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In vitro pharmacological activities of different extracts of *Launaea procumbens* (Roxb.) aerial parts

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Abstract

Introduction: *Launaea procumbens* (Roxb.) (Asteraceae) is a widely distributed herb throughout India. This plant has been utilized as traditional medicine in various traditional systems of medicines. It is used in the treatment of various diseases like rheumatism, kidney disorder, hepato-protection etc.

Methods: Powdered stem and leaves of LP were sequentially extracted with different solvents in Soxhlet extraction apparatus and analysed for their pharmacological activities.

Results and Discussion: The methanolic, acetonic and chloroform extract showed more promising antimicrobial as well as antioxidant activity as compared to petroleum ether extract. Maximum number of phytochemicals was detected in case of methanolic extract followed by the chloroform extract. Total phenolic and flavanoid contents were higher in chloroform and methanol extract, respectively.

Conclusion: As methanolic and chloroform extract showed good antimicrobial and antioxidant potential these extracts can be utilized as an effective and safe antioxidant source, as an ethno-medicine and on a commercial basis for the development of drugs.

Keywords: *Launaea procumbens*, biological, phytochemicals, traditional

1. Introduction

Plants have always played an important role as a source of medicine for treatment of various human ailments. They have been an important source of novel pharmacologically active compounds with many blockbuster drugs being derived directly or indirectly from them [1]. It is estimated that nearly 25% of pharmaceuticals contain plant-derived ingredients yet only a small percentage of the plants have been investigated for potential phytodrugs. Due to the contribution of plants to disease treatment and prevention, in recent years increased efforts to survey more and more plants have stepped up the pace of the discovery of new bioactive compounds. It is often estimated that more than 80% of the world's population are dependent on plants and herbal remedies for their primary source of health care because of their tested safety and efficacy [2]. Therefore, Plants used in ethnomedicine are of great significance and represent a great source of novel leads for drug development.

Launaea belongs to the family Asteraceae and comprises about 40 species. *Launaea procumbens* (Roxb.) is a widely distributed perennial herb and used in folk medicine as hepatoprotective, bitter stomachic, for skin diseases, as antitumor and as insecticides. The plant is a rich source of secondary metabolites and possess triterpenes, sesquiterpene lactones, steroids, and flavonoids [3-4-5-6]. Chemical characterization revealed the presence of salicylic acid, vanillic acid, synergic acid [7], 2- methyl-resorcinol and gallic acid in LP [8] as well as phenolic and polyphenolic compounds [9]. These compounds are reported to have anti-carcinogenic, anti-inflammatory, hepatoprotective and antioxidant properties [10].

Based on the literature survey and its ethanopharmacological importance, the present study was designed to assess the different extracts of aerial vegetative parts of LP for antimicrobial and antioxidant potential in relation to its phytochemicals and total phenolic and flavanoid content.

2. Materials and Methods

2.1 Preparation of plant extracts

Fresh stem and leaves of LP were collected and after washing plant material was dried at room temperature till the constant weight was obtained. 50g powder of plant material was extracted sequentially (1:5W/V) in a Soxhlet assembly by using four different solvents i.e. petroleum ether, chloroform, acetone and methanol. All the plant extracts were then separately concentrated by using a rotary evaporator at temperature (40 - 50°C) and stored at 4°C till further use.

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2.2 Phytochemical Screening

The plant extracts prepared in different solvents were screened qualitatively for the presence of various phytochemicals [11-13].

