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Anti-Oxidant, Anti-Bacterial and Anti-Cancer Activity of *Mentha Piperita* Against Mcf-7 Cells

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The study evaluates the antioxidant, antibacterial and anticancer activities of different extracts of *Mentha piperita* leaves against the MCF-7 cell line. The leaves of *Mentha piperita* were extracted with aqueous, ethyl acetate, and chloroform. These extracts were subjected to qualitative phytochemical screening, antibacterial activity, cytotoxic activity, and AO/ErBr assay for apoptotic effect against the MCF-7 cell line. Qualitative analysis of the leaves' different extracts revealed the presence of glycosides, alkaloids, flavonoids, terpenoids, tannin, and saponin. The antibacterial activity of the leaf extracts was examined against four different bacterial species (*Bacillus cereus, Pseudomonas fluorescens, Aeromonas hydrophila* and *Klebsiella pneumoniae*). The aqueous extract exhibited a high level of antibacterial activity (18.66mm ±1.1 in *Bacillus cereus*). Free-radical scavenging activity of chloroform extractof M. piperita leaves was found to be more than aqueous and ethyl acetate extracts. Further, aqueous, ethyl acetate,and chloroform extracts exerted a cytotoxic effect with the IC50value of 45±1.5μg/ml, 29±1.2μg/ml, and 24±1.0μg/ml, respectively.From this study, we have observed that chloroform extract showed a concentration-dependent apoptotic effect against MCF-7 cells determined by AO/EtBr assay. The results further depicted that the selected traditional *Mentha piperita* could be used as a potential anticancer, antibacterial, and good antioxidant agent against the MCF-7 cell line.

Keywords: Anti-oxidant; Anti-bacterial; Anti-cancer; *Mentha piperita*, MCF-7.

Breast cancer is significant cancer among women; it affects 2.1 million women every year. It is also one of the leading causes of the most significant number of death among women. In 2019, 268600 new cases of invasive breast cancer, and 41,760 women died due to this disease. At the same time, breast cancer rates are higher among women in more developed regions than developing and underdeveloped countries and increasing the incidence of breast cancer in every region globally. Women are more exposed to breast cancer, particularly in the age group of e”50 years, and 90% of deaths occur in this age group (ACS, 2019).Breast cancer begins either in breast tissue made up of a gland called lobules or a duct that connects lobules to the nipple. About 40% of breast cancer in the female is a mutation in BRCA-1, and BRCA-2 genes are the main factor (Zoi*et al.,* 2017).

Elevated estrogen or hormonal replacement therapy levels increase the risk in pre-or post-menopausal women (Peairs *et al*., 2017).

Treatment includes single therapy or combination therapy such as radiotherapy, hysterectomy, surgery, pelvic exenteration, chemotherapy, pelvic lymph node dissection, biological techniques, trachelectomy, chemoradiation, and conization. Despite high treatment benefits, conventional therapies exhibit toxicity against normal cells and are also associated with severe side effects. On the other hand, biologically based therapies include herbs, dietary supplements, and traditional medicine systems, which are now attracting global attention as potential sources of anticancer agents. They are extensively used because of their availability, applicability, affordability, therapeutic efficacy, and with little or no side effects, which, in turn, has accelerated the scientific research on these agents (Joseph *et al*., 2020). Various active compounds (or their semi-synthetic derivatives) derived from medicinal plants have been assessed for their efficacy and tolerability in breast cancer treatment. Some of these plant species, including *Taxus baccata* (paclitaxel, docetaxel), *Podophyllum peltatum* (etoposide), *Camptotheca acuminata* (camptothecin), and *Vinca rosea* (vinblastine, vinorelbine) have well-recognized antitumor activity in breast cancer and have been evaluated in clinical trials.

