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CHARACTERIZATION OF THREE AQUATIC ANGIOSPERMS FOR PHYTOCHEMICALS AND ANTIMICROBIAL ASSAY IN INDIA

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ABSTRACT

The Most aquatic angiosperms possess phytochemical and antimicrobial properties. Phytochemical screening revealed the presence of biologically active substances (flavonoids, tannins, sterols, and resins) at various concentrations. For this purpose, we characterized three aquatic plants *i.e. Nymphoides indica, Nymphoides hydrophilla* and *Hydrilla verticillata* were examined to determine the presence of photochemical properties using standard methods and antimicrobial activity, as were studied against two bacteria *i.e. Bacillus sp.* and *Brevundimonas* sp., which were determined by the agar diffusion method. *N. indica* has been found to be *biologically* active with a few phytochemicals that have been recovered *viz.* saponin, starch, flavonoids, triterpenoids, steroids, glycosides, and tannins. In *N. hydrophilla* alkaloids and carbohydrates were recovered more than *N. indica* and triterpenoids were not found in *N. hydrophilla* likewise presence of starch, carbohydrate, tannin (aqueous extract), and flavonoids were found in *H. verticillata.* zone of inhibition was recorded 1.4 cm for *Bacillus sp*. by ethanol extraction, and *H. verticillata and N. indica* did not show any antimicrobial activity against either bacterial species *i.e. Bacillus sp.* and *Brevundimona sp.* Further research should be carried out to isolate potential antimicrobial agents from these extracts. These aquatic angiospermic plants can also be a source of biologically active compounds and can be helpful in pharmacognosy and modern drug production.
***Keywords*:** Phytochemical, antimicrobial activity, *Nymphoides indica, Nymphoides hydrophilla, Hydrilla verticillata,* aquatic angiosperm.

Introduction

Nature is a source of therapeutic agents for the production of modern drugs. These therapeutic agents are found in plants as secondary metabolites. They do not participate in growth and development, but have some crucial and significant therapeutic properties. They have been studied for their antimicrobial properties and presence of phytochemicals. A small percentage of the total species of plants on earth are used as food by humans and animals, and even more are used for medicinal purposes. Plants contain many useful molecules, including secondary metabolites such as phenolic compounds, *polyphenols, flavonoids, flavones, flavanols, tannins, terpenoids, steroids, alkaloids, quinones, resin, gum*. These secondary metabolites are widely used in medicinal sciences. Most of these are potent bioactive compounds found in plant parts that can be used for the synthesis of useful drugs. Currently, the science of Ethno botany and Ethno pharmacology has led people toward the different sources of these compounds. econdary metabolites are chemically and taxonomically diverse compounds with unknown functions. The active components of many drugs found in plants are secondary metabolites (Ghani 1990; Dobelis 1993).

Present study is based on three aquatic plants *i.e. Nymphoides indica (L.) Kuntze, Nymphoides hydrophilla* (L.) *Kuntze* and *Hydrilla verticillata* (L.f.)

Royle. Nymphoides indica is found in tropical areas worldwide. It is found in pools, pans, marshes, and rivers in most states of India. Other regions where it occurs naturally include Australia, New Zealand, Africa, China, Bhutan, Bangladesh, Indonesia, Japan, Nepal, Spain, Sri Lanka, Taiwan, Switzerland, Mexico, and Malaysia. *Nymphoides hydrophilla* (L.) *Kuntze* is very common throughout India and is gregarious in habit, growing near the margins of tanks and lakes. It is distributed throughout India, Sri Lanka, China, and Malaysia. (Altameme*,* 2015) *Hydrilla verticillata* (L.f.) *Royle. is* an aquatic angiosperm plant that is thought to be native to various tropical and subtropical areas, such as the warmer regions of Asia, Australia, and the United tates. This is common in India.

Aquatic plants have economic and environmental uses depending on their natural characteristics. Aquatic plants are not commonly used phytochemical studies. The aim of the present study was to attract researchers towards aquatic plants because they contain many significant secondary metabolites with antimicrobial properties. Aquatic plants consist of a large number of phytochemicals belonging to several classes and have been shown to have inhibitory effects against some types of microorganisms *in vitro* that making these aquatic plants as significant as many terrestrial plants.

