Research Article

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STANDARDIZATION AND THROMBOLYTIC ACTIVITY OF SOME POTENT AYURVEDIC HERBS

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ABSTRACT

Atherothrombotic diseases such as myocardial or cerebral infarction are severe consequences of the thrombus formed in blood vessels which can be dissolved by the thrombolytic agents. These drugs have various limitations which cause serious and sometimes fatal consequences. To overcome the side effects of the synthetic drugs, the Ayurvedic herbs and their formulations provide a safer and efficacious therapy. The current investigation was carried out to standardize and to evaluate the thrombolytic potential of some very important and potent Ayurvedic herbs. The clot lysis effects of hydro-alcoholic herbal extracts of *Azadirachta indica, Allium sativum* and *Withania somnifera* were studied by *in vitro* thrombolytic model. Whole blood from healthy individual was allowed to form clots in a pre-weighed sterile micro centrifuge tubes; serum was removed, and clot weighed. After lysis by streptokinase, the fluid was removed, and remnants of clot were again weighed along with the tube. Percentage of clot lysis was calculated on the basis of the weight difference of micro centrifuge tubes obtained before and after clot lysis. *Azadirachta indica, Allium sativum* and *Withania somnifera* exhibited clot lysis *in vitro* with reference to streptokinase. However, the *in vivo* studies have yet to be performed and the exploration of the active thrombolytic components is also required. Thus, these popular Ayurvedic herbs can be incorporated as a thrombolytic agent for the improvement of patients suffering from Atherothrombotic diseases.

Keywords: *Azadirachta indica, Allium sativum, Withania somnifera,* Standardization, Thrombolytic

INTRODUCTION

Various severe diseases like ischemic heart disease, stroke, atherosclerosis, hyperuricemia and rheumatoid arthritis are the consequences of thrombosis.[1] Thrombolytic therapy uses drugs called thrombolytic agents, such as streptokinase, urokinase, alteplase and tissue plasminogen activator (TAP) to dissolve clot. Thrombolytic drugs dissolve blood clots by activating plasminogen, which forms a cleaved product called plasmin. Plasmin is a proteolytic enzyme that is capable of breaking cross-links between fibrin molecules, which provide the structural integrity of blood clots.[2, 3] The limitations with synthetic thrombolytic drugs are their associated side effects like bleeding complications, bleeding noted at a catheterization site, lysis of hemolytic plugs, gastroinstestinal and cerebral hemorrhages. Therefore, patients who have experienced trauma injury or who have a history of cerebral hemorrhagic stroke are not usually administered thrombolytics.

Ayurveda is considered as the oldest system of medicine which originated in India about more than 5000 years ago. This medicinal science of healing utilized the herbs for the treatment of various human ailments. Ayurveda has described the utility of various herbs in several diseases. Of these *Azadirachta indica, Allium sativum* and *Withania somnifera* are few of the important drugs that were investigated for their thrombolytic potential.

Azadirachta indica A. Juss. syn. *Melia azadirachta* Linn. (Fam. Meliaceae) also known as Aristah in Sanskrit, Neem in Hindi and Margosa tree in English is a medium to large evergreen tree attaining a height of 15-20m and found throughout the plains of India up to an altitude of 900m.[4] Various parts of the tree like seed oil, leaves, bark, wood and flower are used for various activities such as analgesic, anticholinergic, antihelminthic, antihistaminic, antiprotozoal, antipyretic, antiviral, bactericidal, contraceptives, fungicides, insecticides and insect repellents.[5]

Allium satvium Linn. (Fam. Liliaceae) also known as Rasona in Sanskrit, Garlic in English and Lassun in Hindi is a perennial bulbous plant cultivated as an important condiment crop in the country.[6] Garlic has been used since ages in various conditions of acne, allergies, arthritis, diabetes, diarrhea, emphysema, hypoglycemia, insomnia, pneumonia, rheumatism, ulcers, warts and wounds.[7]

Withania somnifera Dunal. (Fam. Solanaceae), also known as Hayagandha in Sanskrit and Asgandh in Hindi is a perennial shrub found throughout India.[8] Ayurveda mentions it as the best adaptogenic and it is also used as a sedative, diuretic, anti-inflammatory, anti-diabetic, anti-epileptic, anti-stress, blood tonic etc.[9]

The literature does not give very strong evidences that *Azadirachta indica*, *Allium sativum* and *Withania somnifera* are used as thrombolytics in Ayurveda. Thus, the current research was carried out to investigate the thrombolytic potential of these potent Ayurvedic herbs.

