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Influence of cultural conditions on comparative estimation of various phytoconstituents and their effects on antibacterial activity of *Euphorbia hirta* L. leaves extracts

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Abstract

Euphorbia hirta L. (EH) (Family: Euphorbiaceae) is a pantropical weed, native to India. The present study aimed to extract leaves of EH for comparative estimation of various phytoconstituents and their effects on antimicrobial activity, procured from Karnataka and West Bengal geographical locations. Dried leaves were estimated for macro and micronutrient along with other heavy metal contents. Methanol and aqueous extracts were prepared using hot maceration method and screened for presence of various phytochemicals. The results revealed alkaloids, flavonoids, tannins, steroids, glycosides and carbohydrates and phenols in leaves in higher amount collected from West Bengal. Based on that, further total alkaloid, flavonoids and phenolics were estimated and then antibacterial activity was carried out using the agar well diffusion method (Different concentrations of crude drugs such 50 µg/ml, 75 µg/ml, and 100 µg/ml) against two gram-positive bacteria, *viz*., Bacillus subtilis ATCC 6633, *Straphyloccus aureus* ATCC 29726, and *Pseudomonas aeruginosa* ATCC 25619, and *Escherichia coli* ATCC 8739, a group of gram-negative bacteria that frequently cause enteric infections in humans. Minimum inhibitory concentration (MIC) value ranged from 25 to 100 mg/ml. Interestingly, the activity was dependent on content of elements, type of extract, dose as well as source of the collection of leaves. Resulted higher antimicrobial activity of methanolic extract collected from West Bengal soil zone and confirmed its potential broad spectrum antimicrobial activity due to the positive correlation of content of metal ions and soil nature with the sample.

1. Introduction

Medicinal plants have been utilized in medicine from the time immemorial. Global studies have been conducted to test their efficacy, with some of the findings leading to the development of plant-based medicines. The global industry for medical plant products is worth more than $100 billion each year (Sofowora *et al*., 2013). Therefore, the role of medicinal herbs cannot be ignored. They have an important role in illness prevention, and their promotion and usage are compatible with all current disease preventive techniques. India is known for its Ayurvedic treatment from the ancient time onwards and about 90% of traditional medicine remedies contain medicinal plants. Ultimately, it was established that any efficacy of the herbals are mainly due to the presence of the bioactive components in the plant bodies which are commonly known as secondary metabolites.

Of late, *Euphorbia hirta* L. (EH) belongs to the plant family Euphorbiaceae, is very common plant in India and in fact it is considered as a weed which is often found in waste places along the roadsides, distributed throughout the hotter parts in tropical and subtropical countries, especially in India (Kirtikar and Basu, 1998). The plant is often used traditionally for female disorders, respiratory ailments (cough, coryza, bronchitis, and asthma), and worm infestations in children, dysentery, jaundice, pimples, gonorrhea, digestive problems, tumors, and measles (Kirtikar and Basu, 1998). The plant is used as antimicrobial, anti-inflammatory, antiamoebic, antifertility, antimalarial, antioxidant, sedative, cytotoxic, aflatoxin inhibition, larvicidal, immunomodulatory and so on (Verma, 2017). The activities are mainly due to the plant secondary metabolites that are present in EH especially flavonoids (quercetin, quercitrin, quercitol) (Yan *et al*., 2011), terpenoids (triterpenes: α-amyrin, β-amyrin, friedelin, taraxerol) (Baslas and Agarwa, 1980), phenols, tannins (dehydro-ellagi-tannins-euphorbins A, B, C, E, and terchebin, the monomeric hydrolysable tannins-geraniin) (Ogunlesi *et al*., 2009), and essential oil. It is also evident that plant constituents are varied with the geographical location, climatic conditions, soil nature and many other factors (Liu *et al*., 2016; Kumar *et al*., 2017; Bisht *et al*., 2018; Das *et al*., 2019). Not only that, even bioactivity of the plant constituents are also varies with the geographical location (Mangoale and Afolayan, 2020; Vilkickyte and Raudone, 2021). Thereafter, no such reports on related metal ion contents in the leaves in this EH plant and also their correlation with the activity. Based on the concept, the present investigation was carried out to evaluate the impact of geographical location on estimation of bioactive component present and their effects on antimicrobial activity using two different solvents.

