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Essential Oil Composition and Biological Activities of *Aegle marmelos* (L.) Correa Grown in Western Ghats Region-South India

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Abstract: The present study aimed to evaluate the chemical composition of essential oil of *Aegle marmelos* (L.) grown in Western Ghats region. The hydrodistilled essential oil was analyzed by GC/MS and yield of the oil was (0.9 % v/w). A total of 31 components was identified, represents 97.44 % of the oil. The major components of essential oil from Western Ghats were p-mentha-1,4(8)-diene (33.2 %), limonene (13.1 %), p-cymen-α-ol (9.5 %), γ-gurjunene- (7.9 %), β-phellandrene (4.3 %), β-pinene (2.0 %), pinocarvone- (3.5 %), and terpinen-4-ol (1.8 %). The essential oil produces significant anticancer activity against human cervical cancer cell line HeLa and less sensitive against African Monkey Kidney normal cell line Vero with IC_{50} values of 85.6 µg/ml and 120.7 µg/ml respectively. The *in vitro* antioxidant activity against DPPH radicals, the essential oil is very effective to suppress the reactive oxygen species with IC_{50} 28.35 µg/ml and BHT as the standard with IC_{50} values of 19.50 µg/ml. This is the first kind of report on the essential oil composition and *in -vitro* antioxidant and anticancer activities of *Aegle marmelos* (L.) grown in Western Ghats region.

Key words: *Aegle marmelos* (L.), Western Ghats, p-mentha-1,4(8)-diene, limonene, anticancer, DPPH and antioxidant.

Introduction

The Western Ghats is one of the hotspots with many endemic plants in India have vast sources of unexplored medicinal plants with excellent curative medicinal properties which have been used in different traditional health care systems [1]. As far as medicinal plants of Western Ghats are concerned, the phytochemical and biological activity studies are in few numbers only [2]. Particularly the essential oils from most of the plants in Western Ghats are unexplored, due to these facts one of the important tree was selected in this investigation. *Aegle marmelos* (L.) belongs to Rutaceae family, commonly known as Bael tree, indigenous to India [3]. Bael tree is distributed in Srilanka, Bangladesh, Thailand, Burma, Nepal, Cuba, Egypt and China [4] and entire plant parts of the tree are used in many ailments. In India, leaves, bark and roots parts are traditionally used to cure various diseases, such as asthma, fractures, healing of wounds, swollen joints, jaundice, Diarrhoea, typhoid, heart diseases and diabetes [5]. From the literature, *A. marmelos* also have numerous pharmacological uses like antimicrobial [6-8] antifungal [9], insecticidal [10], antioxidant [11], anticancer [12-13], antidiabetic [14], antiproliferative [15], larvicidal and

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nematocidal [16].

In recent years, cancer has become dangerous disease and requires much cost for its treatment, so chemoprevention is very essential to control by means of less or non- toxic chemicals either of natural or synthetic origin with cost effective and without side effects. The plant and plant based products are extensively employed to cure cancer. It is also known that the plant possessing anti inflammatory and antioxidant properties has better results in the suppression of tumors. Therefore, the essential oil obtained from *A. marmelos* leaves were tested for its anticancer and antioxidant potential. There is no report on the essential oil composition and *in vitro* antioxidant and anticancer activities of *A. marmelos* grown in Western Ghats, South India. So the present investigation aimed to evaluate the essential oil composition and its *in vitro* antioxidant and anticancer activities of *A. marmelos*. This is the first kind of report in Western Ghats region for identification of new constituents and its antioxidant and anticancer activities.

Materials and methods

Plant materials

Fresh leaves of *A. marmelos* (1kg) were collected from Udumalpet, (10° 34' 59.'' N and 77° 15' 0.0'' E). Tamilnadu, South India between the periods of October-January. The plant material was identified and authenticated by Dr. P. Sathishkumar Assistant Professor, Department of Botany, NGM College, Pollachi, Coimbatore, Tamilnadu. The Voucher specimen (PCH012) was preserved in the chemistry department.

Isolation of essential oil

About 1kg of fresh leaves were washed with tap water and cut into small pieces, subjected to hydrodistillation using modified Clevenger type apparatus for 3 hours and was repeated three times. The volatile oil obtained was separated from the water. Traces of water in the oil were separated using anhydrous Sodium sulphate and stored in a sealed container which is kept in the freezer at 4°C until further uses.

