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IN VITRO ANTIBACTERIAL, ANTIOXIDANT, HAEMOLYTIC, THROMBOLYTIC ACTIVITIES AND PHYTOCHEMICAL ANALYSIS OF *SIMAROUBA GLAUCA* LEAVES EXTRACTS

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ABSTRACT:

Objective: To study the leaves of *Simarouba glauca* for their antibacterial, antioxidant, haemolytic, thrombolytic activities and to perform phytochemical evaluation. **Methods:** The three extracts (chloroform, methanol, ethyl acetate) of *Simarouba glauca* were screened for antibacterial activity against five pathogenic microorganisms by well diffusion method. *In vitro* antioxidant activity of extract was studied using H_2O_2 radical scavenging assay. The haemolytic activity was determined using agar diffusion techniques on blood agar plate, thrombolytic activity by clot disruption and phytochemical potential by qualitative analysis. **Results:** Among the different extracts tested, the methanol extract of leaves showed significant antimicrobial activities. The most susceptible micro-organisms were found to be Gram negative bacteria (*Stenotrophomonas maltophilia*, *Citrobacter*), Gram positive bacteria (*Enterococcus faecalis*). H_2O_2 scavenging activity of *Simarouba glauca* was found to increase with increasing concentration of the extract. IC_{50} values of H_2O_2 scavenging activity was 6.72±0.1 µg/mL which was found in chloroform extract. The haemolytic activity was found to be higher in ethyl acetate extract than methanol, chloroform. The chloroform and ethyl acetate extracts shows 23.68 % and 21.60 % clot lytic activity whereas standard streptokinase shows 34.86 % in thrombolytic assay. The phytochemical evaluation indicates the presence of chemical constituents. **Conclusions:** This study shows that all the extract of leaves of *Simarouba glauca* has bioactivity. Further compound isolation is in process to confirm the activities of individual compound.

INTRODUCTION: The World Health Organization estimates that 80% of the world's inhabitants rely mainly on traditional medicines for health care [1].

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Natural products and their derivatives have historically been exploited as a valuable source of novel therapeutic agents [2].

Further, a large proportion of plant based compounds are used as lead molecules in drug discovery to produce synthetic molecular analogs, implying that phytochemicals play a critical role in diversity oriented synthesis of natural product like pharma compounds [3]. *Simarouba glauca* (Family: Simaroubaceae) is a medium-sized tree that grows up to 20 m high, with a trunk 50 to 80 cm in

diameter. It produces bright green leaves 20 to 50 cm in length, small white flowers, and small red fruits. This tree species is a native of Central and South America and found under a wide range of conditions and at low to medium elevation from Southern Florida to Costa Rica, Caribbean islands, Bahamas, Jamaica, Cuba, Hispaniola, Puerto Rico, Nicaragua, Mexico, EI Salvador etc. This plant was introduced in India during 1960 [4].

The main active groups of chemicals in Simarouba are called quassinoids, which belong to the main plant chemicals in *S. glauca*.

The previous study of this plant shows that it has antimicrobial and insecticidal activity [5]. It also has been used as febrifuge, antidysentric, antiherpetic, antihelminthic [6] and antiprotozoal [7] activities, but according to the best of our knowledge there is not any scientific detailed report on antioxidant, haemolytic and thrombolytic activities.

So we have selected the chloroform, methanol and ethyl acetate extract of leaves of *S. glauca* to see the antioxidant, haemolytic, thrombolytic potentials as well as phytochemical constituents.

MATERIALS & METHODS:

Plant material: *S. glauca* leaves were collected from distinct region of Coimbatore. The plant was identified by plant taxonomist, Dr. G. V. S. Murthy, Botanical Survey of India, Southern Regional Centre, Coimbatore.

Preparation of plant material: Fresh leaves were collected and dried at room temperature. Dried leaves were powdered mechanically. Powdered leaves were then packed in Soxhlet apparatus and extraction was done [8].

Chloroform: 60 gm of dry powder was subjected to Soxhlet extraction with 300 mL chloroform, extraction was carried out for 3 hrs, 9 cycles and temperature was maintained at 65°C. Colour of extract was green.

Methanol: 60 gm of dry powder was subjected to Soxhlet extraction with 300 mL methanol, extraction was carried out for 3 hrs, 10 cycles and temperature was maintained at 65°C. Colour of extract was dark green.

