Chemical Composition, Antioxidant, Cytotoxic and DNA Damage Protective Activities of *Plectranthus mollis* Essential Oils

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Abstract

The essential oils were obtained by hydro-distillation from *Plectranthus mollis* and subjected to phytochemical screening and gas chromatography-mass spectroscopy (GC-MS) to establish their qualitative and quantitative compositions. Phytochemical investigations confirmed that the essential oils are rich-source of phenols, flavonoids, ascorbic acid and carotenoids. The essential oils were evaluated for their antioxidant and lipid-peroxidation inhibition activities besides their oxidative DNA damage protection potentials. GC-MS data revealed a total of 40 compounds identified, which accounted 98.4% of the total constituents. The major constituents were: Fenchone (31.43%) followed by Piperitone oxide (17.79%), 3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl) (8.19%), *Z*-β-Farnesene (5.98%) and α-Humulene (5.86%). These essential oils exhibited broad-spectrum, dose-dependent antioxidant potencies as evidenced by total-antioxidant-activity (TAA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging, and ferric reducing antioxidant power (FRAP) assays. They showed significant inhibition of lipid peroxidation rate measured in terms of malondialdehyde (MDA) content. These results hold significance, as lipid-peroxidation is considered as biological end-point of oxidative damage and its inhibition as the most important index for antioxidant potential. The oils successfully protected the DNA from Fenton's reagent induced DNA damage. The essential oils of *P. mollis* showed good anti-proliferative activity in a dose-dependent manner against human melanoma cancer M-16 cell line, with only ~10% cell survival at 2 µL concentration.

Keywords - *Plectranthus mollis;* essential oils; antioxidants; cytotoxicity; lipid peroxidation; DNA protection; human cancer cells.

1. Introduction

Various plant extracts and phytochemicals including essential oils are considered strong antioxidative agents and have been advocated as potent lead-compounds for these products. Essential oils are natural, volatile, complex secondary metabolites produced by aromatic plants and are characterized by their strong odour [1]. They are known for their broad-spectrum medicinal and commercial values. *Plectranthus mollis* (family *Lamiaceae*) is an annual erect herb, locally called Lal aghada, found throughout India and is used extensively as a vasoconstrictor, cardiac depressant, respiratory stimulant, fever-reducer, besides being also used for rheumatism, hemorrhage, mental retardation, snakebites as well as a general tonic [2].

Reactive oxygen species (ROS) consisting of both free radical compounds such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals (·OH), as well as non-free-radical compounds hydrogen peroxide (H_2O_2), organic peroxide (ROOH), ozone (O_3) and singlet oxygen ($^1O_2$) are known to induce oxidative stress which in turn is considered as major causative factor of many of today's diseases including diabetes

and cardiovascular diseases [3, 4]. Though these ROS are generated naturally in living organisms as byproducts of endogenous metabolism and are even known to play significant roles in cell signaling. However, their excess amounts are associated with cellular disorders through their actions on proteins, lipids and DNA [5]. ROS-mediated oxidative stress has been regarded as exacerbating factors in DNA damage and mutations, and causes biomolecular oxidation, oxidizes the cellular thiols which eventually affects key enzymes and lipid peroxidation and ultimately underline the aging process [4, 6]. Further, ROS are considered to cause cancer and several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Down's syndrome, inflammation, viral infection and various other digestive disorders such as ulcer and gastrointestinal disorders [7, 8].

Antioxidants play an imperative role in providing protection against ROS-driven oxidative damages and associated peroxidation lipid bilayer membranes as well as DNA strand breaking [9]. Even though many antioxidants of synthetic origin are available and used frequently, however, they are often being associated with toxic and mutagenic effects [10] and therefore have limitations for their medical uses. On this account, their natural counterparts have a clear edge for being less- or non- toxic and hence can serve as potential drug and dietary molecules. Therefore, in recent years the antioxidants of phyto-origin have seen an unprecedented demand in bio-pharmaceuticals, nutraceuticals besides their use as food additives [4]. Medicinal plants offer excellent sources of phytochemicals with therapeutic properties such as phenols, flavonoids, vitamins, tannins, many of which have potent antioxidant activities and therefore can be exploited in drug discovery programs as well as in the preparation of foods and pharmaceutical products [11].

In-vitro anti-inflammatory activity, chemical constituents, protein denaturation and membrane stabilization activities of *P. mollis* were studied [12]. Essential oils of *P. mollis* are reported to have antimicrobial, bronchodialatory [13] as well as mosquito repellent activities [14]. We report herein the phytochemical constituents; GC-MS based chemical composition of *P. mollis* essential oils and their antioxidant, DNA-protective and cytotoxicity potentials against human cancer cells.

