

CHEMICAL COMPOSITION, *IN VITRO* ANTIBACTERIAL, AND ANTIOXIDANT ACTIVITY OF ESSENTIAL OIL FROM LEAVES OF *AJUGA PARVIFLORA* BENTH

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ABSTRACT

Objectives: The aim of our study was to evaluate the phytochemical compounds and assess their antibacterial and antioxidant activity of essential oil of *Ajuga parviflora* Benth. growing in the Himalayan region.

Methods: In the present study, the phytochemical constituents of essential oil were isolated by steam distillation and screened by gas chromatography (GC) and GC-mass spectrometry (GC-MS) analysis from *A. parviflora* is rich in oxygenated monoterpenoids and sesquiterpenoids. The essential oil was further evaluated for their antibacterial by well-diffusion method and antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay at various concentrations.

Results: The major chemical constituents were α -cadinol (21.36%), α -muurolol (14.2%), cubebol (12.76%), germacrene D-4-ol (9.6%), germacrene D (4.32%), farnesyl acetate (3.58%), longifolol acetate (2.76%), and β -bourbonene (2.35 %) whereas monoterpenoids were minor constituents. The zone of inhibition (ZOI) shown by essential oil against test bacterial strains at concentration of 500 μ g/ml to Gram-negative bacteria *Pseudomonas aeruginosa* (5.6 mm), *Escherichia coli* (5 mm), *Salmonella typhimurium* (8.6 mm), and *Proteus vulgaris* (10.7 mm) and Gram-positive bacteria *Bacillus subtilis* (8.3 mm). At the same time, ZOI of standard antibiotic ciprofloxacin against test bacterial strains was *P. aeruginosa* (9.6 mm), *E. coli* (14.3mm), *S. typhimurium* (19.3 mm), *P. vulgaris* (17.8 mm), and *B. subtilis* (20.6 mm), respectively. The free radical scavenging property of *A. parviflora* employed by *in vitro* assay methods like scavenging activity of DPPH was 81% at 500 μ g/ml concentrations, respectively.

Conclusion: Our study showed that α -cadinol, α -muurolol, and cubebol as the major components in this oil which was absent in previous findings of *A. parviflora* and essential oil had potent antibacterial and antioxidant activity, respectively.

Keywords: *Ajuga*, Essential oil, Chemical constituents, Strains, Antibacterial, Antioxidant.

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INTRODUCTION

The valley Jammu and Kashmir is regarded as hub for the medicinal plants. The people there were using these medicinal plants for their cure and preventing various diseases since ancient times. There are about a total of 937 plant species belonging to 129 families have been reported from Jammu and Kashmir having traditional medicinal uses [1]. The genus *Ajuga* belonging to family Lamiaceae represented by 236 genera and up to 7200 species native to Europe, Asia. *Ajuga parviflora* Benth. is a perennial herbaceous plant also called as bugleweed. Conventionally, the plants have been used in fever, toothache, dysentery, diuretic, anti-inflammatory, and antimicrobial agents. *Ajuga* plant contains neo-clerodane diterpenes, flavonoids, triterpenes, sterols, and essential oils which possess medicinal properties [2]. The aerial parts of *Ajuga bracteosa* had characterized by chemical compounds such as hexacosanol, β -sitosterol, tetracosanoic acid, and β -sitosterol- β -D-glucoside [3]. The essential oil of *Ajuga chamaepitys* (L.) is rich in monoterpenes and sesquiterpenes predominantly α -pinene (16.1%), ρ -pinene (34.3%), and germacrene-D (5.6%) was the major components [4]. The gas chromatography (GC) and GC-mass spectrometry (GC-MS) analysis of essential oil from aerial parts of *Ajuga comata* Stapf. were identified as caryophyllene (30.9%), caryophyllene oxide (24.9%), (E)- β -farnesene (12.6%), eudesmol (3.2%), and cadinene (3.1%), and germacrene-D was reported for the first time [5]. Volatile constituents were isolated from hydrodistillation method and were analyzed by GC and GC-MS, namely β -caryophyllene (22.4%), γ -muurolene (12.7%), γ -terpinene (6.3%), α -humulene (5.8%), α -amorphene (3.8%), and β -selinene (2.5%) were found to be major constituents [6]. The essential oil isolated from the *Ajuga austro-*

