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Original Research article

GC-MS Analysis of *Mentha arvensis* L. Essential Oil for its Antimicrobial Activity by Bio-autography Assay

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Abstract: The extraction of essential oil for its analysis and anti-microbial study has been in practice from so many years. The present work in *Mentha arvensis* L. for its anti-bacterial activity using bioautographic technique is for the first time. The essential oil extracted from *Mentha arvensis* L. was investigated for its antimicrobial activities against *Aspergillus niger*, *Klebsiella pneumoniae* and *Escherichia coli*. The essential oil extracted from the sample was analyzed using GC-MS technique to find out the presence of constituents. There are 72 identified bioactive molecules were identified in which menthol was present predominantly followed by isomenthane, menthol acetate and D-Limonene. The sensitivity of the chemical compounds against the microorganisms was determined by the bio-autographic assay. The oil exhibited maximum zone of inhibition as anti-bacterial agent against *Klebsiella pneumoniae* and *Escherichia coli* followed by as anti-fungal agent against *Aspergillus niger*. The anti-microbial activity could be due to the combined activity of the compounds present in the oil mixture extracted from the sample.
Key words: Bio autography, TLC, essential oil, Tetrazolium, Vanillin

Introduction

Plant natural products have been used as a potent antimicrobial agent in the traditional system of medicine. The essential oils from *Mentha* species are in practice to use as antibiotics for treatment of various diseases (Zaidi and Dahiya, 2015). Not only in medicine, its use in culinary and cosmetics also has been seen (Singh and Pandey, 2018).

The genus *Mentha* belonging to the family Lamiaceae has 25-30 species which are ubiquitous in nature (Dorman *et al.*, 2003). *Mentha arvensis* L. is originated from parts of Eurasia and cultivated largely in the Northern parts of India.

Essential oils (EOs) are basically volatile in nature and generally consist of mixture of volatile compounds such as mono, sesquiterpenoids and phenylpropanoids. The essential oils have been used in folk and traditional medicine from ancient time. They are used in variety of areas such as pharmacology, medical microbiology, phyto-pathology and also as potential anti-microbial agents (Horvath *et al.*, 2010).

Bioautography is an assay where the microbial activity can be detected using planar chromatographic technique. In this technique, the TLC plates are used where the biologically active organism is involved in the antimicrobial activity (Morlock and Schwack, 2010 and Moricz and Ott, 2016). It is a fast and subtle screening method for the presence of the antimicrobial compounds (Dewanjee *et al.*, 2015).

The conventional methods like agar diffusion to detect the antimicrobial activity using essential oil is quite challenging

as the EO are viscous, volatile and water non soluble (Moricz *et al*., 2016). The common screening methods like agar diffusion, disc diffusion etc. is inadequate for the antimicrobial study. Therefore, it is important to acclimatize a new technique where a combination of analytical method were used which help in easy detection of the antimicrobial activity.

Direct bioautography with TLC technique is a rapid and sensitive method to detect the antimicrobial activity. From time immemorial the essential oils have shown the antioxidant, antibacterial and antifungal, insecticidal properties (Burt, 2004 and Kordali et al., 2005). These natural products are safe and not reported any side effects compared to other synthetic drugs (Zaidi and Dahiya, 2015).

In view of this, the present study aimed to study the TLC bioautographic assay is used to detect the zone of inhibition against few micro-organisms like *Aspergillus niger*, *Klebsiella pneumoniae* and *Escherichia coli* with the essential oil extracted from *Mentha arvensis* L.

Materials and methods

The plant *Mentha arvensis* L. was procured from Lalbag Botanical Gardens, Bengaluru and were maintained in the Botanical Garden of Mount Carmel College, Autonomous, Bengaluru (Fig.1).

Fig. 1. The herb Mentha arvensis L.

The micro-organisms like fungi and bacteria were used for the antimicrobial studies. The fungal organism *Aspergillu sniger* was collected from Veterinary College, Bengaluru. The cultures were grown on Sabouraud Dextrose Agar (SD) medium and the cultures were also maintained in SD broth.

Similarly, the gram-negative bacteria like *Klebsiella pneumoniae* and *Escherichia coli* were used for the study and the bacteria were procured from Veterinary College, Hebbal, Bengaluru, Karnataka Veterinary Animal and Fisheries Sciences University (KVAFSU) and Department of Microbiology, Mount Carmel College, Autonomous respectively. All bacterial cultures were maintained on Mueller Hinton Agar (MH) at 4°C and cultured on MH broth at 37°C and incubated. Both fungal and bacterial cultures were periodically maintained.

