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Research Article

IN VITRO BIOLOGICAL STUDY OF SEVEN NEPALESE MEDICINAL PLANTS AND ISOLATION OF CHEMICAL CONSTITUENTS FROM *CISSAMPELOS PAREIRA*

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ABSTRACT

Objective: This study aimed to investigate the phytochemical analysis and biological activities of methanol extracts of seven medicinal plants such as *Anisomeles indica*, *Achyranthes bidentata*, *Sphenomeris chinensis*, *Cleistocalyx operculatus*, *Malvaviscus arboreus*, *Cissampelos pareira*, and *Tectaria coadunate* collected from Tanahun district of Nepal.

Methods: Phytochemical analysis was performed by color differentiation methods adopting the standard protocol. Antioxidant activity of plant extracts was evaluated by 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay. Flavonoid content was estimated by aluminum chloride colorimetric method. Antidiabetic activity was evaluated by α-amylase inhibition assay where acarbose was used as standard. Toxic effect was studied by brine shrimp bioassay.

Results: Phytochemical analysis showed the presence of alkaloids, polyphenols, flavonoids glycoside, and terpenoid in most of the extracts. *T. coadunate* and *C. pareira* exhibited high antioxidant activity with IC_{50} 41.84 and 52.03 μg/ml, respectively. Whereas, the plant extracts of *Malvaviscus arboretum*, *S. chinensis,* and *A. bidentata* exhibited moderate antioxidant activity with IC_{50} 76.07, 81.05, and 89.93 μg/ml, respectively. The result of flavonoid content showed the values ranged *A. indica* (1.84 mg quercetin equivalent per gram [mg QE/g]) to *A. bidentata* (5.93 mg QE/g). *C. pareira* and *S. chinensis* exhibited the highest α amylase inhibition activity with IC_{50} 471.68 and 517.59 μg/ml, respectively. Whereas, *A. indica* and *M. arboreus* showed moderate activity with IC_{50} 626.12 and 952.39 μg/ml, respectively. *C. pareira* exhibited against *Staphylococcus aureus* (ATCC 25923) with a zone of inhibition 12 mm/well, and *Escherichia coli* (ATCC 25922) 9 mm/well but, *T. coadunate* showed 14 mm/well against *S. aureus*. The plant extracts of *A. bidentata* and *C. operculatus* showed toxic effect against newly hatched brine shrimp larvae. The chemical compounds isolated from *C. pareira* indicated by gas chromatography-mass spectrometry analysis were 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy) tetrasiloxane, alpha-tocopherol, pentadecanoic acid, and 4,22-stigmastadiene-3-one. The major compound was indicated by percent peak area and base m/z value as alpha-tocopherol.

Conclusion: Present study revealed that plant extracts are the potential source of antioxidant, antidiabetic, and antibacterial agents showing different biological activities. The results of this study provide partial scientific support for the traditional application of medicinal plants to cure diabetes and infectious diseases, although further studies are needed to assess the mechanism of action.

Keywords: Phytochemical, Antioxidant, Antimicrobial, Cytotoxic, Antidiabetic, 2,2-diphenyl-1-picrylhydrazyl, Zone of inhibition, Medicinal plant.

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INTRODUCTION

Nepal is rich in biodiversity and accommodates all types of world agro-climate for cultivation and conservation of a wide variety of biological resources. Peoples have been using medicinal plants for many years to relieve and cure of simple to life-threatening diseases is mentioned in Ayurveda as the oldest repository of human knowledge as the foundation of medicinal science. The use of herbal medicines for the treatment of different diseases has the least side effect and high efficacy. The use of plants as medicines and as food is gaining popularity not only in the developing countries but also in many of the developed countries. The medicinal plants of Nepal may be considered one of the important natural resources to enhance the economic status of the country [1]. Peoples of different communities have been using medicinal plants as anticancer, antidiabetic, antioxidant, and as an anti-infective for many years with limited scientific validation. An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation reaction can produce free radicals. In turn, these radicals can start chain reactions and play a major role in the development of cancer, heart disease, ageing, cataracts, and impairment of the immune system in human beings [2]. Human beings on exposure to various organic compounds such as environmental pollutants and drugs can cause cellular damages through metabolic activation of those compounds to highly reactive substances such as ROS (reactive oxygen species). ROS as superoxide radicals, hydroxyl radicals, and hydrogen peroxides derived from the metabolism of oxygen can damage the cell membrane and DNA, leading to mutation which ultimately causes cancer [3]. Diabetes mellitus is a chronic endocrine, metabolic disorder characterized by altered carbohydrates, lipids, proteins, electrolytes, and water metabolism. It includes a group of metabolic diseases characterized by hyperglycemia, in which blood sugar levels are elevated either because the pancreas does not produce enough insulin or cells do not respond to the produced insulin [4]. Diabetes is of insulin-dependent type 1, insulin-resistant type 2, and gestational.