Ethyl acetate: 60 gm of dry powder was subjected to Soxhlet extraction with 300 mL ethyl acetate, extraction was carried out for 3hrs, 9 cycles and temperature was maintained at 65°C. Colour of extract was dark green. The extracts were then kept in sterile bottles, under refrigerated conditions, until further use. The dry weight of the plant extracts was obtained by the solvent evaporation and used to determine concentration in mg/mL.

Preliminary phytochemical screening: The extracts were subjected to preliminary phytochemical testing to detect for the presence of different chemical groups of compounds. *S. glauca* leaves extract were screened for the presence of alkaloids, flavonoids, carbohydrates, glycosides, phenolic compound, tannins, triterpenoids, cardinolides, saponins, fixed oils as described in literatures [9, 10, 11].

Microbial cultures: *Stenotrophomonas maltophilia, Klebsiella pneumoniae, Enterococcus faecalis, Citrobacter* and *Proteus mirabilis* were chosen based on their clinical and pharmacological importance [9]. All microbial cultures were obtained from Plant Biotechnology Laboratory, Department of Biotechnology, University College of Engineering – BIT campus, Trichy. The bacterial cultures were incubated for 24 hours at 37°C on nutrient agar. The bacterial strains were grown in Mueller-Hinton agar (MHA) plates at 37°C (the bacteria were grown in the nutrient broth at 37°C and maintained on nutrient agar slants at 4°C). The stock cultures were maintained at 4°C.

Antibacterial activity: *In vitro* antibacterial activities were examined for chloroform, methanol and ethyl acetate extracts. Antibacterial activities of plant leaf extracts against five pathogenic bacteria (one Gram positive and four Gram negative) were investigated by the well diffusion methods. Agar plates were inoculated with 100 µL of standardized inoculums (1.5×10^8 CFU/mL) of each selected bacterium (in triplicates) and spread with sterile swabs. Wells of 6 mm size were made with sterile borer into agar plates containing the bacterial inoculums and the lower portion was sealed with a little molten agar medium. The sets of three dilutions (10, 25 and 50 µg/mL) of plant leaves extracts (chloroform, methanol and ethyl acetate solvent) were poured into a different well of inoculated plates.

Control experiments were carried out under similar condition by using cefotaxime as a standard drug. Chloroform, methanol and ethyl acetate were used as a negative control which was introduced into a well instead of plant extract. The zones of growth inhibition around the well were measured after 18 to 24 hours of incubation at 37°C. The sensitivities of the microorganism species to the plant extracts were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks, and values <8 mm were considered as not active against microorganisms.

Scavenging of H_2O_2 radicals: The ability of the extracts to scavenge H_2O_2 was determined according to the reported method [12]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extract of selected concentrations (2-10 µg/ mL) in distilled water were added to H_2O_2 solution (0.6 mL, 40 mM). The absorbance of H_2O_2 at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without H_2O_2. Ascorbic acid was used as a standard antioxidant. The % of H_2O_2 scavenging of both extracts and standard compounds were calculated. % scavenged $[H_2O_2] = [(A_o - A_1)/A_o] \times 100$ where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract. The antioxidant activity of the extract was expressed in IC_{50} values.

Haemolytic assay: The haemolytic activity of the extract was determined using agar diffusion technique on blood agar plate [13]. Blood agar was prepared and well measuring 5 mm were made on the agar using cork borer. The wells were filled with 20 µL of different concentration of plant extracts solution. The plates were then incubated at 37 ºC for 5 hours.

Thrombolytic assay: Whole blood (6 mL) was collected from the healthy volunteers without a history of oral contraceptive or anticoagulant therapy. For each treatment six tubes were taken and experiment was repeated thrice**.** Blood sample (1 mL) was distributed in pre weighed sterile micro centrifuge tubes and incubated at 37 ºC for 90 mins for clot formation. After clot formation, the serum was completely aspirated without disturbing the clot and the tubes were again weighed to determine the clot weight (clot weight = weight of the tube containing clot – weight of the empty tube). To the each Eppendorf tube containing pre weighed clot, 20 µL, 40 µL, 60 µL, 80 µL and 100 µL of chloroform, methanol and ethyl acetate extract were added. 50 µL of sterile distilled water and streptokinase was used as a negative and positive control. All the tubes were incubated at 37 ºC for 18 hrs and observed for clot lysis. The fluid obtained after the incubation was removed carefully and the tubes were weighed again to observe the difference in weight after clot disruption. Difference in the weight taken before and after clot lysis was expressed as percentage of clot lysis.

