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BIOACTIVE POTENTIAL OF CULTIVATED *Mentha arvensis* L. FOR PRESERVATION AND PRODUCTION OF HEALTH-ORIENTED FOOD

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ABSTRACT

Mentha arvensis L. is traditionally used in folk medicine, and pharmacological industry due to presence of active chemical substances. It is also valuable for food industry as additives because of the presence of antioxidant, cytotoxic, antidiabetic and antimicrobial constituents. This study is intended to examine reactive oxygen species (Cvetanović *et al.*) generation, lipid oxidation, cytotoxicity and antimicrobial effect of aqueous extract from *M. arvensis* L. prepared in various solvents i.e. fermented methanol extract (FM.E), distilled water extract (DW.E) and methanol extract (M.E). Phytochemical screening of the extract was qualitatively investigated for the isolation of alkaloids, flavonoids, fats and oils, menthol and quinones. To check the potential of extract as preservative, pH, lipid oxidation and Fourier transformed infrared spectroscopy (FT-IR) analysis was performed. Our results showed FM.E induce ROS generation, cytotoxicity and inhibit *Staphylococcus aureus* (4.20±0.90 mm) and *Pseudomonas aeruginosa* (3.23±0.32 mm) growth. In addition, *in vivo* results showed FM. E and M.E efficiently maintained chicken meat pH and reduced lipid oxidation. The presence of essential phytochemicals was responsible for inhibition of biofilm formation. FT-IR analysis revealed the presence of free OH stretching vibrations at 3878.69 cm^{-1}, free NH at 3459.56 cm^{-1} and H-NH bond stretching 3388.02 cm^{-1} groups in chicken meat which belong to *M. arvensis* L. extracts. These results suggest that menthol from *M. arvensis* L. extract is favorable food additive against resistant pathogens.

Keywords: *Mentha arvensis* L; Phytochemicals; ROS generation; Lipid oxidation; Antimicrobial

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INTRODUCTION

Now a days, eating habits of high-fat foods and less exercise are the main causes of obesity, diabetes and other chronic diseases (House *et al.*, 2018). Maximum taking in of triglycerides containing food causes increasing the number of patients globally (Hamid, 2019). World is currently facing health challenges arising from increase in population and diet related diseases (Cainzos-Achirica *et al.*, 2019). Accelerating population growth rates combined with poor nutritional values, intensify use of natural products like phytochemicals to enhance nutritional values of the food (Al-Rowaily *et al.*, 2019; Sunera *et al.,* 2020; Yousaf *et al.*, 2018). Food with therapeutic benefits for human health are believed to reduce the risk of various lethal health problems (Abou-Hany *et al.*, 2018). Recently food development is consumer demand or due to progress in science and innovation (Brownlee, 2005). Previous research focused on identification of physiological active compounds in plants that are found active against various metabolic disorders (Isgut *et al.*, 2018). Epidemiological studies suggest that regular intake of these compounds is necessary to prevent the body from chronic and infectious diseases (Sharman *et al.*, 2019; Suleman *et al.*, 2019). Phytochemicals and essential metabolites obtained from plants contribute to reduce oxidation, cytotoxicity and inhibit microbial growth in food products (Medina *et al.*, 2017; Tai *et al.*, 2000). There are various ways to use these compounds in food such as additives, and preservatives. Due to ethnopharmacological potentials

medicinal plants offer a variety of alternatives to reduce bacterial growth (Ashmawy *et al.*, 2018).

