Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(4): 15-19
Received: 04-05-2020
Accepted: 06-06-2020

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Comparative study of antibacterial assay of *Mentha piperita* (*in vivo* and *in vitro* cultured) leaves extract on enveloped human pathogenic bacteria and its phytochemical screening

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DOI: https://doi.org/10.22271/phyto.2020.v9.i4a.11662

Abstract
The purpose of this study was to examine the effectiveness of mint extract for the control of growth and survival of pathogenic microorganisms that are common cause of infections. Mint plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, steroids, phenol etc. with the help of certain tests which confirm their presence. The antimicrobial activity of mint is due to the presence of various secondary metabolites. Therefore, mint plants can be used to isolate bioactive natural products that may serve as leads in the development of new pharmaceuticals drugs. We use both polar solvent aqueous and ethanol for the extraction of active components from leaves of the plant. Mentha extract showed antimicrobial activity against many bacterial strains like *Bacillus subtilis*, *Enterococcus feacalis* and *Salmonella typhi*. Mint leaves gaves considerable amount of antimicrobial activity higher with ethanol extract and least with water extract. The presence of useful phytochemicals in the samples has proved it to be very important plant both economically as well as medicinally.

Keywords: Mentha piperita, phytochemicals, leaves extract, antimicrobial activity, bioactive compounds

Introduction
Among several plants, Mint (*Mentha piperita* L.) is a rapid growing perennial herb most widely used in medicinal preparations. It is commonly known as Peppermint, Brandy mint, Candy mint, Lamb mint, Balm mint, Vilayati pudina or Paparaminta (Punit and Mello, 2012) [14]. They are economically important to man due to their multiple applicatons such as pharmaceuticals, flavor, fragrance, insecticides, dyes, food additives, toxins etc. (Mehta *et al*., 2012) [8]. The mint plants widely used for treatment of digestive disorders and nervous system action because of its antitumor and antimicrobial properties, chemo-preventive potential, its renal actions, antiallergenic activities, and also for lessening cramping, digestive complaints, anorexia, nausea and diarrhea in folk remidies and traditional medicine (Loolaie M, *et al*., 2017) [12]. Peppermint oil is used in daily life and it is used peppermint candy, chewing gum, candy care, ments chocolate, shampoo, insects repellants and used for flavouring pharmaceuticals and oral preparations such as toothpastes, dental creams, and mouth washes. Higher and aromatic plants have been traditionally used in folk medicine as well as to extend the shelf life of food, showing inhibition against bacteria, fungi and yeast (Ebenezer *et al*., 2011) [7]. Peppermint oil relaxing action also extended to tropical use, when applied tropically it acts as counterirritant and analgesic with the ability to reduce pain and improve blood flow to the affected area (Suresh kumar et al., 2007) [16]. Peppermint oil used for skin problems such as itching, acne, allergic rash, headache, muscles pain, nerve pain, bacterial and virus infections (Fatiuh *et al*., 2002) [6]. Peppermint constituents (oil, leaf, leaf extract and leaf water) are commercially used as raw materials in toothpaste, toothpowder, mouth freshner, confectionary, chewing gum, candies, perfume, analgesic balms caugh drops, antiseptic mouth rinses, and chewing tobacco industry and also used as a necessary ingredients in Touarag tea, a popular tea in the Northern African and Arab countries (African pharmacopoeia, 1985) [3]. It was also found to reduce the incidence and multiplicity of benzo[a]pyrene-induced lung carcinogenicity and mutagenicity (Samarth *et al*., 2006) [15]. Qualitative tests and phytochemicals analysis were done as to test the presence of terpenoids, flavonoids, and phenolics.

The analysis revealed the presence of alkaloids, flavonoids, steroids, tannins, and phenols (Sujanal *et al*., 2011). The eastern and western traditional medicinal peppermint and its oil have been used as a antispasmodic, aromatic, antiseptic and also in the treatment of cancer, colds, cramps, indigestion, nausea, sore throat and toothache (Briggs, 1993) [4]. Peppermint oil and menthol have moderate antibacterial effect against gram-positive and gram-negative bacteria (Diaz *et al*., 1998) [5]. In clinical trails peppermint oil role in irritable bowel syndrome affirms its effectiveness compared with a placebo with no serious constipation or diarrhea (Kline *et al*., 2001; Liu *et al*., 1997; Pittler an Ernst, 1998) [9, 10, 13]. The study confirms that both aqueous as well as organic solvent leaf extract posses antibacterial properties against various pathogens viz., *Bacillus substilus, Pseudomonas aureus, Serratia marcesens, Streptococcus aureus* (Sureshkumar *et al*., 2007: Pramila *et al*., 2012) [16].