RESULTS:

Preliminary phytochemical investigation: The preliminary phytochemical investigation of the methanolic extract of *S. glauca* showed that it contains alkaloids, flavonoids, carbohydrates, glycosides, phenolic compound, tannins, terpenoids, cardinolides, saponins, fixed oils. Tannins were not present in ethyl acetate extract. Terpenoids and saponins were not detected in methanol extract. Flavonoids, carbohydrates, phenolic compound, fixed oils were present in chloroform extract remains were not detected (**Table 1**).

TABLE 1: PHYTOCHEMICALS SCREENING OF DIFFERENT EXTRACTS OF LEAVES OF *S. GLAUCA*

Phytochemicals	Test performed	Extracts		
		Chloroform	Methanol	Ethyl acetate
Alkaloids	Mayer's test	-	+	+
Flavonoids	Alkaline reagent test	+	+	+
Carbohydrates	Molisch's test, Fehling's test and Benedict's test	+	+	+
Glycosides	Borntragar's test	-	+	+
Phenolic compounds	$Fecl_3$ test, lead acetate test	+	+	+
Tannins	$Fecl_3$ test	-	+	-
Terpenoids	Salkowski test	-	-	+
Cardinolides	$Fecl_3$ test	-	+	+
Saponins	Foam test	-	-	+
Fixed oils	Spot test	+	+	+

Antibacterial activity: Antibacterial activity of three different concentrations of extracts of *S. glauca* has been evaluated *in vitro* against gram positive and negative bacteria that are known to cause infections in humans and plants. The extracts showed varying degrees of antimicrobial activity against tested microorganisms, values which are presented in Table 2. Methanol extracts exhibited higher degrees of antibacterial activity than the other extracts. Among the three extract, ethyl acetate extracts showed least inhibition of growth of microorganisms. The inhibitory effects of the extracts were compared with the standard antibiotics such as cefotaxime.

TABLE 2: ANTIBACTERIAL ACTIVITY OF LEAVES EXTRACTS OF *S. GLAUCA*

Microorganism	Zone of inhibition (mm)											
	Chloroform (µg/mL)				Methanol (µg/mL)				Ethyl acetate (µg/mL)			
	10	25	50	+ve	10	25	50	+ve	10	25	50	+ve
Bacteria												
Gram Negative												
Stenotrophomonas maltophilia	8±0.2	9±0.5	9±0.3	21±0.3	15±0.7	19±0.1	25±0.3	27±0.7	NA	9±0.2	11±0.5	32±0.6
Klebsiella pneumonia	11±0.2	11±0.3	12±0.2	24±0.1	12±0.1	14±0.5	15±0.3	26±0.2	10±0.1	11±0.6	13±0.0	30±0.3
Citrobacter	10±0.2	11±0.1	13±0.3	24±0.5	13±0.2	16±0.3	22±0.6	26±0.0	9±0.3	11±0.1	12±0.0	33±0.1
Proteus mirabilis	18±0.5	10±0.5	12±0.3	23±0.0	11±0.3	13±0.0	17±0.1	25±0.5	11±0.7	13±0.1	12±0.2	33±0.0
Gram Positive												
Enterococcus faecalis	10±0.3	12±0.1	11±0.7	24±0.5	10±0.6	15±0.2	17±0.6	26±0.1	12±0.0	11±0.0	11±0.3	25±0.1

Results represented as means ± standard deviation (n = 3); +ve control: cefotaxime (µg/ml); NA: No activity

\Scavenging of H_2O_2: Five concentrations ranging from 20 to 100 µg/ml of the chloroform, methanol and ethyl acetate extracts of *S. glauca* were tested for their antioxidant potential using H_2O_2. It was observed that free radicals were scavenged by test compounds at different concentrations. The maximum inhibitory concentrations (IC_{50}) were found to be 6.72±0.1, 4.46±0.2 and 4.85±0.3 µg/mL, respectively. The percentage scavenging of each concentration of extracts was shown in **Table 3**.

TABLE 3: ANTIOXIDANT ACTIVITY OF *S. GLAUCA* LEAF EXTRACT

Percentage of H_2O_2 scavenging activity			
Concentration (µg/mL)	**Chloroform**	**Methanol**	**Ethyl acetate**
20	2.1±0.2	8.45±0.3	2.45±0.2
40	7.8±0.4	18.98±0.2	8.85±0.2
60	14.7±0.2	26.69±0.4	19.6±0.2
80	23.5±0.2	49.06±0.2	47.5±0.4
100	38.2±0.3	55.7±0.2	49.18±0.2
IC_{50} µg/mL	6.72±0.1	4.46±0.2	4.85±0.3

Results represented as means ± standard deviation (n = 3).