From last few decades, consistent endeavors are in progress to control metabolic issue through ingestion of dietary sources. The chemical drugs are now accessible for antibacterial applications but exhibit serious side effects (Cuadrado *et al.*, 2019), featuring the need to discover effective natural compounds. Health benefits can be result by taking in a hygienic food containing natural ingredients. Natural products, for example, plants extract, either as pure forms or as adulterated form, give boundless chances to new medicate discoveries due to the unmatched chemical diversity and biocompatible nature (Cláudio *et al.*, 2018; Sasidharan *et al.*, 2011). Recently many of these plants have been recommended for their preservative and food additive ability, playing double role i.e. food flavor and bioactive compounds (Salehi *et al.*, 2018). Plants aqueous extracts can be used as basic material, additive or preservative in food industry such as bakery, confectionary products, ice creams, meat products and desserts (Sun-Waterhouse, 2011). Aqueous extracts usually obtained from aqueous phase through physical process that does not influence their composition (Salehi *et al.*, 2018). But prior use at mass scale thorough investigation such as cytotoxicity, antioxidant, antidiabetic activities and lipid oxidation potential are necessary to ensure their efficacy and safety through research proof-of-concept for potential health claims.

Genus *Mentha* belongs to family Lamiaceae (Labiatae) comprising around 30 species distributed in temperate regions of Eastern Asia (EA), South Asia (SA), Australia, North America and South Africa (Anwar *et al.*, 2019; Ayaz *et al.,* 2020a; Ayaz *et al*. 2020b). These are aromatic perennial herbs, cultivated for their essential oils and culinary purposes. Therefore, many hybrids and numerous cultivars are available. Species of the genus *Mentha* such as *M. arvensis* contain various derivatives such as flavonoids, alkaloids, phenol, terpenes and polysaccharides. Terpenes and quinines are used in eatables and medicine, cosmetics and pesticides industries (Chitrakar *et al.*, 2019). Phytochemical screening allows identification and presence of essential phytochemicals that can specify the role of phytochemical in food industry (Salehi *et al.*, 2018). Various medicinal activities of *M. arvensis* are reported such as, anti-inflammatory, antiallergic, antifungal and antibacterial (Malik *et al.*, 2012). Majority of the food and beverages industries are utilizing artificial *M. arvensis* extract or flavors in dietary products (Joseph *et al.*, 2018). Widespread use of synthetic food additives (Carmoisine, Indigocrarmine, Alkaline phosphatase etc.) and preservatives (Benzoic acid, Sodium nitrite, Vitamin E, and Vitamin C etc.) has led to huge health problems. These issues triggered the search for new and biocompatible strategies to inhibit food borne pathogens and maintain food duration. Therefore, in this study we have determined antioxidant, cytotoxic, antidiabetic, antifungal and antibacterial activities of *M. arvensis* different extracts. Additionally, all prepared extracts were applied on chicken meat to assess lipid oxidation and pH to evaluate *M. arvensis* extract role on food borne pathogens and retain food quality.

MATERIALS AND METHODS

Cultivation and preparation of Extracts: Plants were cultivated in home garden at Tahlian (33.62° N and 73.77° E), Azad Kashmir, Pakistan. The leaves of fully mature plant were washed with tap water, subsequently rinsed in distilled water (DW) and then air dried, eventually ground into fine powder. The material was extracted with three different solvents, D.W extract (DW. E), methanol extract (M.E) and fermented methanol extract (FM. E) (Shoba Thomas, 2001). In brief 50 g of the powder was soaked in the above-mentioned solvents, while fermented extract was prepared by inoculating *Lactobacillus plantarum* (*L. plantarum*) in De Man, Rogosa and Sharpe broth (MRS) at 37 °C for 24 h and diluted to get the initial culture. The *M. arvensis* extract of 5 % containing subculture of fresh bacteria (4 % v/v) was incubated at 37 °C for 24 h. Eventually, the extract was sterilized and filter using Whatman's filter paper. The stock solution was prepared at concentration of 0.100, 0.250 and 0.500 mg/mL and stored at 4 ºC for further use.

Phytochemical analysis: The phytochemical analysis of all (DW.E, M.E and FM.E) prepared extract was performed to confirm the presence of alkaloids, fats and oils, menthol, flavonoids and quinones by following the protocol of Suja , and Williams (2016).

