

Comparative study of antioxidant and antibacterial activities in the methanol and ethyl acetate extract of leaf and stem bark of *Semecarpus anacardium* Linn.

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Abstract

Semecarpus anacardium Linn has been used in Nepal for a long time to treat various malfunctions in the body. Antioxidant and antibacterial activities of the extract of leaf and bark of *Semecarpus anacardium* Linn have been determined and the data have been utilized to relate the importance of the extracts in medical field. Study of DPPH radical scavenging activity displayed the IC_{50} value (30.75 ± 0.93 $\mu\text{g/mL}$) of methanol stem bark extract close to IC_{50} value of standard ascorbic acid, indicating its potency as antioxidant. This fact has been further vouched by their high TPC and TFC values. The ethyl acetate stem bark extract and methanol leaf extract depicted a significant antibacterial activity against *S. aureus* and *E. coli*, respectively. The methanol stem bark extract has been found to have better antibacterial and antioxidant activity than methanol leaf extract.

Keywords: Medicinal plants, flavonoids, Antibacterial activity, Antioxidant activity.

Introduction

Nepal is floristically characterized by six adjoining floristic regions. Till date, 701 species of medicinal plants have been reported in Nepal.¹ About 7,000 species of flowering plants found in Nepal are said to have medicinal use.² Early human civilization develop a medicine system like Chinese medicine, Ayurvedic Medicine, Japanese Kampo, Unani Medicine and traditional Systems of medicine.³ Plants synthesize varieties of unique and complex structure organic compounds are secondary metabolites called natural product viz alkaloids, flavonoids, steroids, tannins, phenolic Compounds etc.⁴ Antioxidants protect cells from reactive oxygen species, prevent free radicals production and helps to function naturally prevailing antioxidants in our body. Oxidation induced by ROs cause oxidative stress, cellular damage and neurodegenerative diseases like Parkinson's disease, Alzheimer's disease, gene mutations & cancers etc.⁵ ROs are highly unstable ions induced due to cell metabolic processes, exogenous pollutants (smoking, radiations), superoxide anion and radicals etc.³ An antibacterial agent is any substances of natural, synthetic and semi-synthetic origin that kills or inhibits the growth of Bacteria but cause little or no damage to the host. Antibiotics recently have become less effective due to drug-resistant bacteria and their toxicity⁶, has led to new antibiotics discovery from medicinal plants.⁷ Polyphenols are secondary

