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Phytochemical and pharmacological investigation of *Teucrium muscatense*

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Abstract

The effect of enzyme inhibition, anticancer, antifungal, antioxidants activities of the extracts of *Teucrium muscatense* obtained with different organic solvents was investigated. Chloroform fraction exhibited promising inhibition (64%) with IC 50 value (390 ± 2.0 μg/mL) against urease, while other fractions displayed moderate activity. In case of α-glucosidase and acetyl cholinesterase enzyme inhibition assays, all fractions were found inactive at a concentration of 1.0 mg/mL. The n-hexane fraction of *Teucrium muscatense* exhibited highest anticancer activity against breast cancer cell (MDAMB231) line at high (100 μg/mL) concentration and also inhibited the growth of *F. oxysporum* up to 64.3% in case of antifungal activity. EtOAc fraction showed highiest DPPH (70.6%) and ABTS (76.7%) radical scavenging activity at highest concentration (1000 μg/mL). Bioassay guided isolation of EtOAc fraction afforded two flavonoids (1 and 2). Both compounds showed highiest ABTS activity and could be a significant markers in the EtOAc fraction. Anti-lipid peroxidation assay was also performed in which aqueous fraction showed highest percentage of 73.2% at higher concentration (1000 μg/mL) followed by n-BuOH (64.1%) and EtOAc (51.4%) fractions. GC/MS analysis of the essential oil of *T. mascatense* showed higher percentage of linalool (34.18%), limonene (13.45%), linalyl acetate (10.04%), and β-eudesmol (9.21%). Proximate composition of *Teucrium muscatense* showed that it contained high amount of ash (19.6%), protein (10.3%), and fiber (17.5%).

Key words: *T. mascatense*, flavonoids, GC/MS, anti-cancer, biological activities

Introduction

The Sultanate of Oman is blessed with many useful plants there are more than 300 species of Teucurium all around the world [1–2]. Moreover about 49 species are endemic to Europe mainly in the Mediterranean region and many of these species are used in the folk medicine viz., as stimulants, tonics, and stomach ache remedy and also as anti-diabetic agents [3]. In some Arab countries the tile plants is used to treat skin diseases, fever, stomach diseases, kidney problems, diarrhea, cough, jaundice and abdominal pain [4–6]. There are various reports related to the pharmacological properties of Teucurium species. They are commonly used in folk medicine for various types of pathological conditions as astringent, diabetes, inflammation, gastric ulcer, 3 intestinal inflammation, diuretic, antiseptic, antipyretic and anti-helmintic [7]. Teucrium species were also found to possess a wide range of biological activities such as antioxidant, antimicrobial, anti-diabetic, anti-inflammatory [8], antipyretic, hepatopotective, hypoglycemic [9], larvicidal [10] and anti-nociceptive [11]. In current study we have investigated the enzyme inhibition, anticancer, antifungal, antioxidants activities of the extracts of *Teucrium muscatense* obtained with different organic solvents was investigated. In addition, we have also investigation the essential oil composition and proximate analysis of different extracts of title plant.

Materials and Methods

Plant Collection and Identification

T. mascatense was collected during their growing season from Jabal Al-Akhdar, Oman in June 2013 and identified by Dr. Syed Abdullah Gillani (plant taxonomist) at the Department of Biological Sciences and Chemistry, University of Nizwa, Oman. After collection, the plant was immediately washed, dried, and chopped into the small pieces and then stored at room temperature for further analysis.

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Extraction and Fractionation

Dried plant material (3.5 Kg) of *T. mascatense* was initially extracted vigorously with methanol (5.5 L) for 45 days at room temperature. The combined methanol extract was evaporated under reduced pressure leaving behind a greenish, syrup residue (104.8 g). The residue was suspended in 1 L distilled water and partitioned successively with n-hexane (13.8 g), dichloromethane (21.4 g), ethyl acetate (4.4 g), n-butane (35.2 g), and aqueous extracts (21.9 g), respectively. The EtOAc fraction was separated into seven fractions (F 1-7) by column chromatography (CC) on silica gel, using gradients of n-hexane/EtOAc (15:85, 30:70, 50:50, 70:30 and 0:100). Subfraction F 4 (0.5 g) obtained using 50% EtOAc/n-hexane was further purified by CC with n- hexane/EtOAc (90:10 to 40:60 as gradient) to give apigenin-4′, 7-dimethyl ether [(1, 8.5 mg; EtOAc/n-hexene (4:6)] and apigenin 7-methyl ether [(2, 6.5 mg; EtOAc/n-hexene (5:5)].

Anticancer activity

MDA-MB- 231 cell grown in Dulbeccos modified eagle's medium with 10% fetal bovine serum and 1% antibiotics in humidified atmosphere at 5% CO_2, 37 C. The viable cells were counted in haemocytometer using trypan blue and 1×10^5 cells/mL were seeded in 96 well plates and incubated overnight in incubator for an attachment. Then cells were treated with respective concentration of different fractions for 24 hrs. The stock solution was prepared in DMSO and diluted in medium before treatment. After treatment period, the cells were treated with MTT reagent (5 mg/mL) for 4 hrs and eventually the violet crystals dissolved in DMSO. The absorbance read at 540 nm (690 nm reference wavelength). The cells without treatment considered as 100% cell survival and based on this cell viability of treated cells quantified. Values are presented as Mean ± SD of four duplicates. Cells (1 10 5 /well) were seeded and allowed to adhere firmly in cell culture medium with 10% FBS [12]. Urease, α- glucosidase, and Acetyl Cholinesterase Enzyme Inhibition Bioassays Urease enzyme inhibition activity was performed according to the method as described [13]. 25 μL solution of Jack bean Urease, 55 μL of 100 mM urea dissolved in phosphate buffer (pH 8.2) and 5 μL (0.5 mg/mL) of various fractions of *T. mascatense* were incubated at 30 ºC for 15 minutes in 96-well plate. The production of ammonia was measured by indophenol method to determine the urease inhibitory activity. The phenol reagent (45 μL, 1% w/v phenol and 0.005% w/v sodium nitroprusside) and alkali reagent (70 μL, 0.5% w/v sodium hydroxide and 0.1% NaOCl) were added to each well and the increasing absorbance at 630 nm was measured after 50 minutes, using a microplate reader (spectrophotometer). The change in absorbance per minute was noted. All the tests were performed in triplicate. The assays were performed at pH 8.2 (0.01 M $K_2HPO_4.3H_2O$, 1.0 mM EDTA and 0.01 M $LiCl_2$). The IC 50 values were then calculated by using EZ-Fit enzyme kinetics program (Perrella Scientific Inc., Amherst, MA, USA). Thiourea was used as a standard inhibitor with 95 ± 1.5%.
The percentage inhibition was calculated from the formula:

$$100 - (\text{OD testwell} / \text{OD control}) \times 100.$$

The α-glucosidase (E.C.3.2.1.20) enzyme inhibition activity was performed according to the previously described procedures [14] with slight modification. In this assay 0.1 mg of α- glucosidase was dissolved in 10 mL of phosphate buffer (pH 6.8). In 96-well plate 20 μL of inhibitor sample of concentration 0.5 mg/mL was premixed with 120 μL of 50 mM phosphate 5 buffer (pH 6.8) and 20 μL of 5 mM p-nitrophenyl α-D-glucopyranoside. The reaction mixture was preincubated at 37 ˚C for 5 min. After that 20 μL α-glucosidase was added in reaction wells and incubated at 37 ˚C for 15 min. In blank sample 20 μL α-glucosidase and 20 μL of inhibitor sample were replaced by 40 μL of phosphate buffer. The control sample wells contain 20 μL of phosphate buffer in place of 20 μL of inhibitor sample. The reaction was terminated by the addition of 100 μL Na_2CO_3 (200 mM). Inhibition activity was determined spectrophotometrically at 400 nm on spectrophotometer (Spectro Max Molecular Devices, USA). Acrabose was used as standard inhibitor. The percentage inhibition was calculated using the equation:

$$\text{Inhibition \%} = 1 \ (\text{OD blank sample}/\text{OD control}) \times 100$$

Inhibition of acetyl cholinesterase enzyme activity was determined using Ellman's colorimetric method as described [15]. In this assay 96-well plate was used and each reaction well contained 25 μL of 15 mM ATCI dissolved in water, 125 μL of 3 mM DTNB in Buffer (50 mM Tris–HCl, pH 8), and 25 μL of plant extract of concentration 0.5 mg/mL dissolved in DMSO. In blank sample 25 μL plant extract was replaced by 25 μL of Tris-HCl buffer. In control sample also 25 μL of Tris-HCl buffer was used in place of 25 μL of plant extract. 96-well plate was then incubated at 25 ˚C for 15 minutes. Thereafter, 25 μL AChE (0.2 μg/mL) was added to the wells and the absorbance measured five times consecutively 45 second. In blank sample 25 μL of AChE were replaced by 25 μL of Tris-HCl buffer. The reaction was monitored for 5 min at 405 nm [16]. The percentage inhibition was calculated using the equation:

$$\text{\% Inhibition} = 1 \ (\text{blank sample}/\text{control}) \times 100$$

Anti-Lipid Peroxidation Bioassay

Anti-lipid peroxidation activity was measured by the thiobarbituric acid (TBARS) assay as described [17]. To carry out the assay 5 μL of Tris-HCL buffer (pH 7.1), 20 μL of substrate (Phosphotidyl choline), 5 μL of ferrous sulphate (1 mM), and 20 μL (0.5 mM) of sample inhibitor were added in 96 well plate and of double distilled water (30 μL) was added. The mixture was then incubated at 37 °C for 15 min. Then 120 μL of TBA (0.35%) and 50 μL of TCA (50%) were added to the reaction mixture and then incubated for 15 minutes at 100 °C water bath. Pink colour chromogen appeared. Readings were taken at 532 nm (spectra Max-340). Activity (%)

was carried out in comparison with a DMSO treated control group and % lipid peroxidation activity was calculated. BHA (1.5 mM) was used as reference compound in lipid 6 peroxidation assay [18]. All tests in this assay were performed three times. The percent inhibition activity was calculated by the formula:

% Inhibition = (A control – A sample / A control) × 100

Where A control is the absorbance of control reaction and A sample is the absorbance of test reaction.

DPPH Radical Scavenging Activity

Free radical scavenging activity of the different fractions of *T. mascatense* was determined according to the method [19]. To 50 µL of 0.1 mM DPPH in methanol, a volume of 50 µL of plant extract (range 20–1000 µg/mL) was mixed and kept in the dark at room temperature for 60 minutes. After incubation, the absorbance was recorded at 490 nm. The results were compared with the control which contained 50 µL of ethanol instead of plant extracts. BHT and Quercetin were used as a positive control in concentration range of 5–250 µg/mL. The antioxidant activity was expressed as follow:

% RSA = (A control – A sample /A control) × 100.

Where RSA is the radical scavenging activity, A control is the absorbance of control reactions and A sample is the absorbance of test reaction.

ABTS Radical Scavenging Activity

ABTS radical scavenging activity was performed according to the method [20]. The stock solution which was allowed to stand in the dark for 16 h at room temperature contained equal volumes of 7 mM ABTS salt and 2.4 mM potassium persulfate. The resultant solution was diluted with methanol until an absorbance of 0.706 ± 0.001 at 734 nm was obtained. Varying concentrations (0.25, 0.5, 1, and 2 mg/mL) of the extract were allowed to react with 2 mL of the solution and the absorbance readings were recorded at 734 nm. The scavenging capacity of the extract was compared with that of trolox and the percentage inhibition calculated as:

% ABTS radical scavenging activity = (A control – A sample / A control) × 100

Where A control is the absorbance of ABTS radical and methanol and A sample is the absorbance of ABTS radical and sample extract/standard. All tests were carried out in triplicates. The extract concentration providing 50 % inhibition was obtained by plotting inhibition percentage versus extract concentration.

Total Flavonoid Contents

The total flavonoid contents were determined using slightly modified method of the assay reported [17]. For analyses different fractions (50 µL) of the plant were mixed with 100 µL of deionized water and 100 µL of 5% $NaNO_2$ solution was added to the sample together with 150 µL of 10% $AlCl_3$. After mixing and incubation for 5 minutes, 100 µL of 1 M NaOH was added to the reaction mixture and a total volume of 200 µL was made up with deionized water. Following vigorous mixing, the absorbance was measured at 510 nm. A standard curve was prepared using 25–1000 mg/L of Quercetin. The results were expressed as mg quercetin equivalent (QE)/g dry extract (dE).