2.3 Total phenolic and flavonoid content (TPC and TFC)

Total phenolic and flavonoid content of the plant extracts was determined by Folin-Ciocalteu method [14]. To the 1mg/ml solution of each plant extract, 5ml of Folin-Ciocalteu and 2ml of Na₂CO₃ was added. The solution was vortexed and incubated in dark for 15min. Absorbance was measured at 620nm. Gallic acid was used as standard. The total phenolic content was calculated from calibration curve ($y = 0.0164x + 0.0557$, $R^2 = 0.9964$) and result was expressed in terms of mg of gallic acid equivalent (GAE)/ gram dry weight of sample. Total flavonoid content of the plant extracts was estimated by Aluminium Chloride Colorimetric method [14]. In the 3ml of methanol, 0.2ml of 10% aluminium chloride, 0.2ml of 1M potassium acetate and 5.6ml of distilled water were added to plant extract of 1mg/ml concentration. The mixture was vortexed and left at room temperature for 30 minutes. The absorbance was taken at 420nm. Quercetin was used as standard. Flavonoid content was determined from the calibration curve ($y = 0.0047x + 0.0391$, $R^2 = 0.996$) and results were expressed as mg of quercetin equivalent (QE)/ gram dry weight of sample. All tests were performed in triplicates.

2.5 Antimicrobial studies

Antimicrobial potential of all the four plant extracts was evaluated by disc diffusion method and micro broth dilution assay against six bacterial and four different fungal strains.

2.5.1 Preparation of plant samples

All the extracts were re-constituted in DMSO to obtain the concentrations of 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml; 200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml for antibacterial and antifungal studies, respectively.

2.5.2 Inoculum preparation

The antimicrobial activity of plant extracts against six bacterial strains (*Staphylococcus aureus* (MTCC-96), *Bacillus subtilis* (MTCC-2057), *E. coli* (MTCC-41), *Streptococcus pyogenes* (MTCC-890), *Pseudomonas aeruginosa* (MTCC-2453) and *Chromobacterium violaceum* (MTCC-2656) was assessed. Four different fungal strains used were *Aspergillus niger* (MTCC- 3002) *Rhizopus oryzae* (MTCC-262) *Fusarium oxysporum* (MTCC- 7392) and *Penicillium expansum* (MTCC- 2818).

Firstly broth was prepared by dissolving the Hi-media nutrient broth (6.5 gm) in distilled water (500ml) and it was sterilized by autoclaving at 121° C for 15 minutes. A loopful sterilized powder of a particular bacterial/fungal strain was added to this 10 ml of sterile nutrient broth and incubated at 37°C for 24 hours. After this, cultures were adjusted to the 0.5 McFarland (1.5×10^8 CFU/ml) and used for the experiments [15].

2.5.3 Disc diffusion method

For assessing the antibacterial activity of plant extracts 6mm sterilized discs (Whatman paper no. 3) were kept over the nutrient agar media (Hi-media) petriplates already spreaded with 100µl bacterial inoculums. 10µl plant extract of different concentration was loaded on the discs. Ampicillin, chloramphenicol (0.1mg/ml) was taken as positive controls and DMSO was taken as negative control. However for the

determining the antifungal activity similar procedure was followed except the media used were CZ and PDA and fluconazole (0.1mg/ml) was used as positive control. The volume of fungal inoculums was 20µl on the media.

Petriplates were then incubated at 37°C in BOD incubator for, after incubation each petriplate was examined and diameter of the ZOI was measured. Each assay was repeated thrice. Method was subjected to antibiotic sensitivity tests by the Kirby-Bauer's disc diffusion method (1996) [16].

2.5.4 Minimum inhibitory concentration (MIC)

MIC's of plant extracts was determined by micro broth dilution assay using two fold serial dilutions [17]. 100 µl of nutrient broth and 100 µl plant extracts was added to each well of a 96 well microtitre plate (12x8). 10µl of standardized inoculums (1.5×10^8 CFU/ml) and 10µl of rezazurin dye (0.2% W/V) were added in each well and petriplates were incubated at 37°C for 24hrs for the bacterial MIC.

However rezazurin was not added in case of fungal MIC and petriplates were incubated for 72 hrs. The lowest concentration that inhibited the test organism was recorded as MIC.

2.6 Non enzymatic antioxidant potential

Non enzymatic antioxidant potential of plant extracts was determined by DPPH and ABTS assays.