MATERIALS AND METHODS

Plant Material

Neem leaves were collected from medicinal garden, KIET School of pharmacy, Ghaziabad. Garlic was purchased from local market, Ghaziabad and Ashwagandha roots were procured from Khari Baoli market, New Delhi. The herbs were dried and coarsely powdered. All the plant materials were authenticated by microscopic methods in Pharmacognosy lab, KIET School of pharmacy, Ghaziabad.

Streptokinase (SK)

To the commercially available lyophilized Streptokinase vial (Polamin Werk GbH, Herdecke, Germany) of 15,00,000 I.U., 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U.) was used for *in vitro* thrombolysis.[10]

Blood Specimen

Whole Blood (4ml) was drawn from healthy human volunteers (n=10) without a history of oral contraceptive or anticoagulant therapy. Written consent was taken from each volunteer before the collection of blood sample. 500 µl of blood was transferred to each of the previously weighed micro centrifuge tubes to from clots.

Standardization of crude drugs[11]

The coarsely powdered plant materials were standardized using the following parameters:

Determination of Ash Values

Total Ash Value

2g of crude drug was weighed accurately in a previously ignited silica crucible. The material was ignited at temperature of 500-600°C until it turns white indicating the absence of carbon. It was then cooled and total ash in mg per gram was calculated.

Acid Insoluble Ash

Using 25 ml of dilute hydrochloric acid, the half of the ash from the dish used for total ash washed into a 100ml beaker. A wire gauge was placed over a Bunsen burner and boiled for five minutes. Filtered through an ashless filter paper, the residue was washed twice with hot water. Crucible was ignited in the flame, cooled and weighed. The acid insoluble ash of the crude drug was calculated with reference to the air-dried sample of the crude drug.

Water Insoluble Ash Value

To the crucible containing the other half of the total ash content, 25ml of hot water was added to it. Then, the whole material was filtered through ash less filter paper. The filter paper along with insoluble matter was transferred to crucible and ignited to constant weight. The residue was then allowed to cool and weighed.

Determination of Extractive Values

Water Soluble Extractive Value

5g of crude drug was accurately weighed in conical flask. 25ml of water was added to it and kept for 24 hours shaking the flask occasionally. The contents were then transferred to china dish and evaporated to dryness on water bath, cooled and finally weighed.

Ethanol Soluble Extractive Value

5g of crude drug was accurately weighed in conical flask. 25ml of ethanol was added to it and kept for 24 hours shaking the flask occasionally. The contents were then transferred to china dish and evaporated to dryness on water bath, cooled and finally weighed.

Chloroform Soluble Extractive Value

5g of crude drug was accurately weighed in conical flask. 25ml of chloroform was added to it and kept for 24 hours shaking the flask occasionally. The contents were then transferred to china dish and evaporated to dryness on water bath, cooled and finally weighed.

Petroleum Ether Soluble Extractive Value

5g of crude drug was accurately weighed in conical flask. 25ml of petroleum ether was added to it and kept for 24 hours shaking the flask occasionally. The contents were then transferred to china dish and evaporated to dryness on water bath, cooled and finally weighed.

Moisture Content (Loss on Drying)

The crude drug was placed in a weighing bottle. It was dried at 105˚C in hot air oven and weighed after 15 minutes. When the weight of the crude drug became constant, then percentage of water loss on drying was calculated.

Swelling Index

1g of crude drug was placed in a stoppered measuring cylinder containing 9ml water and kept aside for 24 hours. The swelling in the crude drug was noticed and swelling index was calculated.

Foaming index

1g of the crude drug was transferred to a 500ml conical flask containing 100 ml of boiling water. It was maintained at moderate boiling for 30 minutes, cooled and filtered into a 100ml volumetric flask. Sufficient water was added through the filter to dilute to volume. The decoction was poured into 10 ml stoppered test tubes in successive portion in 1ml, 2ml, 3ml and the volume of the liquid in each tube was adjusted with water to 10ml. The tubes were stoppered and shaken in a length wise motion for 15 minutes. They were then allowed to stand for 15 minutes and the height of the foam was measured.
Formula used: 1000/A

TLC Profile

The solvent systems used for the Thin Layer Chromatography of the herbs were as follows