iranica Rech.f. was contained pinene (30.5%), trans-verbenol (7.0%), caryophyllene oxide (6.8%), myrtenol (6.3%), 1-octen-3-ol, (6.2%), and β -pinene [7]. The methanolic extract of *A. parviflora* contained alkaloids, namely integerrimine, senecionine, 3 β ,17 β ,20-trihydroxy-1-oxo-(20R,22R)-witha-5,14,24-trienolide, and 28-hydroxy-14,20-epoxy-1-oxo-(22R)-witha-2,5,24-trienolide possessed antifungal activities [8]. The essential oil extracted from the aerial parts of *Ajuga chamaecistus* Ging. spp. *chamaecistus* was analyzed which contain β -pinene (15.0%) and linalool (14.5%) as the major constituents [9]. Methanol, butanol, chloroform, and water extracts of *Ajuga turkestanica* Rgl. Brig contained phytoecdysteroids and iridoids. The IC₅₀ values for antioxidant activity of water and butanol were 7.24 \pm 0.82 and 14.57 \pm 1.64 μ g/ml. The chloroform extract has antimicrobial activities against *Staphylococcus aureus* and *Streptococcus pyogenes*. Further investigation of methanol and chloroform contained volatile components, namely Pregna-4,9(11)-dien-20-ol-3-on-19-oic acid lactone (19.58%), 20-methyl-pregna-5,17-dien-3 β -ol (12.93%), 3,7-dioxocholan-24-oic acid (10.53%), and betulin (10.18%) were detected for the first time [10]. Phytochemical investigation of *Ajuga Forrestii* Diels involves neo-clerodane diterpenes, phytoecdysteroids and stigmastane sterol and iridoid glycosides [11]. Column chromatography, nuclear magnetic resonance spectroscopy, and mass spectroscopy led to isolation and identification of five compounds, namely verbascoside, echinacoside, ajugoside, harpagide, and 8-O-acetylharpagide from *Ajuga tenorei* C. Presl. known to exert peculiar pharmacological activities [12]. Ethanolic extracts of *Ajuga genevensis* were rich in caffeic, p-coumaric, and ferulic acids, luteolin, quercetin hyperoside, and apigenin were identified by liquid chromatography-MS analysis. Further, this extract was evaluated to show better results against antimicrobial and antioxidant effects [13].

The diterpenoids isolated from *Ajuga ciliata* Bunge were elucidated as (12S)-1 β ,6 α ,19-triacetoxy-18-chloro-4 α ,12-dihydroxy-neo-clerod-13-en-15,16-olide, and 12(S)-6 α ,18,19-triacetoxy-4 α ,12-dihydroxy-1 β -triglyoxy-neo-clerod-13-en-15,16-enolide and evaluated for neuroprotective effect against MPP(+)-induced neuronal cell death in dopaminergic neuroblastoma SH-SY5Y cells possessed moderate neuroprotective effects [14]. Our present study reveals the chemical identification and to check their properties of bioactive compounds.

MATERIALS AND METHODS

Plant material

The fresh plant material was collected from tarai altitudes of Jodhpur (Doda) and Jammu and Kashmir (India) at an elevated altitude of 1500 m in the month of August 2016 when plant in good blooming stage. The aerial parts were washed with cold water and their dead skin; dried leaves were skimmed off which were used for the extraction of oil. The preliminary plant identification was done by Prof. P. C. Pandey, Department of Botany, Kumaun University, Nainital. The plant was further confirmed by Botanical Survey of India, Dehradun, Voucher specimen *A. parviflora* Benth. Acc. No. 118084 where herbarium of plant specimens has been deposited of dried specimens.

