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Antibacterial Potential of Beta Vulgaris, Spinacia Oleracea and Trigonella Foenum-Graecum against MDR Clinical Isolate of Salmonella Enterica Serovar Typhi

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Abstract

This study was aimed to assess the efficacy of Trigonella foenum-graecum, Spinacia oleracea, and Beta vulgaris against multidrug-resistant Salmonella enterica serovar Typhi. Medicinal plant extracts were prepared using methanol, aqueous, and ethyl acetate. Standard techniques were used for the phytochemical analysis. Mueller Hinton agar was used to detect the antibacterial activity of plants. Microdilution method were used to determine the minimum inhibitory concentration (MIC) for a bioactive compound. The highest concentration of protein was observed in the leaf and seed portions of Beta vulgaris in methanol and ethyl acetate. The seed portion likewise contained the highest concentration of flavonoids. Beta vulgaris doesn't contain phytosterols. Trigonella faenum-gracum contains the largest concentrations of proteins, flavonoids, and saponins. Spinacia oleracia contained the highest concentrations of protein and flavonoids, but not phytosterols. Spinacia oleracia seeds in ethyl acetate showed high antibacterial activity i-e 10mm, 23mm, 30mm of inhibition zone while Spinacia oleracia leaves showed inhibition of 27mm, 17mm, 9mm against the selected pathogen. Trigonella foenum-graecum leaves in ethyl acetate and roots in methanol showed inhibition of 27mm, 20mm and 25mm, 22mm. Beta vulgaris roots in methanol show higher inhibition off 14mm, 18mm, 23mm while roots in ethyl acetate show inhibition of 15mm, 27mm. The minimum inhibitory concentrations (MIC) of Beta vulgaris roots in methanol and ethyl acetate were 7.4 and 7.6 ug/ml, Spinacia oleracea seeds and leaves had 7 ug/ml, 7.3 ug/ml, 4.7 ug/ml, and 5 ug/ml in ethyl acetate, and Trigonella foenum-graecum roots in methanol had a MIC of 5.8 ug/ml, while the MIC of leaves in ethyl acetate was 6.8 ug/ml. The minimum inhibitory concentrations (MIC) of Beta vulgaris roots in methanol and ethyl acetate were 7.4 and 7.6 ug/ml, Spinacia oleracea seeds and leaves had 7 ug/ml, 7.3 ug/ml, 4.7 ug/ml, and 5 ug/ml in ethyl acetate, and Trigonella foenum-graecum roots in methanol had a MIC of 5.8 ug/ml, while the MIC of leaves in ethyl acetate was 6.8 ug/ml. GC-MS analysis of methanol extract of Beta vulgaris roots confirm the existence of 120 bioactive compounds and the most abundant was 2-PENTACOSANONE. Overall, Trigonella foenum-graecum, Beta vulgaris, and Spinacia oleracea

harboured bioactive compounds that had strong antibacterial effect against Salmonella enterica serovar Typhi and can serve as wonderful source of alternative to antibiotics.

Keywords: *Salmonella Enterica Serovar Typhi, Beta Vulgaris, Bioactive Compounds, Multidrug Resistance, Flavonoids.*

Introduction

Typhoid fever, a global disease affecting 26 million people annually, disproportionately affects children, contracted through environmental contact with pathogen-containing water or food (Britto et al., 2018). Typhoid fever caused 11-21 million cases and 148,000-161,000 deaths worldwide in 2015, with *Salmonella enterica* serover Typhi accounting for 76.3% of cases, and a global mortality rate of 0.95% for 2017 (GBD 2017). Since 2018, five countries with high rates of typhoid fever, antimicrobial resistance, or recent outbreaks have added typhoid conjugate vaccines to their immunization programs. Between 2017 and 2022, the CDC identified seven confirmed outbreaks, including one in Pakistan, three in Zimbabwe, and one in the Philippines (Hancuh et al., 2023). Typhoid fever treatment increasingly involves carbapenems, macrolides, and third-generation cephalosporins due to MDR *Salmonella enterica* serovar Typhi and fluoroquinolone resistance, with ciprofloxacin being the drug of choice (Marchello et al., 2020). Population in Punjab and Sindh provinces are the most susceptible to typhoidal pathogen. According to a study, a new, extensively drug-resistant typhoid outbreak in Hyderabad was reported in 2016 (Jabeen et al., 2023). Resistance to third-generation cephalosporins and azithromycin has recently evolved in low- and middle-income countries (LMICs) including Pakistan, India, and Bangladesh. Outbreaks of extensively drug-resistant (XDR) *S. typhi* strains (resistant to first- and second-line antibiotics as well as third-generation cephalosporins) in Pakistan have highlighted the dangers of inappropriate and unnecessary antibiotic use, as well as the risk of patients with typhoid fever being left with no treatment options (Khanum 2022). This antibiotic-resistant pathogenic pathogen necessitates the exploration of novel antimicrobials from diverse natural sources like microorganisms, plants, and marine animals.

