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Original Article

BIOLOGICAL ACTIVITIES OF SOME SELECTED NEPALESE MEDICINAL PLANTS AND ISOLATION OF CHEMICAL CONSTITUENTS FROM *CALLICARPA MACROPHYLLA*

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ABSTRACT

Objective: The main objectives of this study was to analyze the phytochemicals, determine the total flavonoid content, brine shrimp toxicity, antibacterial activity, evaluate the antioxidant, antimicrobial, anti-diabetic activities of nine medicinal plants *Callicarpamacrophylla, Bauhinia purpurea, Plumeriarubra, Girardiniadiversifolia, Acacia nilotica, Woodfordiafruticosa (Bark) Woodfordiafruticosa (flower), Terminaliaalata,* and *Premnabarbata.*

Methods: The cold percolation method was adopted for the extraction of secondary metabolites in methanol. The preliminary phytochemical analysis was performed by colour differentiation methods. The radical scavenging activity was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The antidiabetic activity was performed by α-amylase enzyme inhibition activity. The chemical constituent was isolated by column chromatography from the biologically active plant fraction.

Results: The phytochemical investigation has shown plants are the rich source of secondary metabolites as quinones, saponins, terpenoids and glycosides. Among the nine tested plants, *Terminaliaatalia* showed the highest radical scavenging activity 96.41±0.47 with IC_{50} value 6.353 µg/ml, followed by *Girardiniadiversifolia* 97.26±0.67 with IC_{50} value 11.52 µg/ml whereas ascorbic acid has 39.85 µg/ml as standard. *Bauhinia purpurea* showed significant inhibition to the α-amylase enzyme having inhibitory concentration IC_{50} 17.05±13.00 SD in a dose-dependent manner. *Woodfordiafruticosa* demonstrated significant toxicity to *A. salina* with LC_{50} value of 457.08 µg/ml. *Callicarpamacrophylla* bark showed a potential inhibitory activity against the growth of *Straphylococcusaureus* as compared to standard chloramphenicol. Active plant extract of *Callicarpamacrophylla* was subjected for column chromatography. Conclusion: Out of nine plant samples *Terminaliaatalia* showed the highest radical scavenging activity. The plant extract of *Bauhinia purpurea* showed significant inhibition to the α-amylase enzyme inhibition. *Woodfordiafruticosa* demonstrated significant toxicity to *A. salina,* whereas *Callicarpamacrophylla* showed the potent antibacterial activity. The active plant extract was subjected for column chromatography and different fractions were collected in solvent polarity basis.

Conclusion: The phytochemical investigations showed that plant extracts are the rich sources of secondary metabolites such as alkaloids, flavonoids, saponins, glycosides, polyphenols, coumarins and reducing sugars which showed they are supposed to be responsible for different biological activities. IC_{50} values showed the varied degree of antioxidant property of which *Plumeriarubra* and *Acacia nilotica* exhibit good antioxidant property with IC_{50} value close to the standard ascorbic acid.

Keywords: Phytochemical, Medicinal plants, Antioxidant, Cytotoxic, Antimicrobial, Antidiabetic, α-amylase

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INTRODUCTION

Nepal is rich in all three levels of biodiversity, namely species diversity, genetic diversity and habitat diversity. In Nepal, large number of medicinal plants are known to have medical values and peoples have been using since many years to cure specific diseases [1]. Nepal has been regarded as the natural showroom of biodiversity because of its geotopography which is reflected in its dramatic contrast of climatic condition, which in turn is reflected in floral and faunal variations. Such biodiversity has supported the live hood of people who live in remote areas of Nepal. These local people of a different ethnic group traditionally acquired a diversity of knowledge regarding the utilization of plant and animal resources for various purposes like food, medicine, clothing construction, dyes, ritual performances, energy, etc. About 80-90% people living in rural areas of Nepal depend directly or indirectly on the formal and informal system of traditional medicine involves the use of plant extracts [2]. Antioxidants are natural or synthetic substances that may prevent or delay oxidative cell damage in human beings. In humans, free radicals have been blamed, at least partially, for the development of several chronic ailments, for example, Alzheimer's disease, atherosclerosis, cancers and many others [3].

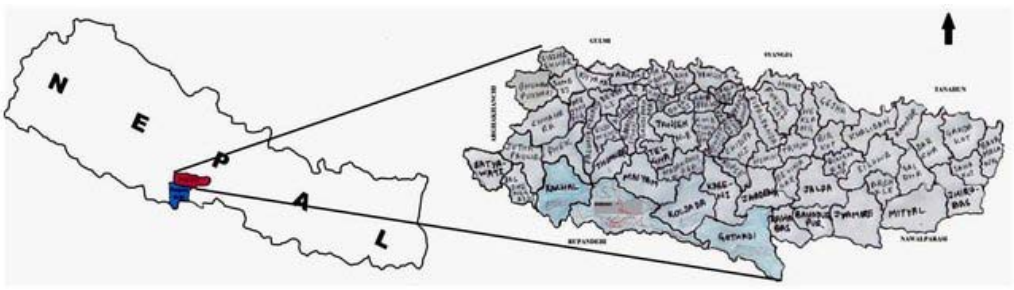

Fig. 1: Location map of study site (medicinal plants collection): Palpa district of Nepal

