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Study of biological activities of seven medicinal plants from Makwanpur district of Nepal

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Abstract

Objective: Popularity of traditionally used medicinal plants have recently increased worldwide. Since Nepal has a great diversity of indigenous (medicinal) plants species, this research investigated seven medicinal plants *Justicia adhatoda*, *Cuscuta reflexa*, *Clerodendrum infortunatum*, *Catharanthus roseus*, *Azadirachta indica*, *Careya arborea* and *Rhododendron arboretum* of Makwanpur District of Nepal to provide scientific evidence for their medicinal properties.

Methods: Plant extracts were prepared by cold percolation method using methanol solvent. Analysis of phytochemical constituents was carried out using standard methods. Antioxidant activity of plant extracts was analyzed by the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. The antimicrobial activity of the plant extracts was carried by the disc diffusion method.

Results: Phytochemical analysis revealed the presence of polyphenols, reducing sugar, glycosides, quinines, coumarins derivatives. Antioxidant activity was determined in the 1,1-diphenyl-2-picrylhydrazyl (DPPH). Antioxidant activity of *C. arborea* shows maximum antioxidant activity, whose IC₅₀ value was less than all other extract. Total flavonoids content of *A. indica* (294.791 ± 5.565 mg/gm quercetin equivalent) was found highest among other extracts.

Total phenol content of *R. arboreum* (159.095 ± 1.731 mg/gm gallic acid equivalent) was found higher than other extracts.

No activity against both bacteria was observed with extract up to the maximum concentration tested, 50 mg ml⁻¹ for *A. indica*, *J. adhatoda*, *C. reflexa*, *C. roseus*, *C. infortunatum*. *R. arboreum* was found active to Gram positive and Gram negative bacteria whereas *C. arborea* shows activity only against Gram positive bacteria.

Conclusions: The experimental findings show that the methanol extracts of *C. arborea*, *C. infortunatum* and *R. arboreum* possess significant antioxidant and antimicrobial activities respectively justifying the use of these plants in traditional medicine, which may be developed as phytomedicines.

Keywords: Total phenolic content; DPPH radical scavenging; antioxidant; antibacterial activity

Introduction

Reactive oxygen species (ROS), such as singlet oxygen, superoxide ion, hydroxyl ion and hydrogen peroxide, are highly reactive, which are generated normally in cells during metabolism. They cause oxidative damage to enzymes, protein and DNA by covalent binding and lipid peroxidation, with subsequent tissue injury^[1]. This has been associated with pathogenesis of various diseases like cancer, aging, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases etc.^[2, 4]. Antioxidants are agents which scavenge the free radicals and avert the damage initiated by ROS. Natural antioxidant agents have fascinated much interest of public because of their ability to scavenge free radicals, less side effect and cost effective^[5]. Majority of the natural antioxidants from plants are secondary metabolites like phenolics and flavonoids. These are found in different parts of the plants such as leaves, fruits, seeds, bark and roots. Many of these phenolic compounds also possess other therapeutical value like antimicrobial, anti-inflammatory, antimutagenic, hypocholesteremic and antiplatelet aggregation properties. Synthetic antioxidants and antimicrobials in use have been limited due to their adverse side effects^[6, 8]; therefore, there is a need for more effective, less toxic and cost effective antioxidants and antimicrobials from natural sources. Consequently, there has been a growing interest to identify natural antioxidants and antimicrobials from traditional medicinal plants from all over the world^[9, 10]. Many plants have been reported with promising antioxidant and antimicrobial activity so far^[11, 12].

Nepal is rich in biodiversity due to its geographical features. Varieties of medicinal plants are found in Nepal. About 1600-1900 species of plants are commonly used in traditional medicinal practices in Nepal^[13, 14].

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They can be the motives for the scientific researchers. Makawanpur district lies in between Mahabharat Prabat series and Chure Prabat the extremely different and important variations in diversity as well as the climate.

Although there is a growing interest in the scientific evaluation of various plants used in traditional medicine of Nepal, many of ethno medicinal plants are still not explored scientifically for their medicinal potential. The biological properties of the plants are greatly influenced by genetic factors, geographical, climate, soil quality and seasonal factors as well as the developmental stages of the concerned plant, its parts/tissues [15, 16].