Beetroot, also known as *Beta vulgaris*, is a deep red vegetable with high antioxidant activity and various health benefits. Its abundance of secondary metabolites influences its therapeutic efficacy and physiological activity (Jhon et al., 2017; El-Beltagi et al., 2018). *Spinacia oleracia*, a green leafy vegetable, is known for its anti-inflammatory, antioxidant, and lipid-lowering properties. Consumption offers health benefits like cardiovascular protection, cholesterol reduction, and anti-cancer effects. Its antioxidant properties are attributed to its abundant phenolic or flavonoid chemicals (Murcia et al., 2020; Zhou et al., 2023). Fenugreek, an annual herb from the Fabaceae family, is used to cure a variety of ailments, including diabetes, menstrual cramps, stomach pain, asthma, pneumonia, microbiological infections, hormonal imbalances, inflammation, cellulitis, boils, tuberculosis, and cancer (Mahbub et al., 2015; Singh et al., 2023). In order to assess the phytochemical profiles of many solvent extracts from several medicinal plant components and their antibacterial activity against the multidrug-resistant typhoidal bacterium, the current study was undertaken.

Methods

Healthy and disease free plants (*Beta vulgaris, Spinacia oleracia and Trigonella foenum-graecum*) were collected from different areas of Haripur and then transferred to microbiology laboratory at University of Haripur, KPK, Pakistan. The plant extracts were prepared using ethanol, methanol, and ethyl acetate in flasks with plant powder. The extracts were stored at 28°C for 72 hours, filtered, and evaporated. The extracts were then dissolved in DMSO at a concentration of 10 mg/mL for further analysis, and dried completely in a dry heat oven at 37°C (Mamman et al., 2013). Salmonella Shigella agar was utilized to reactivate multidrug-resistant Salmonella bacteria. To assess *Salmonella enterica* serovar Typhi's multi-drug resistance, antibiotic sensitivity test was conducted. Gentamycin, erythromycin, cefterixone, vancomycin, azetronanm, pencillin G, cefturoxine sodium and ciprofloxacin were the eight antibiotics used

against *Salmonella enterica* serovar Typhi. The antibacterial activity of chosen plants was assessed using the agar well diffusion method. Muller Hinton Agar plates were labeled with bacterial strains and plant extracts. The plates were incubated at 37°C for 24 hours, and the zone of inhibition against bacteria was determined (Magaldi et al., 2004). The microdilution method was used to determine the MIC values of the extracts against microorganisms in 96-well microplates. The plates were incubated at 37°C for 24 hours (Omogbai et al., 2011). GC-MS analysis of the plant extract was done by using a Thermo Scientific (DSQII) GC, which had a 30-meter TR-5MS capillary column. The carrier gas was helium, flowing at 1 ml/min. The sample volume was injected at 50°C for 2 minutes, then increased to 150°C and 300°C, and held for 5 minutes (Gomathi et al., 2015). The extracts of three selected medicinal plants were screened for phytochemical components using conventional procedures (Mehta et al., 2016).

Results

Qualitative analysis of phytochemicals

The presence of alkaloids, phenols, diterpene, carbohydrate, proteins, phytosterol, tannins, flavonoids, glycosides, saponins in the leaf, seed and roots of *Spinacia oleracia, Beta vulgaris* and *Trigonella faenum-gracum*was was detected in various solvents. The highest amount of protein was detected in the leaf and seed part of *Beta vulgaris* in methanol and ethyl acetate. Flavonoids were also present in maximum amount in the seed part. Phytosterols were not present in *Beta vulgaris* (Table 1). Saponins, Protein and Flavonoids are present in the highest amount in *Trigonella faenum-gracum* (Table 2). Protein and Flavonoids are present in maximum amount while Phytosterols are not present in the *Spinacia oleracia* (Table 3).