GC/MS analysis

GC/MS analysis of the essential oil of *A. marmelos* was carried out using thermo GC-trace ultra-version: 5.0 coupled with thermo MS DSQ II instrument. Compounds were separated on DB-35, MS capillary standard non-polar column (30 m × 0.25 mm), film thickness 0.25 µm. Helium was used as the carrier gas and the temperature programming was set with initial oven temperature at 70°C and the process carried out in three ramps, final temperature of first ramp was set at 150°C with a variation of 10°C/min, the final temperature of second ramp was 220°C hold time was 2 min, rate of change of temperature was 5°C/min, in third ramp the temperature was 260°C hold for 2 minutes and raised 10°C per minute and maximum temperature for the process was 300°C. The sample of 100 mL was dissolved in 1 mL of acetone and 1 µL was injected with split less mode. Mass spectra were recorded over 50-500 amu range with electron impact ionization energy 70 eV, while injector and MS transfer line temperature were set at 230°C and 280°C respectively.

The components were identified by comparison of their mass spectra with those of the NIST mass spectral library version.2.0d, as well as on comparison of their retention indices either with those of authentic compounds or with literature values [17]. The percentage composition of the components was determined by co-injection some of the authentic samples available in the laboratory and from raw peak area percentages of the total compound with individual components "(external standard method)".

In vitro anti-oxidant activity

DPPH radical scavenging activity

The free radical scavenging activity of the essential oil of *A. marmelos* was measured with the stable radical 1, 1-diphenyl-2-picrylhydrozyl (DPPH) in terms of hydrogen donating or radical scavenging activity [18]. 0.1 mM solution of DPPH in ethanol was prepared and 1.0 ml of this solution was added to 3.0 ml of essential oil solution in water at different concentrations (10, 20, 40, 60, 80 and 100 mg/ml). After 30 minutes, the absorbance was measured at 517 nm. The lower absorbance of the reaction mixture indicates higher free radical activity. The control experiment was also carried in the same manner with distilled

water in place of the oil. BHT was used as standard. Radical Scavenging Activity was calculated using the following equation:

Scavenging effect % = $[(A_0-A_1)/A_0]$ x 100

where A_0 was the absorbance of the control and A_1 was the absorbance of the essential oil.

***In vitro* anticancer screening**

An *in vitro* cytotoxicity test was performed for the given test sample as per standard procedure using MTT assay [19]. The culture medium from the HeLa monolayer was replaced with fresh medium. Test sample in duplicates was added to the cells. After incubation at 37±1°C for 18 hrs, MTT was added in all the wells and incubated for 4 hrs. After incubation, DMSO was added in the wells and read at 570 NM using a microplate reader. Cytotoxicity was calculated by using the formula.

Cytotoxicity = [(Control - Treated)/ Control] * 100

Result and discussion

The analysis of essential oil of *Aegle marmelos* leaves was pale yellow in colour and the average yield was about 0.7 % (v/w). The essential oil composition was analyzed by GC-MS and a total of 31 compounds were identified which represents 97.44 % of the detected oil composition. The results are given in the table (1) and GC-MS profile of essential oil was given in figure (1).

The major composition of essential oil from Western Ghats were p-Mentha-1,4(8)-diene (33.2 %), limonene (13.1 %), p-cymen-α-ol (9.5 %), γ-gurjunene- (7.9 %), β-phellandrene (4.3%), L-β -pinene-(2.0 %), pinocarvone (3.5 %), terpinen-4-ol (1.6 %) and other minor compounds were α-acorenol (1.3 %), p-cymen-8-ol (1.2 %), (-) spathulenol (1.0 %), guaia-1(10),11-diene (1.82 %) and phytol (0.5 %). The essential oil mainly consisted of monoterpene hydrocarbons (65.01 %) and sesquiterpenes hydrocarbons (15.0 %), in addition to that oxygenated compound representing (9.5 %) of the total oil. However, the percentage of p-limonene (13.1 %), β-phellandrene (4.3 %) and β-pinene (2.0 %), were quite different from the essential oil obtained from various part of the India and other countries. p-Mentha-1,4(8)-diene (33.2 %) reported here for the first time from *A. marmelos* in Western Ghats, South India. Moreover the essential oil obtained from North India was limonene (67.8 %), α-ocimene (4.8 %), cubedol (3.2 %) as main constituents [20], similar results are obtained limonene (26.8 %), β-phellandrene (16.2 %), α-pinene (6.6 %) [21]. On the other hand, our obtained results were different from the leaves oil isolated from Egypt sources, which constituted mainly of α-phellandrene (20.97 %) α-pinene (17.76 %) and δ-carene (16.37 %) was reported by Ibrahim *et al.*,[6]. Whereas β-phellandrene (7.5 %), α-phellandrene (27.5 %) and α-pinene (7.7 %) are major components in Cairo [22]. But some of the reported oils are quite

