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Phytochemical Analysis and Biological Activity of *Micromeria fruticosa* (L.) Collected from Northern Jordan

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Abstract

Different plant-derived bioactive compounds have the potential to be used for medical applications. One of the native medicinal shrub plant species in the Mediterranean area is *Micromeria fruticosa* from the *Lamiaceae* family. In the present work, the antioxidant activity, antimicrobial activity, phytochemical screening analysis, total phenolic content (TPC) and total flavonoid content (TFC) were determined for plant extracts of *M. fruticosa* collected from Jordan. Furthermore, the identification of phenolic compounds was done using liquid chromatography. Results showed that strong antioxidant activity (93.54%) was obtained from butanol extract while water extract exhibited the weakest antioxidant activity (13.7%). The essential oil extract showed the most potent activities against different types of bacteria compared to butanol and aqueous methanol extracts, with minimum inhibitory concentration (MIC) values of 0.2–0.75 mg/ml. The phytochemical analysis of *M. fruticosa* showed the presence of flavonoid, alkaloids, phenols, and tannins in all solvents extract, with maximal phytochemical compounds obtained from butanol extract. The highest TPC, TFC values were measured butanol extract of *M. fruticosa*, whereas the lowest values were measured for water extract. The ions chromatographic analyses (RP-HPLC) for phenolic compounds present in extracts fraction revealed that the most abundant phenolic acids were gallic acid and ellagic acid. Results indicate that *M. fruticosa* extracts may be used in drug industry as a source of effective compounds.

Keywords: Micromeria fruticosa, Antimicrobial, Antioxidants, Phytochemical Screening, Total Phenolic, Phenolic compounds.

1. Introduction

Plants have been used for treatment of several diseases for long time; more than 90% of prescribed medicines are derived from plants. In Jordan, approximately 2100 medicinal plant species were observed. *Micromeria fruticosa* is an aromatic perennial plant native to the rocky area along the Mediterranean, including Palestine and Jordan, known as Qurnya and Ishbitesh-shai (Abu-Rabia, 2012). *Micromeria fruticosa* is derived from the Greek words micro and morose (meaning small and part), referring to the leaves, stalks, and flowers (Quattrocchi,Umberto, 2000) (Figure 1). It is a member of the genus *Micromeria* of *Lamiaceae* family. It grows up to 70 cm high and spread to 60 cm. The essential oil and extracts of this plant contain an important type of monoterpenes. Levels and composition variation of the monoterpene components with season were noted (Dudai *et al*., 2001). Furthermore, several studies suggested that *Micromeria fruticosa* could contain a reasonable amount of antioxidant components (Telci, 2007) and antimicrobial molecules, which could assist in preventing several types of diseases.

Recently, medicinal plants have been studied and investigated to approve their activities and turn the synthetic drug into new safe alternatives (Najadat, 2018; Anand *et al*., 2019; Es-Safi *et al.,* 2020). Medicinal plants pose low-cost production processes and few environmental hazards, side effects, and toxicity that are lesser than synthetic drugs (De Smet, 2004). Plant phenols (flavones, phenolic acids, tannins, and anthocyanins) represent significant natural antioxidant, anticancer, anti-ulcer, antimicrobial, and anti-inflammatory properties (Nikolic *et al.,* 2012). In addition, traditional medicinal plants were used without a scientific background (Strohl, 2000), but in the ancient world and nowadays medicinal plants have been used for treating several diseases. Recently, ensuring the safety, quality and effectiveness of natural medicinal plants and herbal medicine became a key issue in all developing countries by evaluating the potential of plant-derived compounds (Jamshidi-kia, 2018). As a result, it is noteworthy to investigate the effect of medicinal plants on disease, and pathogenic agents and assess the effectiveness of their active ingredients. Several parts of plants can be used for preparing herbal medicine, such as bark or the quinine bark [Cinchona] (Flatie *et al*., 2009). The preparation of medicinal plants can be conducted in several ways including macerations (cold-soaking), infusions (hot teas), tincture (alcohol and water), decoctions (boiled teas) (Handa *et al.,* 2008), soxhelt extraction (Amid *et al*., 2010), microwave-assisted

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extraction (Trusheva *et al.,* 2007), and sonication extraction (Dhanani *et al.,* 2013).