2.6.1 DPPH and ABTS Assay

The free radical scavenging activity of plant extracts were measured using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay [18] and ABTS(2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay. For assessing antioxidant activity by DPPH assay 1mg/ml stock solution of plant extract in their respective solvents were diluted from 10-100 µg/ml. One ml of 0.3mM DPPH solution was added to 1ml of the plant extract of different concentrations and also to the standard (Ascorbic acid). Samples were incubated in dark for 30minutes. The absorbance was measured at 517nm by UV-Vis spectrophotometer (SHIMADZU 1800, Tokyo, Japan). ABTS assay was also used to carry out the antioxidant potential of plant extracts [19]. ABTS radical (ABTS+) was produced by reacting 7mM ABTS solution with 2.45 mM ammonium per sulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS solution was diluted with ethanol to get an absorbance of 0.700 at 745 nm. A 1mg/ml stock solution of each plant extract in their respective solvents and also the standard (Ascorbic acid) were prepared. These were diluted from 10-100 µg/ml. One ml solution of ABTS was added to the 1ml of plant extracts of different concentration. The absorbance was measured at 745nm.

The assays were performed in triplicates and mean of the data were recorded. The scavenging activity of the extracts was calculated in % inhibition according to the formula given below:

$$\% \text{ Inhibition} = (\text{Abs control} - \text{Abs sample}) / \text{Abs control} \times 100$$

2.7 Enzymatic assays

2.7.1 Extraction of plants for enzymatic Assays

0.5 g fresh plant material (stem and leaves) was collected and immediately it was transferred into liquid nitrogen and brought to the lab. Plant extract was prepared by homogenization of plant material in 5ml of extraction buffer

which contained 100mM potassium phosphate buffer (pH-7.0) and 0.1mM sodium ethylene di-amine tetra acetic acid (EDTA). The homogenate was filtered through muslin cloth and centrifuged at 14000 rpm for 20 minute at 4°C. The supernatant obtained was used for all the enzymatic assays.

2.7.2 SOD assay

3ml reaction mixture contained 50mM potassium phosphate buffer, 13mM methionine, 75 mM NBT, 2µM riboflavin, 0.1 mM EDTA and 0.1ml plant extract was transferred into test tubes. These test tubes were placed in the light intensity of 5000 Lux for 25 min. The absorbance was measured at 560 nm. Reaction mixture placed in dark served as the control. One unit of SOD activity can be defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT per unit time at 560nm [20].

2.7.3 Catalase assay

3ml reaction mixture containing 0.1ml plant extract, H₂O₂ (10mM), and potassium phosphate buffer (50mM) of pH-7.0), absorbance was taken continuously for 5 min. at 240 nm in every 30 seconds. Enzyme activity was expressed as µmoles of H₂O₂ decomposed mg-1 fresh weight min-1. One unit of catalase is the decomposition of 1.0 µmole of H₂O₂ per minute at pH 7.0 at 25 °C [21] (EC of H₂O₂ is 39.4 mM-1cm-1).

2.7.4 GST assay

3 ml reaction mixture contained potassium phosphate buffer (97mM) of pH-6.5, 1mM EDTA (in DW), CDNB (30mM) (prepared in 95% ethanol) and GSH 75 mM (prepared in Buffer) was prepared. The absorbance of the mixture was taken at 340 nm till 5 min. for every 30 sec. One unit of GST activity is the amount of enzyme which produces 1.0 µmol of GS-DNB conjugate per min [22] (EC of GS-DNB conjugate at 340 nm is 9.6 mM-1cm-1).

3. Statistical analysis

Antioxidant activity (IC 50 values) and total phenolic and flavonoid content were correlated using Pearson's correlation coefficient. Statistical analysis was performed using the SPSS version 16.0. Data are presented as means ±SD and P values of less than 0.05 were taken as statistically significant.

4. Results and Discussion

4.1 Phytochemical analysis (Table-1)

All the four plant extracts of LP prepared in different solvents were screened for the presence of various phytoconstituents. Phenols, flavonoids and alkaloids were present in all the plant extracts. Methanolic extract showed the maximum number of phytochemicals.

Table 1: Phytochemical analysis of plant extracts of LP prepared in different solvents.

