INDIAN JOURNAL OF SCIENCE AND TECHNOLOGY

RESEARCH ARTICLE

Evaluation of Haemostatic Potential and HPTLC Fingerprinting of *Annona squamosa* Linn. Bark Extract

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OPEN ACCESS

Received: 14-05-2024

Accepted: 25-06-2024

Published: 19-07-2024

Citation: Bhagwat JA (2024) Evaluation of Haemostatic Potential and HPTLC Fingerprinting of *Annona squamosa* Linn. Bark Extract. Indian Journal of Science and Technology 17(27): 2858-2864. https://doi.org/10.17485/IJST/v17i27.1634

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Funding: R.J.College Seed Grant Ref. 1680 (24/01/2024)

Competing Interests: None

Published By Indian Society for Education and Environment (iSee)

ISSN
Print: 0974-6846
Electronic: 0974-5645

Abstract

Objectives: The present work focuses on exploring and evaluating the hemostatic property of *Annona squamosa* Linn. bark. Being a renewable resource, it has the potential to contribute to the hemostatic agent. Hemostatic efficacy of the cold and hot bark extract at different concentrations of 10, 50, 100 & 250 mg/ml of saline were evaluated using whole blood clotting, platelet aggregation test, prothrombin time CAMAG HPTLC system. The hemostatic efficacy. At 250 mg/ml saline, both the extracts showed excellent hemostatic efficacy by clotting blood in 1.2 s and 3 s, respectively. The prothrombin time of cold and hot were 5 s and 5.1 s which were less than that of Liquiplastin partial prothrombin time which was Liquicelin E (13.85 s). *Annona squamosa* Linn. to be used as a topical hemostatic agent. Saponins, which are known to be antihemorrhagic, were detected by HPTLC. hemostatic efficacy of *Annona squamosa* Linn. bark was evaluated, which can contribute to the development of a fast-acting, cost-effective, easy-to-use, and sustainable topical hemostat.

Keywords: Annona squamosa Linn; Hemostatic activity; Prothrombin time; Activated partial thromboplastin time; HPTLC fingerprint

1 Introduction

Acute blood loss from a damaged blood vessel is known as hemorrhage. It can occur due to trauma caused by injury, surgical bleeding, or genetic disorders related to blood clotting [(1)]. This is one of the major causes responsible for a significant number of deaths each year [(2)]. Hemorrhage can be treated by suturing the damaged area or applying anti hemorrhagic agents. The surgical technique may not always be helpful as the shape and size of the wounds may differ depending on the cause. Usually, the wounds inflicted during road accidents, gunshots, explosions, or assaults have jagged edges deep, leading to uncontrollable bleeding, which may be the cause of death. Under such circumstances, lives could be saved if the blood loss is stopped immediately till the arrival of medical help. Topical hemostatic agents are appropriate for these harsh conditions. An assortment of natural and synthetic hemostatic agents is used to stop excessive bleeding. These topical hemostats have side effects such as triggering immune reactions, requiring specific storage conditions, and being less stable or expensive [(3)]. Consequently, there is an acute need to find new hemostatic agents

that do not require special storage conditions and can be used easily, even under unfavorable circumstances.

According to a recent report, traditional medicine lists 92 medicinal plants belonging to 59 families that show hemostatic activity. Amongst these families, the more commonly studied plants were from the Asteraceae, Moraceae, Poaceae, and Euphorbiaceae [4]. Annonaceae, a widely distributed plant family found in tropical and subtropical regions, was found to be less explored for its hemostatic potential. *Annona squamosa* Linn. belongs to the family Annonaceae. It is a small deciduous tree of about 3-7 m in height, broadly distributed in the tropics. The bark is light brown and slightly rough, with light brown spots [5]. Traditionally, leaves, seeds, roots, flowers, bark, and fruit of *Annona squamosa* Linn. have been used for the treatment of various ailments such as infection, dysentery, haemorrhage, wounds, diabetes, inflammation, fever, epilepsy, thyroid disorder, cancer, tumors, and obesity to name a few [6].

The medicinal properties of plants are attributed to the phytochemicals present in the plants. In *Annona squamosa* Linn., these properties are due to the presence of carbohydrates, glycosides, phytosterols, oils, saponins, tannins, alkaloids, phenols, flavonoids, peptides, and various acetogenin compounds [7]. Each plant has a unique composition of phytochemicals, which can be used to generate a chromatographic fingerprint of the plant for its identification and quality control [8]. High-performance thin layer chromatography (HPTLC) fingerprinting study of different parts of *Annona squamosa* Linn. showed the presence of six, nine, eight, and seven polyvalent phytoconstituents in stem, leaf, fruit cover, and seed, respectively [9]. In a recent study, Shah reported the presence of gallic acid and quercetin in the hydroalcoholic extract of leaves of *Annona squamosa* Linn using the HPTLC technique [10]. Extractable and non-extractable polyphenols were detected from *Annona squamosa* fruit peels using high-resolution mass spectrometry (DART-HRMS) methodology [11].