Therefore, a drug having twofold properties, that is, lowering of blood lipids and glucose together, is in great demand [5]. Higher plants, animals, and microorganisms are found to produce a large number of different protein inhibitors of α-amylases and α-glucosidases [6]. The drugs acarbose and miglitol are competitive inhibitors of intestinal α-glucosidase and reduce the post-prandial digestion and absorption of starch and disaccharides without promoting insulin secretion in diabetes patient [7]. Natural α-glucosidase and α-amylase inhibitors are used as oral antidiabetic drugs for treating type 2 diabetes with minimal side effects [8-10]. The appearance of drug resistance, as well as the development of undesirable side effects of certain antibiotics, has led to the search of new natural antibiotic agents having high efficacy and less side effect from natural sources. Plants from high altitude habitat are a potential source of new and potent anti-microbial agents [11-13]. Thus, this study focused on the chemical and biological activities of some selected medicinal plants collected from Tanahun district of Nepal, which provides scientific validation in using such plants to cure diabetes and infectious diseases.

METHODS

Chemicals

Most of the chemicals and solvents used were of laboratory grade. Methanol (Fisher Scientific), acetone (Fischer Scientific), hexane (Merck), and dimethyl sulphoxide; dimethyl sulfoxide (DMSO) (Merck), Folin-Ciocalteu reagent, α-amylase enzyme, and acarbose were purchased from the local market. Chemicals and reagents such as gallic acid, quercetin, ascorbic acid, iodine trichloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), $NaNO_2$, $AlCl_3$, KOH, and NaOH were available in the laboratory. Reagents and solvents used during phytochemical analysis were prepared in the laboratory with the chemicals provided in the laboratory.

Equipment

Electric grinder, mortar, and pestle, digital weighing balance (GT 210), hot air oven (Griffin-Grundy), and rotatory evaporator (Buchi RE 111) with a water bath (Buchi 461), spectrophotometer (WPA, supplied by Philip Harris Shenstone, England), iodine chamber, cuvettes, and micropipettes (Erba BIHOT) were used during this work.

Collection and identification of plant samples

The plants were collected from the Tanahun district of Nepal. The respective part of the plant used, scientific name, and traditional uses are shown in Table 1. The taxonomic identification of the plants was done at the Central Department of Botany, Tribhuvan University, Kirtipur.

Sample preparation

The collected plant parts were washed in tap water to remove the contaminants. Then, the plant parts were shade dried. The dried parts were grounded into powder form in an electric grinder and stored in clean plastic bags. The plant extracts were prepared by cold percolation using methanol as a solvent. Fifty grams of powder plant samples were kept separately in clean and dry conical flasks. Three hundred milliliter of methanol was added to each flask and kept for each 3 days with frequent agitation. The mixtures were decanted and filtered with the help of cotton plug and thus obtained filtrates were concentrated in a rotatory evaporator. After complete evaporation of the solvent, the percentage yield for each plant extracts was calculated. The plant extracts were kept in the vial for their phytochemicals analysis and biological activities at 4°C.

Phytochemical analysis

The phytochemicals present in different plant extracts were analyzed by following the standard protocol given by Culei [21].

Total flavonoid content

The total flavonoid content of the plant extracts was estimated by aluminum chloride colorimetric assay taking quercetin as standard [22].

Table 1: List of medicinal plants and their traditional uses

Code	Scientific name	Parts used	Traditional uses
MA_1	*Anisomeles indica*	Whole plant	Urine infection [14]
MA_2	*Achyranthes bidentata*	Stem	Worm treatment [15]
MA_3	*Sphenomeris chinensis*	Leaf	Wound healing [16]
MA_4	*Cleistocalyx operculatus*	Bark	Stomachache [17]
MA_5	*Malvaviscus arboreus*	Leaf	Urine infection [18]
MA_6	*Cissampelos pareira*	Stem	Common cold [19]
MA_7	*Tectaria coadunate*	Rhizome	Toothache, stomachache [20]

The average absorbance values obtained for different concentrations of quercetin were used to plot the calibration curve [23]. Total flavonoid content in the plant extract was calculated as, C = cV/m, where, C = total flavonoid content (mg QE/g), c = concentration of quercetin established from calibration curve in mg/ml, and V = volume of the extract (ml).