S.No	Phytochemicals	Plant extracts			
		Methanol	Acetone	Chloroform	Petroleum ether
1	Alkaloids	+	+	+	+
2	Flavanoids	+	+	+	+
3	Glycosides	-	-	-	-
4	Phenols	+	+	+	+
5	Saponins	+	+	-	-
6	Steroids	-	-	+	+
7	Terpenoids	-	-	+	+

** (+) indicate the presence of phytochemicals, (-) indicate the absence of phytochemicals

4.2 Total phenolic and flavonoid content (Table-2)

Maximum TPC (113.94±1.2) and TFC (82.6±0.10) were observed in methanolic extract followed by acetonetic and chloroform extracts (Table -2). The least value of TPC (42.52± 0.21) and TFC (23.08± 0.11) was observed in petroleum ether extract.

Table 2: IC-50 values of different extracts of LP for various antioxidant systems

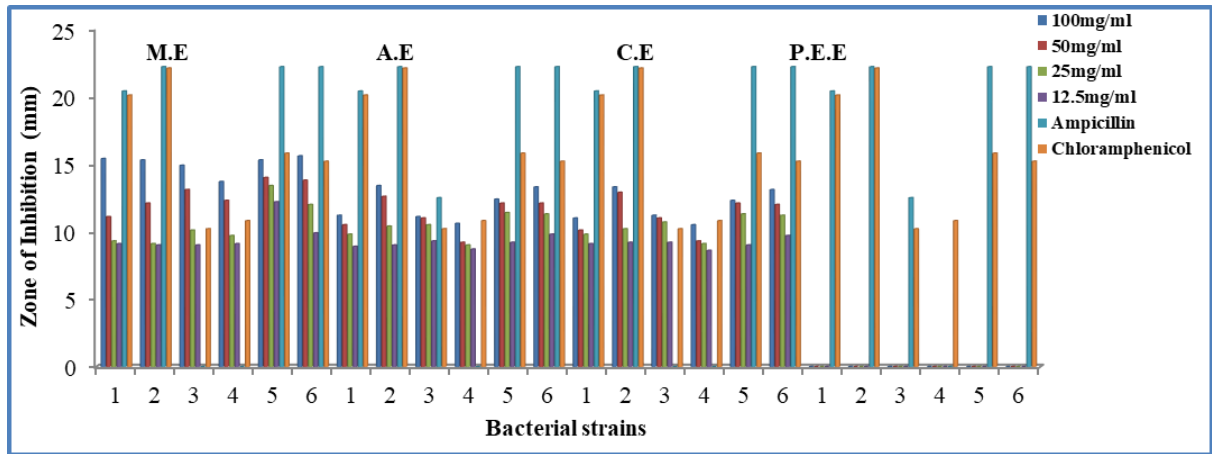
Plant extracts of LP	TPC	TFC
Methanol	113.94±1.2	82.6±0.10
Acetone	85.48±0.4	71.12±0.21
Chloroform	84.98±1.0	69.2±0.11
Petroleum ether	42.52±0.9	23.08±1.3

Each value in the table is represented as three biological mean ± SD (n=3)

4.3 Antibacterial activity (Disc diffusion assay) (Figure-1)

ZOI (mm) obtained with different plant extracts against different bacterial strains are graphically represented in

Figure- 1. Methanolic extract showed strong inhibition against all the bacterial strains. The maximum ZOI was obtained with 100 mg/ml conc. against *Escherichia-coli* (15.6 ± 0.21mm) followed by *Bacillus subtilis* (15.4 ± 1.02mm). The reason for methanolic extract to be most effective could be the presence of diverse phytochemicals. Quantitative analysis also indicated highest phenolic and flavonoid content (table - 2) in the methanolic extract, which comply to the statement that methanol, is undoubtedly considered the best solvent for extraction of some phytoconstituents due to its fair solubility and polarity [23]. Acetonetic and chloroform extract also showed good activity against all bacterial strains with 13.4± 0.02mm and 13.3± 0.06mm ZOI, respectively against *staphylococcus pyogens*. Petroleum ether extract did not show any ZOI against the bacterial strains used in present investigation. Maximum ZOI recorded with the positive controls i.e. Ampicillin and Chloramphenicol was 22.2±0mm and 22.1±0mm against *C. violaceum* and *S. pyogens*, respectively. *P.aeruginosa* and *S. aureus* were found to be resistant with one of the positive control i.e. Ampicillin.