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2. Materials and Methods

2.1 Plant collection and processing

The said EH plant leaves were collected from two different geographical locations, *viz.* Kalyani, West Bengal (WB) (The latitude is 22.9750^0 N, and the longitude is 88.4345^0 E) and Bangalore, Karnataka (KAR) (The latitude is 12.9724^0 N, and the *longitude* is 77.5806^0 E). The leaves of EH was identified and authenticated (Herbarium No.: PCOG-317/KCP/2020-21) by the Principal Scientist, Dr. P.E Rajasekharan, Indian Institute of Horticultural Research, Bangalore.

After collection of the plants, the leaves were cleaned in running water separately and then shade dried for 15 days with proper precaution to avoid microbial contamination. Further, the leaves were grinded separately by mixer grinder and kept for dust proof sealed plastic cover for the experimentation purpose.

Chemicals used

Concentrated H_2SO_4, and $HClO_4$ (LR grade, procured from SD Fine Chemicals, Mumbai, India), Agar (Hi Media), Methanol (procured from SD Fine Chemicals, Mumbai, India), Standard Ampicillin (procured from SD Fine Chemicals, Mumbai, India), Muller Hinton culture medium (Hi Media Lab, India).

2.2 Microorganisms used

Two gram-positive bacterial strains, *viz.*, *Bacillus subtilis* ATCC 6633, and *Straphyloccus aureus* ATCC 29726, and two gram-negative bacterial strains, namely; *Pseudomonas aeruginosa* ATCC 25619, and *Escherichia coli* ATCC 8739 were used as source of microorganism in the present investigation (Das and Trivedi, 2015). All the organisms were collected from Department of Microbiology, Bangalore University, Bangalore, India. They were grown and maintained by subcultured on nutrient agar medium in Dept. of Pharmaceutical Microbiology, Krupanidhi College of Pharmacy, Bangalore. Stock solution of ampicillin as standard was prepared as 25 µg/ml (w/v) concentration in sterile distilled water (Das *et al*., 2011) and 0.1 ml ampicillin was used as standard for antibacterial activity.

2.3 Phytochemical screening

Stored coarsely powdered samples (150 g) were used for the preparation of extracts by soxhlet method using methanol and water as solvents at 40°C for 7 h. Thereafter, extracted liquids were separately filtered with Whatman No. 1 filter paper. The filtrate was then concentrated by evaporated the solvent in a rotary flash evaporator at 40°C and stored in refrigeration condition (at 4°C) in glass bottles for further experimentation. The yield of the extracts (from the both geographical sources) was estimated separately by following expression and the presence of various phytochemicals was screened qualitatively by various chemical tests (Sazada *et al*., 2009; Khan and Das, 2019).

$$\text{Yield (\%)} = (X_1 * 100) / X_0$$

where,

X_1 = The weight of final extract

X_0 = The actual dry weight of the plant powder

2.4 Metal ion determination

The powdered leaves of EH were analyzed for the content of metal ions using spectroscopic and using Atomic absorption spectrophotometer. Digestion method was followed using concentrated acid mixtures (H_2SO_4:$HClO_4$) for the determination of the metal ions especially Zn, Fe, Cu, Ni, Cd, As, Co, Pb, *etc*., at 200°C until dense white fumes were evolved and finally white residue was obtained. Deionized water was added into the digested sample and the volume made up to 50 ml. Final solutions were analyzed for various heavy metals using an AAS (Perkin Elmer Model: A Analyst 100; Australia). Air acetylene gas was used as oxidant in the AAS and the concentration of the above elements was determined using the standard condition. Based on the concentration ranges of the sample and the linear relation between the absorbance (AU), the final wavelength was fixed and concentration of the determined element against the using blank sample. Results were tabulated by carrying out triplicate analyses for the reproducibility of the method used (Das *et al*., 2019).