Haemolytic activity: **Table 4** explains the haemolytic activity of the *S. glauca* leaf extract in various concentrations. The zone of haemolysis was directly proportional to concentration of the extract. Ethyl acetate extract of *S. glauca* showed moderate haemolytic activity than methanol and chloroform. The activity of the extract to lyse the blood cell can be linked with the antimicrobial factors like flavonoids and phenolic compound which has been distributed in *S. glauca*.

TABLE 4: ZONES OF HAEMOLYSIS (mm) OF *S. GLAUCA* LEAF EXTRACT AT DIFFERENT CONCENTRATION

Extract	Zones of haemolysis (mm)		
	Concentration of crude extract (mg/mL)		
	25	**50**	**100**
Chloroform	6±0.2	6±0.1	6±0.3
Methanol	5±0.1	6±0.2	6±0.2
Ethyl acetate	6±0.1	8±0.01	7±0.9

Results represented as means ± standard deviation (n = 3).

Thrombolytic activity: **Table 5** show the haemolytic activity of the extract investigated by measuring the lysis of a human red blood cells suspension in a spectrophotometric assay. Chloroform, methanol and ethyl acetate extracts showed 23.68%, 13.88% and 21.60% clot lysis respectively.

TABLE 5: EFFECT OF *S. GLAUCA* LEAF EXTRACTS ON *IN VITRO* CLOT LYSIS

Extract	% of clot lysis				
	Concentration of crude extract (µg/mL)				
	20	40	60	80	100
Chloroform	2.9±0.4	8.6±0.2	11.0±0.1	16.6±0.4	23.7±0.2
Methanol	2.7±0.1	8.1±0.1	9.5±0.2	10.8±0.4	13.9±0.4
Ethyl acetate	2.9±0.2	8.3±0.4	11.0±0.2	18.9±0.2	21.6±0.6

DISCUSSION: In recent years, the search for phytochemicals possessing antioxidant, anticancer and antimicrobial activities have been on the rise due to their potential use in the therapy of various chronic and infectious diseases.

In the present work, the extracts obtained from *S. glauca* show strong activity against most of the tested bacterial strains. The results were compared with standard antibiotic drugs. In this screening work, extracts of *S. glauca* were found to be active against Gram-positive, Gram-negative strains.

The ability of *S. glauca* leaves extract to scavenge H_2O_2 was determined according to the method of Ruch *et al*. The *S. glauca* chloroform extracts were capable of scavenging H_2O_2 in a concentration dependent manner. IC_{50} for scavenging of H_2O_2 were 6.72±0.1 µg/mL for chloroform extract, 4.46±0.2 µg/mL for methanol extract and 4.85±0.3 µg/mL for ethyl acetate respectively. The IC_{50} values for ascorbic acids were 9.4±0.2 µg/mL. The effectiveness of the leaves might be due to the hydroxyl groups existing in the phenolic compounds chemical structure [14] that can provide the necessary component as a radical scavenger. A potent scavenger of free radicals may serve as a possible preventive intervention for the diseases [15].

The result of phytochemicals in the present investigation showed that the plant contains more or less same components like alkaloids, flavonoids, carbohydrates, glycosides, phenolic compound, tannins, terpenoids, cardinolides, saponins, fixed oils. Many plants used in traditional medicine worldwide contain saponins, which can often account for their therapeutic action including antibacterial, antiviral, anti-inflammatory, antiprotozoal and antitumor activities [16]. However, the most characteristic property of saponins is their ability to cause haemolysis. When added to a suspension of blood, they produce changes in erythrocyte membranes causing haemoglobin diffusion into surrounding medium.

In the present study, ethyl acetate solvent extract of *S. glauca* showed highest haemolytic activity. There are several thrombolytic drugs obtained from various sources. Some are modified further with the use of recombinant technology in order to make these thrombolytic drugs more site specific and effective [17]. Side effects related to these drugs have been reported that lead to further complications [18]. Sometimes the patients die due to bleeding and embolism [19-22].

In our study, the *in vitro* thrombolytic activity results revealed that chloroform, methanol and ethyl acetate extracts showed 23.68%, 13.88%, and 21.60% clot lysis respectively for 100 mg/mL and compared with the negative control (methanol, cyclohexane and chloroform solvent).

CONCLUSION: The present study revealed that the *S. glauca* leaf poses good antimicrobial and antioxidant, haemolytic and thrombolytic activities and the work is still under progress to explore the chemical nature of the active constituents and other pharmacological investigations are also under evaluation.

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