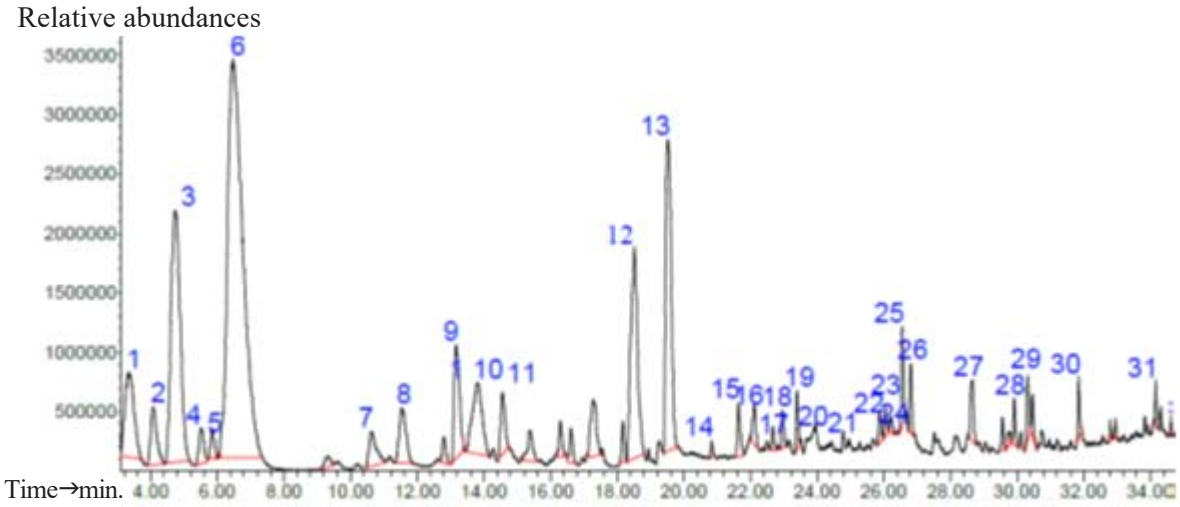


Figure 1. GC/MS chromatogram of leaf essential oil of *A. marmelos*

Table 1. GC-MS analysis of volatile composition of essential oil of *A. marmelos*

No.	Compounds	R.T	RI (estimated)	RI (reported)	(%) Composition
1	L-β-Pinene	3.3	958	961	2.0
2	Ethyl isovalerate	4.0	976	978	0.3
3	Limonene	4.7	1018	1018	13.1
4	β-Phellandrene	5.5	1026	1030	4.3
5	β-*cis*-Ocimene	5.8	1034	1034	0.6
6	p-Mentha-1,4(8)-diene	6.4	1052	1056	33.2
7	β-Linalool	10.6	1082	1091	0.5
8	p-Allyl toluene	11.5	1096	1033	0.6
9	3,9-Epoxy-p-menta-1,8(10)-diene	13.1	1104	1106	3.51
10	Pinocarvone	13.8	1114	1600	3.0
11	Terpinen-4-ol	14.5	1137	1161	1.6
12	γ-Gurjunene	18.5	1191	-	7.5
13	p-Cymen-α-ol	19.5	1197	1197	9.5
14	α-Terpineol	20.8	1198	1198	0.6
15	(Z)-Cinerone	21.6	1238	-	0.3
16	β-Elemene	22.3	1387	1389	0.8
17	2-Methyl-oct-2-enedial	22.6	1461	1469	0.9
18	Guaia-1(10),11-diene	22.9	1490	-	1.8
19	Caryophyllene	23.9	1415	1424	0.8
20	Caryophyllene oxide	24.0	1507	1576	0.6
21	Limonen-6-ol	25.2	1560	1560	0.3
22	Carotol	25.6	1593	1594	0.6
23	p-Cymen-8-ol	26.0	1197	1198	1.2
24	(-)-Spathulenol	26.2	1567	1577	1.0
25	Iso aromadendrene epoxide	26.5	1588	1590	0.5
26	α-Acorenol	26.8	1598	1598	1.3
27	*trans*-Longi pinocarveol	28.6	1599	-	1.5
28	Eudesm-7(11)-en-4-4-ol	29.8	1647	1681	0.7
29	Phytol	30.4	2045	2099	0.7
30	Calcitriol	31.8	3150	-	0.1
31	Fenretinide	34.9	3289	-	0.3

