Research article
J. Adv. Nutri. Sci. Technol. 1(2): 60-70, 2021 **DOI: 10.15228/ANST.2021.v01.i02.p02**

Phytochemical characterization and evaluation of the antimicrobial and antioxidant activity of various fractions of *Malva sylvestris (Pick-cheese)* leaf extracts

[1*]Saima Naz, [2]Muhammad Naveed Rasheed, [3]Muhammad Idrees Jillani
[1*]Division of Science and Technology, University of Education Lahore, Multan Campus 60700 Pakistan
[2]Division of Science and Technology, University of Education Lahore, Multan Campus 60700 Pakistan
[3]Department of Chemistry, University of Sahiwal, Pakistan
***Corresponding Author Email:** saima.naz@ue.edu.pk

Abstract

The methanolic extract fractions of *Malva sylvestris*, collected from Bahawalpur district, were subjected to study the total phenolic contents, total flavonoid contents, antioxidant and antimicrobial activity. The antioxidant activity was measured using the linoleic acid system, DPPH scavenging assay, and determination of reducing power. The antimicrobial activity was evaluated through the Disc diffusion method. The partition fractionation of the methanolic extract of *M. sylvestris* was performed using ethanol, acetic acid, acetone, ethyl acetate, and n-hexane. Yields of polar solvent fractions were higher than other fractions in these plants. HPLC analysis presented three major phenolics, gallic acid (141.51-192.03 µg/g), coumaric acid (112.21-172.11 µg/g), and Ellagic acid (110.31-159.18 µg/g) in *M. sylvestris* with maximum yield in methanol fractions. The total phenolic contents analyzed using Folin- Ciocalteu reagent of the samples varied from 5.58-17.1g/100g, 2.66-7.41g/100g dry weight expressed as gallic acid equivalents (GAE). The determined total flavonoid contents in the fractions ranged from 11.50-32.4g/100g and 24.91-36.36g/100g dry weight expressed as catechin equivalents. The percentage of inhibition of linoleic acid peroxidation and reducing potential was more significant in *M. sylvestris methanol* fractions, i.e., 47.6% and 3.3 mg/100mL, respectively. *M. sylvestris* plants also demonstrated remarkable antimicrobial activity against various pathogens (*E.coli, P.multocida, S.aureus, B. subtillus, A. niger, A. flavus, A, fusarium, F. Solani*). The chemical screening of crude extracts constitutes an efficient complementary approach allowing localization and targeted isolation of new types of constituents with potential activities.

Keywords*: Malva sylvestris* extract; partition fractionation; antioxidant activity; antimicrobial activity; HPLC

Highlights:

- *Malva sylvestris* plants extract selected for total phenolic, total flavonoid, antioxidant and antimicrobial activity
- HPLC analysis validated three major phenolics, gallic acid, coumaric acid, and Ellagic acid
- verified remarkable antimicrobial activity against several pathogens

1. Introduction

Activated oxygen is present in the form of reactive oxygen species or free radicals. Reactive oxygen species (ROS) and free radicals have emerged as challenging species in the food industry (Gülçın et al., 2003). These have a worse influence on aging and injury at the cellular level and medicinal and biological systems (Lai et al., 2001; Büyükokuroğlu et al., 2001). Different enzymatic and non-enzymatic reactions produce oxygen free radicals during typical physiological phenomena (Aruoma, 1998). But as a result of pathological condition, overproduction of species occurs resulting in damage (El-Habit et al., 2000). Due to the presence of antioxidants in nature, scientists are striving to replace synthetic antioxidants to avoid their adverse effects (Kinsella et al., 1993).

Native to Europe, Asia, and North Africa, *M. sylvestris L*. is a class Equisetopsida, subclass Magnoliidae, superorder Rosanae, order Malvales, family *Malvaceae*, and genus Malva. The flowers of *M.sylvestris* are almost odorless and have a mucilaginous taste when chewed. They are 3–5cm wide and have an epicalyx; the rest of the stalk does not exceed 20 mm long (Pljevljakušić et al., 2018; Block et al., 2008; Baser, 2005; Kaileh et al., 2007).

Numerous studies involving medicinal plants have demonstrated the worldwide importance of *M. sylvestris* in traditional medicine. As a medicinal food, *M. sylvestris* has been consumed as a mild laxative, a liver cleansing tonic, and against heartburn. It can be prepared as soup but is most commonly prepared in salads. In pharmaceutical preparations, it can be used to treat conditions such as gastrointestinal disorders, abdominal pain, diarrhea, and respiratory diseases (Idolo

et al., 2010; Leporatti & Corradi 2001; Cornara et al., 2009). The leaves, flowers, and aerial parts of *M. sylvestris* are known worldwide due to their anti-inflammatory properties, mainly against gingivitis, abscesses, and tooth pain (Conforti et al., 2008; Scherrer et al., 2005; Nelly et al., 2008; Pollio et al., 2008).

Additionally, the leaves and flowers have sufficient potential for use in treating urological problems, insect bites, burns, furuncles, and ulcerous wounds (Lardos, 2006; Leonti et al., 2009). It is essential to mention that the use of *M. sylvestris* in association with other medicinal species is a common practice that enhances their expected effects. Due to the significance of the plant *M. sylvestris*, the phytochemical screening of the extract of the plant in the different solvent has been conducted using advanced technology. Furthermore, antioxidant and antimicrobial activities of the plants are also studied and discussed in the relevant section of the paper.

Material and Methods

2.1 Plant material

Plant materials were selected based on their medicinal use. Fresh aerial parts of *M. sylvestris* were collected from Bahawalpur district, Punjab, Pakistan, in September-October 2019. The plant materials were further authenticated and identified by a taxonomist in the Department of Botany, Islamia University Bahawalpur Pakistan.