Isolation of essential oil

The essential oil was obtained by steam distillation of fresh plant material (10 kg roots) using copper still fitted with spiral glass condenser. The distillate was saturated with NaCl and extracted with hexane. The hexane extract is dried with sodium sulfate (Na₂SO₄) and the solvent removed with Rotovap at moderate pressure and 38°C temperature and stored at 4°C for further analysis. All chemical and reagents of analytical grade were obtained from Merck, Mumbai, India.

Chemical and reagents

All chemicals and reagents used were of analytical grade. Hexane, anhydrous Na₂SO₄, dimethyl sulfoxide, ether, ethanol, tween 20, and sodium hypochlorite (NaOCl) were obtained from Merck, Mumbai, India, whereas potato dextrose agar (PDA), potato dextrose broth, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, α -tocopherol, and dextrose (D-glucose) were obtained from Hi-media Pvt. Ltd, Mumbai, India.

Analysis of essential oil

GC and GC-MS analysis

The analysis of the oil was done using a gas chromatograph (Shimadzu GC QP 2010) equipped with RTx-5MS capillary column, 100970 1(30.0 m \times 0.25 mm, film thickness: 0.25 μ m). The oven temperature (50°C–280°C) was programmed at 50°C for first 2 min, then 3°C/min to 200°C, and then 10°C/min to 280°C, after which it was maintained isothermally at 280°C for 8 min. N₂ was used as the carrier gas at 113.0 mL/min. The injector temperature was 250°C, detector temperature 260°C, and the injection volume was 0.5 μ L using a 10% solution of the oil in n-hexane. The GC-MS analysis was carried out with GC-MS QP 2010 (Shimadzu) fitted with RTx-5MS capillary column, 1009701 (30.0 m \times 0.25 mm, film thickness: 0.25 μ m). The oven temperature (50°C–280°C) was programmed at 50°C for first 2 min, then 3°C/min. to 200°C, and then 10°C/min. to 280°C. After which, it was maintained isothermally at 280°C for 8 min. N₂ was used as the carrier gas. The injection volume was 0.5 μ L and split ratio was 1:90. The mass spectra were taken at 70 eV. The percentage of peak area normalization was taken to express the relative percentage of the oil constituents.

Identification of compounds

Identification of different chemical constituents of the essential oil was done by comparing their Retention Indices/Kovat indices in relation to a series of n-alkanes (C₆–C₃₃) indices on the RTx-5MS capillary column, either with those of published data [15] or with authentic samples which were further supported by NIST and WILEY mass spectral library searches. The results are presented in Table 1.

Table 1: Phytochemical composition of aerial parts of essential oil from *Ajuga parviflora*

Compound	% in the oil	Method of identification	
		R.I. ^a	Others
1-octen-3-ol	0.52	984	b, c, d
2-nonanone	0.08	1093	b, c, d
Linalool	1.16	1098	b, c, d
Terpineol	0.14	1136	b, c, d
<i>p</i> -menth-4-en-3-one	0.07	1146	c, d
<i>n</i> -tridecane	0.09	1304	c, d
<i>p</i> -vinyl-guaiacol	0.35	1314	c, d
eugenol	0.31	1361	b, c, d
α -copaene	1.41	1378	b, c, d
β -bourbonene	2.35	1392	b, c, d
<i>E</i> -caryophyllene	0.18	1423	b, c, d
β -copaene	0.06	1434	c, d
γ -elemene	0.61	1438	b, c, d
α -humulene	2.08	1457	b, c, d
9- <i>epi</i> -(<i>E</i>)-caryophyllene	0.50	1469	c, d
germacrene D	4.32	1488	b, c, d
γ -amorphene	0.18	1496	c, d
<i>epi</i> -cubebol	1.78	1499	b, c, d
γ -cadinene	2.05	1516	b, c, d
cubebol	12.76	1519	b, c, d
α -cadinene	0.32	1541	c, d
isospathulenol	0.30	1545	c, d
α -calacorene	0.15	1550	c, d
Maaliol	0.60	1570	c, d
Germacrene D-4-ol	9.60	1577	b, c, d
β -caryophyllene oxide	0.67	1586	c, d
β -oplopenone	0.48	1608	c, d
Humulene oxide	1.26	1612	b, c, d
<i>Epi</i> -cubanol	0.39	1630	c, d
Cubanol	0.80	1646	b, c, d
α -muurolol	14.21	1649	b, c, d
α -cadinol	21.36	1656	b, c, d
Pentadecanal	0.39	1703	c, d
Oplopanone	0.10	1743	c, d
Khusinol acetate	0.92	1823	c, d
Longifolol acetate	2.76	1827	b, c, d
Farnesyl acetate	3.58	1848	b, c, d
<i>E</i> , <i>E</i> -geranyl linalool	0.58	2028	c, d
Abietadiene	0.14	2089	c, d
<i>Trans</i> -totalol	0.08	2317	c, d
<i>Trans</i> -ferruginol	0.36	2334	c, d
Sesquiterpenoids	12.18		
Oxygenated sesquiterpenoids	65.41		
Monoterpenoids	0.09		
Oxygenated monoterpenoids	2.55		
Higher hydrocarbon	7.36		
Total identified	87.50		
Yield	4.8 (4.05% by weight)		

