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Antibacterial, antioxidant potential and functional group analysis of Kashmir grown *Prunella vulgaris* L. root extract

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Abstract
The root of *Prunella vulgaris (*Lamiaceae), was Screened for its *in-vitro* antioxidant and anti-bacterial capacity. The antioxidant results revealed that the methanol extract of *Prunella vulgaris* root (MERE) possess highest scavenging capacity than petroleum ether extract (petroleum ether = PERE). The antimicrobial results revealed that *Staphylococcus aureus*, *Klebisiella pneumonia* and *Escheichia coli* were the main susceptible pathogens and significant activity was also recorded by PERE against *Staphylococcus aureus*, *S. pneumonia*, *Enterococcus faecalis* and *K. pneumonia* strains tested, at different concentrations. Column chromatographic fractions were also tested for antibacterial activity, wherein F4 (fraction 4) out of six fractions showed appreciable antibiotic activity against all the organisms tested. FT-IR analysis was done to confirm functional groups and nutrient type present. Besides the plant has great importance as far as its other clinical applications are concerned. The present investigation can potentially be utilized for evaluating the bioactive components with other species of medicinal plants in other different regions of the world and can be used for preparation of superior combination of this plant in pharmaceutical industries.

Keywords: Antioxidant; *Prunella vulgaris*, antibacterial activities; FT-IR analysis; column chromatography

Introduction
Prunella vulgaris var. lilacina has a wide distribution in in different regions of the world such as: Korea, China, Japan and Europe, and it is applied for the treatment of eye pain, headache dizziness and inflamation[1]. There are about 15 Prunella species dispersed throughout the world both in the temperate and tropical regions [2]. *Prunella vulgaris* L. possess long stem (1-2 feet) and leaves attached at the edge. Flowers consist of purple colour, present at the tip of stem which bloom mostly from June to August. *Prunella vulgaris* L. is known to have great medical importance. The plant is semi-wild and is found in humid and open regions like on banks of streams, in farming lands and shady shrubs. *P. vulgaris* was well-liked in western conventional medicine all through the seventeenth century as a cure for fever, cough, and wound healing [3]. In Kashmir, *P. vulgaris* is populary known as "Kalleh- yeuth" and is used in traditional Unani medicine. During sub-minus temperatures of winters it is used as brain tonic, boiled and the steam was inhaled to clear the mucus from throat and reduces headache. Women use the herb for bathing after giving birth to the baby. It was included in folk medicine as a conventional antipyretic antidote in China [4]. Previously reported studies has revealed that *prunella vulgaris* contains phytochemicals such as saponins, alkaloids,phenols, tannins and anionic polysaccharide prunellin [5]. *Prunella vulgaris* exhibit anti-HIV activity [6], and it also shows anti-HSV type 1 and 2 activity. Moreover aqueous and ethanolic extracts of *P. vulgaris* also showed the scavenger effects on DPPH [7]. The methanol extract of the plant has also been reported to show antimicrobial activity against *S. aureus* and *E. faecalis* has also been recorded [8]. The dried parts of the herb can stored as a beverage, and its leaves are used as vegetable dishes in southeast China [9]. The plant is widely distributed and wildly used medicinal herb across the Kashmir valley.

Materials and methods
1. Collection of plant and preparation of extracts
Prunella vulgaris was collected during the flowering stage in august 2020 from the Aharbal province in Jammu and Kashmir's Kulgam District. The roots were separated from the plant and allowed to be dried in shade for 10 days. The *P. vulgaris* species was identified by faculty of Agriculture AU (Annamalai University India) with specimen no. Hort./Id.2/2020. One voucher specimen was kept at Department of Horticulture Annamalai University. The dried plant material was ground by the electric grinder. The ground powder obtained was extracted with several solvents (methanol and petroleum ether) by using soxhlet extracter.

2. Antioxidant assays
2.1. ABTS radical scavenging assay
The ABTS technique gives the potential of different constituents to hunt the 2, 2- azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation [10].
The antioxidant capacity was observed in a reaction comprising of 0.5 mL of 15 µM H_20_2, 0.5Ml of 7 mM ABTS, and 50Mm sodium phosphate buffer, pH 7.5. The recording of absorbance was done at 734 nm with ascorbic acid taken as comparative standard. IC_{50} value is the quantity of sample necessary to inhibit 50 percent of ABTS production. The percentage inhibition of ABTS was calculated as the following equation:

$$\% \text{ of [ABTS] scavenging} = \frac{A0-A1}{A0} \times 100$$

Where A_0 = absorbance of the control and A1 = absorbance of the sample in presence of ascorbic acid (ABA).