Mentha piperita L., Commonly known as Peppermint, is an essential medicinal plant belongs to the Lamiaceae family, and it is well known for its medicinal values (Rita *et al*., 2010). *Mentha piperita* containing menthol, methyl acetate, and menthone are the major chemical components present. It also consists ofbioactive molecules such as pulegone, menthofuran, limone, flavonoids, glycosides, and polyphenols (Saharkhiz*et al*., 2012). Traditionally, *Mentha piperita*is used as astringent, antiseptic and analgesic, radio-protective, antioxidant, anti-carcinogenic, anti-tumorigenic anti-allergic. Peppermint oil vapour is used as an inhalant for upper respiratory tract diseases. *M. piperita* leaf infusion is used to treat cough, inflammation in oral mucosa and throat infections and also useful to relieve headaches, diarrhea, chickenpox, menstrual cramps, liver, gallbladder, and biliary tract disorders (Rita *et al*., 2010). Peppermint is also popular for its fragrance and used in confectioneries, cosmetics and in therapeutics used since a longback as a flavoring agent (Sujana *et al*., 2011). Hence, in the present study, further in-depth workis carried out to evaluate the anticancer efficacy of the differentextracts of *Mentha piperita* and arrive at the probable mechanismof the selected extract's anticancer action various *in vitro* assays.

MATERIALS AND METHODS

*Mentha piperita*leaves were purchased from Kumbakonam, Tamil Nadu. Identification and authentication were confirmed with the help of Flora of the presidency of Madras by Gamble. The vouchers specimen was deposited at the herbarium of the Department of Centre for Advanced Research in Indian System of Medicine, and SASTRA Deemed to be University, Thanjavur, Tamil Nadu.The seeds were shadedried for 10 days and were powdered with an electronic blender. It wasstored in an airtight container at room temperature till use.

Preparation of Plant extracts

100 g of the dried leaf powder was macerated with 300 ml of chloroform (0.8%), ethyl acetate (1.05%), and aqueous (2.8%) successively and kept at room temperature in a shaker for 72 hours. The extract was filtered and dried using a rotary evaporator, and it was stored at 4°C for further studies such as phytochemical screening, antibacterial assay, *in vitro* cytotoxic assays.

Chemicals and other reagents

4, 6-diamidino-2-phenyl indole, Dulbecco's Modified Eagle's Medium (DMEM), Dimethyl Sulphoxide, 2, 2 diphenyl-2-picrylhydrazyl (DPPH), 3-(4, 5-dimethylthazolk-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Muller Hinton Agar Medium, Ascorbic acid, Acridine orange, Ethidium bromide, Phosphate buffered saline (PBS), 4, 6-diamidino-2-phenyl indole (DAPI) were purchased from Sigma Aldrich, USA.

Qualitative analysis of phytochemical constituents

The phytochemicals present in *Mentha piperita* were analyzed by the method of Harbone (1973).

Antibacterial assay

The standard method of antibacterial activity, well diffusion assay, was followed based on the guidelines of CLSI (CLSI, 2006). Muller Hinton agar (MHA) was prepared, sterilized, and plated. The bacterial test strains such as *Bacillus cereus*, *Pseudomonas fluorescens*, *Aeromonas hydrophila*, and *Klebsiella pneumonia* were prepared by inoculating in nutrient broth, and turbidity was adjusted to 0.5 Mc Farland standards with a final inoculum of 1.5×10^8 CFU/ml and swabbed uniformly on the surface of agar individually. Using a sterile well cutter, wells were made, and 100µl aqueous, ethyl acetate, and chloroform extracts of *M. piperita* were added. The antibiotic chloramphenicol served as a positive control.The zone of inhibition (ZOI) was measured in mm (diameter) after the incubation period of 24 hrs at 37 ºC under aerobic conditions. The formation of a clear zone around theindicates antibacterial activity of the tested extract. The experiment was repeated thrice.

Antioxidant assay

1,1 diphenyl 2 picrylhydrazyl (DPPH) assay was performed by astandard method (Joseph *et al.,* 2020). DPPH was used to measure the free radical scavenging activity of the extract. About 0.3Mm solution of DPPH in 95% methanol was prepared. One milliliter of this solution was added to 3 ml of the fraction dissolved in chloroform, ethylacetate, and aqueous at various concentrations and allowed to stand in a dark roomat room temperature for 30 min. The absorbance was recorded at515 nm in a colorimeter, and the experiment was repeated thrice. The decrease in absorbance of the DPPH solution indicated an increase inantioxidant activity. The freeradical scavenging activity was expressed as a percentage of inhibition of the DPPH radical. The antioxidant activity was expressed as:

$$\% \text{ of disappearance} = (\text{control} - \text{sample})/(\text{control}) \times 100$$

Cell line and culture conditions

MCF-7 is a human breast cancer cell line with estrogen, progesterone, and human epidermal growth factor receptor-2. It is considered a suitable model cell line for breast cancer investigations worldwide. MCF-7 is a poorly-aggressive and non-invasive cell line, normally be considered to have low metastatic potential. It is ER-positive and progesterone receptor (PR)-positive and belongs to the luminal molecular subtype. MCF-7 cells are used universally in research for ER-positive breast cancer cell experiments, with the majority of the investigations into acquired anti-estrogen drug resistance. MCF-7 cells are well-suited for anti-hormone therapy resistance studies as it is easily cultured and retain ER expression when treated with such targeted therapy. To investigate the properties of acquired anti-hormone-resistant breast cancer cells, populations of MCF-7 cells adapted to various anti-hormone environments have been created (Serban*et al.,* 2015).

