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Analysis of chemical composition and evaluation of antigenotoxic, cytotoxic and antioxidant activities of essential oil of *Toddalia asiatica* (L.) Lam.

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ABSTRACT

Objective: To analyse the chemical composition of essential oil isolated from the leaves of *Toddalia asiatica* (L.) Lam. and to test its bioactive properties. **Methods:** Gas chromatography-mass spectrometry (GC/MS) analysis and antigenotoxicity, cytotoxicity and antioxidant studies of isolated oil. **Results:** GC-MS analysis of oil revealed the presence of 42 compounds. The major compound in the oil was β-phellandrene (21.35%). DNA protecting activity of oil was considerably significant against H_2O_2 induced genotoxicity in human lymphocytes. Concentration of oil needed to protect 50% of DNA was calculated as 100 μg/mL. Cytotoxicity of oil against breast (MCF-7) and colorectal (HT-29) cancer cells were observed with the IC_{50} value of (7.80±0.03) μg/mL and (100.00±0.16) μg/mL respectively. Considerable DPPH free radical, hydroxyl radical scavenging, iron chelation and inhibition of lipid peroxidation activities of oil were also studied. **Conclusions:** The results of the present study clearly indicate oil could be a promising candidate for food and drug preparation.

1. Introduction

Toddalia asiatica (L.) Lam. belongs to the family Rutaceae and commonly known as Forest Pepper. It is a prickly climber grows over bushes and hedges in dry areas. The leaves and roots of this plant have been reported to possess antiviral, anti-inflammatory, antimicrobial, antiplatelet and analgesic activities[1-4]. Traditionally the peoples of Kani tribals in Southern India use leaves of forest pepper to cure skin diseases[5]. Flindersine isolated from the leaves showed antibacterial and antifungal activities[6]. Secondary metabolites such as quinoline, protoberberine alkaloids, coumarins, biscoumarins, furanocoumarins, benzopyrans, terpenoids and cyclohexylamides have been reported in the roots[7,8].

Based on medicinal value and rich bioactive secondary metabolites in *Toddalia asiatica (T. asiatica)*, the present study was focused to isolate and analyse the chemical composition of essential oil in the leaves. Biological activities like antigenotoxic cytotoxic and antioxidant activities were also studied.

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2. Materials and methods

2.1. Plant material and essential extraction

T. asiatica (L.) Lam. leaves were collected from natural habitats. The plant material was identified and voucher specimen has been deposited. Freshly collected leaves were hydrodistilled for 3 h using Clevenger apparatus for essential oil extraction. Further extracted essential oil was dried over anhydrous sodium sulphate and stored in a refrigerator (4 ℃) until further analysis.

2.2. Gas chromatography (GC) analysis

GC analysis was carried out using Varian 3800 GC equipped with mass selective detector coupled to front injector type 1079. The chromatograph was fit with DB 5 column (30.00 m×0.25 mm). The injector temperature was set at 280 ℃ and the oven temperature was initially maintained at 45 ℃ then set to 300 ℃ at the rate of 10 ℃/min and finally held at 200 ℃ for 5 min. Helium was used as a carrier gas with the flow rate of 1.0 mL/min. The percentage composition of essential oil was calculated by the GC peak areas.

2.3. Gas chromatography/mass spectrometry (GC/MS)

analysis

GC coupled with mass spectroscopy was performed using Varian 3800 GC equipped with Varian 1200L single quadrupole mass spectrometer. GC condition was same as described earlier and mass spectrometer was operated in the electron impact mode at 70 V. Ion source and transfer line temperature was maintained at 250 ℃. The compounds were identified based on the comparison of retention indices (RI), retention time and mass spectra. Library search was carried out using the NIST and Wiley GC/MS spectral database and by co-comparing with the mass spectral data and RI in the literature[9].

2.4. Comet assay

2.4.1 Cell preparation

Human lymphocytes were isolated from fresh whole blood by adding 15 mL blood to 1mL RPMI 1640 with 10% FBS on ice for 30 min, then underlaying it with 5 mL of Histopaque 1077 before centrifuging at 200 r/min for 3 min at 4 ℃. Lymphocytes were separated as a pink layer at the top of the Histopaque 1077.