Oxidative stress is a pathological state in which reactive oxygen/nitrogen species (ROS/RNS) overwhelm antioxidative defense of the organism, leading to oxidative modification of lipids, proteins, DNA, tissue injury and accelerated cellular death [3]. Commercially available antioxidants are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butylated hydroxyquinone (TBHQ). But, these antioxidants have side effects and toxicity when taken *in vivo*. Hence, their use is being restricted and have an urgent need to find out safer and bioactive natural antioxidant [4, 5]. Diabetes mellitus is a metabolic disorder characterized by loss of glucose homeostasis occurring due to defects in insulin secretion or insulin action resulting from impaired metabolism of glucose, lipids and proteins. Hyperglycemia, the primary clinical diagnosis of diabetes, is thought to be contribute to diabetic complications by altering vascular cellular metabolism in human body. Diabetes is a multifactorial diseases leading to several complications require a multiple therapeutic approach [6, 25]. α-amylase is an enzyme that breaksα bonds of large polysaccharides, such as starch and glycogen yielding glucose and maltose [8, 24]. It is the major form of amylase found in humans and other mammals [7]. α-amylase inhibitory agent is a protein family which inhibits mammalian α-amylases mainly by forming a stoichiometric complex with α-amylase [9].

An antimicrobial agent either kills microorganisms or inhibits their growth. Antimicrobial medicines can be grouped according to the effect caused by microorganisms in human body [10]. Today, numerous antimicrobial agents exist to treat a wide range of infections. The development of new anticancer and antimicrobial therapeutic agents is one of the fundamental goals in medicinal chemistry. One new strategy for the research on new anticancer and antimicrobial therapeutic agents has been the use of metal-containing compounds. The antimicrobials should be selectively toxic to the pathogenic microbes but not toxic to the host tissues [10]. More ethnopharmacological studies have been performed in Nepal but these results are not well documented and explored. The peoples of different communities of Nepal have been using such medicinal plants for cure of simple to life threating diseases but the modes of preparation and administration of traditional herbal medicines are not well known. The evidences that show the relationship between pharmacological and phytochemical uses of plants are not well explored [13].

The most important part of this research is documenting the traditional knowledge and perform scientific validation of traditionally used different natural products, especially medicinal plants. Validation can be performed by different *in vitro* and *in vivo* experiments or by isolating the target secondary metabolites, which is useful for treating particular diseases or any health disorders [11, 12]. Present study focused on the collection of nine medicinal plants, *Callicarpamacrophylla, Bauhinia purpurea, Plumeriarubra*, *Girardiniadiversifolia*, *Acacia nilotica*, *Woodfordiafruticosa* (bark), *Woodfordiafruticosa* (flower), *Terminaliaatalia* and *Premnabarbata* from Palpa district of Nepal based on ethnomedicinal and traditional uses of plants and to perform their scientific validation as the primary source of medicine curing different diseases. Based on their biological activities, one of the plant fraction was selected to isolate the chemical compound by column chromatography.

MATERIALS AND METHODS

Plant materials

The plant materials were collected from the BoughaGumha VDC of Palpa district Nepal. The plants were identified by Dr. Munesh Gubhaju, Tribhuvan Multiple Campus, Tribhuvan University, Tansen, Palpa. The list of medicinal plants with voucher specimen number and their uses are shown in table 1.

Table 1: List of selected medicinal plants and their therapeutic uses

Voucher specimen number	Code	Scientific name	Nepali name	Used part	Altitude (m)	Therapeutic uses
2713	AB_1	*Callicarpamacrophylla*	Dahigola	Bark, fruit and root(Plant juice)	1300-2700	Fever, stomatitis, throat pain [14, 15].
9423	AB_2	*Bauhinia purpurea*	Tanki	Bark (powder, paste)	1300-2500	Diarrhoea, dysentery [15].
1222	AB_3	*Plumeriarubra*	Golaichi	Whole part(fruit)	1300-2000	Anorexia, marasmus [15].
4649	AB_4	*Girardiniadiversifolia*	Chalnisisnu	Root (decoction, paste)	Below 2000	Cooling agent, constipation [14, 15].
10076	AB_5	*Acacia nilotica*	Jukkharat		900-3300	Sprain, cut, ulcer [15].
6038	AB_6	*Woodfordiafruticosa*	Dhayaro	Bark (juice)	Below 1500	Dysentry [15].
6038	AB_7	*Woodfordiafruticosa*	Dhayaro	Flower (juice)	Below 1500	Dysentry, jaundice [15].
5192	AB_8	*Terminaliaalata*	Saaj	Bark (paste, juice)	300-1800	Gastritis [15].
5842	AB_9	*Premnabarbata*	Gineri	Stem bark (juice)	1100-2700	Cooling agents, fever [14, 15].