MTT assay

3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide (MTT)is a colorimetric assay used to evaluate cell viability. Cytotoxic assayon the MCF-7 cell line was performed by a standard method (Ramar *et al.,* 2012). The cells were grown (1×10^4 cells/well) in a 96 well plate for 48 h till 85% confluence. The medium was replaced with a fresh medium containing the serially diluted compound, and the cells were further incubated for 48 h. Theculture medium was removed, and 100 µL of the MTT (Hi Media) solution was added to each well and incubated at 37°C for 4 h. After removing the supernatant, 50 µL of dimethyl sulfoxide was added toeach of the wells and incubated for 10 min to solubilize the formazan crystals. The optical density was measured at 620 nm using an ELISA multiwell plate reader (Thermo Multiskan EX, USA). The OD value was used to calculate the percentage of viability using the following formula,

$$\% \text{ of viability} = (\text{OD value of the experimental sample})/(\text{OD value of theexperimental control}) \times 100$$

The effects of extracts were expressed by the 50% inhibitory concentration (IC50) values. The IC50 was defined as the concentrationthat reduced the treated cells' absorbance by 50% concerning untreated cells.

Acridine orange- ethidium bromide assay

Approximately 1ìL of a dye mixture (100 mg/ml acridine orange (AO) and 100 mg/ml ethidium bromide (EtBr) in distilled water) was mixed with 9 ml of cell suspension (1×10^5

cells/ml) on clean microscope coverslips. The selected cancer and normal cells were collected, washed with phosphate buffered saline (PBS) (pH 7.2), and stained with 1 ml of AO/EtBr. After incubation for 2 min, the cells were washed twice with PBS (5 min each) and visualized under a fluorescence microscope (Nikon Eclipse, Inc., Japan) at 400× magnification with an excitation filter at 480 nm. Likewise, the cells were plated on a glass coverslip in a 24-well plate and treated with complex for 24hours. The fixed cells were permeabilized with 0.2% Triton X-100 (50il) for 10min at room temperature and incubated for 3min with 10ìl of DAPI (4, 6-diamidino-2-phenyl indole) by placing a coverslip over the cells to enable uniform spreading of the stain. The cells were observed under (Nikon Eclipse, Inc., Japan) fluorescent microscope (Renuka *et al.,* 2017).

Statistical analysis

All the in vitro experiments were done in triplicate, and the experiments were repeated at least thrice. The statistical software SPSS version 17.0 was used for the analysis. P-value <0.01 was considered significant.

Table 1. Preliminary phytochemical screening of the various extracts of the *Mentha piperita* leaf

S.No	Secondary Metabolites	Ethyl Acetate	Chloroform	Aqueous
1.	Carbohydrates	+	+	+
2.	Steroids	+	+	-
3.	Protein	-	-	+
4.	Phenols	+	-	+
5.	Glycosides	-	-	+
6.	Alkaloids	+	+	+
7.	Flavonoids	+	+	+
8.	Terpenoids	+	+	+
9.	Saponin	-	-	+
10.	Tannin	-	-	+
11.	Anthraquinone	-	-	-

