethylbutyl)phenoxy]silane	$C_{17}H_{30}PSi$	435	5.78
15.74	Trimethyl[-4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	$C_{15}H_{24}O_2Si$	264	5.91
19.18	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	578	6.10
19.45	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	222	5.61
19.72	Tris(tert-butyldimethylsilyloxy)arsane	$C_{18}H_{45}O_3 Si_3As$	468	18.55

Notes: RT-Retention time

Table 3. Minimum inhibitory concentrations (µg/mL) of crude seed extracts of *T. emetica* against pathogenic fungi.

Microorganism	Methanol extract	Ethanol extract	Ethyl acetate extract	Hexane extract	Chloroform extract	Distilled water	Amphotericin-B
C. albicans	75.45±1.95[a]	-	-	100±2.92[a]	80.12±1.03[b]	-	0.62±0.09[b]
C. krusei	90.41±1.22[a]	-	-	77.40±1.07[b]	100.11±0.95[a]	-	1.25±0.03[a]
C. parapsilosis	37.46±2.35[b]	-	40.62 ± 2.30[a]	40.95 ± 0.82[c]	76.27 ± 2.49[c]	-	1.25 ± 0.06[a]

*Notes: Values labelled with different letters are significantly different when compared within extract type, across species (*ANOVA*; $p<0.05$). Values represent mean ± SD of 3 trials of 3 replicates each.*
"–": no minimum inhibition in the tested range (6.25-200 µg/mL)

Table 4 DPPH radical scavenging activities of crude seed extracts of *T. emetica*

Sample	IC_{50} (µg/mL)
Methanol	5.94 ± 0.75
Ethanol	-
Ethyl acetate	-
Hexane	-
Chloroform	-
Distilled water	-
Ascorbic acid	4.67 ± 0.16

Notes: Values represent mean ± SD of 3 trials of 3 replicates each.
"-": No activity

4. CONCLUSIONS

In the current study, the medicinal plant *T. emetica* was investigated for phytochemical profile, anti-oxidant and antifungal activity. The biological investigation of the methanolic crude extract of the seeds of this species displayed radical scavenging ability. Also, solvent seed extracts (excluding distilled water extract) of *T. emetica* appear to be potential sources of antifungal agents. The results obtained suggest that this plant is very important from the medicinal point of view, and it needs further phytochemical exploitation to isolate phytochemical constituents having antifungal and antioxidant activites. The good degree of correlation of traditional therapeutic claims with specific antifungal activity as observed in the present preliminary results warrant further investigation of the seeds of this species and these studies are underway in our laboratory.

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6. ACKNOWLEDGEMENTS

This work was made possible through financial support from the National Research Foundation (South Africa) and University of KwaZulu-Natal, Durban.

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