2.5 Antibacterial study

2.5.1 Determination of minimum inhibitory concentration (MIC)

The bacterium was inoculated to Muller Hinton culture medium for 24 h to form colony. 1.0 ml of the extract solution at concentrations of 200 µg/ml was added to 1 ml of Nutrient Broth to obtain extract concentrations of 150, 100, 50 and 25 µg/ml for both the separate plants collected from two different geographical locations (Atata *et al*., 2003). In each 9 ml of Nutrient Broth containing standardized test organism of bacterial cells, 0.1 ml of each concentration and 0.5 McFarland turbidity standard (1.0×10^8 cfu/ml) was inoculated (Das, 2014; Saravana Kumar *et al*., 2020) by serial dilution method. All the tubes were then incubated at 37°C for 24 h and finally the growth was observed. Based on the MIC value, further dose was fixed for the study.

2.5.2 Determination of zone of inhibition

All the different extracts were assessed by the agar well diffusion method where each microbe was subculture on the recommended specific media for each microorganism at 35-37°C for 25 h. All the three different concentrated extracts (50 µg/ml, 75 µg/ml, and 100 µg/ml) were sterilized by filtration through a membrane filter. Sterile cock borer (6 mm) was used to prepared discs and 50 µl of each extract were placed separately in the wells of agar plates inoculated with microbial culture. Ampicillin (25 µg/disc) was used as standard. All the plates were incubated at 37°C for 15 h and an antibacterial activity was observed. The zone of inhibition (mm) was calculated by using sliding calipers from the back of the inverted Petri dishes (Kaur *et al*., 2013; Hamid *et al*., 2020). All the readings were taken triplicate to minimize the error.

2.5.3 Correlation study

The percentage yield, metal ion content and the antibacterial activity were correlated for the two different samples and the result was established for the impact of both the cultural conditions on each parameters.

3. Statistical analysis

Analysis of metal ions was calculated by taking mean values of three replicated set of data. Further, antibacterial activities of the two

different zone samples were expressed as the mean ± standard error of mean (SEM) where values of $^{**}p < 0.01$ and $^{*}p < 0.05$ were considered statistically significant based on the one-way ANOVA study. Graph pad prism 5 was used for tabulation of the graphs and antimicrobial activity determination, respectively.

4. Results

4.1 Yield of the extracts

The percentage yields of different extracts procured from two different geographical zones, were separately calculated and the results were depicted in Figure 1. The figure clearly indicated that methanol extract gave more yield in terms of % w/w for both the samples separately [12.3 and 9.8%, respectively for West Bengal (WB) and Bangalore (KAR)].

4.2 Phytochemical screening

Preliminary phytochemical investigations of different extracts of EH leaves were investigated and revealed the presence of alkaloids, flavonoids, tannins, steroids, glycosides and carbohydrates and phenols as major secondary constituents in both the extracts (Table 1).