2.2 Extraction and fractionation

Aerial parts of the plants were rinsed with distilled water, cut into small pieces, and shade dried at room temperature. Extraction was carried out through the Soxhlet extraction method. A total of 70 g of plant material was extracted with solvent (methanol) 500 mL for three hours in multiple experiments. The extracts were separated from the residues by filtering through Whatman No.1 filter paper. The residues were extracted twice with the same fresh solvent and extracts combined. The solvent was evaporated at 45°C under reduced pressure in a Rotary evaporator (EYELIA, SB-651, and Rikakikai Co. Ltd. Tokyo, Japan). The dried crude extracts were weighed to calculate the percentage yield and stored at -4 ^{0}C in a refrigerator and used for analyses.

This crude methanolic extract (CME) of *M. sylvestris* were subjected to solvent fractionation by dissolution in water and sequential partition with ethanol (4×300 mL), acetic acid (4×300 mL), acetone (4×300 mL), ethyl acetate (4×300 mL), *n*-hexane (4×300 mL) and 50% methanol (2×150 mL) as indicated in Fig. 1 (Cho et al., 2010). Each fraction thus obtained, including the final hydro methanol fraction, was evaporated to dryness and subjected to antioxidant bioassays.

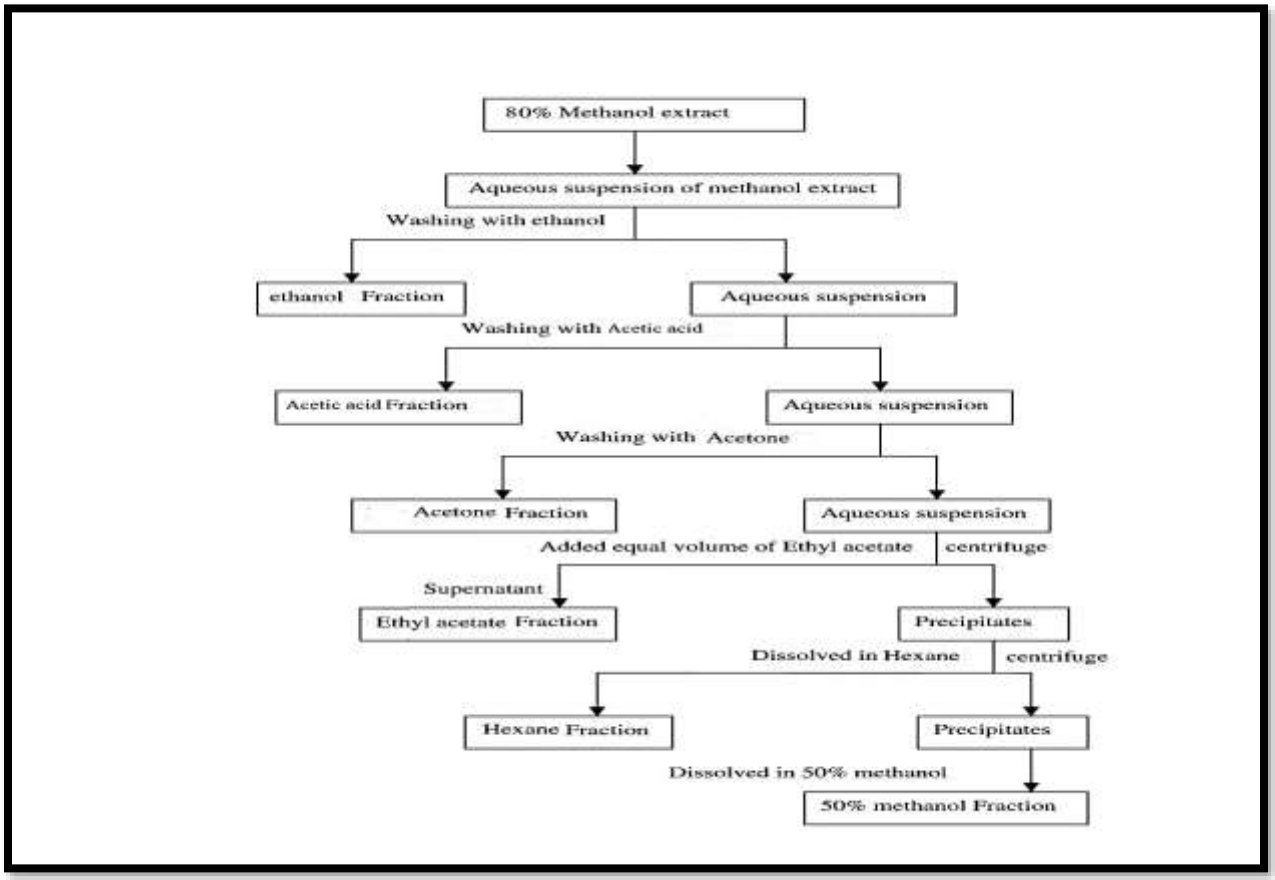

Figure:1. Schematic diagram of Partition fractionation

2.3 Evaluation of the antioxidant activity of plant extracts

2.3.1 Determination of total phenolic content (TPC)

Folin-Ciocalteu reagent was utilized to assess the amount of total phenols (Chaovanalikit & Wrolstad, 2004). Briefly, 50 mg of each crude extract was mixed with 7.5 mL deionized water and 0.5 mL of Folin-Ciocalteu reagent for 10 min, and the mixture was kept at room temperature, then 20 % sodium carbonate (w/v, 1.5 mL) was added. At 40°C for 20 min, the mixture was heated then cooled in an ice bath. Using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan), absorbance was read at 755 nm. The calibration curve was prepared using the Gallic acid, the concentration of total phenols was calculated within the range of 10-100 mg/L (R^2=0.9986). The results expressed as Gallic acid equivalents (GAE) g/100g of dry plant matter. Three readings were taken for each sample, and the results were averaged. Based on dry weight (DW), the results were expressed.