different with limonene chemotype [23, 24]. The volatile oil obtained from Cuba mainly contains δ-cadinene (12.1 %) and β-caryophyllene (10 %) [25]. Seasonal and chemotypic variations was evaluated in North India [26], which revealed that significant variation in their terpenoids compositions. Further the volatile oil from Nepal showed limonene (64.1 %, (E)-β-ocimene, and germacrene B (4.7 %) was main constituents [16]. According to Verma *et al.*,[26], the qualitative and quantitative variations between the essential oil composition may be attributed to the difference in geographical location, seasonal variations, climate conditions, post harvest peroid and time of harvest. The leaf oil from Western Ghats region may be new chemotype, and major composition is quite different from the reported data.

Plants and plant derived products are low-cost, fast, and less toxic as compared with conventional treatment methods. Phytochemicals are selective in their functions and acts specifically on tumor cells without affecting normal cells [27,28]. So the

leaf essential oil was tested against cervical cancer cell line (HeLa) and African Monkey kidney normal cell line (Vero) using MTT assay and the results are given (Table. 2 & 3. Figure. 2 & 3). The toxicity of essential oil in normal cell line was evaluated and the results were compared with cancer cell line (HeLa). The essential oil inhibited both the cells in a concentration dependent manner. The IC_{50} value of the HeLa cell line was 85.6 µg/ml and the Vero cell line has 120.7 µg/ml. The cytotoxic potential may be attributed to the major compounds, p-mentha-1, 4(8) -diene (33.2 %) and limonene (13.1 %). The essential oil was active against brine shrimp lethality assay [29]. *A. marmelos* essential oil demonstrated cytotoxicity against MCF-7 human breast cancer cell line with IC_{50} value of 100 µg/ml [16]. The acetone and methanol extract of *A. marmelos* tested for its anticancer activity against MDA-MB-231, Hep-2 and Vero cells by Seemaisamy *et al.*[13]. Brine shrimp lethality test of methanol extract also showed the anticancer potential of *A. marmelos* [12]. From the available sources the anticancer potential was less studied, moreover the present study indicated that the volatile oil was less sensitive against normal cell line Vero compared to HeLa cancer cell. Similar results were obtained from the extract of *C. sanguicolle*, an endemic species, exhibited highest cytotoxic effect on the HeLa cell line and low cytotoxicity on the Vero cell line [30]. The results revealed that the essential oil obtained in *A. marmelos* has potent anticancer activity.