Allelopathic Activity

Allelopathic potential of the chloroform, n-hexane, n-butanol and water fractions was determined according to the method described [21]. Lettuce seeds (Lectuca sativa, Great Lakes 366, Takii Seed Co. Ltd, Japan) were used in this assay as an indicator species to see the effective concentration at 50 percent inhibition (EC 50) using various concentrations of sub-fractions. Two different concentrations (20 and 100 mg) of each sub-fraction were prepared by dissolving it in 5% DMSO. A filter paper of 27 mm (Type Roshi Kaisha, Ltd, Tokyo) was put in a petri dish. The dilutions were subjected on the filter paper and thus allowed to spread over it. Lettuce seeds (15 numbers) were placed on it and the dishes were sealed and packed for incubation for 72 hours at room temperature. For each sub-fraction, mean, SD variance and standard error were calculated to determine the inhibition pattern at various concentration levels.

Isolation of the Essential Oil

NEOS (essential oil system) was carried out with a Milestone technologies (Model: IEC 60825- 1; 2007) microwave apparatus with a maximum delivered power of 900 W variable in 10 W increments and 650 nm wave length. During experiment, time, pressure and power were controlled with the "easy-WAVE" software. Fresh sample of *T. mascatense* (100 g) was heated using a fix power of 400 W for 50 min at 100 °C in water. The extraction was continued at 100 °C until no more essential oil was obtained. The essential oils were collected, dried over anhydrous sodium sulphate and stored at 4 C until used. Extractions were performed at least three times and the mean values were reported. Incense (Bukhor) was produced in a form of smoke by heating the sample in a hotplate, collected and concentrated in a sample vial with a septum. It was then injected into the GC-MS using a syringe.

Gas chromatography-Mass spectrometry (GC-MS) Analyses

GC-MS analysis was performed on a Perkin Elmer Clarus 600 GC System, fitted with a Rtx-5 MS capillary column (30 m 0.25 mm i.d. 0.25µm film thickness; maximum temperature, 350 ˚C), coupled to a Perkin Elmer Clarus 600C MS. Ultra-high purity helium

(99.9999%) was used as carrier gas at a constant flow of 1.0 ml/min. The injection, transfer line and ion source temperatures were 290, 250 and 200 °C, respectively. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from auto tune. All data were obtained by collecting the full-scan mass spectra within the scan range 40-550 amu. The injected sample volume was 1 μl with a split ratio of 200:1. The oven temperature program was 60 ºC at a rate of 3–280 ºC/min hold for 2 minutes. The unknown compounds were identified by comparing the spectra obtained with mass spectrum libraries (NIST 2011 v.2.3 and Wiley, 9 th edition).

Proximate Analysis

By using the standard methods of the AOAC [22], determination of moisture, ash, and crude fibers (on dry basis) was carried out. The determination of proteins in terms of nitrogen was done by micro Kjeldahl method [23]. The nitrogen value was converted to protein by multiplying to a factor of 6.25. The lipid content of the samples was done using soxhlet type of the direct solvent extraction method. The solvent used was petroleum ether (boiling range 40-60 ºC) [24]. The crude fiber was also determined by the method described by [22]. The energy values (Kcal/100 g) were determined by multiplying the values of carbohydrates, lipids and proteins by a factor of 4, 9, and 4 respectively, and taking the sum expressed in kilocalories. The total carbohydrates were determined by difference method [24]. All the proximate values were reported in percentage.

Antimicrobial activity of different extracts of T. muscatense

Antifungal activity was carried out using well-diffusion method [13]. Zone of Inhibition (ZIC) and fungal growth was tested against 10000 ppm concentration of extracts by taking 60 mg from each extract fraction in 6 mL of DMSO solvent. Media for antifungal test was prepared using potato Dextrose Agar (approximate 38 g per liter), 19.5 g dissolved in 500 mL of distilled water, and then autoclaved at 121 ºC for 2 hours to be completely dissolved. Petri plates were prepared with 25 mL of sterile PDA medium and left for solidification for 30 min. Samples from two concentrations and samples from fungi were transferred into the plates to assess the effect of fungal growth and zone of inhibition. Eighteen plates for 6 extracts with 3 fungi (Asperigillus niger, Fusarium oxysporum, and Alternaria altemate) were done. The plates were incubated for one week at 37 ºC. ZIC was recorded in centimeters. Antibacterial activity was carried out using well-diffusion method [13]. The nutrient agar media was prepared, 28 g powder to 1000 mL distilled de-ionized water, and then autoclaved at 121 ºC for 15 min in autoclaved machine. Petri plates were prepared with 25 mL of sterile nutrient medium and left for solidification for about 30 min. Three wells in each nutrient agar plates were made by using sterile Weller. The wells were loaded with 10000 ppm extracts concentrations and bacterial pads (E-coli) were transferred into the centre of plates to assess the effect of bacterial growth and zone of inhibition. The plates were incubated for one week at 27 ºC.

Results and Discussion

The structures (Figure 1) of apigenin-4′, 7-dimethyl ether (1) [25-28] and apigenin 7-methyl ether (2) [29,30] were identified using spectroscopic techniques (1 H, 13 C NMR, COSY, HSQC, HMBC and ESIMS) and comparison with previously published data

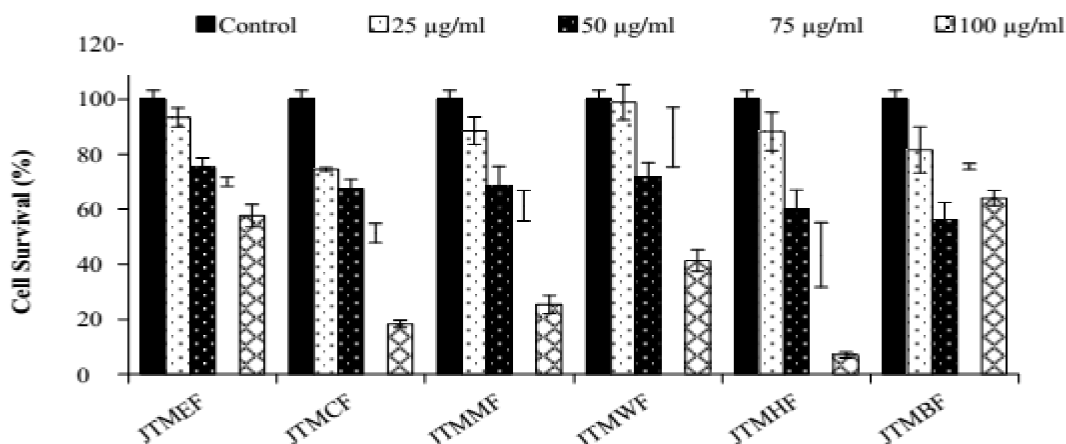

Figure 1: Isolation of two flavonoids 1 and 2 from chloroform fraction of *T. muscatense*.