2.3.2 Determination of total flavonoid contents (TFC)

Total flavonoid contents were determined by following the standard method (Dewanto et al., 2002). Each plant extract (1mL)(0.1 mg/mL) was diluted with 4mL water in a 10 mL volumetric flask. Firstly, to each volumetric flask, 0.3 mL of 5% $NaNO_2$ solution was added; followed by 0.3 mL of 10% $AlCl_3$ and 2mL of NaOH (1.0 M). Then 2.4 mL distilled water was added to the reaction flask and mixed well. Absorbance was read at 510 nm. Total flavonoid contents were determined as catechin equivalents g/100g of dry weight. The samples were analyzed thrice, and results were averaged.

2.3.3 Determination of reducing power

According to the earlier method (Yen et al., 2000), the reducing power of the extracts was measured. Each concentrated extract (2.5-10 mg) was mixed with 5.0 ml sodium phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0%); the mixture was incubated at 50°C for 20 min. Then 10% trichloroacetic acid (5ml) was added, and the mixture was centrifuged at 980g for 10 min at 5°C in a refrigerated centrifuge (CHM-17; Kohusan Denki; Tokyo, Japan). The upper layer of the solution, 5.0 mL, was diluted with 5.0 mL distilled water and 1.0 mL of ferric chloride 0.1%. Using a spectrophotometer (U-2001, Hitachi Instruments Inc, Tokyo, Japan), the absorbance was read at 700 nm. Three readings were taken for each sample, and the results were averaged.

2.3.4 DPPH scavenging assay

The antioxidant activity of extracts was also evaluated by measuring their radical scavenging ability using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as a reducing agent according to the reported procedure (Iqbal et al., 2005). To each extract (1.0 mL) containing 25 microgram /mL of dry matter in methanol, a freshly prepared solution of DPPH (0.025 g/L, 5.0 mL) was added. Then absorbance at 515 nm was measured at different time intervals 0, 0.5,1,2,5, and 10 min. The following equation applies to calculate free radical inhibition by DPPH:

I%= 100[(Ac - As)/Ac]

Ac= absorbance of the control reaction (containing all reagents except the test extract)

As= absorbance of the sample

2.3.5 Determination of antioxidant activity in a linoleic acid system

According to the procedure, by measuring the inhibition of linoleic acid peroxidation, the antioxidant activity of the extracts was determined. 5 mg of each plant extract was mixed with 0.13 mL of linoleic acid, 10mL of 99.8% ethanol, and sodium phosphate buffer (10 mL, 0.2 M, pH 7), with distilled water, made the volume up to 25 mL and incubated for 360h at 40 °C. By applying the thiocyanate method, the extent of oxidation was determined. In short, ammonium thiocyanate 0.2 mL (30% w/v), 10 mL ethanol (75% v/v), 0.2 mL sample solution and 0.2 mL ferrous chloride ($FeCl_2$) (20 mM in 3.5 % HCl; v/v) were added in a consecutive manner. Absorbance was measured at 500 nm using a spectrophotometer after 3 min of stirring compared to control where the extract was not added. As a positive control, butylated hydroxytoluene was used. By using the following formula, the percent inhibition of linoleic acid was determined.

100- [(sample absorbance increased at 360h/control absorbance increased at 360h) 100]

2.4 Evaluation of antimicrobial activity

Disc diffusion method was used to determine the antimicrobial properties of isolated compounds.

2.4.1 Disc diffusion method

The disk diffusion method (d'Azevedo et al., 2004) is used to determine the antimicrobial activity of plant extracts. The discs (6mm in diameter) were soaked with 50 µL extract and placed on the inoculated agar. Standard antibiotics like *Rifampicin* (30 µg/disc) and *Fluconazole* (30 µg/disc)were used to compare the activity as positive references for bacteria and fungi, respectively. Test discs and standard discs were placed in separate Petri dishes. Petri dishes were then incubated at 37°C for 24h for bacterial and 25°C for 3 days for fungal growth. Antimicrobial activity was evaluated by measuring the inhibition zone (mm).

2.5 Determination of phenolic compounds by HPLC

Phenolic analysis was performed using high-performance liquid chromatography. HPLC (model LC-10A, Shimadzu, Kyoto, Japan), equipped with two LC-10 AS pumps, Rheodyne injector, SCL-10A system control unit, SPD-10A UV–vis detector, CTO-10A column oven, and data acquisition class LC-10 software was used. 20µL of the filtered sample was injected into an analytical Supelco (Supelco Inc., Supelco Park, Bellefonte, PA, USA) ODS reverse phase (C18) column (250×4.6 mm; 5µm particle size). Two solvent systems, A: containing water and Acetic acid (94:6 v/v) and B: containing 100% acetonitrile, were used to separate polyphenolic components. The separation was achieved by isocratic elution of the mobile phase (0-15min= 15%B, 15-30=45%B, 30-45min=100%B) at a flow rate of 1.0 mL/min at room temperature. Detection was measured at a wavelength of 280nm. Identification of phenolics was carried out by comparing their retention times with those of authentic standards (Sigma Chemicals Co., St Louis, MO, USA). Quantitative determination was carried out by using calibration curves of the standards.

