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Reserch Article

EVALUATION OF ANTI MICROBIAL, ANTI OXIDANT ACTIVITY AND ESTIMATION OF TOTAL FLAVONOID CONTENT IN *SAPINDUS TRIFOLIATUS* SEED EXTRACTS

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ABSTRACT

Objective: The present study aims at evaluation of antimicrobial activity and *in-vitro* anti oxidant activity of n-hexane and ethyl acetate extracts of seeds of *Sapindus trifoliatus.* Total flavonoid content was also estimated.

Methods: For evaluating antibacterial activity disc diffusion method was followed using different gram positive and gram negative bacterial strains. For evaluating anti fungal activity different fungal strains were selected. For evaluating anti oxidant activity 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) method and reducing power method was used. For DPPH method Butylated Hydroxy Anisole (BHA) was used as a positive control and for reducing power method ascorbic acid was used as a standard reducing agent. All the analysis was made with the use of UV-Visible Spectrophotometer. The content of total flavonoids was measured spectrophotometrically by using the aluminium chloride colorimetric assay where catechin was used as a standard.

Results: The extracts showed good DPPH radical scavenging activity and reducing power activity which was found to increase with the increasing concentration of the extracts. The total flavonoid content was found to be 103.9 mg CE/100g.

Conclusion: Ethyl acetate extract of seeds of *Sapindus trifoliatus* showed more prominent activity when compared to n-hexane extract which could be attributed to the presence of flavonoid content.The study could be further extended to isolate and characterize phytoconstituents from these extracts.

Keywords: *Sapindus trifoliatus*, n-hexane extract, Ethyl acetate extract, Disc diffusion method, DPPH Method, Reducing power method, Total flavonoid content.

INTRODUCTION

Infectious diseases account for a high proportion of health problems in the developing countries like India. Micro organisms have developed resistance to many antibiotics and this has created immense clinical problem in treatment of infectious diseases. The resistance of the organisms increased due to indiscriminate use of commercial anti microbial drugs commonly used for the treatment of infectious diseases. This situation forced the scientists to search for new anti microbial substances from various sources including medicinal plants **[1].**

Antioxidants are radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neuro-degenertion, Parkinson's diseases, mongolism, ageing process and perhaps dementias **[2]**. Flavonoids and flavones are widely distributed secondary metabolites with antioxidant and antiradical properties **[3]**.

Plants are potent biochemical factories and have been components of phytomedicine since times immemorial; man is able to obtain from them a wondrous assortment of industrial chemicals. Plants based natural constituents can be derived from any part of plant like bark, leaves, flowers, roots, fruits, seeds, etc **[4]** i.e. any part of the plant may contain active components. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. The medicinal actions of plants are unique to particular plant species or groups are consistent with this concept as the combination of secondary products in a particular plant is taxonomically distinct **[5]**. Antioxidant-based dugs/formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease, and cancer have appeared during the last 3 decades **[6]**.

This has attracted a great deal of research interest in natural antioxidants. Subsequently, a worldwide trend towards the use of natural phytochemicals present in berry crops, tea, herbs, oilseeds, beans, fruits, and vegetables has increased. Several herbs and spices have been reported to exhibit antioxidant activity, including rosemary, sage, thyme, nutmeg, turmeric, white pepper, chili pepper, ginger, and several Chinese medicinal plants extracts **[7].** The majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, and isocatechins. In addition to the above compounds found in natural foods, vitamins C and E, beta-carotene, and tocopherol are known to possess antioxidant potential **[8]**.