Materials and Methods

This research work was conducted at the Plant Biotechnology Research Laboratory, Department of Biotechnology, S.G.R.R University, Patel Nagar, Dehradun Uttarakhand (248001).

Materials

MS Media: Murashige and skoog medium (MSO or MS-zero) is a plant growth medium used in the laboratories for cultivation of plant cell culture. It contains macronutrients, micronutrients, vitamins, plant growth hormones are essential for the growth and development of culture tissue. PH was adjusted by pH paper or electrical pH meter at 5.8 pH. Agar is a gelling agent, it provide support to explants for growth.

A carbon source is essential for the cells, tissue or organ culture for *in vitro* regeneration. Sucrose is almost universally used for micro propagation purpose, as it readily utilizable by cells. Sucrose concentration of 30g/l was found to be optimal for the growth of *Mentha piperita.*

Sterilization

Sterilization of MS media

Plant tissue culture media are generally sterilized by autoclaving at 121^0c and 1.05kg/cm^2 (15-20 psi). There is evidence that medium exposed to temperature in excess of 121^0c may not properly gel or may result in poor cell growth. Minimum autoclaving time required for the liquid volume to reach the sterilizing temperature 121^0c for 15 minutes.

Several medium components are considered thermolabile and should not be autoclaved. Stock solution of the heat labile components are prepared and filter sterilized through a 0.22um filter into a sterile container and then filtered solution is aseptically added to the culture medium.

Sterilization of explants

Explants collection: Small pieces of stems from healthy plants of mint (*Mentha piperita*) were cut with the help of a steel cutter and then packed in the zip lock polythene bags. Bags should be wiped with little amount of alcohol. The bags were brought to the plant tissue culture lab.

Explants sterilization: The cut stems and nodal explants were taken out from zip lock bags and all the leaves were removed with the help of surgical knife and further cut into smaller pieces. All the cut stems (nodal explants) were put in the beaker and a plastic mesh was placed on the mouth of beaker with the help of a rubber band. The beaker was then kept under running tap water for 5- 10 minutes.

Then, the explants were washed by adding Tween 20 which were kept for 15-20 minutes. Rinsed with distilled water 4-5 times. Then, 0.2% Bavistin (w/v) was taken in the beaker containing explants and these were allowed to stand for 15-20 minutes. Now again, the explants were rinsed with distilled water 4-5 times. After, antifungal treatment 0.1% PVP (w/v) was added for 30 minutes to remove phenolic compounds which are present on the surface of the explants.

Table 1: Chemicals used for surface sterilization

S. No.	Chemicals Name	Objective
1.	Tween 20	Acts as detergents and surfactant
2.	Bavistin	To remove fungi
3.	$Hgcl_2$	To remove microbes. It has antimicrobial activity
4.	Formaldehyde	As a fumigant to sterilize the incubation room inoculating chamber, laboratory.
5.	Ethanol	As disinfectants to sterilize the glasswares, hands and explants, working area in laminar air flow etc.
6.	PVP	Antioxidant

Inoculation

The whole inoculation process is done in laminar air flow to avoid contamination. The surface of laminar air flow is wiped with 70% ethanol and all the tools are heat sterilized with the help of a burner. Now all the explants, are sterilized by solution containing $HgCl_2$ and distilled water, after these explants are washed with autoclaved distilled water. Cut the explants from both the ends with surgical knife, take a flask containing solidified media and sterilize it by keeping its neck upon the burner for short time period, now take explants and inoculate one by one with the help of forceps. One flask may contains 2-3 explants. Now transfer all the inoculated flasks in the incubation chamber, where we can provide proper environment to the explants for better growth.

Collection of plant material

Healthy mint plant were collected, washed thoroughly in tap water and shade dried for 15 days. Then the dried leaves were grinded with an ordinary grinder and then sieved through siever. The powder was stored in an air tight container and kept for further use.

Preparation of Plant Extract (Ethanol and water extract)

The powder was divided into equal parts. Each 5gm sample dissolved in 50ml of solvent (ethanol and water) separately. The flasks were covered with cotton plugs. The suspensions were shaken vigorously in shaker for 2 days and then suspensions were filtered using cheese cloth and again filtered through Whatman no.1 filter paper. The filtrate was then evaporated on a water bath. The concentrated extract were weighted and then dissolved in 2ml of DMSO (Dimethyl sulphoxide) and then stored in airtight sample bottles in a refrigerator at 4^0c for their antimicrobial and phytochemical screening.