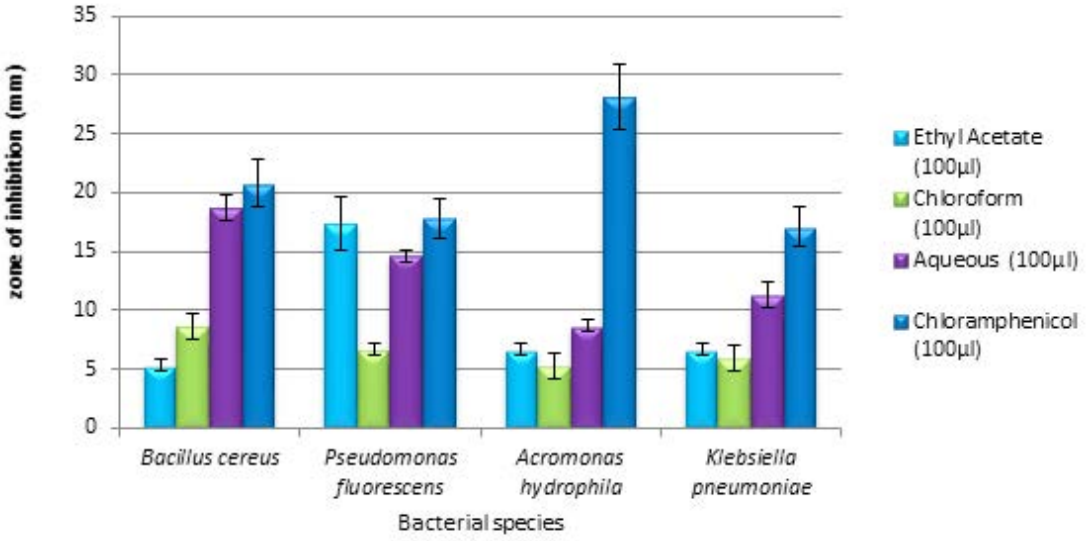

Fig. 1. Antibacterial effect of various extracts of *M.piperita* on *B.cereus*, *P. fluorescens*, *A. hydrophila* and *K. pneumoniae*

RESULTS

In the present work, a traditional anticancer plant source *M. piperita* belonging to the family Lamiaceae, is selected and evaluated for its*in vitro* cytotoxic potential, and anticancer molecules present in the test extract was also identified (Ganesan M and Laiq R 2020). Table-1 represents the preliminary phytochemical screening of various extracts of *Mentha piperita* leaf. Ethyl acetate extract showed carbohydrates, steroids, phenols, alkaloids, flavonoids, and terpenoids. The chloroform extract revealed the presence of carbohydrates, steroids, alkaloids, flavonoids, and terpenoids. The aqueous extract of *M. piperita* contains carbohydrates, steroids, protein, phenols, glycosides, alkaloids, flavonoids, terpenoids, saponin, and tannin. Anthraquinone is absent in all three extracts.

The antibacterial activity of different extracts of *M. piperita* was assessed by measuring the ZOI in the agar well diffusion method. It was observed that among the three extracts tested, aqueous extracts were found active against *B.*

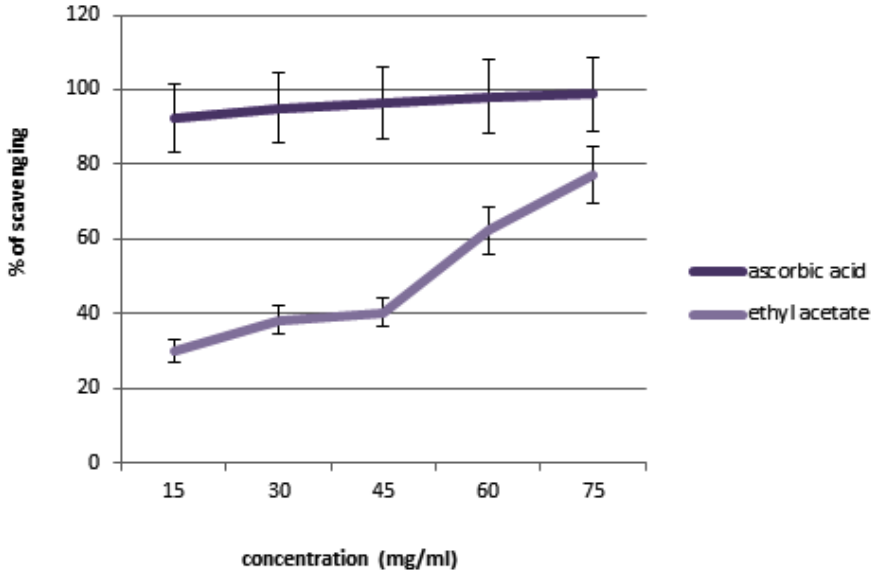

Fig. 2. DPPH radical scavenging activity of ethyl acetate extract of *Menthapiperita*
Values are expressed as mean ± SD