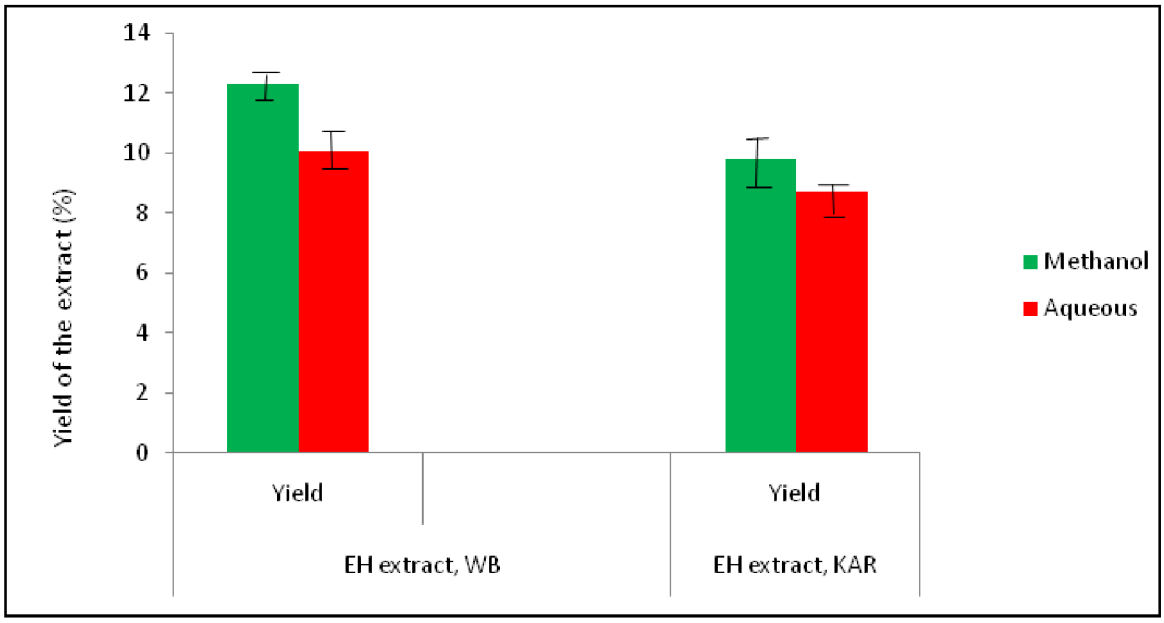

Figure 1: Yield of the extracts in two different solvents; WB = West Bengal; KAR = Karnataka.

Table 1: Phytochemical screening of the extracts

Phytochemicals	EH extract (WB)		EH extract (KAR)	
	Methanol	Aqueous	Methanol	Aqueous
Alkaloids	++	+	+	+
Glycosides	+	+	+	+
Flavonoids	++	+	+	+
Tannins	++	+	+	+
Resins	- -	- -	- -	- -
Steroids	++	++	+	+
Phenolic acids	++	+	+	- -
Proteins	- -	- -	- -	- -
Gums	- -	- -	- -	- -

• (++) = Actively present; (+) = Present faintly; (- -) = Not present

4.3 Elemental analysis

Elements such as Zn, Fe, Cu, Ni, Cd, As, Co, Pb were estimated separately by AAS for the dried leaves of EH, collected from the two different geographical locations and the result was tabulated in Table 2. As per the result, Fe content was higher in West Bengal soil (12.24 mg/kg), followed by Zn and Cu, whereas the same was lesser (Fe, 9.21 mg/kg) and the same trend followed with the Bangalore soil sample. In case of content of non-essential heavy metals such as Cd, Ni, As, Co and Pb, both the samples showed very less or below detectable limits which are very less risk factor (risk assessment code (RAC) of metals in leaves sample was performed as per the procedure described by Singh *et al.* (2005).

Table 2: Elemental analysis of EH leaves from two different soil zones

Parameters	EH leaves (WB)	EH leaves (KAR)
Zn (mg/kg)	7.25 ± 0.02	5.74 ± 0.13
Fe (mg/kg)	12.24 ± 0.01	9.21 ± 0.10
Cu (mg/kg)	0.76 ± 0.04	0.61 ± 0.20
Ni (mg/kg)	0.01 ± 0.11	0.02 ± 0.24
Cd (mg/kg)	ND	0.01 ± 0.04
As (mg/kg)	ND	ND ± 0.10
Co (mg/kg)	ND	0.03 ± 0.03
Pb (mg/kg)	ND	ND

- ND = Not detectable; mean ± SEM; WB = West Bengal; KAR = Karnataka

4.4 Antibacterial study

Both the extracts of West Bengal and Karnataka samples were separately studied for antibacterial activity based on the MIC at 25 µg/ml, and resulted significant dose dependent inhibition (p <0.05) against both the gram-positive and gram-negative microorganisms. The results were slightly lesser than that of standard ampicillin. Methanol extract showed maximum inhibition against gram-positive organism which was similar trend for both the samples but the EH leaves sample procured from Kalyani, West Bengal, showed maximum inhibition against all the pathogens than Bangalore, Karnataka sample. The maximum zone of inhibition showed by WB sample against *Bacillus subtilis* ATCC 6633 (14.2 ± 0.01*), followed by *Straphyloccus aureus* ATCC 29726 (11.4 ± 0.13*) and lowest activity against *Pseudomonas aeruginosa* ATCC 25619 (9.13 ± 0.10*), followed by *Escherichia coli* ATCC 8739 (8.27 ±0.05*) at the concentration of 100µg/ml against standard ampicillin (19.20 ± 0.01). In case of the extracts of KAR sample, the methanol extract showed higher inhibition against *Bacillus subtilis* ATCC 6633 (11.01 ± 0.21*), followed by *Straphyloccus aureus* (10.30 ± 0.01*) and lowest activity showed for *Escherichia coli* ATCC 8739 (9.01 ± 0.20) followed by *Pseudomonas aeruginosa* ATCC 25619 (8.86 ± 0.10) (Figures 2, 3) against standard ampicillin (19.20 ± 0.01) (Table 3).