A study showed that leaves of *Annona senegalensis* (wild custard apple), native to Western Africa, show hemostatic efficacy [12]. However, there are no reports of haemostatic activity in the bark of *Annona squamosa* Linn. So, the need to explore the anti-haemorrhagic potential of *Annona squamosa* Linn. bark extract *in vitro* was felt. Physiological blood clotting involves intrinsic, extrinsic, and common pathways consisting of a series of cascading reactions that terminate in fibrin clot formation. Identification of an appropriate pathway affected by bark extract will determine the future utility of the extract. It was, therefore, essential to select assays representing each blood coagulation pathway. The whole blood clotting assay, prothrombin time, activated thromboplastin time, and platelet aggregation test were used to analyze the effect of the extract on blood coagulation.

Quality control of herbal drugs is of the utmost importance. Hence, an HPTLC fingerprint of *Annona squamosa* Linn. bark was generated using a different solvent system, which was not previously used. Thus, a reference standard was made for further work to ensure the reproducibility of the extracts. The efficacy of herbal extract is due to the presence of secondary metabolites. In this case, saponins have been reported to exhibit haemostatic efficacy [13]. So, to substantiate this claim, the presence of saponins was detected in the bark using HPTLC.

2 Methodology

2.1 Collection and Identification of Plant Material

The bark of *Annona squamosa* Linn was collected from a farm in the Raigad district of Maharashtra. An expert botanist identified the plant material, and a voucher specimen was deposited in the Department of Biological Sciences at Ramniranjan Jhunjhunwala College, Ghatkopar West, Mumbai 400 086, India.

2.1.1 Preparation of Plant Extracts

The bark of *Annona squamosa* Linn. was shade-dried for 8 days and powered using an electrical blender (Murphy Richards). The hot extract was prepared by extracting 30 g of powdered bark with 350 ml of alcohol for 8 hours using the Soxhlet apparatus. The cold extract was obtained by soaking 30g of bark powder in 350 ml of alcohol for 72 hours. Both the extracts were completely dried to remove the solvent using a Rota evaporator (Superfit). The solvent-free extracted powder was used to test hemostatic efficacy. The solvent-free cold extracted powder will be referred to as ASC (*Annona squamosa* Linn. Cold), and the solvent-free hot extracted powder will be referred to as ASH (*Annona squamosa* Linn. Hot) in this paper.

2.1.2 General Phytochemical Tests

The secondary metabolites present in the bark contribute to their bioactivity. The phytochemical tests were carried out on ASH and ASC to detect the presence of common secondary metabolites like tannins, alkaloids, saponins, carbohydrates, proteins, and lipids [14].

2.2 Collection of Blood

Prior consent was taken from the volunteers, and necessary precautions were adopted before collecting blood. A fresh blood sample was collected by venipuncture with a disposable 20 SWG needle and transferred to plastic vials containing trisodium citrate as an anticoagulant.

2.2.1 Determination of Clotting Time

The modified Lee-White method was used to determine the clotting time. ASC and ASH were redissolved in saline to get concentrations of 10 mg/ml, 50 mg/ml, 100 mg/ml, and 250 mg/ml. In all, 0.2 ml of extract was mixed with 0.2 ml of whole blood in a test tube and swirled gently at 37°C in a water bath. The time required to form a clot was noted. The control tube contained 0.2 ml saline. The extract was considered inefficient if the clotting time exceeded 900 seconds (15 minutes)[15]. All the experiments were carried out in triplicates.

2.2.2 Preparation of Platelet Poor Plasma and Platelet Rich Plasma

Platelet-rich normal plasma (PRP) and platelet-poor normal plasma (PPP) were obtained by differential centrifugation of fresh citrated blood using standard protocol[16]. The plasma samples were transferred to siliconized tubes for further use.

2.2.3 Platelet Aggregation Test (PAT)

The platelet aggregation test (PAT) was conducted to determine the ability of the extracts to clump the platelets. A total of 0.2 ml of the extracts (ASC and ASH) at concentrations of 10 mg/ml, 50 mg/ml, 100 mg/ml, and 250 mg/ml of saline were added to 0.2 ml of PRP maintained at 37°C. The control tube was made using 0.2 ml of saline. ADP sodium salt, a platelet aggregating agent, was used as standard (200 μg/ml in barbatone buffer). Thus, the standard tube had 0.2 ml of ADP solution instead of extract. This test was performed according to the protocol provided in the Tulip Diagnostic Kit.