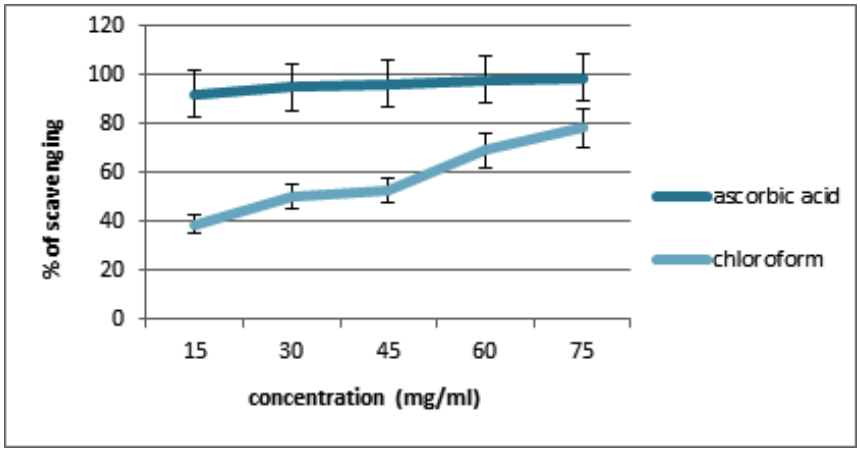

Fig. 3. DPPH radical scavenging activity of chloroform extract of *Mentha piperita*
Values are expressed as mean ± SD

cereus. All three extracts were found ineffective against *A. hydrophila*. Chloroform extracts were effective against all the tested bacterial isolates. Ethyl acetate extract showed the lowest inhibition zone against *B.cereus*(5.32mm) and the highest zone of inhibition against *P. fluorescens*(17.32mm). Chloroform extracts showed the lowest inhibition zone against *A. hydrophila*(5.32mm) and maximum inhibition against *B.cereus*(18.66mm). In the aqueous extract, the lowest inhibition zone was observed against *A. hydrophila* (8.66mm), and the highest activity was found against *Bacillus cereus* (18.66mm). The chloroform and aqueous extract were effective against *B.cereus*. Ethyl extract was effective against *P. fluorescens. A. hydrophila* was resistant against all the extracts. From the above result, it was observed that the aqueous extract exhibited better antibacterial activity compared to other extracts tested (Fig. 1).

Figure-2, 3, and 4 represent the DPPH antioxidant activity of ethyl acetate, chloroform, and aqueous extract of *Mentha piperita,* respectively. The results revealed that the ethyl acetate (77.2±7.7), chloroform (78.2±7.8), and the aqueous (77.9±7.7) extracts showed an increased scavenging activity. The scavenging activity of DPPH radical by these extracts was compared with the standard ascorbic acid. The antioxidant activity of ethyl acetate,chloroform, and methanol extracts of *Mentha piperita* increased with

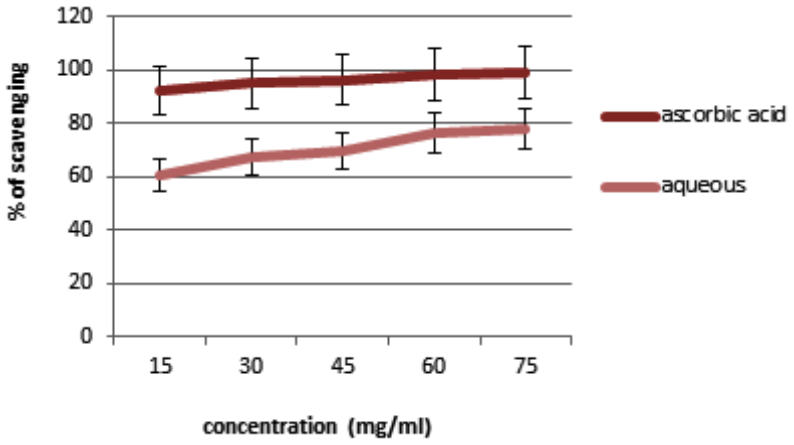

Fig. 4. DPPH radical scavenging activity of aqueous extract of *Mentha piperita*
Values are expressed as mean ± SD

