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BIOCHEMICAL STUDY OF AN ENDANGERED ETHNOMEDICINAL PLANT *CURCULIGO ORCHIOIDES* GAERTN. OCCURRING IN PURULIA DISTRICT OF WEST BENGAL, INDIA

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ABSTRACT: Ethnic peoples from Purulia district of West Bengal in India use a lot of plants for their primary health care without studying phytochemical analysis and biological activities in detail. Main objectives of the present study are to evaluate the antibacterial and antioxidant activities of the whole plant extract of *Curculigo orchioides* using polar and nonpolar solvents. Total phenol content (TPC) was determined by Folin-Ciocalteu assay using Gallic acid (GA) as standard and total flavonoid content (TFC) by $AlCl_3$ method using quercetin as standard. The antibacterial screening was carried out by agar well diffusion method. Methanol extracts exhibit higher phenol (82.93 ± 2.74 mg of GA E/gm of the sample), flavonoid (48.41 ± 1.94 mg of QE/gm of the sample) and alkaloid (5.56 ± 1.56%) content in comparison to other solvents. Similarly, methanol extracts show higher free radicals scavenging property and there was a positive correlation between IC_{50} values with total phenol and flavonoid content. A positive correlation exists in total phenol and flavonoid with antioxidant activities. The plant contains high phenolic and flavonoid content and exhibits antibacterial and antioxidant activities support the ethnomedicinal value of the plant.

INTRODUCTION: Plants have been used medicinally since prehistoric times. Although, after the discovery of the synthetic drug, the use of natural medicines has decreased drastically. However, synthetic drugs have some side effects [1], allergic reactions [2]. So, humans seek some alternative source of medicine. Tribals from a rural area of Purulia district are mainly dependent on medicinal plants for their primary health care. One such traditionally used plant is *Curculigo orchioides* Gaertn. (Hypoxidaceae) commonly known as kalimusli, known in Purulia it as talmuli, is an important endangered medicinal plant used by the tribal people from all over the district for the treatment of carbuncle and cancer.

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The plant is a small annual herb characterized by the yellowish flower, blackish tuberous primary root with dense lateral roots, aerial part possessing numerous linear leaves arranged in rosettes. According to WHO 80% world population relies on traditional medicines for the treatment of common illness [3]. At present, phytochemicals are more in demand in comparison to synthetic drugs due to its fewer side effects, lesser immuno-suppressive activity and wide use for the treatment of several diseases [4]. The rhizome of *C. orchioides* possesses immune stimulant potential [5], hepatoprotective [6], antioxidant [7], and platelet regeneration effect [8]. The present study emphasizes on antibacterial activity against three strains of bacteria, antioxidant activity, total phenol and flavonoid content of the whole plant of *Curculigo orchioides*.

MATERIALS AND METHODS:

Plant Materials: Plant **Fig. 1** is obtained from the forest area of Bandwan in Purulia district, West Bengal, India and is identified with the help of

books and literature, authenticated by Dr. Basanta Kumar Sing, Botanical Survey of India, Kolkata. A voucher specimen (AMC123) was documented in the herbarium of A.M. College, Jhalda.

FIG. 1: ***CURCULIGO ORCHIOIDES***

Extract Preparation for Total Phenol, Flavonoid, Alkaloid, Antioxidant and Antibacterial Activities: Freshly collected whole plant of *Curculigo orchioides* (1800 grams) was washed with running tap water and then shade dried for eleven days. Dried plants crushed to form a powder (780 grams) and extracted successively with soxhlet extractor at 60 ºC by using methanol, ethyl acetate, n-hexane and water for 24 h. After that solvent extracts are removed, filtered and concentrated by using a rotary evaporator at 60 ºC under reduced pressure and 14 gm, 9 gm, 5 gm and 10 gm of the extract obtained respectively which were kept in a refrigerator at 4 ºC for further study.

Determination of Total Phenol Content: Total phenol content was determined using Gallic acid as standard. Plant powder obtained from respective solvent extracts taken for Folin-ciocalteu assay with a slight modification of standard method [9]. The total phenol content was expressed as Gallic acid equivalents (mg of GAE/g sample) through the calibration curve of Gallic acid. Linearity in calibration curve was 10 to 100 µg/ml (r = 0.99).