Dry seeds of *Sapindus trifoliatus* L., is one of the oldest cultivated medicinal plants in the world. In fact botanists traced it to the period of Vedas about 5000 years ago. It is a medium sized deciduous tree growing wild in South India. *Sapindus trifoliatus* is belonging the family Sapindaceae are rich in saponins **[9, 10]**

The preliminary phytochemical investigation of methanolic and aqueous extracts of seeds of Sapindus trifoliatus indicated the presence of steroids, carbohydrates, flavonoids, triterpenoids and saponins. The seeds contain fatty acid. The fatty acids are of rachidic acid, Behenic, Linoleic, Oleic, Palmitic, Stearic, Oleanolic acid and Sapindic acid. They also contain Trifoloside A, Sapindoside C, D, E, Glucopyranosides of Stigmasterol,Kaempferol,Quercetin,β-sitosterol,Hederagenin,Protein,Carbohydrate and Starch **[11]**. Leaves of *Sapindus trifoilatus* can be used to as expectorant, eczema, aphrodisiac, abotrifacient, migraine, psoriasis and inflammation **[12].**

MATERIALS AND METHODS

Chemicals

All the solvents used were of analytical grade. N-hexane, ethyl acetate, methanol, aluminium chloride was obtained from Merck. Butylated hydroxyl anisole(BHA),potassium ferricyanide, trichloro acetic acid, ferric chloride, ascorbic acid, were purchased from R & M chemicals.1,1-Diphenyl picryl hydrazyl(DPPH) and catechin were purchased from Sigma-Aldrich chemical.

Plant Material

Seeds of selected plant materials were collected from local areas of Hyderabad and further authenticated by botany department of Osmania University. Voucher specimens were deposited at the same department. Seeds were dried under shade for five days. Dried seeds were ground into a uniform powder using a pulveriser and stored in polythene bags at room temperature.

Preparation of extracts

The shade dried plant materials were extracted successively with n-hexane, chloroform, ethyl acetate and ethanol using a soxhlet extractor. The extracts obtained were concentrated using rota evaporator and stored in a refrigerator.

Phytochemical Investigation

The preliminary phytochemical investigation of the extracts showed the presence of triterpenoids, saponins and steroids in n-hexane extract and flavonoids in ethyl acetate extract as major constituents. The details of the chemical tests are as follows **[13].**

Test for sterols

a) Salkowaski test : When few drops of concentrated sulphuric acid is added to the test solution, shaken and allowed to stand, lower layer turns red indicating the presence of sterols.

b) Liebermann Burchardt test: The test solution treated with few drops of acetic anhydride and mixed well. When concentrated sulphuric acid is added from the sides of the test tube, it shows a brown ring at the junction of the two layers and the upper layer turns green.

c) Sulphur test: Sulphur when added into the test solution, it sinks in it.

Test for flavonoids

a) Ferric chloride test **:** Test solution with few drops of ferric chloride solution shows intense green colour.

b) Shinoda test: Test solution with few fragments of magnesium ribbon and concentrated hydrochloric acid, shows pink to magenta red colour.

c) Alkaline reagent test: Test solution when treated with sodium hydroxide solution shows increase in the intensity of yellow colour which becomes colourless on addition of few drops of dilute acid.

d) Lead acetate solution test: Test solution with few drops of lead acetate solution (10%) gives yellow precipitate.

Test for Saponins

a)Foam test: Saponins when mixed with water and shaken shows the formation of foam which is stable at least for 15 minutes.

b) Haemolysis test: 2ml of 18% sodium chloride in two test tubes were taken. To one test tube distilled water was added and to the other test tube 2ml of filtrate and then few drops of blood was added to both the test tubes. Mixed and observed for haemolysis under microscope.

c) Raymond's test: Test solution treated with dinitrobenzene in hot methanolic alkali gives violet colour.

d) Bromine water test: Test solution dissolved in Bromine water gives yellow precipitate.

e) Legal's test: Test solution when treated with pyridine (made alkaline by adding sodium nitroprusside solution) gives pink to red colour.

Test for Triterpenoids

a) Salkowaski test: When a few drops of concentrated sulphuric acid is added to the test solution, shaken and allowed to stand, lower layer turns yellow.

b) Liebermann Burchard test: The test solution treated with acetic anhydride, mixed well and concentrated sulphuric acid is added from the sides of the test tube. Deep red colour forms.

