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Evaluation of antioxidant, membrane stabilizing, antimicrobial, cytotoxic and thrombolytic properties of the crude extracts of *Stixis suaveolens* growing in Bangladesh

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ABSTRACT

The current study was conducted to investigate anti-oxidative, membrane stabilizing, antimicrobial, cytotoxic and thrombolytic properties of Methanol, Aqueous, Pet-Ether, Dichloromethane, and Ethyl Acetate soluble fractions of *Stixis suaveolens*. To determine the antioxidant activity, free radical scavenging activities (antioxidant capacity) of the plant extracts on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and total phenolic content was determined. Ascorbic Acid & BHT were used as standards in this studies. To evaluate cytotoxicity, the brine shrimp lethality bioassay was used.Vincristine sulfate (VS) was used as positive control. Antithrombolytic ability of the plant extracts were determined on bloods drawn from healthy volunteers. Membrane stabilizing potential were assessed by evaluating their ability to inhibit haemolysis of human erythrocytes. Antimicrobial investigation by disc diffusion method revealed no anti-microbial potential. The amount of total phenolic content differed in different extractives and ranged from 4.9375mg of GAE/gm of extractives to 70.125mg of GAE/gm of extractives of Fruits of *S. suaveolens* Roxb. The antioxidant activity of IC_{50} values in DPPH method are differed in different extractives and ranged from (75.67µg/ml) to (5634.00µg/ml). Methanol Soluble Fraction (MESFF) of Fruits of *S. suaveolens* Roxb. exhibited highest thrombolytic activity of 26.85%. Furthermore, the Aqueous Soluble Fraction (AQSF) inhibited 11.06%, Ethyl Acetate Soluble Fraction (EASF) inhibited 9.16%, Methanol Soluble Fraction (MESF) inhibited 8.84%, Dichloromethane Soluble Fraction (DCMSF) inhibited 7.92%, and Pet-Ether Soluble Fraction (PESF) inhibited 5.86% of hemolysis of RBC. The highest brine shrimp lethality was given by EASF with 0.99µg/ml followed by PESFwith 1.10µg/ml, DCMSFwith 4.77µg/ml, MESF with 8.37µg/ml and AQSFwith 24.26µg/ml. Hence, that *S. suaveolens* extracts and its fractions possess anti-oxidant, membrane stabilizing, cytotoxic and thrombolytic properties.

Keywords: antioxidant, membrane stabilizing, antimicrobial, cytotoxic and thrombolytic.

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INTRODUCTION

Plants have been used for thousands of years in many countries of the world that have formed the basis of traditional medicinal systems. The plant is a biosynthetic laboratory and the remedial phyto-elements produced through a cascade of biochemical reactions inside a plant significantly contribute to the traditional and modern medicines.[1] Use of therapeutics derived from plants have been increased tremendously with more than 80% of medicines are plant-derived. Although plant derived medicines are promising as remedial agents, most of the plants are not tested for their potential as therapeutic agents.[2] Therefore, scientific evaluation of the biological activities of medicinal plants could help to justify their use in health care system.[3]

Stixis suaveolens Roxb is a flowering plants in the family Capparaceae. It is distributed in the tropical and sub-tropical regions of native to southeastern Asia and the Indian subcontinent. Some species are widely cultivated and naturalized in other tropical and subtropical regions. It is viewed as invasive in Queensland, Cuba, Costa Rica and many of the Pacific Islands. It is also widely available in different area of Bangladesh. It has been estimated that this plant contains a number of biologically active compounds which may be precisely valuable for various medical purposes.[4] The present study was aimed to study antioxidant, membrane stabilizing, antimicrobial, cytotoxic and thrombolytic potential of the crude extracts of *Stixis suaveolens* and it's fractionate.

METHODOLOGY

Material Collection and Preparation: The Fruits of *S. suaveolens* were collected from market. The leaves were sun dried for several days and then oven dried for 24 hours at considerably low temperature and powdered using high capacity grinding machine

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in the Phytochemical Research Laboratory, State University of Bangladesh. The powdered material (300gm) was taken in a cleaned, ambered color reagent bottle (2.5 liters) and soaked in 2.0 L of methanol. The container with its content was sealed by bottle cap and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixtures were then filtered through a fresh cotton plug and finally with a WhatmanNo.1 filter paper. The volume of the filtrate was then allowed to evaporate at ambient temperature until approximately 70% solvent was evaporated. Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen *et al* 1993.