1. Materials and methods

1.1 ***Plant material***

The *P. mollis* plants were collected from Western Ghats, Eastern area of Baramati and Nashik. The botanical identification of plant was carried out at Ananatrao Pawar College and specimen was submitted to MACS Agharkar Herbarium (AHMA Ref. No. 25402).

1.2 ***Essential oil extraction***

Essential oils were extracted from various parts of the plant like flowers, roots, stem and berries by hydrodistillation method. Steam distillation was employed for the extraction of essential oils. In this method, steam was passed through the plant material containing the desired oils. The essential oil yield was ~0.3 % (w/w).

1.3 ***Phytochemical investigation***

Total phenols were estimated using Foline Ciocalteu method [15] and expressed as mg gallic acid equivalents (GAE) g^{-1} extract. Total flavonoids were estimated using modified method by Marinova *et al*. [16] and expressed as mg quercetin equivalents/g extract. Total ascorbic acid was estimated by 2, 4-dinitrophenylhydrazine method [17] Carotenoids were estimated by following Jensen [18] and concentration was expressed as mg β-carotene equivalents/g extract.

1.4 ***DPPH radical scavenging activity***

The antioxidant activity of the plant extract was examined on the basis of the scavenging effect on the stable 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical activity as described by Braca *et al*. [19]. Ethanolic solution of DPPH 0.05 mM (300 µl) was added to 40 µl essential oil with 0.5 - 2 µg/ml concentrations. After 5 min, absorbance was measured at 517 nm. The radical scavenging activity of the plant extract was expressed as % inhibition against control.

1.5 ***Total antioxidant activity (TAA)***

The assay is based on the reduction of Mo(VI) to Mo(V) by sample compound and formation of green colored phosphate/ Mo(V) complex at acidic pH (4.0) [20] 0.1 ml of essential oil from varying concentrations (0.5-2 µg/ml) was added to 1 ml reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated at 95 °C for 90 min. After cooling the samples absorbance was measured at 695 nm and TAA was expressed as GAE.

1.6 ***FRAP (ferric reducing antioxidant power) assay***

The spectrophotometric method is based on reduction of Fe^{3}þ-tetra(2-pyridyl)pyrazine (TPTZ) complex to Fe^{2}þ-tripyridyltriazine at low pH [21]. FRAP reagent contained 300 mM acetate buffer, 10 ml TPTZ dissolved in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in 10:1:1 ratio. Five hundred µl standard was added to 1 ml reaction mixture and incubated at 37°C for 30 min. Absorbance was taken at 593 nm against blank and FRAP values were expressed as GAE.

1.7 ***Determination of inhibition of lipid peroxidation***

Lipid peroxidation inhibition was evaluated using modified Halliwell and Gutteridge method [22]. Freshly excised goat liver was minced using glass Teflon homogenizer in cold phosphate buffered saline (pH 7.4). 10% homogenate was prepared and filtered to obtain a clear homogenate and this process was carried on ice. Varying concentrations (0.5- 2 µg/ml) of the essential oil were added to the liver homogenate and lipid peroxidation was initiated by adding 100 µl ferrous sulphate (15 mM) to 3 ml of the tissue homogenate. After 30 min, 100 µl aliquot was taken in a tube containing 1.5 ml of 10% TCA. After 10 min, tubes were centrifuged and supernatant was mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated for 30 min in a boiling water bath. The intensity of the pink colour complex was measured at 535 nm. The degree of lipid peroxidation was assayed by estimating the TBARS (TBA reactive species) content and results were expressed as percentage inhibition.

1.8 ***DNA damage protection activity***

The ability of different extracts to protect DNA (pBR322, Merck, India) from damaging effects of hydroxyl radicals generated by Fenton's reagent (FR) was assessed by modified DNA nicking assay [23]. The reaction mixture contained 2.5 µl of DNA (0.25 µg) and 10 µl FR (30 mM H_2O_2, 500 µM ascorbic acid and 800 µM $FeCl_3$) followed by the addition of 5 µl of essential oil and the final volume was made 20 µl with DW. The reaction mixture was then incubated for 45 min at 37°C and followed by addition of 2.5 µl loading buffer (0.25% bromophenol blue, 50% glycerol). The results were analyzed on 0.8% agarose gel electrophoresis using EtBr-staining.