Extraction of Essential Oil (EO)

The essential oil from *M. arvensis* was extracted by hydro distillation process using Clevenger apparatus under optimal operational conditions with a temperature of 40°C (Elyemni *et al*., 2019). 100g of fresh leaves of *M. arvensis* were placed in a round bottom flask on a heating mantle. The ratio of the material and solvent were taken in the ration 1: 8. It was kept for 3 hours for condensation of essential oils. At the end of distillation, two phases were observed. The upper organic phase where the essential oils is present and the lower aqueous phase with aromatic water (a saturated aqueous solution formed after distillation). The essential oil was then collected, dried under anhydrous sodium sulphate. The water removed oil was stored in air tight vials in the dark at 4°C till further use (Fagbemi *et al*., 2021 and Fadil *et al*., 2015).

Yield of essential oil

The percentage of essential oil were calculated and expressed in percentage using the formula (Elhmenyi *et al*., 2019).

$$Yield\ of\ oil(\%) = \frac{amount\ of\ extracted\ oil}{amount\ of\ sample} X100$$

Preparation of essential oil concentrations

The extracted oil mixture was dissolved in hexane and from that three different concentrations like 1µl, 2.5µl and 5µl were prepared. These concentrations were used to study the

antimicrobial activity. The amount of essential oil present per µl is 0.01%,

Bioautography Assay

For this assay, commercially available TLC Silica Gel 60 F_{254}Aluminum sheet TLC plates (Catalogue number 1.05554.0007) were used. The TLC plates of size 20cm x 20cm were cut and a line was drawn 2cm from one end of the plate. The extracts were applied on the TLC plates and developed according to the method used for essential oil (Wagner and Baldt, 1996). 10µl of extracted oil was spotted with capillary tube on the TLC plate for about 20-30 times. Then the plates were air dried and run in the mobile phase. The mobile phase used for hexane and ethyl acetate in the ratio of 19:1. After reaching the solvent front, the plates were sprayed with vanillin sulphuric acid solution for visualization of the compounds.

For the bioautographic assay of essential oil, the bacterial and fungal culture grown in 100ml nutrient broth at 37°C and SD fungal broth were used respectively. The bacterial suspension was diluted with 20ml of fresh nutrient broth to 4 x 10^{-7} CFU/mL (Horvath *et al*., 2011). Similarly, the fungal cultures grown on SD broth were also taken at the concentration of 4 x 10^{6} CFU/mL separately. The cultures were sprayed on the TLC plates and kept for drying. Later the plates were incubated for 24 hours at 36°C.

The bioautogram was then incubated at 37°C for 48 hours under humid conditions. The plates were then sprayed with tetrazolium salt solution for visualization of the microbial growth (Wagner and Baldt, 1996). The appearance of the white zones against the purple -pink background on the chromatogram indicates the zone of inhibition (Valgas *et al*., 2007).

GC-MS Analysis

The essential oil extracted in its crude mixture form was used to study the different compounds present in it. Gas Chromatographic analysis was carried out at Azyme Biosciences Ltd, Bengaluru, to find out the compounds present in the essential oil extracted from the leaves of *Mentha arvensis* using Varian 450GC. The column used was CP-SIL C18 with a dimension of 30m x 0.25mm x 0.25µm. Flame ionization detector was used and nitrogen gas was used as mobile phase. The flow rate was 1.0ml/min. The injector temperature was 220°C and the detector temperature was 250°C. Oven temperature was 90°C for 3 minutes, then raised to 180°C at the rate of 6°C/min, held for 5 minutes. Components were identified according to databases and quantified by the comparison with the certified standards.

Statistical Analysis

The data obtained for the antimicrobial activity of oil was represented by clear zone of growth inhibition. The clear zones were measured in mm and the data were subjected to Least Significant Difference (LSD) (SPSS version 16.0) with the Probability (p) values $p < 0.05$ to determine the statistical differences. (Snedecor and Cochran, 1994)

Results

The yield of the essential oil extracted was 0.57%. From this oil, different concentrations like 1µl, 2.5µl and 5µl were prepared by dissolving the extracted oil in hexane. Each microliter consists of 0.01% concentration of essential oil .These concentrations were used to study the antimicrobial activity.

