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Assessment of bioactive potential of ethyl acetate extracted leaves fraction of *Semecarpus anacardium* (Linn.): A potent indigenous ethno-medicinal plant of Jharkhand, India

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Abstract- *Semecarpus anacardium* (vernacularly called "Bhelwa") has been utilized in various forms by the traditional healers, practitioners and physicians of Indian Systems of Medicine in their clinical practices. The traditional usage mainly involves the application of its nuts in treating rheumatism, diabetes, and urinary diseases as well as cancer of esophagus and leukemia. The current study presents a comprehensive evaluation of *S. anacardium* leaves as an alternative to its nuts, with previously reported remarkable medicinal property but mainly delimited by their toxicity. To validate this, various solvent fractionates of *S. anacardium* leaves were assessed for their preliminary phytochemical profile. The results depicted the ethyl acetate (SA(L) EA) extract as the optimal extract with highest phytochemical abundance. The extract also exhibited notable antimicrobial potential against *Aeromonas hydrophila* (bacterial) and *Fusarium oxysporum* (fungal). The study thus highlights the importance of *S. anacardium* leaves and their antimicrobial applications to contribute to its overall pharmacological and therapeutic potential.

Key words: *Semecarpus anacardium* leaves; ethnomedicinal, bioactive metabolites; antibacterial, antifungal

INTRODUCTION

Semecarpus anacardium is a tropical ethnomedicinal plant commonly known as the 'Marking nut' or the 'Oriental cashew'.[1] It is widely distributed in the tropical, subtropical and central parts of India including Assam, Bihar, Jharkhand, Bengal, Orissa, Madhya Pradesh, Maharashtra, Karnataka, Konkan, Kanara Forests of Tamil Nadu etc.

Each individual plant of *S. anacardium* exists as a medium-sized deciduous tree with large, simple, crowded, alternate, oblong-shaped leaves, with about 30-60 cm long and 12-30 cm wide lamina.[1,2] The leaves appear light to dark green in color with a glabrous and dull surface and are obviate, apiculate or obtuse and rounded at the apex.[3] They are usually stiff, large and leathery in texture. In addition, the leaves also possess 1.2-3.8 cm long petioles.[4]

Pharmacologically, the plant *S. anacardium* forms an essential component of all the three major treatises of traditional indigenous Ayurveda, Siddha and Unani system of medicine practiced in India.[5] The traditional usage of the plant mainly involves the application of its nuts in treating gout, rheumatism, diabetes, urinary diseases, leukemia and esophageal cancer.[5,6] The nuts have been employed either alone or as an ingredient of many polyherbal formulation such as "Bhallatakarasayana," "mrithabhallataki," "Brihatbhallatakalehya," and

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"Kalpaamruthaa" for treating various ailments. Overall, the established potency of *S. anacardium* has helped it acquire the title of a "Wonder drug" or "Panacea" (Universal remedy) in ayurvedic literature.[7] The plant is also a well-known indigenous ethnomedicinal plant of Jharkhand. However, it has been reported as a rare and endangered species in the Chotanagpur plateau of Jharkhand.[8] On ethnomedicinal grounds, *S. anacardium* is considered as a highly nutritious and hardy fruit crop that thrives well under stress conditions.[4] A close survey of the past literature reveals an exceeding bioactivity in the nuts as compared to the other plant parts. However, a major proportion of the nuts is highly toxic for human consumption and thus, need to be completely detoxified before utilization.

Owing to the potential toxicity of *S. anacardium* nuts and the inconvenient conventional detoxification process, leaves of *S. anacardium* were chosen as an alternative in the current study. The studies on *S. anacardium* leaves conducted so far, exhibit a trend of green synthesis of metal nanoparticles with different fractions of its extract. A previous study by Raju *et al.,* (2011)[9] demonstrated the green synthesis of gold NPs using aqueous extract of *S. anacardium* leaves. Subsequent study by the same group of authors reported the green synthesis of silver NPs using the boiled aqueous extract of *S. anacardium* leaves.[10] Another study assessed the antimicrobial and anti-biofilm potential of biocompatible silver NPs synthesized from the aqueous extract of *S. anacardium* leaves, against several human pathogens (both Gram positive and gram negative).[11] On this background, the current study has thus been undertaken to screen the optimal extract of *S. anacardium* leaves based on the preliminary phytochemical screening of its different solvent fractionates and subsequent estimation of the antimicrobial properties of the optimal extract.