*1. *Bacillus subtilis*, 2. *Streptococcus pyogenes*, 3. *Staphylococcus aureus*, 4. *Pseudomonas aeruginosa* 5. *Chromobacterium violaceum* 6. *Escherichia coli*.

** M.E methanol extract; A.E- acetic extract; C.E- chloroform extract; P.E.E- petroleum ether extract.

Fig 1: ZOI obtained with different extract of LP and positive controls against different bacterial strains.

4.4 MIC values of different extract of LP (mg/ml) (Table-2)

MIC values shown in table-2 are in the range of 3.125-6.25 mg/ml in case of methanolic extract where as comparatively higher MIC range was obtained in case of acetic (3.125-6.25mg/ml) and chloroform extract (1.56-3.125 mg/ml). MIC

values of petroleum ether were of maximum and didn't show satisfactory values against the bacterial strains. Positive control ampicillin showed MIC ranging from 0.025-0.1mg/ml. *P. aeruginosa* was found to be resistant against to ampicillin. MIC values obtained with chloramphenicol were in the range of 0.025-0.1mg/ml.

Table 2: MIC values of plant extracts of LP and positive controls i.e. Ampicillin and Chloramphenicol against different bacterial strains.

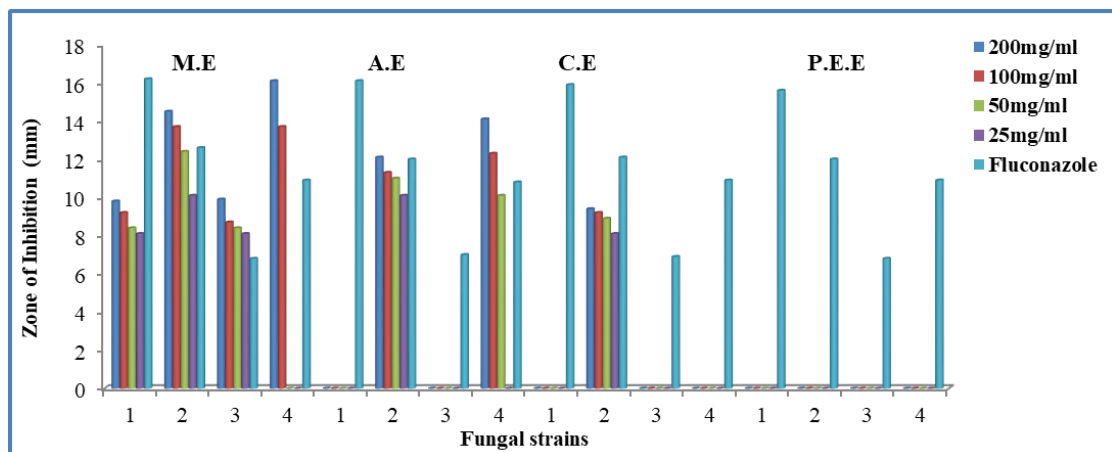
Microrganisms	M.E	A.E	Extracts C.E	P.E.E	A	C
<i>B. subtilis</i>	3.125	3.125	3.125	50	0.025	0.1
<i>S. pyogenes</i>	3.125	6.25	3.125	50	0.025	0.1
<i>S. aureus</i>	6.25	6.25	3.125	NA	N.A	0.1
<i>C. violaceum</i>	6.25	6.25	12.5	NA	0.1	0.1
<i>E. coli</i>	3.125	3.125	1.56	50	0.1	0.025
<i>P. aeruginosa</i>	6.25	6.25	3.125	NA	N.A	0.05

**MIC (Minimum Inhibitory Concentration) values are expressed as mean (n=3), Unit is mg/ml; M.E methanol extract; A.E- acetic extract; C.E- chloroform extract; P.E.E- petroleum ether extract; A- Ampicillin; C- Chloramphenicol; NA- no activity; Ampicillin and Chloramphenicol were used as dilutions from 0.1mg/ml up to all 12 wells.