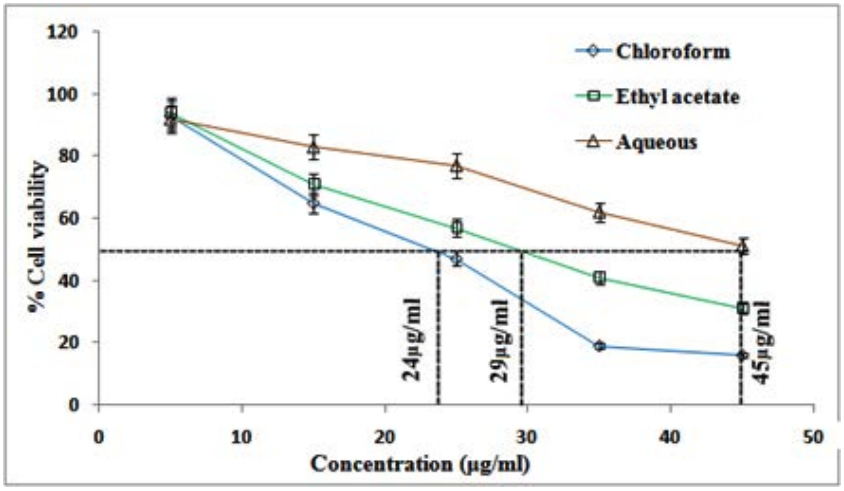

Fig. 5. Cytotoxic effect of ethyl acetate, chloroform and aqueous extract of *Mentha piperita* on MCF-7 cell line.
Values are expressed as mean ± SD

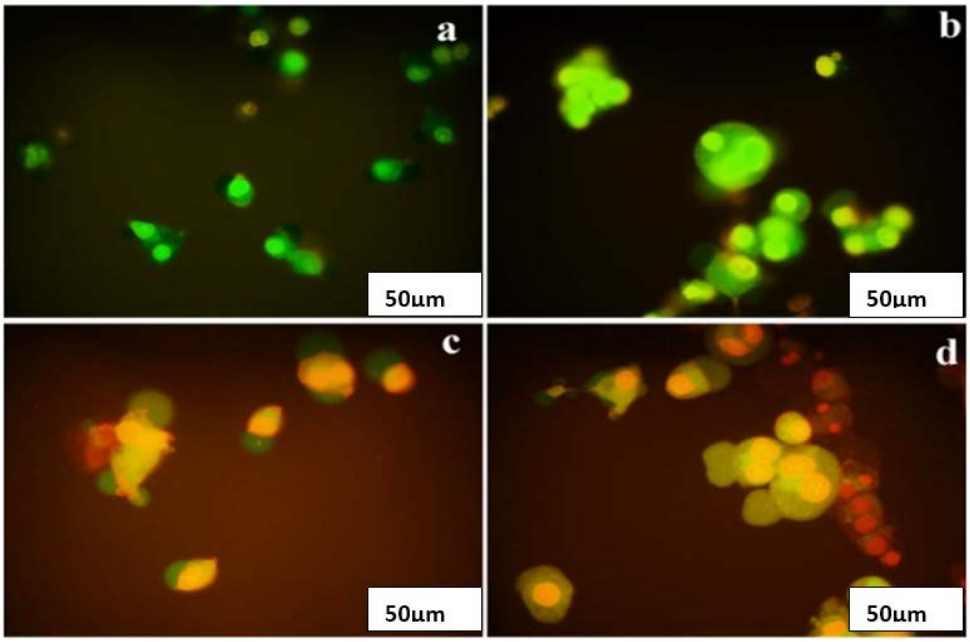

Fig. 6. Apoptotic induction effect of chloroform extract of *Menthapiperita* on MCF-7 (AO/EtBr). a) Control b) 10 µg/mL c) 25 µg/mL and d) 50 µg/mL for 72hrs.

increasing concentration of the extract. Thus, the present results of the DPPH assay suggested that the selected plant *Mentha piperita* have potent antioxidant property.

Figure-5 represents ethyl acetate's cytotoxic effects, chloroform, and aqueous *Mentha piperita* leaf extract against the human breast cancer cell line (MCF-7). From the results, it was observed that the inhibitory activity (IC_{50}) for chloroform, ethyl acetate, and aqueous was found to be 24 ± 1.0, 29 ±1.2, and 45 ±1.5ìg/ml respectively. Of these three extracts, the chloroform extract of the plant showed marked cytotoxic activity against MCF-7 cell line

% of viability = OD value of experimental sample / OD value of experimental control × 100

Figure-6 represents the Acridine orange ethidium bromide assay of chloroform extract of *Mentha piperita*on the MCF-7 cancer cell line. From the images, untreated MCF-7 cancer cells (control) did not show any significant adverse effect than cells treated with chloroform extract of *Mentha piperita*. It has also been observed a concentration-dependent change in the apoptotic effect, which is substantial.