Table: 1: Bioactive components of Beta vulgaris extracts in various solvent

Beta vulgaris				
Bioactive components	**Plant Parts**	**Methanol**	**Ethyl-acetate**	**Chloroform**
Saponin	**Leaf**	+	+	+
	Root	+	+	+
	Seed	+	+	+
Alkaloids	**Leaf**	+	+	+
	Root	+	+	+
	Seed	+	+	+
Phenols	**Leaf**	+	+	+
	Root	+	+	+
	Seed	+	+	+
Carbohydrates	**Leaf**	+	+	+
	Root	+	+	+
	Seed	+	+	+
Proteins	**Leaf**	++	+++	+
	Root	+	+	+
	Seed	+++	+	+
Diterpenes	**Leaf**	+	+	+
	Root	+	+	+
	Seed	+	+	-
Phytosterols	**Leaf**	-	-	-
	Root	+	-	+
	Seed	+	+	-
Flavonoids	**Leaf**	+	+	-
	Root	+	+	+

	Seed	++	-	-
Tannins	Leaf	+	+	-
	Root	+	+	+
	Seed	+	+	+
Glycosides	Leaf	+	+	+
	Root	+	+	+
	Seed	+	+	+

Table 2: Bioactive components of Trigonella faenum gracum extracts in various solvent

Trigonella faenum-gracum				
Bioactive components	Plant Parts	Methanol	Ethyl-acetate	Chloroform
Saponin	Leaf	+	+	+
	Root	+	+	+
	Seed	+++	+++	+++
Alkaloids	Leaf	+	+	+
	Root	+	+	+
	Seed	+	+	+
Phenols	Leaf	+	+	+
	Root	+	+	+
	Seed	+	+	+
Carbohydrates	Leaf	+	+	+
	Root	+	+	+
	Seed	+	+	+
Proteins	Leaf	+	+++	+
	Root	+	+	+
	Seed	+	+	+
Diterpenes	Leaf	+	+	+
	Root	+	+	+
	Seed	+	+	+
Phytosterols	Leaf	+	+	-
	Root	+	+	+
	Seed	+++	+	-
Flavonoids	Leaf	+++	+	+++
	Root	+	++	+
	Seed	+	++	+
Tannins	Leaf	+	+	-
	Root	+	+	+
	Seed	+	+	+
Glycosides	Leaf	+	+	+
	Root	+	+	+
	Seed	+	+	+

Table 3: Bioactive components of Spinacia oleracia extracts in various solvent

Spinacia oleracea				
Bioactive components	**Plant Parts**	**Methanol**	**Ethyl-acetate**	**Chloroform**
Saponin	**Leaf**	+	+	+
	Root	+	+	+
	Seed	+	+	+
Alkaloids	**Leaf**	+	+	+
	Root	-	+	+
	Seed	+	+	+
Phenols	**Leaf**	+	+	+
	Root	+	+	+
	Seed	+	+	+
Carbohydrates	**Leaf**	+	+	+
	Root	+	+	+
	Seed	+	+	+
Proteins	**Leaf**	++	+++	+
	Root	+	+	+
	Seed	+++	+	+
Diterpenes	**Leaf**	+	+	+
	Root	+	-	+
	Seed	+	+	-
Phytosterols	**Leaf**	-	-	-
	Root	-	-	-
	Seed	+	+	-
Flavonoids	**Leaf**	+	+	-
	Root	+	+	+
	Seed	++	-	-
Tannins	**Leaf**	+	+	-
	Root	+	+	+
	Seed	+	-	+
Glycosides	**Leaf**	+	-	+
	Root	+	+	+
	Seed	+	+	+

Antibiotic sensitivity testing

Multidrug resistant strain of *Salmonella enterica* serovar Typhi show resistant against seven antibiotic including erythromycin, cefterixone, vancomycin, azetronanm, pencillin G, cefturoxine sodium, ciprofloxacin as shown in (Fig. 1)

