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Research Article

Evaluation of *In vitro* Anti-inflammatory and Thrombolytic Activities of *Anisomeles indica* Kuntze

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ABSTRACT

Anisomeles indica Kuntze (A. indica) is among the most common aromatic plants with a heritage of being employed as an ethnomedicinal potential. The therapeutic efficacy of the plant is well known for being antioxidant, antifungal, antioxidant, and anti-inflammatory attributes. In this present study, solvent extraction was carried out by employing the soxhlet extraction method. The phytoconstituents analysis revealed the variation and diversification in the phytochemical content at the qualitative level. The phytochemical screening investigation of A. indica explores the occurrence of flavonoids, glycosides, alkaloids, phenolics, saponins, terpenoids, steroids, carbohydrates, and proteins. A. indica extracts were employed for TLC analysis and further employed to ascertain its biological activity, such as anti-inflammatory and thrombolytic activity. Among all extracts (methanol, hexane, chloroform, and ethyl acetate), the methanol extract of A. indica exhibits potent anti-inflammatory activity and also showed significant thrombolytic activity.

Keywords: *Anisomeles indica Kuntze, Thin layer chromatography, anti-inflammatory activity, thrombolytic activity.*

INTRODUCTION

Medicinal plant species have been employed to treat ailments since antiquity to the present day. The biological attributes of the plant are typically derived from active substances produced during secondary metabolism (Sing, 2015). The ethnomedicinal utilization of numerous diverse and endemic plants for the amelioration of different kinds of illness has turned into a remunerative endeavour for people. Investigators have determined that these herbal plants' inherent phytochemical components significantly contribute to their efficacy in managing a wide range of illnesses (Zhang et al., 2021).

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Due to their ability to produce certain bioactive compounds, various communities utilized plants as medicines and as a source of many effective medications by the pharmaceutical industry (Gopal Krishna & Udyakumar, 2014). Secondary metabolites play no significant part in normal vital functions such as growth and development; the secondary metabolites that plants produce are extremely vital to their capability to defend themselves (Kaushik et al., 2021). In developed nations, plants are the major source of more than 20% of drugs, either directly or indirectly. People have been utilizing some herbal medicine remedies to combat a diverse range of illnesses. Even now, various tribal groups and village residents have used folk medicines for thousand for a diverse range of chronic infections. *Anisomeles indic*ia, widely recognized as Indian catmint and a member of the Lamiaceae family, is an aromatic, perennial shrub with substantial ethnomedical benefits; it is found throughout the world in both tropical and temperate climates, including Malaysia, India, Thailand, Indonesia, China, Taiwan, Vietnam, the Philippines, Africa, and Australia (Arisawa et al., 1986). *Anisomeles indica* Kuntze is a plant with a therapeutic value that contains substantial amounts of biologically significant phytocompounds (Ekor, 2014; Khan et al., 2019; & Bagchi et al., 2019). The Labiatae family, also known as the mint family, is among the most significant families of medicinal plants. It is a medicinal plant with carminative, aromatic, astringent, and tonic properties (Batish et al., 2007). The *A. indica* plant can be administered fresh or dried as a wash for skin infections, as a snakebite remedy, and as a cure for so many kinds of animal poisons (Sebastian & Bhandari et al., 1984). *A. indica* leaves are chewed to relieve toothaches. Leaves of *A. indica* has been employed widely to treat a diverse range of illness, including liver and gastrointestinal disease (Lien et al., 2022; & Lien et al., 2022), inflammatory skin disease, immune system deficiencies, and hypertension., additionally *A. indica* aerial parts such as leaves are employed to treat ailments, including rheumatism, epilepsy (Alam et al., 2000), paralysis, convulsions, spasms, pregnancy, fever, dyspepsia, stomach issues, and intermittent fever. These leaves were presumed to be more advantageous for psoriasis, chronic rheumatism, and skin eruptions. It has also been shown to possess insecticidal (Gul et al., 2022), analgesic, antipyretic, and antiphlogistic properties (Dharmasiri et al., 2003). Prior studies on *A. indica* showed radical scavenging, cyclooxygenase inhibitory activity, anti-inflammatory, anticancer and acetylcholine esterase inhibitory activity (Wang et al., 2021; Hsieh et al., 2008; Rao et al., 2009; Huang et al., 2012; & Basappa et al., 2016), extracts of *A. indica* also possess anti-epileptic, anti-nociceptive, anxiolytic, and sedative effects (Uddin et al., 2018). It has also been shown to possess antidepressant, antidiarrheal, and thrombolytic activities (Nasrin et al., 2022). The prior research was solely concerned with the extractions of leaves and other aerial parts employing various solvents. In light of the ethnobotanical and pharmacological attributes of the plant, the current investigation was designed to evaluate the phytochemical evaluation, TLC analysis, and biological activities exploration of *A. indica* extract, such as anti-inflammatory and thrombolytic activities.