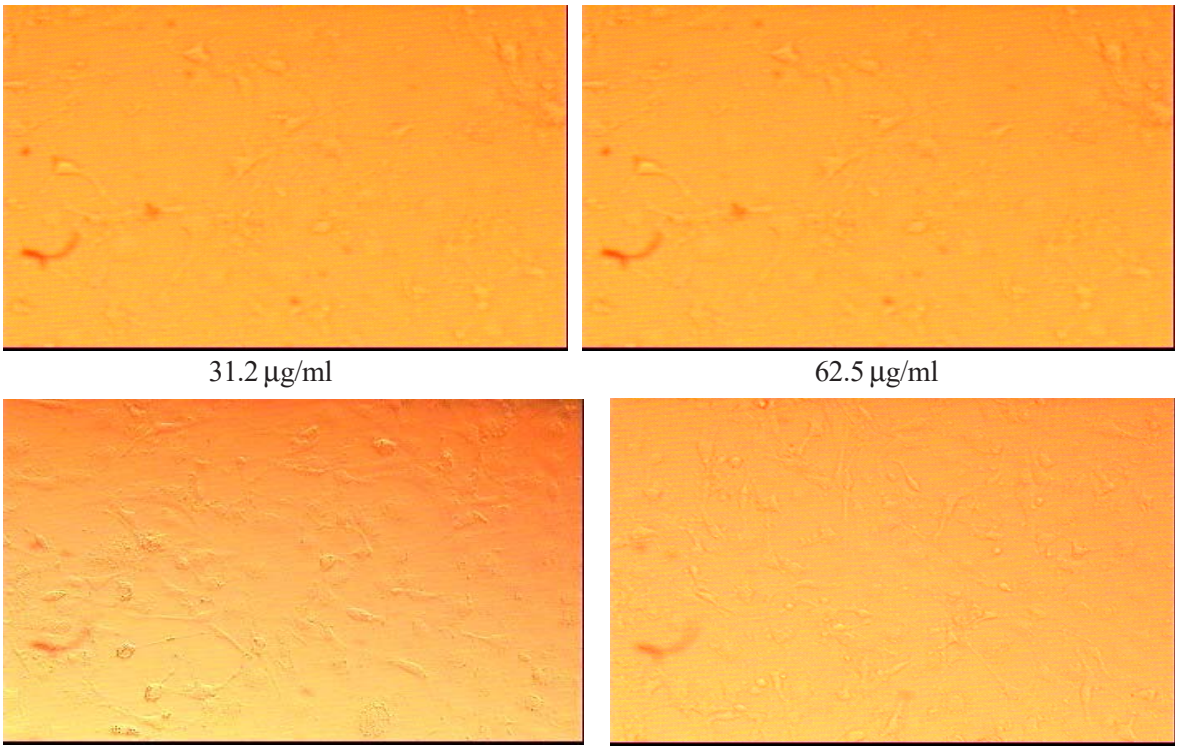
The present study examines antioxidant activity of essential oil of *A. marmelos* and showed a concentration dependent antiradical activity (Figure.4) by inhibiting DPPH radical with IC_{50} values of 28.35µg/ml and BHT as the standard with IC_{50} values of 19.50 µg/ml. DPPH is a purple colored free radical which on reaction with the plant extracts changes to the yellow coloured stable compound and the extent of the reaction is depending on the hydrogen releasing capacity of the antioxidant [17]. The essential oils of different plants have been studied for their antioxidant capacities [31-33], which can be attributed to the presence of terpenes, besides the phenolic compounds that contribute to the free radical scavenging activity [34]. Similarly the essential oil of *A. marmelos* showed a significant antioxidant activity which may be due to the presence of high content of limonene, p-cymen-α-ol in the essential oil [35, 36]. Thus the oil can be used as antioxidant compound that scavenge the free radicals and reactive oxygen species. Previous studies reported the antioxidant potential of various extracts of *A. marmelos* [37-. 39]. Further more, this is the first kind of report on its *in vitro* antioxidant activity of essential oil of *A. marmelos* in the Western Ghats region.

Table.2 Percentage of Cytotoxicity of essential oil of *Aegle marmelos* in Normal Cell Line (Vero)

No.	Concentration (µg/ml)	Cytotoxicity (%)
1	31.2	21.5
2	62.5	34.7
3	125	51.7
4	250	65.2

Table 3. Percentage of cytotoxicity of essential oil of *Aegle marmelos* in HeLa Cell line

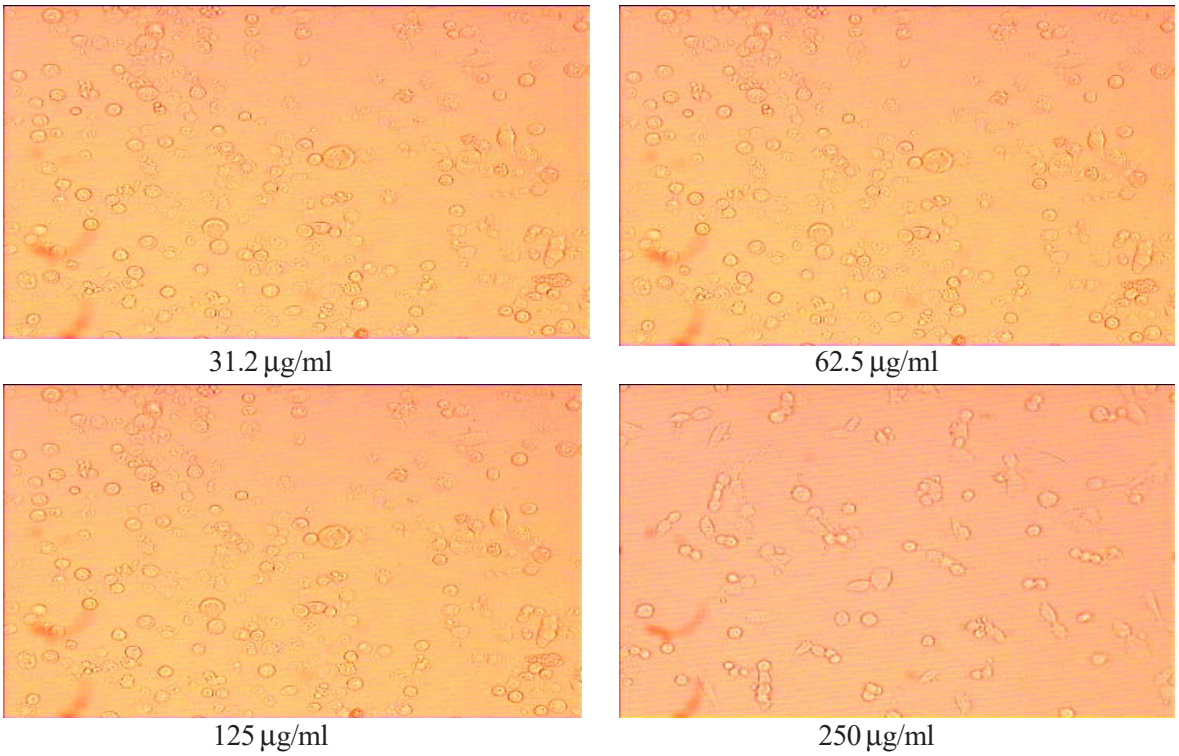
No.	Concentration (µg/ml)	Cytotoxicity (%)
1	31.2	12.7
2	62.5	39.7
3	125.0	70.7
4	250.0	81.2