Sample preparation

The bark of *Callicarpamacrophylla, Bauhinia purpurea, Plumeriarubra, Acacia nilotica, Woodfordiafruticosa, Terminaliaalata, Premnabarbata,* and flower of *Woodfordiafruticosa* and the root of *Girardiniadiversifolia* were collected and washed with tap water to remove the contaminants. Then the collected plant parts were shade dried. The dried plant parts were grinded into powder form in electric grinder and stored in clean plastic bag at 4 °C until to perform different biological activities.

Extract preparation

The phytochemicals were extracted by cold percolation method using methanol as a solvent. Powdered plant parts (150 g) of mentioned plants were kept separately in the clean and dry conical flasks. Methanol (400 ml) was added to each nine different flask and kept for 72 h with frequent shaking. The mixtures were decanted and filtered with the help of cotton plug and thus obtained filtrates were concentrated with the help of rotatory evaporator by distillation at temperatures below 60 °C. The concentrated filtrates were kept in a beaker wrapping with aluminum foil containing small pores to facilitate the evaporation of the solvent. After complete evaporation of the solvent extracts were obtained. These plant extracts were stored at 4 °C until doing biological activities. Biological activities were performed after sudden extraction. Percentage yield for each plant extracts was calculated.

Phytochemical analysis

This method involves the selective and successive extraction of phytochemicals where the method adopted was primarily based on the standard procedure. The analysis of the presence of main groups of natural compounds in the different plant extracts was done by the color reaction using different specific reagents [16].

Antioxidant activity

This method is rapid, simple and inexpensive to measure antioxidant capacity involves the use of the free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH). The ability of different plant extracts to scavenge DPPH free radicals was performed by adopting the standard protocol described by Jamuna *et al.* 2012 [17].

Different concentrations of test samples of 20, 40, 60, 80 and 100 µg/ml were made from stock solutions. Then 2 ml of each plant extracts were mixed with 2 ml of DPPH solution. The test tubes were

shaken vigorously for the uniform mixing then the solutions was kept for 30 min in the dark at room temperature. After 30 min, absorbance was measured at 517 nm using a UV-visible spectrophotometer. Ascorbic acid of same concentrations was used as a standard.

The percentage of the DPPH free radical scavenging activity was calculated by using the equation,

$$Radical\ scavenging\ (\%) = \left[\frac{A0 - As}{A0}\right] * 100$$

Where, A_0= Absorbance of the control (DPPH solution+methanol), A_s = Absorbance of test sample

The IC_{50} indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radical. IC_{50} values were calculated using the inhibition curve by plotting extract concentration versus the corresponding scavenging.

Antidiabetic activity (α-amylase inhibition assay)

The antidiabetic activity of plant extracts was determined by using the α-amylase inhibition assay proposed by Kusano *et al.* 2011with few modification. The undigested starch due to enzyme inhibition was detected through the blue starch iodine complex at 630 nm [18].

1000 µg/ml of stock solution of different dry extracts were prepared by dissolving 17 mg dry mass of extract in 17 ml dimethyl sulphoxide (DMSO). This stock solution was further used to prepare 5 different concentrations of each extracts viz. 640 µg/ml, 320 µg/ml, 160 µg/ml, 80 µg/ml and 40 µg/ml. Substrate was prepared by dissolving 200 mg of starch in 25 ml of NaOH (0.4M) by heating at 100 °C for 5 min. After cooling, pH was adjusted to 7.0 and the final volume was made up to 100 ml using distilled water.

400 µl of substrate was pre-incubated with 200 µl of varying concentrations (640 µg/ml, 320 µg/ml, 160 µg/ml, 80 µg/ml and 40 µg/ml) of plant extracts and acarbose separately at 37 °C for 5 min. After this 200 µl of α-amylase solution was added to each of them and then again incubated for 15 min at 37 °C. After incubation the enzymatic reaction was quenched with 800 µl of HCl (0.1M). Then, 1000 µl of iodine reagent was added, and the absorbance was measured at 630 nm. Then experiment was carried out in triplicate. Percentage of enzyme inhibition was calculated by using formula,

$$\%\ inhibition = 1-[Abs2-Abs1/Abs4-Abs3] \times 100$$

Where,

Abs1 = absorbance of incubated mixture containing plant extract, starch and amylase, Abs2 = absorbance of incubated mixture containing plant extract and starch, Abs3 = absorbance of incubated mixture containing starch and α-amylase, Abs4 = absorbance of incubated solution containing starch only. Graph was plotted by taking the concentration on the x-axis and percentage inhibition on the y-axis. With the help of this graph, IC50 values of each samples were calculated. The species having the lowest IC50 was considered to have the best α-amylase inhibition property.

