Sharma et al., Vegetos 2016, 29:4
10.5958/2229-4473.2016.00098.7

Vegetos- An International Journal of Plant Research

Research Article

A SCITECHNOL JOURNAL

Screening and Bioactivity Measurement of High altitude Medicinal Plants of Nepal

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Abstract

Ten different plants collected from various area of Khaptad National Park, one of the famous National Parks renowned for the source of medicinal herb located at an altitude from 1,000 m to 3,300 m, *P. edgeworthii*, *B. petiolaris*, *E. strobilifera* were found to have potent cytotoxicity effect while three others, *T. foliolosum*, *B. diffusa* and *A. spectabilis* with LC50 of 64.50, 13.59, 6.14 respectively showed very strong cytotoxicity effect against brine shrimp nauplii. The DPPH scavenging assay of all those plants was found to vary from very high to low. *B. petiolaris* (13.49%) showed almost the same scavenging activity to that of Ascorbic acid (17.20%) followed by *E. strobilifera* (6.35%) and *H. salicifolia* (2.38%) at a concentration of 0.5 µg/ml. The antimicrobial activity against five microorganisms (*Bacillus cereus, Bacillus subtillis, Klebsiella pneumonia, Bacillus thuringiensis and Enterobacter*), showed a remarkable Zone Of Inhibition (ZOI). *H. salicifolia* showed 6.5 mm ZOI against *Bacillus cereus*, 7 mm against Bacillus thuringiensis and 6 mm against *Klebsiella pneumonia* at 25 µg/ml whereas *A. spectabilis* showed 9 mm as highest ZOI against Enterobacter at 25 µg/ml.

Keywords

Abies spectabilis; Thalictrum foliolosum; DPPH; Antioxidant activity; Antimicrobial activity; Brine shrimp bioassay; Phytochemical activity

Introduction

World Health Organization defines a medicinal plant as any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis [1]. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, working in the control or treatment of a disease condition and hence contains chemical components that are medically active [2,3].

It has been ages since medicinal plants were discovered and they have been in use all the way through human history. Plants have the ability to synthesize a wide variety of chemical compounds which are used to perform important biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals. It is estimated that less than 10% of the total such compounds, at least 12,000 have been isolated so far. Chemical compounds in plants mediate their effects on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs; thus herbal medicines do not differ greatly from conventional drugs in terms of how they work. The medicinal properties of plants could be based on the cytotoxicity antioxidant, antimicrobial [4,5] antipyretic effects of the phytochemicals in them [6].This enables herbal medicines to be as effective as conventional medicines, but also gives them the same potential to cause harmful side effects.

The uses of plants as medicines exist before written human history. Ethnobotany (the study of traditional human uses of plants) is accepted as an effective way to discover future medicines [5]. In 2001, researchers identified 122 phytochemical [7] compounds used in modern medicine which were derived from "ethnomedical" plant sources; 80% of these have had an ethnomedical use identical or related to the current use of the active elements of the plant. Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including aspirin, digitalis, quinine, and opium.

Different plant parts and components (roots, leaves, stem barks, flowers or their combinations, essential oils) have been employed in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal and biliary systems, as well as on the skin [3,4].

The use of medicinal plants offers poorer populations the ability to fight diseases at low costs. The uses of traditional medicine and medicinal plants in most developing countries, as a normative basis for the widely observed [8] (Table 1).

Materials and Methods

Collection of plant parts from various sited

The collected plants were dried and then finely powdered using pestle and mortar and finely grinded. The powdered plants then processed for extraction.

Preparation of plant extracts

Cold methanol extraction: The finely powdered samples were taken 15gm and mixed with 60ml of methanol. The mixture was stirred for about half an hour. Then the mixture was kept for 48 hours. After 48 hours ,the solution was filtered and fresh methanol was added to crude extract. The filtrate extract was evaporated using rotary evaporater. The final extract was weighted to and yield of extract was calculated.

Phytochemical analysis of sample crude extract

Tests for coumarin: 4 ml extract solution was taken; 1-2 drops of water (hot) was added. Volume was made half (UV fluorescence). 10% NH4OH was added to another half volume (strong fluorescence), presence of green fluorescence indicated the presence of Coumarin.