Anticancer activity

The cytotoxic effect of crude methanol and different fractions of *T. mascatense* on cancer cell line, namely MDA-MB- 231 (breast cancer), was evaluated using different concentrations. After treating the cells with varying concentrations (25, 50, 75 and 100 µg/mL) for 24 hours, an MTT assay was performed and cell viability was measured. The n-hexane fraction of *T. mascatense* exhibited

highest anticancer activity against breast cancer cell line at higher concentration (100 µg/mL) only, followed by chloroform and methanol fractions. The inhibitory properties of these fractions were compared with standard doxorubicin for breast cancer cell line. Interestingly, n- hexane fraction showed highest cell death cause than standard doxorubicin at the highest concentration (100 µg/mL) (Figure 2). Chloroform, methanol and aqueous fractions also displayed moderate activity at highest concentration. This indicates that n-hexane fraction is relatively more cytotoxic towards breast cancer cell as compared to other fractions.

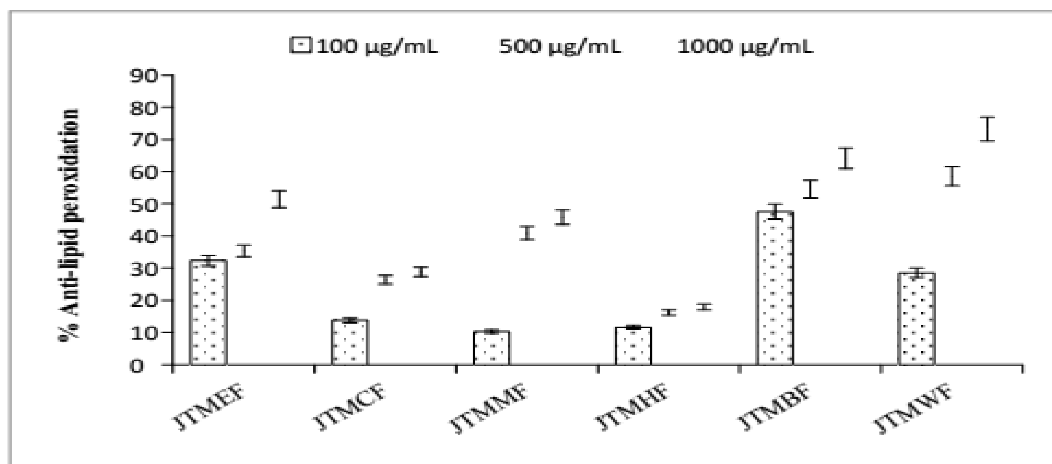

Figure 2: Anticancer activity of *T. mascatense* against breast cancer cells (MDA MB 231); nhexane (JTMHF), chloroform (JTMCF), EtOAc(JTMEF), n-BuOH (JTMBF), H2O (JTMWF)

Urease, - glucosidase, and Acetyl Cholinesterase Enzyme Inhibition Bioassays

The inhibitory activities of crude extract and various fractions of *T. mascatense* were determine at a concentration of 1.0 mg/mL against three enzymes including urease, α-glucosidase and acetyl cholinesterase (Table 1). $CHCl_3$ soluble fraction exhibited promising inhibition (64%) with IC 50 of 390 ± 2.00 µg/mL, against urease enzyme followed by EtOAc (52%). Urease enzyme is involved in stomach ulcer and serves as a virulence factor in human and animal infections of urinary and gastrointestinal tracts [31]. Recently, natural products have gained much interest for the safe inhibition of urease as potential new antiulcer drugs [32]. All fractions along with crude extract were found inactive against α-glucosidase and acetyl cholinesterase enzymes activity. Thiourea, Acrabose and galantamine were used as standard inhibitor against urease, α- glucosidase and acetyl cholinesterase enzymes respectively (Table 1).

Table 1: Different fractions of *T. mascatense* investigated for urease, acetyl cholinesterase and 11 - glucosidase enzymes at 1.0 mg/mL concentration.

Sample Code	Urease Enzyme (%) Inhibition	IC_{50} (µg /mL)	α- glucosidase % Inhibition	Acetyl cholinesterase % inhibition
JTMMF[a]	NA	-	NA	NA
JTMWF	40 ± 1.50	-	NA	NA
JTMBF	35 ± 1.00	-	NA	NA
JTMCF	64 ± 2.50	390 ± 2.00	NA	NA
JTMEF	52 ± 1.00	-	NA	NA
JTMHF	30 ± 1.00	-	NA	NA
Thiourea	95 ± 1.50	21 ± 1.50 mM	-	-
Acrabose			72 ± 2.00	-
galantamine				92 ± 2.5

[a]methanol (JTMMF) extract viz., *n*-hexane (JTMHF), chloroform (JTMCF), EtOAc (JTMEF), n-BeOH (JTMBF), and aqueous (JTMWF)

Anti-lipid peroxidation

Lipid peroxidation is a toxic metabolic process caused due to the production of free radicals from compounds including hydro peroxides, redox cycling compounds and iron-containing

compounds. In TBARS assay used for the measurement of lipid peroxidation activity of plant extracts and compounds, TBA reacts with malondialdehyde (MDA) produced as a secondary product of lipid peroxidation to give red chromogen which is determined spectrophotometrically [33]. Different fractions of *T. mascatense* were tested for anti-lipid peroxidation using Thiobarbituric Acid (TBARS) Assay. The present results showed that approximately all the tested samples, dose dependently, inhibited lipid peroxidation. However, aqueous fractio showed the highest scavenging potential (73.2%) followed by n-butanol (64.1%) and EtOAc (51.4%) fractions at the highiest concentration (1000 µg/mL), while chloroform and n-hexane fractions showed the least anti-lipid peroxidation activity (Figure 3). BHA was used as standard had 91.2 ± 0.54% inhibition at the same concentration. The results indicated that the compounds contributing towards the anti-lipid peroxidation activity are chemically diverse from each other ranging from non-polar to highly polar fractions. The extracts can be used as preventive measure against damaging effects of free radicals on lipoproteins, DNA, sugars, proteins and amino acids in living bodies.