Qualitative screening and evaluation of an antibacterial activity

Sterile Muller-Hinton Agar (MHA) plates were dried to remove excess of moisture from the surface of the media. The agar plates for the essay were prepared by labeling them with the name of the bacteria and the name code of the disc. The inoculums of bacteria were transferred into petri disc containing solid nutrient media of agar using sterile swab. The plate was rotated through an angle of 60 °after each swabbing. The swab was passed around the edges of the agar surface. The inoculated plates were left to dry for minutes at room temperature with a lid closed. Four wells were made in each incubated media plates with the help of sterile cork borer no.6. So, the diameter of a well was 6 mm and labeled properly. Then 50 µl of the working solution of the plant extract, DMSO as negative control and 25 µl of ofloxacin as a positive control at the same time were loaded into the respective wells with the help of micropipette. The plates were then left for half an hour with the lid closed so that the extract diffused into media. The plates were incubated overnight at 37 °C. After 24 h of incubation, the plates were observed for the presence of inhibition of bacterial growth indicated by a clear zone around the wells. The size of the zone of inhibition was measured and the antibacterial activity expressed in terms of the average diameter of zone of inhibition in millimeters. The absence of zone of inhibition was interpreted as the absence of activity. The ZOI were measured with the help of a millimeter ruler and the mean was recorded [19].

Brine shrimp bioassay (Toxicity test)

The eggs of brine shrimp are readily available at low cost and they remain viable for years in the dry state. Upon being placed in a brine solution, the eggs hatch within 48 h providing large number of larvae (nauplii). It determines the LC_{50} value (S) (µg/ml) for the crude extract (s). Extracts having LC_{50}values less than 1000 ppm (µg/ml) are considered as pharmacological active. Compounds/ extracts having LC_{50} values less than 1000 ppm (µg/ml) are considered as pharmacological active. The assay was carried out by adopting the standard protocol of Meyer *et al.* 1982 [20].

LC_{50} value is the lethal concentration dose required to kill 50% of the shrimps. It can be determined as follows,

If 'n' is the number of replicates (here three), 'x' is the log of constituents in mg/ml (log10, log100 and log1000 for three dose level respectively), y is prohibit for average survivor of all replicates.

$$\alpha = \frac{1}{n}[\sum y - \beta \sum x]$$

$$\beta = \frac{\sum xy - \sum x \ \sum y /n}{\sum x^2 - (\sum x)^2 /n}$$

Where,

From prohibit regression,

$$Y = \alpha + \beta X$$

$$X = (Y - \alpha)/\beta$$

Where Y is constant

LC_{50} = Antilog x

In the present work, brine shrimp bioassay of different plant extracts was carried out and the lethal concentration value was calculated.

Extraction and isolation of pure compounds

On the basis of biological activities, the plant extract of *Callicarpamacrophylla* was selected as an active sample for the isolation of compounds by chromatographic technique. Bark of *C. macrophylla* was dried and powdered. 150 g of powdered plant material was extracted with methanol by cold percolation. The solvent was filtered and evaporated in a rotatory evaporator to get methanol extract. The yield of the methanolic extract obtained was 15.56 g. The methanolic extract was then fractionated with different solvents such as hexane, dichloromethane, ethyl acetate and methanol-based on polarity.

Chromatographic separation

The hexane fraction weighing 8.01 g was adsorbed on 20 g silica gel and loaded on to a silica gel (120 g, Qualigens, and 60-120 mesh) packed column having an internal diameter of 3 cm with the adsorbent height 32 cm. The column was initially eluted with hexane and then the gradient of hexane in ethylacetate of increasing polarity and finally reported upto 100% ethyl acetate. Different fractions were collected and analyzed by thin-layer chromatography (TLC). Based on TLC report hexane fraction was selected for isolation of chemical constituents by column chromatography.

RESULTS AND DISCUSSION

Yield percentage of plant extracts

Quantitative estimation of plant extracts showed different yield percentage shown in table 2.

Table 2: The yield percentage of extracts of plant samples

Name of sample plants	Sample taken (g)	Extract (g)	Percentage yield
Callicarpamacrophylla	150	15.56	10.37
Bauhinia purpurea	150	11.10	7.4
Plumeriarubra	150	8.25	5.5
Girardiniadiversifolia	77	6.45	8.37
Acacia nilotica	150	5.12	3.41
Woodfordiafruticosa (bark)	150	10.78	7.18
Woodfordiafruticosa (flower)	150	9.65	6.43
Terminaliaatalia	150	13.12	8.74
Premnabarbata	150	12.75	8.5

The plant sample *Callicarpamacrophylla* showed the highest yield percentage (10.37%), indicating the plant extract is the rich source of secondary metabolites. The plant extract of *Acacia niloticca* showed the lowest yield percentage indicating the extract is the poor source of secondary metabolites as phytoconstituents.

Phytochemical screening

The results of the phytochemical analysis is shown in table 3.