The present research was carried out in three aquatic angiosperms *N. indica (L.) Kuntze, N. hydrophilla* (L.) *Kuntze* and *H. verticillata (L.f.) Royle.* These aquatic plants *N. indica* (L.) *Kuntze, N. hydrophilla* (L.) *Kuntze, H. verticillata* (L.f.) *Royle* are commonly found in pond, rivers. These plants are also found in ponds on the university campus of Guru Ghasidas Vishwavidyalaya, Bilaspur (C.G.), India. The objective of the present study was to characterize the phytochemical and antimicrobial activities of these aquatic plants. Bacterial samples of *Bacillus sp.* and *Brevundimonas* sp. were used for the antimicrobial study.

Materials and Methods

During the experiment, the following methods were adopted for the performance of the present study.

Plant materials

The healthy and fresh plants *i.e. N. indica* (L.) *Kuntze, N. hydrophilla* (L.) *Kuntze* and *H. verticillata* (L.f.) *Royle.* were collected from ponds on the university campus of Guru Ghasidas Vishwavidyalaya, Bilaspur, C.G. India.

Selection of solvents

The nature of the solvent was determined by the nature of the extraction sample used. Some solvents for biologically active compounds were selected for the study based on the nature of the compounds.

Water

Water is a universally used solvent. It is generally used for tannins, terpenoids, saponins, and starches.

Alcohol

Alcohol is more efficient in cell wall degradation,has a non-polar character, and causes polyphenols to be released from cells. It is more efficient than water because of the water extract enzyme polyphenol oxidase, which degrades polyphenols in the water extract, but alcohol does it more gently. However, water is a better medium for the growth of microorganisms than alcohol. The higher concentration of ethanol due to the polarity of the solvent was increased by adding a certain amount of water.

Ethanol

It is commonly used for tannin, terpenoid, alkaloid and polyphenols.

Methanol

It is commonly used for tannin, terpenoid, saponin and polyphenols.

Chloroform

It is commonly used for flavonoid and terpenoids.

Acetone

It is commonly used for phenols.

Ether

It is commonly used for alkaloid and terpenoids.

Preparation of plant extract

Fresh and healthy plants were then removed and washed with water. Leaves, stems, roots, and other parts of the plants were separated and kept shadedried separately. The dried plant parts were pulverized into a fine powder using a mortar, pestle, and strainer (filter). Fifty grams of powder from each plant were taken, and samples were prepared and kept in airtight boxes. Phytochemical analysis and extraction were performed using powdered samples. The dried powder samples were dissolved in appropriate solvents and heated in a water bath for 5-6 minutes at 40 ^{o}C. Heating is important for neutrality of the toxicity of solvents. Different sample extractions were performed using different solvents. After heating, the extract was filtered through Whatman filter paper. Some of the extracts of each solvent were used for qualitative phytochemical screening to identify the various classes of active chemical constituents using standard prescribed methods.

Methods of phytochemical screening

Standard methods were carried out for the eight phytochemicals, in which alkaloids were tested using Hager's reagent and Dragendorff's reagent test (Ghani, 1998). carbohydrates tested by the Molisch's and Barfoed test (Foulger, 1931). Tannin was tested by ferric chloride and chlorogenic acid test (Clifford,1999) saponin by saponin test (Rao, and Gurfinkel, 2000), starch by iodine test (Cochran *et.al* 2008) glycoside by Keller-killiani test and Baljet test (Keller, 1895), steroids and triterpenoids by Salkowski and Sulfur powder test (Deepak *et.al* 2013) and flavonoids by nitric acid test (Chaplan, 2005).

Antimicrobial assay

The antimicrobial activity of aquatic plants *N. indica* (L.) *Kuntze, N. hydrophilla* (L.) *Kuntze, H. verticillata* (L.f.) *Royle* against bacteria *Bacillus sp.* and *Brevundimonas* sp. was determined by agar diffusion method. Two bacterial cultures *Bacillus sp.* and *Brevundimonas* sp, were used in this study. These bacteria are cultured in MS medium (Mineral salt medium) (O'Loughlin et.al., 1999) both bacterial samples were provided by Department of Botany, (microbiological laboratory), Guru Ghasidas Vishwavidyalaya. Bilaspur-495009 (C.G.)