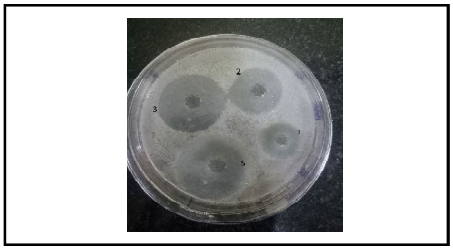

Figure 2: EH extract against *Bacillus subtilis* (WB sample).

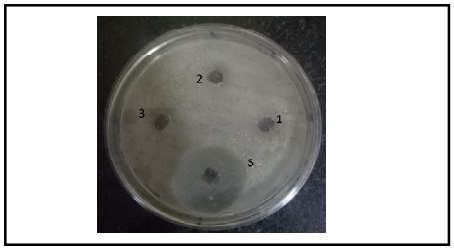

Figure 3: EH extract against *Bacillus subtilis* (KAR sample).

(S = standard ampicillin; 1 = 50 µg/ml, 2 = 75 µg/ml, and 3= 100 µg/ml)

Table 3: Antibacterial activity of EH extracts

Microorganisms	Concentration (µg/ml)	Zone of inhibition (mm)			
		EH of WB (Meth)	EH of WB (Aqu)	EH of KAR (Meth)	EH of KAR (Aqu)
Bacillus subtilis	50	11.6 ± 0.13***	9.68 ± 0.03**	10.42 ± 0.21***	9.20 ± 0.11
	75	13.8 ± 0.22***	10.08 ± 0.20***	10.94 ± 0.01***	10.02 ± 0.30
	100	14.2 ± 0.01***	10.85 ± 0.31***	11.01 ± 0.21***	10.48 ± 0.21
Straphyloccus aureus	50	9.11 ± 0.20**	9.07 ± 0.30**	9.78 ± 0.34**	9.03 ± 0.31**
	75	10.0 ± 0.11***	9.57 ± 0.22**	10.21 ± 0.20***	9.56 ± 0.14**
	100	11.4 ± 0.13***	10.02 ± 0.21***	10.30 ± 0.01***	10.0 ± 0.01**
Pseudomonas aeruginosa	50	8.42 ± 0.03	8.01 ± 0.30	8.06 ± 0.02	ND
	75	8.67 ± 0.11**	8.33 ± 0.01**	8.21 ± 0.04*	8.02 ± 0.11*
	100	9.13 ± 0.10**	9.04 ± 0.22**	8.86 ± 0.10**	8.40 ± 0.04*
Escherichia coli	50	7.86 ± 0.24	ND	7.98 ± 0.23	ND
	75	8.01 ± 0.01**	7.89 ± 0.26	8.78 ± 0.21*	8.11 ± 0.23
	100	8.27 ± 0.05	8.20 ± 0.01	9.01 ± 0.20**	8.45 ± 0.01*
(Ampicillin)	25	19.20 ± 0.01	19.20 ± 0.01	19.20 ± 0.01	19.20 ± 0.01

- The results represented as mean ± standard error of mean (n = 6). Data were analyzed by one-way ANOVA, followed by Dunnett comparison test against standard ampicillin. Values were considered significant at $^{***}p<0.001$; $^{**}p< 0.01$; $^{*}p<0.05$

4.5 Correlation study

The yield of the methanolic EH leaves extracts was correlated with the elemental content and antibacterial activity. The result revealed the positive correlation with among them and the same trend followed with the both geographical location samples. The yield of methanol extract of EH sample of WB and KAR locations showed positive correlation (R^2 = 0.926) with antibacterial activity and elemental content (Zn and Fe) and depicted in below Figures (Figures 4, 5, 6).