2.2.4 Prothrombin Time (PT)

The effect of extracts on the extrinsic and common blood coagulation pathways was analyzed using the prothrombin time (PT) test. A volume of 0.2 ml of PPP was added to 0.2 ml extracts (ASC and ASH) at different concentrations, namely, 10 mg/ml, 50 mg/ml, 100 mg/ml, and 250 mg/ml of saline, and the tubes were placed in a water bath at 37°C[17]. Liquiplastin, a calcium thromboplastin reagent (Tulip Diagnostics), was used as a positive standard. The control tube contained 0.2 ml saline. Clotting time was recorded for all the tubes. The tests were performed in triplicates. The protocol provided by the kit purchased from Tulip Diagnostics, Goa, India, was followed.

2.2.5 Activated Partial Thromboplastin Time (aPTT)

aPTT test is used to evaluate the effect of extracts on the intrinsic pathway of blood coagulation. This test was performed by using a standard kit procured from Tulip Diagnostics, Goa, India. A volume of 0.2 ml of extracts of ASC and ASH (10 mg/ml, 50 mg/ml, 100 mg/ml, and 250 mg/ml of saline) was added to different tubes containing 0.2 ml of PPP. The tubes were kept in a water bath at 37°C for 3 minutes. Pre-warmed 0.2 ml $CaCl_2$ solution was added to these tubes forcefully and placed in a water bath at 37°C. Liquicelin–E, an activated cephaloplastin reagent (Tulip diagnostics), was used as standard. The control tube contained 0.2 ml saline instead of extracts. The time required to form gel was noted. All the tests were performed in triplicates, and the results were recorded.

2.3 HPTLC Analysis

2.3 1 Sample preparation

The sample for HPTLC was prepared by sonicating 1gm of dried bark power in HPLC grade methanol for 30 minutes, followed by centrifugation at 3000 rpm and filtration to remove the suspended particles.

2.3.2 Methodology

The chromatographic fingerprint of *Annona squamosa* Linn. bark was generated using CAMAG HPTLC (Switzerland). Linomat IV Spotter was used to load the sample and Scanner II attached to CAMAG CATS 3 software was used to analyse the data. As mentioned earlier, saponins contribute to the hemostatic activity of plants. Hence, HPTLC was used to detect the presence of saponins in the alcoholic bark extract. A sample of 20 μl was applied on an HPTLC plate of Silica gel 60 F254, and a run was carried out using Chloroform: Acetic acid: Methanol: Water (6.4:3.2:1.2:0.8) as mobile phase for saponins. The plates were

visualized under visible 550 nm, UV 254 nm, and fluorescence 366 nm before and after derivatization. Anisaldehyde sulphuric acid was used as a derivatising agent for saponins [18].

2.4 Statistical Analysis

The statistical analysis was conducted using Microsoft Excel. The average values and the standard deviation were found for all the test results. Single factor Analysis of Variance (ANOVA) was used to find the difference between the clotting time of the extract and standard at the level of significance $p<0.05$.

3 Results and Discussion

The secondary metabolites impart various efficacies to the plant extract. In the current study, both the hot and cold extracts of *Annona squamosa* Linn. bark, as depicted in Table 1, showed the presence of alkaloids, tannins, saponins, phenols, flavonoids, carotenoids, and gum. However, the tests could not detect the presence of starch and protein in the extracts. These results are in alignment with the results obtained by Marahatta [19], who reported the presence of alkaloids, flavonoids, polyphenols, glycosides, carbohydrates, saponins, and tannins in the methanolic bark extract of *Annona squamosa* Linn.

During the present study, it was observed that the cold and hot bark extracts showed concentration-dependent hemostatic efficacy. At the low concentration of 10 mg/ml of saline, both extracts were ineffective against whole blood coagulation and did not affect the prothrombin time, activated partial thromboplastin time, or platelet aggregation either.