Test for alkaloids: 5 ml of extract was concentrated to yield a residue. Residue was dissolved in 3 ml of 2% (v/v) HCL. Few drops of Mayer's reagent were added. Appearance of the dull white precipitate indicated the presence of basic alkaloids.

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Received: July 27, 2016 **Accepted:** September 29, 2016 **Published:** September 30, 2016

Table 1: Zone of inhibition of Antibiotics

Antibiotics	ZONE OF INHIBITION (mm)				
	B.cereus	*K.pneumonia*	*S.aureus*	*B.thuringiensis*	*Enterobacteria*
P^{10}	0	0	0	0	0
GEN^{10}	26	32	33	22	30
CF^{30}	32	39	22	29	35
TE^{30}	24	21	22	24	27

P^{10}: Penicillin – 10 mcg/disc
GEN^{10}: Gentamicin-10 mcg/disc
CF^{30}: Ciprofloxacin-30 mcg/disc
TE^{30}: Tetracyclin-30 mcg/disc

Test for saponins: 2 ml extract was shaken vigorously for 30 seconds in a test tube. Persistence of thick forth even after 30 minutes indicated the presence of saponins.

Tests for glycosides: 1 ml of the extract was dried with NH4OH and shaken. Appearance of cherish red color indicated the presence of glycosides.

Tests for reducing sugar (reducing & non reducing): 0.5 ml of extract was taken and1 ml distill water was added. 5-8 drops of Fehling's solution (hot) was added. Presence of brick red precipitation indicated the presence of reducing sugar.

Tests for sterols and triterpenes: 1 ml extract was dissolved in 10 ml chloroform. Equal volume of conc. H_2SO_4 was added by the side of test tube. Upper layer turned red and Sulphuric acid layer turned yellow with green fluorescence. This indicated the presence of steroids.5 ml of extract was taken and mixed with 2 ml of chloroform.3 ml of conc. H_2SO_4 was added to form a layer. Reddish brown precipitate formation at the interface formed indicated the presence of terpenoid.

Tests for flavonoids: When Test solution treated with few drops of Ferric chloride solution confirm the result by the formation of blackish red color indicating the presence of flavonoids.

Tests for tannis and polyphenols: Liebermann Burchard test: Crude extract after mixing with few drops of acetic anhydride was boiled and cooled. Concentrated sulphuric acid was then added from the sides of test tube which resulted in brown ring at the junction of two layers. Green coloration of the upper layer and deep red color in the lower layer indicate a positive test for steroids and triterpenoids respectively [10].

Antimicrobial screening

Preparation of plant extract: Stock solution of 400 mg/ml was prepared by weighing 200 mg of plant extract in 1.5 ml eppendorf tube and the final volume of 0.5 ml of DMSO [Di methyl sulpho oxide] was added by micropipette. Extract was completely dissolved by vortexing for 5-10 minutes. Test solution of 200 mg/ml, 100 mg/ml, 50 mg/ml, and 25 mg/ml concentration was prepared.

Preparation of inoculums: Stock solution of 400 mg/ml was prepared by weighing 200 mg of plant extract in 1.5 ml Eppendroff tube and the final volume of 0.5 ml of DMSO was added by micropipette. Extract was completely dissolved by vortexing for 5-10 minutes. Different test solution of 200 mg/ml,100 mg/ml, 50 mg/ml and 25 mg/ml concentration was prepared for the analysis..

Inoculation procedure: Each culture to be tested was streaked onto nutrient agar to obtain isolated colonies. Overnight incubation was done at 37°C. Then isolated colonies were transferred by the help of sterile loop onto Nutrient Broth. Overnight incubation was done at rotary shaker at 37°C.

Antimicrobial disc and plant extract disc: For inoculation, a sterile cotton swab was dipped into the suspension and was pressed firmly against the inside wall of the tube just above the fluid level and the swab was rotated to remove excess liquid. The cotton swab was streaked over the entire surface of the medium three times, rotating the plate approximately 60°C after each application to ensure an even distribution of the inoculum. Finally, swabbing was done all around the edge of the agar surface.

Recording and interpreting results: After the discs were placed on the plate, the plate was inverted and incubated at 37°C for bacteria. After incubation, the diameter of the zones of complete inhibition was measured (including the diameter of the disc) and recorded it in millimeters. The measurements were done with a ruler on the undersurface of the plate without opening the lid.