2.2. DPPH free radical scavenging assay

The DPPH free radical scavenging capacity of methanol and petroleum ether extracts of *P.vulgaris* was determined by the method followed by [11] with little modifications. DPPH is considered as a stable free radical and upon reduction becomes stable diamagnetic molecule. DPPH donates hydrogen after reacting with antioxidant agent and is reduced, to give a colour change from violet to light yellow which depends proportionally to the nature and amount of radical scavengers present. The percentage inhibition of DPPH was calculated as the following equation:

$$\% \text{ of [DPPH] scavenging} = \frac{A0-A1}{A0} \times 100$$

2.3. Antimicrobial studies

2.3.1. Microorganisms and culture maintenance

Antimicrobial potential of *Prunella vulgaris* root extract was performed against three gram positive and three gram negative pathogenic bacterial strains. *Staphylococcus aureus*, *Streptococcus pneumonia*, *Enterococcus faecalis*, *Klebsiella pneumonia* and *Escherchia coli* bacterial strains were tested, obtained from Department of Microbiology Annamalai University, India. Muller-Hinton agar was used as media for the maintenance of stock cultures in Department of Zoology Annamalai University, India. The strains of bacteria were grown-up on an agar media at 37ºC until the visibility of bacterial colonies on the agar slants for 24 h. The colonies of different microbial strains were cultures in Muller Hinton Broth (MHB) then allowed to grow until late log phase.

2.3.2. Well diffusion assay

The stock cultures were utilized then for pathogen culturing on petri-plates; one strain per plate, using agar well diffusion method to check the antimicrobial potential. Sterile cork borer was used to make wells of 4 mm diameter for the different samples and the plates were kept at 37ºC for 24 hours and zones of inhibitions were observed. Different concentrations (75, 100, 125 and 150 µg) of the sample extracts were impregnated in the wells (4mm). Chloramphenicol (15µl/well) was used as positive control.

2.4. Column chromatography

The MERE crude extract obtained after soxhlet extraction was subjected to column chromatography. The bioactive secondary metabolites were separated with a normal phase packed silica gel column by the method followed by [21] with slight modifications. The silica column was prepared by using silica gel of 12.5 g . The dried MERE was solubilised in DMSO. Then, 0.5 g of silica was added, and allowed to dry so as to make a dry band, and then added to the top of the packed silica column. A total of five fractions were collected, each fraction containing 10 ml. The similarity of the collected fractions was checked using thin-layer chromatography (TLC) plates with ethyl acetate and hexane in the ratio of 4: 6. The collected fractions were screened for antimicrobial activity using the well diffusion method.

2.5. Phytochemical investigation

2.5.1. Total phenolic content (TPC)

Total phenolic content of MERE, and PERE was revealed based on Folin–Ciocalteu (FC) method followed by [12]. 500 µL of sample extracts were mixed with 2.5 mL of FC reagent and were allowed to settle for 5 min. consequently 1.8 mL of (8% w/v) sodium carbonate solution were supplemented and mixed thoroughly. Absorbance at 750 nm was measured by using spectrophotometer (Shimadzu UV-Visible 160A, Kyoto Japan), after the incubation of 1 h at room temperature. Gallic acid was taken as standard. The total phenolics (TPC) was expressed as mg gallic acid equivalents (GAE) pergram of sample.

2.5.2. Total flavonoids content (TFC)

TFC of MERE and PERE was estimated by using aluminium chloride method as followed [13] with minor modifications. In brief 500 µLo f 2% ALCL3 (aluminium chloride) with methanol added to 500 µL of sample extract. Absorption was recorded at 430nm spectrophotometer (Shimadzu UV-Visible 160A, Kyoto Japan) after incubating for 15 minutes at room temperature. Quercitin was used as standard. The total flavonoids were expressed as mg quercitin equivalents per gram of the dry weight of sample. The values are represented as triplicate analysis.

