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Impact Factor: 4.428

Study of Antimicrobial and Anti-Thrombolytic Effects of Chloroform Fraction Obtained from Ultrasound Treated Fresh Leaves of *Mikania cordata*

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Abstract
Mikania cordata belongs to the family Asteraceae is locally known as Asham lata recognised as one of the worst weeds of the world. Traditionally the plant is a popular medicinal plant among different tribal medicinal practitioners for its many proven pharmacological effects. In the present study the chloroform fraction from aqueous fresh leaves extract obtained by ultrasound treatment was used for antimicrobial and anti-thrombolytic study. . Ultrasound assisted extraction is a comparatively novel extraction method which complies with most of the green extraction principles. Phytochemical study indicated that the chloroform fraction successfully isolated alkaloid, flavonoid, glycoside, terpenoids etc. from the wider mixing observed in its aqueous extract. The crude extracts showed promising sensitivity on the shigella group of bacteria which are generally responsible for GIT related discomfort including diarrhea, dysentery etc. The chloroform extracts also showed a promising clot lysis effect on humans. The plant may have a promising source for developing antimicrobial and thrombolytic medicine.

Key words: *Mikania cordata, Asham lata, Jerman lata, Green extraction, Ultrasound*

Introduction:

The plant *Mikania cordata* (Burm. f.) Robinson of the family Asteraceae is termed as Bitter vine in English and locally recognised as Asham lata [1-2], German lata [3], Kelakhi [4], Rifjhe [5], Tara lata [3], Zaganlau [2] etc. in Bangladesh. The plant is widely distributed throughout the tropical regions of Asia, Africa, America etc. [3, 6-7] which is listed in the top three among the worst weeds of the world [8]. This plant is well recognised due to its devastating spreading nature and suppresses the growth of other plants by shading light and smothering them. Sometimes this plant inhibits and slows the seedling growth and germination in some crops by releasing chemicals of high allelopathic properties [8].

The plant *M. cordata* is a well-recognised medicinal plant used by the folk medicinal practitioners in Bangladesh for the treatment of gastric ulcer and local antiseptic, however literature survey indicates several other medicinal applications including jaundice, fever, colds, dysentery, rheumatism, respiratory diseases, and scorpion stings and also reported as good haemostatic agents [9]. Several ethnobotanical survey in Bangladesh found the popular uses of *M. cordata* in different tribes such as Chakma [10-11], Garo [5], Khumi [12], Lushai [2], Marma [12], Santal [1], Tripura [4,12] etc. Most of the above tribes prefer the leaves paste on cuts and wounds to stop bleeding and the leave juice for chest pain after eating, acidity, and dysentery.

The objective of the present study was to compare the antimicrobial properties of the chloroform fraction and aqueous fraction obtained by ultrasound treated aqueous extraction from fresh leaves of *M. cordata*. Ultrasound assisted extraction (UAE) is a comparatively noble well accepted green extraction method first introduced at the end of the last century. The extraction mechanism is comparatively simple as the ultrasound treatment breaks down the cell wall of the plant components and facilitates the extraction of all components in the cell to the surrounding solvents [13-16]. Several studies indicated that better extraction may be possible in the aqueous system from the fresh plant parts with the help of ultrasound [17-21]. The limitation of this process is that mixing of all possible components inside the cell may be present in the crude extracts, making it inappropriate for medicinal application. Fractional separation by using chloroform, ethyl acetate, dichloromethane, diethyl ether etc. may easily separate the particular type of components from the crude extract on the basis of their solubility index [22]. In this study chloroform was used for separation and pharmacological efficacy was compared with the aqueous crude extracts by antimicrobial sensitivity study on different pathogenic microorganisms connected to the GI tract discomfort. The chloroform fraction was also used to see its thrombolytic (blood clot lysis) properties [9, 23], as several previous studies found such properties in their ethanol extract from *M. cordata* leaves. The ultimate goal of this technique is to improve the existing green extraction procedure for isolating a defined class of phytochemicals from the plants within a short period of time.

Materials and Methods:

Preparation of Crude Extracts:

Healthy fresh leaves of *Mikania cordata* (or Asham lota) were collected from the field before sunrise and immediately washed by the running tap water and then distilled water. The surface water from the clean leaves dried under circulating air. Within 6 hours 100 gm of leaves were taken in a conventional juice blender machine and a uniform fine juice was prepared by adding distilled water q.s. to 500 ml [17-21]. The whole juice was passed through a 20 mesh size net and transferred to a 500 ml conical flask. The whole juice was treated by ultrasound in an ultrasonic bath (Power Sonic 405) for 30 minutes at 40^{o}C bath temperature.

The mixture was then filtered by three layers of cloth and the filtrate was equally divided into Part-A and Part-B. Solution of Part-A were dried at 55^{0}C temperature in a water bath and named the extraction process is Aqueous Ultrasound Assisted Extract (UAE) from fresh leaves of *M. cordata*. Crude extracts obtained from this method basically contain all possible components present in the cell.