1.9 ***Cytotoxicity of essential oils against human cancer cells***

The essential oil was tested for its cytotoxicity against human melanoma cancer cells (M-16) using standard MTT (methylthiazole tetrazolium) test as described earlier by our group [24]. Briefly, the cells were seeded at the density of 15,000 per well into 96-well plates and allowed to adhere for 24 h at 37 °C. Next day, cells were treated with various concentrations (0.25, 0.50 and 2.0 µl) of essential oil of *P. mollis* in 1% dimethyl sulfoxide (DMSO) for 24 h in triplicates. Absorbance was taken at 570 nm using 630 nm as reference filter. Absorbance given by untreated cells (without DMSO and essential oil) was taken as 100 % cell survival.

1.10 ***Statistical analyses***

All the experiments were conducted in triplicate to check the reproducibility of the results obtained. The graphs were plotted using Microcal Origin 6.0.The results are presented as means ± standard error (SE) and means were compared using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$, using MSTAT-C statistical software package.

2. Results and discussion

Results depicted in Table 1 revealed that the essential oil of this plant is a rich source of phenols,

flavonoids and ascorbic acid. Several reports have shown a correlation between higher amounts of polyphenols in plants and correspondingly their higher antioxidant potential as they inhibit free radical formation and/or interrupt propagation of autoxidation [3-4, 25] and our results supported these hypotheses.

Table 1 Total phenols, flavonoids, ascorbic acid and carotenoid contents of *P. mollis* essential oil.

Essential oil	Phenols*	Flavonoids^	Ascorbic acid#	Carotenoids$
P. mollis	1.1 ± 0.02	2.9 ± 0.04	44.1 ± 0.9	2.5 ± 0.2

*gallic acid; ^quercetin; #ascorbic acid and $β-carotene equivalents mg/g extract. Each value represents the mean of three replications ± SE.

The GC-MS method for analyzing the essential oil is very common and broadly used. This method reveals the composition of essential oil and also detects the presence of compounds which are present in micro amount. The GC-MS analysis of *P. mollis* showed the major components are Fenchone, Piperitone oxide and 3-cyclopenten-1-0ne, 2-hydroxy-3-(3-methyl-2-butenyl)- as 31.43%, 17.79% and 8.19% respectively, followed by Coaene, (Z)-a-farnesene and a-humulene as around 5%. The remaining components were found in the range of 0-3%.

Fig. 1. GC–MS chromatogram of the hydrodistilled essential oil of *P. mollis*

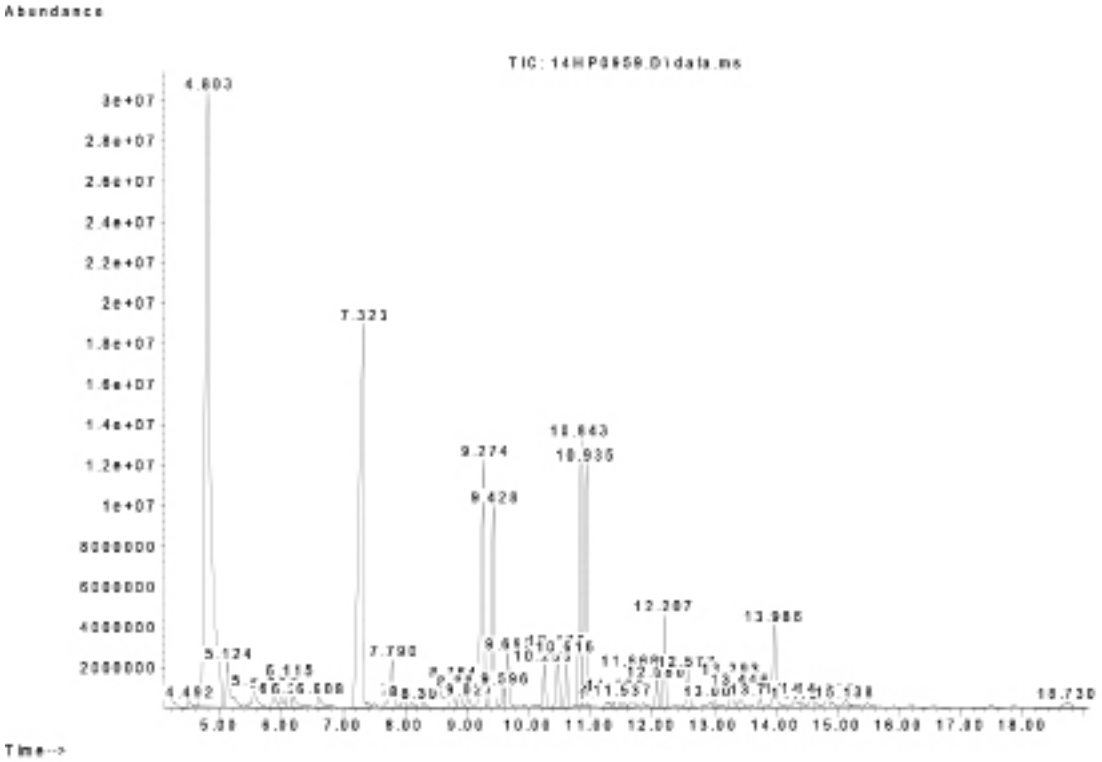


Table 1 Constituents as identified by GC-MS in the hydrodistilled essential oil of *P. mollis*.