Recent studies conducted on medicinal plants suggest that active ingredients of these plants are available (Stoyanova *et al*., 2020; Subasi, 2020). Effective extracts of medicinal plants can combat human pathogenic bacteria with the least adverse impacts. Several plants have been investigated regarding antimicrobial and antioxidant activity, total phenolic content, and toxicity effect (Al-Qudah *et al.,* 2022, Imtara *et al.,* 2019; Cock *et al.*, 2017; Diky *et al.,* 2021). *M. fruticosa* is an important plant used worldwide to treat several types of diseases. *M. fruticosa* is a perennial aromatic naturally grown plant used in phytotherapy to treat colds, heart disorders, eye infections, high blood pressure, and abdominal pains. In addition, it is used as a flavoring substance in the food industry. Essential oil of this plant contains different components such as Menthol, Pulegone, Menthofuran, Linalool, 1,8-Cineole, and Piperitenone and other metabolites after oxidation-reduction reaction in the liver (Al-Hamwi *et al*., 2011; Dudai *et al*., 2001; Telci *et al*., 2007). The aim of this study to assess the phytochemical screening, antioxidant and antibacterial activity of *Micromeria fruticosa* plants collected from northern Jordan.

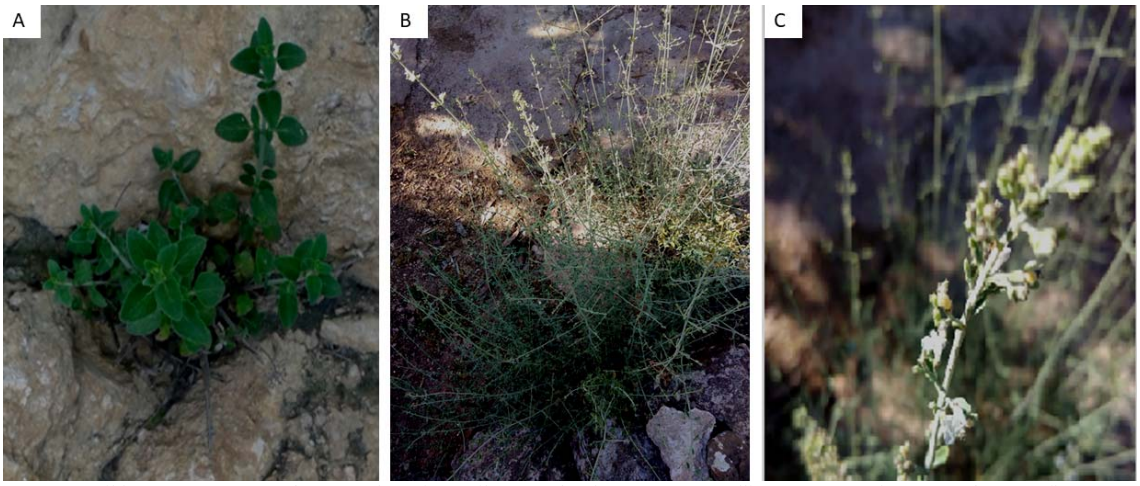

Figure 1. Wild-grown *Micromeria fruticosa* plants from Ajloun area/Jordan. A: young seedling. B: mature plant. C: *M. fruticosa* flowers.

2. Materials and methods

2.1. Plant Materials

Fresh *Micromeria fruticosa* plants were collected from Halawah and the Osarah regions from Ajloun, northern Jordan in April and May 2021. It was identified at the Department of Biological Sciences, Yarmouk University by Professor Ahmad El-Oqlah. *Micromeria fruticosa* samples (leaves and stems) were air dried in the shade for one week, grinded into powder, and stored in the refrigerator in a closed bottle until use.