Brine shrimp bioassay for cytotoxicity

Preparation of test sample: Stock solution was prepared by dissolving the collected extract of different amount in 10 ml DMSO. The stock was further diluted to 1000, 100 and 10 ppm. The control was prepared in the same way.

Hatching of brine shrimp: 50 mg of Brine Shrimp eggs were sprinkled in a beaker containing 300 ml of sea water. The sample was allowed to incubate at 32-35°C for 24 hrs for the shrimp eggs to hatch and mature as nauplii.

Bioassay

Test tubes were divided into four groups each containing three test tubes. After 24 hrs of incubation, the nauplii were recovered with a dropper and 10 nauplii were transferred in each test tube. The groups were administered with different dilutions of sample. The test tubes were then incubated at 32-35°C overnight. The numbers of survivals usually swimming was counted for each test tube at the end of 24 hr. Thus, the number of death was computed and graph was plotted for death percentage versus Log of concentration of the extract. This gives linear equation in the form of y= mx+c.

Calculation of LC_{50}

The extract concentration was converted to the log (to make the wide range of values easier to deal with). Calculate % death (death/initial*100) Correct the % death for any control death (%death test-% control) Plot (XY scatter, don't plot the line, just plot the points) % death corrected vs. log extract conc (ppm). Determine the Lethal Dose 50 (LD_{50}) from the graph from the equation of straight line (Table 2).

Antioxidant activity (DPPH free radical scavenging assay) [2, 2-dipheny-1-picrylhydrazyl]

The DPPH assay method is based on the reduction of DPPH, a stable free radical. This free radical DPPH [11] with an odd electron gives a maximum absorption at 517 nm (purple color). In reaction of DPPH with antioxidants [12], it becomes paired off in the presence of a hydrogen donor and is reduced to the DPPHH and as a result [13] the absorbance is reduced from the DPPH. When DPPH solution is mixed with a substance which can give hydrogen atom, then this gives rise to the reduced form (Diphenylpicrylhydrazine, non-radical) with the loss of this violet color [14].

doi: 10.5958/2229-4473.2016.00098.7

Table 2: Result of brine shrimp assay and calculation of LC_{50}

Extract	Conc In ppm(A)	Log A	Initial nauplii	Nauplii Survival			Mean survival	Death	% death	Corrected % death	LC_{50}
				N=1	N=2	N=3					
A.sarmentosa	10	1	10	9	10	9	9.33	0.67	6.67	6.67	14681.63
	100	2	10	8	8	7	7.67	2.33	23.33	23.33	
	1000	3	10	8	6	6	6.67	3.33	33.33	33.33	
	Control		10	10	10	10	0	0	0	0	
P.edgeworthii	10	1	10	7	8	8	7.67	2.33	23.33	23.33	257.17
	100	2	10	6	8	6	6.67	3.33	33.33	33.33	
	1000	3	10	2	4	4	3.33	6.67	66.67	66.67	
	Control		10				10	0	0	0	
S.daltoniana	10	1	10	9	10	10	9.67	0.33	3.33	3.33	14064.79
	100	2	10	8	9	8	8.33	1.67	16.67	16.67	
	1000	3	10	7	6	7	6.67	3.33	33.33	33.33	
	Control		10				10	0	0	0	
B.petiolaris	10	1	10	10	10	10	10	0	0	0	257.17
	100	2	10	7	8	8	7.67	2.33	23.33	23.33	
	1000	3	10	2	0	2	1.33	8.67	86.67	86.67	
	Control		10				10	0	0	0	
Rhododendron sp	10	1	10	10	10	10	10	0	0	0	14064.79
	100	2	10	9	8	8	8.33	1.67	16.67	16.67	
	1000	3	10	7	8	7	7.33	2.67	26.67	26.67	
	Control		10				10	0	0	0	
B.diffusa	10	1	10	2	4	4	3.33	6.67	66.67	46.67	13.59
	100	2	10	0	2	2	1.33	8.67	86.67	66.67	
	1000	3	10	0	0	0	0	10	100	80	
	Control		10				8	2	20	0	
E.strobilifera	10	1	10	8	9	7	8	2	20	0	223.46
	100	2	10	2	5	4	3.67	6.33	63.33	43.33	
	1000	3	10	0	3	0	1	9	90	70	
	Control		10				8	2	20	0	
H.salicifolia	10	1	10	6	10	8	8	2	20	10	6814.29
	100	2	10	6	8	4	6	4	40	30	
	1000	3	10	6	4	6	5.33	4.67	46.67	36.67	
	Control		10				9	1	10	0	
A.spectabilis	10	1	10	2	5	4	3.67	6.33	63.33	43.33	6.14
	100	2	10	0	2	3	1.67	8.33	83.33	63.33	
	1000	3	10	0	0	0	0	10	100	80	
	Control		10				8	2	20	0	
T.foliolosum	10	1	10	5	5	6	5.33	4.67	46.67	26.67	64.5
	100	2	10	2	2	1	1.67	8.33	83.33	63.33	
	1000	3	10	0	0	2	0.67	9.33	93.33	73.33	
	Control		10				8	2	20	20	