Procedure for antimicrobial study

Pure cultures of *Bacillus* sp. and *Brevundimonas* sp. were used in this study. To prepare the suspension of each culture, 1 ml of sterile distilled water was used. Nutrient broth agar (NA) medium was prepared for bacterial culture. Twelve plates of NA medium were prepared for antimicrobial assays. Each bacterial culture suspension was spread on the surface of the medium using sterile swab in aseptic conditions under a laminar airflow hood. The suspension was allowed to uniformly distribute microbial cells in the medium plates. Four plates were prepared as controls. Vancomycin, Ciprofloxacin, Gentamycin, Streptomycin, Neomycin, Penicillin, Ampicillin and Tetracycline were used as controls in the antimicrobial assay.

Four wells were cut in each plate using cork borer. Aqueous, ethanol, and chloroform sample extracts were poured separately into different culture wells. 0.5 ml of each extract was added. The plates were incubated at 32^0C for 48 h. After incubation, the plates were observed for the presence of a zone of inhibition. If present, the diameter of the zone of inhibition was measured using scale or a Vernier caliper.

Results

A comparative analysis of Phytochemicals for the screening *of N. indica, N. hydrophilla* and *H. Verticillate*

A comparative analysis was carried out to screen for phytochemicals in three aquatic angiosperms *i.e. N. indica, N. hydrophilla* and *H. Verticillate*. A total of nine parameters, *viz.* saponin, starch, flavonoids, triterpenoids, steroids, alkaloids, glycosides, carbohydrates, and tannin, were tested using standard methods for all three aquatic angiosperms. (Table-1) Saponin was found in *N.indica* and *N. hydrophilla* but not in *H. verticillate* whereas starch and flavonoids were present in all three aquatic plants. Terpinoids and steroids are present in *N. indica* whereas alkaloids have only been reported in *N. hydrophilla*. Glycosides are present in two species of aquatic plants *i.e. N. hydrophilla* and *H. verticillate.* Tanin was present in all three aquatic plants. (Table 1, Plate 1A, Plate 1B & Plate 1C)

Study of Antimicrobial assay

An experiment was also performed for the study of antimicrobial assays using two bacteria *i.e. Bacillus* sp. and *Brevundimonas* sp., where *Brevundimonas* sp. was found to be highly significant for zone inhibition during the study of the three species of ethanol extract. *Bacillus* sp. was found to be less effective in the zone of inhibition, except in the case of *H. verticillate* ethanol extract where 1.4 cm zone of inhibition was recorded. However, the zone of inhibition was not significant for *N. indica* and *N. hydrophilla* by using both bacteria. In the aqueous extracts of N. *hydrophilla, H. verticillata*, *N. indica* and *N. hydrophilla* ethanol extracts, the zone of inhibition was highly significant for *Brevundimonas* sp. The graph represents the zone of inhibition of bacteria in *Hydrilla verticillata* extract that showed maximum antimicrobial activity towards *Bacillus* sp. *Hydrilla verticillata* has a prominent antimicrobial activity. The plant extract was tested with *Bacillus* sp. and *Brevundimonas* sp. and comparatively it is observed that *Bacillus* sp. is more sensitive than *Brevundimonas* sp.

Fig. 2. represents the zone of inhibition toward *Brevundimonas sp.* by the above-mentioned extract. *Brevundimonas sp.* is a highly sensitive bacterial species. All five extracts of the three plants were tested against *Brevundimonas sp.*, and all extracts except the extract of *Nymphoides indica* exhibited antimicrobial activity with different degrees of effect, which is represented in a graph for clarity. Only the *Nymphoides indica* plant did not show antimicrobial activity against

the bacterial strain *Brevundimonas sp.* (Table 2, Fig.1, Fig. 2, Plate-2A, Plate 2 B). In another set of experiments, eight antibiotics *viz.* Gentamycin, Neomycin and Ampicillin, Vanomycin, Penicillin, streptomycin, Ciprofloxacin and Tetracycline, were tested for antimicrobial activity towards *Bacillus* sp. and *Brevundimonas* sp. The results are highly significant for the screening of antibiotics against both bacteria. (Plate 3 A & 3B)