Preparation of Extracts for Phytochemical Analysis

The collected leaves of *Mentha piperita* were washed and then dried under shade. The coarse powder was soaked in 10 ml of distilled water and extracted in the cold for 3 days with occasional shaking. The solvent from the total extract was filtered and concentrated on a water bath for 8 hours. The remaining was used for the analysis of phytochemicals test, same procedure was followed for ethanolic extraction using ethanol.

Antimicrobial Assay

Collection of Test Organisms

The test organisms (*E. coli*, *Pseudomonas sp*. *Staphylococcus aureus, Baccillus subtilis, Salmonella typhi*, *Enterococcus faecalis)* used were isolate and identified in the Department of Biotechnology, SGRR University, Dehradun. The bacterial isolates were first subcultured in a nutrient broth and incubated at 37^0c for 24 hours. All the test organisms were maintained in refrigerator at 4^0c in nutrient broth.

Determination of Antibacterial Activity

The antibacterial activity of the leaf extracts was determined using agar well diffusion method. Nutrient broth media was inoculated with the given microorganisms by spreading the bacterial inoculums (0.2ml) on the solidified media. Wells (8 mm diameter) were punched in the agar with a cork borer and filled with plant extracts. Control wells containing neat solvents (negative control) or standard antibiotic solution (positive control) Cefixime were also run parallel in the same plate. The plates were incubated at 37ºC for 24 hours. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition for the respective drug. The relative antibacterial potency of the given preparation was calculated by comparing its zone of inhibition with that of the standard drugs Cefixime. Clear inhibition zones around the wells shows antimicrobial activity.

Results and Discussion

The shoot buds emerged on 8th and 10th day of culture. The surface sterilization method followed in the present study produced 60% infection free cultures and PVP was used to prevent browning of the medium. Shoot induction was observed in all the different concentrations tried but varied with concentrations of sucrose, agar and PGRs taken.

Table 2: Shoot induction in nodal explants of *M. piperita*

S. No.	Sucrose (g/L)	PGRs (mg/L)	Agar (g/L)	No. of Explants	Percent Shoot induction
1.	30	1.5 NAA + 3.0 BAP	6.0	4	25
2.	35	1.5 NAA + 4.0 BAP	8.0	4	50
3.	40	2.0 NAA + 5.0 BAP	4.5	4	75
4.	35	2.0 NAA + 6.5BAP	5.0	4	50

The nodal explants showed average shooting response in different concentrations of BAP and NAA taken. Best response was observed on MS medium containing 40 g/L sucrose and agar concenartion of 4.5 g/L supplemented with 2.0 NAA + 5.0 BAP (mg/L) (Average number of shoots 3 to 4). When the nodal explants of *Mentha* were propagated by 1.5 NAA and 3.0 BAP, Sucrose 15gm/500ml and Agar 3gm/500ml it showed good growth. But when MS were supplemented by higher PGRs concentration 2.0 NAA and 5.0 BAP, high sucrose 20gm/500 ml and low Agar 2.25gm/500ml, the explants showed efficient growth.

According to different plant species have different potentials for absorbing and metabolizing the endogenous hormones and compounds of the nutrient medium, thus presenting different responses to *in vitro* cultivation.

Table 3: Effect of varying sucrose and agar concentrations on shoot initiation in nodal explants of *Mentha piperita*

S. No.	Sucrose (g/L)	PGRs (mg/L)	Agar (g/L)	No. of Explants	No. of Shoots/explants	Shoot Formation
1.	30	1.5 NAA + 3.0 BAP	6.0	4	1-2	+
2.	35	1.5 NAA + 4.0 BAP	8.0	4	2-3	++
3.	40	2.0 NAA + 5.0 BAP	4.5	4	3-4	+++
4.	35	2.0 NAA + 6.5 BAP	5.0	4	1-3	+

+++ = Good; ++ = low; + = Poor

Phytochemical Analysis of Plant extract

The extract were analyzed for various phytoconstituents like carbohydrates, tannins, amino acids, flavonoids, steroids, alkaloids, terpenoids, glycosides and saponins. The aqueous and ethanolic extracts of the plant were analyzed for the qualitative and quantitative phytochemicals analysis. A number of biological activities that protect most of chronic diseases are exhibited by different extract of phytochemicals such as alkaloids, flavonoids, steroids, glycosides, phenols, saponins and (Abbas Ibrahim *et al*., 2019). Different studied have illustrated the health benefits of saponins among which are their effect on blood cholesterol levels, cancer, bone health and stimulation of immune system (Mercy Gospel Ajuru, *et al.*, 2018). Phytochemicals tests shows that mentha contains a wide variety of constituents. Ethanolic extract shows phytochemical screening more active constituents than aqueous extract.