Preparation of inoculums: DPPH solution of 100 µM was prepared by dissolving 3.94 mg of DPPH in 100 ml of methanol. It was protected from light by covering the bottle with aluminum foil [15].

Preparation of standard solution: 10 mg/ml stock solution of Ascorbic acid was prepared by dissolving 10 mg Ascorbic acid in 1 ml methanol. Test solution 0.5,2,4,8,16, and 32 µg/ml of ascorbic acid was prepared from stock solution by dilution.

Preparation of test Sample: 100 mg of plant extract was dissolved in 1ml of methanol to prepare stock solution of 100 mg/ml. Test solution 0.5, 1, 2, 4,8,16 and 32 µg/ml was prepared from stock solution by dilution. Experiments were done in triplicate.

Estimation of DPPH activity: Estimation was done on 1:1 ratio 0.75 ml methanol and 0.75 ml DPPH solution were mixed as control 1.5 ml methanol was taken in eppendoff tube as blank 0.75 ml ascorbic acid was mixed with 0.75 ml DPPH as standard. 0.75ml plant extract was mixed with 0.75 ml DPPH as sample. All these mixtures were immediately kept in dark to prevent from light. After 30 minutes, absorbance was noted in 517 nm.

Calculation of IC_{50}: IC50 was calculated from % scavenging activity. Absorbance at 517 nm was determined after 30 minutes using UV-visible Spectrophotometer and IC50 was also determined. Lower the absorbance of the reaction mixture indicated higher free radical scavenging activity. IC50 value denoted the concentration of sample required to scavenge 50% of the DPPH free radical.

Table 3: DPPH free radical scavenging assay: Calculation of % scavenging activity of DPPH by ascorbic acid and plant extracts.

Sample	Conc(ug/ml)	A_0	A_1	%Scavenging Activity =((A_0-A_1)/ A_1).100
Ascorbic acid	0.5	0.378	0.313	17.2
	1	0.378	0.291	23.02
	2	0.378	0.228	39.68
	4	0.378	0.18	52.38
	8	0.378	0.14	62.96
	16	0.378	0.075	80.16
	32	0.378	0.035	90.74
P.edgeworthii	0.5	0.378	0.374	1.06
	1	0.378	0.369	2.38
	2	0.378	0.356	5.82
	4	0.378	0.33	12.7
	8	0.378	0.327	13.49
	16	0.378	0.311	17.72
	32	0.378	0.29	23.28
S.daltoniana	0.5	0.378	0.374	1.06
	1	0.378	0.371	1.85
	2	0.378	0.342	9.52
	4	0.378	0.33	12.7
	8	0.378	0.311	17.72
	16	0.378	0.269	28.84
	32	0.378	0.245	35.19
B.petiolaris	0.5	0.378	0.327	13.49
	1	0.378	0.308	18.52
	2	0.378	0.286	24.34
	4	0.378	0.266	29.63
	8	0.378	0.244	35.45
	16	0.378	0.15	60.32
	32	0.378	0.127	66.4
A.spectabilis	0.5	0.378	0.372	1.59
	1	0.378	0.35	7.41
	2	0.378	0.339	10.32
	4	0.378	0.325	14.02
	8	0.378	0.31	17.99
	16	0.378	0.272	28.04
	32	0.378	0.199	47.35
B.diffusa	0.5	0.378	0.375	0.79
	1	0.378	0.373	1.32
	2	0.378	0.368	2.65
	4	0.378	0.356	5.82
	8	0.378	0.339	10.31
	16	0.378	0.326	13.76
	32	0.378	0.317	16.14
H.salicifolia	0.5	0.378	0.369	2.38
	1	0.378	0.361	4.5
	2	0.378	0.351	7.14
	4	0.378	0.323	14.55
	8	0.378	0.302	20.11
	16	0.378	0.244	35.45
	32	0.378	0.146	61.38
E.strobilifera	0.5	0.378	0.354	6.35
	1	0.378	0.307	18.78
	2	0.378	0.282	25.4
	4	0.378	0.273	27.78
	8	0.378	0.263	30.42
	16	0.378	0.176	53.44
	32	0.378	0.066	82.54