Cytotoxic Analysis: To confirm cytotoxic effect of all extracts 3T3 L1 mouse preadipocytes were obtained from Sigma Aldrich, Germany. The cells were sub-cultured in Dulbecco's modified Eagle's medium enrich with fetal bovine serum (10 %) and *Penicillium* (1 %) was deposited in 96 well plate. Cells were treated with 200 µl respective extract and incubated at 37 °C for 4 h in 5 % Co_2. Toxicity was checked by adding 20 µl sterilized 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent in separate wells and incubated at 37º C for 2 h. Eventually, 0.100, 0.250 and 0.500 mg/mL (200 µl) of all extracts (DW. E, M.E and FM.E) was added to respective wells and mixed with the cells for the thorough suspension of formazan crystal (Lee *et al.*, 2015). Distilled water treated cells were considered as a control. The quantity of viable cells was studied at 490 nm in a microplate ELISA spectrophotometer reader. The cell's sustainability was measured as a percentage of the viability of the control.

ROS generation: Antioxidant activity was determined by assessing free radical scavenging activity practicing 2,2-diphenyl-1picrylhydrazyl (DPPH) assay (Kang *et al.*, 2011). Samples were prepared with 0.100, 0.250 and 0.500 mg/mL (200 µl) concentrations. For positive control Vitamin C was used. The samples were kept at 25 ºC for half hour and determined free radical scavenging activity by adding 50 µM DPPH solution (1:1) and incubated at dark for 30 min. Finally, absorbance was measured by spectrophotometer at 517 nm. The IC_{50} value of the extracts was analyzed by following the formula (MubarakAli *et al.*, 2018):

Scavenging effect (%) = [100- (AC-AS/AC)]

In this equation AC stands for absorbance of the control reaction, whereas AS is the absorbance of the tested samples.

Antibacterial activity: Antibacterial activity of all extracts was performed against pure culture of food bacterial strains *Staphylococcus aureus* (*P. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) received from department of Biotechnology Kotli university, Azad Jammu and Kashmir. Well diffusion method was used to check their antibacterial activity with some modifications (Salehi *et al.*, 2015). The inoculum of the selected strains was incubated for 24 h in nutrient broth at 37 ºC. To perform antibacterial activity entire agar surface was inoculated with bacterial inoculum. Cork borer of 6 mm was punched on the solid agar surface to make wells and filled with DW. E, M.E and FM. E (200 µl) of all concentrations (0.100, 0.250 and 0.500 mg/mL). As a control potato dextrose agar (PDA) dishes were supplemented with same amount of DW. The experimental data was obtained from three independent experiments and average was calculated.

***In vivo* effect of menthol:** All extracts (DW. E, FM. E and M.E (250 mg/mL) were applied on chicken meat to determine the potential of extracts in meat preservation. To check the effect of all extracts, chicken meat (300 ± 20 g weight) was purchased and packed in polystyrene boxed with flaked ice. After that visible dark meat, skin and bones were removed and cut into 0.4 x 3-4 cm sized pieces. Subsequently, chicken meat samples were given dip treatment for 2 h separately in DW. E, FM. E and M.E (250 mg/mL) concentration, D.W was used as a control. all treatments were well drained and packed in separate airtight beakers containing polyvinyl dichloride at 4 ºC for 30 d to assess lipid oxidation and pH of the samples.

pH analysis: The pH of the samples was measured on every 5th d post treatment of all extracts (250 mg/mL). Readings were taken by sticking the probe into the sample's beaker.

Lipid oxidation assay: Lipid oxidation of all extracts using 250 mg/mL extracts was analyzed by 2-thiobarbituric acid (TBA) test. TBA reactive substances (TBARS) values were expressed as mg malonaldehyde/Kg sample. The samples were observed randomly after every 5 d.

FT-IR analysis: FT-IR spectroscopy was performed to confirm the presence of organic compounds on post treated chicken meat using FT-IR spectrometer (Shimadzu, Japan) coupled with attenuated total reflectance (Amaral *et al.,* 2018). A small piece of post treated 30 d chicken from all treatments was placed individually on ATR cell and the spectra was measured as % treatment in the range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1} applying 16 scans per sample.