On the other hand, 25 ml chloroform was added to the filtrate of Part-B, mixed vigorously and transferred to a separating funnel (250 ml capacity). The poor solubility of chloroform (0.795×10^{6} µg/L water at 25^{0C}) [24] in the water allows them a rapid separation after mixing. The solution was kept undisturbed and the organic portion was collected minutely. A drastic variation of polarity index (Pi) of water (Pi = 10.2) [25] and chloroform (Pi = 4.1) [26] make drastic changes in their absorption pattern. So, by applying the fractional separation of chloroform may successfully separate a particular group of components from the aqueous mixture. The process was repeated three times and the organic phases (chloroform) were added together and dried under a fume hood. The process was named as the chloroform fraction of Aqu. UAE from fresh leaves of *M. cordata*. Through this extraction procedure terpenoids, flavonoids, alkaloids etc. related bioactive components may be separated out from the mixture [22]. Extraction yield was calculated as per equation 1. [27]

$$\% \text{ Extraction yield} = \frac{W2}{W1} \times 100 \ldots\ldots\ldots \text{Equation 1}$$

Where, W1: Weight of starting plant materials

W2: Weight of crude extract after drying

Phytochemical Screening Test:

Qualitative phytochemical tests for alkaloids (Dragendroff test and Mayer test), anthraquinones (addition of ammonia solution to the sample create a bright pink color in the aqueous layer), flavonoids (addition of H_2SO_4 to the sample develop a yellow color which may be disappeared on standing), glycosides (addition of glacial acetic acid, ferric chloride and H_2SO_4 create a brown ring in the interface), saponins (vigorous

shaking create a stable persistent froth), steroids (addition of acetic anhydride and concentrated H_2SO_4 to the sample changes the color from violet to blue), tannins (addition of ferric chloride turns blue-black coloration of the sample), terpenoids (addition of $CHCl_3$ and H_2SO_4 to the solution make a reddish brown coloration in the interface) and vitamin C (addition of sodium nitroprusside, NaOH and HCl in the solution turns blue color) were performed as per the procedure of Allen and Harborne with slight modification and successfully applied in different previous publications [17,19-21].

Antimicrobial study:

In the present study four pathogenic microorganisms connected to the GIT related disease such as *Shigella boydii*, *Shigella flexneri*, *Shigella sonnei* & *Shigella dysenteriae* are normally responsible for abdominal cramping, diarrhea, watery diarrhea, dysentery, nausea, rectal pain, blood, mucus, or pus in the stool etc. Disc diffusion method [17, 29-31] was used for studying the sensitivity of the crude extract. For comparison, both extracts were applied in the same plate with two different doses of 300µg/disc and 500µg/disc in the surface of the inoculated nutrient agar with the aid of sterilized pair of forceps. A pre-diffusion time of 30 minutes was allowed for the extracts to diffuse from the discs into the agar medium before incubation. The degree of sensitivity of the organisms to the extracts was determined by measuring the diameter of visible zones of inhibition to the nearest millimeter.

Thrombolytic assay:

Thrombolytic effect of the chloroform fraction of Aqueous UAE from fresh leaves of *M. cordata* was studied in comparison to the standard thrombolytic drug streptokinase (SK). Commercially available lyophilized SK vial of 15,00,000 I.U was dissolved in 5 ml of distilled water and used 100 µl at a dose 30000 I.U./100 µl to the 1 ml equivalent blood clot for lysis-which was termed as the positive control [23]. Similarly 30 mg crude drug of CF was suspended in 10 ml of distilled water, shaken vigorously on a vortex mixture and the suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a filter paper. 100 µl of CF crude extract at a dose 300 µg / 100 µl was applied to the 1 ml equivalent blood clot for lysis-which was termed as the experimental group. Only 100 µl of distilled water was applied to the 1 ml equivalent blood clot-which was termed as control.

Blood samples (5 ml) were collected from a group of healthy volunteers (n=4, 2 male and 2 female) without a history of oral contraceptives or anticoagulant therapy. 3 ml of blood was transferred to the previously weighted and 3 (marked in positive control, experiment and the control) sterile eppendorf tubes (1 ml in each). The eppendorf tubes were placed in the incubator at 37°C for 45 minutes to form a clot. After clotting, the serum was completely removed without disturbing the clot and calculating the weight of the clot as per equation-2 [23]. 100 µl aqueous solutions of crude extract (300µg / 100µl CF), 100 µl of streptokinase (30,000 I.U/100 µl) solution add to the positive control, and 100 µl distilled water to the negative control. All the eppendorf tubes were then incubated at 37°C for 90 minutes and the released fluid was removed carefully and again calculated the weight of the clot as per equation-2. Difference obtained in weight taken before and after clot lysis was expressed in percentage as per equation 3 [23].