Determination of Total Flavonoid Content: Total flavonoid content was determined by $AlCl_3$ method using quercetin as standard [10]. A mixture of 10% of 100 µl of $AlCl_3$, 100 µl $NaNO_3$(5%), 670 µl of 1 mM NaOH and 100 µl of the sample was vortexed and incubated in the dark at room temperature for 25 min. The O.D. value was measured at 510 nm. The experiment was repeated thrice.

Estimation of Alkaloid Content: Alkaloid content was determined by using a suitable method with slight modification [11]. Five grams of the powdered methanol and aqueous plant extracts dissolved in 20 ml of 20% acetic acid prepared in methanol (v/v), then filtered after four hours. The filtrates were kept in a water bath for 30 min at boiling temperature. Ammonium hydroxide was poured into the extract, dropwise and produced some precipitation (ppt). The collected ppt was washed with dilute ammonium hydroxide and filtered. The residues obtained in the filter paper, dried and weighed.

$$\% \text{ of alkaloid} = \frac{\text{Weight of residues}}{\text{Weight of sample}} \times 100\ \%$$

Antioxidant Activities:

DPPH Antioxidant Activity: DPPH antioxidant activity was carried out by procedure Zhu *et al*., [12] with slight modification. By using respective solvents prepare a stock solution, from the stock solution make various concentrations (50-400 µg/ml). DPPH mixed with different concentrations, after 30 min incubation O.D. value at 517 nm taken by jasco V-630, USA. Ascorbic acid was measured in the same procedure. Antioxidant scavenging capacity was estimated by calculating IC_{50} values.

$ABTS^{\bullet+}$ Antioxidant Activity: For $ABTS^{\bullet+}$ antioxidant assay, the method of Re *et al*., [13] was followed with slight modification. The $ABTS^{\bullet+}$ radical cation was prepared by mixing an equal volume of 7 mM of $ABTS^{\bullet+}$ stock solution with 2.45 mM of potassium persulfate and incubated in dark condition for 12-16 h at room temperature. Before experimental processing, the $ABTS^{\bullet+}$ radical reaction mixture was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm. The requisite amount of plant extract (50-400 µg/ml) was mixed with 2 ml of $ABTS^{\bullet+}$ radical reaction mixture and incubated in the dark for 6 min. After that absorbance at 734 was recorded by UV-Vis spectrophotometer and percent free radical scavenging activity was determined by the following formula

$$\% \text{ free radical scavenging activity} = \frac{A_{blank} - A_{sample}}{A_{sample}} \times 100$$

Where A_{sample} and A_{blanks} are the respective absorbance's of tested samples and $ABTS^{\bullet+}$ reaction mixture.

Hydrogen Peroxide Scavenging Activity: Hydrogen peroxide scavenging activity was determined by using a method of Harborne [14] with modifications. Aliquots of 0.1 ml from different concentrations of extracts were taken into the test tubes and make the volume up to 0.4 ml by adding with 50 mM phosphate buffer (pH 7.4) then add 0.6 ml of hydrogen peroxide solution, vortexes the mixtures and absorbance measured at 230 nm after 10 min, against a blank. Hydrogen peroxide scavenging abilities were calculated using the following equation:

Hydrogen peroxide scavenging activity = (1- absorbance of sample/absorbance of the mixture) × 100

***In-vitro* Antibacterial Assay:**

Bacterial Strain and Culture Conditions: Authentic, pure cultures of pathogenic bacteria like *Escherichia coli (E. coli* MTCC 443), *Staphylococcus aureus (S. aureus* MTCC 3160) were provided by the microbiological laboratory and clinical detection center Paschim Medinipur and *Bacillus subtilis* from Vidyasagar University Microbiology Department Paschim Medinipur, India. They were cultured in tryptone soy broth or agar (TSB or TSA) in aerobic condition at 37 °C.

Well Diffusion Method: The good diffusion method was used to study the antibacterial activity as described by Bauer *et al*. [15] Lawn bacterial cultures were spread on the Muller Hinton agar using a spreader. The wells were cut on the agar plates using a cork borer; plant extracts were poured into the well using sterile micropipette [16]. The plates were incubated at 37 ºC for 24 h. After incubation, the diameter of the zone of inhibition was measured by using a scale.