^aRetention index (RI) relative to homologous series of n-alkenes (C₆–C₃₂) on RTx-5MS capillary column. ^bCompound checked by authentic standards compounds. ^cRetention index (RI). ^dMS, NIST08.LIB and WILEY8.LIB libraries spectra and the literature

Pathogenic bacteria and their culture

The bacterial strains were obtained from MTCC, IMTEC, Chandigarh for this study. The pure culture of bacterial species was maintained in PDA and stored below 4°C. The pathogenic bacteria *Pseudomonas aeruginosa* (MTCC 424), *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 443), *Proteus vulgaris* (MTCC 1771), and *Salmonella typhimurium* (MTCC 3224) were cultured on Potato dextrose agar medium in sterilized Petri dishes. Pure culture of bacterial strains was prepared by emulsifying single colony in 10ml of sterilized required broth in tubes and inoculated bacterial cultures were incubated overnight for 24 h.

In vitro antibacterial activity

For antibacterial activity, well-diffusion method was used to check the antibacterial activity to check the potency of bacterial strains that were

Table 2: ZOI shown by essential oil from aerial parts of *Ajuga parviflora* against bacterial strains

Bacterial strain	M.T.C.C. No.	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml	600 µg/ml
<i>Pseudomonas aeruginosa</i>	424	2.2 mm	2.8 mm	3.2 mm	4.3 mm	5.6 mm	7.2 mm
<i>Escherichia coli</i>	443	2.8 mm	3.5 mm	4.1 mm	4.6 mm	5.1 mm	6.9 mm
<i>Bacillus subtilis</i>	441	4.2 mm	5.8 mm	6.7 mm	7.2 mm	8.6 mm	10.9 mm
<i>Salmonella typhimurium</i>	3224	4.3 mm	5.2 mm	6.5 mm	7.0 mm	8.3 mm	10.1 mm
<i>Proteus vulgaris</i>	1771	5.2 mm	5.7 mm	6.8 mm	8.0 mm	9.1 mm	10.7 mm

ZOI: Zone of inhibition

Table 3: Absorbance shown by the *Ajuga parviflora* and control (ascorbic acid and Vitamin E) at different concentrations

Concentration (µg/ml)	Absorbance		
	Essential oil	Ascorbic acid	α-tocopherol
100	0.32±0.001	0.236±0.022	0.921±0.021
200	0.44±0.003	0.280±0.032	1.320±0.004
300	0.56±0.260	0.520±0.002	1.890±0.002
400	0.89±0.040	0.826±0.001	2.348±0.006
500	1.23±0.160	1.890±0.043	2.613±0.013
600	1.67±0.008	2.520±0.001	3.124±0.042

Values in the column are men of three absorbance results±SD. SD: Standard deviation

cultured in nutrient agar medium at 4°C for further use. The cultured medium was autoclaved at 121°C and 15 lbs for 20 min. Autoclaved medium when cooled and poured in sterilized Petri plates under aseptic conditions until it got solidified. After solidification, the Petri plates were incubated for sterility testing at 28°C in incubator. Well was created in dried plates and pipette was used to place 30 µl of essential oil into each well. The plates were placed in an incubator at 28°C for 24 h. The diameter of zone of inhibition was measured for both bacteria and antibiotic and recorded in mm, held on the back of inverted plates [16].