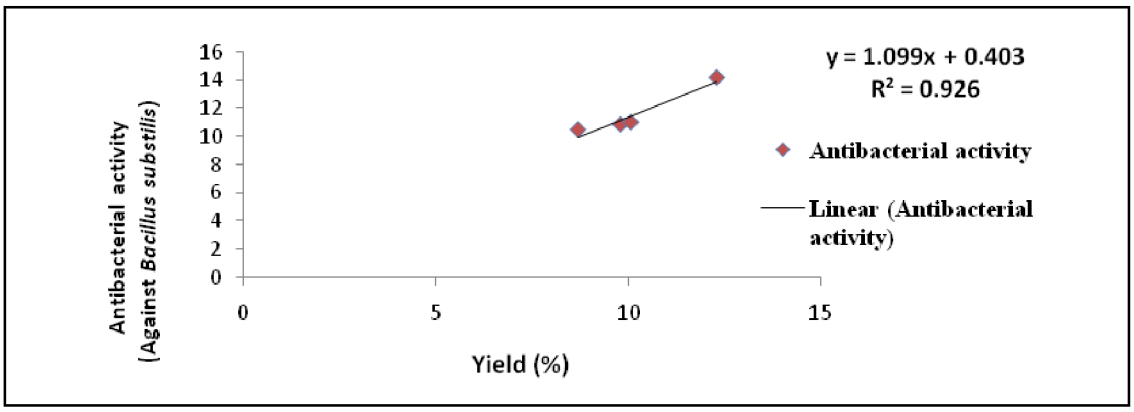

Figure 4: Correlation study of antibacterial activity with yield (WB and KAR samples).

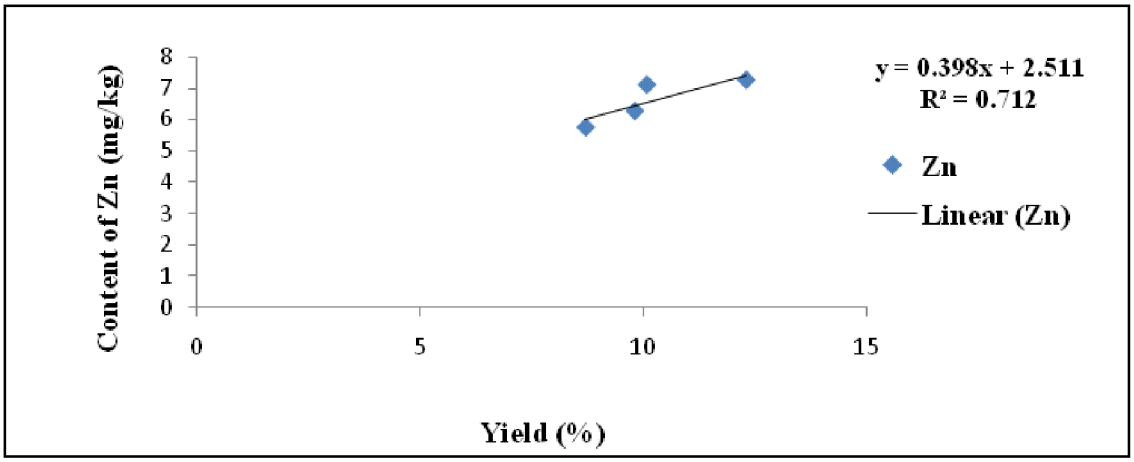

Figure 5: Correlation study of Zn content with yield (WB sample).