Results of the phytochemical analysis showed that quinoneis present in all the extracts except *terminaliaalata,* saponins and terpenoids are present on most of the plant extracts. Glycosides are present in *Callicarpamacrophylla*, *Plumeriarubra*, *Woodfordiafruticosa* (flower) and *Premnabarbata,* whereas there is an absence of reducing sugars in all plant extracts. Alkaloids are present only in *Plumeriarubra* and *Woodfordiafruticosa* (flower). The result of the phytochemical analysis slightly differs due to variation in altitude, different environmental conditions, method and time of sample collection, extraction procedure and also due to lab setup and chemical grades.

Table 3: Phytochemical screening of plant extracts

S. No.	Groups of compounds	AB_1	AB_2	AB_3	AB_4	AB_5	AB_6	AB_7	AB_8	AB_9
1	Basic Alkaloids	-	-	+	-	-	-	+	-	-
2	Coumarins	+	+	+	+	-	-	-	-	+
3	Flavonoids	+	+	-	-	+	+	+	-	-
4	Glycosides	+	-	+	-	-	-	+	-	+
5	Polyphenols	+	+	-	+	-	-	+	-	-
6	Quinones	+	+	+	+	+	+	+	-	+
7	Reducing sugars	-	-	-	-	-	-	-	-	-
8	Saponins	+	+	-	+	+	+	+	+	+
9	Terpenoids	+	+	+	+	+	+	+	+	+

Where, '+' represents presence and '-'represents absence, AB_1= *Callicarpamacrophylla*AB_2=*Bauhinia purpurea*AB_3= *Plumeriarubra,* AB_4= *Girardiniadiversifolia* AB_5= *Acacia niloticaAB*$_6$= *Woodfordiafruticosa* (bark), AB_7= *Woodfordiafruticosa* (flowerAB_8= *Terminaliaalata* AB_9= *Premnabarbata*

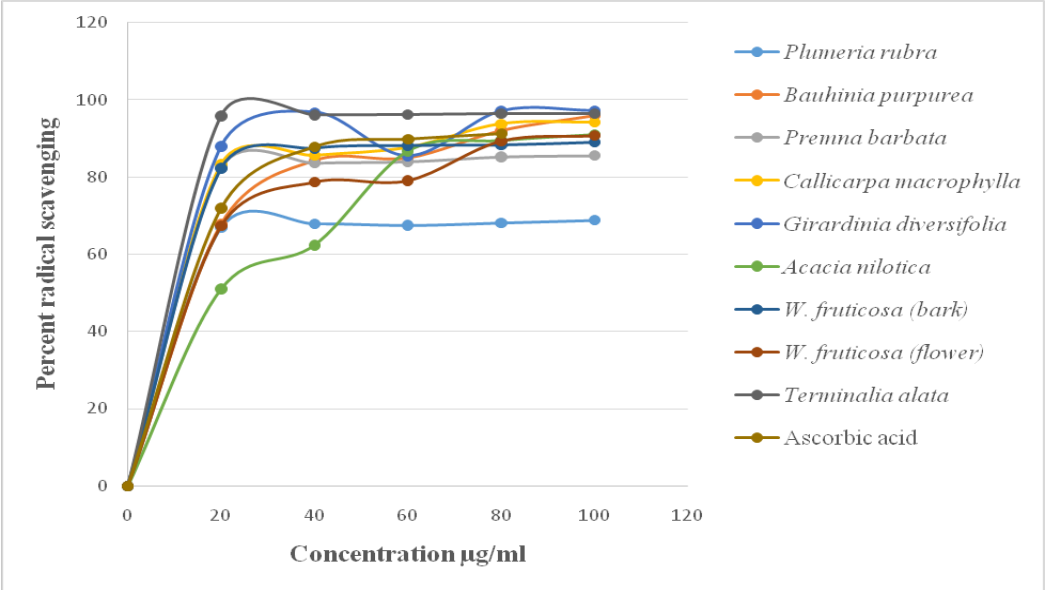

Fig 2: Percent free radical scavenging against the concentration of plant extracts and ascorbic acid (Values are expressed as mean±SD with n=3)

Antioxidant activity

Antioxidant activity of each plant extract were measured by using DPPH free radical scavenging. DPPH radical is scavenged by the plant antioxidants in which the donation of proton forming the reduced DPPH takes place. DPPH solutions show a strong absorbance band at 517 nm, appearing as deep violet color.

The color changes from purple to yellow indicates reduction, which can be measured by its decrease of absorbance at wavelength 517 nm.

The degree of decolorization indicates the free radical scavenging potentials i.e. antioxidant potentials of the sample.

Percentage scavenging of the DPPH radical was gradually increased with the increase in the concentration of the methanolic plant extract from 20-100 µg/ml. The percentage inhibition of DPPH free radical of methanolic extract of bark of *Callicarpamacrophylla* and *Terminaliaalata* was found almost equal to the standard ascorbic acid taken whereas nearly equal to the bark of *Acacia nilotica* and flowers and barks of *Woodfordiafruticosa*. Graphical representations of DPPH assay of all the extracts is shown in fig. 1.