Discussion

Phytochemical screening was performed by some of the standard methods, including the Hager test and Keller–Kilani test, and the results of the phytochemical screening state that these plants contain secondary metabolites. *N. indica* represents the presence of some active components in extracts prepared using different solvents. The biologically active components are saponins, starches, flavonoids, triterpenoids, steroids, glycosides, and tannins (Plate 1A). *N. hydrophilla* plants contain saponins, starches, flavonoids, alkaloids, glycosides, carbohydrates, and tannins (Plate 1B). *H. verticillata* contained starch, carbohydrates, tannins (aqueous extract), and flavonoids (Plate 1C). The antimicrobial activity of this plant was evaluated using two bacterial strains. As summarized in Table-2, the extracted plants showed promising antimicrobial activity against the selected bacterial species. Only *H. verticillata* ethanol extract showed antimicobial activity against *Bacillus sp.* as a zone of inhibition. The zone of inhibition is an area around the well where no growth of bacteria is observed. The diameter of the zone of inhibition was 1.4 cm for *Bacillus sp.* bacteria after ethanol extraction of *H. verticillata* (Plate 2 A).

N. indica did not show any antimicrobial activity against either *Bacillus sp.* or *Brevundimona sp.* (Plate 2 B). Aqueous and ethanol extract of *N. hydrophilla* showed antimicrobial activity against *Brevundimonas sp. Bacillus sp.* The results showed that two of the three aquatic plants, *Hydrilla verticillata* against *Bacillus sp.*, *Brevundimonas sp.* and *N. hydrophilla* inhibited *Brevundimonas sp.*

These plants have potential antimicrobial activity; therefore, further research should be conducted to isolate potential antimicrobial agents from the extract. These aquatic angiospermic plants can also be a source of biologically active compounds and can be helpful in pharmacognosy and modern drug production.

Conclusion

The present study was carried out on three aquatic angiospermic plants, *N. indica, N. hydrophilla* and *H. verticillata* and the results revealed the presence of biologically active compounds. The results are summarized in Table-1.

The selection of the solvent system largely depends on the specific nature of the target bioactive compounds. Different solvents have been used for the extraction of biologically active compounds. A good solvent should have some significant properties, such as low toxicity, solubility, ease of evaporation at low heat, ease of absorption of the extract, preservative action, and inability to dissociate the extract.

The extraction of hydrophilic compounds uses polar solvents, such as water, methanol, and ethanol. For the extraction of more hydrophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in a ratio of 1:1 was used.

The choice of solvent is influenced by what is intended for the extract. At the end of the extraction, the solvent should be non – toxic and should not interfere with the bioassay.

It has been widely observed that the medicinal value of plants lies in their bioactive phytocomponents present in the plants. The antimicrobial activities of the extracts of *N. indica, N. hydrophilla* and *H. verticillata* were evaluated *in vitro* against two bacteria, *Bacillus sp.* and *Brevundimonas* sp.

Table 1 : Phytochemicals screening of *N. indica, N. hydrophilla* and *H. verticillate*

S. No.	*N. indica*				*N. hydrophilla*				*H. verticillate*			
	Secondary metabolites	**Test Method**	**Extraction of leaf**	**Results**	**Secondary metabolites**	**Test Method**	**Extraction of leaf**	**Results**	**Secondary metabolites**	**Test Method**	**Extraction of leaf**	**Results**
1.	Saponin	Foam test	Aqueous	+	Saponin	Foam test	Aqueous	+	Saponin	Foam test	Aqueous	-
							Ethanol	+			Ethanol	-
2.	Starch	Iodine test	Fresh leaf	+	Starch	Iodine test	Fresh leaf	+	Starch	Iodine test	Fresh leaf	+
											Ethanol	+
3.	Flavonoid	Nitric acid test	Chloroform	+	Flavonoid	Nitric acid test	Chloroform	+	Flavonoid	Nitric acid test	Aqueous	-
											Ethanol	-
											Chloroform	+
4.	Triterpinoid	Salkowaski test	Chloroform	+	Triterpinoid	Salkowaski test	Chloroform	-	Triterpinoid	Salkowaski test	Aqueous	-
											Ethanol	-
											Chloroform	-
5.	Steroid	Salkowaski test	Chloroform	+	Steroid	Salkowaski test	Chloroform	-	Steroid	Salkowaski test	Aqueous	-
											Ethanol	-