Statistical Analysis: Statistical analysis was performed using Microsoft Excel-2010 software. Data are expressed as mean ± SD from three replicates. EC_{50} values were calculated by regression analysis. A probability of $P<0.05$ was considered significant.

RESULTS:

Total Phenol Content: Due to the presence of hydroxyl groups phenolic compounds are very important in plants as they can scavenge free radicals. TPC of *C. orchioides* was solvent dependent and expressed as milligrams of GA equivalent. **Fig. 2** summarize the TPC and ranging from 35.7 ± 3.32 to 82.93 ± 2.74. The methanol extract exhibited the highest total phenol content in comparison to other solvent extracts.

Total Flavonoid Content: Total flavonoid content also depend upon the solvent types as shown in **Fig. 2** varied from 12.46 ± 2.08 to 48.47 ± 1.94 mg of quercetin equivalent/gm of extract. The methanol extract showed the highest amount of flavonoid content **Fig. 2** followed by aqueous, ethyl acetate and n-hexane.

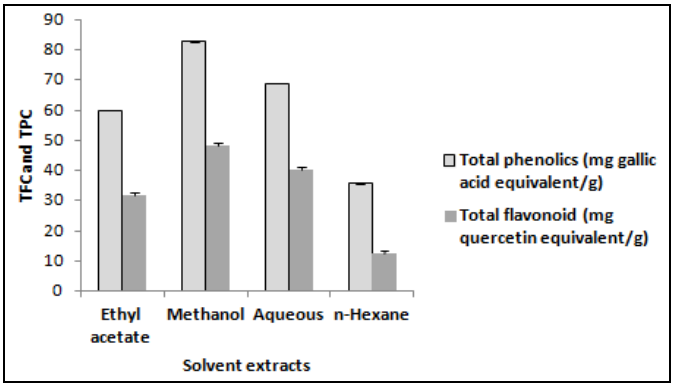

FIG. 2: TOTAL FLAVONOID CONTENT (TFC) AND TOTAL PHENOL CONTENT (TPC) IN VARIOUS SOLVENT EXTRACTS. Each value is represented as the mean ± standard deviation (n=3)

Estimation of Alkaloid Content: Alkaloid content shows higher in methanol extract (5.56 ± 1.56%) in compare with water extract (2.25 ± 1.4%) **Fig. 3.**

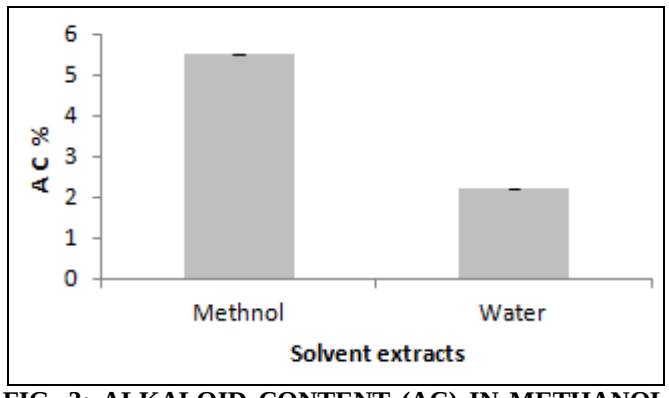

FIG. 3: ALKALOID CONTENT (AC) IN METHANOL AND AQUEOUS EXTRACT

***In-vitro* Antioxidant Activities:**

DPPH Radical Scavenging Activity: Antioxidants present in the plant extracts decrease the absorbance of DPPH radical at 517 nm because of the reaction between antioxidants and radicals. It is noticeable by seeing the color changes from purple to yellow.

The scavenging effects of various solvent extract on DPPH radical **Fig. 4** shown in the following order: methanol > aqueous > ethyl acetate > n-hexane. The IC_{50} value was shown in **Table 1**. Though the capability of scavenging the free radicals is very low in comparison with vit - C but plants have the potentiality to scavenge the free radicals and may be used as natural antioxidants.