Statistical analysis

Data were recorded as absorbance for each concentration, from which the linear correlation coefficient (R^2) value was calculated. The regression equation is given as, y = mx + c, using this regression equation, the concentration of the extract was calculated. Thus with the calculated value of the concentration of extract, the flavonoid content was calculated by equation. Where, y = absorbance of the extract, m = slope from the calibration curve, x = concentration of the extract, and c = intercept. The inhibitory concentration was calculated from the regression equation graphically.

Antioxidant activity

A rapid, simple, and inexpensive method to measure antioxidant capacity involves the use of the free radical DPPH. The ability of different plant extracts to scavenge DPPH free radicals was evaluated by adopting the standard protocol [24].

The percentage of the DPPH free radical scavenging activity was calculated using the following equation:

$$\text{Radical scavenging (\%)} = [(A_0\text{-}As)/A_0]*100$$

Where, A_0 = Absorbance of the control (DPPH solution + methanol), A_s = Absorbance of test sample. The IC_{50} (50% inhibitory concentration) value is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radicals. IC_{50} values were calculated using the inhibition curve by plotting extract concentration versus the corresponding scavenging effect.

Antidiabetic activity

The antidiabetic activity of plant extracts was calculated by using the α-amylase inhibition assay by adopting the standard protocol with some modifications [25]. The undigested starch due to enzyme inhibition was detected through the blue starch iodine complex detected at 630 nm.

$$\%\ \text{inhibition}=[Abs_2 - Abs_1/(Abs_4 - Abs_3)]*100$$

Where, Abs_1 = absorbance of the incubated mixture containing plant extract, starch, and amylase, Abs_2 = absorbance of the incubated mixture containing plant extract and starch, Abs_3 = absorbance of an incubated mixture containing starch and amylase, and Abs_4 = absorbance of an incubated solution containing starch only. Standard graph was plotted by taking the concentration on the x-axis and percentage inhibition on the y-axis. Based on this graph, IC_{50} values of each sample were calculated and compared. The species having the lowest IC_{50} is considered to have the best α amylase enzyme inhibition property or antidiabetic property.

Antibacterial activity

Antibacterial activity of the plant extracts was evaluated by agar well diffusion method**.** The bacterial strain *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 25923 were grown on nutrient agar media. Effectiveness of antimicrobial substance was evaluated by determination of the zone of inhibition (ZOI) [11]. 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 ofloxacin as positive control 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 ZOI was measured and the antibacterial activity expressed in terms of the average diameter of the ZOI in millimeters. The absence of the ZOI was interpreted as no activity [26,27].

Toxicity test
The newly hatched brine shrimp larvae (nauplii) are used for biological screening. This method is rapid, inexpensive, simple, and in-house approach to know the toxic effect of plant extracts. It determines the LC_{50} values in μg/ml for the crude extracts. Compounds having LC_{50} values less than 1000 ppm are considered as pharmacologically active. The toxicity was performed by adopting the standard procedure [17]. LC_{50} value is the lethal concentration dose required to kill 50% of the organisms used in bioassay.

Extraction and isolation of compounds
Cissampelos pareira was selected as the potent plant source to isolate the chemical constituents. About 400 g of bark powder of the plant was extracted in methanol by cold percolation. The content was filtered and concentrated in a rotatory evaporator. The methanol extract was fractionated by solvent-solvent extraction based on solvent polarity as hexane, dichloromethane, and ethyl acetate. The hexane fraction (8 g) was adsorbed on silica gel and loaded on to silica (120 g, qualigens, and 60–120 mesh) packed column having an internal diameter of 3 cm with height 32 cm. The column was initially eluted with hexane and then the gradient of hexane-ethyl acetate of increasing polarity. Different fractions collected through column were analyzed by Thin-layer chromatography (TLC) which guides for changing solvent polarity. The purity of the fractions was tested by TLC and subjected to gas chromatography-mass spectrometry (GC-MS) analysis.