MATERIALS & METHODS

Collection and authentication of plant material

Fresh mature leaves of *S. anacardium* (Linn.) were collected from the 'Chatra' forest range in Barkagaon, Hazaribag, Jharkhand (23.8069°N, 85.1687°E) during April 2020 (Fig. 1). The leaves were taxonomically identified and authenticated in the Department of Botany, Vinoba Bhave University, Hazaribag, Jharkhand.

Preparation of plant extract

Collected leaves were washed thoroughly in running water followed by a rinse with sterile distilled water to remove the impurities, microbes or attached soil particles. They were then shade-dried at room temperature for 20 days and coarsely ground in an electrical grinder. The powdered crude extract was sieved using a wire mesh and stored in airtight glass containers at room temperature for further use. Initially, 20 g of the powdered crude extract was defatted with petroleum ether in a Soxhlet extractor. The defatted extract was then re-extracted in 200ml of different solvents of increasing polarity, namely chloroform, ethyl acetate, ethanol, methanol and water at room temperature for 3-4 days. The process was repeated thrice for each solvent. The filtrates were pooled for each solvent and de-solventized in a rotary evaporator (BUCHI rotavapor R-210) at 40-45°C under reduced pressure and *in vacuo*, for distilling off the solvents. The residual fractions were finally recovered as highly concentrated extracts, labelled as SA(L) Chl, SA(L) EA, SA(L) Eth, SA(L) Meth and SA(L) Aq for chloroform, ethyl acetate, ethanol, methanol and aqueous extract respectively. The phyto-extracts were then stored at 4°C until further use for phytochemical investigation.

Determination of extraction yield

The extraction yield was measured by pouring the obtained solvent fractionates in pre-weighed petri-plates. By subtracting the weight of empty petri plates from their weight along with the phytoextracts, the resultant yield of the individual extract was calculated. The calculations were made based on the following formula.[12] :-

$$\text{Yield (\%)} = \frac{X_1}{X_0} \times 100$$

Where, X_1 refers to the weight of extract after evaporation of solvent and X_0 refers to the dry weight of the plant powder before extraction.

Qualitative phytochemical profiling of *S. anacardium* leaves extracts

Preliminary qualitative screening was conducted to identify the occurrence of phytochemical groups in various solvent fractionates of *S. anacardium* leaves. Phyto chemical tests were performed for tannins, saponins, quinones, anthraquinones, alkaloids, flavonoids, phenols, steroids, terpenoids, carbohydrates and proteins. The assessment was carried out using standard protocols described by Adegoke *et al*., (2010)[13].

Determination of antimicrobial activity

Based on the results of the preliminary screening, the best-obtained optimal extract with the highest

phytochemical abundance was proceeded for further evaluation of the antimicrobial activity.

Antibacterial activity

Antibacterial activity against *Aeromonas hydrophila* was performed to suggest the possible usage of the plant extracts for disease management in aquaculture, which is the further extension of the research work. *Aeromonas hydrophila*, is a Gram-negative bacterium and the most encountered fish pathogen. The antibacterial activity was assessed using agar well diffusion method.[14] The bacterial inoculum was prepared with strain *A. hydrophila* 0637P, procured from HiMedia. The bacteria were initially cultured in nutrient broth (NB) overnight in an incubator shaker at 37°C. Subsequent sub culturing was performed and the culture was preserved on nutrient agar slants until the completion of the experiments. Agar plates were prepared in triplicates for each concentration. by adding 0.5 ml of prepared inoculums at the center of sterile petri dish