Figure: 1: Antibiotic sensitivity testing by disc diffusion method

Antibacterial activity of selected plants extract

Detection of antibacterial activity of plants was done by using Mueller Hinton agar. *Spinacia oleracia* seeds in ethyl acetate show high antibacterial acticity i-e 10mm, 23mm, 30mm of inhibition zone while *Spinacia oleracia* leaves show inhibition of 27mm, 17mm, 9mm against Salmonella selected pathogen.as shown in (Table 4). *Trigonella foenum-graecum* leaves in ethyl acetate and roots in methanol show inhibition of 27mm, 20mm and 25mm, 22mm (Table 5). *Beta vulgaris* roots in methanol show higher inhibition off 14mm, 18mm, 23mm while roots in ethyl acetate show inhibition of 15mm, 27mm as shown in (Fig 2 & Table 6)

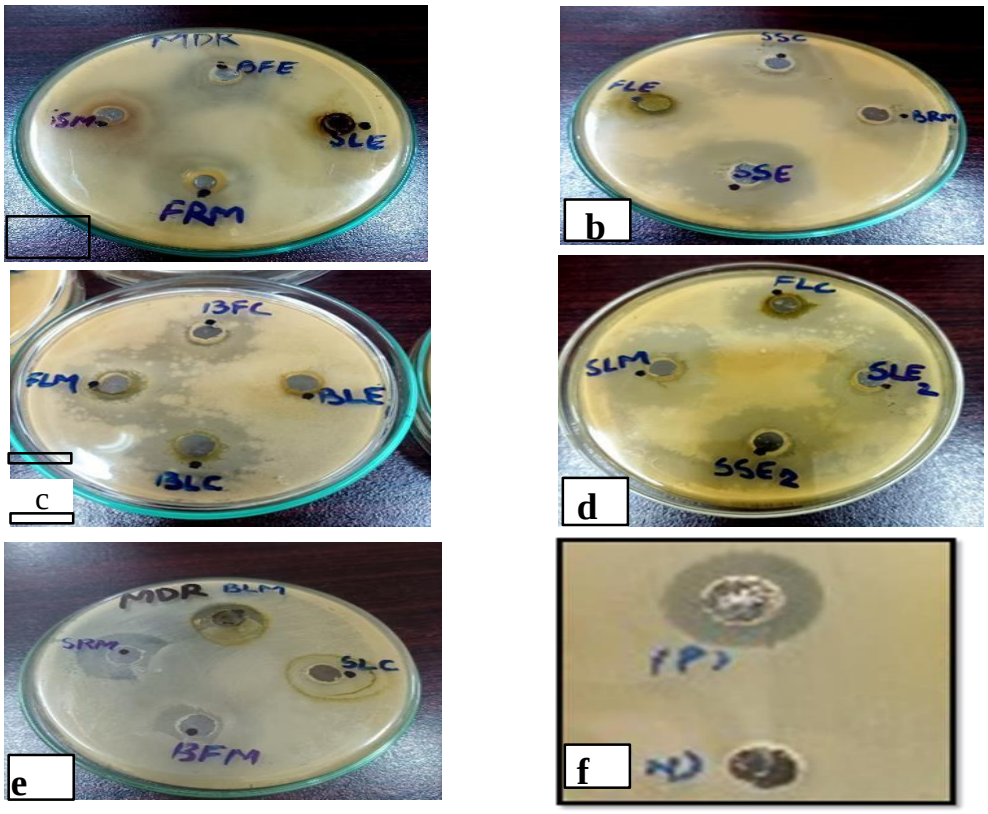

Figure 2: Antibacterial activity of chloroform, ethyl acetate and methanolic extract of *Beta vulgaris*, *Spinacia oleracea* and *Trigonella foenum-graecum* (a) Antibacterial activity of ethyl acetate extract of *Beta vulgaris* roots(BRE), methanolic extract of *Spinacia oleracia* seed(SSM) and *Trigonella foenum-graecum* roots(FRE) (b) Antibacterial activity of *Spinacia oleracia* seed extract in ethyl acetate(SSE) and chloroform(SSC), *Beta vulgaris* roots extract in methanol(BRM), *Trigonella foenum-graecum* leaves extract in ethyl acetate(FLE) (c) Antibacterial activity of *Beta vulgaris* leaves extract in chloroform(BLC) and roots extract in chloroform(BLC), *Trigonella foenum-graecum* leaves extract in methanol(FLM) (d) *Spinacia oleracia* seeds and leaves extract in ethyl acetate (SSE) and (SLE), *Spinacia oleracia* leaves extract in methanol(SLM) and *Trigonella foenum-graecum* leave extract in chloroform(FLC) (e) Antibacterial activity of *Spinacia oleracia* roots extract in methanol(SRM) (f) Positive and negative control.