Therefore, this study was conducted to determine the total phenolic and flavonoid content, the antioxidant and antimicrobial activity of methanolic extract of Seven ethno medicinal plants i.e *Justicia adhatoda*, *Cuscuta reflexa*, *Clerodendrum infortunatum*, *Catharanthus roseus*, *Azadirachta indica*, *Careya arborea*, *Rhododendron arboretum* from Makawanpur District.

Experimentals

Chemicals

The solvent used for extraction was methanol (Merck, Germany) and DPPH, Ascorbic acid (Sigma Aldrich, USA). All other chemicals used were of the commercially available highest grade.

Collection and processing of plants

All plants were collected from Makwanpur District based on their medicinal uses by local people. Among them *Justicia adhatoda*, *Catharanthus roseus*, *Clerodendrum infortunatum*, *Careya arborea*, *Azadirachta indica* and *Cuscuta reflexa* were collected from Hatiya VDC of Makawanpur district and *Rhododendron arboretum* were collected from Palung and Bhutandevi temple side respectively. All collected plants were identified by Assistant curator Mr. Mukti Ram Poudel, Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu. All collected plant materials were washed by water, shade dried and powdered by grinding machine. Extraction was carried out by soaking 50 g of dried powdered samples in about 300 ml of methanol (Analar grade) for 3 days. The extracts were filtered first through cotton wool, then through Whatman filter paper no. 42 (125 mm). The collected extract was dried using a rotary evaporator.

Phytochemical screening

The method employed for the phytochemical screening was based on the procedure given by Prof. Culie (1982) with slight modification [16,17]. It is the process by which the presence of main groups of natural constituents in different extracts is analyzed by using different specific reagents. The different phytochemicals in the various extracts were identified by the color reaction with different reagents.

Antioxidant activity

DPPH radical scavenging activity (RSA) assay

The free radical scavenging activity of sample and standard ascorbic acid solution in methanol was determined based on their ability to react with stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical [18, 19]. The plant sample at various concentrations (15- 250 µg/ml) was added to a 100 µM solution of DPPH in methanol. After incubation at 37 °C for 30 min, the absorbance of each solution was determined at 517 nm. The measurement was performed in triplicates. The

antioxidant activity of the sample was expressed as IC₅₀ (inhibitory concentration), which was defined as the concentration (in µg/ ml) of sample required to inhibit the formation of DPPH radicals by 50%. Ascorbic acid was used as positive control. Free radical scavenging activity was calculated by using following equation.

$$\% \text{ of free radical scavenging activity} = \frac{A_0 - A_T}{A_0} \times 100$$

Where

A₀ is the absorbance without samples extract and A_T is the absorbance of samples extract.

The % scavenging was then plotted against concentrations used and from the graph IC₅₀ was calculated.

Statistic: All the analysis was carried out in triplicate and the results are expressed as mean ±SD.

Antibacterial assay

The antibacterial assay of plant extracts was carried by using Agar well diffusion method based on the procedure given by Dingle *et al* [20]. The wells were made in the incubated media plates with the help of sterile cork borer (6 mm) and labeled properly. Then 50 µl of the working solution of the extract were loaded into the respective wells with the help of micropipette. The solvent (DMSO + water) was tested for its activity as a control at the same time in the separate well. The plates were then left for half an hour with the lid closed so that extracts diffused to the media. The plates were incubated overnight at 37 °C. The microorganisms (*Staphylococcus aureus* and *Escherichia coli*) used in this study were identified strains obtained from Central Department of Microbiology, TU, Nepal.