Statistical analysis: All experiments were repeated thrice. Average mean values for all replicates were analyzed by ANOVA whereas, significant difference was separated by applying Bonferroni test set at $P<0.05$.

RESULTS AND DISCUSSION

Taxonomy of *Mentha arvensis* L.: The plant morphology was carefully examined that shows stems erect or ascending, with retrorse hairs on surface. Leavers arranged in opposite pairs and simple, ovate to ovate elliptic having 2.5 x 1.2-2 cm size and serrulate to serrate eglandular hairs on both surfaces (Figure 1A). The root stock spread in creeping manner from which the plant body arise and grow erect. The plant habit is herbaceous with creeping rhizomes (Figure 1B) from which the plant body arise.

Phytochemicals identification: Phytochemicals test showed the presence of alkaloids, flavonoids, fats and oils, quinones and steroids in M.E and FM. E while DW. E lack quinones (Table 1). In FM. E the presence of quinones may be because of microbial metabolites secretion. The phytochemical analysis showed the presence of essential compounds which can help to protect the food material. In addition, use of fermented plant extracts in dietary products will enable pharmaceutical and food industry to produce health-oriented food products (Kieliszek *et al.*, 2018). Previous studies showed that addition of solvents like methanol, chloroform, ethanol, and hexane enhance the antimicrobial potential due to easy release of phytochemicals (Alam *et al.*, 2016; Johnson *et al.*, 2011). The presence of alkaloids, flavonoids, fats and oils, quinones and steroids indicate that *M. arvensis* can be use as significant cytotoxic and antioxidant agent (Bouyahya *et al.*, 2020).

Cells viability: Cytotoxic effect of *M. arvensis* extracts showed significant activity against 3T3 L1 mouse cell lines (Figure 2). FM. E was more active and showed potential inhibition against cell lines. Minimum viability

(IC_{50}) was showed by FM. E 0.21±0.02 followed by M.E 0.19±0.01 Mean±SD and DW. E 0.25±0.01 Mean±SD respectively. We found that FM. E was significantly different from DW. E and comparatively more active against 3T3 L1 mouse cell lines then DW. E and M.E. The basic mechanism against cancerous cells toxicity was generated due to apoptosis by stimulating reactive oxygen species (Cvetanović *et al.,* 2015) resulting in DNA damage and causing cell death (Wang *et al.*, 2019). Our results are consistent with the study of Cvetanović *et al.* (2015). These results indicated that fermentation enhance the cytotoxic activity of phytoextract by releasing active biological compounds in the host cell (Shen *et al.*, 2018). Cell division is a complex mechanism and can be treated by targeting multiple signaling pathways by using beneficial plant products in our daily dietary products (Tamang Kailasapathy, 2010). The use of plant extract in food production and preservation can help to maintain the quality of food. The benefit of beneficial microbes in plant extract neutralize free radicals and prevent cell division (Shen *et al.*, 2018). Thus, fermented extract through beneficial microbes effects the food quality and prolong the food life.

Figure 1. *M. arvensis* L. morphology: A) Leaf's structure B) Root Morphology

Table 1. Preliminary phytochemicals analysis of *M. arvensis* L.

S. No	Constituents	Methanol extract	Fermented extract	Distilled water extract
1.	Fats and Oils	+	+	+
2.	Quinones	+	+	-
3.	Terpenoids	+	+	+
4.	Alkaloids	+	+	+
5.	Steroids	+	+	+

*The positive (+) sign indicates the presence of phytochemicals whereas negative (-) sign indicates absence of phytochemicals