4.5 Antifungal activity of LP (Disc Diffusion Assay) (Figure-2)

Methanolic extract showed significant ZOI (shown in figure - 2) against all the strains followed by acetic and chloroform extract whereas petroleum ether extract exhibited no activity against any fungal strains. The most susceptible strain was *R. oryzae* as it showed ZOI of 16.1 ± 0.26 mm. *A. niger* and *F. oxysporum* were found to be most resistant to all the extracts

except methanolic extract. Yam, Nostro and Duarte [24-25-26] also reported that fungi and gram negative bacteria are more resistant to plant extracts than gram negative bacteria. Yam *et al.*, (1997) reported antibacterial activity of camellia against pathogenic bacteria, including methicillin-resistant *S. aureus*. Tea extracts were bactericidal to *Staphylococci* and *Yersinia enterocolitica* even at very low concentrations.



*1. *Aspergillus niger* 2. *Penicillium expansum* 3. *Fusarium oxysporum* 4. *Rhizopus oryzae*.

** M.E methanol extract; A.E- acetic extract; C.E- chloroform extract; P.E.E- petroleum ether extract.

Fig 2: ZOI obtained with different extract of LP and positive control against different fungal strains.

4.6 MIC values of plant extracts of LP (mg/ml) (Table -3)

MIC's of different extracts of LP obtained are presented in table -3. Methanolic, acetic and chloroform extract exhibited the significant antifungal activity against all the fungal strains displaying values in range of 3.125-12.5mg/ml, 3.125-6.25 mg/ml and 3.125-6.25 mg/ml, respectively. Petroleum ether extract showed activity (25mg/ml) only against *R. oryzae* out of four fungal strains and acetone didn't show any activity.

Table 3: MIC values of plant extracts and of positive control (Fluconazole) against different fungal strains

Microorganism	Extracts				Fluconazole
	M.E	A.E	C.E	P.E.E	
<i>Penicillium expansum</i>	6.25	6.25	6.25	NA	0.00625
<i>Fusarium oxysporum</i>	3.125	NA	NA	NA	0.00078125
<i>Rhizopus oryzae</i>	6.25	3.125	3.125	25	0.00078125
<i>Aspergillus niger</i>	3.125	NA	NA	NA	0000.05

**MIC (Minimum Inhibitory Concentration) value are expressed as mean (n=3), Unit is mg/ml; M.E methanol extract; A.E- acetic extract; C.E-chloroform extract; P.E.E- petroleum ether extract; NA- no activity; Fluconazole was used as dilutions from 0.1mg/ml upto all 12 wells.

4.7 Non enzymatic assays

4.7.1 DPPH assay [Table -4]

The use of ABTS and DPPH assay provides an easy and rapid way to evaluate the non enzymatic antioxidant activity. The highest antioxidant activity was obtained with the methanolic extract as it showed least IC- 50 value i.e. 58.7 ± 0.09 (DPPH); 41.7 ± 1.1 (ABTS) (Table-4) in both the antioxidant system followed by acetic extract and chloroform extract. The least antioxidant activity with highest IC-50 value (91.9 ± 0.52 DPPH); (108.2 ± 0.29 ABTS) was exhibited by the petroleum ether extract. The results suggests that the strong antioxidant

activity of methanolic extract might be due to the presence of phenolic and flavonoid compounds in higher amount as compare to the other extracts. The scavenging ability of the phenols is mainly, because of the presence of hydroxyl groups. Flavonoids are a large group of compounds occurring ubiquitously in food plants. They occur as glycosides and contain several phenolics hydroxyl groups in their ring structure, capable of antioxidant activities [7]. Methanolic extract of LP is more potent compared to other extracts and is in accordance with previous reports also [27-28-29]. From our results, it is inferred that highest TPC (113.94 ± 1.2 ; Table- 4) and TFC (82.6 ± 0.10) were present in methanolic extract of LP. Our results were on the same pattern of a previous report of Cao *et al*, 2009 [30]. On a similar pattern of antimicrobial results, antioxidant potential was also least in case of petroleum ether extract.