Fig. 1: Morphology of *Semecarpus anacardium*(A) Tree located in a local forest area (site of collection) (B)Enlarged view of leaves (C) Crude extract of dried plant leaves

followed by pouring molten nutrient agar. The petri plates were shaken carefully to ensure proper mixing of the inoculum into the poured media and were allowed to solidify. Upon solidification, 3 wells of diameter about 6 mm, were made on each agar plate using a sterile cork-borer. The 3 wells represented the positive and the negative control as well as the test sample. A stock of commercially available purified streptomycin, 'Ambistyrin', procured from Abbott Rx, was prepared to be taken as the positive control. Different concentrations such as 1 mg ml^{-1} and 5 mg ml^{-1} of the active extract were tested. Sterile distilled water was chosen as the negative control. Equal volumes of positive and negative control along with the test sample were loaded to their respective wells. The plates were refrigerated for about 2 h to enhance the diffusion of the extracts into the agar. Finally, the plates were subjected to incubation for 48 h at 37°C. Inhibition of bacterial growth was determined by measuring the clear zone formed around the wells, inclusive of the well diameter which became visible after the incubation period. The size of the zone of inhibition (ZOI) was measured using a millimeter ruler, and the antibacterial activity was quantified in terms of average diameter of ZOI in millimeters.

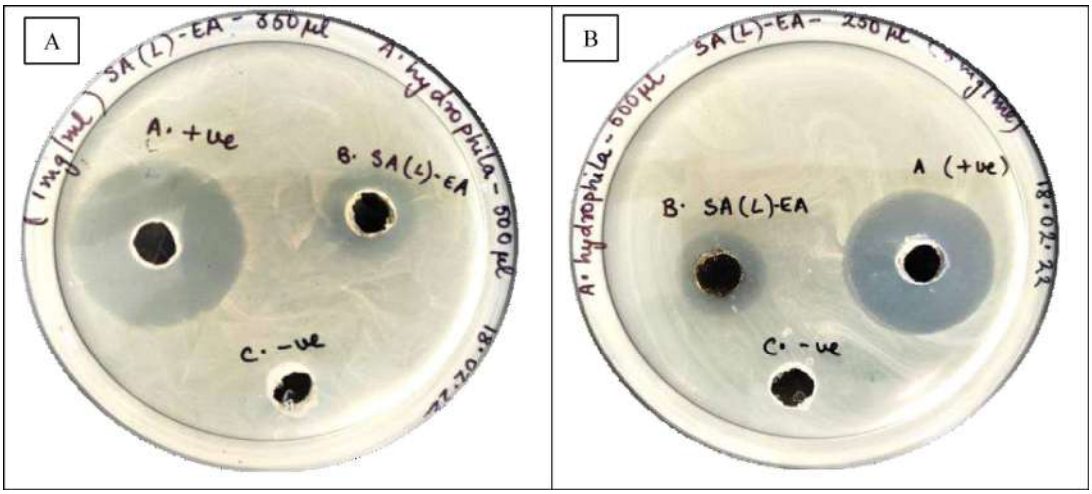

Fig. 2: Shows the zone of inhibition of different concentrations A) 1 mg ml^{-1} B) 5 mg ml^{-1} of SA(L) EA extract against *A. hydrophila*

Fig. 3: Shows the antifungal activity of SA(L) EA with reference to PDA control against *Fusarium oxysporum* at 5 mg/ml concentration

Antifungal activity

The antifungal activity was determined via the poisoned food technique performed using the method described by Falck (1907)[15]. The test pathogen *Fusarium oxysporum* F. (ITCC 4814) was collected from Department of Mycology and Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi.

The plant extract (5 $mgml^{-1}$) was thoroughly mixed with PDA, and its concentration was maintained to desired values. The medium was poured into 90-mm petri plates. A 7^{th} day-old actively growing culture of *Fusarium oxysporium* was carefully cut with the help of the micro tip, aseptically shifted to the center of each petri plate containing the poisoned medium and grown in PDA. The plates without SA(L) extracts were maintained as control. After 10 days of incubation, the radial growth of the mycelia was measured and the percentage of inhibition over control was calculated using the formula:

$$\text{Percentage of growth inhibition} = \frac{\text{Mycelial growth in control} - \text{Mycelial growth in treatment}}{\text{Mycelial growth in control}} \times 100$$

Statistical analysis

All the graphical representations of the observations were prepared and organized using Graph Pad Prism version 9.3.1 (471). The results were all, expressed as mean ± standard error (SE), obtained from three separate observations. The entire data was analyzed by one way analysis of variance (ANOVA) for detection of differences between means. The comparison of means differing significantly was made using Duncan's multiple range test (DMRT) with 5% probability level ($p \leq 0.05$) as the confidence limit. All the statistical analyses were carried out in SPSS version 22.0.