Table 1. General Qualitative Phytochemical Tests

Sr. no	Constituent	Test	Inference	
			ASC	ASH
1	Alkaloids	Dragendroff	+	+
		Wagner	+	+
		Hager	+	+
2	Phenols	Ferric Chloride	+	+
3	Saponins	Foam Test	+	+
4	Tannins	Gelatin	+	+
5	Flavones	Diluted Sulphuric Acid	+	+
6	Carotenoids	Antimony Chloride	+	+
7	Gums	Lead Acetate	+	+
8	Proteins	Ninhydrin	-	-
9	Starch	Iodine	-	-

All the tests were carried out in triplicates.
*ASC- Annona squamosa Linn bark cold extract
*ASH- Annona squamosa Linn bark hot extract

However, the highest concentration tested, 250 mg/ml, for both extracts showed maximum hemostatic efficacy. It was observed that the ASC and ASH bark extracts effectively formed blood clots with whole blood with anticoagulant at the concentration of 250 mg/ml in 1.2 seconds and 3 seconds, respectivelyTable 2. This difference was found to be statistically significant at $p<0.05$. The extract led to clot formation in the blood with an anticoagulant. It was also observed that the clot became loose after 15 minutes which may be due to fibrinolytic activity shown by the extract. This observation is significant with respect to the application of extract as a hemostat. Most of the synthetic hemostats are not biodegradable and lead to embolism [20].

The platelet aggregation test analyses the ability of the extracts to initiate platelet aggregation, which is an important parameter in the formation of a blood clot [21]. ADP, which is the activator of platelet aggregation, was used as standard. Both extracts showed better platelet aggregation activity Table 2 than the standard compounds, which was statistically significant at $p<0.05$. Prothrombin time is a parameter used to evaluate the effect of extracts on the extrinsic factors of coagulation [22]. It was found that the ASC and ASH bark extracts, when tested for PT Table 2, showed gel formation in 5 seconds at 250 mg/ml concentration, which was much less than the time taken by the positive control Liquiplastin. This difference was statistically significant at $p<0.05$. Activated partial thromboplastin clotting time indicates the activity of extract to affect coagulation factors of intrinsic and common coagulation pathways [23]. Both extracts showed excellent activity with platelet-poor plasma (PPP) used

for measuring Activated partial thromboplastin clotting time Table 2. This result showed that extracts affected the intrinsic and common pathway clotting factors positively. Both the extracts exhibited activity better than standard Liquicelin E, which was found to be statistically significant at $p<0.05$.

Since the alcoholic extract synergistically shows hemostatic efficacy, it can be used without isolating a specific compound, which will significantly reduce the cost of the hemostatic preparation.

Table 2. Hemostatic activity of Different Concentrations of *Annona squamosa* Bark Extracts

	Time required for Clot/Gel Formation (in seconds)							
Concent-ration in mg/ml	**Whole Blood**		**PT**		**aPTT**		**PAT**	
	ASC	**ASH**	**ASC**	**ASH**	**ASC**	**ASH**	**ASC**	**ASH**
10	No clot	No clot	No gel	No gel	No gel	No gel	No gel	No gel
50	429.6 (±0.15)	672 (±0.01)	615.6 (±0.25)	600 (±0.11)	7.03 (±0.06)	3.31 (±0.27)	12.23 (±0.25)	15.1 (±0.11)
100	321 (±0.08)	444 (±0.01)	420 (±0.05)	375.6 (±0.25)	2.03 (±0.06)	1.6 (±0.15)	3.0 (±0.05)	3.1 (±0.2)
250	1.2 (±0.05)	3.1 (±0)	5 (±0.1)	5.1 (±0.05)	1.12 (±0.03)	1.1 (±0.1)	1.52 (±0.02)	1.53 (±0.01)
Negative Control	No clot formation		No gel formation		No gel formation		No gel formation	
Positive Control	NIL		Liquiplastin 15.08 (±0.07)		Liquicelin-E (±0.06)	13.85	ADP 20.28 (±0.02)	

Note: All the values in the table were averages of three readings.
± Indicated standard deviation (SD)
*ASC- (*Annona squamosa* Linn. Cold) bark powder obtained by cold extraction.
*ASH- (*Annona squamosa* Linn. Hot) bark powder obtained by hot extraction.
*PT- Prothrombin Time
*aPTT- Activated Partial Thromboplastin Time
*PAT- Platelet Aggregation Time

Table 3 and Figure 1 represent the chromatographic fingerprint of *Annona squamosa* Linn. bark. This fingerprint can be used as a measure for quality control and to ensure the proper extraction of phytochemicals. The HPTLC fingerprint showed 12 peaks with the area under the curve represented in Table 3. The range of the peaks was between 0.02 to 0.90. The prominent peaks were at position number 2 (Rf 0.06, 10.92% area), number 3 (Rf 0.11, 11.35% area), number 10 (Rf 0.66, 35.28% area), and number 12 (Rf 0.90, 15.58% area). Clearly, the peak at position number 10 had the maximum area under the curve. The chromatographic plate was visualized in visible light at 550 nm and under long and short UV. Mona Agrawal et al.[24] have reported HPTLC of leaves and seeds of *Annona squamosa* Linn. Sandeep and Mittal[25] reported the detection of two new compounds along with three known alkaloids from the defatted ethanolic extract of *Annona squamosa* Linn. bark. They used TLC, IR, NMR, and LC\MS for their study.