Table 4: Phytochemicals present in different extract of *Mentha piperita* leaves extract

	In vivo extract		*In vitro* extract	
Phytochemical Test	**Aqueous**	**Ethanol**	**Aqueous**	**Ethanol**
1. Mayer test(Alkaloids)	–	+	_	+
2. Ferric chloride test (Tannins)	+	_	_	+
3. Conc HCL+ Mg (Flavonoids)	+	_	_	+
4. Sulphuric acid test (Steroids)	_	+	_	+
5 Ferric chloride test (phenols)	_	+	_	+
6. Forth test (saponins)	+	_	+	_
7. Molisch's test (Carbohydrates)	_	+	_	+

Antibacterial Activity Estimation

The mint plant extracts showed antimicrobial activities against all tested bacterial strains. The results of the antimicrobial activity obtained using agar well diffusion

methods.

As compared with the bacterial strains the best antibacterial activity were shown with *E. faecalis* and lesser observed with *B. subtilis* and least or no activity were observed with *S. typhi* strain.

In table 5 showed that extract of *Mentha piperita* leaves (*in vivo*) make a maximum zone of inhibition on bacterial strains including *B. Subtilis* (12mm), *E. faecalis* (16mm), *S. typhi* (4mm) in ethanol extract and in table 6 *B. Subtilis* (10mm), *E. faecalis* (14mm), *S. Typhi* (3mm) in aqueous extract were determined.

Table 5: Antibacterial activity at different concentration of leaves extract of *Mentha piperita* (*in vivo*) by Agar well diffusion method.

Test organisms	Solvent extract	Zone of inhibition as observed in different conc. of extracts (1mg/ml) 50ul/ml	25ul/ml	12.5ul/ml
B. subtilis	Ethanol	12mm	10mm	8.5mm
E. feacelis	Ethanol	16mm	14mm	11mm
S. typhi	Ethanol	4mm	2.5mm	1mm

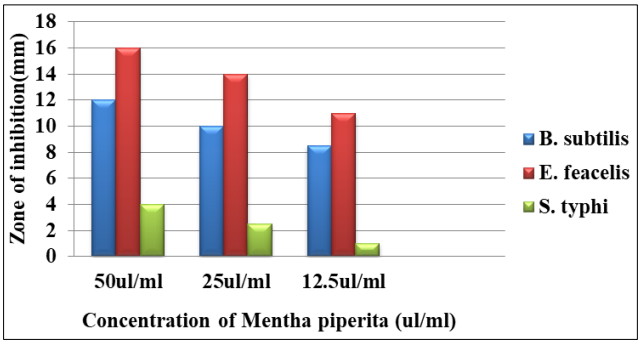

Fig 1: Graphical representation of Antibacterial activity at different concentration (Ethanol extract)

Table 6: *B. Subtilis* (10mm), *E. faecalis* (14mm), *S. Typhi* (3mm) in aqueous extract were determined

Test organisms	Aqueous extract	Zone of inhibition as observed in different conc. of extracts (1mg/ml) 50ul/ml	25ul/ml	12.5ul/ml
B. subtilis	Water	10mm	8.5mm	7mm
E. feacelis	Water	14mm	12mm	10mm
S. typhi	Water	3mm	1.5mm	1mm

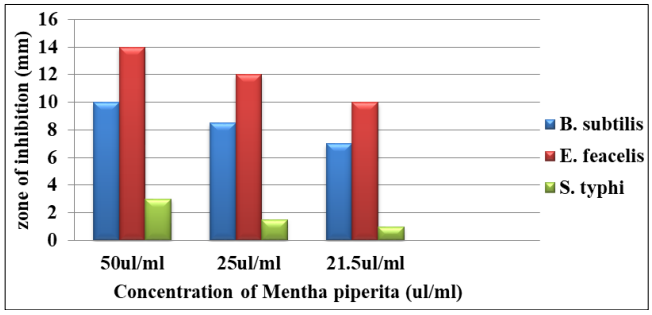

Fig 2: Graphical representation of Antibacterial activity at different concentration (Aqueous extract)

In table 7 showed that extract of *Mentha piperita* leaves (*in vitro*) make a maximum zone of inhibition on bacterial strains including *B. Subtilis* (14mm), *E. Faecalis* (15mm), *S. Typhi* (6mm) in ethanol extract and in table 8 *B. Subtilis* (11.5mm), *E. faecalis* (17.5mm), *S.typhi* (4mm) in aqueous extract were determined.