TLC detection

The bioautographic assay is generally used to identify compounds for antimicrobial activity by separating components onto the surface of TLC plates. The TLC plates when were sprayed with vanillin solution, the compounds were visualized. DMSO was taken as control (Fig. 3a) and the anti- microbial activity was expressed as the diameter (mm) of inhibition zones (Horvarth *et al*, 2010). The identification of specific compounds was limited by the unavailability of reference standards

The inhibition zones were measured in mm from the TLC bioautographic plates for *M. arvensis* essential oil against *A. niger, K. pneumoniae* and *E.coli* with different concentrations like 1 µl, 2.5 µl and 5µl. . Inhibition zones observed were 1.2 mm, 3.1mm and 1.7 mm and 2.4 mm, 7.2 mm and 2.5mm against the growth of *E. coli* and *K. pneumoniae* for the concentration 1 µl, 2.5 µl and 5µl

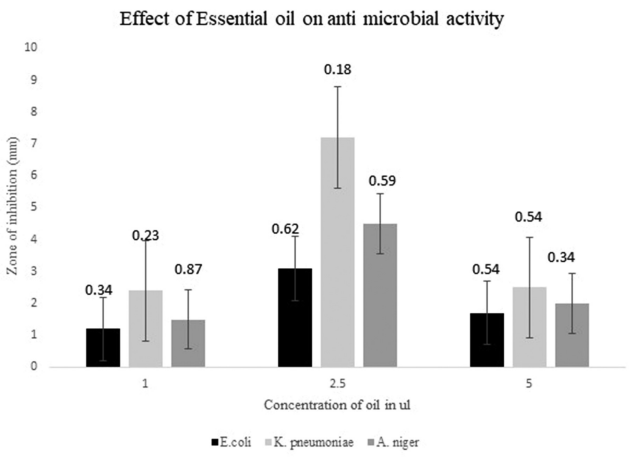

Fig. 2. Antimicrobial activity by essential oil from *M. arvensis*

Table 1. Antimicrobial activity from *M. arvensis* showing the inhibition zones.

	Zone of inhibition (mm)		
Concentration (µl)	*E.coli*	*K. pneumoniae*	*A.niger*
1	1.2 ± 0.34[a]	3.1 ± 0.23[b]	1.7 ± 0.87[c]
2.5	2.4 ± 0.62[a]	7.2 ± 0.18[b]	2.5 ± 0.59[a]
5	1.5 ± 0.54[a]	4.5 ± 0.54[b]	2.0 ± 0.34[c]

respectively. For the fungus *A. niger*, it was observed that 1.5 mm, 4.5 mm and 2.0 mm for the oil concentrations 1 µl, 2.5 µl and 5µl respectively (Table 1 and Fig.2).

The inhibition zones against the growth of bacteria *K. pneumoniae* were observed as it showed clear zones on the purple pink background, followed by *E. coli* and anti-fungal activity against *A. niger*.

The superscribed alphabets in the same row indicates the number of ranges indicating zone of inhibition in mm and values having same alphabet did not differ significantly as determined by LSD ($p<0.05$). the statistical significant differences of the various concentrations were compared and superscript alphabets indicates the p value. The value of a = p 0.006, b = p 0.05 and c = p 0.001.

These zones were observed on the TLC plates of essential oil as clear white spots on pink background when sprayed with tetrazolium salt solution. The Rf values varied from0.27cm to 0.88cm (Fig. 3b). (Reference standard not run for the Rf values). But the Rf values were compared with the earlier research work carried out in *Mentha* species (Zaidi and Dahiya, 2015) . An inhibition zones were observed between the Rf values 0.38 to 0.88. A big inhibition zone was seen between 0.82 to 0.88 another two zones were observed between 0.82 and 0.61 and 0.47 and 0.38 (Fig. 3c). Below the Rf value 0.38 no inhibition zones were observed, for the assayed crude oil. The maximum inhibition of growth of *K. pneumoniae* could be due to the presence of the compound menthol at the concentration of 2.5 µl.