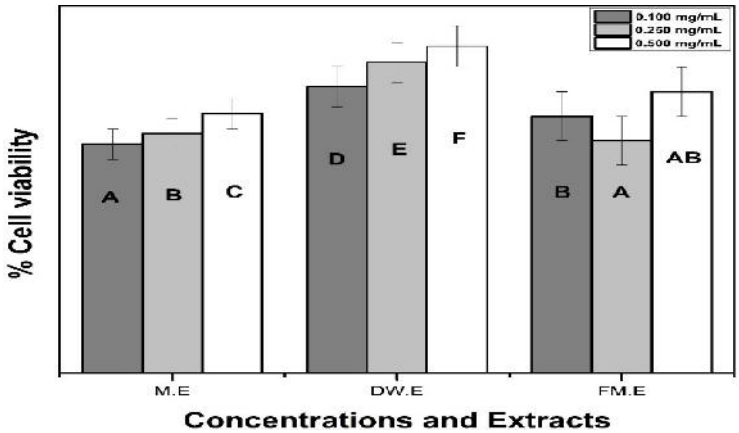

Figure 2. Effect of *M. arvensis* L. extract on 3T3-L Cell's viability. M.E represent menthol extract, DW. E represent distilled water extract and FM.E represent Fermented menthol extract

Antioxidant potential: Antioxidant activity of different concentrations on the reducing power of DW. E, M.E and FM. E showed in (Figure 3). Our results revealed that reducing power of all extracts increased with increase in concentration, but FM. E was found more active (1.47±0.03) than DW. E (1.68±0.06) and M.E (1.64±0.13). Hence it is confirmed that DPPH activity of fermented plant extract increased two times as compared to other solvents. Usually antioxidant activity of different compounds is linked with cytotoxicity because many compounds revealed both antioxidant and cytotoxic activities (Benabdallah *et al.*, 2018). These results suggest that phytochemicals of *M. arvensis* has outstanding potential to donate electron to free radicals resulting in production of stable non-reactive species and restricting the free radical chain reaction (Bardaweel *et al.*, 2018).

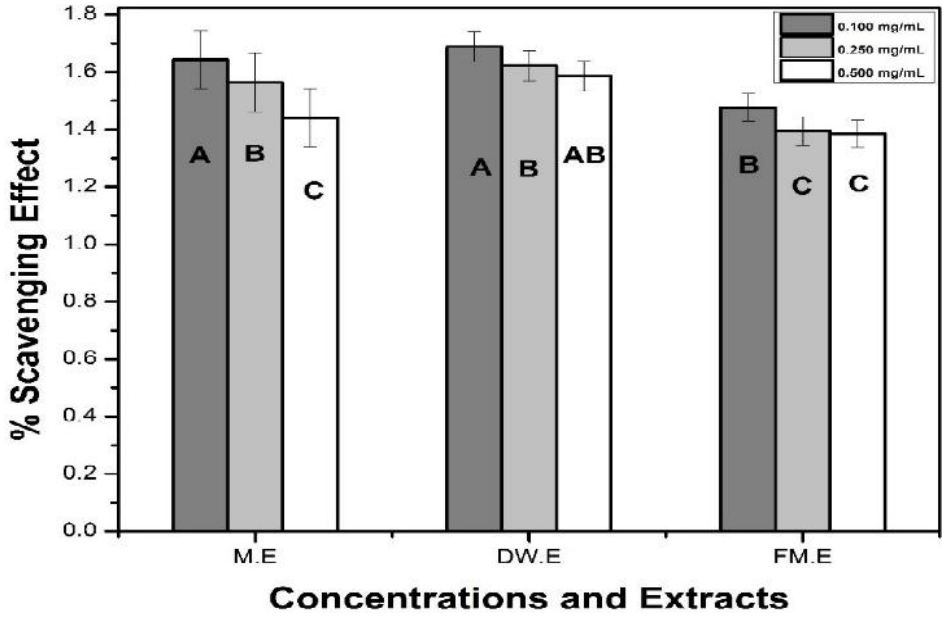

Figure 3. DPPH radical scavenging activity of *M. arvensis* L. extracts. M.E represent menthol extract, DW. E represent distilled water extract and FM.E represent Fermented menthol extract.