Table 4: Measurement of zone of inhibition of *Spinacia oleracea* leaf, seed, root in mm

Spinacia oleracea **Zone of inhibition(mm)**									
	METHANOL			**ETHYL ACETATE**			**CHLOROFORM**		
	5mg/ ml	**10mg/ ml**	**20mg /ml**	**5mg/ ml**	**10mg/ ml**	**20mg/ ml**	**5mg/ Ml**	**10mg /ml**	**20mg /ml**
Leaf	**-**	**15mm**	**25mm**	**9 mm**	**17mm**	**27mm**	**-**	**-**	**22mm**
Seed	**-**	**13mm**	**19mm**	**10mm**	**23mm**	**30mm**	**-**	**-**	**20mm**
Root	**11mm**	**24mm**	**27mm**	**-**	**-**	**-**	**-**	**-**	**-**

Table 5: Measurement of zone of inhibition of Trigonella foenum-graecum leaf, seed, root in mm

Trigonella foenum-graecum **Zone of inhibition (mm)**									
	METHANOL			**ETHYL ACETATE**			**CHLOROFORM**		
	5mg/ml	**10mg/ ml**	**20mg/ Ml**	**5mg /ml**	**10mg/ ml**	**20mg/ ml**	**5mg/ Ml**	**10mg/ Ml**	**20mg/ ml**
Leaf	**-**	**5mm**	**11mm**	**-**	**20mm**	**27mm**	**-**	**22mm**	**23mm**
Seed	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Root	**-**	**22mm**	**25mm**	**-**	**-**	**-**	**-**	**-**	**-**

Table 6: Measurement of zone of inhibition of *Beta Vulgaris* leaf, seed, root in mm

Beta Vulgaris **Zone of inhibition (mm)**									
	METHANOL			**ETHYL ACETATE**			**CHLOROFORM**		
	5mg/ Ml	**10mg/ ml**	**20mg/ ml**	**5mg/ ml**	**10mg/ ml**	**20mg/ ml**	**5mg/ Ml**	**10mg/ Ml**	**20mg/ Ml**
Leaf	**-**	**19mm**	**20mm**	**-**	**-**	**-**	**-**	**18mm**	**23mm**
Seed	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Root	**14mm**	**18mm**	**23mm**	**-**	**15mm**	**27mm**	**-**	**16mm**	**18mm**

Minimum inhibitory concentration determination against bacterial strains

MIC value of *Beta vulgaris* root in methanol was 7.4ug/ml and 7.6ug/ml and roots in ethyl acetate was 6.5ug/ml and 6.8ug/ml against *Salmonella entterica* serovar Typhi as shown in (Fig 3). MIC value of *Spinacia oleracea* seeds and leaves in ethyl acetate was 7ug/ml, 7.3ug/ml and 4.7ug/ml, 5ug/ml as shown in (Fig 4). MIC value of *Trigonella foenum-graecum* leaves in ethyl acetate was 6.5ug/ml and 6.8ug/ml and MIC value of *Trigonella foenum-graecum* roots in methanol was 5.4ug/ml and 5.8ug/ml as shown in (Fig 5)