Peak	Retention time (min)	Constituents	Content in oil (%)
1	4.492	Terpineol, cis-á-	0.21%
2	4.803	Fenchone	31.43%
3	5.124	Fenchol, exo-	1.58%
4	5.56	Camphor	0.80%
5	5.867	.(-)-Borneol	0.49%
6	6.004	4-Terpineol	0.34%
7	6.115	.p-Cymen-8-ol	0.84%
8	6.2	à-Terpieol	0.40%
9	6.608	Phenol, 4-methyl-2-(2-propenyl)-	0.45%
10	7.323	Piperitone oxide	17.79%
11	7.79	Thymol	1.39%
12	8.108	: 2-Oxabicyclo[2.2.2]octan-6-one, 1,3,3-trimethyl-	0.22%
13	8.764	Berbenone	0.75%
14	8.883	à-Cubebene	0.49%
15	9.027	Eugenol	0.31%
16	9.274	3-Cyclopenten-1-one, 2-hydroxy-3-(3- ethyl-2-butenyl)-	8.19%
17	9.428	Copaene	5.01%
18	9.596	à-Farnesene	0.44%
19	9.663	á-Cubebene	1.45%
20	10.255	á-Caryophyllene	1.03%
21	10.476	à-Bergamotene	1.42%
22	10.616	á-Sesquiphellandrene	1.15%
23	10.843	.(Z)-á-Farnesene	5.98%
24	10.935	.à-Humulene	5.86%
25	11.299	ç-Muurolene	0.20%
26	11.366	.à-Curcumene	0.18%
27	11.426	á-Cubebene	0.37%
28	11.537	Eremophilene	0.22%
29	11.668	.2-Vinylnaphthalene	0.93%
30	12.08	Germacrene D-4-ol	0.65%
31	12.207	á-Cadinene, (-)-	2.09%
32	12.577	.(+)-Ledol	0.99%
33	13.007	à-Calacorene	0.19%
34	13.293	Widdrol	0.77%
35	13.986	.1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	2.30%
36	14.306	Cubenol	0.37%
37	14.42	tau.-Muurolol	0.17%
38	14.837	à-Cadinol	0.32%
39	15.138	Bicyclo[4.4.0]dec-2-ene-4-ol, 2-methyl-9-(prop-1-en-3-ol-2-yl)-	0.28%
40	18.73	Hexahydrofarnesyl acetone	0.41%

The antioxidant activity is commonly evaluated by measuring the inhibition rate of an oxidation processes in the presence of an antioxidant [26]. Antioxidant efficiency is often linked to the antioxidant ability to scavenge stable free radicals [27]. DPPH radical scavenging assay has been most extensively used to study the antioxidant potential of the extract/essential oil. The DPPH radical occurs in stable form in the solution which imparts it a violet colour. When these radicals are scavenged by the antioxidant compounds present in the essential oil the intensity of violet colour reduces which is measured spectro-photometrically at 517 nm. The *P. mollis* essential oil exhibited very good activity with IC_{50} 1.85 µg/ml (Table 2). This value is almost similar to the standard antioxidant compound ascorbic acid. The IC_{50} value is comparatively very less than the other essential oils, (EC 50-7146 µg/ml for *Ajuga chamaepitys,* 80.21 µg/ml for parsley, 5.8 µg/ml for *M. longifolia*) [28-30] which makes it a potent antioxidant essential oil.

FRAP is an important indicator of reducing potential of an antioxidant which is associated with the presence of compounds responsible for donation of hydrogen atom to break the free radical chain. The FRAP assay is based on the reduction of Fe^{3+} to Fe^{2+} by the antioxidant phytochemicals. The *P. mollis* essential oil showed concentration-dependent activity which was expressed as gallic acid equivalents and the results are listed in Table 2. The FRAP assay provides a reliable method for evaluation of antioxidant activities of various plant extracts, essential oils and compounds as antioxidant capacity is directly correlated with its reducing capacity [21] and our results are in conformity of these findings.