Microbial growth inhibition: We found that all *M. arvensis* extracts are active against bacterial strains *S. aureus* and *P. aeruginosa* used in this study (Figure 4A and B). FM. E showed maximum inhibition against both strains *S. aureus* and *P. aeruginosa* and significant difference as compared to control, M.E and DW. E (Table 2). M.E showed significant inhibition at all concentrations (0.1, 0.250 and 0.500 mg/mL) against both strains *S. aureus* and *P. aeruginosa* whereas, DW. E revealed significant difference as compared to control (Table 2). Previous results indicated that bactericidal effects of plant extracts usually because of the presence of active constituents (Murugan *et al.*, 2018). Apart from this modifying plant extracts with fermentation showed synergetic effect in controlling microbial growth. It releases cellulase enzyme which break down the plant cellulose and activate secondary metabolites more efficiently (Kadhim *et al.*, 2016). Therefore, the use of plant extracts specifically fermented plant extracts can help to prolong the preservation period of the food. Fermented plant extracts prevent oxidation in the food material eventually preventing the food material from bacterial attack.

Table 2. Antibacterial activity of *M. arvensis* L. extracts against food pathogens.

	S. aureus				***P. aeruginosa***			
Extracts	**DW. E**	**M.E**	**FM. E**	**Control**	**FM. E**	**M.E**	**DW. E**	**Control**
0.100 mg/mL	2.15±0.47^{a}	3.96±0.45^{b}	4.20±0.90^{c}	0.26±0.15^{d}	3.23±0.32^{b}	3.06±0.31^{ab}	2.77±0.27^{e}	0.12±0.06^{d}
0.250 mg/mL	1.36±0.16^{b}	3.47±0.49^{a}	4.17±0.24^{ab}	0.19±0.17^{c}	2.86±0.39^{d}	2.73±0.24^{d}	2.72±0.24^{d}	0.19±0.17^{c}
0.500 mg/mL	1.47±0.23^{b}	3.34±0.52^{a}	3.26±0.66^{a}	0.3±0.1^{c}	3.08±0.29^{a}	3.11±0.27^{a}	2.85±0.07^{d}	0.19±0.11^{c}

Antibacterial activity was assessed by measuring the zone of inhibition (mm). Different letters in the same row indicate significant ($P<0.05$) differences

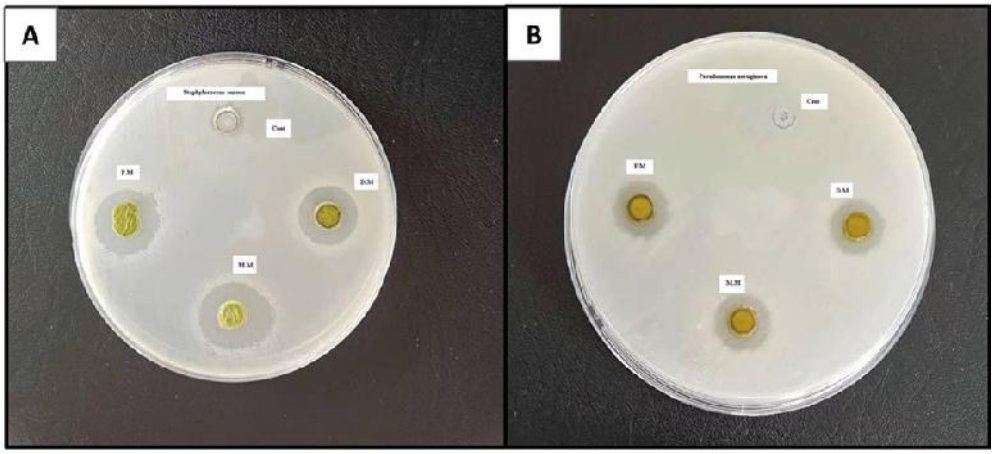

Figure 4. Antibacterial activity of *M. arvensis* L. extracts against bacterial strains. (A) *Staphylococcus aureus* activity (B) *Pseudomonas aeruginosa* activity