2.2. Preparation of plant extract

The fine powdered plant material of *Micromeria fruticosa* was defatted with petroleum ether using Soxhlet extraction and then dissolved in methanol. The methanolic extract was evaporated under vacuum to get a crude extract. The obtained extract was steeped in distilled water and was extracted with chloroform by using a separating funnel. The organic layer was partitioned between 10% methanol and hexane. The polar organic species were extracted from the aqueous layer using n-butanol.

2.3. Determination of antioxidant activity of Micromeria fruticosa from Jordan using 1,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Capacity Assay

The scavenging activities of different prepared extracts of *Micromeria fruticosa* were screened and tested using 2,2-diphenyl-picrylhydrazyl radical (DPPH) according to the literature (Braca *et al*., 2002; Berset *et al*., 1995; Al-Saleema *et al*., 2019). Different extracts dilutions of the plant extract (0.005, 0.02, 0.04, 0.06, 0.08 and 0.1 mg/ml) were dissolved in 10 ml methanol. Methanol was used for preparing DPPH solution with the addition of 100 ml methanol to 6.0 mg of DPPH for each type of solvent. Briefly, 2 ml of DPPH solution was combined with 1 ml of each dilution of plant extract. The mixtures were incubated shackled and then allowed to settle in a dark area for 30 min and the absorbance was measured at 515 nm after 30 min at room temperature using a spectrophotometer. The scavenging activity of each extract on DPPH radical was calculated using the following equation:

Scavenging activity (%) = (1-Absorbance of sample / Absorbance of control) x 100%

2.4. Antibacterial activity of Micromeria fruticosa extracts

Four Gram-positive strains (*Staphylococcus* aureus ATCC (29213, *Micrococcus luteus* ATCC (9341), *Bacillus cereus* ATCC (11778), and *Staphylococcus epidermidis* ATCC (12228) and five Gram-negative strains (*Serratia marcescens* ATCC (27117), *Escherichia coli* ATCC (25922), *Proteus vulgaris* ATCC (29905), *Salmonella typhimurium* ATCC (13311), and *Klebsiella pneumoniae* ATCC (13883) were used. These bacterial strains were supplied from Laboratory of Microbiology at Yarmouk University in Jordan.

2.4.1. Agar Well Diffusion (AWD) Assay

Bacterial species were fecundated with suspensions of 0.5 McFarland standard and poured on the surface of Mueller-Hinton agar plates (Imtara, 2018). The plates were dried by placing the plates in an incubator for 24 h at 37 °C. The extract of the plant was dissolved in DMSO. Bacterial suspension (100 µL) was then smeared on agar

plates having 2 mm diameter. The plates were incubated at 37°C for 24 hours and the diameters of the inhibition zones were measured after incubation. The minimum inhibitory concentration (MIC) was calculated.

2.5. Phytochemical screening analysis of different extracts of Micromeria fruticosa from Jordan.

The qualitative phytochemical screening of the prepared *Micromeria fruticosa* plant extracts was used to identify the various major groups of chemical constituents such as alkaloids, glycosids, steroids, flavonoids, tannins, phenolic compounds, and saponins present in the extracts using the following standard procedures (Alamzed *et al.,* 2013; Thusa and Mulmi, 2017; Talukdar and Chaudhary, 2010). Results are addressed according to the presence (+) and absence (-) of phytochemical compounds.

2.6. Determination of Total phenolic Content (TPC)

TPC of *Micromeria fruticosa* was determined following Imtara *et al.* (2018) method. Absorbance was measured at 725 nm. The TPC in different extracts was expressed as mg gallic acid equivalents (GAE) per g dry mass.

2.7. Determination of Total Flavonoid Content (TFC)

TFC of different *Micromeria fruticosa* solvent extracts was determined according to the procedure described in the literature (Al-Humaidia *et al.,* 2019). The absorbance was measured at 410 nm. The results were expressed as mg quercetin/g of dry extract.