Antimicrobial activity of various extracts

The antimicrobial activity of all the extracts were carried out by Disc diffusion method using different gram positive and gram negative bacterial strains **[14,15].**The basic idea of diffusion assay is as follows: A suspected antimicrobial compound or treatment is presented within a reservoir created on an inoculated plate of agar medium; Following diffusion of the compound(s) through the agar, "zone of inhibition" forms where concentrations of the diffused molecules are sufficient to inhibit microbial growth. Diffusion of antimicrobial compounds from a reservoir over time produces an outward gradient of decreasing concentration of the compound. Where concentration of the compound is sufficient to inhibit the growth of the microbes, the growth is blocked; resulting in the observed zone, which extends outward from the reservoir (with a corresponding decrease in concentration) to the distance from the reservoir at which the minimum concentration required for inhibition exists.

Strains used

The antimicrobial activity of seeds of *Sapindus trifoliatus* extracts was studied against bacterial strains viz. *E. coli* (Gram-negative), *S. aureus* (Gram -positive) and *B. substilis* (Gram-positive). The fungal strains used were *C. albicans* and *A.niger*. For antibacterial activity tetracycline was used as standard, while for antifungal activity Griseofulvin was used as the standard.

Composition of Media

The medium should be such that it will promote aster growth of microorganisms. (Table **1**& **2**)

Table 1: Nutrient agar media (for bacterial activity)

S. No.	Ingredient	Quantity (g)
1	Beef extract	10
2	Peptone	10
3	Sodium chloride	5
4	Agar	12
5	Water	Up to 1000 ml

Table 2: Potato Dextrose Agar Media (For Antifungal activity)

S. No.	Ingredients	Quantity (g)
1	Potato Infusion	0.2
2	Dextrose	20
3	Agar	15
4	Water	Up to 1000 ml

P**rocedure**

Screening of antimicrobial activity was carried out using the disc diffusion method. This method depends on the diffusion of drug from disc through the solidified agar layer of a petridish to an extent such that growth of the inoculated microorganism is prevented entirely in a circular area "zone" around the cup containing the solution of the compound under test. One loopful of the stock culture was inoculated at 10 ml of agar slant previously in sterilized test tubes, and incubated at 37^{o} for 24 h and 20^{o} for 48 h respectively for bacteria and fungi.

About 3 ml of distilled water was added to the test tube and a suspension of the culture was obtained by shaking for few minutes. The solutions were made by dissolving the test compound in minimum amount of DMSO, and volume was made with sterilized water to produce a concentration of 100 µg/ml. All the operations were carried out under aseptic conditions. Respective sterile medium was melted on water bath and kept at 45^{o} in constant temperature water bath. In each sterile petridish 25 ml of molten medium was added and 10^{7}/ml of sub cultured organism under study were inoculated. The culture and agar medium were mixed and allowed to solidify. Four disc of whatmann No.41 (4 mm

diameter), which were dipped in test samples were then kept on the upper surface of the medium. Solution was allowed to diffuse in the medium for 2 h by keeping the petridish at room temperature and then incubated for about 24 h at 37° (for bacteria) and 48 h at 20° (for fungi).

Screening of anti oxidant activity

a) DPPH Radical scavenging activity

According to the method **[16, 17, 18],** 0.2 ml of methanolic solution of plant extract samples at different concentration (50-250µg/ml) was mixed with 0.8 ml of Tris HCl buffer (100Mm, pH 7.4). One ml DPPH (500 M in methanol) solution was added to above mixture. The mixture was shaken vigorously and incubated for 30min in room temperature. Absorbance of the resulting solution was measured at 517nm using UV-Visible Spectrophotometer (Systronics UV-Visible Spectophotometer 117, INDIA). All the assays were carried out in triplicates with BHA (Butylated Hydroxy Anisole) as a positive control. Blank was prepared without the addition of DPPH and for control 0.2 ml of methanol (without plant extract) was added. Percentage of DPPH scavenging activity determined as follows.

% scavenging DPPH free radical = 100 × (1-AE/AD)

Where AE is absorbance of the sample solution and AD is the absorbance of the DPPH solution with nothing added (blank, without extract).

Purified sample 2mg/ml of *Sapindus trifoliatus* extracts were taken for antioxidant activity with a standard BHA (Butylated Hydroxy Anisole) antioxidant. Decreased absorbance of the reaction mixture indicates stronger DPPH radical-scavenging activity. In this study n- hexane and ethyl acetate extracts of *Sapindus trifoliatus* seeds were used.