2.6 Statistical analysis

All the experiments were performed thrice, and the investigated data were reported as mean ($n = 3 \times 3$) ± SD ($n = 3 \times 3$). Analysis of variance (ANOVA) was performed using Minitab 2000 Version 13.2 statistical software (Minitab Inc., PA. USA).

3. Results and Discussion

3.1 Estimation of Percentage yield

The extractive components in plants vary from season to season or depend on the nature of solvents for the same plant. Plant bioactive components are extracted mainly by polar solvents, and methanol is considered best in this regard (Flórez et al., 2015). Fractionation yields of *M. sylvestris* depict that the polar solvents like methanol, ethanol, and acetic acid showed high extract yield (14%, 11%,10%), respectively, followed by less polar solvent fractions, i.e., acetone (5%) and ethyl acetate (3%) respectively as shown in Fig.1.The non-polar solvent n-hexane (4%) resulted in a lower yield. It showed that the sugar-related compounds (glycosides, carbohydrates), polar alkaloids, and phenolic compounds are better extracted with polar solvents, as reported by Menichini et al. (2011).

3.2 High-Performance liquid chromatography

The HPLC fingerprints of *M. sylvestris* partitioned fractions show various flavonoids and phenolic acid compounds, including gallic acid, coumaric acid, ellagic acid, vanillic acid, 4-hydroxy-3-methoxy benzoic acid, cinnamic acid, caffeic acid, syringic acid, quercetin, rutin, kaempferol, and myricetin. These were present in varying amounts. Figure 1 showed a schematic diagram of Partition fractionation. The major phenolics i.e. gallic acid (192.03±0.02), (175.15±0.02), (141.51±0.03), coumaric acid (172.11±0.04), (143.49±0.03), (112.21±0.01), ellagic acid (159.18±0.01), (135.44±0.03), (110.31±0.04), vanillic acid (133.17±0.02), (111.96±0.01) (101.24±0.03) and cinnamic acid (108.27±0.02), (101.23±0.01), (97.24±0.03) µg/g quantity in methanol fraction. The minor phenolic acid components (<100 µg/g) in the fractions were 4-hydroxy-3-methoxy benzoic acid (54.97±0.02), (36.26±0.03), (12.18±0.05), caffeic acid (95.23±0.02), (75.16±0.01), (83.54±0.03), and Syringic acid (76.35±0.02), (34.34±0.01), (49.22±0.03) µg/g quantity in methanol fraction respectively. Three major phenolics were gallic acid (175.03±0.02), (161.15±0.02), (145.51±0.03), Chlorogenic acid (121.11±0.04), (102.49±0.03), (81.21±0.01and Ferulic acid (140.33±0.03), (127.83±0.01), (107.30±0.03) µg/g quantity in methanol fraction respectively. The minor phenolic acid components (<100 µg/g) were coumaric acid (71.18±0.01), (31.44±0.03), (57.31±0.04), syringic acid (34.03±0.03), (22.12±0.04), (41.25±0.02), and caffeic acid (11.01±0.04), (25.10±0.02) (10.63±0.02) µg/g quantity in methanol fraction respectively. The maximum concentration of component was gallic acid followed by ferulic acid>chlorogenic acid >coumaric acid >syringic acid>caffeic acid in all solvent fractions of *A.arvensis.* Similarly, the major flavonoids detected in *M. sylvestris* were naringenin (111.97±0.02), (102.26±0.03), (106.18±0.05), quercetin (197.07±0.03), (183.23±0.01), (165.33±0.02), catechin (232.97±0.02), (204.18±0.05), (212.26±0.03), and kaempferol (155.17±0.02), (132.96±0.01), (115.24±0.03) µg/g quantity in methanol fraction respectively. The minor flavonoid components (<100 µg/g) in the fractions were apigenin (90.95±0.03), (71.17±0.05), (80.87±0.01) and rutin (73.05±0.01), (54.44±0.06), (32.87±0.02) µg/g quantity in methanol fraction respectively. A similar trend in antioxidant activities showed the direct relationship of phenolic contents and antioxidative potential of various *M. sylvestris* fractions.

3.3. Determination of total phenolic contents

The determined phenolic acid contents are represented as (GAE) Gallic acid (mg/g) equivalent. The amount of total phenolic contents ranged from 5.58-17.1/100g in *M. sylvestris* leaves, respectively. Due to the quickness and lower interference, the Folin-Ciocalteau method was selected to quantify phenolics compounds (Sultana et al., 2007). The amount of total phenolic contents in *M. sylvestris* leaves are higher (17.1/100g). Fig. 2 represents that in the leaves of *M. sylvestris*, the maximum

TPC quantity obtained by methanol fraction followed by ethanol>Acetic acid>Acetone>*n*-Hexane>Ethyl acetate. Generally, it speculated that there lies the relationship between antioxidant activity and total phenolic contents of plant extracts because the antioxidant activity depends on phenolic or polar components (Terpinc et al., 2012; Roidaki et al., 2015; Kiprovski et al., 2015).