Total Phenolic content

The total phenolic content of methanolic extract of different selected plants were determined using the Folin–Ciocalteu phenol reagent with slight modification. 0.1 ml of each extract (2.5 mg/ml) was separately mixed with the 1 ml of Folin–Ciocalteu phenol reagent (1:10 dilution with the distilled water) and 0.8 ml of aqueous 1 M Na₂CO₃ solution [21]. The reaction mixture was allowed to stand for about 15 minutes and the absorbance of the reactants was measured at 765 nm using the UV- visible spectrophotometer. The calibration curve was obtained using the solution of gallic acid as standard in methanol and water (50:50 v/v) using the concentration ranging from 10-100 µg/ml. Based on this standard graph, the concentration of the individual samples was calculated. The total polyphenol content was expressed in terms of the milligrams of the gallic acid equivalent per gram of the dry mass (mg GAE g⁻¹). For each extract, three replicates were performed for the reproducibility of results.

Total flavonoids content estimation

The total flavonoid content in the plant extract was estimated using the Aluminium chloride (AlCl₃) colorimetric method with slight modifications. 0.25 ml of extract (10 mg/ml) was separately mixed with the 0.75 ml of methanol, 0.05 ml of the 10% aluminum chloride, 0.05 ml of the 1 M potassium acetate (CH₃COOK) and 1.4 ml of the distilled water. The reaction mixture was allowed to stand for about 30 minutes in room temperature. The absorbance of the mixture was measured at 415 nm using the UV – visible spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The calibration curve

was obtained with the help of the quercetin (Sigma) standard solutions in methanol with the concentration ranging from the 10-100 µg/ml. The total flavonoid content was expressed in terms of the milligram of quercetin equivalent per gram of the dry mass (mg QE/g) [22]. For each experiment the tree replication was used for the accuracy and reproducibility of results.

Results and Discussion

Phytochemical Screening

Extracts of plants were dried and phytochemical screening of all plant materials was done on the basis of Alamzeb Muhammad *et al*, (2013) [16, 17]. The results obtained are shown in table 1.

Table 1: Screening Test of Methanol Extract

Group of compounds	J. (L)	C. (P)	Cl. (F)	Az. (L)	Co. (B)	Ca. (L)	R. (F)
Polyphenols	+	+	+	+	+	+	+
Reducing compounds	+	+	+	+	+	+	+
Alkaloids	+	+	-	-	-	+	-
Glycosides	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+
Quinones	+	+	+	-	+	-	+
Steroids	+	+	+	-	+	-	-
Saponins	+	-	-	-	-	+	-
Coumarin derivatives	+	+	+	-	+	+	+

+ = Present - = Absent

Where,

J. (L) = Leaf of *J. adhatoda*

C. (P) = Plant of *C. reflexa*

Cl. (F) = Flower of *C. infortunatum*

Az. (L) = Leaves of *A. indica*

Co. (B) = Bark of *C. arborea*

Ca. (L) = Leaves of *C. roseus*

R. (F) = Flower of *R. arboreum*.

During the phytochemical screening of methanol extract of the all plants extracts shows presence of reducing compounds, glycosides, flavonoids and polyphenols. Alkaloids were found to be present in *J. adhatoda*, *C. reflexa* and *C. roseus*. Saponin was found in *J. adhatoda*, *C. roseus*, *R. arboreum*. Presence of steroid was found in *J. adhatoda*, *C. reflexa*, *C.*

infortunatum and *C. arborea* plant extracts. Quinone was present in all extract except *A. indica* and *C. roseus*. Coumarin derivative was found to be present in all extract except *A. indica*.

However, the natural products profile and consequently the bioactivity are known to vary with climate and geographical location of the plants. The present study also correlates with little variation [23, 27].

Phenolic and flavonoid contents

The total phenolic content of the methanolic extracts of *C. infortunatum*, *C. arborea*, *A. indica*, *C. reflexa*, *C. roseus*, *J. adhatoda* and *R. arboreum*, calculated from the calibration curve (Figure. 1) ($R^2= 0.9815$), were 150.55 ± 0.49 , 134.22 ± 0.55 , 132.88 ± 0.68 , 116.55 ± 1.04 , 109.42 ± 1.23 , 93.75 ± 0.80 , 159.51 ± 1.24 gallic acid equivalents /gm (Figure. 2) and the total flavonoid content ($R^2= 0.9946$) were 156 ± 21.6 , 93 ± 8.33 , 294.66 ± 13.95 , 98.37 ± 1.27 , 181.86 ± 13.6 , 154.29 ± 1.66 and 93.64 ± 8.33 quercetin equivalents/g (Figure 3 and 4) respectively. Phenolic compounds have free radical scavenging ability so the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. Flavonoids are major plants secondary metabolites such as flavones, flavanols and condensed tannins. The antioxidant activity of which depends on the presence of free OH groups, especially 3-OH. It is reported that plant flavonoids having antioxidant activity *in vitro*, also act as antioxidants *in vivo* [21, 22].