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metabolites, involved in defense against UV radiations or pathogens. The antioxidant activity of phenolic is mainly due to their redox property, allowing them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators.⁸ Flavonoids, are the polyphenolic compound having 15-carbon skeleton with two phenyl rings and one heterocyclic rings. Antioxidant property of flavonoids depends upon their molecular structures, hydroxyl group position and other features in the chemical structure.⁹ *Semecarpus anacardium Linn.* belongs to Anacardiaceae family, mostly distributed in Sub-Himalayan tract and hotter parts with height of 15-25 meter. It has been employed traditionally to treat disease like rheumatism, asthma, urinary discharges, cancer & psoriasis, diabetes, diarrhoea, skin diseases, ulcer, gout, piles, Alzheimer's disease etc. Bhelwa (Hindi), Bhallataka (Sanskrit), Bhalayo (Nepali) etc. are some other names it is addressed with. Besides, Marking nut tree and Oriental cashew nut tree are the next representative names for *Semecarpus anacardium Linn.*¹⁰⁻²¹ The stem bark color of this plant is grey desquamating in rough flakes while leaves are simple, alternate, obovate oblong, apex is rounded and main nerves are in 15-25 pairs. Flowers are greenish white in color.¹³ Fruits are thickened disc¹³, kidney shaped and drupaceous nut commonly known as marking nut.¹⁷ Fruits are orange red in color, smooth, fleshy and 2.5 cm long with sweet and hot taste and contains corrosive juice, used for both internal and external treatments.¹³ Sahoo et al.¹¹ isolated a bright yellow solid crystal, Butein^{11,14} compound from EtOAc stem bark extract of SA plant. EtOAc extract of stem bark was reported to exhibit highest TPC (686.7 µg Gallic acid equivalents per gram of SA extract) and a potent antioxidant with IC₅₀ value 44.03±4.12 µg/mL. Naveen Kumar et al.¹² reported, MeOH SB extract have highest DPPH radical scavenging activity (74.58%) compared to natural antioxidant Quercetin (78.35%). Mohmad et al.¹⁵ investigated on different SA leaves extracts and found MeOH leave extract among others to be potent antioxidant with the radical scavenging activity of 84.28%. C.Selvam et al.¹⁸ isolated Butein & 7,3',4'-Trihydroxyflavone from the EtOAc SB extract. Pednekar et al.¹⁹ found MeOH leaf extract of SA as a potent antioxidant with IC₅₀ value 916.58±2.14 µg/mL. Despite the multi uses of Bhalayo, its toxicity should be handled with intense care due to its corrosive juice and vesicant nature.¹⁶ The tarry oily in the fruit nut contains anacardic acid has Urushiol (allergen), is toxic.^{10,21} The symptoms of Urushiol-induced contact dermatitis includes itching, redness, burning sensation, swelling, papules, vesicles and blisters. SA toxins lead to acute renal failure due to hemodynamic effects.¹⁰ In case inhaled, vomiting, excessive thirst, decrease in WBC count, less urine output with colored urine & sometimes blood in urine (Haematuria) are noticed.¹⁸ Nevertheless, those side effects can be suppressed by consuming bland and cooling diet like rice, milk, coconut oil, coriander leaves pulp, ghee, butter mixed with musta (*Cyperus rotundus*), amlika leaves juice etc. are antidotes. Neem (leaves) paste can be used externally to cure rashes and vesicles. Salt & spices consumption and exposure to heat and sun, is strictly prohibited. It should not be used in small children, very old persons and pregnant women.²¹ To the best of our knowledge, no study has been performed on comparing these properties between stem bark and leaf extracts of *S. anacardium Linn.* The present study mainly focuses on comparative study of methanol and ethyl acetate extracts of stem bark and leaves regarding antioxidant and antibacterial activities.

Experimental Methods

Chemicals

All reagents and solvents used were of laboratory reagent grade purchased from E. Merck, T. Fisher Scientific and Qualigens Chemical Companies, India like silica gel of 60-120 mesh, "Silica gel-G", 0.2

mm thick pre-coated TLC aluminum plates, iodine chamber, conc. H₂SO₄ & Folin-coicalteu reagent. Other chemicals and reagents like DPPH, ascorbic acid, gallic acid, quercetin were provided by Central Department of Chemistry. Chemicals like aq. Na₂CO₃, NaNO₂, NaOH, AlCl₃, Ethanol etc. were available in laboratory.

Equipments

Soxhlet, rotatory evaporator, spectrophotometer, micropipettes, digital weighing machine, column chromatography, water bath and other apparatus like conical flasks, Round bottom flask, Beakers etc.

Collection of plant materials

The plant materials were collected from Rajahar-16, Devchuli municipality of Nawalparasi district and was identified by Central Department of Botany, Tribhuvan University.

Extraction of plant materials

The collected fresh plant materials were rinsed with clean water, shade dried for about a month and eventually grinded to powder form to prepare extracts. 100 g powder form of SA plant leaves was extracted by cold percolation method (with frequent agitation for 48 hours) and 190 g of stem bark of SA by soxhlet method in four different solvents (300-400 mL) based on their increasing polarity viz hexane, chloroform, ethyl acetate and methanol successively at room temperature. The mixture from cold percolation was filtered repeatedly for about 6-8 times using whatman filter paper to obtain filtrate. Similarly, the solvents used for soxhlet extraction, had undergone several cycles for about 11-13 hours were collected and the obtained solvents (soxhlet) and filtrates (percolation) were concentrated by rotary evaporator under reduced pressure to obtain crude extracts, are further employed to investigate total phenol content, total flavonoid content, antioxidant activity and antibacterial activity.