2.4.2. Essential oil and hydrogen peroxide treatment

Cells were incubated with different concentrations (25, 50, 75 and 100 μg/mL) of essential oil for 60 min in a dark incubator together with untreated control samples. After pre treatment with essential oil, the cells were treated with 100 μmol of hydrogen peroxide for 1 h on ice.

2.4.3. Comet assay

The procedure described by Singh *et al* was adopted with minor modifications[10,11]. Briefly, a base layer of 1.5% NMP agarose was placed on a microscope slide and 10 μL of the cells suspended in 120 μL of 0.5% LMP agarose at 37 ℃ were then spread on the base layer. A coverslip was added and the agarose was allowed to solidify at 4 ℃ for 15 min, after which the coverslip was gently removed and the slide immersed in freshly made lysing solution composed of 89 mL stock solution (2.5 mol NaCl, 100 mmol EDTA, 10 mmol tris pH 10.0 and 1% sodium dodecyl sulphate), 10mL of DMSO, 1mL of triton X-100; pH 10.0 at 4 ℃ for at least 1 h, protected from light. At the end of lysing period, slides were transferred to an electrophoresis box containing a high pH (13.0) buffer (300 mmol NaOH, 1 mmol EDTA) and incubated at 4 ℃ for 20 min to allow the DNA to unwind. A current of 25 V was applied for 20 min, after which, the slides were submerged in a neutralization buffer (0.4 mol Tris HCl, pH 7.5) for 15 min, dried at room temperature and fixed in 100% ethanol for 10 min. The slides were stored overnight, briefly rinsed in distilled water, stained with 20 mg/mL ethidium bromide and covered with a coverslip. The slides were immediately evaluated at 400× magnification using a Nikon fluorescence microscope fitted with a 515-560 nm excitation filter and a 590 nm barrier filter. The comet tail length was measured using eyepiece micrometer as described in the literature[12]. DNA damage was calculated as: comet tail length (μm)=maximum total length-head diameter.

2.5. Cell lines and culture

The human breast (MCF-7) and colorectal cancer (HT-29) cell lines were cultured in a T25cm2 cell culture flask containing DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL).

The cell culture in the T25cm2 flask was harvested using trypsin and the cell number was counted using a hemocytometer. 10^4 cells/100 μL were added in each well of a 96 well plate and incubated for 24 h. Then the cells were treated with various concentrations of essential oil dissolved in medium and further incubated for 48 h. A 20 μL of MTT (5 mg/mL) in phosphate buffered saline was added to each well and the plate was incubated at 37 ℃ for 4 h. The medium was removed and 100 μL of DMSO was added to each well. After 10 min of incubation at 37 ℃ the plate was read at 570 nm using a microplate reader[13].

2.6. Antioxidant studies

2.6.1. DPPH radical scavenging activity

Different concentrations (25, 50, 75, 100 and 125 μg/mL) of essential oil was dissolved in DMSO and mixed individually with 0.1 mmol DPPH and 50 mmol Tris-HCl buffer (pH 7.4). Reaction mixture was incubated at 37 ℃ for 30 min and then absorbance was measured at 517 nm[14].

2.6.2. Hydroxyl radical scavenging activity

Reaction mixture includes 7.5 mmol $FeSO_4$, 7.5 mmol of 1, 10-phenanthroline, 0.2 mol phosphate buffer (pH 7.8), 30 mmol H_2O_2 and different concentrations of essential oil (200, 250, 300, 350 and 400 μg/mL). The reaction was started by adding H_2O_2. After incubation at room temperature for 5 min, absorbance was read at 536 nm was measured[15].

2.6.3. Metal chelating activity

Briefly, 2 mmol $FeCl_2$ was added to different concentrations of essential oil (200, 300, 350, 400 and 450 μg/mL). Further, reaction was initiated by addition of 5 mmol ferrozine. The mixture was vigorously shaken and left to stand at room temperature for 10 min. Absorbance was measured at 562 nm after 10 min[16].