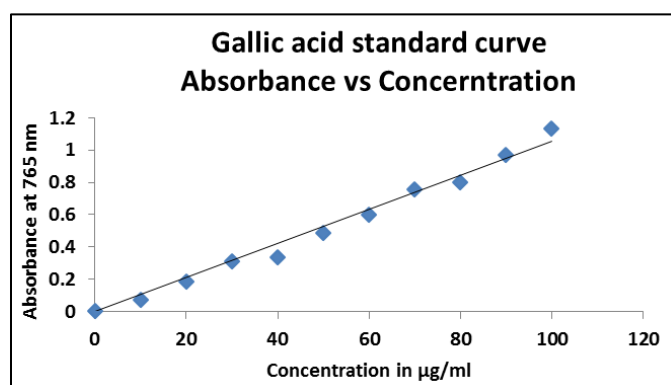


Fig 1: Graph of absorbance versus Concentration of Gallic acid.

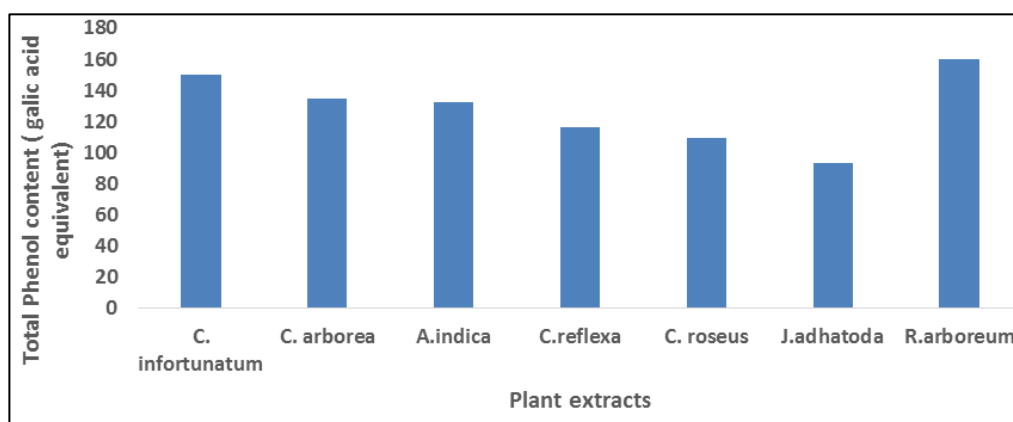


Fig 2: Total phenol content of plant extracts in Gallic Acid equivalent mg/gm

From above data it is found that total phenol content of *R. arboreum* is high than *C. infortunatum*, *C. arborea*, *A. indica*, *C. reflexa*, *C. roseus* and *J. adhatoda*. Total phenol content of methanolic: water leaf extract of *C. roseus* was reported [28].

Total Phenolic content of other selected plant have not been reported.

Different in the amount of TPC in different plants and their parts could be explained by the fact that the presence of

phenolics is affected by maturity at harvest, growing condition, soil condition and post-harvest treatment. Many studies confirmed that amount and composition of phenolics compound is diversified at sub-cellular level within the tissues. Concentration of simple phenolics compound such as

caffeic acid, ferulic acid etc are generally higher in younger tissues, later than different phenolic acids condense to form complex phenolic compounds such as flavonoids, tannins and lignins.