A0 =Absorbance of DPPH solutionA1= Absorbance of DPPH along with different concentration of extracts: Comparison of % scavenging of DPPH by Ascorbic acid and different plant extracts (concentration range: 0.5-32 µg/ml)

The capability to scavenge the DPPH radical was calculated using following equation.

Percentage scavenging = A0-A1X 100A0

Where, A0 =Absorbance of DPPH solution

A1= Absorbance of DPPH along with different concentration of extracts

Result and Discussion

The yield percentage of plant extract from the warm extraction was calculated. Yield percentage was calculated by using following formula:[16]

Yield = (weight of the extract obtained)/(total weight of the sample loaded) x 100%

Phytochemical screening result

Based on our phytochemical screening test, Rhododendron, *B.diffusa, E.strobilifera, B.petiolaris*, shows the presence of Flavonoids, Tannins and Polyphenol was not found in *E.strobilifera* & *A.sarmentosa*, which play a role in protection from predation, and as pesticides, and in plant growth regulation, In higher plants, flavonoids are involved in UV filtration, symbiotic nitrogen association. Basic alkaloids were found in all test species except H.salicifolia, A.spectabilis, T.foliolosum, Alkaloids have a wide range of pharmacological activities including anti-malarial (e.g. quinine), antiasthma (e.g. ephedrine), anticancer (e.g. homoharringtonine), cholinomimetic (e.g. galantamine). Triterpines was present in all test species except *Rhododendron*, *A.spectabilis, T.foliolosum* which indicate it toxic nature. Glycoside was only found in *B.diffusa*.

Saponin [9] serve as anti-feedants, and to protect the plant against microbes is present in Rhododendron. Reducing sugar was only found in *P.edgeworthii & Rhododendron*, capable of acting as a reducing agent because it has a free aldehyde group or a free ketone group, due to this it affects the cell membrane's fluidity and serves as secondary messenger in developmental signaling.

Antimicrobial assay

Antimicrobial assay was also performed with all plant extracts from by agar disc diffusion method. The assay was performed using four standard antibiotic disc, plant extracts of four different concentration (200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml) and five bacterial strain. The antimicrobial activity was assessed by measuring the diameter of ZOI [Zone of Inhibition]. Among 4 standard discs (P10, GEN10, CF30, TE30) used, P10 didn't show any antimicrobial activity.

GEN10 showed greatest activity against *S.aureus* of 33 mm and lowest against *B.thuringinesis* of 21 mm, CF30 showed greatest activity against *K.pneumoniae* of 39 mm and lowest against S.aureus of 22 mm and TE30 showed the greatest activity against Enterobacter of 27 mm and lowest against *K.pneumoniae* of 21 mm.