RESULTS AND DISCUSSION

Phytochemical analysis
The result of phytochemical analysis for each plant extract is shown in Table 2.

The results showed all most the plant extracts were found rich in secondary metabolites. Flavonoid was present in all most all the plant extracts. On the other side, alkaloids were present in *Achyranthes bidentata*, saponins in *Anisomeles indica*, glycosides in all plant extracts, and terpenoids were present in all plant extracts except *A. indica* and *Tectaria coadunate*.

Total flavonoid content
Total flavonoid content was estimated by constructing a calibration curve taking quercetin as standard. The results of flavonoid content are shown in Fig. 1. The flavonoid content was expressed as mg QE/g.

The results showed that almost all extracts were found rich in flavonoid content as compared to standard quercetin. *A. bidentata* exhibited the highest total flavonoid contents (TFC) 5.93 mg QE/g, whereas *Malvaviscus arboreus*, *Cleistocalyx operculatus*, *C. pareira*, *T. coadunate,* and *Sphenomeris chinensis* are moderate in flavonoid content. The plant extract of *A. indica* exhibited the lowest TFC content. The results of the present study were found comparable to the previously reported results [26]. Flavonoid compounds are capable of effectively scavenge free radicals because of their phenolic hydroxyl group. Their antioxidant properties depend on their structure, particularly hydroxyl position in the molecule. Although, quantitative determination of flavonoid compounds in plant extracts is influenced by their structural complexity, diversity, nature of analytical assay method, selection of standard, and presence of interfering substances.

Table 2: Phytochemical analysis of methanol extract of all plants

Groups of compounds	MA_1	MA_2	MA_3	MA_4	MA_5	MA_6	MA_7
Basic alkaloids	+	+	−	−	+	+	−
Coumarins	−	−	+	−	−	−	+
Flavonoids	−	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+	+
Polyphenols	−	+	+	+	+	+	+
Quinones	+	−	−	−	−	+	−
Reducing sugars	−	+	−	−	+	+	+
Saponins	+	+	−	+	−	+	+
Terpenoids	+	+	+	+	+	+	−

"+" represents presence and "−" represents absence

Antioxidant activity
The results of antioxidant activity are shown in Figs. 2 and 3. Antioxidant activity of plant extract is expressed as percent radical scavenging against concentration. The radical scavenging activity is in a dose-dependent manner. With the help of a graph plotting the concentration against the radical scavenging activity, the inhibitory concentration (IC_{50}) was calculated and the result is displayed in Fig. 4.

Scavenging of DPPH free radical exhibited by *T. coadunate* was found to be highest in comparison with other extracts with an IC_{50} 41.84 μg/ml. Similarly, *C. pareira* and *M. arboreus* showed moderate antioxidant potential IC_{50} 52.03 μg/ml and 76.07 μg/ml, respectively. The rest of the plant extracts were poor antioxidants as compared to the standard ascorbic acid. A previous study reported that plant samples rich in phytoconstituents were found responsible for the antioxidant activity [11]. DPPH scavenging assay showed that the plant extracts exhibited dose-dependent percentage scavenging. The antioxidant potential was expressed as the amount of the extract needed to decrease 50% of the initial concentration of the free radical. The extracts were found to be active antioxidants in the concentration range of 20-100 μg/ml to scavenge free radical. The results of this study were found comparable to the results reported by Ebrahimzadesh *et al*. (2010) as IC_{50} of *H. officinalis* L. 311±14.5 μg/ml, *V. odorata* leaves 245.1±9.6 μg/ml, *B. hyrcana* leaves 113.1±8.9 μg/ml, and *C. speciosum* flower 585.6±21.2 μg/ml. The IC_{50} for BHA, Vitamin C, and quercetin as standard was reported 53.96±3.1, 5.05±0.1, and 5.28±0.2 μg/ml, respectively [28].