The linear regression of the percentage of radical scavenging versus concentration was used for the calculation of the concentration of each plant extract required for 50% inhibition of DPPH activity (IC_{50}). The antioxidant potential has an inverse relation with IC_{50} value, lower the IC_{50} indicates high antioxidant potential. The IC_{50} values of the plant extracts along with the standard ascorbic acid is shown in table 4.

Table 4: Comparison of IC_{50} values of different plant extracts with standard ascorbic acid

Plant sample/ascorbic acid	IC_{50} (µg/ml)
Standard Ascorbic acid	39.85
Callicarpamacrophylla	17.771±1.568
Bauhinia purpurea	23.57±1.491
Plumeriarubra	36.027±0.762
Girardiniadiversifolia	11.526±4.027
Acacia nilotica	34.195±5.079
Woodfordiafruticosa(bark)	15.853±1.886
Woodfordiafruticosa(flower)	24.452±2.982
Terminaliaalata	6.353±0.485
Premnabarbata	17.167±0.673

Values are expressed as mean±SD with n=3

The inhibitory concentration of *Terminaliaalata*, *Girardinia-diversifolia* and *Callicarpamacrophylla* showed low IC_{50} value with high antioxidant potential. These plant samples are the good sources of natural antioxidants. The rest of the plant extracts are moderate towards antioxidant activity with respect to the standard ascorbic acid. The antioxidant potential of plant sample was found comparable to the previously reported results [21, 22]. The results perform the scientific validation to the plant extracts that have been using by the peoples since many years to cure simple and life threating diseases.

Antibacterial activity

The diameter of zone of inhibition (ZOI) produced by plant extracts on particular bacteria was measured for the estimation of their antimicrobial activity. The methanolic extact of *Plumeriarubra*, *Bauhinia purpurea*, *Premnabarbata*, *Woodfordiafruticosa* and *Terminaliaalata* did not show any zone of inhibition at 10 mg/ml. Further, extracts of *Callicarpamacrophylla*, *Girardiniadiversifolia*and *Acacia nilotica* were found not to be resistant against *E. coli,* whereas the same extract were found to be resistant against *S. aureus*. The extract of *Callicarpamacrophylla, Girardiniadiversifolia and Acacia nilotica* found active for the inhibition of the growth of *S. aureus* only whereas negative response towards *E. coli*. The *Callicarpamacrophylla* showed the highest ZOI (12 mm) against *S. aureus. Terminaliaalata* against *E. coli* showed 20 mm of ZOI.

α-amylase inhibition activity

The absorbance of different test samples was recorded by spectrophotometer. The graph was plotted concentration of plant extract against the percentage α-amylase inhibition, where acarbose was used as the positive control. The IC_{50} values of each extracts were calculated with the help of plot.

The comparisons of percentage α-amylase inhibition between different plant extracts and acarbose as standard are shown in the fig. 2.

Table 5: The results of antimicrobial screening of different plant extracts

S. No.	Plant extracts	Bacteria	ZOI (mm) of extracts at concentration 10 mg/ml	ZOI (mm) of chloramphenicol as control at 100 mg/ml
1	*Callicarpamacrophylla*	*E. coli*	-	14
		S. aureus	**12**	18
2	*Bauhinia purpurea*	*E. coli*	-	14
		S. aureus	-	18
3	*Plumeriarubra*	*E. coli*	-	14
		S. aureus	-	18
4	*Girardiniadiversifolia*	*E. coli*	-	14
		S. aureus	**7**	18
5	*Acacia nilotica*	*E. coli*	-	14
		S. aureus	**10**	18
6	*Woodfordiafruticosa*	*E. coli*	-	14
		S. aureus	-	18
7	*Woodfordiafruticosa*	*E. coli*	-	14
		S. aureus	-	18
8	*Terminaliaalata*	*E. coli*	-	14
		S. aureus	-	18
9	*Premnabarbata*	*E. coli*	-	14
		S. aureus	-	18

(-) = No effective antibacterial activity, ZOI = Zone of Inhibition, *E. coli:* Gram-negative organism, *S. aureus:* Gram-positive organism

Table 6: Comparison of IC_{50} values of different plant extracts with standard acarbose

S. No.	Plant samples/extracts	IC_{50} value (µg/ml)
1	Acarbose	361.01
2	*Callicarpamacrophylla*	475.00
3	*Bauhinia purpurea*	17.05
4	*Plumeriarubra*	133.50
5	*Girardiniadiversifolia*	4308.25
6	*Acacia nilotica*	329.57
7	*Woodfordiafruticosa (bark)*	76.78
8	*Woodfordiafruticosa (flower)*	366.52
9	*Terminaliaalata*	664.13
10	*Premnabarbata*	777.36