(b) Reducing power

According to the method **[19, 20],** 1 ml of plant extract solution (final concentration 100-500 mg/l) was mixed with 2.5 ml phosphate buffer(0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [$K_3Fe(CN_6)$] (10g/l), then mixture was incubated at 50 degree C for 20 minutes. A 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, a 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml Fecl3 (1g/l) and absorbance measured at 700nm in UV-Visible Spectrophotometer (Systronics UV-Visible Spectophotometer 117, INDIA). As a control, ascorbic acid was used (final concentration 15 mg/ml).

Increased absorbance of the reaction mixture indicates stronger reducing power.

Determination of total flavonoids [21]

Sample preparation

A ground, freeze dried sample of 0.5 g was weighed and flavonoid compounds were extracted with 50 ml 80% aqueous methanol on an ultrasonic bath for 20 min. An aliquot (2 ml) of the extracts was ultracentrifuged for 5 min at 14000 rpm.

Total flavonoid assay

The total flavonoid content was measured by the aluminium chloride colorimetric assay.An aliquot of extracts or standatrd solution of catechin(20,40,60,80 and 100 mg/l) was added to 10 ml volumetric flask containing 4 ml of dd H_20. To the flask was added 0.3ml 5% $NaNO_2$.After 5 min,0.3 ml 10% $AlCl_3$ was added. At 6^{th} min, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with dd H_20. The solution was mixed well and the 250 µg/ml of extract absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of the plant was expressed as mg catechin equivalents (CE)/100g fresh mass. Samples were analysed in duplicates.

RESULTS AND DISCUSSION

The in-vitro antimicrobial activity was carried out by Disc diffusion method using different bacterial strains, which showed that these extracts possess prominent activity compared with that of standard. Zone of inhibition were found to be prominent in concentration of 5µg / ml for bacterial strains and 45µg / ml for fungal strains. The n-hexane and ethyl acetate extracts of seeds of *Sapindus trifoliatus* showed significant anti bacterial.**(**Table **3)** and anti fungal activity **(**Table **4)**.

Table 3: Antibacterial activity of seeds of *Sapindus trifoliatus* extracts against different bacterial strains

Microorganism	Zone of Inhibition(diameter in mm)		
	n-hexane	Ethyl acetate	Tetracycline
B.subtilis	20.33 ± 2.41	21.44 ± 3.20	40.00 ± 2.25
E.coli	18.34 ± 2.88	20.38 ± 3.14	33.60 ± 2.31
S.aureus	18.8 ± 3.30	19.33 ± 2.50	33.85 ± 1.80

Table 4: Antifungal activity of seeds of *Sapindus trifoliatus* extracts against different fungal strains

Microorganism	Zone of inhibition (diameter in mm)		
	n - hexane	Ethyl acetate	Grieseofulvin
C.albicans	16.60 ± 2.23	19.66 ± 1.50	35.00 ± 0.25
A.niger	15.6 ± 2.14	16.33 ± 2.14	30.60 ± 2.31

Antioxidant Assay

DPPH scavenging activity

The percentage of DPPH radical scavenging activity of n-hexane extract of *Sapindus trifoliatus* presented (Table **5)**. The DPPH radical scavenging activity of the extract increases with increasing concentration. Only 17.65% DPPH radical scavenging was present for 250µg/ml of extract and it was 69.18% in the presence of 100 mg/l BHA. Although this plant extract shows lower scavenging activity in comparison to BHA, plant extract exhibited antioxidant potential and increased concentration of plant extract has shown increased antioxidatnt potential.

The percentage of DPPH radical scavenging activity of ethyl acetate extract of *Sapindus trifoliatus* is presented *(*Table **6)**.The DPPH radical scavenging activity of the extract increases with increasing concentration.Only 26.57% DPPH scavenging activity was exhibited by 250µg/ml of extract and 78.82% in the presence of 100 mg/l BHA. These results suggest that ethyl acetate extracts exhibited better activity than n-hexane extract.