MATERIALS AND METHODS

Chemicals

Solvents such as methanol, ethyl acetate, chloroform, and hexane were procured from SD Fine-Chem, India. Bovine serum albumin and Streptokinase were purchased from Sigma-Aldrich, Germany. Tris base was purchased from Lobachemie, India. In this current study, the analytical grade chemicals and solvents have been employed, and when needed, all laboratory chemical regent solutions are freshly prepared.

Plant collection and authentication

Pre-flowering *A. indica* plant leaves were harvested during monsoon from the field of Davangere University, Shivagangothri, Davangere, Karnataka state. Pusphalatha, professor of the Department of Botany,

Sahyadri Science College, Kuvempu University, Shivamogga, India, identified and validated the plant. For future use, the voucher specimen was deposited. The aerial part, like leaves, has been employed for the extraction.

Preparation of crude extracts

The leaves of *A. indica* are exploited for hot extraction. Fresh leaves were separated, washed, semi-shed, sun-dried for seven days, crumbled in a blender, ground into coarse powder material, sieved, and employed for solvent extraction (Basappa et al., 2016). 30 grams of leaf powder was employed for soxhlet extraction, obtained extracts were filtered, and recovered resultant filtrate was concentrated in Rota-evaporator (Evator, Medica Instruments, and Mumbai, India). The resultant concentrated filtrate was freeze-dried, resulting extracts were weighed, and the yield percentage was quantified and exploited to study its biological activity. The percentage of yield of different extracts, consistency, and colour were recorded (table 1).

Preliminary phytoconstituents evaluation of *A. indica* extracts

The standard test outlined in the literature were employed to validate the existence of phytoconstituents, including glycosides, alkaloids, phenolic, saponins, terpenoids, and steroids present in *A. indica* extract. The outcome of these qualitative tests was based on colour intensity and precipitate formation (Sonam Pandey, 2015 & Harborne, 1987).

TLC analysis (Thin layer chromatography)

TLC (Thin layer chromatography) is widely employed for separating phytochemical compounds in extracts as a fingerprint analytical technique. TLC can be utilized for identifying and quality control medicinal preparations and other applications in pharmaceutical industries. Separation of bioactive components in *A. indica* extracts was achieved by TLC (Thin layer chromatography) analysis (Nutan & Veena et al., 2019). The sample was spotted by using a micropipette on the precoated silica gel 60 F254 TLC (20X20 cm with 0.5mm thickness) plate along with the standards such as quercetin, tannic acid, and vanillin. The solvent system was standardized by PRISMA optimization procedure, the optimized mobile phase was ethyl acetate: methanol: chloroform (1:1:8), and the retention factor (Rf) of *A. indica* extracts are compared with standards tannic acid, quercetin and vanillin, the retention factor calculated (Rf) by the following formula.

$$RF = \frac{\text{Distance moved by solute front}}{\text{Distance moved by the solvent front}}$$