All the plant extract showed some antimicrobial activity however the activity was less than the activity of standard antibiotic disc. The largest zone of inhibition

(13 mm) was obtained with *B.petiolaris* against *K.pneumonia*. *B.petiolaris* showed activity against all test organisms and showed significant ZOI against *K.pneumonia* (ZOI-13 mm at 200 mg/ml, 10 mm at100 mg/ml,7.5 mm at 50 mg/ml)and Enterobacter(ZOI-12 mm at 200 mg/ml,10 mm at 100 mg/ml, 8 mm at 50 mg/ml and 8 mm at 25 mg/ml) . Among 10 plant extract, A.sarmentosa showed the least activity against the test organism as it showed no activity against *B.thuringinesis*, *S.aureus* and *K.pneumonia* and show little activity against *B.cereus* (ZOI-7 mm at 200 mg/ml extract concentration) and Enterobacter(ZOI-6.5 mm at 200 mg/ml extract concentration). *P.edgeworthii* showed greatest activity S.aureus (ZOI-10 mm at 200 mg/ml extract concentration) against among test organism used. *S.daltoniana*, *Rhododendron*, *B.diffusa* and *T.foliolosum* showed activity against all test organisms. However, the activity was not significant as ZOI<10 against all test organism. *E.strobilifera* showed ZOI (11 mm) against *B.thuriengenesis* at 200 mg/ml concentration. *H.salicifolia* showed activity against all test organisms and greatest against *B.cereus* (ZOI- 11 mm at 200 mg/ml, 9 mm at 100 mg/ml ,6.5 mm at 50 mg/ml and 6.5 mm at 25 mg/ml), *B.thuringinesis* (ZOI-11 mm at 200 mg/ml, 9 mm at 100 mg/ml, 8 mm at 50 mg/ml and 7 mm at 25 mg/ml) and *K.pneumoniae* (ZOI-10 mm at 200 mg/ml , 7 mm at 1000 mg/ml, 6.5 mm at 50 mg/ml and 6 mm at 25 mg/ml). *A.spectabilis* showed greatest activity against Enterobacter i.e. ZOI of 11 mm at 200 mg/ml, 10 mm at 100 mg/ml, 10 mm at 50 mg/ml and 9 mm at 25 mg/ml. Zone size interpretative standards for selected antimicrobial discs (P10, GEN10, CF30, TE30) and their observed ZOI during experiment.

Brine shrimp bioassay

Brine shrimp bioassay was performed to evaluate toxic effect of our sample towards the test animal i.e brine shrimp which provides idea of potential cytotoxic effect of our sample. The assay was done by calculating the 50% lethal concentration (LC50). LC50 value was different for the different plants. The LC50 value were A.sarmentosa-14681.63 ppm, *P.edgeworthii*-257.17 ppm, *S.daltoniana*-14064.79 ppm, *B.petiolaris*-257.17 ppm, *Rhododendron*-14064.79 ppm, *B.diffusa*-13.59 ppm, *E.strobilifera*-223.46 ppm , *H.salicifolia*-6814.29 ppm, *A.spectabilis*-6.14 ppm and *T.foliolosum*-64.50 ppm. The different LC50 value could be due to difference in amount and kind of cytotoxic compound (like tannins, triterpenoids, flavonoids or coumarin present in the extract.

From the result of brine shrimp bioassay, *A.spectabilis* was found to have the greatest effect against the test animal i.e. it has LC50 value of 6.14 ppm i.e. (6.14 ppm extract concentration could kill 50% of brine shrimp). Four plants *A.spectabilis, B.diffusa, T.foliolosum and E.strobilifera* were found to have LC50 less than 250 ppm i.e. there might be presence of potent cytotoxic and insecticidal compound.

DPPH free radical scavenging assay

Antioxidant activity of the selected plant extracts (seven out of ten) by calculating the IC50 value (50% Inhibitory concentration). The IC50 value of reference standard ascorbic acid was found to be 3.84 µg/ml. The IC50 value of extracts were found to be A.sarmentosa-17.04 µg/ml, S. daltoniana-11.82 ug/ml, B.petiolaris-5.62 ug/ml, *A.spectabilis*-10.10 ug/ml, *B.diffusa*- 24.84 ug/ml, *H.salicifolia*-8.39 ug/ml and *E.strobilifera*-5.46 ug/ml. *E.strobilifera* showed most effective antioxidant activity with IC50 value of 5.46 .This study revealed that selected plant extracts possessed significant antioxidant activity and this could be due to the presence of phenolic compounds (Table 3).

DPPH free radical scavenging assay

Calculation of % scavenging activity of DPPH by ascorbic acid and plant extracts.

Acknowledgement

This part of the work has been supported by KOICA-1-1 project supported by KOICA Nepal.

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