Antimicrobial activity: The antimicrobial activity of methanolic crude extract of *S. suaveolens* and its Kupchan fractions were investigated by disc diffusion method[5] using standard ciprofloxacin (30 µg/disc) as references.

Antioxidant Activity

Total phenolics analysis: The antioxidant effect of phytochemicals is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes.[6,7,8] Total phenolic content of *S. suaveolens* extractives was measured by employing published method by Skerget et al., 2005 involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as standard.[7,8]

Determination of free radical scavenging activity: The antioxidant activity (free radical scavenging activity) of the methanolic crude extract of *S. suaveolens* and its Kupchan fractions on the stable radical 1,1- diphenyl-2-picrylhydrazyl (DPPH) was determined by the method developed by Brand-Williams et al., 1995.[9]

Determination of Thrombolytic activity: Aliquots (5 ml) of venous blood were drawn from healthy volunteers who were distributed in ten different pre weighed sterile eppendorf tubes (0.5 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each eppendorf tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each eppendorf tube containing pre-weighed clot, 100 µl aqueous solutions of different partitionates along with the crude extracts was added separately.[10,11] As a positive control, 100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control eppendorf tubes. All the eppendorf tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released of fluid was removed and eppendorf tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

% clot lysis = (Weight of the lysis clot / Weight of clot before lysis) × 100

Determination of membrane stabilizing activity: The membrane stabilizing activity of the crude ethanolic and its soluble partitionates was assessed by evaluating their ability to inhibit hypotonic solution and heat-induced haemolysis of human erythrocytes following the method developed by Sikder et al., 2012. Whole blood was collected from male human under standard condition. EDTA was used to prevent clotting. The blood was washed three times with isotonic solution (154 mMNaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifuge action for 10 min at 3000 g. Thus the suspension finally collected was the stock erythrocyte (RBC) suspension.[12,13]

i)Hypotonic Solution- Induced Hemolysis: The experiments were carried out with hypotonic solution. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) with 4.5 ml of hypotonic solution (50 mMNaCl) in 10 mM sodium phosphate buffer saline (pH 7.4) containing either the different methanolic extract (2.0 mg/mL) or Acetyl Salicylic Acid (0.10 mg/mL). The Acetyl Salicylic Acid was used as a reference standard. The mixtures were incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance (O.D.) of the supernated was measured at 540 nm.

The percentage inhibition of either hemolysis or membrane stabilization was calculated using the following equation:

% inhibition of hemolysis = 100 × {(OD1-OD2)/ OD1}

Where,

OD1 = Optical density of hypotonic-buffered saline solution alone (control) and

OD2 = Optical density of test sample in hypotonic solution.

ii) ***Heat- induced hemolysis:*** Aliquots (5 ml) of the isotonic buffer containing 1.0 mg/mL of different extractives of plants were put into two duplicate sets of centrifuge tubes.[13,14] The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 µL) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54°C for 20 min in a water bath. The other pair was maintained at 0-5°C in an ice bath. The reaction mixture was centrifuged for

3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition or acceleration of hemolysis in tests andwas calculated according to the equation:

$$\% \text{ Inhibition of hemolysis} = 100 \times [1- (OD_2\text{-}OD_1/ OD_3\text{-}OD_1)]$$

Where,

OD_1 = test sample unheated, OD_2 = test sample heated and

OD_3 = control sample heated

Determination of cytotoxic activity: It was determined by performing brine shrimp lethality bioassay following the procedure of Meyer et al.[15,16] The lethality of the extractives to brine shrimp was determined & the results are given in Table 8.3. The lethal concentration (LC_{50}) of the test samples after 24 hours was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. Vincristine sulfate (VS) was used as positive control.

RESULT AND DISCUSSION

The present study was designed to evaluate the antimicrobial, antioxidant, thrombolytic, membrane stabilizing, cytotoxic properties of *S. suaveolens* and the results have been summarized in tables 1-5.