2.6.4. Inhibition of linoleic acid peroxidation

The different concentration of essential oil (300, 350, 400, 450 and 500 μg/mL) mixed with tris-HCl buffer (pH 7.5), 20 mmol linoleic acid and 4 mmol $FeSO_4$. The peroxidation was started with addition of 5 mmol ascorbic acid. The reaction mixture was incubated for 60 min at 37 ℃. Then 2 mL of 10% ice cold TCA was added and 1 mL aliquot of the samples was added with 1 mL of 1% TBA. The TBA/sample mixture was heated in a water bath at 95 ℃ for 60 min. The absorbance was read at 532 nm[17].

2.7. Statistical analysis

Data obtained from the *in vitro* experiments were analysed using SPSS (16.00) for IC_{50} and students *t* test calculations.

3. Results

3.1. Chemical composition of essential oil

A total of 42 compounds, representing 91.25% have been identified from the essential oil obtained from the leaves of *T. asiatica*. RI and percentage of compounds present

are shown in Table 1. Major compounds of the oil were cyclic monoterpene, β-phellandrene (21.35%) followed by *cis*-ocimene (12.87%), α-phellandrene (9.01%), viridiflorol (6.74%), β-bisabolol (5.24%) and α-pinene (4.49%).

3.2. Antigenotoxic activity

Table 1
Chemical composition analysis of essential oil of *T. asiatica*.

RI*	Compounds	%
700	Heptane	0.10
834	Isovalericacid	0.13
939	α-pinene	4.49
976	Sabinene	1.03
980	β-pinene	0.17
991	Myrcene	1.92
993	Butanoicacid. butylester	1.53
1001	δ-2-carene	0.08
1005	α- phellandrene	9.01
1011	δ-3-carene	0.29
1022	Ortho-cymene	1.88
1031	β-phellandrene	21.35
1033	1,8 cineole	0.81
1040	cis β-ocimene	12.87
1050	trans β-ocimene	2.71
1062	γ-terpenene	0.03
1089	p-cymene	1.59
1098	Linalool	1.35
1102	Nonanal	0.14
1199	Dodecane	0.60
1204	Decanal	0.68
1220	Decylacetate	0.60
1235	Myrtenyl acetate	1.53
1243	Hexyl 3-methyl butanoate	0.23
1339	δ-elemene	1.50
1383	Geranyl acetate	0.20
1404	*cis*-caryophyllene	0.84
1407	Dodecanal	0.28
1415	*cis*-α- bergamotene	0.07
1418	trans-caryophyllene	2.14
1433	γ-elemene	1.17
1495	α- zingiberene	0.13
1524	β-sesquiphellandrene	0.60
1545	cis-sesquisabinene hydrate	1.14
1556	Germacrene B	0.36
1574	GermacreneD-4-ol	0.22
1583	Globulol	2.40
1590	Viridiflorol	6.74
1640	α -cadinol	1.01
1642	Cubenol	0.36
1671	β-bisabolol	5.24
1683	α-bisabolol	0.73
	Total Identified	91.25

RI*: RI obtained on DB-5 column.

The effect of DNA protection of essential oil was analyzed on human lymphocytes. Comet assay was used to analyze the DNA damage where the lengths of the comets were used to calculate DNA damage. Hydrogen peroxide (100 μ mol) induced DNA damage in lymphocytes cells were produced a tail length of (9.15±0.25) μ mol (Figure 1). The lymphocytes pretreated with essential oil showed a decrease in comet tail length after treatment with hydrogen peroxide. The result of comet assay showed that dose dependent decrease in comet tail length in all the concentrations of essential oil studied. A maximum of 50% of DNA was protected by oil at 100 μ g/mL (Figure 2).

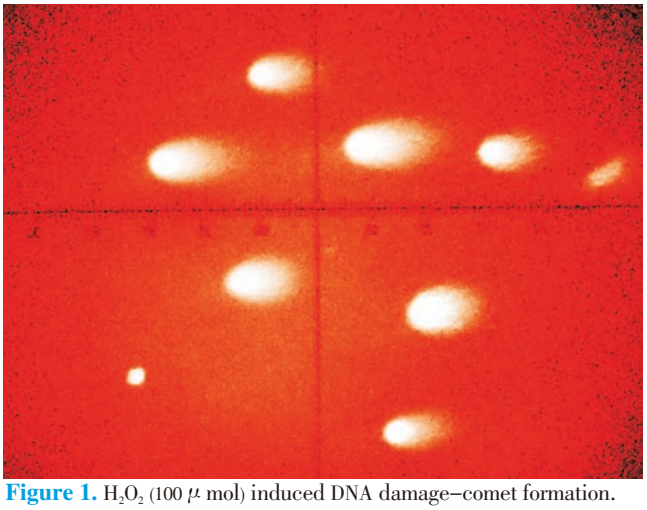

Figure 1. H_2O_2 (100 μ mol) induced DNA damage-comet formation.