Table 3. Analysis of Peak and Area under the Curve for Chromatographic Fingerprint of Bark of *Annona squamosa* Linn

Peak	Max Rf	AUC	% Area
1	0.02	502.4	1.27
2	0.06	4307.9	10.92
3	0.11	4476.4	11.35
4	0.25	998.0	2.53
5	0.31	659.5	1.67
6	0.40	862.6	2.19
7	0.43	929.1	2.36
8	0.50	2086.7	5.29
9	0.57	4158.9	10.55
10	0.66	13912.9	35.28
11	0.80	393.8	1.00
12	0.90	6143.7	15.58

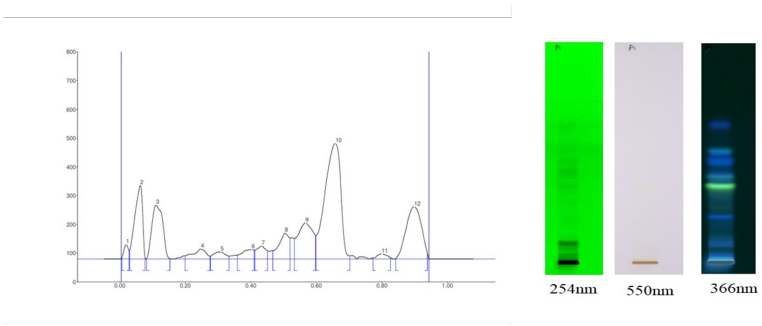

Fig 1. HPTLC Fingerprint of Bark of *Annona squamosa* Linn and its visualization at different nm

Further, HPTLC was carried out to detect the presence of saponins. After the derivatization with the anisaldehyde-sulphuric acid reagent, two distinct blue bands were observed under long UV (366 nm), and blue, yellow, green, and violet bands were observed at 550 nm, indicating the presence of saponins, as seen in Table 4 and Figure 2. The hemostatic efficacy of the *Annona squamosa* Linn. bark extract may be due to the presence of saponins. However, further studies on the isolated fraction of saponins are required and are currently underway.

Table 4. Analysis of Peak and Area under the Curve for Saponins of *Annona squamosa* Linn Bark

Peak	Max Rf	AUC	% Area
1	0.06	3336.7	11.81
2	0.1	525.8	1.86
3	0.16	1669.8	5.91
4	0.36	10637.0	37.65
5	0.46	7629.8	27.00
6	0.54	1311.7	4.64
7	0.57	2066.2	7.31
8	0.64	1078.8	3.82

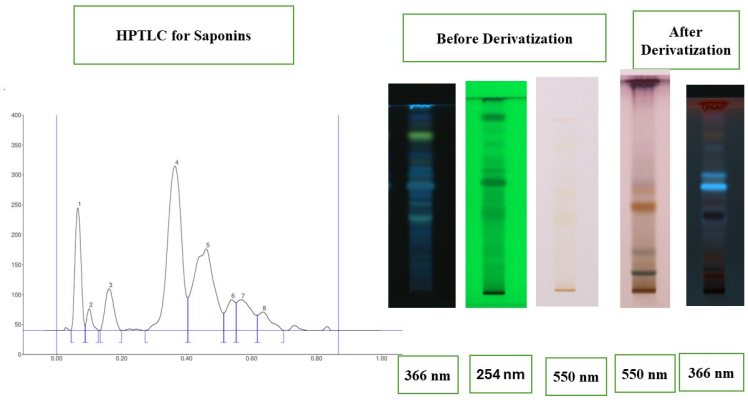

Fig 2. HPTLC for *Annona squamosa* Linn. Bark Saponins before and after Derivatization

4 Conclusion

The study concluded that the bark of *Annona squamosa* Linn has excellent hemostatic activity, which has not been reported elsewhere as per the literature survey. Since the bark is a renewable natural resource and its alcoholic extract can be used without

purification, it is a sustainable and cost-effective solution. Thus, evaluation of hemostatic efficacy will contribute to developing an economical, easy-to-use, fast-acting topical hemostatic agent/antihemorrhagic agent in the form of a tincture, gel, or ointment. The extracts could form a retractable clot in blood containing anti-coagulant, thus making it a lucrative option for patients undergoing anticoagulant treatment. *In vivo*, studies are recommended to evaluate its efficacy as an injectable hemostatic agent that can be used to treat internal haemorrhage.

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