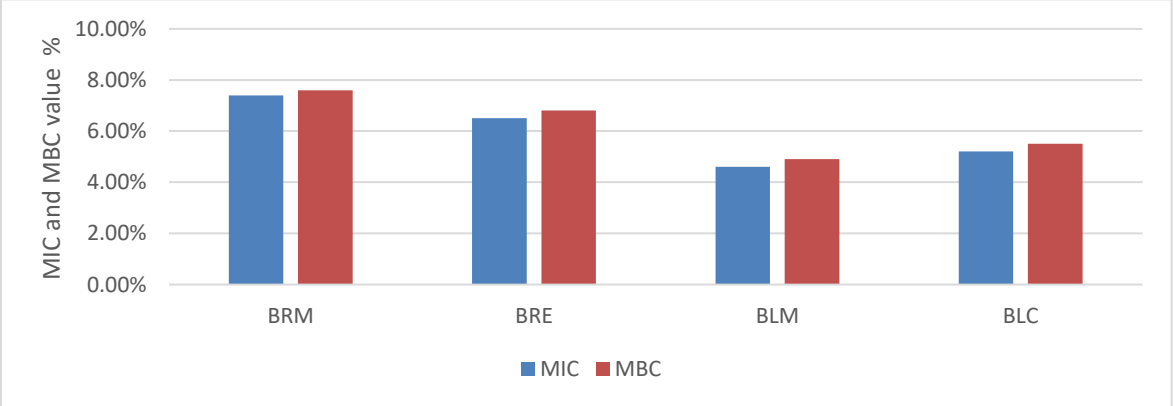

Figure 3: Minimum inhibitory concentration (MIC) of ethyl acetate and chloroform and methanolic extract of *Beta vulgaris roots, leaves and seeds*

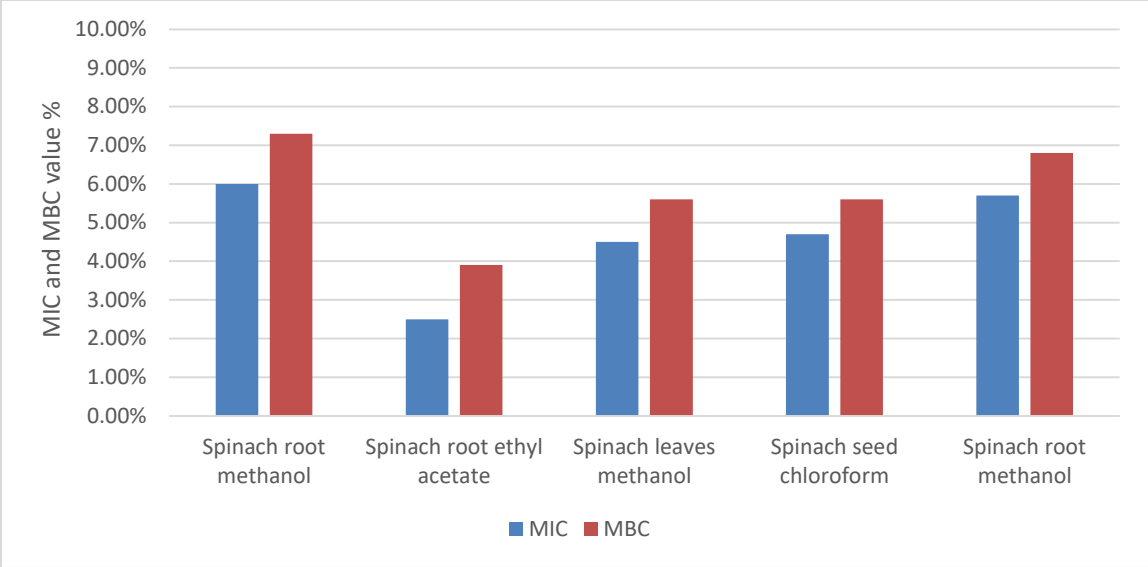

Figure 4: Minimum inhibitory concentration (MIC) of ethyl acetate and chloroform and methanolic extract of *Spinacia oleracia* roots, leaves and seeds