Table 1 **Fractions obtained from Fruits of *S. suaveolens* Roxb. Extract**

Plant Part	Sample Code	Fraction
Fruits of *S. suaveolens* Roxb.	MESF	Methanol Soluble Fraction
	AQSF	Aqueous Soluble Fraction
	PESF	Pet-Ether Soluble Fraction
	DCMSF	Dichloromethane Soluble Fraction
	EASF	Ethyl Acetate Soluble Fraction

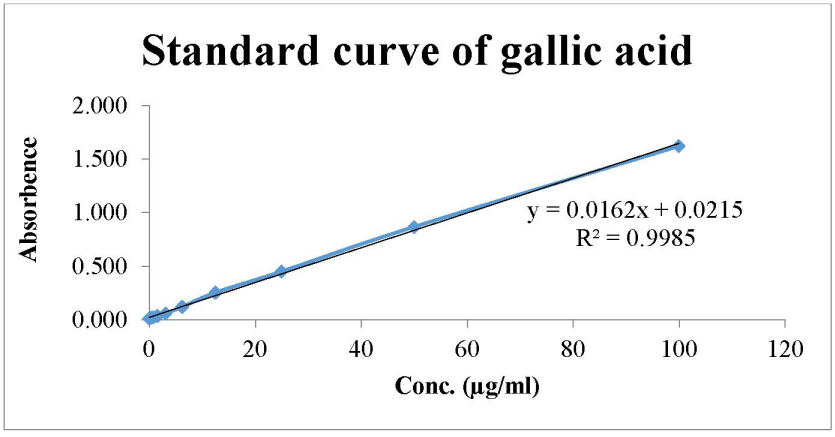

Figure 1 Standard curve of gallic acid for total phenolic determination

In the present study the crude extracts as well as fractions were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required.[5,7] The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The diameter of zone of inhibition expressed in millimeter is then measured to determine antimicrobial activity of the test agent.[5,8] Study of antimicrobial activity al the fraction exhibited no antimicrobial activity against the listed microorganisms as compared to Ciprofloxacin. The results are given in the Table 2

The free radical scavenging activities (antioxidant capacity) of the plant extracts on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method of Brand-Williams *et al.* (1995). Total phenolic content of Fruits of *S. suaveolens* Roxb. extract was measured employing the method as described by Skerget *et al.* involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard.[15,16]

The amount of total phenolic content differed in Total phenolic content of the samples are expressed as mg of GAE (Gallic acid equivalent)/ gm of extractives. The amount of total phenolic content differed in different extractives and ranged from 4.9375mg of GAE/gm of extractives to 70.125mg of GAE/gm of extractives of Fruits of *S. suaveolens* Roxb. Among all extractives of Fruits of *S. suaveolens* Roxb. The highest phenolic content was found in DCMSF (70.13mg of GAE/gm of extractives) followed by EASF (53.75mg of GAE/gm of extractives), MESF (46.44mg of GAE/gm of extractives), PESF (37.44mg of GAE/gm of extractives) and AQSF (4.94mg of GAE/gm of extractives). The antioxidant activity of IC_{50} values in DPPH method are differed in different extractives and ranged from (75.67µg/ml) to (5634.00µg/ml). Among all extractives of *S. suaveolens* Roxb. the highest free radical scavenging activity was given by DCMSF (75.67) followed by EASF (120.66µg/ml), MESF (1065.88µg/ml), PESF (2613.54µg/ml) and AQSF (5634.00µg/ml) as compared to Ascorbic Acid & BHT was 3.05µg/ml &16.44µg/ml.