In vitro antioxidant activity

The DPPH assay was done according to the method described by Brand William *et al.* 1995 [17]. The DPPH 0.0039 g was dissolved in ethanol and made up to 100 ml with double-distilled water. The ethanol (20%) 20 ml and 80 ml double-distilled water were prepared. The 100 µM DPPH (50 µl) was added to equal volume of 20% ethanol to generate 400 µl DPPH. The oil samples of different concentrations were taken in different test tubes and added DPPH 400 µl and make volume up to 100 µl with double-distilled water. Then, it was shaken vigorously and taken in the dark for 20 min at room temperature. The reduction in absorbance was recorded at 520 nm in UV-VIS spectrometer. Ascorbic acid and α-tocopherol were used as standard and controlled absorbance of DPPH was taken without adding oil sample, and all the assays were carried out in triplicate. Scavenging effect (%) of free radical DPPH was calculated as:

$$\text{Scavenging effect \%} = \frac{\text{Absorbance of control} - \text{Absorbance of oil sample}}{\text{Absorbance of control}} \times 100$$

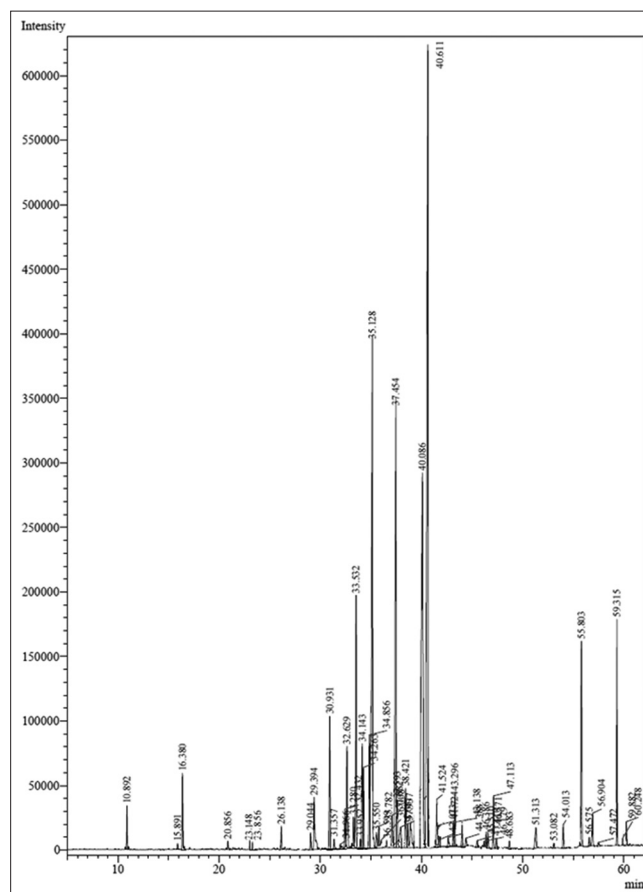
IC₅₀ assay was calculated graphically using curve by plotting antioxidant capacity or percentage inhibition versus corresponding sample concentrations.

Statistical analysis

For all the tests, the mean values and standard deviations were calculated and data were analyzed using SPSS 16.0 statistical software. The one-way analysis of variance was applied for calculating the results. The means were compared by Duncan tests at level of significance of $p \leq 0.05$.