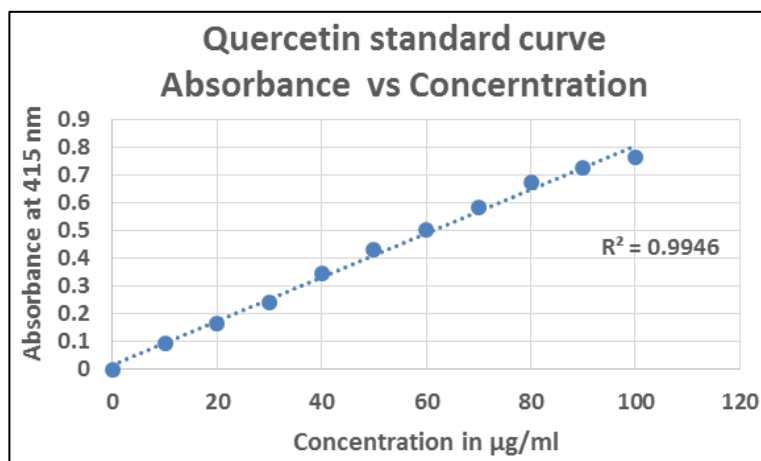


Fig 3: Graph of Absorbance versus concentration of Quercetin

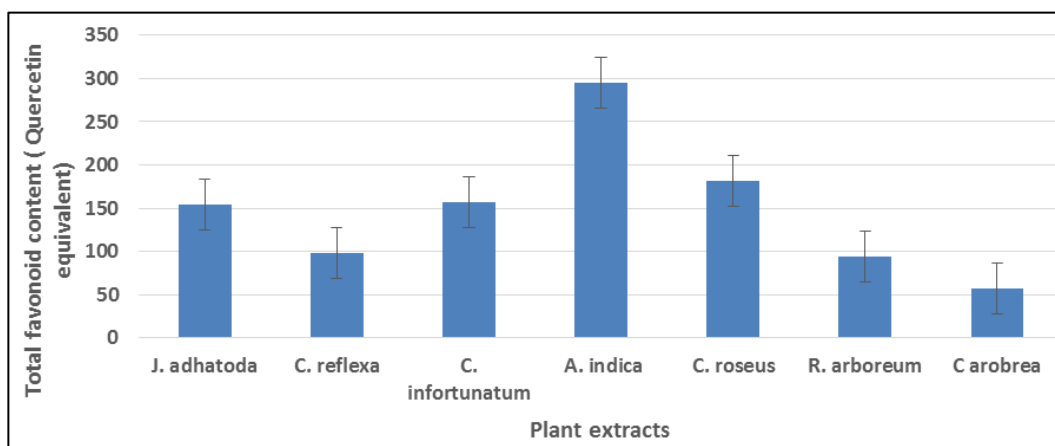


Fig 4: Total flavonoids content of plant extracts quercetin equivalent in mg/gm.

Above data shows that total flavonoids of *A. indica* is higher among other plant. Higher to lower total flavonoids content is as follows; *A. indica*, *C. roseus*, *C. infortunatum*, *J. adhatoda*, *C. reflexa*, *R. arboreum* and *C. arborea* (Fig. 4). TFC of methanolic extract of flower and fruits of *J. adhatoda* and leaf extract of *R. arboretum* had been reported [29, 30]. Total flavonoid content of other selected plants has not been reported. Variation of TFC from plants to plants and parts to parts can be justify as the fact that the time of maturity at harvest, growing condition, soil condition, altitude of plant growth and post-harvest treatment [31].

Antioxidant Activity

DPPH assay

The antioxidant activities of crude methanolic extract of six plants were measured and the results are given below in table 3. The result of DPPH assay shows that almost all the methanol extracts showed potential radical scavenging capacity. IC₅₀ of the extract indicates the corresponding concentration in which the radical scavenging capacity is 50%. The IC₅₀ of the extract and standard are determined graphically as given by Subedi *et al* 2012 (Fig 1). From the

table (3), it is found that the IC₅₀ of extracts of all the plants except *J. adhatoda* is less than 100. The IC₅₀ of the *J. adhatoda* is 234.457 µg/ml. Among all the extracts tested, *C. arborea* (bark), and *R. arboreum* have the lower IC₅₀ value. IC₅₀ of *C. arborea* was found to be near to ascorbic acid.