Antibacterial activity
The results of antibacterial activity are shown in Table 3.
C. pareira demonstrated the highest antibacterial activity against *S. aureus* with ZOI 12 mm/well and *E. coli* with ZOI 9 mm/well as compared to positive control ofloxacin which showed inhibition toward *S. aureus* 18 mm/well and *E. coli* 14 mm/well. On the other hand, *T. coadunate* showed

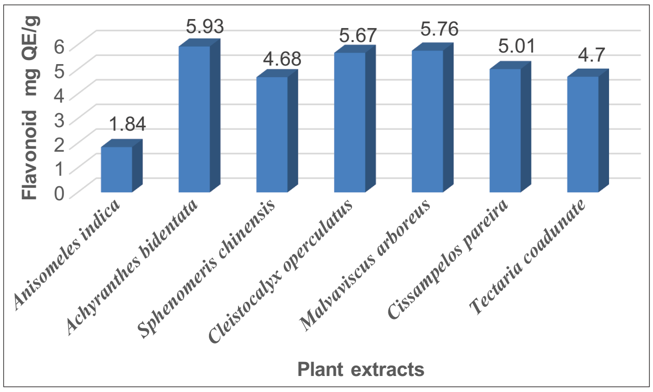

Fig. 1: Flavonoid content in plant extracts

Table 3: Antibacterial activity of plant extracts

Bacteria	Plant extracts	ZOI (mm) at 100 μg/ml	
		Plant extracts	Positive control
E. coli	*Cissampelos pareira*	9	14
S. aureus	*Cissampelos pareira*	12	18
E. coli	*Tectaria coadunate*	-	14
S. aureus	*Tectaria coadunate*	14	18

ZOI: Zone of inhibition. *E. coli*: *Escherichia coli. S. aureus: Staphylococcus aureus*

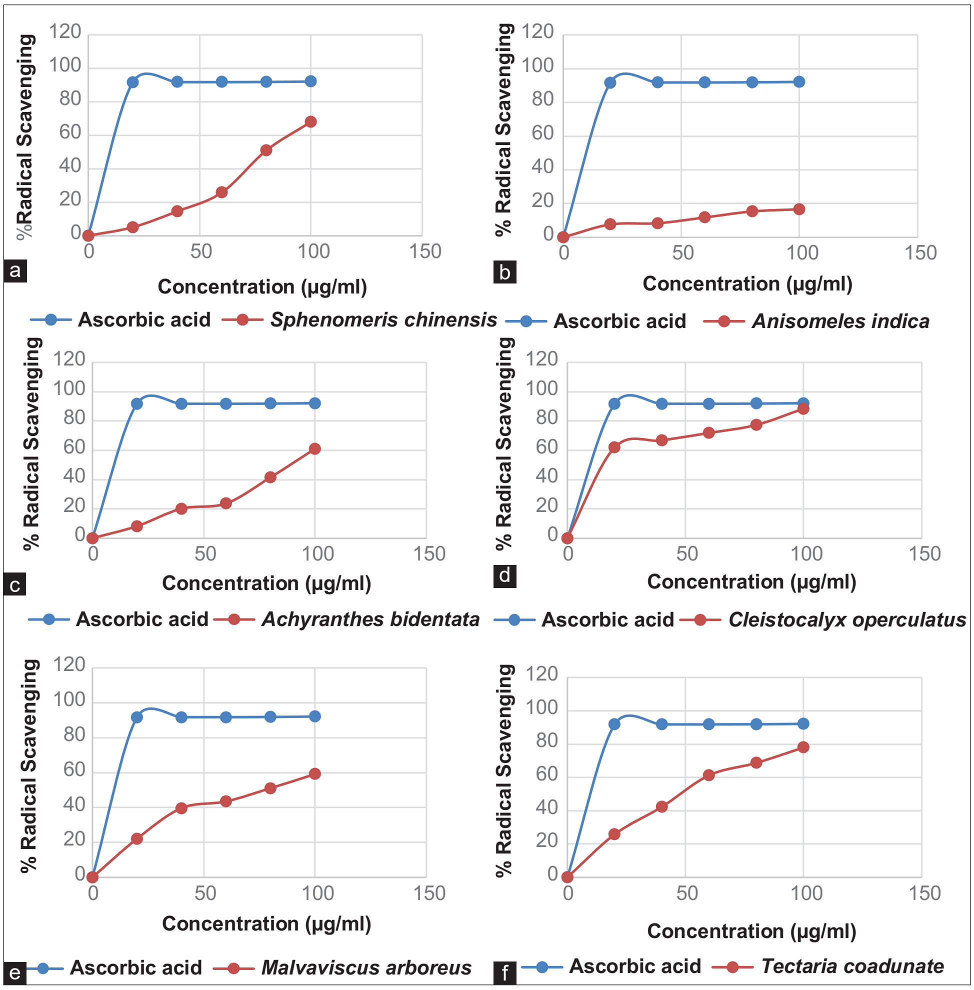

Fig. 2: (a-f) Comparison of percentage radical scavenging against concentration of ascorbic acid and plant extracts