Effect on chicken meat lipid oxidation and pH: Application of *M. arvensis* showed significant potential to maintain chicken meat lipid oxidation and pH.

pH control: Initial pH of all treatments was observed from 5.8-6.1 (Table 3), whereas all samples showed increase in pH values from day first of storage to the last day. Our results showed that control showed increase in pH after day 10 to end of the storage significantly higher than *M. arvensis* all extracts. It means that treatment of food with phytogenic extracts can prevent pH increase in food and prolong storage time. In addition, FM. E showed decrease in pH as compared to DW. E and M.E which proved that presence of microbial enzymes in extracts result in pH decrease. It is understood that pH and antioxidant concentration strongly influence the storage ability of the food. The decrease in the pH might be due to the release of antioxidant compounds present in the extract or transformation into new compounds due to fermentation process (Arabshahi-D *et al.*, 2007). Our results indicated that FM.E can be use as potential pH control agent in the food industry.

Table 3. Effect of menthol from *M. arvensis* L. extracts on the pH.

Extracts	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
M.E	5.96+0.02^{a}	5.93+0.01^{a}	5.91+0.06^{a}	6.08+0.02^{a}	6.18+0.04^{a}	6.18+0.01^{a}	6.2+0.04^{a}
DW.E	5.87+0.05^{a}	5.87+0.01^{b}	5.86+0.03^{a}	5.27+0.05^{c}	6.38+0.01^{a}	6.38+0.01^{a}	6.44+0.01^{a}
FM.E	6.18+0.01^{a}	6.18+0.01^{a}	6.18+0.04^{a}	6.21+0.01^{a}	6.24+0.01^{a}	6.25+0.01^{ab}	6.26+0.02^{d}
Control	5.93+0.05^{b}	5.93+0.04^{b}	5.88+0.02^{b}	6.24+0.04^{bc}	7.13+0.03^{b}	7.24+0.03^{b}	7.44+0.03^{b}

*Different letters across the same column show indicate significant difference (P<0.05)

Lipid metabolism: All tested extracts screened were effective as antioxidants yield lower percentage of TBARS values over the 30 d period as compared to control (Figure 5). The TBARS values of F.ME and M.E were significantly lower than DW. E and control. The phenomena involve plant extract slow down oxidation and lipid free radicals are more stable due to the presence of organic substances, which prolong the reaction time (Amaral *et al.,* 2018). Lipid oxidation is the main process responsible for declining nutritional values and taste and aroma of food (Font-i-Furnols Guerrero, 2014). In food industry, it is mainly controlled by synthetic antioxidants due to low price but cause serious health problems (Abootalebian *et al.*, 2016). Therefore, *M. arvensis* extract used in this study provide can be used as a natural antioxidant in food industry. Application of plant extracts and fermentation played synergistic role in controlling chicken meat pH and lipid oxidation which provide an avenue for the use of fermented plant extracts in food industry to produce health-oriented food.

FT-IR spectroscopy: To verify the effect of all plant extracts on chicken meat and variations within plant extracts FT-IR spectra was obtained. The FT-IR spectra measured from all treatments are shown in Figure 6. The major and common spectra observed in all samples including control associated with the presence of free OH stretching vibrations at 3878.69 cm^{-1}, free NH at 3459.56 cm^{-1} and H-NH bond stretching 3388.02 cm^{-1} and these peaks are attributed to water and principal organic compounds of these samples (Rodiles-López *et al.*, 2019). Another common band (2892.69-2699.06 cm^{-1}) in all samples associated with O-CH_3 asymmetrical stretching vibration (Figure 6 A-C) (Prakash *et al.*, 2018).