											Chloroform	-
6.	Alkaloid	Hager's reagent test	Aqueous	-	Alkaloid	Hager's reagent test	Aqueous	+	Alkaloid	Hager's reagent test	Aqueous	-
			Ethanol	-								
7.	Glycoside	Keller-killiani test	Aqueous	+	Glycoside	Keller-killiani test	Aqueous	+	Glycoside	Keller-killiani test	Aqueous	-
			Ethanol	+			Ethanol	+			Ethanol	-
8.	Carbohydrate	Barfoed test	Aqueous	-	Carbohydrate	Molisch reagent test	Aqueous	+	Carbohydrate	Molisch reagent test	Aqueous	+
			Ethanol	-			Ethanol	+			Ethanol	-
9.	Tannin	Feric chloride test	Ethenol	+	Tannin	Feric chloride test	Aqueous	+	Tannin	Feric chloride test	Aqueous	+
		Chlorogenic acid test	Ethenol	+						Chlorogenic acid test	Aqueous	-

(+) indicates the presence of metabolites.
(–) indicates absence of metabolites.

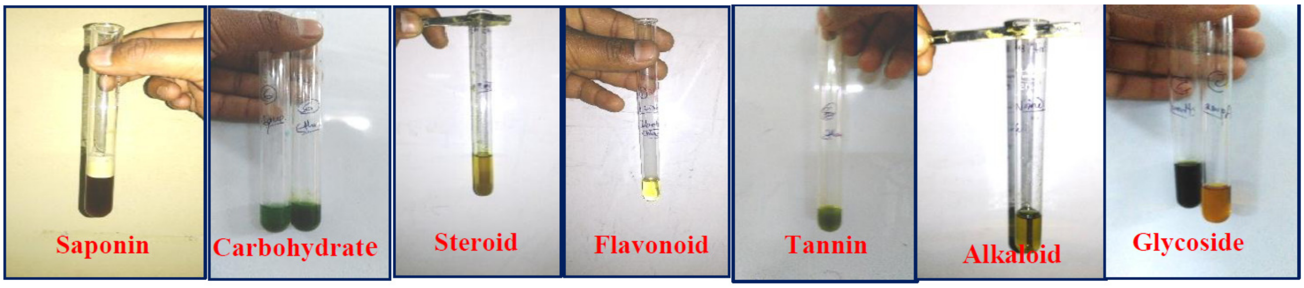


Plate 1 A : Phytochemical screening on *Nymphoides indica*

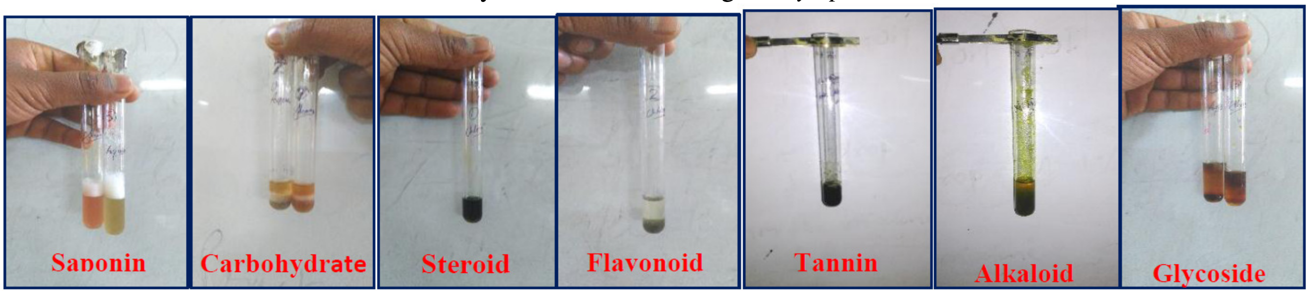


Plate 1 B : Phytochemical screening on *Nymphoides hydrophilla*

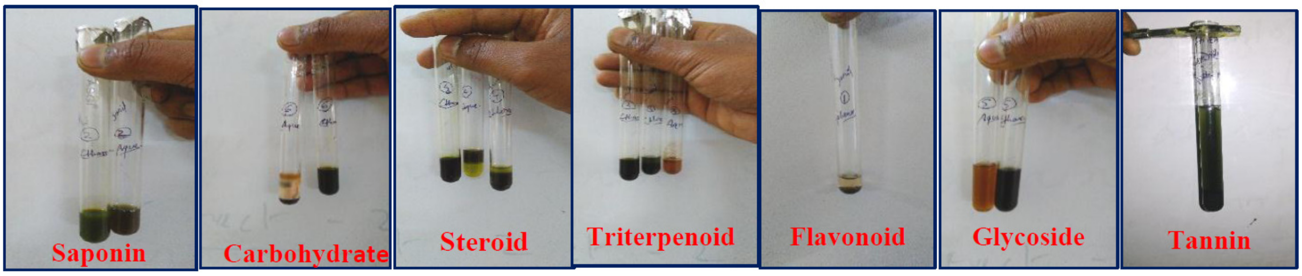
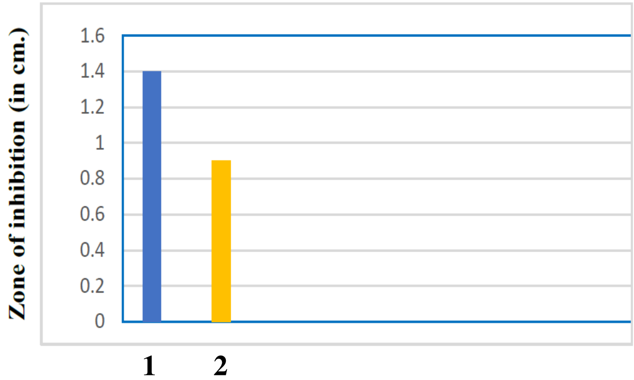


Plate 1 C : Phytochemical screening on *Hydrilla verticillata*

Table 2: Antimicrobial assay

Name of bacteria	Zone of inhibition (in cm.)				
	Ext. -1	**Ext.-2**	**Ext.-3**	**Ext.-4**	**Ext.-5**
Bacillus sp.	-	-	1.4cm.	-	-
Brevundimonas sp.	1.1cm	1.1cm.	0.9cm.	-	1.2cm.

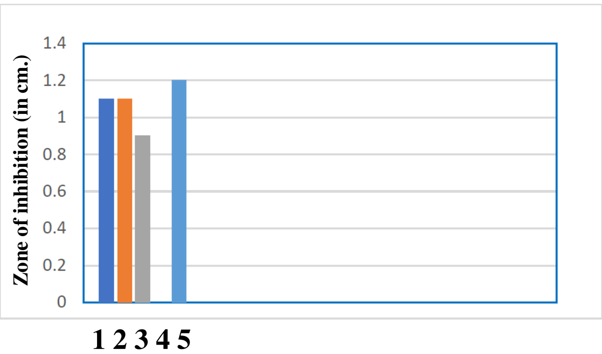
(-) indicates absence of zone of inhibition, **Ext.-1** stands for *N. hydrophilla* aqueous extract, **Ext.-2** stands for *H. verticillata* aqueous extract, **Ext.-3** stands for *H. verticillata* ethanol extract, **Ext.-4** stands for *N. indica* ethanol extract, **Ext.-5** stands for *N. hydrophilla* ethanol extract.