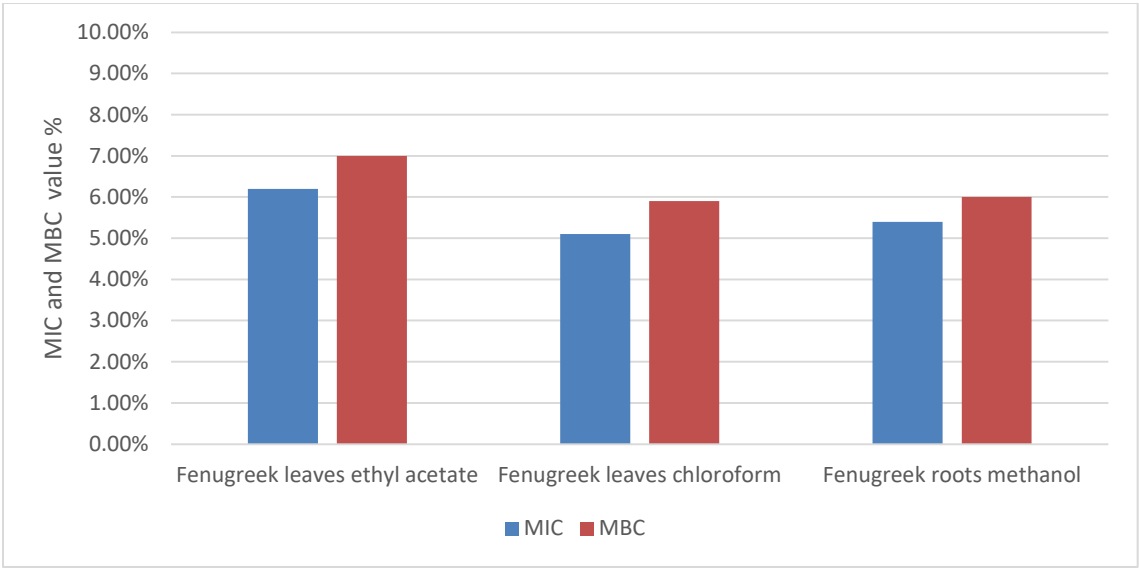

Figure 5: Minimum inhibitory concentration (MIC) of ethyl acetate and chloroform and methanolic extract of Trigonella foenum-graecum seeds roots and leaves

Gas Chromatography-Mass Spectrometry (GCMS)

The GC-MS analysis of methanol extract of *Beta vulgaris* roots revealed the existence of bioactive compounds (Table 7).

Table 7: Show GC-MS Analysis of extract of *Beta vulgaris* roots

Name	RT	Area	% comp
SILANE, CYCLOHEXYLDIMETHOXYM ET	1.169	4998635	0.601917
CARBAMIC ACID, HYDROXY-, ETHYL	1.329	68810.648	0.008286
2-PENTACOSANONE	3.805	412396288	49.65922
TETRAPENTACONTANE, 1,54-DIBROM	14.674	57687.246	0.006946
3-METHYLDOTRIACONTANE	14.95	89697.805	0.010801
1-DECANOL, 2-HEXYL-	15.17	14356.719	0.001729
BIS(TRIDECYL) PHTHALATE	16.26	48461.863	0.005836
1,1,3,6-TETRAMETHYL-2-(3,6,10,	16.315	103942.367	0.012516
BIS(TRIDECYL) PHTHALATE	16.475	236095.047	0.02843
1-BROMOEICOSANE	20.852	401821184	48.38581
CARBONIC ACID, OCTADECYL VINYL	25.604	4089.828	0.000492
HENTRIACONTANE	25.619	5161.045	0.000621

Bioactive compounds identified from the methanol extract of *Beta vulgaris* roots contained 120 active compounds Among the 120 compound the most aboundant were 2-PENTACOSANONE (Peak area: 49.65922%;RT:3.805) and 1-BROMOEICOSANE (Peak area:48.38581; RT:20.852) (Fig. 6).

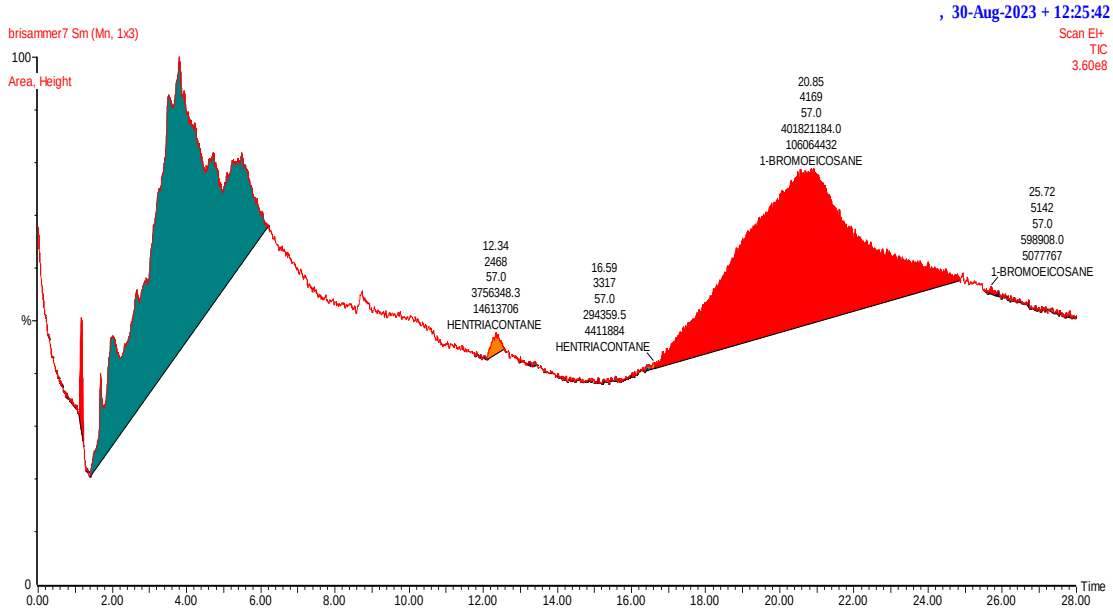

Figure 6: Shows graph peak values of GC-MS Analysis of *Beta vulgaris* roots extract