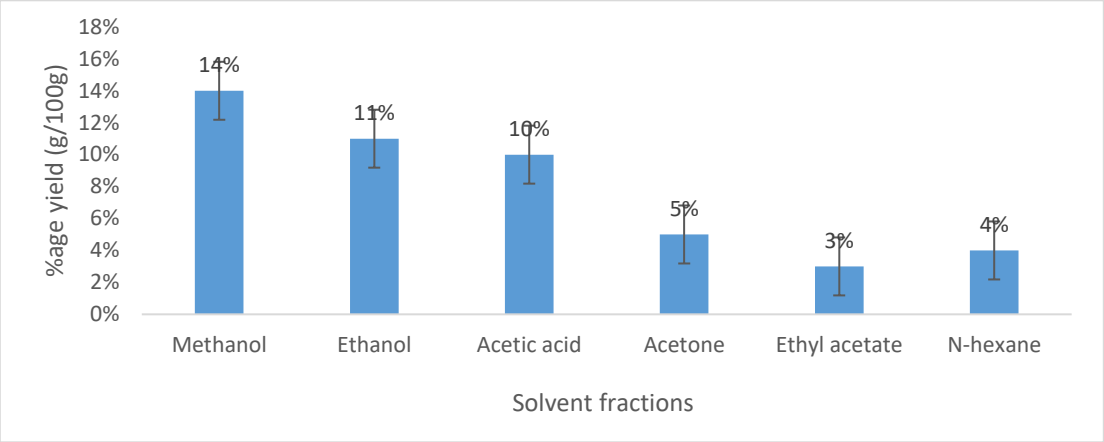

Figure 2. The percentage yield of *M. sylvestris* solvent fractions

3.3. Estimation of Total Flavonoid Contents

The total flavonoid contents of *M. sylvestris* solvent fractions ranged from 11.5-32.4g/100g, respectively. Fig. 3 depicts that in *M. sylvestris* leaves, maximum TFC was achieved from ethanol fraction (32.4/100g), which was found to be higher than that of other fractions followed by methanol>*n*-Hexane>Acetic acid>Acetone >Ethyl acetate. Considerable variation ($p<0.05$) was perceived in total flavonoid contents of various solvent fractions of leaves.

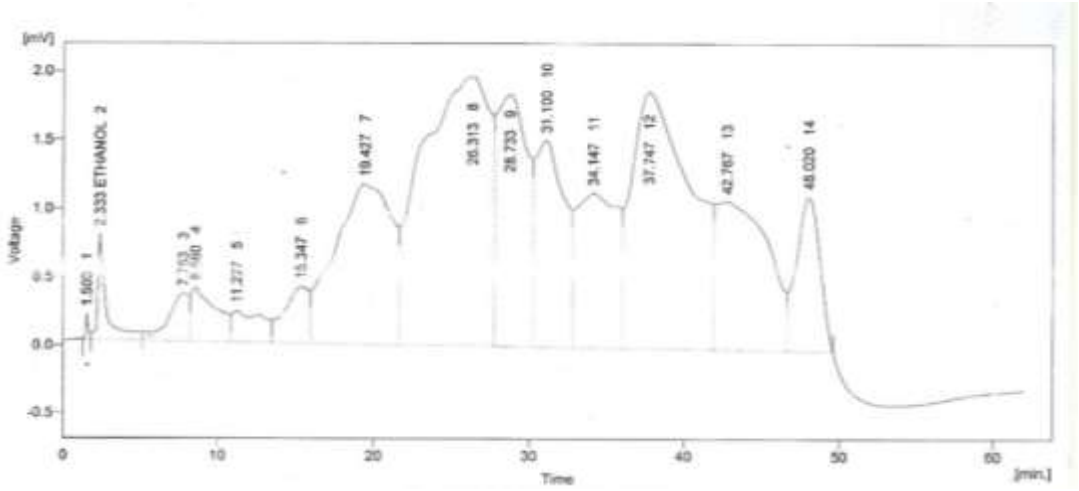

Figure 3. Methanol extract chromatogram by HPLC of *M. sylvestris* leaves

3.5 Estimation of reducing the power of *M. sylvestris*

Reducing power was measured over a range of concentrations 2-8mg/100mL. Different solvent fractions show higher reducing potential at 8mg/100mL concentration ranged from 0.53-0.97 mg/100mL 0.56-1.14 mg/100mL shown in Fig.4-6 respectively. The results show that as extract concentration increased, the reducing potential also increased (Kim et al., 2015). More polar solvent fractions ethanol, methanol, acetic acid presented higher reducing potential than other fractions. In *M. sylvestris*, ethanol fraction showed higher reducing capacity followed by methanol>Acetic acid>Acetone>n-Hexane>Ethyl acetate.