Where, **1** – *Bacillus sp.*, **2** - *Brevundimonas sp.*

Fig. 1: Graphical representation of comparative study of both bacteria on *Hydrilla verticillata*

The graph represents the zone of inhibition of bacteria in *Hydrilla verticillata* extract that showed maximum antimicrobial activity towards *Bacillus* sp. *Hydrilla verticillata* has a prominent antimicrobial activity. The plant extract was tested with *Bacillus* sp. and *Brevundimonas* sp. and comparatively it is observed that *Bacillus* sp. is more sensitive than *Brevundimonas* sp.



where **1 –** *Nymphoides hydrophilla* aqueous extract, 2– *Hydrilla verticillata* aqueous extract, **3** – *Hydrilla verticillate* ethanol extract, and **4-** *Nymphoides indica* ethanol extract. **5-***Nymphoides hydrophilla* ethanol extract

Fig. 2: Antimicrobial activity of all extract toward *Brevundimonas sp.*

The graph represents the zone of inhibition toward *Brevundimonas sp.* by the above-mentioned extract. *Brevundimonas sp.* is a highly sensitive bacterial species. All five extracts of the three plants were tested against *Brevundimonas sp.*, and all extracts except the extract of *Nymphoides indica* exhibited antimicrobial activity with different degrees of effect, which is represented in a graph. Only *Nymphoides indica* did not show antimicrobial activity against the bacterial strain *Brevundimonas sp.*