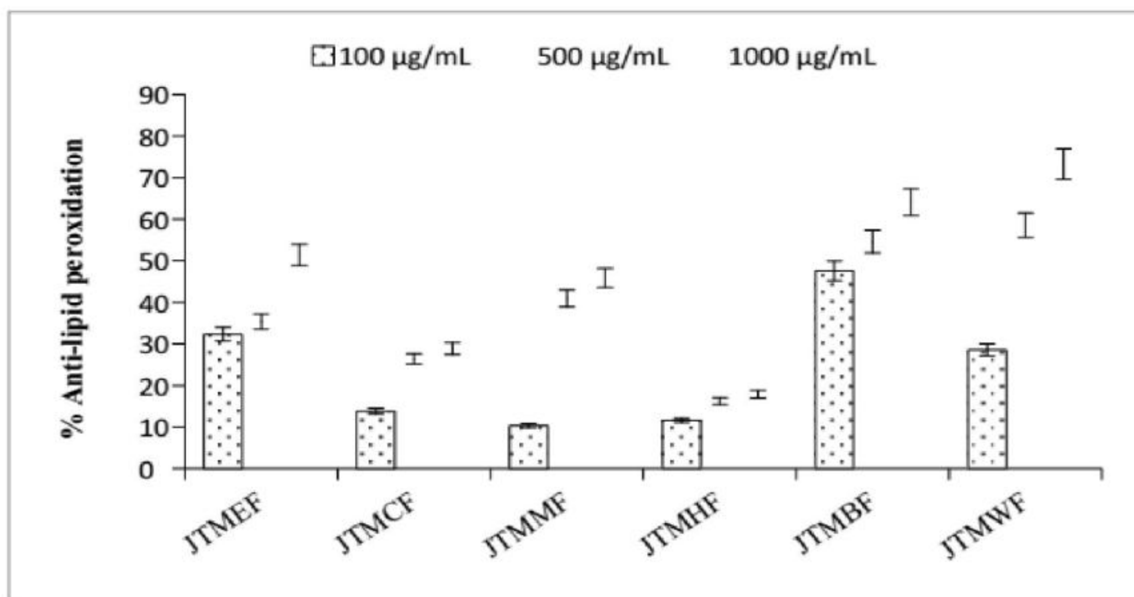

Figure 3: Anti-lipid peroxidation of different fractions of T. mascatense.

DPPH Radical Scavenging Activity

DPPH is a stable radical used for estimation of antioxidant activity of plant extracts. In the present study significant antioxidant activity in terms of % inhibition was observed against DPPH with highest activity observed for EtOAc fraction (70 ± 2.14%) followed by n-butanol and methanol fractions (35.6 ± 1.35% and 31.6 ± 1.42% respectively). Lowest anti-radical activity was observed for n-hexane fraction which had least total flavonoid contents (Figure 4). Traditionally, synthetic antioxidants such as BHT, BHA, and ascorbic acid have been widely used as antioxidants in the food industry [34]. Antioxidants have been detected in a number of agricultural and food products including cereals, fruits, vegetables and oil seeds [35]. The strong radical scavenging activity of polar extracts can be attributed to the high phenolic and flavonoid contents found in these extracts as compared to non-polar extracts. However, polar extracts can be used as a source for obtaining pure compounds with antioxidant activity in sub micromolar range. In the present study, the radical scavenging effects are high in EtOAc fraction, suggesting the presence of bioactive metabolites which has oxidative stress mitigation properties.

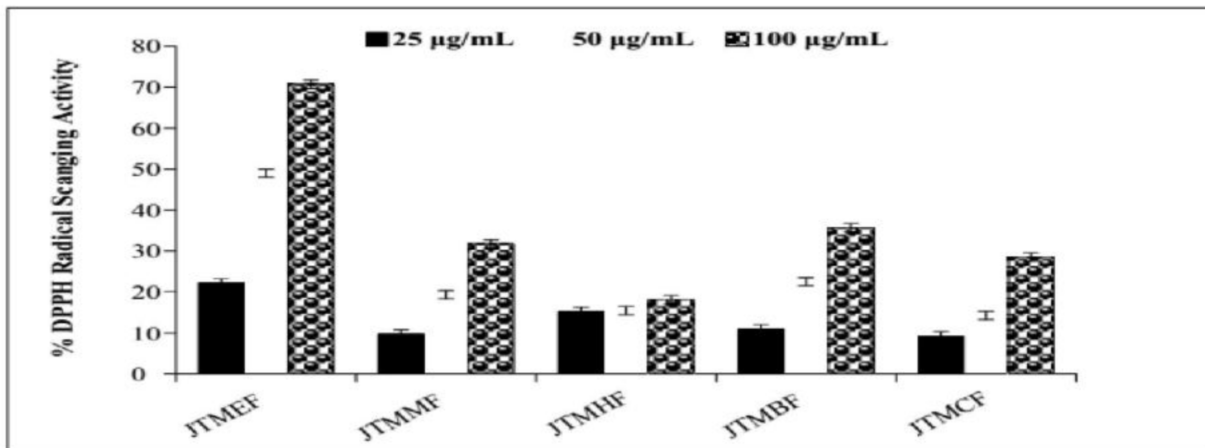

Figure 4: DPPH Radical Scavenging Activity of the different fractions of T. mascatense.

ABTS Radical Scavenging of the Fractions

Different fractions of the *T. mascatense* were subjected to the ABTS radical scavenging activity. EtOAc fraction showed highest activity (76.7 %) at higher concentration (1000 µg/mL), which was gradually decreased to 67.0 and 28.0% with the decrease in concentration (Figure 5). Lowest concentration was observed in the n-hexane fraction (15-28%) when compared with the other fractions. Ascorbic acid was used as a standard which showed 92.31% activity. ABTS Radical Scavenging Activity of the Pure Compounds (1 and 2) Compounds 1 and 2 were isolated from the EtOAc fraction of *T. mascatense* and subjected to the ABTS radical scavenging activity using ascorbic acid as a standard. Different concentrations (10, 20, 50. 100 and 200 µg/mL) were prepared. Interestingly, compound 2 showed higher activities than compound 1 at all concentrations and displayed almost similar level to the standard (ascorbic acid) at the highest concentration (200 µg/mL; Figure 6). The highest activity of compound 2 may be due to the presence of additional hydroxyl group at C-4′ position.