Antibacterial activity

Antibacterial activity of the plant extracts was performed by agar well diffusion method following standard protocol provided by Cavalieri S. J., et al.⁶ Four labeled wells of diameter 6 mm were made in each incubated media plates. Then 50 µL of the working solution of the plant extract, DMSO as negative control (N) and 25 µL of chloramphenicol (antibiotic) as positive control (P) at the same time in the separate well were loaded into the respective wells by micropipette. The plates were then left for half an hour with the lid closed so that the extract diffused into media and then incubated overnight at 37 °C. After 24 hours of incubation, the plates were observed for the inhibition of bacterial growth indicated by a clear zone around the wells. The size of the zone of inhibition was measured by millimeter ruler and the antibacterial activity is expressed in terms of the average diameter of ZOI in millimeters.

DPPH free radical scavenging activity

The free radical scavenging activity of different plant extracts were measured by using DPPH assay described by Jamuna S. et.al, (2012).²² Initially, 10 mg of the samples to be tested was dissolved in 10 mL methanol to prepare stock solution of concentration of 1 mg/mL (1000 µg/mL). Stock solution of each extracts were screened preliminary for their antioxidant activity by adding 0.5 mL of 0.2 mM DPPH in 1 mL of extract solutions differently and was left for 30 minutes in dark. After 30 minutes, discolorations (color change from purple to yellow) were taken for further testing at 517 nm using a UV-visible spectrophotometer. The control was prepared without the plant extracts (methanol + DPPH). Ascorbic

acid of same concentrations was prepared as a standard and its absorbance was taken spectrophotometrically at 517 nm. Calibration curve was constructed. Percent radical scavenging activity by sample treatment was determined by comparison with methanol treated control group; ascorbic acid was used as positive control. Measurement was carried out at least in triplicates. The percentage of the DPPH free radical scavenging activity was calculated by using the following equation:

Radical scavenging (%) = $[(A_0 - A_s)/A_0] \times 100$ (Where, A_0 = Absorbance of the control (DPPH solution + methanol), A_s = Absorbance of test sample)

Total Phenol content (TPC) determination

The total polyphenol content of plant extracts were estimated using Folin-Ciocalteu reagent involving gallic acid standard based on redox reaction following the standard protocol provided by Kim et al.²³ The stock solutions of all the extracts were prepared by dissolving 10 mg in 1 mL of methanol (10 mg/mL). Serial dilutions were carried out to prepare concentration of 0.125, 0.25, 0.5 and 1.0 mg/mL. To these diluted solutions, 10% FCR and 7% Na_2CO_3 were added and incubated for 30 min. and absorbance was measured at 760 nm against blank for each concentration using UV-visible spectrophotometer. The average absorbance values obtained at different concentrations of gallic acid were used to plot calibration curve. With the help of this standard graph of gallic acid, the concentrations of the individual samples were 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) of extract. TPC can be calculated using following formula;

$$C = cV/m \dots\dots\dots (A)$$

Where, C = Total phenolic content compounds in mg GAE/gm

c= Concentration of gallic acid established from the calibration curve in mg/mL, V = Volume of extract in mL, M = Mass of plant extract

Total flavonoid content (TFC) Determination

The total flavonoid content of the plant extracts were determined according to aluminum chloride colorimetric method involving quercetin as standard, described by Kalita, P. et.al, (2013).²⁴ The stock solution of all the extracts was prepared by dissolving 10 mg in 1 mL of methanol (10 mg/mL). Prepare Serial dilute solutions of concentration 0.125, 0.25, 0.5 and 1.0 mg/mL. To these dil. Solutions, 2 mL double distilled water was mixed followed by 0.15 mL 5% NaNO_2 . After 5 min, 0.15 mL of 10% AlCl_3 was added. 1 mL of 1 M NaOH was added after 6 min. Finally 1.2 mL of double distilled water was added to the solution and absorbance for each concentration of extracts was measured at 510 nm using spectrophotometer. Measurements were carried out in triplicate. The calibration curve is plotted by average absorbance values obtained at different concentrations of quercetin. The total flavonoid content was expressed in terms of the milligrams of the quercetin equivalent per gram of the dry mass (mg QE/g) of extract. TFC can be calculated using the same formula as used for TPC.

Result and Discussion

The data from the phytochemical screening of MeOH and EtOAc extracts of *S. anacardium* Linn are depicted in table-1.