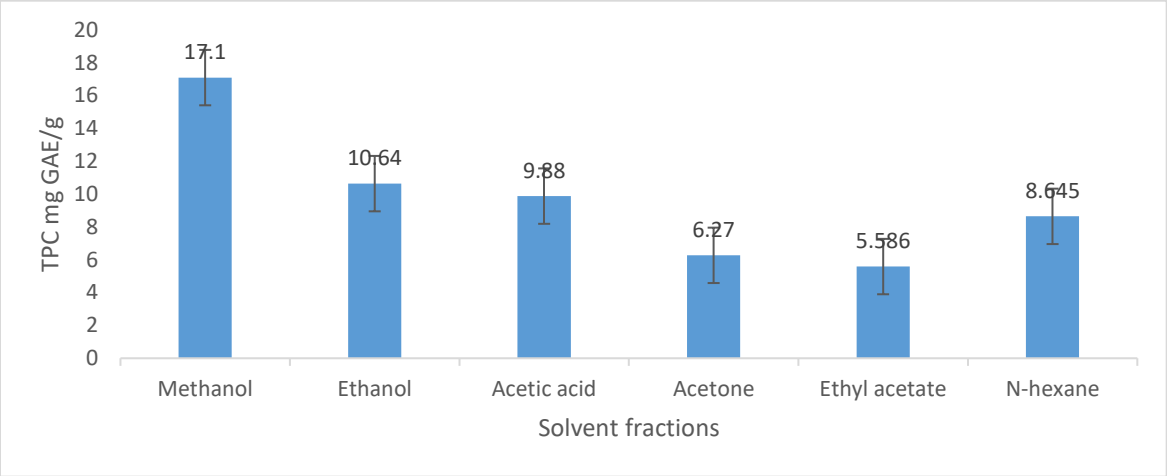

Figure 4. TPC values in different solvent fractions of *M. sylvestris*

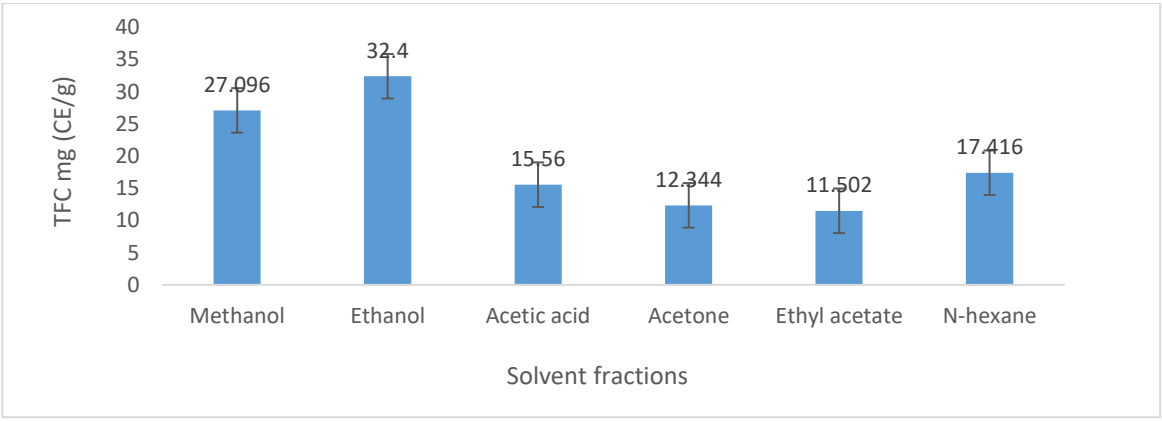

Figure 5. TFC values in different solvent fractions of *M. sylvestris* leaves

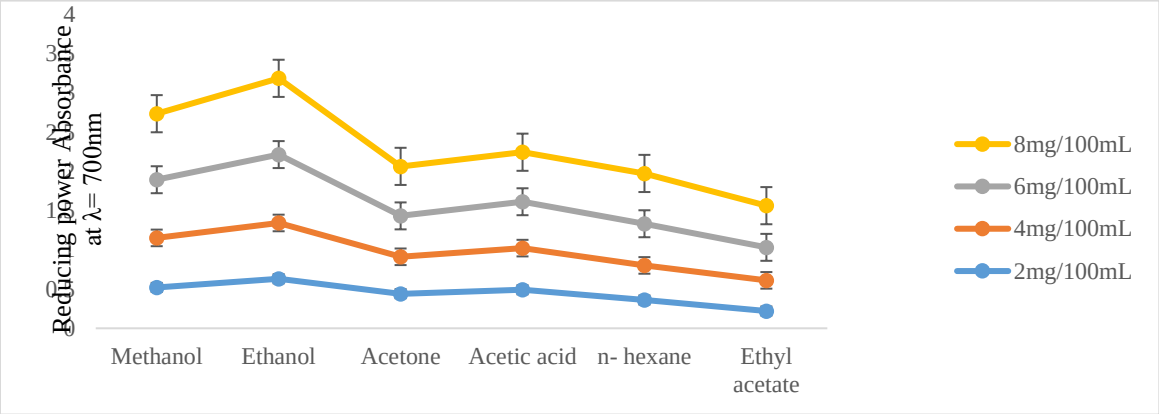

Figure 6. Reducing power of solvent fractions of *M. sylvestris* leaves

3.7 Inhibition of Linoleic acid peroxidation

Different solvent fractions of *M. sylvestris* were estimated for linoleic acid peroxidation inhibition assay, and Fig. 7 shows the %age inhibition of linoleic acid after 15 days (360 h) incubation. All fractions for polar solvent show linoleic acid peroxidation ranging from 47-28.01%, 13-18% for less polar fractions, and 27% for a non-polar solvent fraction of *M. sylvestris*. It was established that there was a significant polarity effect on linoleic acid oxidation ($P<0.005$). Overall, result shows that methanol gave a high percentage of linoleic acid peroxidation followed by ethanol>Acetic acid>*n*-Hexane>Ethyl acetate>Acetone. The decrease in polarity of solvent fractions results in the decreasing of antioxidant activity due to the less amount of phenolic compounds responsible for this activity (Iqbal et al., 2015).