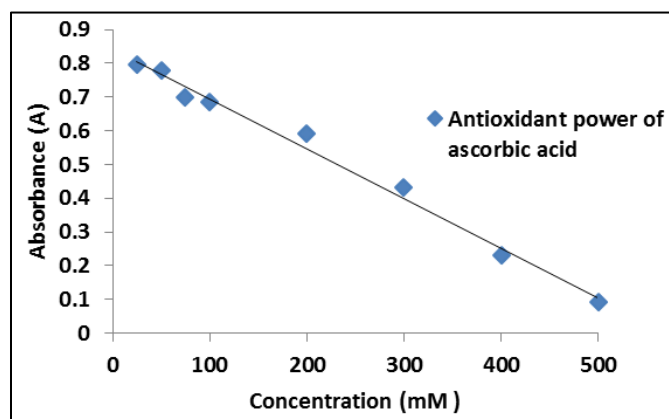


Fig 5: Graph Absorbance versus Concentration of Ascorbic acid

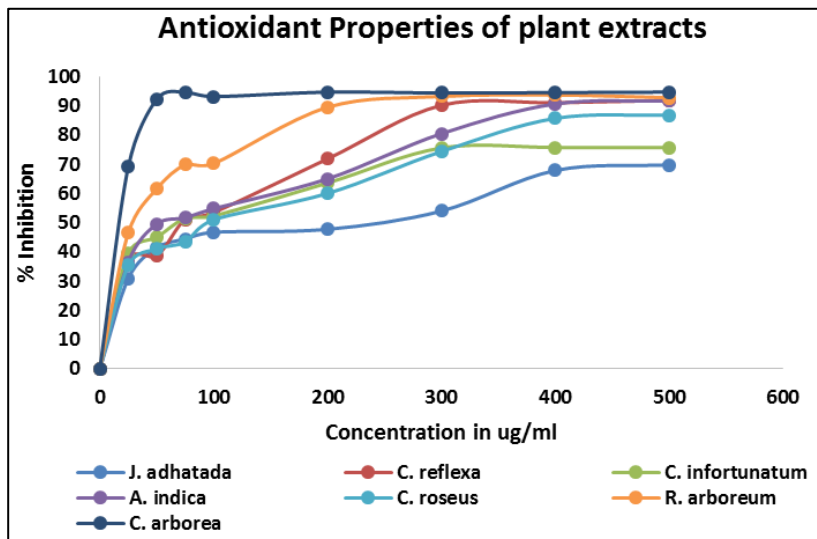


Fig 6: Graph of % inhibition versus concentration

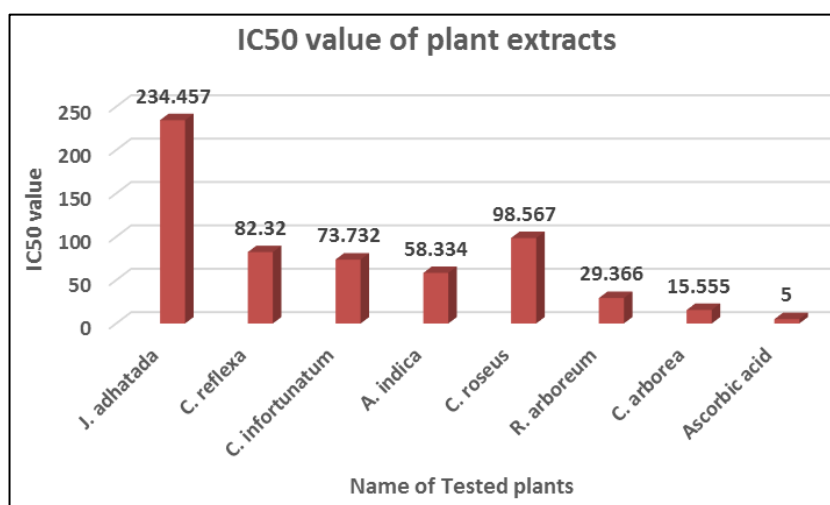


Fig 7: Graph of IC₅₀ versus plant extracts

The result obtained here is in good correlation with published literature. Viji kumar *et al*, (2011) reported that methanolic extract of *C. reflexa* have IC₅₀ value 359.48 µg/ml. Modi *et al*, (2010) reported that ethanolic extract of leaf of *C. infortunatum* shows 80% inhibition against DPPH at 0.10 mg/ml concentration. Kumar *et al*, (2014) reported that acetone extract of leaf of *R. arboreum* shows maximum inhibition 78.60% against DPPH at 500 µg/ml concentration. The result obtained here are in good correlation the reported literatures [28, 29].