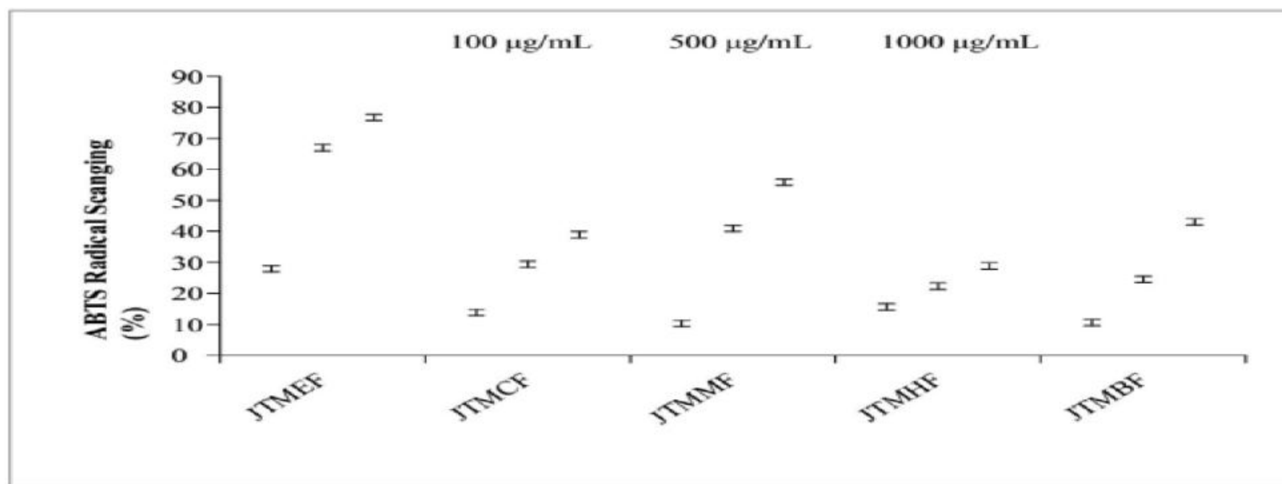

Figure 5: ABTS scavenging activity of different fractions of *T. mascatense*

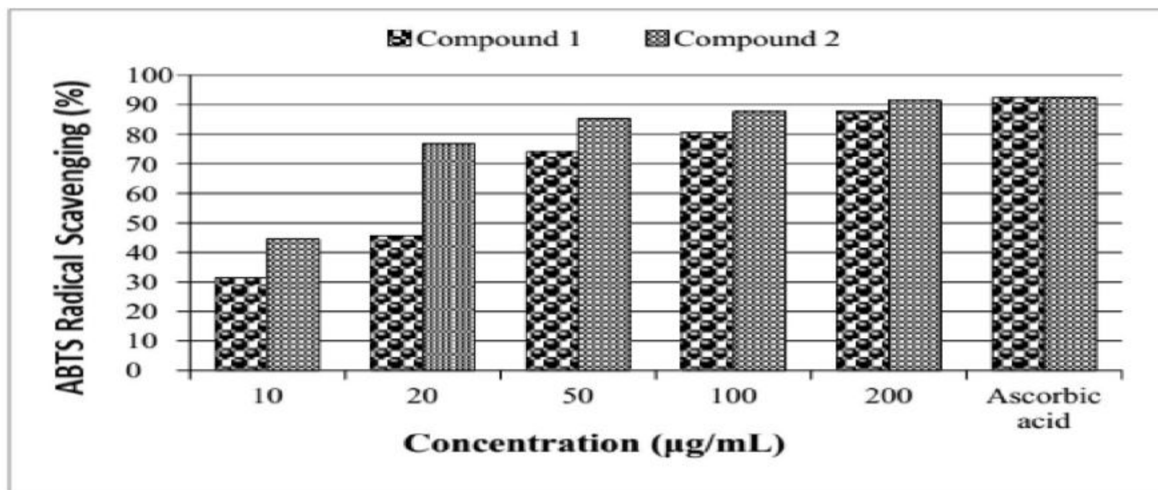

Figure 6: ABTS activity of compounds 1-2isolated from ethyl acetate fraction of T. mascatense.

Total Flavonoid Contents

The total flavonoid contents in the different fractions of *T. mascatense* showed that the n-butanol fraction (55.13 µg/g) had comparatively higher total flavonoid contents followed by EtOAc (52.0 µg/g) and aquoues fractions (46.5 µg/g) at higher concentration (1000 µg/mL) (Figure 7). Crude extracts of medicinal plants, rich in phenolics and flavonoids, are of increasing interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food [13]. Previously nine flavonoids such as 5-hydroxy- 3,6,7,4-tetramethoxyflavone, salvigenin, 5- hydroxy-6,7,3,4- tetramethoxy flavone, chrysosplenetin, cirsilineol, cirsimaritin, cirsiliol, apigenin and luteolin were isolated from the 70% aqueous methanol fraction (using defatting procedure) and of *Teucrium barbeyanum* [36]. Similarly Cakir et al. (2006) isolated one flavonoid and three flavonoid glycosides namely cirsilineol, luteolin 7-O-rutinoside, luteolin 7- O-glucoside, and hesperetin 7-O-rutinoside from the acetone fraction of *Teucrium orientale* L [37]. Ethyl acetate and n-butanol fractions of *T. barbeyanum* also showed comparable antioxidant activity to known antioxidants (trolox and ascorbic acid) and the highest total phenolic and flavonoid contents [36]. Previous report on the aqueous extracts of *Teucrium polium* showed remarkable antioxidant activity in vitro [38]. Our findings were found in close proximity to the reported values of *T. barbeyanum* and *T. polium*. However, n-butanol and EtOAc fractions of *T. mascatense* have not been subjected to such analysis, which could be interesting to search for bioactive secondary metabolites.