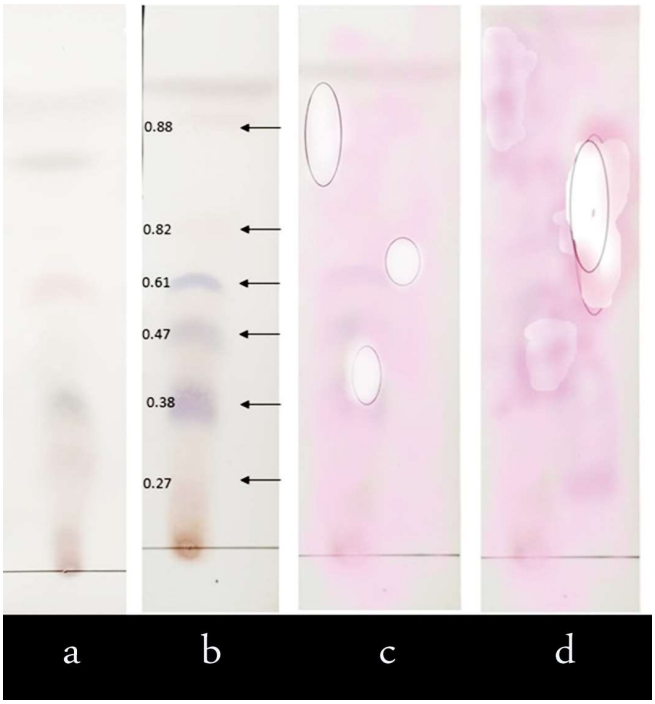

Fig. 3. TLC plate of *Mentha arvensis* oil.
(a) Control with DMSO
(b) showing the separation of compounds
(c) Essential oil showing anti-bacterial activity ; big size white circle is *K. pneumoniae* and smaller 2 white zones are *E.coli*
(d) Essential oil showing anti-fungal activity against *A. niger*

Detection of compounds by GC Analysis

A total 72 compounds were identified by GC-MS analysis. The principal compounds like menthol, isomenthane, menthyl acetate and D-limonene were detected by gas chromatography. The compounds identified were by comparing their Retention time and peak height. Menthol (43.08 peak with % area 61.93 RT 16.84) was the predominant component in the essential oil of *Mentha arvensis* followed by isomenthane, I-mentone and D-limonene with 16.5, 10.18 and 7.31 with % area 11.44,

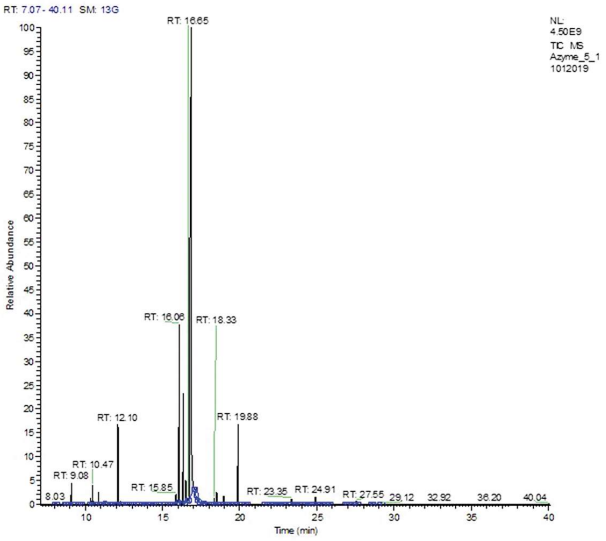

Fig. 4. Chromatogram showing the different compounds analysed through GC-MS.

Table 2. Relative percentage of peak area of *Mentha arvensis* L. essential oil.