The different methanolic extracts of Fruits of *S. suaveolens* Roxb. at concentration 2.0 mg/mL significantly protected the lysis of human erythrocyte membrane induced by hypotonic solution, as compared to the standard acetyl salicylic acid (0.10 mg/mL) (Table 5). The methanolic extract and its different fractionates of Fruits of *S. suaveolens* Roxb. were effective in the membrane

Table 2 **Antimicrobial activity of test samples of Fruits of *S. suaveolens* Roxb**

	Diameter of Zone of Inhibition (mm)					
Test Microorganisms	**MESF**	**PESF**	**DCMSF**	**EASF**	**AQSF**	**Ciprofloxacin**
			Gram positive bacteria			
Bacillus sereus	0	0	0	0	0	40
Bacillus megaterium	0	0	0	0	0	40
Bacillus subtilis	0	0	0	0	0	38
Staphylococcus aureus	0	0	0	0	0	41
Sarcinalutea	0	0	0	0	0	40
			Gram negative bacteria			
Escherichia coli	0	0	0	0	0	45
Pseudomonas aureus	0	0	0	0	0	41
Salmonella paratyphi	0	0	0	0	0	41
Salmonella typhi	0	0	0	0	0	38
Shigellaboydii	0	0	0	0	0	37
Shigelladysenteriae	0	0	0	0	0	38
Vibrio mimicus	0	0	0	0	0	50
Vibrio parahemolyticus	0	0	0	0	0	50
			Fungi			
Aspergillus niger	0	0	0	0	0	45
Candida albicans	0	0	0	0	0	30
Sacharomycescerevacae	0	0	0	0	0	40

Table 3 **Standard curve preparation by using gallic acid**

Si. No:	Conc. Of the Standard (µg / ml)	Absorbance	Regression line	R^2
1	100	1.620		
2	50	0.866		
3	25	0.450		
4	12.5	0.253		
5	6.25	0.120	y = 0.0162x + 0.0215	0.9985
6	3.125	0.059		
7	1.5625	0.034		
8	0.78125	0.022		
9	0.3906	0.020		
10	0	0.011		

Table 4 **IC_{50} values of the standard and partitionates of Fruits of *S. suaveolens* Roxb**

Plant part	Sample Code	(IC_{50}µg/ml)	Test Sample
Fruits of *S. suaveolens* Roxb.	BHT	16.44	Tert-butyl-1-hydroxytoluene (STD.)
	AA	3.05	Ascorbic Acid (STD.)
	MESF	1065.88	Methanol Soluble Fraction
	AQSF	5634.00	Aqueous Soluble Fraction
	PESF	2613.54	Pet-Ether Soluble Fraction
	DCMSF	75.67	Dichloromethane Soluble Fraction
	EASF	120.66	Ethyl Acetate Soluble Fraction

Table 5 **Effect of different extractives of Fruits of *S. suaveolens* Roxb. on hypotonic solution-induced hemolysis of erythrocyte membrane**

Sample code		Concentration	
Hypotonic medium	Absorbance	50 mM	% inhibition of hemolysis
MESF	1.681	2 mg/mL	8.84
AQSF	1.640	2 mg/mL	11.06
PESF	1.736	2 mg/mL	5.86
DCMSF	1.698	2 mg/mL	7.92
EASF	1.675	2 mg/mL	9.16
ASA	0.703	0.10 mg/mL	61.90

Table 6 **Effect of different extractives of Fruits of *S. suaveolens* Roxb. on heat induced hemolysis of erythrocyte membrane**

	Absorbance			
Sample code	Heat	Cold	Concentration	% inhibition of hemolysis
MESF	1.758	0.494	2 mg/mL	21.30
AQSF	1.671	0.075	2 mg/mL	21.19
PESF	1.702	0.605	2 mg/mL	26.62
DCMSF	1.786	1.661	2 mg/mL	71.53
EASF	1.264	0.130	2 mg/mL	42.44
ASA	1.428	0.500	0.10 mg/mL	42.00
Control	2.100			

Table 7 **Thrombolytic Activity (in terms of % of clot lysis) of the extractives of Fruits of *S.suaveolens* Roxb**