Anti-inflammatory activity *A. indica* extracts

The *A. indica* extracts of various concentrations, 20 µg to 100µg, were added to the test tube containing 1 ml of 10 mg/ml concentration of bovine serum albumin in PBS (Chandra et al., 2012). Using a small amount of 1N HCl, the reaction mixture pH was adjusted to 6.4. The reaction mixture was heated at 57^{o} C for 10 minutes, after the incubation at 37^{o} C for 20 minutes. The reaction mixture was brought to ambient temperature (25°C). The turbidly was measured spectrophotometrically at 660 nm along with the positive control aspirin. The control contains 2ml of PBS containing BSA. Triplicates of the test have been run. The results were expressed in terms of IC50 value, ascertained by plotting the percentage of inhibition of the protein denaturation inhibition against concentration.

$$\text{Percent of inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Thrombolytic activity of *A. indica* extracts

Healthy volunteers provided 5 ml of venous blood, which was collected in five distinct pre-weighed clean and sterile microcentrifuge tubes and incubated for 40 minutes at ambient temperature (37^o C). Following the development of a clot or thrombus, fluid has been drained off from each microcentrifuge tube without disturbing it, and the residual clot in every tube was then measured by weighing again in order to determine the actual clot weight (W_3) by subtracting the weight of clot-containing tube (W_2) from the weight of the tube alone (W_1). Negative control consisting of 100 µl of sterile deionized water was utilized. 100 µl of Streptokinase (SK) was employed as a positive control. 100 µl of each *A. indica* extracts in different concentrations (25, 50, 100 µg) was then separately added to the sterile micro-centrifuge tubes. Then, each tube was incubated for an additional 90 minutes at 37^o C to assess the clot lysis. Following incubation, the released fluid was completely discarded, and the microcentrifuge tubes were weighed once again to ascertain whether there had been a weight differential just after the clot was broken. The following formula has been employed to determine the per cent of clot lysis (Ogugofor et al., 2022).

$$\% \, clot \, lysis = \frac{Weight \, of \, clot \, released}{Clot \, weight} \times 100$$

Data analysis

Each test has been assessed thrice; data were tabulated as mean ± SD (standard deviation). IC_{50} (The IC_{50} value is the 50% of inhibition of its activity under the assay conditions) values from the *invitro* data were ascertained by regression analysis using Graph pad Prism software, MS-excel program.

RESULTS

***A. indica* extracts yield**

The impact of different solvents employed in the extraction had a different impact on the extractable yield. Chloroform, hexane, and ethyl acetate extracts were found to be 0.3 ± 0.1 g/ 30g, 0.9 ± 0.07 g/ 30g, and 0.6 ± 0.1 g/ 30g dry weight of plant material, respectively, which were lesser yields contrast to methanol extract of *A. indica*. After freeze-drying, it was revealed that 3.3 ± 0.18 g of *A. indica* methanol extract was obtained for every 30 per gram dry weights of plant material. The percentage of yield of extracts, consistency, and colour of *A. indica* extracts are represented in Table 1.

Table 1: Percentage of the yield of extracts

***A. indica* extracts**	**Percentage of yield** (g/ 30 **g of Dry weight)**	**Consistency**	**Colour**
Hexane	0.9 ± 0.07	Greasy	Black
Chloroform	0.3 ± 0.1	Sticky	Deep Green
Ethyl acetate	0.6 ± 0.1	Power	Dark brown
Methanol	3.3 ± 0.1	Sticky	Brown

Phytochemical screening of *A. indica* extracts

Bioactive phytoconstituents evaluation of *A. indica* extracts illustrated the existence of glycosides, alkaloids, tannins, phenolics, flavonoids, steroids terpenoids, and saponins, based on the visual appearance and precipitation formation resulting from the addition of chemical reagent, results were depicted in table 2.