Sl.No.	Apex RT	%Area	%Height	Identification
1	8.03	0.04	0.05	2,5-Diethyltetrahydrofuran
2	8.67	0.01	0.01	â-Pinene
3	8.86	0.02	0.04	á-Phellandrene
4	9.08	1.2	1.98	á-Pinene
5	9.59	0.03	0.04	Camphene
6	9.73	0.01	0.01	Thuja-2,4(10)-diene
7	10.32	0.34	0.57	Sabinene
8	10.47	1.13	1.82	â-Pinene
9	10.85	0.66	1.14	â-Myrcene
10	11.2	0.1	0.15	3-Octanol
11	11.3	0.01	0.02	(-)-â-Pinene
12	11.7	0.01	0.02	á-Terpinene
13	11.96	0.06	0.09	o-Cymene
14	12.1	4.61	7.31	D-Limonene
15	12.24	0.18	0.27	1,8-Cineole
16	12.33	0.01	0.01	trans-â-Ocimene
17	12.65	0.02	0.04	trans-â-Ocimene
18	13.01	0.03	0.04	ã-Terpinene
19	13.51	0.02	0.03	Linalool oxide
20	13.85	0.05	0.07	Terpinolene
21	14.03	0.02	0.02	p-Cymenene
22	14.37	0.07	0.1	Linalool
23	14.49	0.01	0.02	Nonanal
24	14.92	0.01	0.01	â-Thujone
25	15.08	0.01	0.02	trans-p-Menth-2,8-dien-1-ol
26	15.52	0.01	0.02	cis-p-Mentha-2,8-diene-1-ol
27	15.85	0.8	0.87	Isopulegol
28	16.06	11.44	16.5	Isomenthone
29	16.33	6.66	10.18	l-Menthone
30	16.49	1.66	2.05	(-)-Menthol
31	16.65	0.11	0.22	Isopulegone
32	16.84	61.93	43.08	(±)-Menthol
33	17.08	0.14	0.24	Levomenthol
34	17.27	0.15	0.24	á-Terpineol
35	17.62	0.04	0.06	Caprylyl acetate
36	17.83	0.03	0.05	Ethyl maleate
37	18.19	0.02	0.03	cis-3-Hexenyl-á-methylbutyrate
38	18.33	0.39	0.59	cis-3-Hexenyl isovalerate
39	18.49	0.71	1.06	Pulegone
40	18.67	0.01	0.01	D-Carvone
41	18.96	0.51	0.74	Piperitone
42	19.38	0.11	0.13	Menthol, acetate
43	19.7	0.01	0.01	Geranyl acetate
44	19.88	4.8	7.37	Menthol, acetate
45	20.31	0.09	0.15	Isomenthol acetate
46	20.42	0.08	0.12	Isopulegyl acetate
47	21.65	0.02	0.03	m-Eugenol
48	22	0.01	0.01	Vertenex
49	22.18	0.01	0.01	Copaene
50	22.41	0.09	0.14	â-Bourbonene
51	22.55	0.01	0.03	â-Elemen
52	22.76	0.01	0.01	Jasmone
53	23.05	0.06	0.1	Decyl acetate
54	23.35	0.36	0.52	Caryophyllene
55	23.61	0.01	0.02	â-Copaene
56	23.65	0.01	0.01	Octyl isovalerate
57	23.8	0.03	0.05	Octyl isovalerate
58	24.08	0.01	0.02	Geranyl acetone
59	24.28	0.02	0.03	Humulene
60	24.6	0.01	0.01	Germacrene D
61	24.74	0.01	0.01	ã-Cadinene
62	24.91	0.45	0.7	Germacrene D
63	25.28	0.08	0.1	ã-Elemene
64	25.7	0.01	0.02	ã-Cadinene
65	25.81	0.05	0.08	ä-Cadinene
66	26.86	0.01	0.02	Nerolidol
67	27.31	0.01	0.01	Spathulenol
68	27.43	0.03	0.04	Caryophyllene oxide
69	27.55	0.27	0.32	Diethyl Phthalate
70	28.54	0.03	0.06	cis-3-Hexenyl phenyl acetate
71	28.62	0.01	0.01	(-)-Spathulenol
72	29.12	0.02	0.03	á-Cadinol
42				

6.66 and 4.61 RT 16.06, 16.33 and 12.1) respectively (Fig. 4 and Table 2).

Discussion

The traditional use of plants as medicine, from ancient time suggests that the use of *Mentha* EO as antibiotics to treat many infectious diseases. Essential oils are volatile in nature and a complex mixture of isoprenoids with low molecular weight (Sharifi-Rad *et al*., 2017). The EOs are being used as topical antiseptics which exhibit antimicrobial properties using them as topical antiseptics (Zaidi and Dahiya, 2015and Gende *et al*., 2014). Menthol which is present almost 40% -50% is used as carminative, refrigerant, stimulant, etc. Essential oil

extracted from leaves of *M. arvensis* can be diluted and used in treating various skin diseases (Thawkar *et al*., 2016).

The essential oil present in *Mentha* species showed the presence of terpenes such as menthol, neomenthol, isomenthol, isomenthone, pulegone, carvone etc. (Singh and Pandey, 2018 and Thawkar *et al*., 2016).

In our current research work, the percentage of oil obtained was 0.57% from the fresh leaves. Similar kind of results was observed by Thawkar *et al*., in 2016 as 0.62% from the leaves of *M. arvensis*. The biologically active compounds separated on TLC followed by TLC bioautography of EO against the three microorganisms were studied. The anti-microbial activity was well exhibited by the oil mixture against both bacteria and fungus.