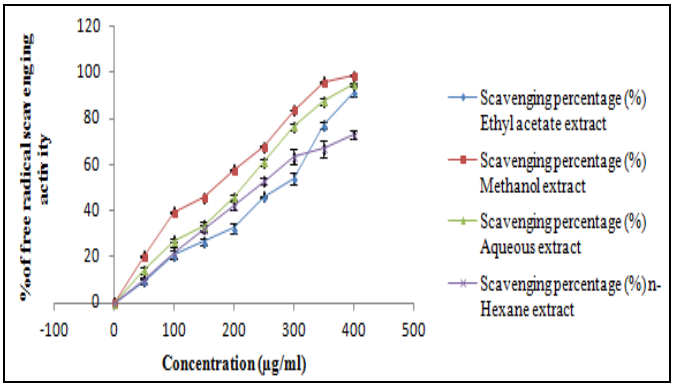

FIG. 4: DPPH ANTIOXIDANT ACTIVITY OF FOUR SOLVENT EXTRACTS. Each value is represented as the mean ± standard deviation (n=3)

ABTS Radical Scavenging Activity: Fig. 5 shows the antioxidant capacity of various solvent extracts decrease in this order, methanol > aqueous > ethyl acetate > n-hexane. The IC_{50} value of methanol extract was most pronounced in compare with other solvent extracts **Table 1.**

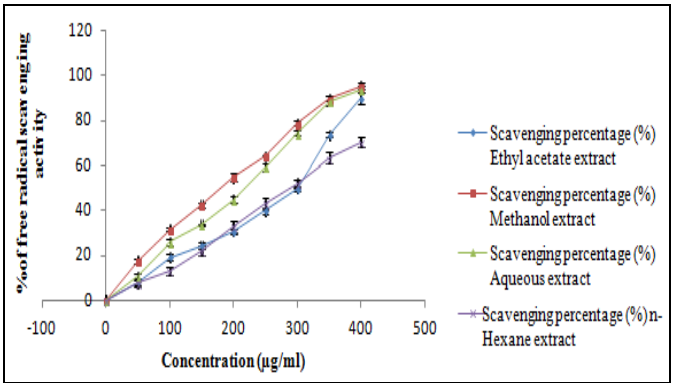

FIG. 5: ABTS$^{\bullet+}$ ANTIOXIDANT ACTIVITY OF FOUR SOLVENT EXTRACTS. Each value is represented as the mean ± standard deviation (n=3)

Hydrogen Peroxide Radical Scavenging Activity: The effect of different solvent extracts on hydrogen peroxide radical was concentration dependent (50-500 µg/ml) as shown in **Fig. 6**. In this study, results showed that various solvent extracts had strong potential in eradicating hydrogen peroxide at all concentrations. The IC_{50} value of different solvent extracts shows in **Table 1**.

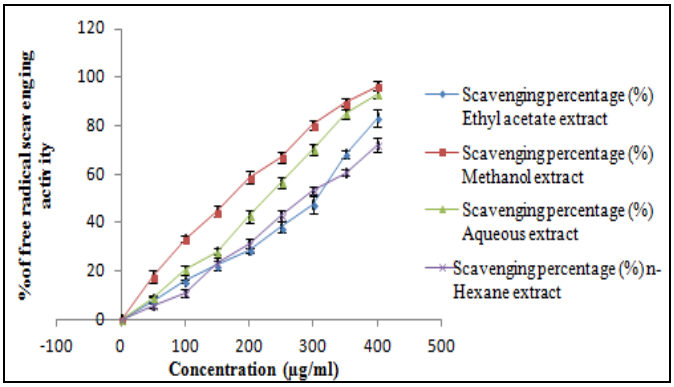

FIG. 6: HYDROGEN PEROXIDE RADICAL SCAVENGING ACTIVITY OF FOUR SOLVENT EXTRACTS. Each value is represented as the mean ± standard deviation (n=3)

IC_{50} Value: Inhibitory concentration (IC_{50}) parameter was used for the interpretation of the results of antioxidant activities. The discoloration of the sample was plotted against sample concentration to determine the IC_{50} value. Its value in four solvents extracts shown in **Table 1** by using three different assay techniques.