RESULTS & DISCUSSION

The extraction yield varied significantly among the different solvent fractionates of *S. anacardium* leaves (≤ 0.05) (Figure 2). It can be observed that the lowest resultant yield % was achieved for SA(L) PE while the highest was obtained for SA(L) Eth extract. In addition, the yield % was seen to differ non-significantly (≥ 0.05) between the SA(L) Chl and SA(L) Aq fractions, as shown in Table 1. The extraction yield, depicted in terms of the extractive values, determines the number of bioactive constituents available in a given plant extract, that can be extracted using a particular solvent. Appropriate solvent systems are generally employed, either alone or in combination with other solvents, to isolate and extract these diverse molecules.[16] In the present study, the higher alcohol-soluble portion suggests a higher number of polar metabolites in the plant extract indicating ethanol as a better solvent for their extraction than water.[17] A study determining the extractive value for *S. anacardium* nuts reported a lower yield of about 1.46 % for the ethyl acetate residue.[18] Similarly, another study reported an extractive value of 0.25 % for the ethyl acetate fraction of *S. anacardium* bark.[19] In contrast to these, the current study revealed a higher yield (5.43 %) for the ethyl acetate fraction of the leaves, which might be correlated to the differential accumulation of the metabolites in different parts of *S. anacardium*.

Phytochemical investigation

Different phytochemical groups were detected in various solvent fractionates of *S. anacardium* leaves based on change in color or precipitate formation. The ethyl acetate extract SA(L) EA was identified as the best-obtained optimal extract pertaining to the highest abundance of the phytochemical groups in it. The results reveal a significant presence of tannins, saponins, alkaloids, flavonoids, phenols, steroids and terpenoids in SA(L) EA fraction. The non-polar fractions of *S. anacardium* leaves revealed the qualitative absence of distinct groups of secondary metabolites. A pharmacognostic study conducted on *S. anacardium* leaves demonstrated the similar presence of phytochemical groups in different solvent fractionates.[17] The results of preliminary qualitative assessment of the solvent fractionates of *S. anacardium* leaves has been outlined in Table 2.

Determination of antibacterial activity of SA(L) EA against *A. hydrophila*

The plant extract at different concentrations was tested for its effectiveness against a selected strain of *A. hydrophila*, as tabulated in Table 3 and visually presented in Fig. 2. It can be deduced from the findings of the antibacterial assay that SA(L) EA at a concentration of 5 mg ml^{-1} effectively controlled the growth of *A. hydrophila*, with a zone of inhibition of $12.5 \pm 0.29^{***}$ mm (≤ 0.001). The extract at this concentration also showed better efficacy towards pathogen inhibition than its effect at 1 mg ml^{-1}. This reflects a positive correlation of the inhibitory activity with the concentration of the active extract, SA(L) EA. Next, antimicrobial activity in plant extracts are fundamental in inhibiting the growth of pathogenic and spoilage microorganisms for further administration or

supplementation to animal health diets.[20] In similar line of the findings of the current study, ethyl acetate fraction of *S. anacardium* leaves was reported to induce a zone of inhibition of 15 mm against a Gram-negative bacteria *Escherichia coli* in a previous study by Bhatt and Chhetry, (2018)[21]. Besides, a notable work relates the occurrence of amentoflavone in the methanolic extract of stem bark to broad range of antimicrobial activities, particularly antibacterial and antifungal activity, thoroughly supported by *in-silico* evidences.[22] In addition, notable antibacterial activity of n-butanolic extract of *S. anacardium* nuts was also demonstrated against specific strains of Gram-positive and Gram-negative bacteria. The activity was correlated to a novel acyclic isoprenoid isolated from the nuts and

Table 1- Extractive value of different solvent fractions of *S. anacardium* leaves, SA(L)

Solvent fraction	Extraction yield
SA(L) PE	2.41c ± 0.10
SA(L) Chl	6.78b ± 0.24
SA(L) EA	5.43bc ± 0.22
SA(L) Eth	12.61a ± 0.27
SA(L) Meth	7.55b ± 0.29
SA(L) Aq	6.36bc ± 0.22

PE: petroleum ether; Chl: Chloroform; EA: Ethyl acetate; Eth: Ethanol; Meth: Methanol; Aq: Aqueous. Data represented as Mean ± SE, (n = 3). Different superscript letters (a, b, c) within the same column are significantly different (≤ 0.01)