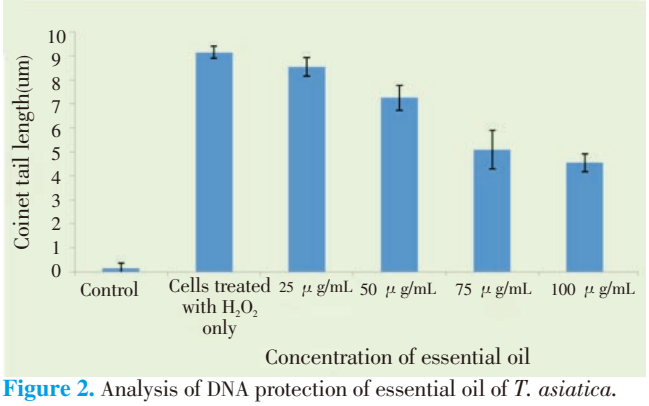

Figure 2. Analysis of DNA protection of essential oil of *T. asiatica*. All the concentrations tested showed statistically significant ($P<0.05$) data when analyzed in students *t* test.

3.3. Cytotoxic activity

The concentration range of 4.0 μ g/mL to 20.0 μ g/mL of oil showed significant cytotoxicity against MCF-7 cells with IC_{50} value of (60.80±0.03) μ g/mL and concentration needed to inhibit the growth of HT-29 cells was calculated as (100.00±0.16) μ g/mL.

3.4. Antioxidant activity

The essential oil showed potent DPPH free radical scavenging activity with the IC_{50} value of (64.80±0.13) μ g/mL. The quantity of oil required to inhibit hydroxyl radical formation by 50% was calculated as (281.30±0.18) μ g/mL. At a

Table 2
Antioxidant activity of essential oil of *T. asiatica*.

Assays	IC_{50} (μ g/mL)*
DPPH free radical scavenging	64.08±0.13
Hydroxyl radical scavenging	281.31± 0.18
Ferrous ion chelation	319.12±0.10
Inhibition of lipid peroxidation	372.11±0.12
BHT	30.14±0.01

*IC_{50} values were expressed as the mean±SD of three replicates.

concentration of (319.10±0.10) μg/mL oil showed potent iron chelating activity. The capacity to prevent linoleic acid peroxidation was observed at (372.10±0.12) μg/mL. However the observed antioxidant activity of the oil was lesser than that of positive control such as BHT (Table-2).

4. Discussion

Essential oils isolated from plants have been reported to have numerous compounds with various percentages. Most of the essential oils contain mono and sesquiterpenes as predominant compounds. In the present study, chemical composition of essential oil from the leaves of *T. asiatica* showed that β-phellandrene (21.35%) was major compound but the previous results showed linalool as major compound[2]. This result confirms that chemical and percentage composition of individual compounds present in the essential oils of same species may vary depending on the growing environment, soil, climate, availability of water and genetic differences *etc*. Meanwhile, β-phellandrene has been reported as a major compound in the essential oil of rutaceae members such as *Thamnosma montana* and *Haplophyllum tuberculatum*[18,19]. The possible mechanism by which the oil inhibited oxidative damage in human lymphocytes may be due to the presence of antioxidant compounds in the oil. Natural compounds may protect against oxidant induced DNA damage directly either by free radical scavenging activity or by decreasing free radical production[20]. This shows that the oil inhibit or scavenge the free radicals produced by hydrogen peroxide and protects the DNA. The observed cytotoxicity of the oil might be due to the presence of mono and sesquiterpenes. The cytotoxic activities of some mono and sesquiterpenes are reported in the literature[21,22]. Result of the antioxidant study shows a general trend that essential oils which contain monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpenes have high antioxidant properties.

Conflict of interest statement

We declare that we have no conflict of interest.

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