Table 1: Phytochemical Screening of MeOH and EtOAC extracts of *S. anacardium* Linn.

SN	Plant Extracts	Alk	Cou	Flav	Gly	Quin	PP	Sug	Sap	Terp
1	MeOH SB extract	+	+	+	+	+	+	-	+	+
2	MeOH leaf extract	+	-	+	+	-	+	-	+	-
3	EtOAC SB extract	-	+	+	-	+	+	-	+	-
4	EtOAC leaf extract	-	+	+	+	-	+	-	+	+

Alk = Alkaloids, Cou = Coumarins, Flav = Flavonoids, Gly = Glycosides, Quin = Quinones, PP = Polyphenols, Sug = Reducing sugars, Sap = Saponins, Terp = Terpenoids, SB=stem bark

Antibacterial Activity

The diameter of zone of inhibition (ZOI) produced by MeOH and EtOAC extracts of both stem bark and leaves on particular bacteria were measured for the estimation of their antibacterial activity. Results obtained from the antibacterial analysis of different extracts are tabulated in table-2.

Table 2: Antibacterial analysis results of MeOH & EtOAC extracts of SA stem bark and leaf.

SN	Plant Extracts (<i>S. anacardium</i> Linn.)	Bacteria	ZOI (mm) at conc. of 100 mg/mL	ZOI (mm) of Chloramphenicol as control at 100 mg/mL
1	Methanol stem bark	<i>E.coli</i>	14	24
		<i>S. aureus</i>	19	29
2	Methanol leaf	<i>E.coli</i>	16	24
		<i>S. aureus</i>	19	29
3	Ethyl acetate stem bark	<i>E. coli</i>	14	24
		<i>S. aureus</i>	27	29
4	Ethyl acetate leaf	<i>E. coli</i>	15	24
		<i>S. aureus</i>	22	29

(-) control= DMSO (No effective antibacterial activity), ZOI = Zone of Inhibition,

E. coli: Gram-negative organism, *S. aureus*: Gram-positive organism

The above results indicate, all the four different extracts depicted considerable inhibition of growth against both the bacteria. Nevertheless, the EtOAC extract of the SA stem bark and MeOH extract of SA leaf showed more efficacy against *S. aureus* and *E.coli* with ZOI value of 27 mm and 16 mm respectively. Gram positive bacteria were inhibited effectively than the gram negative bacteria as, the unique outer membrane that excludes the extract from penetrating the cell in gram negative bacteria, is absent in gram positive type.

Total phenolic and flavonoid content

Both the total phenolic and flavonoid content were shown maximum by MeOH stem bark extract with the values of 6104.94±4.97 mg GAE/g and 184.23±2.07 mg QE/g respectively, displayed strongest

antioxidant activity. While total phenolic content for EtOAC stem bark extract and total flavonoid content for EtOAC leaf extract were recorded to be 5251.08±3.78 mg GAE/g and 162.18±1.86 mg QE/g respectively.

DPPH Free Radical Scavenging Activity

The DPPH radical scavenging assay suggested, the IC₅₀ value of MeOH extract of SA stem bark was 30.75±0.93 µg/mL while EtOAC stem bark extract, EtOAC leaf extract & MeOH leaf extract of SA had IC₅₀ values 32.83±0.51, 36.30±0.25, 37.76±0.38 µg/mL respectively, close to standard ascorbic acid (25.18±3.91 µg/mL). The results suggested the fact that, these extracts of various plants can be good alternatives in the medicinal field based on their antioxidant properties.