Table 4: IC-50 values of different extracts of LP for various antioxidant systems

Plant extracts of LP	DPPH (IC-50)	ABTS (IC-50)
Methanol	58.7 ± 0.09	41.7 ± 1.1
Acetone	74.2 ± 0.21	47.1 ± 0.5
Chloroform	81.1 ± 0.39	49.9 ± 0.49
Petroleum ether	91.9 ± 0.52	108.2 ± 0.29

Each value in the table is represented as three biological mean \pm SD (n=3)

4.8 Correlation between antioxidant activities IC₅₀ Values, total phenolic and flavonoid content (Table- 5)

Correlation (Pearson correlation coefficient = r) between the IC₅₀ values and total phenolic and total flavonoid content of the extracts are shown in the Table -5. There is a positive correlation between the TPC and TFC and negative correlation is there between IC- 50 values and TPC/TFC which means a low IC-50 value is having high antioxidant potential and vice versa.

Table 5: Correlation between IC₅₀ Values, total phenolic and flavonoid contents

Pearson correlation coefficient (r)				
TPC & TFC	TPC & IC ₅₀ of ABTS	TFC & IC ₅₀ of ABTS	TPC & IC ₅₀ of DPPH	TFC & IC ₅₀ of DPPH
0.968*	-0.930	-0.993**	-0.953*	-0.870

* Correlation is significant at the 0.01 level (2 tailed).

** Correlation is significant at the 0.01 level (2 tailed).

4.9 Enzymatic assays

The enzyme activity was found to be 0.301 ± 0.16 μ mole of H₂O₂ per minute FW for Catalase and 1.210 ± 0.31 μ mol of GS-DNB conjugate/min FW for Glutathione-S- transferase while in case of SOD value was found to be 36.12 ± 0.21 SOD(Unit) FW for Superoxide dismutase respectively (Unit activity definitions are mentioned in materials and methods section. Catalase, GST and Superoxide dismutase are the key enzymes that become involved in cellular defence against reactive oxygen species in living organisms; hence these are an important indicator of antioxidant capacity [31]. In a previous report of Khan, (2012) activity of basic antioxidant enzymes like Catalase, SOD and GST in rats was measured with and without supplementation of LP for 7 days, where LP was given it improved the activity of these enzymes as compared where it was lacking, showing protection against free radicals. Our activity level of this enzyme in LP is also comparable to this finding [32].

5. Conclusion

The results obtained in the present investigation have

significant value with respect to the antioxidant potential and antimicrobial activities especially of methanolic extract. The presence of these activities may be attributed to the high amount of phenolic and flavonoid compounds present in this extract. Our study suggested that the methanolic extract can be utilized as an effective and safe antioxidant source, as an ethno-medicine and on a commercial basis for the development of drugs. Further, some bioactive elements from the plant should be isolated and assessed for their medicinal properties.

6. Summary

- LP also called as jungle booti is a species of plant in the family Asteraceae. The plant also has been utilized as traditional medicine in various traditional systems of medicine, used in the treatment of rheumatism, kidney disorder, hepato-protection etc.
- LP was extracted in different solvent (Methanol, acetone, chloroform, petroleum ether) by using the soxhlet apparatus.
- These extracts were further analysed for various biological activities like antimicrobial activity,

antioxidant potential in relations to the phytochemicals, total phenolic and flavonoid contents of LP.

- The methanolic and chloroform extract showed more promising antimicrobial activity as well as antioxidant potential as compared to acetonic and petroleum ether extract. Maximum number of phytochemicals were detected in case of methanolic extract followed by chloroform extract. The total phenolics and flavanoids were higher in chloroform and methanol extract, respectively.
- The present investigation provides information on the antioxidant and antimicrobial activities in relation to the phytochemicals, total phenolic and flavonoid contents of LP.

Abbreviations used:

LP: *Launaea procumbens*

TPC-Total phenolic content

TFC-Total flavonoid content

MIC-Minimum inhibitory concentration

ZOI-Zone of inhibition

PDA- Potato dextrose agar

CZ- Czapek dox

P.E-Plant extracts

DMSO-Dimethyl sulfoxide

EC-Extinction coefficient

FW- Fresh weight

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