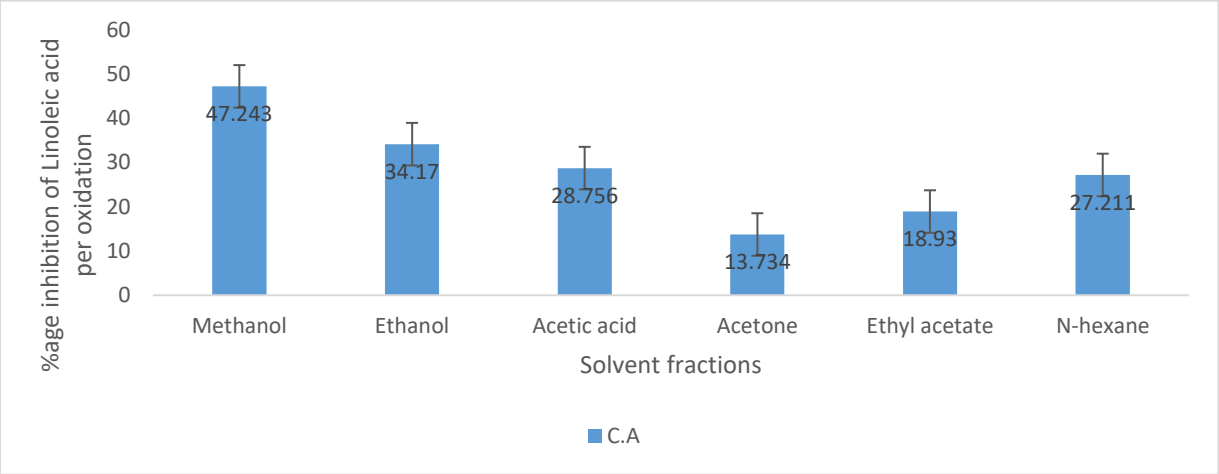

Figure 7. %age of inhibition in different solvent fractions of *M. sylvestris*

3.8 Antimicrobial activity

The solvent fractions of *M. sylvestris* leaves were used to define their antimicrobial activity. A Vernier caliper was used to measure the zone of inhibition (mm). Results show that in some solvent fractions, the inhibition zone is extensive, while in other fractions, the average inhibition zone shows less activity against microorganisms. Four different strains of bacteria and fungi were used for antimicrobial assay determination.

The data presented in Table 1 and Figure 7 are the mean of three independent experiments. Results show that in an antibacterial assay, the MF has a broad zone of inhibition against *B. subtilis* strain nHF shows less inhibition zone against *E. coli* bacterial strain. MF displayed a wide inhibition zone against A. fusarium fungal strain and EAF showed less inhibition zone than the same fungal strain in the antifungal case. Results specify that methanol fractions show good results.

Results reported in Table 1 and Figure 8 indicate that in methanol fraction, antibacterial activity showed a broad zone of inhibition against S. aureus bacterial strain than that of other fractions that show less inhibition zone. Similarly, in antifungal activity, methanol fraction showed broad inhibition against *A. fusarium* fungus strain, and the rest of another solvent fraction inhibits less zone. It revealed that the methanolic fractions show good results while root and fruit juice extract .showed antifungal activity (Fig.9). Furthermore, *C. albicans* was resistant to all tested plant samples. Among all tested plant samples, leaf and peel extracts have shown less antimicrobial activity (Sah et al., 2011).

Table 1. Antimicrobial activity of *M. sylvestris* solvent fractions zone of inhibition in mm

Micro organism (Bacteria)	**MF**	**EF**	**AF**	***n*.HF**	**EAF**	**AAF**	**Rifampicin**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
E. coli	16±0.01	20±0.03	14±0.02	9±0.02	12±0.03	22±0.02	26±0.01
P. multocida	23±0.03	25±0.02	20±0.01	11±0.01	17±0.02	19±0.01	29±0.02
B. subtilis	29±0.01	25±0.03	23±0.02	16±0.03	20±0.02	25±0.02	32±0.03
S. aureus	17±0.02	19±0.01	14±0.01	10±0.02	13±0.01	18±0.01	23±0.01
Micro organism (fungi)	**MF**	**EF**	**AF**	***n*.HF**	**EAF**	**AAF**	**Fluconazole**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
A. niger	10±0.01	16±0.03	18±0.03	11±0.03	13±0.01	19±0.01	22±0.01
A. flavus	22±0.03	24±0.04	18±0.01	15±0.05	14±0.07	25±0.02	28±0.03
A. fusarium	26±0.01	22±0.01	18±0.03	11±0.01	09±0.03	19±0.01	30±0.02
F. Solani	17±0.01	19±0.03	15±0.02	12±0.02	10±0.04	22±0.05	25±0.03

MF: Methanol fraction, EF: Ethanol fraction, AF: Acetone fraction, *n*-HF: *n*- heaxane fraction, EAF: Ethyl acetate fraction, AAF: Acetic acid fraction (each 25µg/disc)