Reducing power

Different extracts of seeds of *Sapindus trifoliatus* exhibited good reducing power. The reducing power of n-hexane extract of *Sapindus trifoliatus* (100-500mg/ml*)* concentrations is presented (Table **7)**. High absorbance indicates high reducing power. However, this reducing power is lower than that of ascorbic acid which was used as control. Therefore, the absorbance of ascorbic acid at concentration 10 mg/l was 0.86 while at the 500mg/l n-hexane extract concentration it was 0.56.

Table 5: DDPH scavenging activity of n-hexane extract of *Sapindus trifoliatus* seeds

Absorbance at 517 nm				% of activity	
Sample µg/ml	OD	BHA (mg/l)	OD	Sample	Standard
50	1.093	2	0.891	1.53	19.72
100	1.071	4	0.729	3.51	34.32
150	1.002	6	0.624	9.72	43.78
200	0.943	8	0.583	15.04	47.47
250	0.914	10	0.342	17.65	69.18

OD: Optical density
Control OD AT 517 nm------1.110

Table 6: DPPH scavenging activity of ethyl acetate extract of *Sapindus trifoliatus* seeds

Absorbance at 517 nm				% of activity	
Sample µg/ml	OD	BHA (mg/l)	OD	Sample	Standard
50	1.008	2	0.804	9.18	27.56
100	0.985	4	0.732	11.26	34.05
150	0.944	6	0.498	14.95	55.13
200	0.832	8	0.389	25.04	64.95
250	0.815	10	0.235	26.57	78.82

OD: Optical density
Control OD AT 517 nm------1.110

Table 7: Reducing power of n-hexane extract of *Sapindus trifoliatus* seeds

Sample	Concentration(mg/ml)	Absorbance(700nm)
*Control	0	0.03±0.012
Psoralea corylifolia	100	0.15±0.023
	200	0.34±0.043
	500	0.56±0.006
Ascorbic acid	5	0.73±0.061
	10	0.86±0.024
	15	1.32±0.048

*The control was test sample without plant extract.High absorbance indicates high reducing power

The reducing power of ethyl acetate extract of *Sapindus trifoliatus* is presented in (Table **8)** .The absorbance of ascorbic acid in a sample (10mg/l) was 0.78 while at the 500mg/l ethyl acetate extract concentration it was 0.71.The reducing power of ethyl acetate extract of *Sapidnus trifoliatus* seed extract has shown good reducing power than n-hexane extract. As the amount of extract increases, the reducing power also increases. In both cases of n-hexane and ethyl acetate extracts of *Sapindus trifoliatus* there is a remarkable concentration dependent reducing power was observed. This variation in reducing activity may be due to crude nature of plant extracts and availability of different phytochemicals in these plants.

Table 8: Reducing power of ethyl acetate extract of *Sapindus trifoliatus* seeds

Sample	Concentration(mg/ml)	Absorbance(700nm)
*Control	0	0.09±0.021
Psoralea corylifolia	100	0.32±0.112
	200	0.64±0.003
	500	0.71±0.071
Ascorbic acid	5	0.425±0.014
	10	0.78±0.016
	15	1.21±0.001

*The control was test sample without plant extract.High absorbance indicates high reducing power

Total flavonoid content

The total flavonoid content of the plant material was found to be 103.9 mg CE/100g.

CONCLUSION

The present investigation indicates that *Sapindus trifoliatus* seed extract has shown very good anti oxidant activity. Total flavonoid content was measured in methanol extract of plant material. The total flavonoid content was found to be 103.9 mg CE/100g. As ethyl acetate extract showed very prominent activity, it was attributed to the flavonoids present in the extract. The results of the present study suggests that *Sapindus trifoliatus* seed extracts contain potential antioxidant bioactive compounds and there is prospectus for the commercial utilization.It also shows its great importance as therapeutic agent in preventing or curing the diseases caused due to oxidative damage. Attempts would be made to isolate and characterize phytoconstituents in n-hexane and ethyl acetate extract to ascertain lead compounds

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