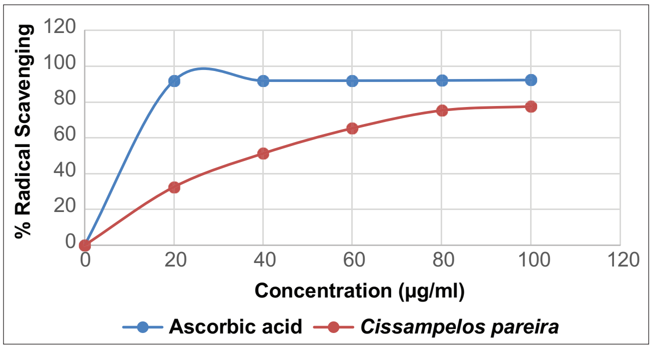

Fig. 3: Radical scavenging against the concentration of *Cissampelos pareira*

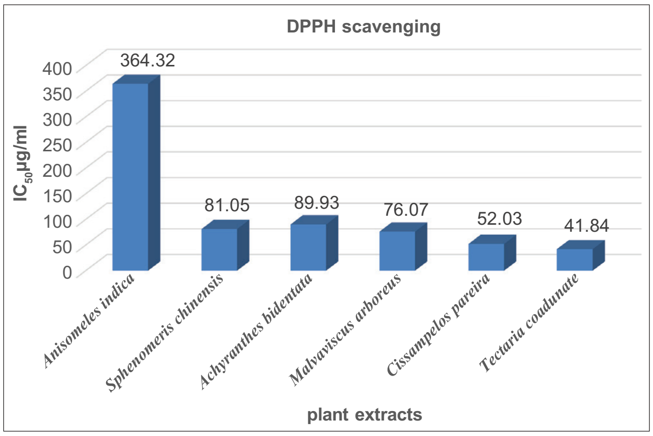

Fig. 4: Antioxidant activity (IC_{50}) values of active plant extracts

inhibition only against *S. aureus* 14 mm/well but not against *E. coli*. The rest of the plant extracts were found inactive against these organisms.

Alpha-amylase inhibition activity

The results of α amylase inhibition are displayed in Fig. 5, where the graph showed α amylase inhibition against the different concentrations of plant extracts.

The results of α-amylase inhibitory activity of plant extracts *C. pareira* showed high α amylase inhibition IC_{50} 471.68 µg/ml (Fig. 6). The plant extracts of *S. chinensis*, *A. indica*, and *M. arboreus* showed moderate α amylase enzyme inhibition activity IC_{50} 517.59, 626.12, and 952.39 µg/ml, respectively. The rest of the plant extracts exhibited poor α amylase enzyme inhibition as compared to the standard acarbose. The results of this study showed some similarities to the results reported by the previous researchers for the antidiabetic activity of *C. pareira* extract and some other medicinal plants [26].

Toxic effect

The degree of lethality was found to be directly proportional to the concentration of the extracts that are maximum mortalities of the brine shrimp larvae that took place at the concentration of 1000 µg/ml

Table 4: Fractions collected in column chromatography and TLC report

Solvent system	Fractions	Eluent (ml)	TLC solvent system	TLC spots
100% hexane	1–10	1000	2% EtOAc in hexane	No spots
1% EtOAc in hexane	11–20	1000	2% EtOAc in hexane	No spots
3% EtOAc in hexane	21–30	1000	5% EtOAc in hexane	No spots
5% EtOAc in hexane	31–40	1000	8% EtOAc in hexane	Single spot
10% EtOAc in hexane	41–50	1000	15% EtOAc in hexane	Tailing
13% EtOAc in hexane	51–60	1000	15% EtOAc in hexane	Single spot
20% EtOAc in hexane	61–70	1000	25% EtOAc in hexane	Tailing
30% EtOAc in hexane	71–80	1000	35% EtOAc in hexane	Two spots
50% EtOAc in hexane	81–90	1000	60% EtOAc in hexane	Tailing
70% EtOAc in hexane	91–100	1000	80% EtOAc in hexane	Tailing
100% EtOAc	101–110	1000	2% MeOH in EtOAc	Tailing
1% MeOH in EtOAc	111–115	500	5% MeOH in EtOAc	Single spot
5% MeOH in EtOAc	116–120	500	10% MeOH in EtOAc	Tailing
20% MeOH in EtOAc	121–125	500	25% MeOH in EtOAc	Tailing
50% MeOH in EtOAc	126–130	500	60% MeOH in EtOAc	Tailing