2.8. RP-HPLC analysis of phenolic compounds

Identification of phenolic compounds in the extract was determined using HPLC Agilent 1100 series HPLC system (Agilent Technologies, Waldbrom, Germany) liquid chromatography on C18 (250 × 4.6 mm,5 µm) analytical column at room temperature with a flow rate of 1.3 ml/min. Gradient elution of two solvents was used: water with 0.2% formic acid (solvent A) and 100% methanol (solvent B), with a linear gradient starting at 10% methanol within 45 min; and were then repeated over 20 min. Rosemerinic acid, caffeic acid, gallic acid, syringic acid, hesperidin, quercetin, rutin, luteolin, and epicatechin were used as reference standard solution to quantify the phenolic compounds in the sample.

3. Results and discussion

3.1. Phytochemical Screening Analysis

The qualitative results of phytochemical analysis for *Micromeria fruticosa* in aqueous methanol, n-butanol, and water extracts revealed that flavonoids, alkaloids, phenols, and tannins were present in all plant extracts. Glycoside and steroids were detected in all extracts except in the water fraction. Additionally, the results of the qualitative phytochemical analysis indicate that the butanol extracts were wealthy in phytochemical species compared to methanol and water extracts. Mucilage was also not present in all crude extracts as shown in Table 1. Flavonoids reduce the risk mostly from cardiovascular diseases and cancer and the high amount could be beneficial as antibacterial agents (Ballard and Marostica, 2019). Plants containing phytochemicals species such as flavonoids, alkaloids, and tannins showed cytotoxic effects (Chowdhury *et al.*, 2017). Moreover, lower cholesterol levels, as well as cytotoxic qualities, anti-bacterial, and anti-viral properties, are valued to the presence of saponin (Bailly and Vergoten, 2020). Tannins play a role as an anticancer agent that is perceptible from its inhibitory activity towards growth (Mazni, *et al*., 2016).

Table 1. Phytochemical screening in different extracts of *Micromeria fruticosa.*

Groups	Aqueous methanol	n-Butanol	Water
Flavonoids	+	++	+
Alkaloids	+	++	+
Glycosids	+	+	-
Phenols	+	++	+
Saponins	+	+	-
Tannins	+	++	+
Steroids	+	+	-
Mucilage	-	-	-

(+) indicated the presence of a small amount of phytochemicals, (++) indicated the presence of a large amount of phytochemicals, and (-) indicated the absence of phytochemicals.

3.2. Antioxidant potential of Micrmeria fruticosa extract

Aqueous Methanol, n-butanol, and water extracts of *M. fruticosa* were used to assess antioxidant activity using 2,2-diphyenyl-picrylhydrazyl radical (DPPH) according to Braca *et al*., (2002). Scavenging activity (%) of Aqueous Methanol, n-butanol, and water extracts of *Micromeria fruticosa* in different concentrations is presented in Table 2. In general, the highest radical scavenging activity was observed in butanol extract. Moreover, the DPPH scavenging power of the butanol extract showed strong scavenging effects (93.54%) compared to other crude plant extracts. Furthermore, an increase in the concentration level for each extract obtained from *M. fruticosa* had a significant increase in radical scavenging activity. *M. fruticosa* extracts contain a large amount of reduction agents, that may react with free radicles to stop the radical chain reaction completely.

Table 2. Antioxidant activity (Radical scavenging %) of *M. fruticosa* aqueous methanol, n-butanol, and water extracts measured by DPPH method.

Concentration (mg/ml)	Aq. Methanol extract	n-butanol extract	Water extract
0.005	14.80±0.35	15.60±0.25	13.70±0.15
0.02	38.07±0.64	40.32±0.70	24.12±0.55
0.04	56.42±1.10	62.56±1.22	33.56±0.92
0.06	77.83±1.52	81.24±2.15	48.40±0.82
0.08	86.24±2.35	89.75±2.28	54.36±1.32
0.1	89.41±2.43	93.54±3.15	61.16±1.35

Values are expressed as means ± SD.