A strong band observed in all treatments except control at 1992.22-1892.69 cm^{-1} revealed the presence of C═O stretching vibrations which is attributed to organic compounds of plant extracts because this band is not visible in control (Figure 6 D). Whereas a short band at 2374.80 cm^{-1} associated with C═N stretching vibrations show the presence of microbial compounds of FM.E (Figure 6 C) (Babu *et al.*, 2010). The spectra result clearly indicate that presence of C═O and C═N stretching that effect of plant extracts is viable for long duration and can prolong the food storage. The FT-IR analysis proved that *M. arvensis* contain many functional groups and have the capability to retain the food products like chicken meat for long time. Therefore, the use of *M. arvensis* extracts in food industry is cheap and sustainable for production of health-oriented food.

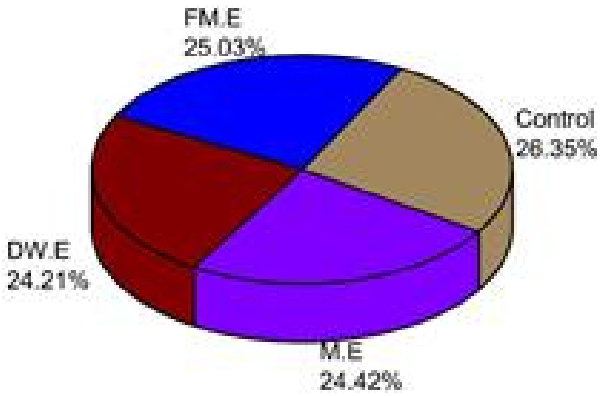

Figure 5. Lipid oxidation percentage of different extracts TBARS values during storage. FM.E represent fermented menthol extract, M.E represent menthol extract, DW.E represent distilled water extract

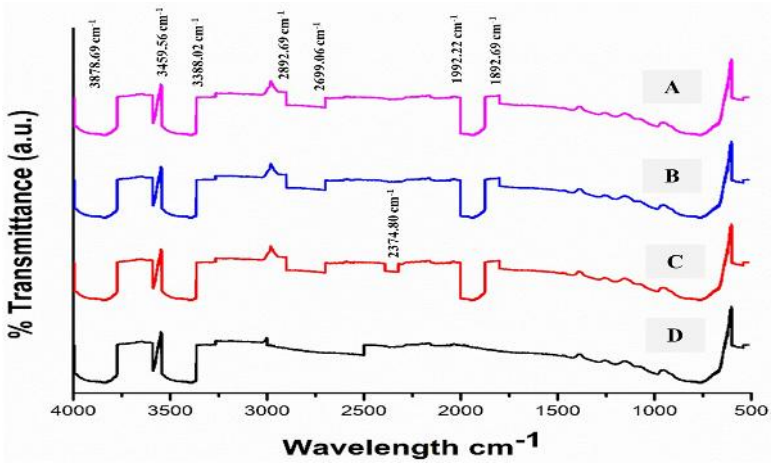

Figure 6. Comparative FT-IR spectra of various plant extracts (A) M.E treated chicken meat spectra (B) DW. E treated chicken meat spectra (C) FM. E treated chicken meat spectra (D) Control Chicken meat treated spectra

Conclusion: Genus *Mentha* offers extensive opportunity to food and nutraceutical industries because of well-developed cultivation and potential medicinal values. Traditionally *Mentha* extract used in food and contain active chemical compounds. Presence of essential oils and aromatic compounds enhance flavor and become a great alternative to artificial preservatives. Our results showed fermented *M. arvensis* L. extract as an ecofriendly and cost-effective alternative to artificial preservatives and applicable in a wide range of industrial processes to inhibit food borne pathogens. It is necessary to evaluate and differentiate among different extracts of plants. Extracts can be obtained in various ways and treated with different processes to ensure their safety. The ROS generation, cytotoxic, antioxidant, antidiabetic, antibacterial and antifungal activities of fermented *M. arvensis* extracts support its utility as potential element for the development of pharmaceuticals and health-oriented food without side effects. Furthermore, the compounds present in *M. arvensis* could be isolate, and characterize in future and used as food additives to control the oxidative deterioration of food products.

Conflict of Interest: There is no conflict of interest.

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