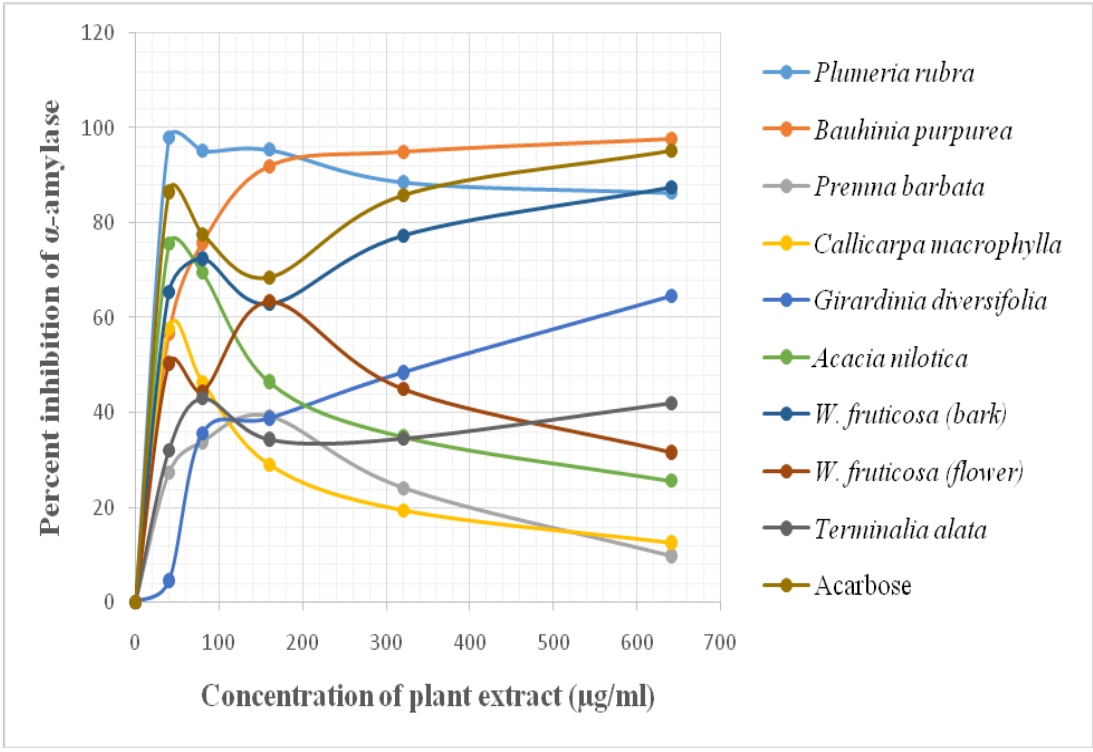

Fig 3: Percentage α-amylase inhibition against the concentration of plant extract (values are expressed as mean±SD with n=3)

The IC_{50} values of different plants extract along with standard acarbose were evaluated and found that the value ranges from 17.05 µg/ml to 4308.25 µg/ml. From the data the extract of *Woodfordiafruticosa (flower)* having IC_{50} value 366.52 µg/ml which is close to the standard acarbose with 361.01 µg/ml IC_{50} value. The plant extracts of *Bauhinia purpurea, Woodfordiafruticosa (bark), Plumeriarubra,), and Acacia nilotica* are found potent than the acarbose. The rest of the plant extacts showed poor inhibitory activity against the α-amylase inhibition activity. Previous research reported that the aqueous leaves extracts of *P. Americana* possess hypoglycemic activity. Similarly, different fractions of *R. Ellipticus* fruits were reported for its antidiabetic activity on alloxan-induced diabetes and glucose tolerance test in rats. The results showed the similarity in α-amylase inhibition activity as reported by the previous researchers [23].

Brine shrimp bioassay (Toxicity test)

The toxicity of different plant extracts were evaluated for their toxicity towards newly hatched Brine Shrimp Larvae (*A. salina* leach) adopting the protocol Mayer *et al.* 1982. In this study, the lethal concentration that kills 50% of the exposed population of *A. salina* (LC_{50}) values in µg/ml for different concentrations of plant extracts was determined and results obtained during these studies were recorded.

The degree of lethality was found to be directly proportional to the concentration of the extracts that is maximum mortalities of the brine shrimp larvae took place at the concentration of 1000 µg/ml and least mortalities were at 10 µg/ml. Those having LC_{50} values less than 1000 µg/ml are supposed to be pharmacologically active. It is cleared that the plant extract of sample AB_6 was found toxic towards the brine shrimp larvae whereas rest of the plant extracts were found nontoxic. Although this method does not provide any adequate information regarding the mechanism of toxic action, it is a very useful method for the assessment of the toxic potential of various plant extracts. This method provides preliminary screening data that can be backed up by more specific bioassays once the active compounds have been isolated.

Isolation of compounds

On the basis of antioxidant activity and anti-diabetic nature of *Callicarpamacrophylla* extract was selected to separate the chemical constituents by column chromatography. The hexane fraction of methanolic extract weighing 3.5 g was adsorbed on 20 g of silica gel to make a slurry and loaded on silica gel packed column. The column was eluted in increasing order of solvent polarity and different fractions were collected/examined by thin-layer chromatography. The results of TLC examination for different fractions collected after elution is shown in table 7.