3.3. Antimicrobial activity of different extracts and essential oil of M. fruticosa.

The antimicrobial activities of the hexane, n-butanol, and aqueous methanol fractions and essential oil of *M. fruticosa* were tested by agar well diffusion method against nine bacterial strains. Results from Table 3 showed that Hexane extract did not have any influence on all tested

bacterial strains. However, the butanol and aqueous methanol extracts have moderate antibacterial activity and showed remarkable activity against all Gram-positive (*Bacillus cereus*, *Micrococcus Luteus*, *Staphylococus aureus*, *staphylococus epidermidis*) and three Gram-negative bacteria strains (*Serratia marcescens*, *Escherichia coli*, *Klebsiella pneumoniae*), but both extracts did not have inhibition effect against *Salmonella typhimurium* and *Proteus vulgaris*. The antibacterial activities of both methanol and ethanol extracts were similar. The antibacterial activity of the essential oil was also tested on the nine bacterial species. Moderate activities against all tested strains compared to standard antibiotic Gentamycin were observed. Comparatively, the essential oil extract demonstrated the highest level of inhibition and was shown to be antibacterial against all Gram-positive and all Gram-negative bacteria.

Table 3. Inhibition zones (mm) diameters produced by plant extracts in an agar well diffusion test against bacterial strains.

bacterial strains	Butanol extract	Aq. methanol extract	Essential oil	Hexane extract	Gentamycin
Bacillus cereus	17±2.21	12±1.87	16±2.16	NA	38±3.12
Micrococcus luteus	16±2.16	14±1.88	19±1.88	NA	29±2.98
Staphylococcus aureus	23±2.78	21±1.88	25±2.95	NA	33±3.01
Serratia marcescens	10±1.76	14±1.88	18±2.24	NA	22±1.92
Escherichia coli	21±1.88	23±2.78	28±2.93	NA	35±3.11
Salmonella typhimurium	NA	NA	17±2.21	NA	23±2.78
Klebsiella pneumoniae	16±2.16	18±2.24	23±2.78	NA	30±2.96
Proteus vulgaris	NA	NA	26±2.94	NA	29±2.98
Staphylococcus epidermidis	14±1.88	14±1.88	18±2.24	NA	26±2.94

Inhibition zone (mm), NA: no activity, tested concentration of the standard antibiotic and extracts in (mg/ml)

MIC values for all *M. fruticosa* and essential oil extracts were determined against all bacterial strains as shown in Table 4. MIC values for the butanol fraction ranged from 0.48-0.75 mg/ml, for the butanol fraction, 0.4-0.7 mg/ml for the aqueous methanol portion, and 0.2–0.75 mg/ml for the essential oil portion. Essential oil extract showed the most potent activities against different types of bacteria compared to butanol and aqueous methanol extracts. MIC values obtained are agreed upon and supported by the value findings of the agar well diffusion method. The MIC values of the aqueous methanolic and essential oil extracts revealed the best inhibition influence versus *Staphylococus aureus*, *Escherichia coli, Klebsiella pnuemoniae,* and *Protues vulgeris.* In contrast, none of the bacteria screened were susceptible to hexane extracts at all the tests.

Table 4: Minimal inhibitory concentration (MIC) (mg/ml) of extracts and essential oil of *M. fruticosa*.

Hexane extract	Essential oil	Aq. methanol extract	Butanol extract	bacterial strains
Bacillus cereus	0.70	0.65	0.65	-
Micrococcus luteus	0.65	0.60	0.55	-
Staphylococcus aureus	0.48	0.45	0.30	-
Serratia marcescens	0.75	0.60	0.65	-
Escherichia coli	0.55	0.40	0.20	-
Salmonella typhimurium	-	-	0.60	-
Klebsiella pneumoniae	0.62	0.70	0.35	-
Proteus vulgaris	-	-	0.25	-
Staphylococcus epidermidis	0.64	0.75	0.60	-