Table 7: Antibacterial activity at different concentraction of leaves extract of *Mentha piperita* (*in vitro*) by Agar well diffusion method.

Test organisms	Solvent extract	Zone of inhibition as observed in different conc. of extracts (1mg/ml) 50ul/ml	25ul/ml	12.5ul/ml
B. subtilis	Ethanol	14mm	12mm	9.5mm
E. faecalis	Ethanol	17mm	15mm	13mm
S. typhi	Ethanol	6mm	3.5mm	2mm

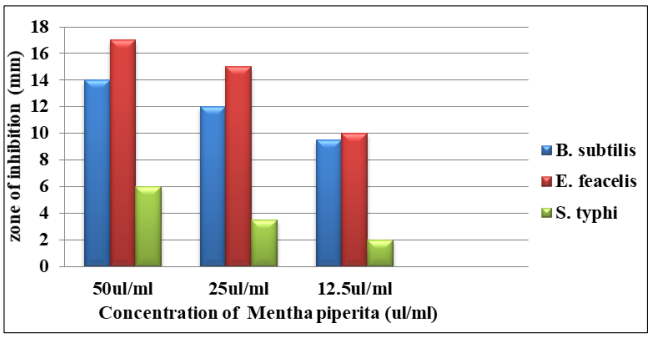

Fig 3: Graphical representation of Antibacterial activity at different concentration (Ethanol extract)

Table 8: *B. Subtilis* (11.5mm), *E. faecalis* (17.5mm), *S.typhi* (4mm) in aqueous extract were determined

Test organisms	Aqueous extract	Zone of inhibition as observed in different conc. of extracts (1mg/ml) 50ul	25ul	12.5ul
B. subtilis	Aqueous	11.5mm	10mm	8mm
E. feacelis	Aqueous	15mm	12.5mm	10mm
S. typhi	Aqueous	4mm	2mm	1mm

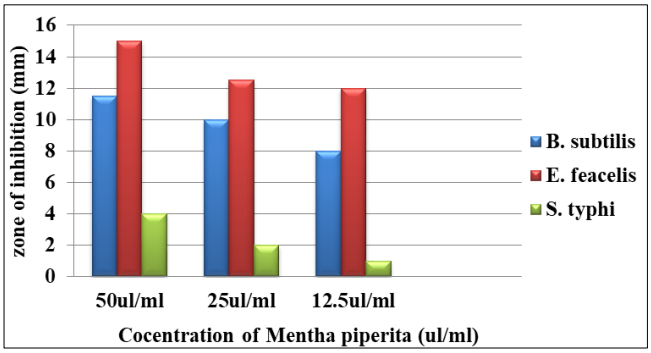

Fig 4: Graphical representation of Antibacterial activity at different concentration (Aqueous extract)

Conclusion

As the work for the development of herbal medicines is in the progress worldwide, the present report of *Mentha piperita* is useful in the treatment of various pathogenic diseases or infections and as it will help in the innovation of new chemical classes of antibiotics or drugs. Finally, it can be concluded that the bioactive chemical compounds present in *Mentha piperita* should find place in treatment of various bacterial infections and indicate this herb should be studied more extensively to explore its potential in treatment of infectious diseases.

Therefore it is essential to research further by identification of biologically active compounds, characterization and purification of the extract of this plants. *Mentha piperita*

leaves could be a possible alternative to chemicals as it can be harnessed as antimicrobial, antioxidant and flavouring agent as spice. The study consequently provides further evidence on the traditional usage of this plant extract as beverage and in treating diseases.

There is a rapid advancement in the tissue culture related to *Mentha* in the areas of agriculture to successfully propagate the plant in very short period of time and providing its benefit to the human walfare.

Acknowledgement

Authors are grateful express thanks to Dr. Kumud Saklani, Principal, Department of Biotechnology, SGRR School of Basic and Applied Sciences, Patel Nagar, Dehradun Uttarakhand, for her kind support and suggestion and providing all facilities for this work. We also express thanks to Dr. Naveen Gaurav Srivastava, Head of the Department of Biotechnology, SGRR School of Basic and Applied Sciences, Patel Nagar, Dehradun Uttarakhand, for his helpful discussion and advice during this work.

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