31.2 µg/ml 62.5 µg/ml

125 µg/ml 250 µg/ml

Figure 2. *In vitro* anticancer activity of essential oil of *A. marmelos* on Vero cell line (images for different concentrations)



31.2 µg/ml 62.5 µg/ml

125 µg/ml 250 µg/ml

Figure 3. *In vitro* anticancer activity of essential oil of *A. marmelos* on HeLa cell line (images for different concentrations)

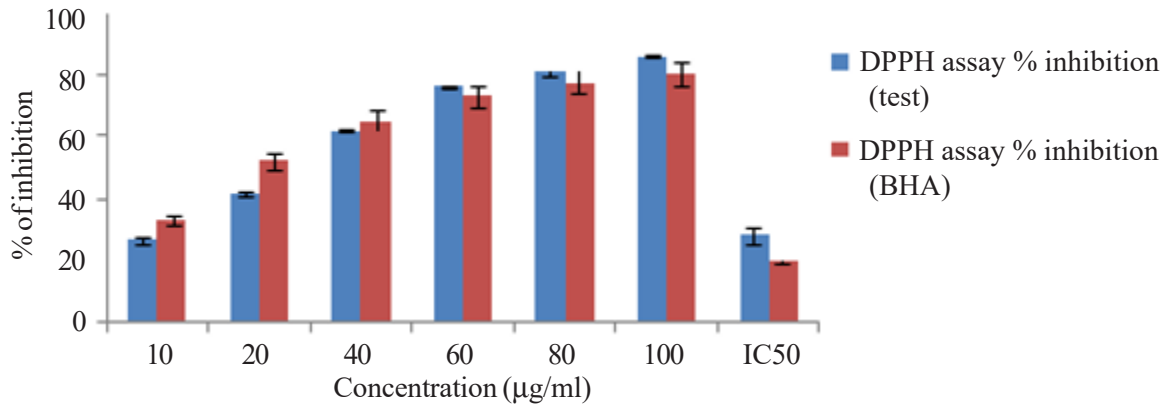


Figure 4. *In vitro* antioxidant activity of essential oil of *A. marmelos* for DPPH assay

Conclusions

The chemical composition of essential oil of *A. marmelos* was analyzed by GC-MS method. A total of 31 components was identified. p-mentha-1,4(8)-diene (33.2 %), limonene (13.1 %), p-cymen-α-ol (9.5 %), β-phellandrene (4.3 %) were the major compounds. The essential oil was tested for its *in vitro* anticancer activity using MTT assay against the HeLa cell line and normal cell line Vero demonstrated IC_{50} value 85.6 µg/ml and 120.7 µg/ml respectively. *In vitro* anti-oxidant study was performed by DPPH assay and the IC_{50} value of essential oil was 28.35 µg/ml and BHT as the standard with IC_{50} values 19.50 µg/ml. More over, the results demonstrated that the essential oil of *A. marmelos* has good anticancer and antioxidant potential and which may be utilized as a natural source of antioxidant and anticancer agents.

Declaration of interest

The authors declare no conflicts of interest in this work.

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