Previously published articles inform that extracts with minimum inhibitory concentration lower than 100 µg/ml can be used to have an excellent antibacterial influence (Kuete, 2010). Hence, this principle shows that the essential oil extract showed high activity against *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae,* and *Proteus vulgaris* bacteria with a range of 0.20-0.35 mg/ml. Aqueous methanol and butanol extracts showed medium influence against all Gram-positive and three Gram-negative bacteria (MIC = 0.40-0.75 mg/ml, 0.48-0.70 mg/ml) respectively. The minimum level of inhibition in all extracts was observed against *Serratia marcescens*, *Staphylococcus epidermidis,* and *Bacillus cereus* with an MIC value of 0.75 mg/ml. The reason could be attributed to the resistance of Gram-negative bacteria because of the complicated structure of their cell wall, which contains a double membrane as opposed to the unique glycoprotein/teichoic acid membrane of Gram-positive bacteria (Ndamane *et al.,* 2013). Antimirobial activity results indicate the potential application of *M. fruticosa* extracts in antibiotic resistance and as a potential replacement of antibiotics to treat drug-resistant infections.

3.4. Determination of Total Flavonoid content (TFC) and total Phenolic content (TPC):

Total phenolic contents in different extracts of *M. fruticosa* were measured by Folin-Ciocalteu technique (Table 5). TPC and TFC values also appeared to have comparable trends in all extracts. The highest TPC and TFC values were noted in the butanol extracts (227.8±1.1, 178.5±3.5) followed by aqueous methanol (184.4±1.2, 94.7±1.4) and then water extracts (133.4±1.5, 72.2 ±1.6) respectively. The obtained results evaluated the lowest values according to both TPC and TFC assays for water extract. In a recent study, TFC and TPC of ethanolic extracts of *M. fruticosa* was 56.78 ± 0.49 mg/g and 8.03 ± 0.01 mg/g, respectively (Sadeq *et al.*, 2021). These values are lower than that of our study. Moreover, these results agree with a recent study that has identified the phenolic and flavonoid compounds in *M. fruticosa* pollens, and they

have been shown to have antioxidant properties (Bakour *et al.,* 2019). In addition, the polarity of the extraction solvents affects the concentration of phenols and flavonoids (Jing *et al.,* 2015). The presence of Flavonoids and Phenolics in *M. fruticosa* extracts indicates the extract's activity as a powerful antioxidant component. These results support the ethno-pharmacological use of *M. fruticosa* in Reactive oxygen species related diseases.

Table 5. TPC and TFC values in different extracts of *M. fruticosa*.

Extracts	TPC (mg/g gallic acid)	TFC (mg/g of quercetin)
n-Butanol	227.8±1.1	178.5±3.5
Aq. methanol	184.7±1.2	94.7±1.4
Water	133.4±1.5	72.2±1.6

3.5. HPLC analysis

In the extraction of *M. fruticosa* using RP-HPLC, nine phenolic compounds were found: Rosemerinic acid, caffeic acid, gallic acid, syringic acid, hesperidin,

quercetin, rutin, luteolin, and epicatechin. All phenolic compounds were present in all solvent extracts. Gallic acid is one of the most important hydroxybenzoic acids, and it was present in large contents in aq. methanol, butanol, and water extracts with percentages of occurrence of 29%, 30%, and 29%, respectively, as shown in Figure 2. Low gallic acid content was observed in the water extract. On the other hand, a high gallic acid amount was observed in samples extracted in aqueous methanol and butanol solvent (Figure 2). A similar trend was observed for syringic acid. Moreover, high caffeic acid amount was observed in water extract and low in aq., methanol and butanol extracts. Rosemerinic acid, quercetin, luteolin and

epicatechin were present in the same amounts in aq. methanol and water extracts with percentage of 3.8%, 14.95, 7.6% and 11.7%, respectively (Figure 2). These phenolic acid results agree with previously published data for other biological samples (Kelebek *et al.,* 2009). Many articles documented the valuable effects of such phenolic compounds on human health, such as their antioxidant properties (Yang *et al.,* 2008; Aytekin *et al.,* 2011; Erkan *et al.*, 2008).