The phenolic compounds containing free hydrogen are responsible for these antioxidant activities. It becomes evident that the antioxidant activities of all plant extracts are due to presence of flavonoids and polyphenols in all the plants. Here in this result antioxidant property of bark of *C. arborea* is high this is may be due to the reduction of hypoperoxides, inactivation of free radicals, chelation of metal ions and combination thereof.

Antibacterial Activity

The antibacterial potential was studied against *E. coli* and *S. aureus* using Agar well diffusion method. The zone of inhibition (ZOI) shown by the extracts at four different concentrations (5 mg/disc, 10 mg/disc, 25 mg/disc and 50 mg/disc) were observed (Fig. 5). The results are shown in

table 2. The standard (positive control) used were streptomycin and DMSO was used as negative control.

Table 2: Results of Antibacterial Screening of Different Plant Extracts

S.N	Plant extract	Bacterial organism	Zone of inhibition			
			Extract			
			5 mg/mL	10 mg/mL	25 mg/mL	50 mg/mL
1.	Co. (B)	<i>S. aureus</i>	0	0	0	8
2.	R. (F)	<i>E. coli</i>	0	0	0	8
2.	R. (F)	<i>S. aureus</i>	0	0	8	9

Table 3: Mean Zone of inhibition (ZOI) shown by different concentrations of streptomycin against tested bacteria

Bacterial Species	(Zone of inhibition in mm)			
	Concentration of Standard antibiotic (Streptomycin)			
	0.25 mg/well	0.1 mg/well	1.25 mg/well	2.5 mg/well
<i>S. aureus</i>	16	18	21	29
<i>E. coli</i>	16	21	24	25

Where,
Co. (B) = Bark of *C. arborea*
R. (F) = Flower of *R. arboreum*.

For the estimation of the potential effect of plant extracts on particular bacteria, the diameter of Zone of Inhibition (ZOI) was measured. From the above table, it was found that flower of *R. arboretum* exhibited moderate antibacterial activity against *S. aureus* and *E. coli*. The result is well supported by literature [29]. But bark of *C. arborea* shows mild anti-bacterial activity against *S. aureus* only. Rest of the tested plant did not produce antibacterial activity although the antimicrobial activity of *A. indica*, *C. roseus*, *C. infortunatum*, *J. adhatoda* had been reported [30]. Sometimes it is difficult to compare results obtained, when dealing with plant extracts, with published results in the literature because several variables influence the results, such as the environmental and climatic conditions under which the plant grew, choice of plant extracts, choice of extraction method, antimicrobial test method and test microorganisms. The antibacterial activity of the plant extracts might be attributed to the presence of bioactive plant compounds such as tannins, phenolic compounds, polyphenols and flavonoids. Thus the results of

antibacterial activities obtained in the present study for the *R. arboretum* extracts were correlated to their total phenolic contents and antioxidant activity (Fig 2 and 7). Standard criteria for evaluation of plant antimicrobial activity are lacking and results greatly differ between authors. The beneficial medicinal effects of plant materials typically result from the secondary products present in the plant although it is usually not attributed to a single compound but a combination of the metabolites. The antimicrobial actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct. They also vary between tissues (higher concentrations occur in bark, heartwood, roots, branch bases and wound tissues), among species from tree to tree and from season to season. That extracts are generally richest in antibacterial agents after the flowering (sexual) stage of their growth is complete, and that plants taken from stressful environments were particularly active.