RESULT AND DISCUSSION

The oil was dominated by oxygenated sesquiterpenoids (65.41%) and sesquiterpenoids (12.18%) whereas monoterpenoids (0.09%)

Fig. 1: Gas chromatogram of *Ajuga parviflora*

and oxygenated sesquiterpenoids (2.55%) constituted as minor components. The essential oil with major constituents is α-cadinol (21.36%), α-murolol (14.2%), cubebol (12.76%), germacrene-4-ol (9.6%), germacrene D (4.32%), fernesyl acetate (3.58%), longifolol acetate (2.76%), and β-bourbonene (2.73%) (Fig. 1 and Table 1). The essential oil of herb *A. parviflora* contains α-cadinol and α-murolol terpenoids as major constituents which were absent in previous analysis *A. parviflora* [6]. This change in composition is due to environmental adaptation climatic conditions and altitude factor of the plants.

The pathogenic bacterial strains incubated for 1 day for *P. aeruginosa* (MTCC 424), *B. subtilis* (MTCC 441), *E. coli* (MTCC 443), *P. vulgaris* (MTCC 1771), and *S. typhimurium* (3224) at 28±2°. The potency of oil was tested against Gram-positive and Gram-negative bacteria. The study shows that the oil has varied effect ranges from 2.2 mm to 10.9 mm in both Gram-positive and Gram-negative bacteria. The oil shows highest effect against *B. subtilis* (10.9 mm) and *P. vulgaris* (10.7 mm) whereas the standard antibiotic ciprofloxacin against test bacterial strains was *P. aeruginosa* (9.6 mm), *E. coli* (14.3 mm), *S. typhimurium* (19.3 mm), *P. vulgaris* (17.8 mm), and *B. subtilis* (20.6 mm), respectively The minimum inhibitory concentration of oil from *A. parviflora* in comparison to standard antibiotic

Table 4: Percentage scavenging activity of different concentration of essential oil and controls

Concentration (µg/ml)	%age of DPPH scavenged by essential oil	%age of DPPH scavenged by ascorbic acid	%age of DPPH scavenged by α-tocopherol
100	21.4	23.4	35.2
200	40.3	41.2	56.4
300	52.4	55.6	60.2
400	70.1	68.3	71.8
500	81.0	71.2	84.0
600	89.1	89.3	94.3

DPPH: 2,2-diphenyl-1-picrylhydrazyl

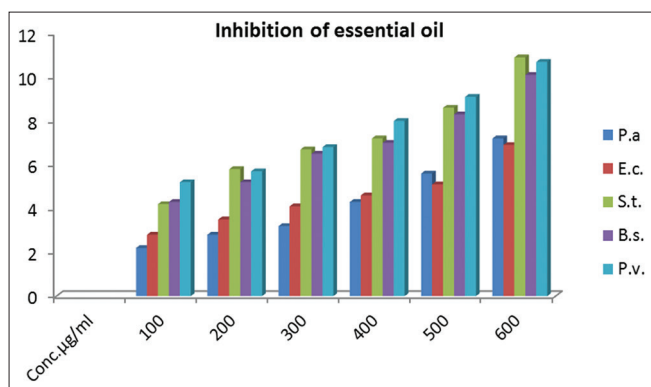


Fig. 2: Effect of essential oil on test bacterial strain at different concentrations (P.a.: *Pseudomonas aeruginosa*, E.c.: *Escherichia coli*, B.s.: *Bacillus subtilis*, S.t.: *Salmonella typhimurium*, P.v.: *Proteus vulgaris*)

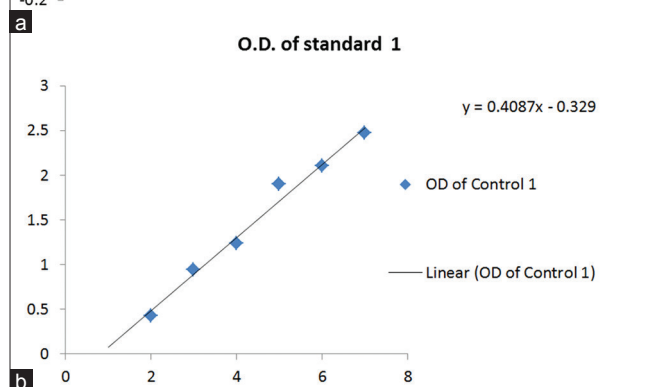
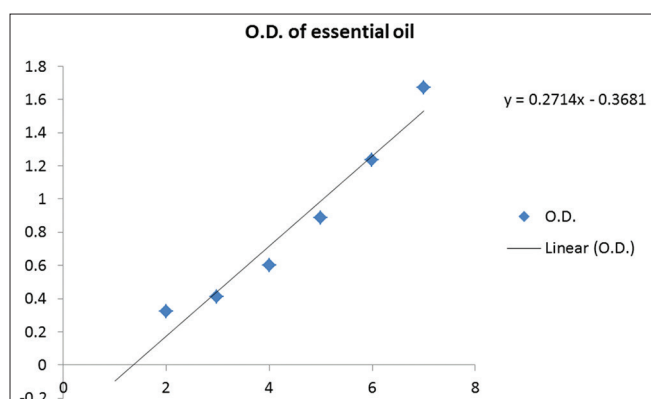