Discussion

A study conducted by Maraie et al. 2014, identified that aqueous and methanolic extracts of fresh and dried leaves of *Beta vulgaris* consisted of flavonoids, glycosides, saponin, tannins, and phenolic components. The high concentration of flavonoids and phenol molecules operate as antibacterial and antioxidants, providing intriguing options, particularly in light of the developing resistance to conventional anti-microbial agents. Another investigation on the leaf extracts of *Beta vulgaris* in methanol and chloroform by Patel and Patel, yielded comparable results to ours, indicating that these extracts contain secondary metabolites such as alkaloids, phenolics, flavonoids, steroids, and terpenoids. Our findings were also supported by Soni and Sosa's study on a methanolic extract of the plants *Spinacia oleracea* and *Trigonella foenum-graecum*, which revealed the presence of saponins, alkaloids, flavonoids, tannins, proteins, and flavonoids that were identified using various techniques. Phytochemical screening of *Spinacia oleracea* leaf extracts in aqueous, ethanol, and ethyl acetate by Olasupo et al. displayed a favourable phytochemical profile, including alkaloids, flavonoids, saponins, phenols, and steroids, tannis, which is consistent with the findings of our study. Alwhibi and Soliman conducted a study on the antimicrobial effects of *Trigonella foenum-graecum* chloroform and methanolic extracts, which showed antibacterial activity with zones of 34 mm in chloroform extract, 22 mm in ethyle acetate extract, and 28 mm in methanolic extract, while the zones of inhibition in our study against *Salmonella enteric*a serovar Typhi were 25 mm in methanol extract and 23 mm in chloroform extract at 20 ug/ml.

Using ethanolic, sonicated n-hexane, and hexane spinach extracts, Khan and Akhtar found that *Salmonella typhi* was inhibited. In their study, the zones of inhibition were 10 mm, 7.3 mm, 10 mm, and 14 mm, whereas in our study, the zones of inhibition were 30 mm in ethyle acetate and 27 mm in methanol. A study conducted by EI-Beltagi *et al*., revealed that ethanolic extract of *Beta vulgaris* root show week antibacterial activity against *Salmonella typhi*. In their study, the zone of inhibition were 7.11mm, where as in our study, the zone of inhibition were 15mm and 27mm.Our findings are also related to the Olasupo et al. (2018), which revealed the presence of saponins, tannins, phenol, flavonoids in the ethyl acetate extract of *Spinacia oleracia* leaf that is similar to our finding. Issazadeh *et al*. conducted a study on the antibacterial effect of the alcoholic extract of *Spinacia oleracia* leaf, which showed the antibacterial activity with the zone of 7.5mm and 7mm against *S. enteritidis*

Conclusion

Antibiotic resistance has grown to be a big global health problem, according to a recent study. Due to self-medication and drug imbalance, *Salmonella enterica* serovar Typhi has developed resistance to a wide range of antibiotics including fluoroquinolones. As a result, third-generation antibiotics like cephalosporin (cefixime and ceftriaxone) and azithromycin are now used to treat XDR (extensively drug-resistant) *S. enterica* serovar Typhi. This study demonstrated the presence of bioactive compounds with significant antibacterial activity in the spinach, beetroot, and fenugreek plants. Medicinal plants are a good source and an alternative to drugs that are resistant to infection because they contain higher concentrations of antimicrobial agents such as tannins, alkaloids, flavonoids, phenolic, antioxidants, and different enzymes that can degrade oxygen reactive species, which can cause damage to DNA, RNA, and proteins. More study is needed to shed light on the basic concepts, refine extraction innovations, carry out clinical studies and evaluate industrial uses.

Conflict of Interest: Authors declare no conflict of interest.

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