Table2: Phytochemical profile of different solvent fractionates of *Semecarpus anacardium* Leaves

Phyto-chemical tested	PE	Chl	EA	Eth	Meth	Aq	Observation
Tannins	–	–	+	+	+	+	Formation of dark blue or greenishblack color
Saponins	–	–	+	+	+	+	Formation of stable foam
Quinones	–	–	–	–	–	+	Formation of reddish blue or greenprecipitate
Anthra-quinones	–	–	–	–	–	–	Presence of brownish red color layerphase
Alkaloids	–	–	+	+	+	+	Formation of yellow precipitate
Flavonoids	–	–	+++	++	++	+	Formation of yellow colour thatdisappears on addition of dilute acid
Phenols	–	–	++	+	+	–	Formation of bluish green or blackcolour
Steroids and terpenoids	++	++	+	–	–	–	Formation of reddish-brown precipitate
Carbohydrates	–	–	–	++	–	–	Reddish- violet ring formed at the Interface
Proteins	–	–	–	–	–	+	Formation of yellow precipitate

PE: Petroleum ether, Chl: Chloroform; EA: Ethyl acetate; Eth: Ethanol; Meth: Methanol; Aq: Aqueous

Table 3: shows the antibacterial activity of different concentrations of SA(L) EA

Test strain	Concentration	Volumeof extract taken (µl)	Zone of inhibition (mm)		
			SA(L)	Positive control	Negative control
			Ethyl acetate		
A. hydrophila	1 mg/ml	300	13 ± 0.01	23 ± 0.01	6 (N)
		350	12 ± 0.01	20 ± 0.02	6 (N)
	5 mg/ml	200	7 ± 0.02	18 ± 0.01	6 (N)
		250	10 ± 0.01	19 ± 0.01	6 (N)
		300	12 ± 0.01	20 ± 0.02	6 (N)
	100 mg/ml	250	8 ± 0.03	18 ± 0.01	6 (N)
		300	8 ± 0.01	19 ± 0.02	6 (N)
		350	8 ± 0.01	20 ± 0.03	6 (N)

Values are means of triplicate determination (n = 3) ± standard deviations. N= no zone of inhibition

Table 4: Day-wise measurement of radial mycelial growth of *Fusarium oxysporum* in control and SA(L) EA plates

Treatment	Day 3	Day 7	Day 10
Control	27.1± 0.16	61.03 ± 0.38	68.06 ± 0.38
SA(L) EA	16.3± 0.08	32.36 ± 0.25	34.4 ± 0.29

was directly proportional to the concentration of the isoprenoid, evident in the increasing zone of inhibition. The extract showed a remarkable activity at a concentration of 15 µg/mL with the maximum inhibition[23] Corroborating these findings, the antibacterial effect obtained in the present study could also be correlated to the characteristic presence of an important biflavonoid amentoflavone in the optimal extract, SA(L) EA.

Antifungal activity

The mycelial growth in control and treatment plates incorporated with SA(L) EA extract has been tabulated in Table 4. The percentage of growth inhibition of the optimal SA(L) EA extract has been calculated as 49.45%. The extract, SA(L) EA was seen to significantly inhibit the mycelial growth of the test pathogen *Fusarium oxysporum* at a concentration of 5 mg ml^{-1}, post 10 days of incubation. The resultant antifungal activity of SA(L) EA achieved in the present study (Fig. 3) could be attributed to the presence of diverse bioactive metabolites in the extract such as flavonoids, phenolic compounds, phytosterol, saponins, tannins and terpenoids. In a similar line of our finding, substantial antifungal activity against *Candida albicans* was recorded in the methanol extract of SA stem bark.[22]

CONCLUSION

The findings of the present study revealed significant accumulation of the tested phenolics in the active extract, SA(L) EA, which seemed to predominate the other solvent fractionates in terms of quantified phenolics. The extract with its potent antimicrobial activity to important pathogens, holds its potential as a novel pharmaceutical drug. Overall, the results of the present study also portray the inestimable importance of the plant as a native and untapped resource with chemical and pharmacological potential, especially for the pharmaceutical and nutritional sectors.

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