2.6. Fourier transform infrared (FT-IR) spectroscopy analysis

FT-IR spectrum was obtained using FT-IR Spectrophometer (Agilent technologies, Cary 630 FT-IR). All the spectra were recorded in the range from 4000-500 cm^{-1} region, with a KBr beam splitter, nicrome source and DTGS detector with a data acquirement rate of 2 cm^{-1}.

2.7. Statistical analysis

All the experiments were performed in triplicates, and the values were presented as the mean ± standard deviation using SPSS (Statistical package for social science, version 1.9).

Results and discussion

Antioxidant activities of two root extracts of *Prunella vulgaris*, DPPH and ABTS assays were evaluated (Table 1). The DPPH and ABTS assays are based on transfer of electrons between the sample and the reagent radical and measured by means of their colour changes in spectrophotometer. Both the extracts of *Prunella vulgaris* showed significant free radical scavenging activities against DPPH and ABTS assays, comparison to that of maximum antioxidant activity of ascorbic acid. However, MERE showed the maximum antioxidant activity at a sample concentration of 100 µL with IC_{50} value of 21.32 ± 3.43 µg/ml for DPPH and 23.16 ± 0.44 µg/ml for ABTS. While PERE revealed the highest antioxidant activity at a sample concentration of 100 µL having IC_{50} value of 35.65 ± 2.19 µg/ml for DPPH and µg/ml for 61.29 ± 0.45 ABTS. The free radical scavenging capacity of different sample extracts of *Prunella vulgaris* are comparared with previous reported studies on flower and whole plant extracts [14].

The estimation of TPC was performed by the method of Folin-Ciocalteu method, shown in Table 1 to supplement the antioxidant potential of root extract of *Prunella vulgaris*. MERE showed the maximum TPC (116.43 ± 5.16 mg/g DM) in comparison to that of PERE (mg/g DM), which

83.10 ± 4.42 exhibited similarity to that of previously reported TPC (115.7 mg GAC/g) of Prunella vulgaris methanol extracts [16].

Flavonoids have several health advantages such as antioxidant, anti-inflamatory and antimicrobial activities [17]. MERE showed highest TFC (85.12 ± 3.43 mg/g DM) values (Table 1), compared to PERE (78.00 ± 3.50 mg/g DM) which is noteworthy than reported previously in methanol extract of whole plant of *Prunella vulgaris* where TFC was 82.8 mg QE/g [17].

Table 1: DPPH and ABTS radical scavenging activities of MERE and PERE extracts

Extract	DPPH and ABTS Antioxidant activity as IC_{50}(µg/ml)							
	100 µg/ml DPPH ABTS		200 µg/ml DPPH ABTS		300 µg/ml DPPH ABTS		Contents in mg/g DM TFC TPC	
MERE	21.32 ± 3.43	23.16 ± 0.44	35.41 ± 1.43	29.54 ± 0.37	46.26 ± 2.22	52.47 ± 0.43	85.12 ± 3.43	116.43 ± 5.16
PERE	35.65 ± 2.19	61.29 ± 0.45	69.45 ± 0.79	79.32 ± 0.53	87.71 ± 2.78	97.48 ± 1.80	78.00 ± 3.50	83.10 ± 4.42
ABA	46.51 ± 0.8	47.57 ± 0.50	49.80 ± 0.55	48.65 ± 1.10	51.00 ± 0.10	51.55 ± 1.65	-	-