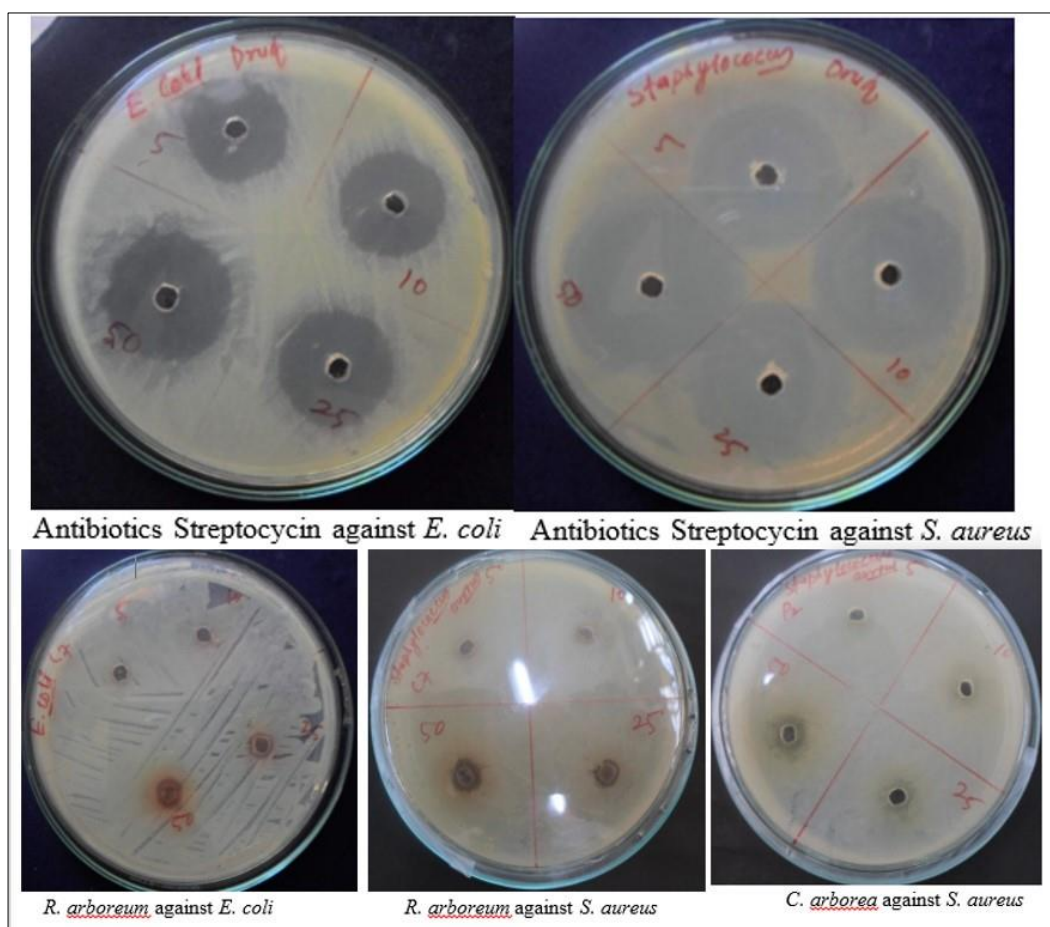


Fig 5: Agar well diffusion test demonstrating inhibition zones by *Streptomycin* against *E. coli* and *S. aureus*, *R. arboretum* against *E. coli* and *S. aureus*; *C. arborea* against *S. aureus*

Conclusions

Phytochemical screening of methanolic extract of all plants exhibit the presence of polyphenols, reducing sugar, glycosides, quinines, coumarins derivatives.

Antioxidant activity of *C. arborea* shows maximum antioxidant activity, whose IC_{50} value was less than all other extract. Total phenol content of *R. arboretum* (159.095 ± 1.731 mg/gm gallic acid equivalent) was found higher than other extracts. Antimicrobial activity of *R. arboretum* was found active to Gram positive and Gram negative bacteria whereas *C. arborea* shows activity only against Gram positive bacteria. We can isolate compound from bark of *C. arborea*

which show potent antioxidant activity in DPPH assay. We can isolate flavonoid from *R. arboretum* which have high total phenolic content.

Statistics

All the analysis was carried out in triplicate, and the results are expressed as mean \pm SD.

Conflict of Interests

The authors have not declared any conflict of interests.

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