TABLE 1: IC_{50} VALUES (µg/ml) FOR RADICAL SCAVENGING

Solvent extracts	DPPH radical	Hydrogen peroxide	ABTS radical
Ethyl acetate	247.321	277.43	260.98
Methanol	173.761	182.74	190.17
Aqueous	204.264	220.83	208.06
n-Hexane	252.22	289.58	289.14

Correlation (R) Between IC_{50} Values of Antioxidant Activities with Total Phenol and Flavonoid Contents: There was a positive correlation (R) between IC_{50} values of antioxidant activities with total phenol and flavonoid contents as shown in **Table 2**. R values from total phenolic with IC_{50} vary from 0.737 to 0.795 and in case of flavonoids are 0.823 to 0.844.

TABLE 2: CORRELATION BETWEEN IC_{50} VALUES WITH TOTAL PHENOL AND FLAVONOID CONTENT

IC_{50}	Total phenolics	Total flavonoid
DPPH	0.785	0.823
H_2O_2	0.795	0.844
ABTS	0.737	0.839

Antibacterial Activity: Methanol extract shown highest inhibition zone **Table 3** in comparison with other solvent extracts. Activity index measured to show the suitable solvents chosen to increase the biological activity.

TABLE 3: ANTIBACTERIAL ACTIVITY (ZONE OF INHIBITION, mm) OF VARIOUS SOLVENT EXTRACTS OF *C. ORCHIOIDES*

Bacteria		Methanol	Ethyl acetate	Aqueous	n-Hexane	Standard
S. aureus	IZ	13.12 ± 0.12	9.28 ± 0.42	11.32 ± 0.46	7.14 ± 0.32	25.56
	AI	0.51	0.36	0.44	0.27	
E. coli	IZ	18.32 ± 0.16	8.62 ± 0.26	13.67 ± 0.76	7.54 ± 0.21	24.89
	AI	0.73	0.34	0.54	0.30	
B. subtilis	IZ	14.54 ± 0.14	8.75 ± 0.24	12.46 ± 0.48	7.32 ± 0.12	21.52
	AI	0.67	0.40	0.57	0.34	

IZ = inhibition zone (mm) includes the diameter of disc (6 mm); AI = activity index = IZ of test sample/ IZ of standard; Standard: Ampicillin (1 mg/disc). Values are mean of triplicate replicates (mean ± S.D.)

DISCUSSION: Studies have revealed that medicinal plants are very good sources of antioxidant and play a significant role in the treatment of several diseases globally [17]. The plant *C. orchioides* contains a higher amount of phenolic compounds in the methanolic extract in comparison to other solvent extracts. Due to the presence of phenolic compounds plant exhibit antioxidant properties and methanolic extract shows more potential on free radical scavenging properties [18]. There was a positive correlation between phenolic compounds and IC_{50} values; this study corroborates the findings of previous authors [19, 20]. The phenolics and polyphenols are the largest groups of secondary metabolites to have antimicrobial and antioxidant properties [21, 22].

Naturally occurring plant flavonoids possess antimicrobial activities [23, 24]. The variation in the antibacterial activity of flavonoids and phenolics is due to the number and positions of methoxy and phenolic groups within their structures [25, 26]. The alkaloid extracts obtained from medicinal plant species have a multiplicity of host-mediated biological activities, including antimalarial, anti-microbial, antihyperglycemic, anti-inflammatory, and pharmacological effects [27, 28]. With respect to different liver marker enzymes, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) methanol extract of *C. orchioides* exhibit hepatoprotective properties. Nagesh and Shanthamme [29] reported the antibacterial property of rhizome extract against pathogenic bacteria. Mehta *et al.*, [30] identified some fatty acid from the root of *C orchioides.* Anticancer phenolic compound curculigoside identified by Kubo *et al.*, [31] from the rhizome of the plant. Whole plant extract and uses of different solvents vary the biological activities as reported by previous authors.

Further study on phytochemical analysis and isolation of bioactive components urgently needed for the development of natural medicine.

CONCLUSION: Due to the presence of secondary metabolites (Phenolic and flavonoid compounds) the studied plant exhibits antioxidant and antibacterial properties. Out of the four solvents used methanol has the more potential to extract the bioactive components from plant powder. This study supports the ethnomedicinal uses of the plant in Purulia district. Further research on phytochemical analysis and isolation of bioactive components urgently needed for the development of natural medicine.

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