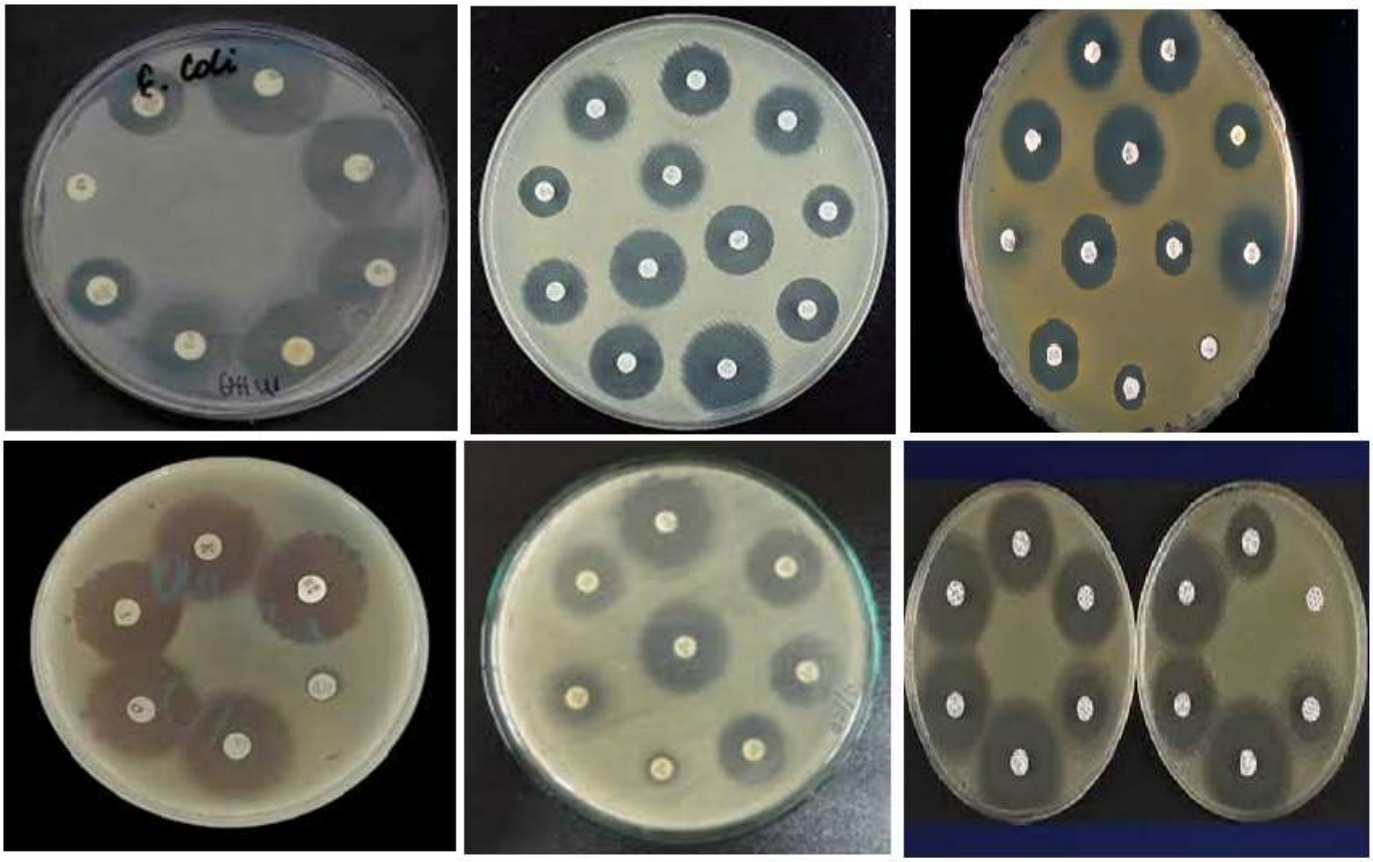

Figure 8. Antibacterial activity of *M. sylvestris* extract fractions by Disc diffusion method against *E. coli* and *P.multocida, S aureus and B.subtilis*

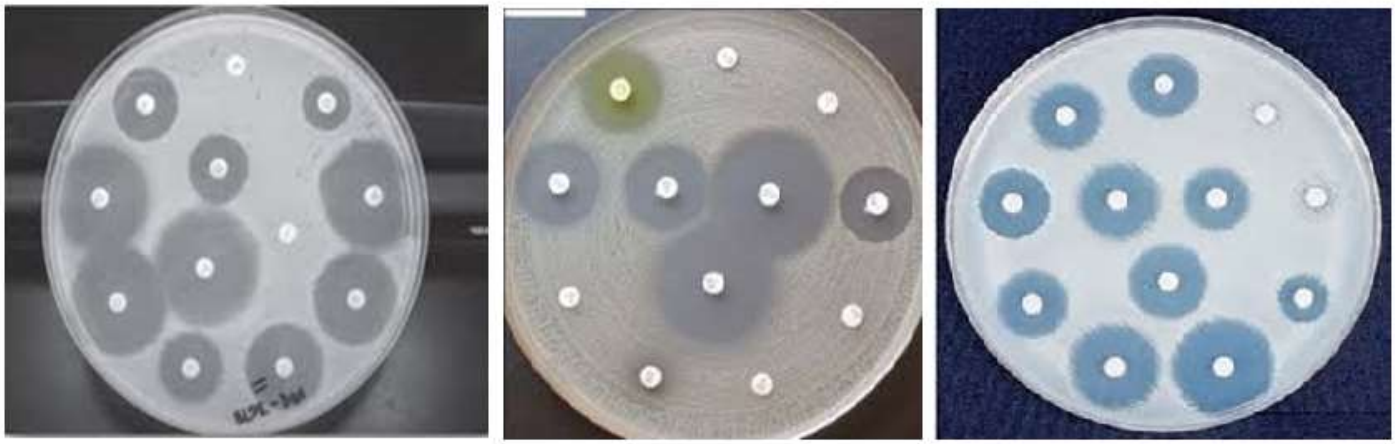

Figure 9. Antifungal activity of *M. sylvestris* extracts fractions by Disc diffusion method against *A. niger* and *A. flavus*

4. Conclusions

Crude methanolic extract fractions of *Malva sylvestris* i.e., methanol, ethanol, and acetic acid, showed significant antioxidant activity in DPPH free radical scavenging activity assay, Linoleic acid inhibition assay, reducing power activity. Antimicrobial activity was presented by all the solvent fractions of these plants. The results of HPLC analysis showed the presence of a higher amount of phenolic and flavonoid components in the methanolic fraction. Further isolation and purification of active compounds from these fractions in the future may reveal the presence of strong novel bioactive agents from *M. sylvestris*

Acknowledgments

The authors delightedly acknowledge the help of Dr. Surrya Manzoor at the University of Education Lahore, Multan campus, and the Institute of Chemical Sciences, Bahauddin Zakariya University Multan, for their esteemed help in the completion of work.

Conflict of interest

The corresponding author declares that there is no conflict of interest among Co-Authors

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Received: 21th February, 2019 **Accepted: 28th March, 2021**