1.1. Antimicrobial activity

Well diffusion assay was used to evaluate the antimicrobial potential of root extract of *Prunella vulgaris* against three gram +ve and two gram -ve bacterial strains (Table 2). The agar diffusion results showed that the root extract of *Prunella vulgaris* exhibited appreciable antimicrobial activity against all the tested strains of bacteria. Gram +ve bacteria were found to be more vulnerable to root extract of *P.vulgaris* than gram –ve bacteria and the zones of inhibition were different at different concentrations of the sample taken. The MERE showed premier antimicrobial activity (Fig.1)with the maximum zone of inhibition values of 20 mm, 17 mm, 15 mm and 8 mm against *streptococcus pneumonia* followed by 17 mm, 12mm, 10 mm and 7 mm against *E.coli*, however the activity of MERE against *E. faecalis* was also moderate (16 mm, 13mm, 12mm and 11mm) and the lowest activity was against *K. pneumonia* (15 , 12, 10 and 9 mm) at different concentrations (150 µl, 125 µl, 100 µl and 75 µl respectively). The PERE showed maximum activity (Fig.2) with highest zones of inhibition against *K. pneumonia* (18 mm, 13 mm, 12 mm, and 10 mm) and moderate activities against followed by 16 mm, 13 mm, 12 mm and 10 mm against *E. coli*. While moderate activity of PERE was observed against *S. aureus* (14 mm, 12 mm, 8 mm and 6 mm) and lowest activity was observed against *S. pneumonia* (12 mm, 10mm, 9 mm and 7 mm) at different concentrations (150 µl, 125 µl, 100 µl and 75 µl respectively). Yoon *et al*. has recently studied that whole plant methanol extract of *Prunella vulgaris* show an efficient anti-fungal and anti-oomycete activity on *Phytophthora infestans*, rice blast fungus, red pepper anthracnose and wheat leaf rust fungus [19]. Mahboubi et al. reported that the methanol extract of *P. vulgaris* exhibited the best activity against *S. aureus*, *S. epidermidis*, *S. sobrinus*, S. *sanguis*, *S. salivarius*, *S. dysenteriae*, *S. flexeneri*, *P. aeruginosa*. *S. saprophyticus*, *S. pneumoiae*, *S. pyogenes*, *E. faecalis*, *E. faecium*, *S. agalactiae*, *K. pneumoniae*, *E. aerogenes*, *A. flavus*, *A. niger* [20].

Several researchers revealed that the extracts of *Prunella vulgaris* exhibited significant anti-microbial activity. Our results revealed that the MERE extracts show significant antibacterial activity than PERE extracts, reason being different subspecies and/or the geographical origin of the plants tested.

Detection of antibiotic potential of crude extract of ethyl acetate column chromatography fraction on silica gel using n-hexane and ethyl acetate as a solvent with steady increase in polarity up to100% ethyl acetate [22], which resulted in six fractions (Fractions 1–6), the column fractions were screened, with fourth fraction (F_4, dark green in colour) showing a wide range of antibacterial activity against all the strains tested (Table 3, Fig. 3).

Table 2: Zones of inhibition (mm) of root extract of *Prunella vulgaris L.*

Sample concentration (µl/ml)							
Extract	**Bacterial strains**	**75**	**100**	**125**	**150**	**Pc**	**Nc**
MERE	*S.aureus*	05	7	9	12	17	ND
	E. facaelis	11	12	13	16	36	ND
	S. pneumonia	8	15	17	20	25	ND
	K. pneumonia	9	10	12	15	32	ND
	E. coli	7	10	12	17	35	ND
PERE	*S.aureus*	6	8	12	14	27	ND
	E. facaelis	6	11	12	12	25	ND
	S. pneumonia	7	9	10	12	19	ND
	K. pneumonia	10	12	13	18	37	ND
	E. coli	10	12	13	16	40	ND