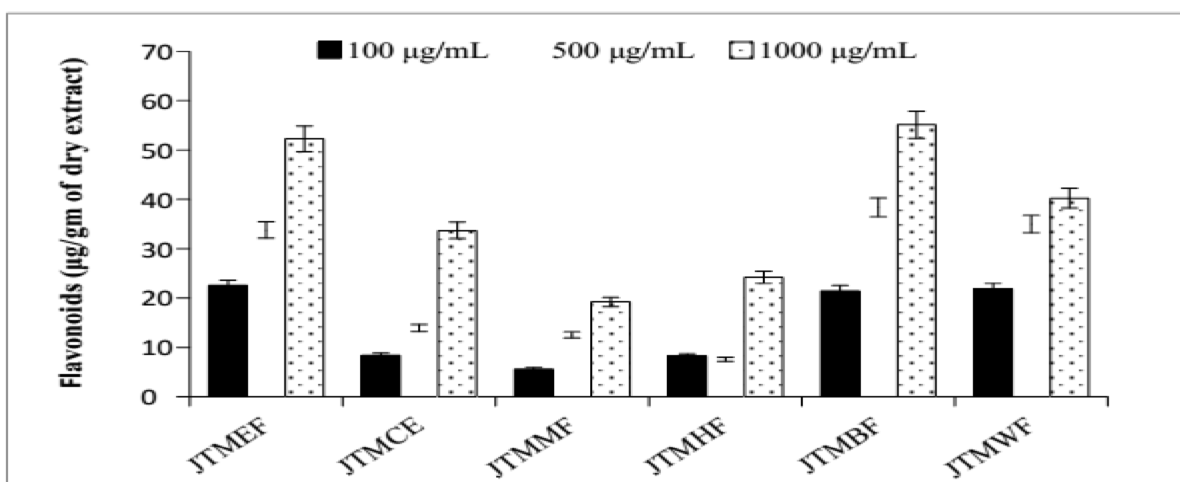

Figure 7: Total flavonoid contents of *T. mascatense* expressed in µg/g of dry extract.

Allelopathic Activity

In the present study, the allelopathic effect of dried leaves of *T. mascatense* showed significant inhibitory effect towards the growth of lettuce seeds. When 20 and 200 mg dried leaves of the tested

plant were tested against lettuce seeds, significant inhibitory effects were observed on root and hypocotyl growths of lettuce seeds (Figure 8, Table 2). At the higher concentration (100 mg), *T. mascatense* completely inhibited the germination of lettuce seeds, due to which no root growth and hypocotyl growth was observed. Similar results were shown by Fujii et al. [39]. According to his reports increased concentrations of leaf extracts suppressed the growth of some vegetable species. However, this was also observed that reduced concentrations of such extract of leaves can also stimulate the germination and growth of seeds [40]. The inhibitory effect suggests that the extract of the leaves may comprise of allelopathic compounds which are affecting the germination potential of seeds. The reason for an inhibitory and stimulatory effect on the germination is due to the presence of different allele-chemicals present in the leaves rich in secondary metabolites including: alkaloids, glycosides, coumarins, flavonoids, and steroids etc. [13]. In cropping system, the biological control of weeds is essential to enhance crop productivity. Such methods have been highly appreciated and practiced to obtained higher yields from present crops with little application of synthetic fertilizers and herbicides [21]. The results from this study suggested that the secondary metabolites from *T. mascatense* can be potential candidates for the phytotoxicity (Allelopathy) and can be utilized on commercial scale for the development of new herbicides or weedicides.

Table 2: Effects of dried leaves on the growth of lettuce seeds. The values of roots (R) and hypocotyls (H) for the selected plant represent growth percentage.

Plant Name	20 mg		100 mg	
	R (%)	H (%)	R (%)	H (%)
Teucrium muscatense	80	77	0	0

Figure 8: Allelopathic effects of dried leaves of T. mascatense.

Essential Oil Composition

Essential oil of *T. muscatense*, extracted through an apparatus NEOS (New Essential Oil System), was analyzed by GC-MS and identified 24 chemical constituents (88.14%) while eleven unknown compounds were also present with 11.86% of the chemical composition (Table 3). The major chemical constituents in the essential oil of *T. mascatense* were Linalool (34.18%), Limonene (13.45%), Linalyl acetate (10.04%), and β-Eudesmol (9.21%). In earlier study by Al Oraimi et al. (2012) on GC-MS analysis of essential oil of *T. mascatense* from Oman used hydrodistillation method of extraction [1]. The study identified 22 compounds (78.4%) in addition to ten unknown compounds (21.6%). The major constituents were Limonene (17.2%), Linalool (12.4%), α-pinene (10.1%), and β-Eudesmol (10.1%) (Al Oraimiet al., 2012). Our result also showed additional chemical constituents which were not reported by using hydrodistillation method used [1]. Six compounds such as Z-β-ocimene (0.08%), Pinocarveol (0.18%), Myrtenal (0.14%), trans-Carveol (0.21%), Nerol (0.15%), and Neryl acetate (1.19%) were observed in our study but not reported through hydrodistillation method. On the other hand, five compounds such as δ-Cardinene (1.6%), Nerolidol (1.2%), tau-Cadinol (0.8%), α-Eudesmol (0.6%), and Torreyol (1.3%) were reported in Hydrodistillation method, but not in our study using NEOS method of essential oil extraction. On the basis of these results, we can conclude that both the methods may be used together to get complete chemical composition profile of essential oil of *T. mascatense* which will make minimum list of 38 chemical compounds (Table 3).

Table 3: Essential oil composition of T. muscatense

S. No.	Compounds Name	RT (min.)	(%)
1	α-Pinene	5.43	2.17
2	β-Pinene	6.57	1.05
3	β-Myrcene	6.9	0.02
4	dl-Limonene	8.13	13.45
5	Z-β-ocimene	8.75	0.08
6	Linalool	10.63	34.18
7	Pinocarveol	12.15	0.18
8	Verbenol	12.39	0.52
9	α-Terpineol	14.23	1.85
10	Myrtenal	14.47	0.14
11	trans-Carveol	15.39	0.21
12	Nerol	15.77	0.15
13	Carvone	16.42	0.68
14	Linalyl Acetate	16.92	10.04
15	Bornyl Acetate	18.18	0.11
16	Terpinyl Acetate	20.81	0.45
17	Neryl Acetate	22.24	1.19
18	trans-Caryophyllene	23.66	0.75
19	Trans-α-bergamotene	24.33	4.77
20	Aromadendrene	25.32	0.41
21	α-Selinene	26.32	1.92
22	Caryophyllene Oxide	30.04	2.16
23	Spiro[4.5]dec-8-en-7-one, 1,8-dimethyl-4	30.95	2.45
24	β-Eudesmol	32.53	9.21
	Unidentified		11.86
			88.14/100