Table 2: Phytochemical evaluation studies of *A. indica* extracts

Phytochemical tests	Hexane	Chloroform	Ethyl acetate	Methanol
Alkaloids	+	-	+	+
Steroids	+	+	+	+
Terpenes	+	+	-	-
Flavonoids	+	+	+	+
Tannins and phenolics	-	+	+	+
Saponins	-	-	+	+
Carbohydrates	-	-	+	+
Glycosides	-	-	+	+
Proteins and aminoacids	-	-	+	+

Table note: +: presence; -: absence

TLC (Thin layer chromatographic) analysis

The results of thin layer chromatographic analysis (TLC) have been depicted in Figure 1 and Table 3. The characteristic bands observed are denoted with band number. The degree of retardation factor for tannic acid was 0. 46, quercetin was 0.43, and vanillin was found to be 0.94. *A. indica* methanol extracts exhibit 8 distinct bands, and the Rf value was 0.05, 0.11, 0.18, 0.25, 0.41, 0.48, 0.56, and 0.97. Ethyl acetate extract showed 5 characteristic bands with Rf values such as 0.07, 0.22, 0.36, 0.72, and 0.89. Hexane extract possesses 2 bands with Rf values of 0.91 and 0.96. Each characteristic band represents one or more bioactive compounds present in the *A. indica*. The results confirmed the presence of bioactive components such as flavonoids, phenolics and other phytoconstituents.

Table 3: Thin layer chromatography analysis of *A. indica* extracts (Hexane, chloroform, ethyl acetate, and methanol)

Sr. No	Phytochemical extracts		Bands	Rf value
	Standard			
	Quercetin		0.46	
	Tannic acid		0.43	
	Vanillin		0.94	
	Hexane	Band H_1	0.91	
		Band H_2	0.96	
	Chloroform	-	-	
	Ethyl acetate	Band -E_1	0.07	
		Band –E_2	0.22	
		Band –E_3	0.36	
		Band –E_4	0.72	
		Band –E_5	0.89	
	Methanol	Band – M_1	0.05	
		Band - M_2	0.11	
		Band – M_3	0.18	
		Band – M_4	0.25	
		Band – M_5	0.41	
		Band – M_6	0.48	
		Band – M_7	0.56	
		Band – M_8	0.97	

Table note: Rf: Retardation factor

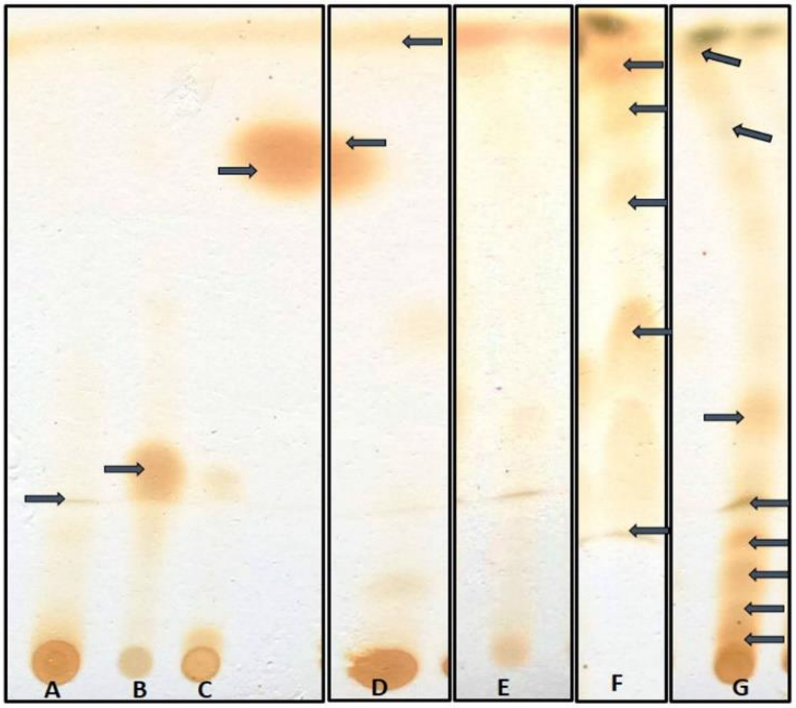

Fig. 1: TLC chromatogram of *A. indica* Chloroform extracts (a) Tannic acid; (b) Quercetin (c) vanillin; (d) Hexane extract; (e) Chloroform extract; (f) Ethyl acetate extract; and (g) Methanol extract.