TLC: Thin layer chromatography

Table 5: Peak report (GC-MS analysis)

Peak	Retention time	Area %	Name	Base m/z
1	9.477	7.95	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane	73.10
2	10.841	4.88	Cyclooctasiloxane, hexadecamethyl ester	73.05
3	12.779	7.03	Pentadecanoic acid, 14-methyl-, methyl ester	74.05
4	13.942	3.79	9,12-Octadecadienoicacid, methyl ester	67.10
5	15.504	71.22	Alpha-tocopherol	165.15
6	19.504	5.14	4,22-Stigmastadiene-3-one	69.10

GC-MS: Gas chromatography-mass spectrometry

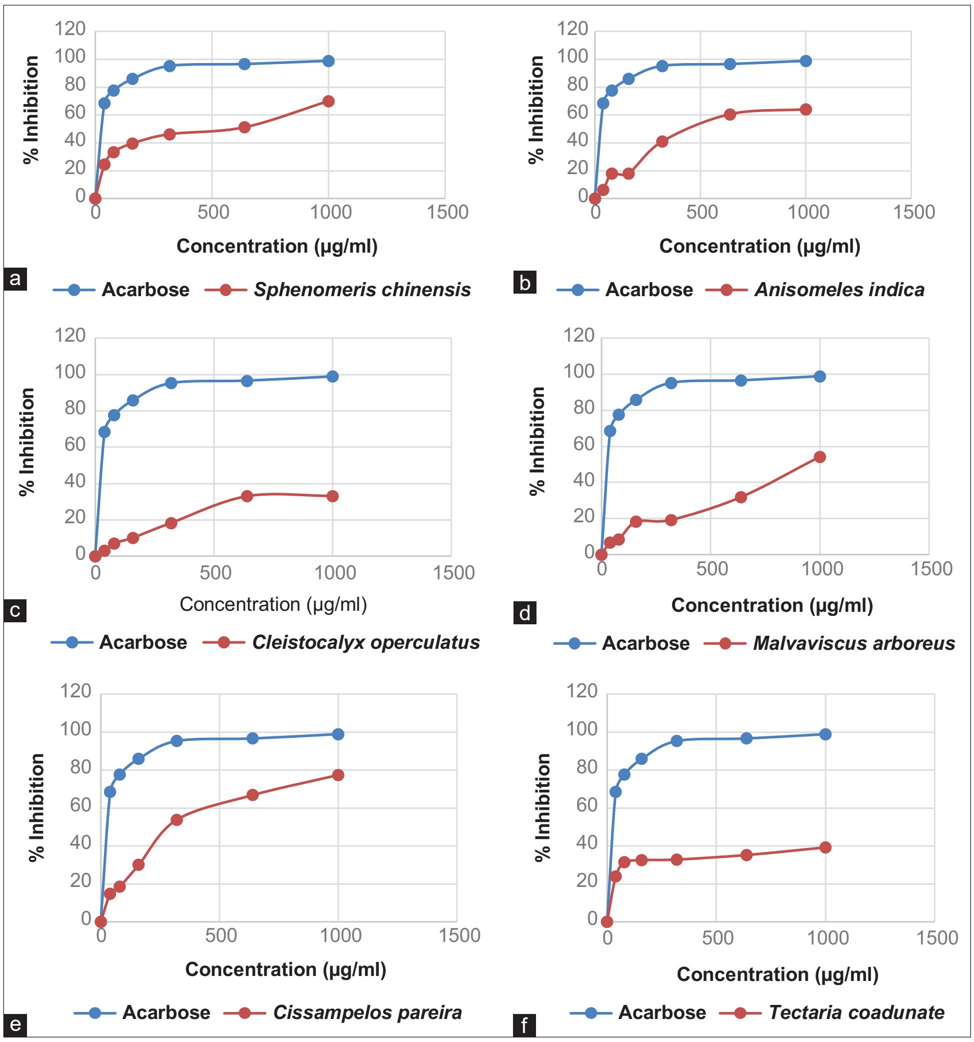

Fig. 5: (a-f) Percent inhibition of α-amylase against the concentration of plant extract as compared to acarbose