Presently in our study, the inhibitory activity was more against *K. pneumoniae* compared to *E.coli*. The results obtained in the current study were on par with the earlier studies against gram negative bacteria (Singh *et al*., 2011). The earlier reports also said that the anti-bacterial activity of the mint EO was effective against *E. coli* and *Klebsiella* species (Jeyakumar *et al*., 2011 and Sujana *et al*., 2013).

Not only the anti-bacterial activity, the EO of mint also possess the antifungal property against *A. niger.* The similar kind of findings were observed by Iscan *et al*., (2002) in *Mentha piperita* plant and in *Pipe rbetel* plant (Valle Jr. *et al*., 2016). The inhibitory effect of *Mentha* oil against *Aspergillus niger* was also reported (Suleiman *et al*., 2011). Menthol which is present in the essential oil showed inhibition activity towards the growth of *A. niger* (Dzamic *et al*., 2010).

It was observed that the inhibition of microbial growth may be due to the presence of one or more active compounds which may combine and have the effect on the growth of the micro-organisms (Zaidi and Dahiya, 2015). The antibacterial activity of the essential oil may be due to the presence of isoprenoid compounds like menthol and carvacrol (Dorman and Deans, 2000). The inhibitory activity of the EO may be due to the presence of terpenoids which were confirmed when the TLC plates were sprayed with vanillin solution. It was known from the past investigation, the phenolic compounds from complexes with the bacterial enzymes and proteins, which makes the inhibition in growth of bacteria (Rhouma *et al*., 2009). This anti -microbial activity could be due to the presence of menthol which is a monoterpenoid compound as its present in large amount. It was observed in the previous studies also that the menthol was having a promising antifungal (Samber *et al*., 2014) and anti bacterial activity (Padmini *et al*., 2010) and having an effective pharmacological activity (Makkar *et al*., 2018).
Similar kind of results were observed by Zaidi and Dahiya(2015) when they conducted work on *Mentha spicata* and *Mentha piperita* The maximum inhibitory activity was observed between the Rf values 0.88 and 0.61. Based on the previous studies conducted on TLC of plant natural compounds, the Rf value of 0.86 were usually attributed to flavonoids and terpenoids (Karthika *et al*., 2014). The oil mixture in the study showed more terpenoid compounds, hence the inhibitory activity could be due to the presence predominant monoterpenoid compounds like menthol, isomenthane.

Our results are par with the research findings by Singh (2010) and Pandey and Tripathi (2011). They also observed *M. arvensis* essential oil was found to be inhibited the growth of *Aspergillus* species. This kind of activity may be due to the action of several monoterpenes which act on the cell membrane by affecting the lipid fraction, causing leakage of intracellular membrane, there by affecting respiratory enzymes of fungi (Trombetta *et al*., 2005 and Cox *et al*., 2000). Similarly the compound having Rf value of 0.86 exhibited significant anti-bacterial activity against *K. pneumoniae* with oil extracted from *Piper betel* (Valle Jr. *et al*., 2016). The antifungal activity against *A.niger*was observed between the Rf values 0.82 and 0.61 (Fig.3d). Earlier studies by Makkar *et al*., (2018) showed that the essential oil extracted from *M. arvensis* showed a good anti fungal activity against *Fusarium moniliforme* and *Rhizoctonia solani*. Another previous study by Zaidi and Dahiya (2015) were also prove that the essential oil extracted from *Mentha spicata* showed an excellent anti-fungal activity against *A. niger*.

In the present study it was observed that the oil extracted from *Mentha arvensis L.* has its effect on the infestation of bruchids on *Vigna radiata L.* Plant essential oils are an important source of natural pesticides. Certain aromatic plant families in particular are rich in essential oils, these can be exploited for obtaining natural biopesticides, one of these families being Lamiaceae. Though they can be expensive when compared to synthetic pesticides they are very safe to the environment and the ecosystem as opposed to the synthetic pesticides. Their quick biodegradation increases their specificity, thereby protecting the beneficial and non-target organisms as well as preventing their accumulation in the environment. Additionally, the EOs are also can be used as a natural pesticide to stored grains rather than using a synthetic pesticides.

This essential oil is further screened for the antimicrobial activity using bio autography assay. Further the chemical compounds present in the oil need to be isolated and each compound can be analyzed for its specific anti microbial activity. Therefore, essential oils are definitely an efficient, safe and viable alternative to synthetic chemical pesticides.

The observations made from the current research work we can conclude that the oil mixture extracted from *Mentha arvensis* can be used as a strong antimicrobial agent.

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