DISCUSSION

Cancer is one of the leading causes of mortality and multistep development, resulting in an uncontrolled and rapid cell division. More than 3000 species of plants have been reported for anticancer treatment in many countries, and a plant comprises a widespread source for cancer treatment (Sumitra C and Krunal N, 2013). In old times, plants have been a rich source of affordable natural compounds in the form of secondary metabolites such as alkaloids, flavonoids, terpenoids, phenols, steroids, and saponins. These metabolites are mostly small organic molecules that often can be excellent leads for the development of a drug. Numerous novel cytotoxic secondary metabolites are secluded every year and constitute a potential source to explore to fight against malignant diseases (Ana and Diana 2018).

The antibacterial assay is carried out to study the effect of plant extracts against various pathogens. The bioactive compounds from plant extracts are characterized and utilized for antibacterial drug designing. The bacterial strains selected for the study include*A. hydrophila, B.*

cereus, K. pneumonia, P. fluorescens, which are associated with various human diseases. The extracts of *M. piperita* showed some level of effectiveness against the test of bacterial strains. The water extracts of *M.piperita* were found effective against *B. cereus. K. pneumonia* was sensitive to ethyl acetate, aqueous, and chloroform extracts. *K. Pneumonia* was highly sensitive towards the ethyl acetate and chloroform leaf extract of *M.piperita* (Sujana*et al*., 2013; Singh *et al*., 2015).

The extracts exhibited antibacterial activity against the bacteria tested may due to the presence of alkaloids, terpenoids, and flavonoids. The supporting results were reported by Mohammed Helmy *et al*., (2017) against various multidrug-resistant bacterial human pathogens by M. piperita. Especially ethyl acetate extract of *M. piperita* was potential and showed activity against *K. pneumonia* isolates.The bioactive compounds present in the extracts may be used as alternative approaches for treating various bacterial ailments.

DPPH radical scavenging activity of ethyl acetate, chloroform, and aqueous extract of *Mentha piperita* leaf extract was found to increase with increasing concentration of all three extracts. This assay was based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants, which denotes the plant has good antioxidant potential.Our results are following the report of Singh *et al*.,(2015). They reported that aqueous extract of *M. piperita* showed the least DPPH scavenging activity (70.3 ±6.1) and chloroform and ethyl acetate extract showed 91.8±5.8 and 84.9±4.2, respectively. Sun *et al*., (2014) reported that the essential oil of *M.piperita* reached 80% at 1000 µg/ml in the DPPH scavenging assay.Thus, the DPPH free radical scavenging assay's present results suggest that the leaf of *Mentha piperita* has a potent antioxidant property.

MTT assay is the most widely used to check the proliferation and effect of any drug can be assessed . The present research data clearly explains that the chloroform extract of the leaf of *Mentha piperita* has potential anticancer activitybased on IC50 value, as mentioned in the result section. Berdowska *et al*., (2013) examined the effect of *Mentha piperita* leaf extracts on the different cancer cell lines, including MCF-7 cells. They reported that aqueous extract showed the least cytotoxicity effect (IC50 value of 752mg/l). Abirami *et al*., (2014) reported that *Mentha piperita* leaf treated Hep-2 Cell lines have a minimum IC50 value of 94. Jain *et al*., (2014) reported that chloroform and ethyl acetate leaf extracts of *M. piperita* treated against MCF-7 and other different cancer cells exhibited good anticancer effects. Based on this report, the chloroform extract of *Mentha piperita* might be due to rich bioactive compounds such as alkaloids, flavonoids, tannins, phenols, and terpenoids.

CONCLUSION

The present study suggests that the chloroform extract of *Mentha piperita* possesses a antiproliferative effect on breast cancer cell line MCF-7. Phytochemical screening revealed the presence of alkaloids, flavonoids, phenols, tannins, and terpenoids. The aqueous extracts of *Mentha piperita* also possess antibacterial activity. Ethyl acetate, chloroform, and aqueous showed good antioxidant properties. Hence, *Mentha piperita* can be subjected to the isolation of novel active compounds to investigate *in vivo* anticancer property.

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Conflict of interest

There is no conflict of interest

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