Table 7: Isolation by column chromatography and TLC report of different fractions collected after elution

S. No.	Elution solvent system	Fraction number	Volume of eluent (ml)	Solvent system for TLC	Remarks of TLC spots
1	100 % hexane	1 to 5	150	1 % EtOAC in hexane	No spots
2	100 % hexane	6 to 9	150	1 % EtOAC in hexane	No spots
3	1% EtOAC in hexane	10 to 13	400	3 % EtOAC in hexane	No spots
4	1% EtOAC in hexane	14-16	300	3 % EtOAC in hexane	No spots
5	3% EtOAC in hexane	17-19	300	5% EtOAC in hexane	Tailing
6	3% EtOAC in hexane	20-21	200	5% EtOAC in hexane	No distinct spot
7	3% EtOAC in hexane	22-26	500	5% EtOAC in hexane	No spots
8	3% EtOAC in hexane	27-28	200	5% EtOAC in hexane	No spots
9	5% EtOAC in hexane	29-31	300	5% EtOAC in hexane	**Single spot**
10	5% EtOAC in hexane	32-33	200	7% EtOAC in hexane	**Single spot**
11	5% EtOAC in hexane	34-35	200	7% EtOAC in hexane	**Single spot**
12	5% EtOAC in hexane	36-38	300	7% EtOAC in hexane	**Single spot**
13	10% EtOAC in hexane	39-40	200	15% EtOAC in hexane	No clear spot
14	10% EtOAC in hexane	41-42	200	15% EtOAC in hexane	No clear spot
15	10% EtOAC in hexane	43-44	200	15% EtOAC in hexane	Tailing
16	15% EtOAC in hexane	45-46	400	20% EtOAC in hexane	No clear spot
17	25% EtOAC in hexane	47-48	200	30% EtOAC in hexane	No distinct spots
18	25% EtOAC in hexane	49-51	300	30% EtOAC in hexane	No distinct spot
19	40% EtOAC in hexane	52-54	300	50% EtOAC in hexane	No spots
20	40% EtOAC in hexane	55-57	300	50% EtOAC in hexane	No distinct spots
21	40% EtOAC in hexane	58-59	200	40% EtOAC in hexane	Multiple spots
22	60% EtOAC in hexane	60-62	300	70% EtOAC in hexane	No distinct spots
23	60% EtOAC in hexane	63-64	200	70% EtOAC in hexane	Multiple spots
24	80% EtOAC in hexane	65-67	300	90% EtOAC in hexane	No distinct spots
25	80% EtOAC in hexane	68-69	200	90% EtOAC in hexane	No distinct spots
26	80% EtOAC in hexane	70-71	200	90% EtOAC in hexane	Tailing
27	100% EtOAC	72-74	300	1% MeOH in EtOAC	Multiple spots
28	100% EtOAC	75-77	300	1% MeOH in EtOAC	Tailing

EtOAC = Ethylacetate, MeOH = Methanol

Single spots were observed in thin layer chromatography in fraction no. 29-31, 32-33, 34-35 and 36-38, indicating the pure compounds. In this study, the characterization of these isolated compounds is not included. By elucidating the structures of these isolated compounds, their *in vivo* and *in vitro* study can be performed, which supports the scientific validation of this plant to the people who have been using as medicine since many years.

CONCLUSION

The phytochemical investigations showed that plant extracts are the rich sources of secondary metabolites such as alkaloids, flavonoids, saponins, glycosides, polyphenols, coumarins and reducing sugars which showed they are supposed to be responsible for different biological activities. IC_{50} values showed varied degree of antioxidant property of which *Plumeriarubra*and *Acacia nilotica* exhibit good antioxidant property with IC_{50} value close to the standard ascorbic acid. The greater antioxidant property showed by plant extract is credited to secondary metabolites like phenols and flavonoids. Study of antibacterial activity against *Straphlococcusaureus* and *Escherichia coli* showed *Callicarpamacrophylla* have high inhibitory activity against the growth of *Straphylococcusaureus*in comparison with standard chloramphenicol followed by *Girardiniadiversifolia* and *Acacia niloticas*howing moderate activity whereas no significant ZOI was observed in other plant extracts. Among the different plant extracts screened against the larvae of brine shrimp, flower of *Woodfordiafruticosa* was found toxic towards them, whereas other plant extracts were found non-toxic. The most effective antidiabetic plant extract was of *Woodfordiafruticosa* (flower) with IC_{50} almost near to standard acarbose. The plant extract with lower IC_{50} value will be greatly beneficial to reduce the rate of digestion and absorption of carbohydrate which thereby contribute for effective treatment of diabetes against hyperglycemia.

AUTHORS'S CONTRIBUTIONS

Dr. Khaga Raj Sharma analyzed the data and wrote the manuscript, whereas KhimendraRana carried out the laboratory work. Both the authors read and approved the final manuscript.

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CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interest for publishing this research article.

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