- *Azadirachta indica* - Acetonitrile: Methanol: Triethylamine (60:40:1)
- *Allium sativum* - Hexane: Isopropanol (92:8)
- *Withania somnifera* -Toluene: Ethyl Acetate: Formic acid (5:5:1)

Rf value was calculated by this formula-Distance travel by solute/Distance travel by solvent

Preliminary Phytochemical Screening

The coarsely powdered plant materials of *Azadirachta indica*, *Allium sativum* and *Withania somnifera* were tested for the presence of alkaloids, steroids, tannins, saponins and glycosides using the standard procedures for preliminary phytochemical screening[12]. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

Herbal Preparation

100g of each of the plant materials: *Azadirachta indica*, *Allium sativum* and *Withania somnifera* were separately extracted by maceration in Methanol: Water (1:1) to give 2.5g dark greenish extract of *A. indica*, 3.8g light yellowish extract of *A. sativum* and 1.2g light brownish extract of *W. somnifera.* 10 mg of each of the extract was suspended in 10ml distilled water and the suspension was shaken vigorously on the vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22- micron syringe filter. 100 µl of this aqueous preparation of herbs was added to the micro centrifuge tubes containing the clots to check thrombolytic activity.[10]

Thrombolytic Activity

This test was performed according to the method described by Prasad *et al*.[10] In the commercially available lyophilized streptokinase vial (15,00,000 I.U.), 5ml sterile distilled water was added and mixed properly. This suspension was used as a stock solution from which appropriate dilution was made. 5 ml of venous blood withdrawn from the healthy volunteers (n=10) without the history of contraceptive or anticoagulant therapy and was distributed (0.5 ml/tube) to each 10 previously weighed sterile micro centrifuge tubes and incubated the clot and each tube having clot was again weighed to determine the clot weight. A volume of 100 µl of the extract (10 mg/ml) was added to each micro centrifuge tube containing previously weighed clot. As a positive control, 100 µl of streptokinase and as a negative control 100 µl of distilled water were separately added to the control tube numbered. All the tubes were incubated at 37˚C for 90 min and observed for clot lysis. After incubation, fluid released was removed and 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.

RESULT AND DISCUSSION

Standardization of crude drugs

The Ayurvedic herbs were standardized as per the WHO guidelines for standardization of crude drugs. Since ashing process involves the complete oxidation of components of product, an increase in ash value indicates contamination, substitution and adulteration. The total ash value is an indicative of total amount of inorganic material after complete incineration.[5] 3.2%, 2.8% and 1.66% was calculated as the total ash values of Ashwagandha, Neem and Garlic respectively. The extractive values aid in estimating the nature of phytoconstituents and also helps in establishing the number of active constituents present in a medicinal plant material. The water soluble extractive values and methanol soluble extractive values of the individual herbs were similar, thus the solvents chosen for extraction were water and methanol in the ratio of 1: 1. The moisture content was determined to establish any increase in weight caused by moisture absorption. Loss on drying or moisture content of the herbs was 2.1%, 0.08% and 0.11% respectively for Ashwagandha, Neem and Garlic. The swelling index test was negative for all the three Ayurvedic herbs under study indicating the absence of the mucilaginous substances in these medicinal plants. The saponins exhibit the lather forming characteristic, thus foaming index is measure of their frothing property. Saponins possess surface active or detergent properties because the carbohydrate portion of the molecule is water soluble, while the sapogenin is fat soluble. All the three herbs of current investigation possessed the frothing characters and the foaming index of Ashwagandha, Neem and Garlic were 0.8, 0.9 and 0.6 respectively. The quantitative analysis of the standardization of crude drugs is mentioned in Table 1.

Preliminary Phytochemical Screening

Ashwagandha responded positively towards the identification tests for non-reducing sugars, proteins, steroids, saponins, coumarin glycosides, flavonoids and alkaloids. Neem showed the presence of non-reducing sugars, saponins, coumarin glycosides, alkaloids, tannins and acidic compounds. Garlic was also found to possess various phytoconstituents like carbohydrates (pentose, hexose and non-reducing sugars), proteins, saponins, coumarin glycosides, flavonoids and alkaloids. The results of the preliminary phytochemical screening are mentioned in Table 2.