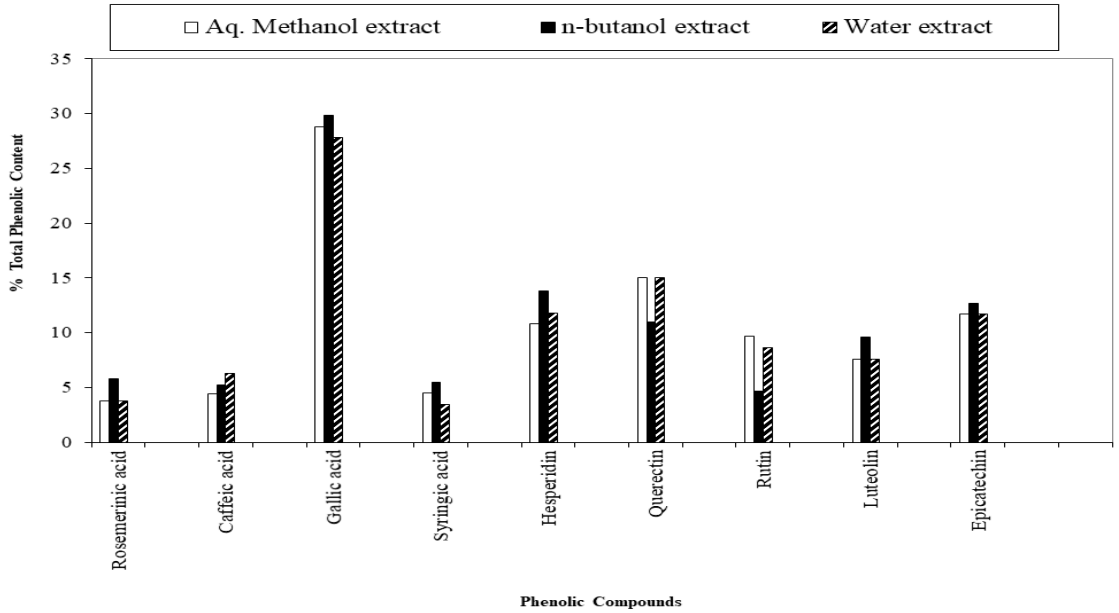

Figure 2: phenolic compounds content of *M. fruticosa* aqueous methanol, butanol, and water extracts using RP-HPLC.

4. Conclusions

The different fractions of *M. fruticosa* showed activities against different strains of bacteria. The minimum inhibition concentration (MIC) values of the *M. fruticosa* extracts were less than the MIC value of the standard, gentamycin. Furthermore, the essential oil extract showed the most potent activities against different types of bacteria compared to butanol and aqueous methanol extracts. The antioxidant data showed that *M. fruticosa* extracts are effective as their antioxidant values are comparable with the standard. Most *M. fruticosa* extracts showed a high antimicrobial activity with high potential to be used in drug industry. This study showed that all Gram- positive and Gram- negative tested bacteria were significantly inhibited by different *M. fruticosa* extracts. No inhibition activity was observed against tested bacteria from hexane extract, due to seasonal variation in the chemical composition of the plant and oil materials. In addition, MIC of *M. fruticosa* were measured for tested microorganisms. The results showed that the essential oil extract manifested the most potent activities against different types of bacteria compared to butanol and aqueous methanol extracts. Identified phenolic compounds separated from *M. fruticosa* extract by HPLC analysis have been tested. With a powerful analytical HPLC technique, the identification and quantification of nine phenolic compounds were achieved for *M. fruticosa.* Findings of this study show that *M. fruticosa* may be used as a safe substitute for food additives and as a potential treatment for diseases caused by antibiotic-resistant bacterial strains and for diseases caused by ROS. Further

study is needed to assess the toxicity of *M. fruticosa* on human and animals.

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