Fractions name	Weight of empty eppendorf tube W_1 mg	Weight of clot containing eppendorf tube before clot disruption W_2 mg	Weight of clot containing eppendorf tube after clot disruption W_3 mg	Weight of clot before lysis ($W_4=W_2-W_1$) mg	Weight of lysis clot ($W_5=W_2-W_3$) mg	% of clot lysis (w5/w4)x100
Methanol extract and different fractions						
MESF	4761.4	4946.9	4897.1	185.5	49.8	26.85
AQSF	4729.2	4897	4870.6	167.8	26.4	15.73
PESF	4619.8	4889.2	4871	269.4	18.2	6.76
DCMSF	4798.6	5050.4	5029.4	251.8	21	8.34
EASF	4824.4	5014.1	4978.4	189.7	35.7	18.82
Blank (water) and Streptokinase						
Blank	4715.1	5002.4	4998.9	287.3	3.5	1.22
SK	4836.7	5324.8	5004.5	488.1	320.3	65.62

stabilizing activity as the extractives prevented the lysis of erythrocytes induced by hypotonic solution. The Aqueous Soluble Fraction (AQSF) inhibited 11.06%, Ethyl Acetate Soluble Fraction (EASF) inhibited 9.16%, Methanol Soluble Fraction (MESF) inhibited 8.84%, Dichloromethane Soluble Fraction (DCMSF) inhibited 7.92%, and Pet-Ether Soluble Fraction (PESF) inhibited 5.86% of hemolysis of RBC. For membrane stabilizing activity Acetyl Salicylic Acid was used as standard drug that exhibited 61.90% inhibition of hemolysis at normal condition.

The different methanolic extracts of Fruits of *S. suaveolens* Roxb. at concentration 2.0 mg/mL significantly protected the lysis of human erythrocyte membrane induced by heat induced, as compared to the standard acetyl salicylic acid (0.10 mg/mL) (Table 6). The methanolic

Table 8 LC_{50} values of the test samples of *S. suaveolens* Roxb

Test samples	Regression line	R^2	Log x	LC_{50}
VS	y = 30.8x + 60.64	R^2 = 0.972	-0.345	0.451
MESF	y = 40.064x + 13.023	R^2 = 0.8949	0.923	8.37
AQSF	y = 44.695x - 11.754	R^2 = 0.9084	1.385	24.26
PESF	y = 20.737x + 49.132	R^2 = 0.5314	0.042	1.10
DCMSF	y = 38.655x + 23.781	R^2 = 0.7273	0.678	4.77
EASF	y = 26.374x + 50.1	R^2 = 0.5903	-0.004	0.99

Here, VS = Vincristine sulphate, MESF= Methanol Soluble Fraction, AQSF= Aqueous Soluble Fraction, PESF= Pet-Ether Soluble Fraction, DCMSF= Dichloromethane Soluble Fraction, EASF= Ethyl Acetate Soluble Fraction.

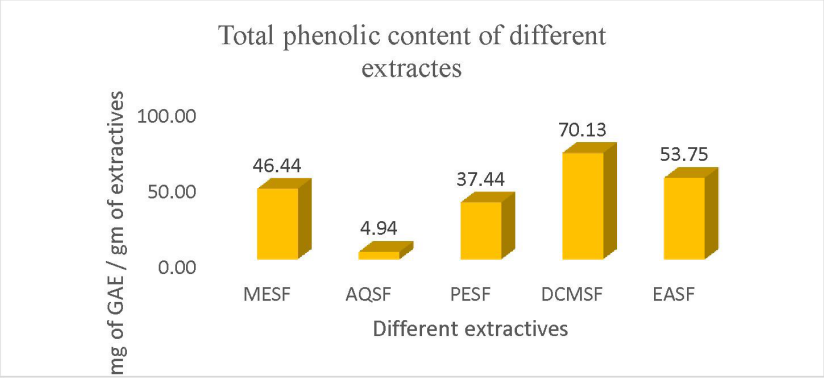

Figure 2 Total phenolic content (mg of GAE / gm of extractives) of different extractives of Fruits of *S. suaveolens* Roxb