Table 1: Standardization of Crude Drugs

S. No	Parameter	Ashwagandha	Neem	Garlic
1.	Total Ash	3.2%	2.8%	1.66%
2.	Acid Insoluble Ash	0.8%	1.4%	0.85%
3.	Water Insoluble Ash	2%	1.8%	0.5%
4.	Water Soluble Extractive Value	24%	15.2%	11.4%
5.	Alcohol Soluble Extractive Value	23.2%	17.6%	12.8%
6.	Chloroform Soluble Extractive Value	2.4%	3.8%	2.8%
7.	Ether Soluble Extractive Value	1.2%	1.0%	1.5%
8.	Moisture Content	2.1%	0.08%	0.11%
9.	Swelling Index	Negative	Negative	Negative
10.	Foaming Index	0.8	0.9	0.6
11.	R_f values	0.29, 0.32, 0.37, 0.57, 0.61, 0.66, 0.74	0.31, 0.42, 0.49, 0.51, 0.72, 0.83	0.36, 0.41, 0.55, 0.59, 0.68, 0.70, 0.76, 0.84

Table 2: Preliminary Phytochemical screening of crude drugs

S.No	Chemical test	Ashwagandha	Neem	Garlic
1.	Tests for carbohydrates			
	Molish's test	-	-	+
2.	Tests for Reducing sugars			
	Fehling's test	-	-	-
3.	Tests for pentose sugars	-	-	+
4.	Tests for hexose sugars			
	Tollen's phloroglucinol test for galactose	-	-	+
5.	Tests for non-reducing Sugars	+	+	+
6.	Tests for gum	-	-	-
7.	Tests for mucilage	-	-	-
8.	Tests for protein			
	Biuret test	-	-	-
	Xanthoprotein test	-	-	-
	Test for protein containing Sulphur	+	-	+
	Precipitation test	+	+	+
9.	Test for amino acid			
	Ninhydrin test	+	-	+
	Test for tyrosine	-	-	-
	Test for cysteine	-	-	-
10.	Test for steroid			
	Salkowski reaction	+	+	+
	Liebermann – Burchard Reaction	+	-	-
	Liebermann's reaction	-	-	-
11.	Test for cardiac glycoside			
	Legal's test	-	-	-
	Test for deoxysugars (killer-killiani test)	+	-	-
	Liebermann's test (Test for bufadenolids)	-	-	-
	Raymond's test	-	-	-
12.	Test for anthraquinone glycoside			
	Borntrager's test	-	-	-
13.	Test for saponin			
	Foam test	+	+	+
14.	Test for coumarin Glycosides	+	+	+
15.	Test for flavonoids			
	Sulphuric acid test	+	+	+
16.	Test for alkaloid			
	Dragendroff's test	+	+	+
	Mayer's test	+	-	+
	Wagner's test	+	+	+
	Tannic acid test	+	-	+
17.	Test for tannins and phenolic compounds			
	5% Ferric chloride solution	-	-	-
	Lead acetate solution	+	+	+
	Acetic acid solution	-	-	-
	Dilute HNO_3	-	+	-
	Dilute NH_4OH	-	-	-
18.	Test for acidic compounds	-	+	-

Table 3: Thrombolytic activity of Control and Standard

	Weight of tube (A) g	Weight of tube + clot (B) g	Weight of clot (C=B-A) g	Weight of tube with clot after lysis (D) g	Weight of clot release (E=B-D)	% clot release
Water (Control)	0.83	1.22	0.39	1.12	0.1	25.6%
Streptokinase (Standard)	0.89	1.18	0.29	0.93	0.25	86.20%

Table 4: Thrombolytic Activity of *Azadirachta indica*

Concentration	Weight of tube (A) g	Weight of tube + clot (B) g	Weight of clot (C=B-A) g	Weight of tube with clot after lysis (D) g	Weight of clot release (E=B-D) g	% clot release	Average % of clot release
1mg/ml	0.85	1.28	0.43	1.14	0.14	32.5%	21.27%
500µg/ml	0.84	1.28	0.44	1.23	0.05	11.3%	
250µg/ml	0.84	1.39	0.55	1.30	0.09	16.3%	
100µg/ml	0.87	1.36	0.49	1.18	0.18	36.7%	
50 µg/ml	0.83	1.21	0.38	1.11	0.1	26.3%	
20 µg/ml	0.85	1.29	0.44	1.27	0.02	4.5%	