Proximate Analysis

T. mascatense was subjected to the proximate analysis including moisture, ash, crude fat, crudefiber, carbohydrate and energy values. The results obtained showed that the moisture content was found 8.47 % in *T. mascatense*. Hussain et al. (2009) determined the same amount in the *N. sauvis* (8.44%) [41]. Ash contents of *T. mascatense* were found 19.66%. In comparison, ash content of *T. mascatense* was found higher than *N. sauvis* (7.91%), *Datura alba* (18.80%), *Phlomis cashmeriana* (17.66%) and *Calotropis procera* (17.62%), while ash values of fruit was also in good agreement with *Dalbergia sisso* (12.33%), *Phlomis bracteosa* (10.83%), and slightly lower than *Avera javanica* (14.23%) [42–44]. The protein content was calculated on the basis of the available nitrogen using Kjeldahl method and was observed 10.31% in *T. mascatense* (Figure 9). Hussain et al. (2010) carried out the same experiments on Phlomis bracteosa (10.61%) and Phlomis cashmeriana (9.51%) belong to the family Labiateae, and found close resemblance to *T. mascatense* [45]. Nutritionally, it is beneficial as proteins contain amino acids utilized by the cells of the body to synthesize all the numerous proteins required for the function of the cell and also to furnish energy [46]. The carbohydrate content of the *T. mascatense* was found to be 39.63% which is slightly lower than the recommended value of WHO (55 to 75%) [47]. Fat contents of *T. mascatense* showed close similarity with Rhiza stricta (3.98%) and Dalbergia sisso (3.35%) [45], however, lower to that of Avera javanica having 1.15% fats [41]. Highest amount of crude fibers (17%) was observed in the *T. mascatense* plant. Nutritionally, this

is of beneficial effect since it had been reported that food fiber aids absorption of trace elements in the gut [48] and reduce absorption of cholesterol [49]. A high intake of dietary fiber also improves glycemic control, decreases hyper insulinemia, and lowers plasma lipid concentrations [50]. The results of proximate analysis clearly showed that the plant (*T. muscatense*) is rich in nutrients, hence qualify as good food material and can be used in poultry feed as an energy source.

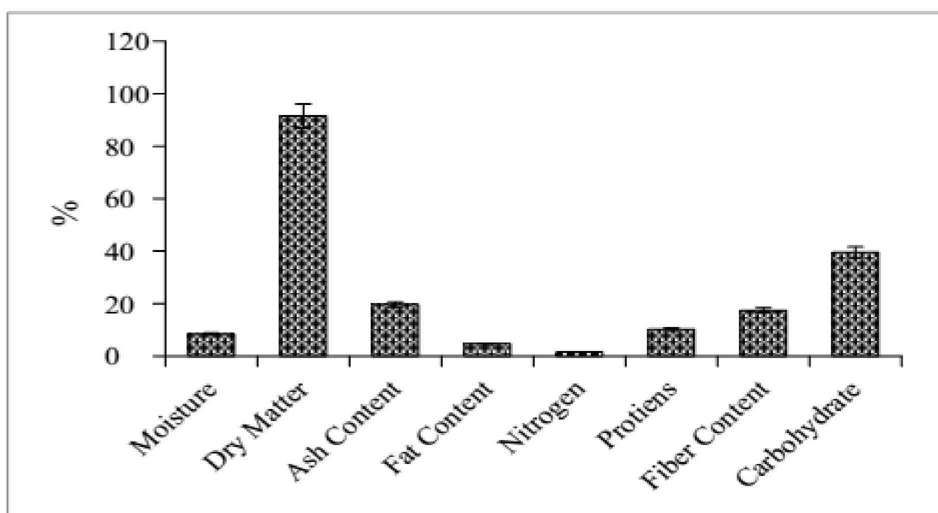

Figure 9: Proximate parameters of dried powder material of *T. mascatense*

Antifungal Activity

Plant extracts and their components have been known to exhibit biological activities, especially antifungal, antibacterial, and antioxidant [51]. Inhibiting their growth through naturally occurring metabolites is an environmental friendly strategy. In present study, we assessed the effects of various fractions of the *T. mascatense* on these noxious microbes and their growth. The effects of n-hexane, chloroform, n-butanol, and aqueous fractions on the growth of fungal (*C. globosum, F. oxysporum*, and *A. niger*) strains were determined using the highiest concentration of 10 mg/mL per well. All the fractions showed weaker growth inhibition (64.3–92.8%) (Table 4), however, some fractions stimulated the growth of the fungal strains. *C. globosum*: The aqueous fraction of *T. mascatense* is weakly inhibited the growth of *C. globosum* upto 91%, while 136.4–163.6% growth enhancement was observed in rest of the fractions. *F. oxysporum*: n-Hexane fraction inhibited the growth of *F. oxysporum* up to 64.3%. *A. niger*: In case of *A. niger*, none of the plant fractions inhibited the fungal growth rather than all the fractions enhanced the growth of fungus ranging from 162.5% to 325% (Table 4). Antibacterial activity of the crude extracts and various sub fractions of *T. mascatense* was tested against gram positive (Staphylococcus aureus) and gram negative bacteria (*Escherichia coli*) at 10 mg/mL concentration due to the least sensitivity. The crude extract of methanol and all the fractions (n-hexane, $CHCl_3$, n-BeOH, and aqueous) were found inactive against *S. aureus* and *E. coli* (Table 2).

Table 4: Antifungal activity of different fractions of *T. mascatense* against three fungal microbes.

C. globosum	Growth	Area	% Growth
Control	11	102	100
JTMHF	18	254.3	163.6
JTMCF	16	200.96	145.5
JTMBF	15	176.6	136.4
JTMWF	10	78.5	90.9
F. oxysporum			
Control	14	153.9	100
JTMHF	9	63.6	64.3
JTMCF	17	226.9	121.4
JTMBF	23	132.3	164.3
JTMWF	18	254.3	128.8
A. niger			
Control	8	50.2	100
JTMHF	13	42.3	162.5
JTMCF	26	531	325
JTMBF	14	153.9	175
JTMWF	15	176.6	187.5

Conclusion

From the results it can be concluded that EtOAc and n-BeOH fractions of *T. mascatense* can be a good source of antioxidants, total flavonoids, and anti-lipid peroxidation, while the n-hexane and chloroform fractions can be used as a source of anticancer, antifungal and urease enzyme inhibition activities. The compounds isolated from EtOAc soluble fraction showed highest ABTS radical scavenging activity. From the results it might be concluded that all these effects could be due to the bioactive components of this plant and need to be identified for further research about this plant. In future extensive studies through further isolation and identification of the bioactive component(s) might produce some breakthrough leads for future biomedical and biopharmaceutical research.

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Conflicts of interests

The authors declare no conflicts of interests.

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