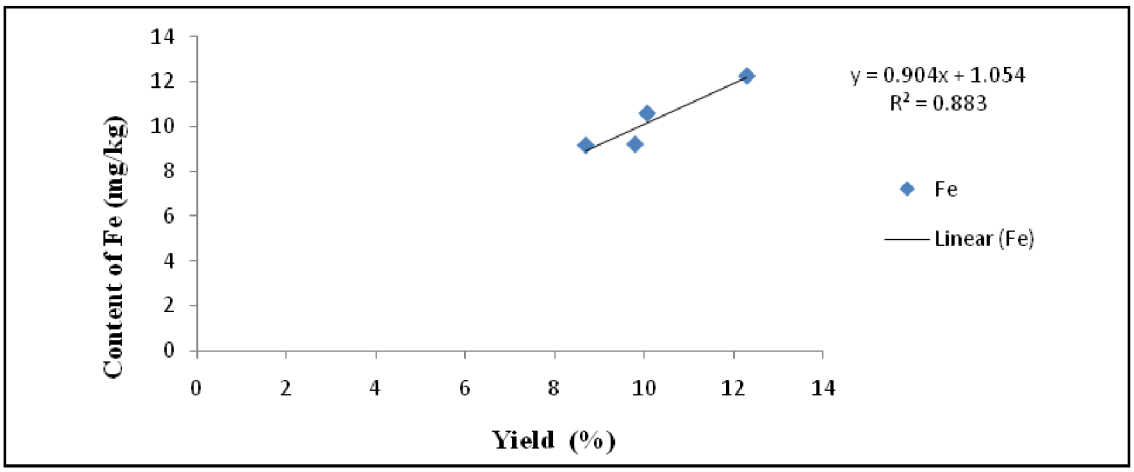

Figure 6: Correlation study of Fe content with yield (WB sample).

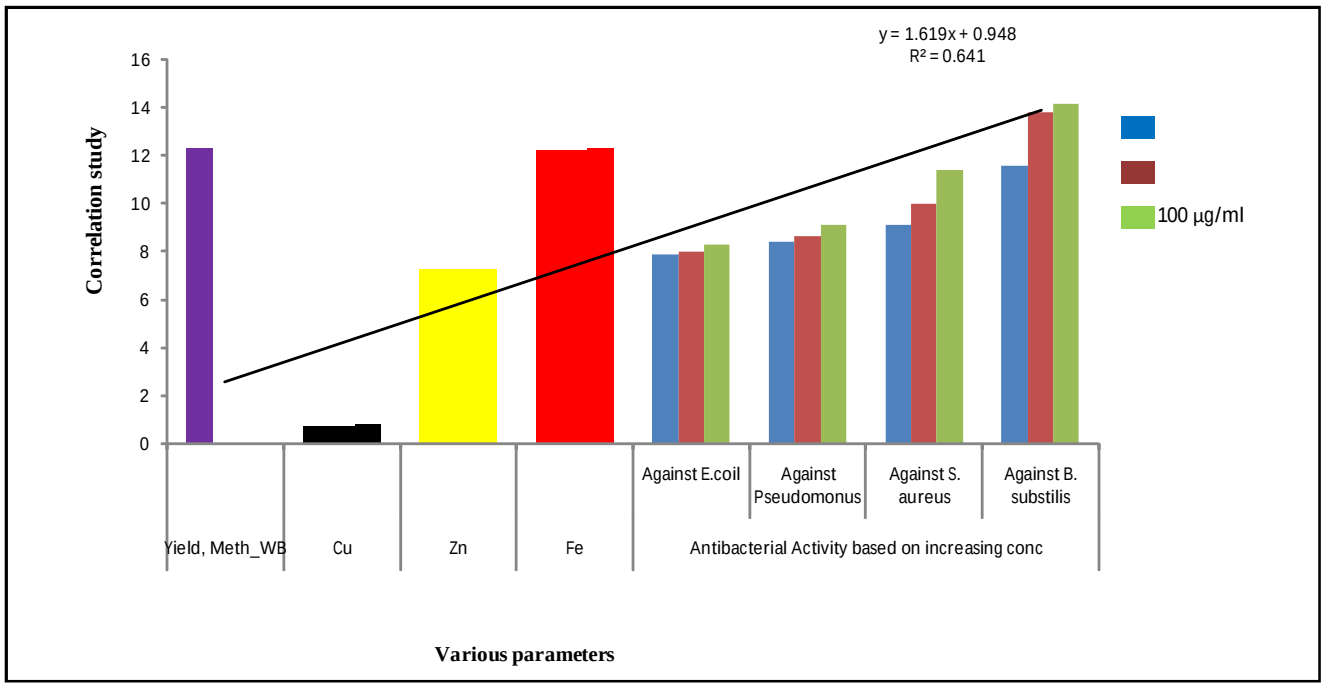

Figure 7: Positive correlation among the yield, elemental content and antibacterial activity based on increased concentration (WB sample).