Table 5: Thrombolytic Activity of *Withania somnifera*

Concentration	Weight of tube	Weight of tube + clot	Weight of clot	Weight of tube clot release	Weight of clot release	% clot release	Average % of clot release
1mg/ml	0.92	1.37	0.45	1.34	0.03	6.6%	15.23%
500µg/ml	0.88	1.39	0.51	1.33	0.06	11.7%	
250µg/ml	0.86	1.28	0.42	1.20	0.08	19%	
100µg/ml	0.89	1.31	0.42	1.27	0.04	9.5%	
50 µg/ml	0.85	1.25	0.4	1.29	0.04	9.5%	
20 µg/ml	0.84	1.38	0.54	1.19	0.19	35.1%	

Table 6: Thrombolytic Activity of *Allium sativum*

Concentration	Weight of tube	Weight of tube + clot	Weight of clot	Weight of tube clot release	Weight of clot release	% clot release	Average % of clot release
1mg/ml	0.89	1.19	0.3	1.23	0.04	13%	22.23%
500µg/ml	0.88	1.18	0.3	1.13	0.05	16.6%	
250µg/ml	0.89	1.24	0.35	1.16	0.08	22.8%	
100µg/ml	0.90	1.19	0.29	1.31	0.12	41.3%	
50 µg/ml	0.91	1.20	0.29	1.15	0.05	17.2%	
20 µg/ml	0.88	1.28	0.4	1.19	0.09	22.5%	

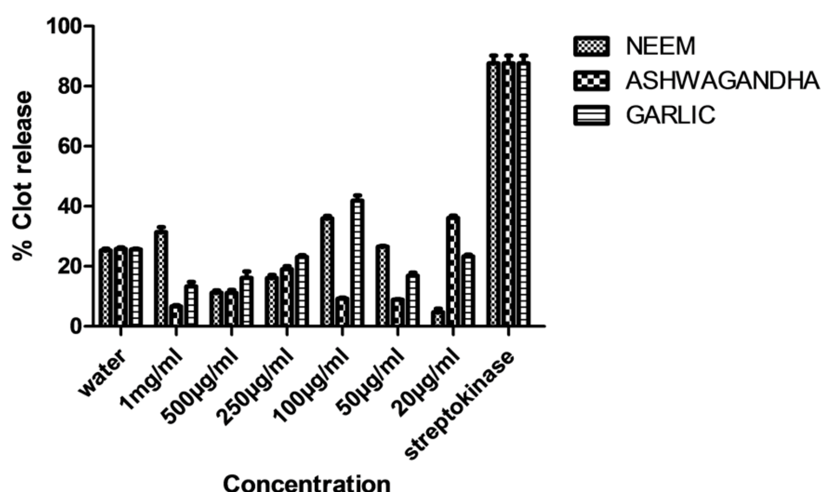

Figure 1: Thrombolytic activity of *Azadirachta indica, Allium sativum* and *Withania somnifera,* water and streptokinase

Thrombolytic Activity

Numerous research investigations have been carried out for the thrombolytic activity of several medicinal plant extracts. Not much literature is available to reveal the thrombolytic potential of *Azadirachta indica*, *Allium sativum* and *Withania somnifera.* All these are highly potent commonly used Ayurvedic herbs beneficial for numerous diseases. An attempt was made to investigate their utility as anticoagulant and antiplatelet agents. The results of the thrombolytic assay indicated that these extracts exhibited very significant thrombolytic activity. 25.6% was the clot release for the control group (water), 86.20% for the standard (streptokinase), 21.27% for *Azadirachta indica,* 15.23% for *Withania somnifera* and 22.23% for *Allium sativum.* The thrombolytic activity of control and standard are mentioned in table 3 and table 4, 5 and 6 respectively contain the results of the thrombolytic assays of *Azadirachta indica, Withania somnifera* and *Allium sativum.* Thus these herbs possess thrombolytic activity and may be used for the prevention of coronary events and strokes. Figure 1 depicts the comparative thrombolytic activity of the control, standard, *Azadirachta indica*, *Allium sativum* and *Withania somnifera*.

CONCLUSION

Through our study it was found that *Azadirachta indica*, *Allium sativum* and *Withania somnifera* contain thrombolytic properties that could cause lysis of blood clots *in vitro*: however, *in vivo* clot dissolving properties, active components of *A. indica*, *A. sativum* and *W. somnifera* for clot lysis and the mechanism for the thrombolytic activity are yet to be discovered. Thus, these potent Ayurvedic herbs could be incorporated as a thrombolytic agent for the improvement of patients suffering from Atherothrombotic diseases and the current research investigation provides an opportunity for the upcoming researchers to explore the pharmacology involved in the thrombolytic action and also the phytoconstituents responsible for the activity.

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