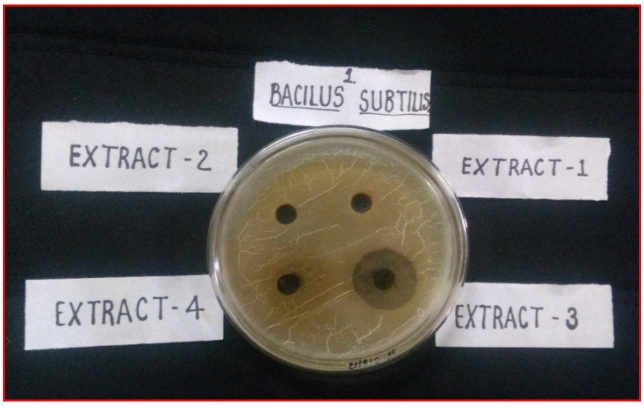


Plate 2 A : Antimicrobial study against *Bacillus* sp

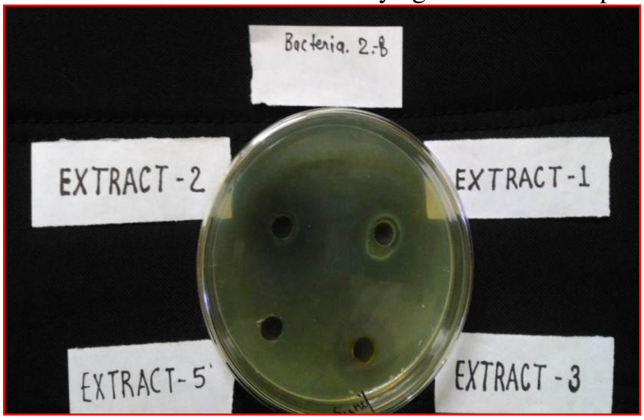


Plate 2 B : Antimicrobial study against *Brevundimonas sp.*

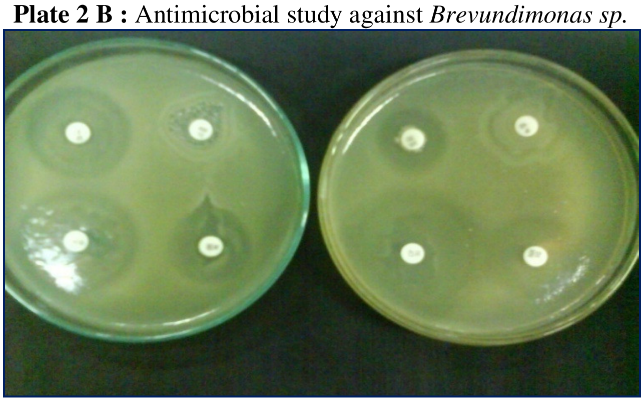


Plate 3 A : Antimicrobial activity of antibiotics towards *Brevundimonas* sp.

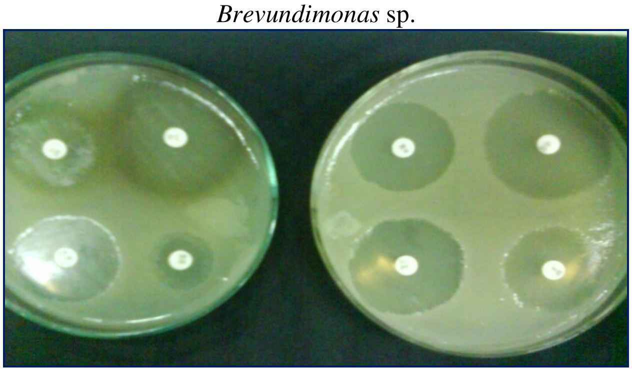


Plate 3 B : Antimicrobial activity of antibiotics towards the *Bacillus* sp.

Gn: Gentamycin, **Nr**: Neomycin, **Am**: ampicillin,**Va**: Vanomycin, **Pn**: Penicillin, **St**: Streptomycin, **Ci** – Ciprofloxacin, **Tt**: tetracyclsine.

Data Availability

The datasets generated and analyzed during the current study were obtained from Guru Ghasidas Vishwavidyalaya, Bilaspur-495009 (C.G.), India.

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