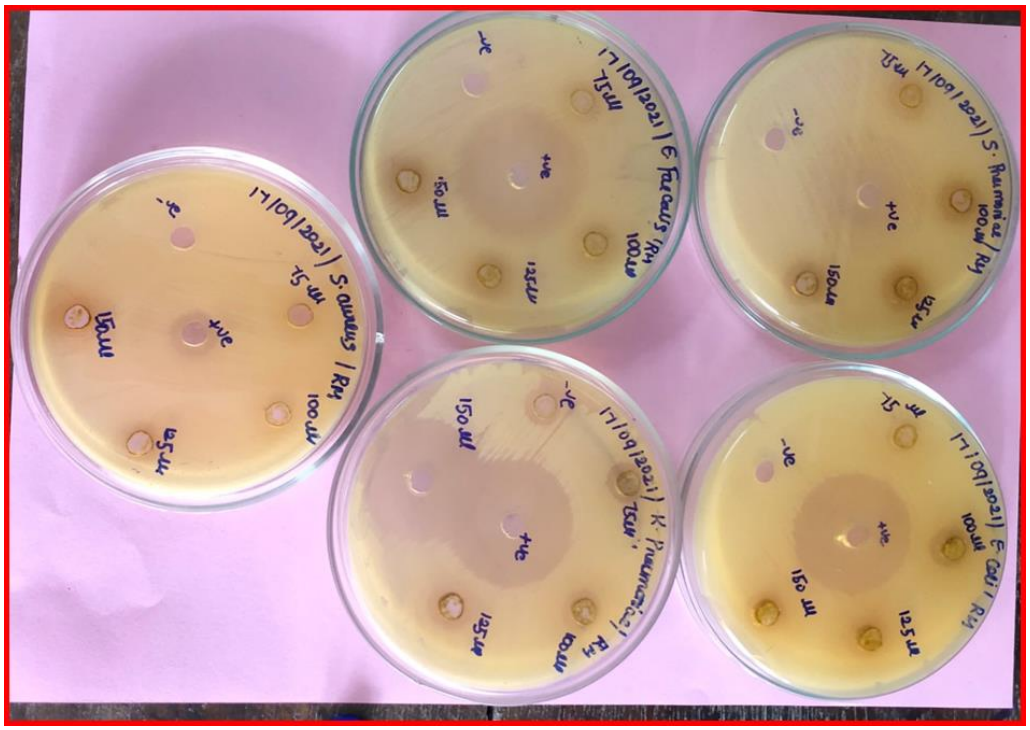

Fig 1: zones of inhibition of MERE against *E.faecalis, S.pneumonia, K.pneumonia, S. aureus* and *E.coli.*

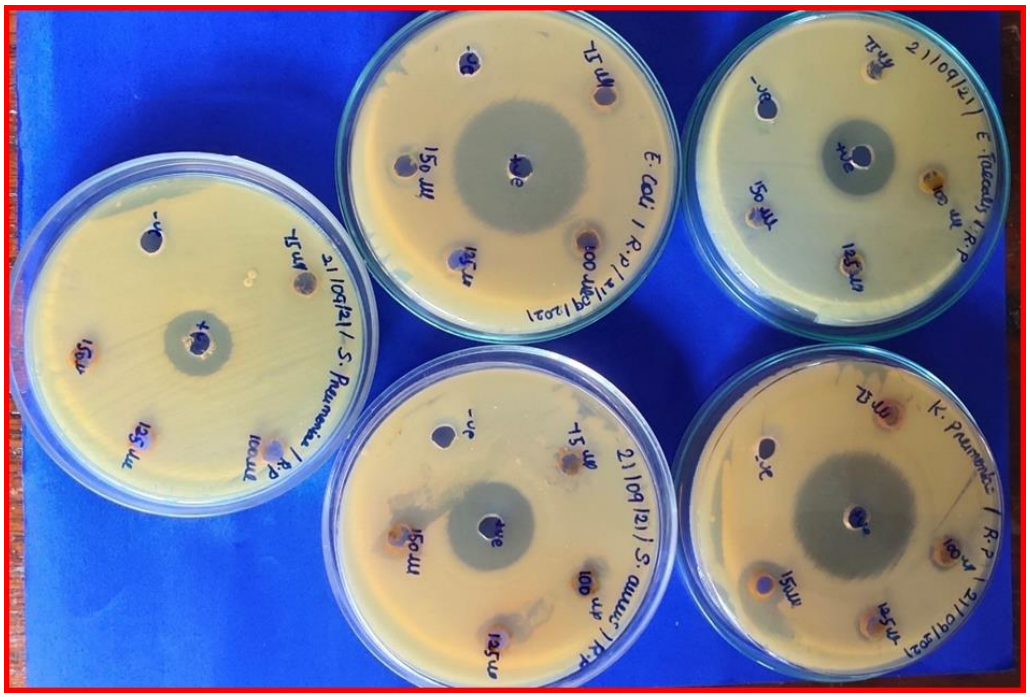

Fig 2: zones of inhibition of PERE against *E.faecalis, S.pneumonia, K.pneumonia, S. aureus* and *E.coli.*

Table 3: Zones of inhibition of F4 of MERE.

Sample concentration (µl/ml)							
Extract	Bacterial strains	75	100	125	150	Pc	Nc
MERE	*S.aureus*	9	11	12	15	22	ND
	E. facaelis	6	7	9	14	32	ND
	K. pneumonia	6	8	9	12	30	ND
	E. coli	6	8	9	12	30	ND
	S. pneumonia	7	10	12	15	23	ND