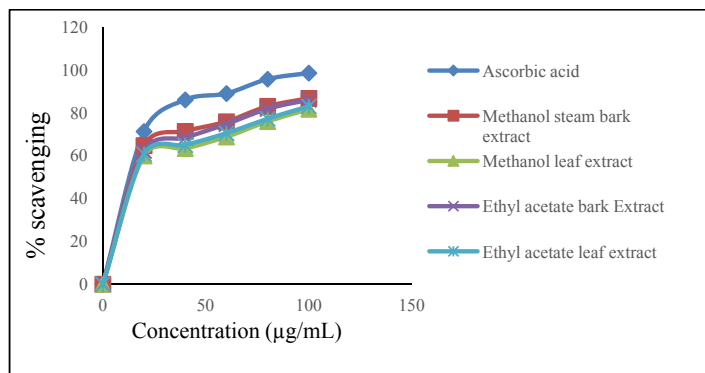


Figure 1: A plot of percentage radical scavenging activity vs concentration of ascorbic acid, methanol & ethyl acetate extracts of stem bark & leaf

Table 3: Comparison of IC₅₀ values of different plant extracts with standard Ascorbic acid

Plant extracts (<i>S. anacardium</i> Linn.)	Free radical scavenging (IC ₅₀)	Total phenolic mg GAE/g	Total flavonoid mg QE/g
Methanol stem bark extract	30.75±0.93	6104.94±4.97	184.23±2.07
Methanol leaf extract	37.76±0.38	2965.50±2.89	103.50±0.87
Ethyl acetate stem bark extract	32.83±0.51	5251.08±3.78	149.041±1.15
Ethyl acetate leaf extract	36.30±0.25	3825.64±2.65	162.18±1.86

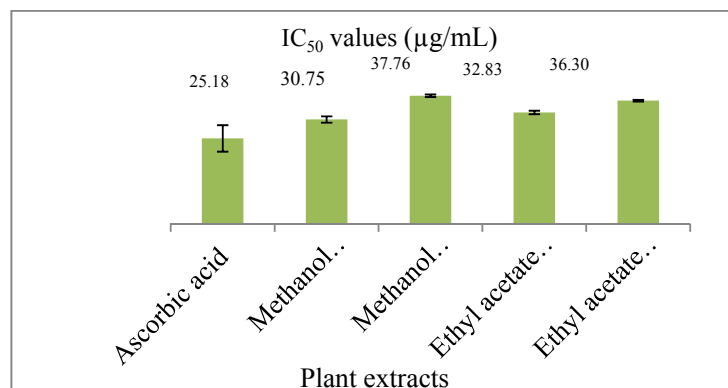


Figure 2: Free radical scavenging activity (IC_{50}) of MeOH and EtOAC extracts of SA represented as $mean \pm S.D$ with average value of triplicate experiments.

Conclusion

DPPH radical scavenging assay displayed the IC_{50} value of MeOH stem bark extract was 30.75 ± 0.93 $\mu\text{g/mL}$ close to IC_{50} value of std. ascorbic acid, made it the potent antioxidant. The antibacterial activity studied by disc diffusion method, invented EtOAC stem bark and MeOH leaf extract possess the highest ZOI value of 27 mm/disc and 16 mm/disc against *S. aureus* & *E. coli* respectively. MeOH stem bark extract bears the highest value for both TPC and TFC i.e. 6104.94 ± 4.97 mg GAE/g extract and 184.23 ± 2.07 mg QE/g extract respectively. This shows that bark and leaf extracts of *Semecarpus anacardium* Linn. are potent antioxidants with high TPC and TFC values that rationalize the logic behind employing medicinal plant to treat disease induced by ROs. Since MeOH SB extract's efficacy over EtOAC SB extract seems superlative, MeOH SB extract of *S. anacardium* Linn. can be handy to contrive new drugs. The possible antioxidant property is due to the presence of phytochemicals like polyphenols, flavonoids etc. MeOH extract of stem bark exhibited highest total phenolic and flavonoid content followed by EtOAC extracts. The results above prove these extracts to be potent antioxidant seedbed and can be employed to isolate active compounds to contrive new drugs. The high TPC & TFC justified the reason behind mobilizing medicinal plants to cure several diseases since ages.

The EtOAC stem bark extract and MeOH leaf extract of *S. anacardium* Linn. depicted a significant antibacterial activity against *S. aureus* and *E. coli*. respectively. The MeOH stem bark extract of *S. anacardium* Linn. discovered sound antibacterial, antidiabetic and antioxidant activity than MeOH leaf extract. Hence the further scrutiny of MeOH stem bark extracts of *Semecarpus anacardium* Linn. can be brought into action to predict its possibility as a potent antioxidant and antibacterial drug.

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