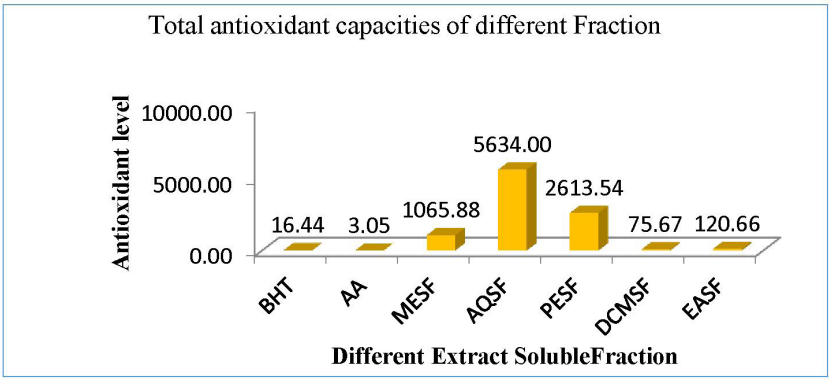

Figure 3 IC_{50} values of the standard and partitionates of Fruits of *S. suaveolens* Roxb

extract and its different fractionates of Fruits of *S. suaveolens* Roxb. were effective in the membrane stabilizing activity as the extractives prevented the lysis of erythrocytes induced by heat. Dichloromethane Soluble Fraction (DCMSF) inhibited 71.53%, Ethyl Acetate Soluble Fraction (EASF) inhibited 42.44%, Pet-Ether Soluble Fraction (PESF) inhibited 26.62%, Methanol Soluble Fraction (MESF) inhibited 21.30%, and Aqueous Soluble Fraction (AQSF) inhibited 21.19% of hemolysis of RBC. For membrane stabilizing activity Acetyl Salicylic Acid was used as standard drug that exhibited 42.00% inhibition of hemolysis at normal condition.

The results showed that the extracts were potent on human erythrocyte adequately protecting it against hypotonic induced and heat induced lysis compared to that of standard anti-inflammatory drug (Acetyl Salicylic Acid).

As a part of discovery of cardio protective drugs from natural sources of the methanol extracts and their different organic soluble fractions i.e. Methanol Soluble Fraction (MESF), Aqueous Soluble Fraction (AQSF), Pet-Ether Soluble Fraction (PESF), Dichloromethane Soluble Fraction (DCMSF) and Ethyl Acetate Soluble Fraction (EASF) of Fruits of *S. suaveolens* Roxb. were assessed for thrombolytic activity and the results are presented in Table 7.2. Addition of 100µl SK, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37°C, showed 65.62% lysis of clot.[18] On the other hand, distilled water was treated as negative control which exhibited negligible percentages of lysis of clot 1.22%. The mean difference in clot lysis percentages between positive and negative control was found statistically very significant. In this study the Methanol Soluble Fraction (MESFF) of Fruits of *S. suaveolens* Roxb. exhibited highest thrombolytic activity 26.85% followed by Ethyl Acetate Soluble Fraction (EASF) 18.82%, Aqueous Soluble Fraction (AQSF) 15.73%, Dichloromethane Soluble Fraction (DCMSF) 8.34% and Pet-Ether Soluble Fraction (PESF) 6.76% exhibited thrombolytic activity.

From this experiment, it can be concluded that the extractives of Fruits of *S. suaveolens* Roxb. showed medium clot lysis activity than the standard substance streptokinase (SK) (65.62%).

In the present bioactivity study all the crude extracts showed positive results indicating that the test samples are biologically active. Each of the test samples showed different mortality rates at different concentrations. Plotting of log of concentration versus percent mortality for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the samples.

The methanol extracts of Fruits of *S. suaveolens* Roxb. and its different partitionates i.e. Methanol Soluble Fraction, Aqueous Soluble Fraction, Pet-Ether Soluble Fraction, Dichloromethane Soluble Fraction, and Ethyl Acetate Soluble Fraction were tested for The lethality of the extractives to brine shrimp was determined & the results are given in Table 8. The lethal concentration (LC_{50}) of the test samples after 24 hours was obtained by a plot of percentage of the shrimps died against the