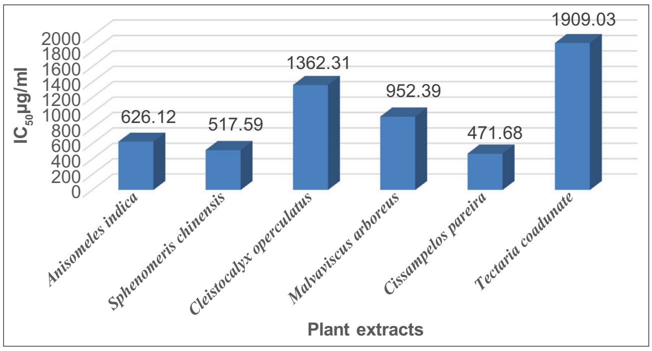

Fig. 6: Comparison of IC_{50} values for α amylase inhibition of different plant extracts

and least mortalities were at 10 µg/ml. Those having LC_{50} values <1000 µg/ml are supposed to be pharmacologically active. The extract of *A. bidentata* was found cytotoxic against brine shrimps showing LC_{50} 15.49 µg/ml whereas *C. operculatus* with LC_{50} 31.63 µg/ml showing mild toxic toward brine shrimp larvae. On the other side, *A. indica*, *S. chinensis*, *C. pareira,* and *T. coadunate* were found nontoxic.

Isolation of chemical compounds and GC-MS analysis

The chemical constituents were isolated from the active plant extract *C. pareira* which was selected based on biological activity. The list of hexane fractions collected after the column chromatography was analyzed by thin-layer chromatography. The list of fractions collected after elution is shown in Table 4.

Fraction collected at 5% ethyl acetate in hexane was yellow viscous seems single spot in TLC was subjected for GC-MS analysis. The fraction showed the following results.

Six compounds were detected by GC-MS analysis of partially purified fraction collected in 5% ethyl acetate in hexane. The list of compounds with peak report is shown in Table 5. Out of six compounds, the alpha-tocopherol is the major compound with a high peak area (71.22%) having a base m/z ratio 165.15. The molecular formula of alpha-tocopherol can be established as ($C_{29}H_{50}O_2$). The structure of alpha-tocopherol is shown in Fig. 7.

CONCLUSION

The phytochemical analysis showed that plants are the rich sources of secondary metabolites from which the active chemical compounds can be isolated. The plant extract *A. bidentata* found rich in flavonoid content. Out of seven medicinal plants, extracts of *T. coadunate*, *C. pareira,* and *M. arboreus* showed promising antioxidant activity as compared to ascorbic acid. The plant extracts of *C. pareira* and *S. chinensis* exhibited the strong α amylase inhibitory activity as compared to the standard acarbose. The plant extracts of *C. pareira* and *T. coadunate* showed significant antibacterial activity against *E. coli* and *S. aureus*. The toxic effect against brine shrimp nauplii was shown by *A. bidentata* and *C. operculatus,* whereas the rest of the plant extracts do not show any toxic effect. The partially purified fraction of *C. pareira* showed the presence of six compounds indicated by GC-MS analysis. Among these six compounds, α-tocopherol showed a high percentage peak area with base m/z 165 indicates the major compound. In this way, the study provides partial scientific support for the traditional application of these medicinal plants to cure diabetes and bacterial infections. Further studies are needed to isolate the active natural conpounds for establish the precise mechanism of action by *in-vivo* experiments.

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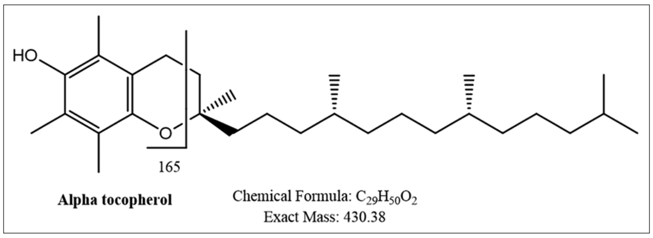

Fig. 7: Major compound from partially purified fraction of *Cissampelos pareira* extract

AUTHORS' CONTRIBUTIONS

Sharma *et al*. wrote the manuscript, whereas Md Amit carried out the laboratory work. Both the authors read and approved the final manuscript.

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Nil.

CONFLICTS OF INTEREST

All authors have no conflicts of interest.

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