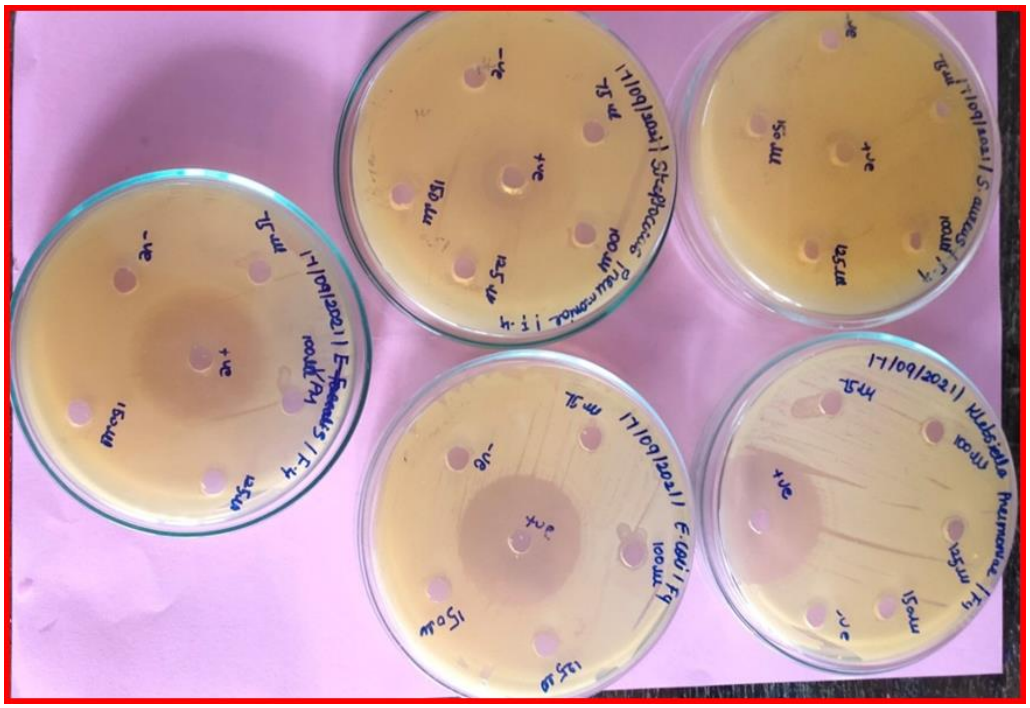

Fig 3: Zones of inhibition of F4 against *E.faecalis, K.pneumonia, S. aureus, S. pneumonia* and *E.coli.*

3.1.2. FT-IR spectral analysis

FT-IR spectra and functional group of compounds present in *Prunella vulgaris* methanol and petroleum ether extract are shown in figure (4) and (5) respectively. Spectral analysis of methanol and petroleum ether extracts revealed almost identical functional groups. Spectral data revealed the presence of bioactive functional groups like alkanes (C–H stretching, C–H rock), alkenes (–C=C– stretching, =C–H bending), alkynes (–C≡C–H: C–H stretching), 1˚, 2˚ amines and amides (N–H stretching), α,β–unsaturated aldehydes and ketones (C=O stretching), 1˚ amines (N–H bending), aromatic (=C–C in–ring), aliphatic amines (C-N stretching), alkyl halides (C–Cl stretching). IR absorption frequencies and the representative spectra are shown in Table 4 and Fig 4, 5. However, it is premature to mention that results obtained in FT-IR alone are adequate to prove the existence of compound classes.

Table 4: Major bands observed in the FT-IR spectra of MERE and PERE.