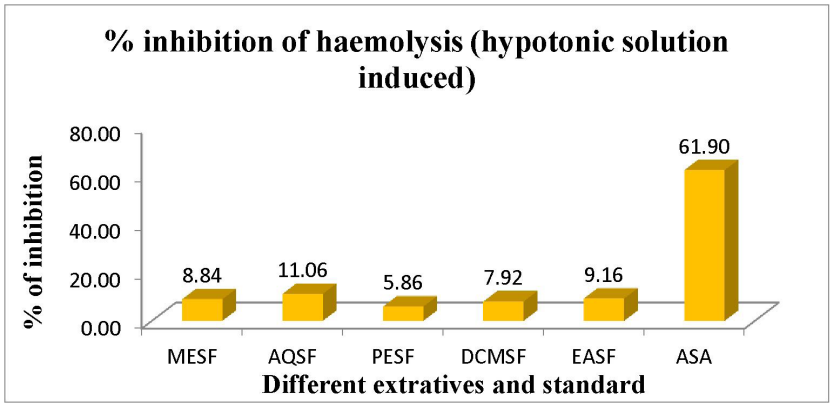

Figure 4 % inhibition of hemolysis of different extractives of Fruits of *S. suaveolens* Roxb

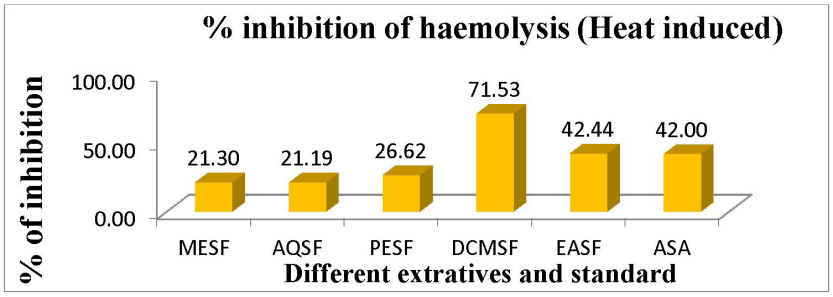

Figure 5 % inhibition of hemolysis of different extractives of Fruits of *S. suaveolens* Roxb

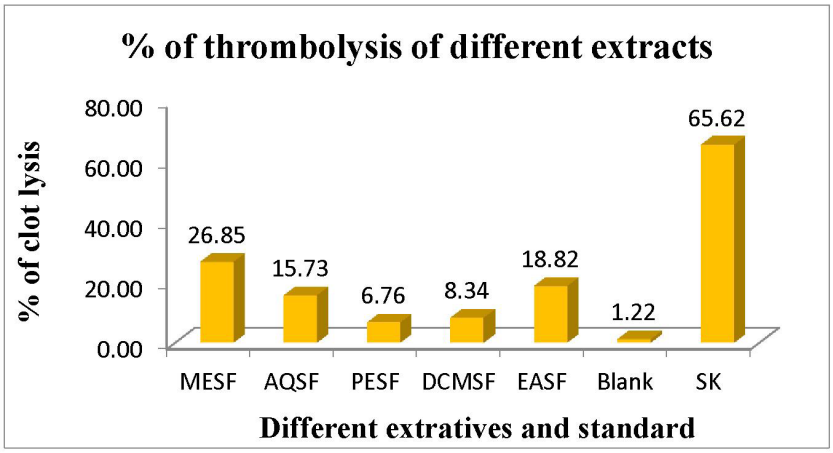

Figure 7.1 Thrombolytic activity of the extractives of Fruits of ***S. suaveolens*** Roxb

logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. Vincristine sulfate (VS) was used as positive control and the LC_{50} was found 0.451µg/ml.

In Brine shrimp lethality bioassay, among all extractives of Fruits of *S. suaveolens* Roxb. The highest brine shrimp lethality was given by Ethyl Acetate Soluble Fraction0.99µg/ml followed by Pet-Ether Soluble Fraction 1.10µg/ml, Dichloromethane Soluble Fraction 4.77µg/ml, Methanol Soluble Fraction 8.37µg/ml and Aqueous Soluble Fraction24.26µg/ml. Here the pet-ether soluble fraction showed medium lethality activity whereas the methanol and ethyl acetate showed mild lethality activity as compared to Vincristine sulphate.

CONCLUSION

The present study revealed that *S. suaveolens* extracts and its fractions possess anti-oxidant, membrane stabilizing, cytotoxic and thrombolytic properties. However, more in-vivo studies and phytochemical screening should be performed to determine its chemical constituents and its further therapeutic potential.

CONFLICT OF INTEREST

Authors have no conflict of interest.

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