Anti-inflammatory assay of *A. indica* extracts

In the anti-denaturation exploration of anti-inflammatory activity, bovine serum albumin was utilized. When BSA is heated, it undergoes denaturation, and autoantigens are expressed that are linked to a type-III hypersensitivity reaction, which is attributed to conditions like serum sickness, glomerulonephritis, rheumatoid arthritis, and systemic lupus erythematosus. One of the causes of rheumatoid arthritis and other chronic inflammatory diseases is protein denaturation. Protein denaturation may be the consequence of autoantigen production. A dose-dependent impact was observed in the *invitro* anti-inflammatory activity by the *A. indica* extracts. The IC_{50} ranged from 42.97 to 256 µg/ml. Significant anti-denaturation activity was observed in methanol and ethyl acetate extracts compared with the standard drug aspirin. IC_{50} value hexane, chloroform, ethyl acetate, methanol, and aspirin were 256 µg/ml, 52.16 µg/ml, 44.72 µg/ml, 42.97 µg/ml, and 42.87 µg/ml, respectively, suggesting that *A. indica* extracts exhibited potential anti-inflammatory activity depicted in figure 2. The results of this study are tabulated in Table 4. The *A. indica* extract's promising therapeutic effects lend credence to the claims made in folk medicine that can be utilized in rheumatism, arthritis, and other chronic inflammatory diseases.

Table 4: Anti-inflammatory activity of *A. indica* extracts.

Sr. No	Extract	IC_{50} (µg/ml)
	Anti-inflammatory activity of extracts	
1	Aspirin (Standard)	42.87
2	Hexane	256
3	Chloroform	52.16
4	Ethyl acetate	44.72
5	Methanol	42.97

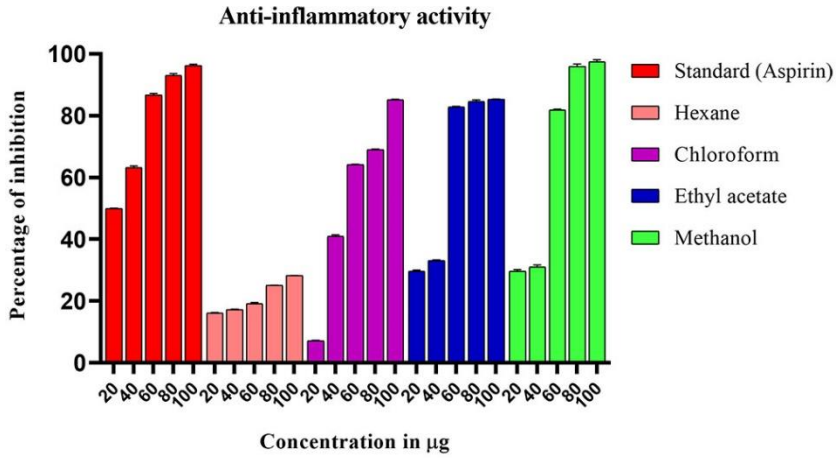

Fig. 2: Anti-inflammatory activity of extracts of *A. indica* at different concentrations (20-100 µg/ml) compared with standard drug Aspirin

Anti-thrombotic/ thrombolytic activity of *A. indica* extracts

The different extracts of *A. indicia* were investigated for thrombolytic potential by assessing the potential of clot lysis. Streptokinase (SK) was employed as a positive control, and it was revealed 45 ± 1.7% lysis per µg/µl was observed as contrasted to 3.4 ± 0.2% for distilled water served as a non-thrombolytic negative control. *A. indicia* extracts in this study displayed varying levels of thrombolytic capability, ranging from 2.6 ± 0.3% to 35.94 ± 0.2% (figure 3). Significant clot lysis activity was found in methanol extract (35.94 ± 0.2%), followed by ethyl acetate extract, hexane extract, and chloroform extract. Therefore, it can be concluded that the extracts of *A. indicia* showed lower thrombolytic activities compared to standard Streptokinase (45 ± 1.7%). The findings of this study are tabulated in Table 5.