Major bonds					
S. No	**IR ν_{max}(cm^{-1}) (Vibration mode)**			**Possible nutrient**	**Phytochemicals**
	Frequency (m^1)	**Bond**	**Functional groups**		
1	670-780	C–H rock, C–Cl stretch, C–H oop, N–H wag	Alkanes, alkyl halides, aromatics, 1˚, 2˚ amines	carbon	Alkaloids, flavonoids, tannins, poly phenols and carboxylic acid containing phytochemicals
2	810-880	C–Cl stretch, C–H oop, N–H wag, =C–H bend	alkyl halides, aromatics, 1˚, 2˚ amines, alkenes	carbon	
3	1030-1090	C–H wag(–CH2X), C– stretch, C–N stretch	alkyl halides, alcohols, carboxylic acids, esters, ethers, aromatic amines	carbohydrate	
4	1110-1165	C–H wag(–CH2X), C–O stretch, C–N stretch	alkyl halides, alcohols, carboxylic acids, esters, ethers, aromatic amines	protein	
5	1245-1260	C–N stretch, C–H wag (–CH2X), C–O stretch	aromatic amines, alkyl halides, alcohols, carboxylic acids, esters, ethers,	protein	
6	1375-1396	C–H bend	Alkanes	Protein and collagen	
7	1435-1460	C–H bend,= C–C stretch (in–ring)	Alkanes, aromatics	Protein and collagen	
8	1520-1595	N–H bend	1˚ amines	protein	
9	1680-1685	N–H bend, –C=C– stretch	1˚ amines, alkenes	protein	
10	1705-1710	C=O stretch,	α, β–unsaturated aldehydes, ketones	fat	
11	2109-2110	C≡C stretch	Alkyne	Amino related component	
12	2340-2345	NH stretch	Amino related component	Amino related component	
13	2850-2855	C–H stretch, O–H stretch	Alkanes, carboxylic acids	Carbon	
14	2919-2925	C–H stretch, O–H stretch	Alkanes, carboxylic acids	Carbon	
15	3244-3280	N–H stretch, O–H stretch (H–bonded)	1˚, 2˚ amines, amides, alcohols, phenols	water	
16	3500-3505	O-H stretching	Hydrogen bonding typical	water	

Fig 4: FT-IR spectrum of MERE

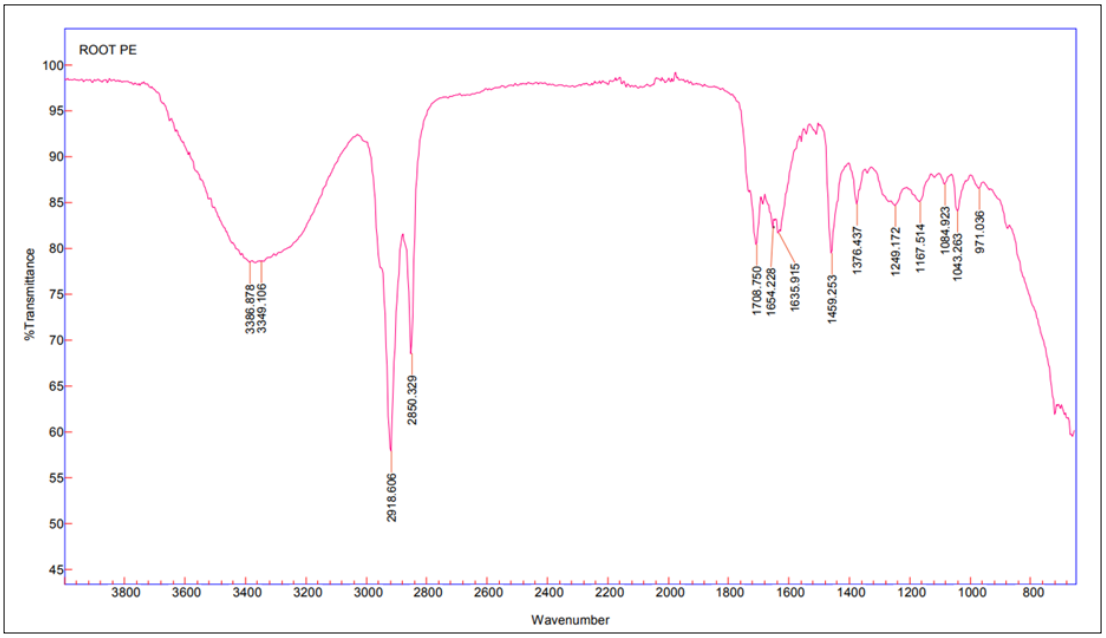

Fig 5: FT-IR spectrum of PERE

Conclusion

Results from the study revealed that MERE and PERE of *Prunella vulgaris* contain significant antioxidant and antimicrobial potential, hence is a competent and easily available source of natural bioactive compounds. However, vulnerability against the pathogens was observed to be more in MERE as compared to PERE and radical scavenging potential was also found to be more in MERE than that of PERE. Hence, in the present study it was reported that the root extract of *Prunella vulgaris* possess an appreciable amount of new antioxidants and antimicrobial compounds. Hence, the fractions showing better purity on TLC and maximum antimicrobial activity were selected for further experimentation to isolate the anti-bacterial and antioxidant compounds from MERE using different spectroscopic techniques.

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