Fig. 3: (a and b) O.D. graph of essential oil and control 1

(ciprofloxacin) showed moderate effect (Table 2 and Fig. 2). The results are indicating that *A. parviflora* have potential use in medicinal and phytotherapy.

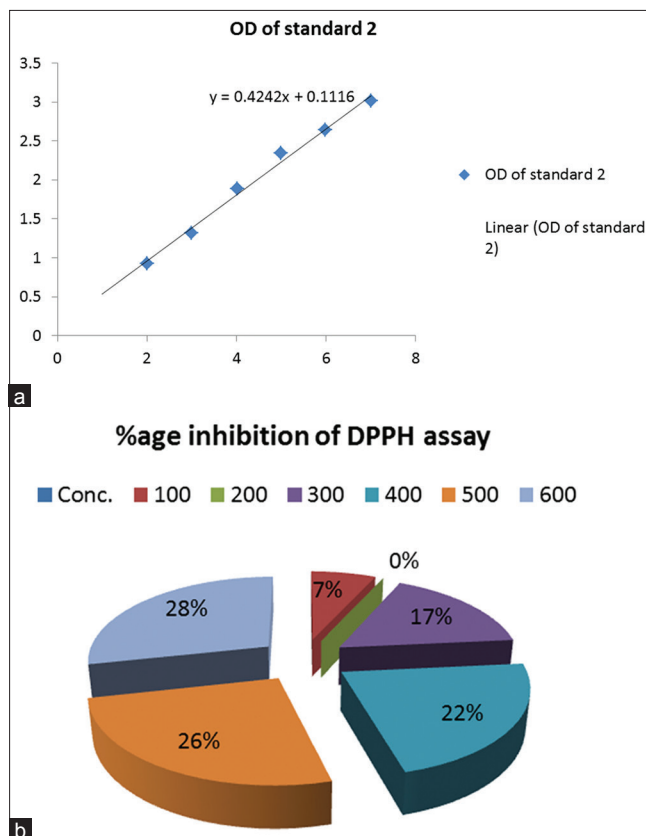


Fig. 4: (a and b) O.D. graph of control 2 and pie chart showing %age inhibition of 2,2-diphenyl-1-picrylhydrazyl assay at various concentrations

This study affirms that *in vitro* antioxidant activity of essential oil from aerial parts of *A. parviflora* was comparable to those of standard ascorbic acid and Vitamin E [18]. Higher absorbance indicated higher reducing power. The present study indicated that the oil at concentration of 100–600 µg/ml range from 21.4% to 89.1% could play an important role in the management of oxidative stress (Tables 3 and 4). Thus, it was considered that the essential oil had antioxidant activity against DPPH radical (Figs. 3 and 4).

CONCLUSIONS

This study showed that α-cadinol, α-muurolol, and cubebol as the major components in this oil which was absent in previous findings of *A. parviflora*. The essential oil from this plant has been responsible for its antidiuretic, anticancer, anti-inflammatory, antioxidant, and antibacterial activities [19]. However, the synergic effect is found to be responsible for its bioactivity. As a result of its activity, it can be used as bactericides and antioxidant which is more safe and eco-friendly as compared with synthetic chemicals. Thus, we hope that the phytochemical investigation helps in coping different diseases of our region.

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