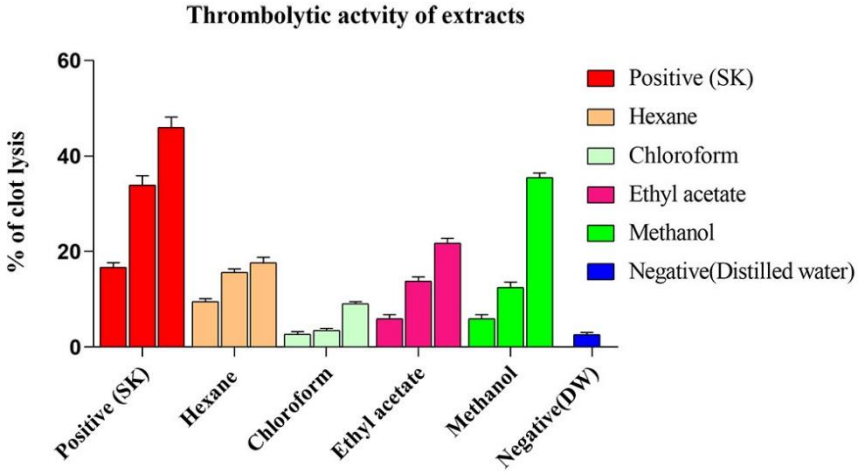

Fig. 3: Thrombolytic activity of extracts of *A. indica* at different concentrations (25, 50, and 100 µg/ml) compared with Streptokinase (Positive control) and Distilled water as non-thrombolytic (Negative control).

Table 5: Thrombolytic activity of A. indica extracts

Sr. No	Extract	Concentrations (µg/ml)	% of clot lysis
1	Distilled water	100 µl	3.1 ± 0.2
2	Strepto kinase	25	16.7 ± 0.7
		50	33.9 ± 1.6
		100	45.94 ± 1.7
3	Hexane	25	9.5 ± 0.4
		50	15.6± 0.5
		100	17.67± 0.9
4	Chloroform	25	2.6 ± 0.4
		50	3.5 ± 0.3
		100	9.0 ± 0.3
5	Ethyl acetate	25	5.9 ± 0.7
		50	13.7 ± 0.7
		100	21.7 ± 0.8
6	Methanol	25	5.9 ± 0.9
		50	12.4 ± 0.9
		100	35.47 ± 0.2

DISCUSSION

The percentage of the yield of *A. indica* extracts is shown in Table 1. Among all the extracts, *A. indica* methanol solvent extraction gives the highest per cent of the yield of extract due to its significant bioactive phytoconstituents extracting efficacy from the plant material. Phytochemical screening of *A. indica* extracts results in the existence of bioactive phytocompound such as terpenoids, carbohydrates, proteins, alkaloids, tannins, phenolics, flavonoids, steroids saponins, and glycosides and others illustrated in table 2. These preliminary phytochemical studies are supported by TLC analysis. Thin layer chromatography is an efficient, rapid, inexpensive analytical technique employed in the determination of the number of bioactive components in a mixture of compounds, determining the solvent composition for preparative analyses, confirming the identity and purity of a compound, following the development of a reaction, separation and analyses the fractions derived from column chromatography separations. The outcomes from TLC are trustworthy and repeatable. All the extracts were subjected to TLC profiling, and the results are remarkable and point to the presence of numerous phytochemicals. In optimized solvent systems, different phytochemicals produce different Rf values. A bioactive compound with a lower Rf value has quite high polarity, and a bioactive compound with a higher Rf value has lower polarity. Among all *A. indica*, methanol extract gives 8 characteristic bands contrary to other extracts such as ethyl acetate, methanol, and chloroform (Nutan & Veena et al., 2019).