Table 2. Antioxidant activities of *P. mollis* essential oil as revealed by DPPH, TAA and FRAP assays

Essential Oil Concentration µg/ml	DPPH (% Scavenging by *P. mollis* essential oil)	DPPH (% Scavenging by std. ascorbic Acid)	TAA (GAE) of essential oil of *P. mollis*	FRAP (GAE) of essential oil of *P. mollis*
0.5	6.02 ± 0.4^{a}	16.42 ± 1.2^{a}	187 ± 5.5^{a}	115 ± 4.5^{a}
1.0	19.3 ± 2.1^{b}	24.31 ± 1.9^{b}	220 ± 5.1^{b}	125 ± 5.6^{b}
1.5	33.9 ± 2.8^{c}	38.66 ± 3.0^{c}	222 ± 8.2^{b}	$130 \pm 7.2^{b,c}$
2.0	54.7 ± 3.1^{d}	50.93 ± 3.7^{d}	255 ± 8.6^{c}	132 ± 6.1^{c}
IC_{50}	1.80	1.945		

GAE: gallic acid equivalents. Each value represents the mean of three replications ± SE. Means within a column for each extract followed by different superscript letters were significantly different from each other according to Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

Free radicals are the most common initiators of oxidative reactions that may result in numerous deleterious effects on the cellular and genetic level. Natural antioxidants can scavenge and react with free radicals, and hence terminate the free radical chain reaction. Recently, herbal medicines containing free radical scavengers are gaining attention of the pharmaceutical research for their importance in preventing and treating several diseases and disorders [3,4]. In this reference, anti-lipid peroxidation activity of an essential oil is of great importance. The inhibition of lipid peroxidation is considered as the most valuable index of antioxidant activity as reported by various investigators [31, 32]. In this investigation the lipid peroxidation was induced in freshly excised goat liver by ferric chloride solution. The capacity of essential oil to inhibit the peroxidation was analysed by the reduction in end product formation, which is MDA. The efficiency of essential oil was 1.625 µg/ml IC_{50} against 1.2 µg/ml of standard ascorbic acid (Table 3).

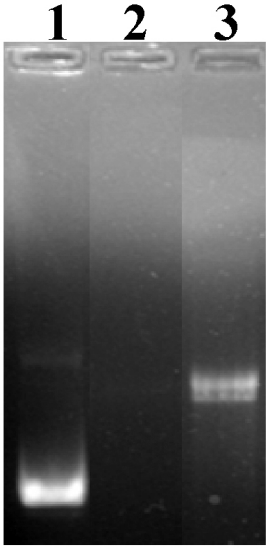
Table 3. Inhibition of lipid peroxidation in goat liver by *P. mollis* essential oil

Concentration (µg/ml)	Inhibition of lipid peroxidation (%) by *P. mollis* essential oil	Inhibition of lipid peroxidation (%) by Std. ascorbic Acid
0.5	14.8 ± 0.4^{a}	22.5 ± 0.6^{a}
1.0	24.5 ± 1.1^{b}	42.0 ± 1.5^{b}
1.5	43.2 ± 2.6^{c}	55.4 ± 3.1^{c}
2.0	54.2 ± 2.9^{d}	58.8 ± 2.8^{c}
IC_{50}	1.625	1.275

Each value represents the mean of three replications ± SE. The lines with the same color and symbol with different letters are significantly different from each other at $P \leq 0.05$ according to Duncan's Multiple Range test.

P. mollis essential oil was evaluated for their oxidative damage protective activity against a model DNA pBR322 and the results are illustrated in Fig. 2. Hydroxyl radicals generated by Fenton's reaction are known to cause DNA damage by causing breaks in it [3] as in the present investigation, lane 2 in Fig. 2 showed absence of DNA band indicating degradation of DNA. The lane no. 3 shows the protection of DNA damage by OH radicals due to presence of essential oil. Free radicals are known for DNA strand breaking and damage which eventually contributes to carcinogenesis, mutagenesis and cytotoxicity. Our group has previously reported the similar results and used plant extracts and fractions for DNA protection against oxidative damage [3, 4].

Fig. 2. DNA damage protecting activities of *P. mollis* essential oil. Lane 1- pBR322 DNA; Lane 2- DNA + Fenton's reagent (FR); Lane 3- essential oil + FR+ DNA



MTT assay clearly showed that the *P. mollis* essential oil has potent cytotoxicity and showed noteworthy anti-proliferative activities against human melanoma cancer M-16 cells, and the activities were dose-dependent with 47.8, 30.4 and 10 % cell survival at 0.25, 0.5 and 2.0 µl concentration of essential oil, respectively.

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