Thereafter, all the parameters such as yield, element contents showed positive correlation (R^2 = 0.641) with the antibacterial effect (WB methanol EH extract) but not statistically significant. The same trend followed for the KAR sample (Not shown in the graph).

5. Discussion

5.1 Yield and phytochemical screening

The results showed that the yield of the extract was higher with methanol extract, followed by aqueous extract and the same results reveled with the both samples collected from the different geographical locations (WB and KAR). This was due to the choice of solvent. In our study, methanol and aqueous solvents were used and among those two solvents, methanol extract enhanced solubility of the bioactive compounds as a result, the content of extract also enhanced. Many earlier literatures were also reported the same (Ajanal *et al*., 2012; Mahdi-Pour *et al*., 2012; Truong *et al*., 2019). The higher yield may be due to the content of the major and minor elements present. Interestingly, element such as Cu content was less in both the EH leaves extracts and the enhanced content of Fe, followed by Zn. This trend increased the leaf biomass with the enzymatic activity and the result was also similar to that of earlier report of Kumar *et al*. (2009). Furthermore, the amount of extract in the present investigation was resulted direct correlation with the leaf biomass and choice of solvent which was the same as reported earlier in the scientific research (Dent *et al*., 2013; Dzah *et al*., 2020).

The preliminary phytochemical screening of both the EH leaves extracts showed the presence of some important bioactive compounds (such as alkaloids, flavonoids, tannins, steroids, glycosides and carbohydrates and phenols) as major constituents, but the tests were slightly different with respect to colors. This may be due to the presence of bioactive compounds in the solvents. In the present study, methanol solvent was used which enhanced solubility of the secondary metabolites and provided presence of the said components prominently with chemical tests. The report was also correlated with the earlier research evidences (Akinsulire *et al*., 2007; Yisa, 2009).

5.2 Elemental analysis

Results revealed that sample procured from West Bengal showed high content of Fe, Zn and Cu than that of Karnataka sample. This is mainly due to the soil nature and uptake of the plant by the root. The collected samples were from two different soil zones, *viz*. Kolkata (West Bengal) which is more acidic soil than Bangalore soil (Karnataka). It was revealed that Fe and Zn contents were higher in acidic soil which showed the same trend in case of Fe and Zn contents in leaves samples (Kumar *et al*., 2009; Das, 2014; Aref, 2012).

5.3 Antibacterial activity

Based on MIC study the dose of extracts was selected and three different concentrations were applied along with broad spectrum antimicrobial standard ampicillin. The results were compared against standard and resulted statistically significant antibacterial activity in terms of zone of inhibition for methanol EH leaves extracts collected from both the geographical locations. It was observed that the antibacterial activity of plant extract generally differs by influence of multiple factors, namely; climatic effect, soil composition, plant age, solvent used for extraction, extraction conditions, composition of extracted product, bacterial strains and so on (Bedi *et al*., 2010; . Bakht *et al*., 2011; Shamna *et al*., 2021). Even recently, the influence of elements present in plant canopies and in soil for bactericidal potentiality was recognized which cannot be ignored (Sharma *et al*., 2011; Osowole and Balogun, 2012) and the same report was revealed in the present investigation. Recently, various metal nanoparticles are also applied especially with Zn metal and revealed potential antimicrobial efficacy which also supported our results (Claudel *et*

al., 2020). The results also revealed zone of inhibition of methanol EH leaves extract of WB zone was more in case of gram-positive followed by gram-negative bacteria. This result may be due to higher amount of Cu content in WB sample than KAR sample which also correlated with the earlier literature (Daboor and Haroon, 2012).

5.4 Correlation study

Finally, correlation study showed the positive relation with the yield, element content and their impact on antibacterial activity but not statistically significant but the correlation was higher in methanol extract than aqueous extract and the same trend followed by both the different geographical zone samples.

6. Conclusion

Sample procured from West Bengal showed significant concentration dependent potential antibacterial activity. The methanol leaf extract gave high activity against gram-positive pathogens due to the presence of more bioactive components and the content of microelements, *viz*., Fe, Zn and Cu. This study confirmed that potent antibacterial activity not only depends on the choice of solvents but also depends on the geographical source and element contents in the plant and the same was correlated which showed positive relationship but was not statistically significant.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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