In the anti-denaturation exploration of anti-inflammatory activity, bovine serum albumin was utilized. BSA denatures when heated, and denaturation of protein leads to auto-antigens production, which can induce conditions including systemic lupus erythematous, glomerulonephritis, rheumatoid arthritis, and serum sickness. Therefore, the ability of plant natural remedies like extracts to prevent the denaturation of protein tends to make them potentially useful for the formulation of anti-inflammatory drugs. The *A. indica* extracts exhibit a dose-dependent response in the BSA denaturation inhibition. The *A. indica* methanol extract effectively suppresses protein denaturation (IC_{50} = 42.97 µg/ml) (Nasrin et al., 2022). These effects are significant compared to those associated with the standard anti-inflammatory drug, aspirin (IC_{50} = 42.87 µg/ml). This outcome could be attributed to the abundance of bioactive constituents and polyphenols existence in these extracts of *A. indica,* which have been demonstrated to assist the anti-inflammatory

activity. Suggesting that A. *indica* extract's promising therapeutic potential lends credence to the claims made in folk medicine that can be utilized in rheumatism, arthritis, and other chronic inflammatory diseases.

The thrombolytic activity of extracts of *A. indica* was a part of the investigation into prospective cardioprotective compounds from plant reserves. In treating and preventing thromboembolic disorder, thrombolytic drugs, such as anticoagulants, are essential. A widely employed thrombolytic agent, Streptokinase acts by transforming more plasminogen into plasmin. However, Streptokinase has many undesirable health consequences, which influence the research to look for substitutes. Consequently, the investigation into plant-derived thrombolytic compounds has attracted much attention (Ogugofor et al., 2022). It can be shown that our outcomes may have important ramifications for cardiovascular health in the case of the thrombolytic test. The thrombolytic activity study aimed to examine the potential of *A. indica* extracts. A well-known thrombolytic drug called Streptokinase was employed as positive control. Contrarily, water was preferred as a negative control. The comparison of negative controls clearly illustrated that adding water to the clot did not cause it to dissolve. After treating the clots with extracts of *A. indica*, a significant amount of thrombolytic activity was observed when compared to the positive and negative control. The results are shown in the figure. 3, which displays 45 ± 1.7% clot lysis after 90 minutes of subsequent incubation at 37^{o} C, where 100 µl/ml streptokinase was added as a positive control contrasted to 3.4 ± 0.2% for distilled water served as a non-thrombolytic negative control. *A.indica* extracts in this study displayed varying degrees of thrombolytic potential, ranging from 2.6 ± 0.3% to 35.94 ± 0.2%. Significant clot lysis activity was observed in methanol extract (35.94 ± 0.2%), which was similar results to the earlier reports (Nasrin et al., 2022). Therefore, the outcome of the study suggests that the extracts of *A.indica* showed lower thrombolytic activities compared to standard Streptokinase (45 ± 1.7%).

CONCLUSION

In the proposed study, it was discovered that the variation and diversification in the phytochemical content have been attributed to the biological characteristics of plants. Phytochemical evaluations explore that *A. indica* covers a broad array of secondary metabolites, some of which have a wide range of therapeutic potential, including antioxidant and anti-inflammatory properties. The occurrence of diverse secondary metabolites, including alkaloids, tannins, phenolics, flavonoids, steroids, terpenoids, saponins, and glycosides in hexane, chloroform, ethyl acetate, and methanol, connected to ethnomedicinal importance. Anti-inflammatory and thrombolytic potentials of *A. indica* would gain attention in therapeutic management.

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Conflict of interest

The author has no conflict of interest

Author contribution

MT: performed work, VK: manuscript revision and design, DVP: Data analysis, PHM: Experimental designing, SRM: Statistical analysis, RF: TLC analysis, VE: Plant collection, we ensure and hereby declare that all authors have read and approved the manuscript.

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