Clot weight = (Weight of clot containing tube) – (Weight of tube alone) …….. Eq. 2
% clot lysis = (Weight of the lysis clot / Weight of clot before lysis) × 100 ….Eq. 3

Result and discussion:

Crude extract obtained from Aqueous Ultrasound Assisted Extraction from fresh leaves of *M. cordata* (Part-A) and its chloroform fraction (Part-B) were studied in the present study. It was observed 20.52±2.77% yield from Part-A, whereas only 3.16±1.11% yield obtained from Part-B (chloroform fraction) which was around 15% of the total aqueous extract (Table 1). After drying the crude extract obtained from Part-A had a dark green color whereas its chloroform fraction had a light black color. Both dried extracts (Part-A and Part-B) were found hard and very much sticky to the beaker and needed methanol assistance to withdraw from the beaker surface. Phytochemical screening observed the presence of alkaloid, anthraquinones, flavonoid, glycoside, saponin, tannin, terpenoid, vitamin-C in the Part-A, whereas only the presence of alkaloid, flavonoid, glycoside and terpenoids were observed in the Part-B (Table 2). Results indicated that chloroform fraction successfully separated the desired components in a considerably large amount. Andrian

et al., and Matawali et al., also observed several variations of components in their ethanol, n-hexane, ethyl acetate, n-butanol and water fraction [32]. The antimicrobial activities of both parts were assessed on four gram negative bacteria in the Shigella class generally responsible for diarrhoea and dysentery related GIT discomfort. Both extracts showed considerable desired antimicrobial sensitivity, however, chloroform fraction (Part B) performed better compared to the aqueous fraction (Part A) at two distinct doses (300 µg/disc and 500 µg/disc) level (Table 3). The present study indicated that both the aqueous and chloroform fraction obtained from ultrasound treated fresh leaves of *M. cordata* have considerable pharmacological action (Chart 1) on gram negative bacteria specially on Shigella class. Several previous study observed that methanol extract [33], ethanol extract [32], ethyl acetate [34] of *M. cordata* leaves had a promising antimicrobial effect on several gram positive and gram negative bacteria such as *P. aeruginosa* [33], *S. aureus* [33-34], *S. pyogenes* [34], *E. coli* [32-33], *S. pneumonia* [33], *S. typhii* [33]. Patar and Yaliaya discovered the presence of acetic acid contents in the *M. cordata* through GCMS and presumed the reason behind the high antimicrobial properties of that extract [35]. The chloroform fraction of aqueous UAE from *M. cordata* was also shown to have thrombolytic properties. In the present study 30µg extract was used to lysis 1 ml blood equivalent clot and compared to the 30000 I.U of standard drug streptokinase. Though the crude extracts showed promising clot lysis effects, it was not equivalent to the standard drug streptokinase. Dose of crude extracts may be increased and need adjustment with the standard drug is desirable in the further study.

Table 1: Extraction (%) yield obtained from ultrasound treated fresh leaves of M. cordata

Trial No.	Starting material (gm)	Part A: Aqueous Extract		Part B:Chloroform Fraction from Part A		
		Dry Weight of crude extract (gm)	Percentage of yield	Dry Weight of crude extract (gm)	Percentage of yield	Respective percentage of the Part A
1	50	9.2	18.4	1.6	3.2	17.39
2	50	10.2	20.4	1.2	2.4	11.76
3	50	12.4	24.8	2.3	4.6	18.55
4	50	10.6	21.2	1.9	3.8	17.92
5	50	8.9	17.8	0.9	1.8	10.11
			20.52±2.77		3.16±1.11	15.15±3.91

Table 2: Presence of phytochemicals in the crude extracts obtained from ultrasound treated fresh leaves of *M. cordata*

Extract	Alka-loids	Anthra-quinones	Flavo-noids	Glycol-sides	Sapo-nins	Ster-oids	Tann-ins	Terpe-noids	Vita-min C
Part A	+	+	+	+	+	-	-	+	+
Part B	+	-	+	+	-	-	-	+	-

Here, Part-A: Aqueous Ultrasound Assisted Extraction from fresh leaves of *M. cordata*
Part B: Chloroform fraction from Part-A

Table 3: Antimicrobial activities of the crude extracts obtained from ultrasound treated fresh leaves of *M. cordata*

Microorganism (Gram –ve bacteria)	Extract contents (300 µg/disc)		Extract contents (500 µg/disc)	
	Part A	Part B	Part A	Part B
S. boydii	12.33±3.79	13.33±4.51	18.00±3.61	21.67±5.86
S. flexneri	11.67±3.06	14.67±3.79	19.67±3.79	21.00±6.24
S. sonnei	11.00±3.61	15.33±5.13	16.67±3.21	22.67±5.13
S. dysenteriae	12.67±3.51	16.33±4.51	17.67±4.73	21.67±5.03

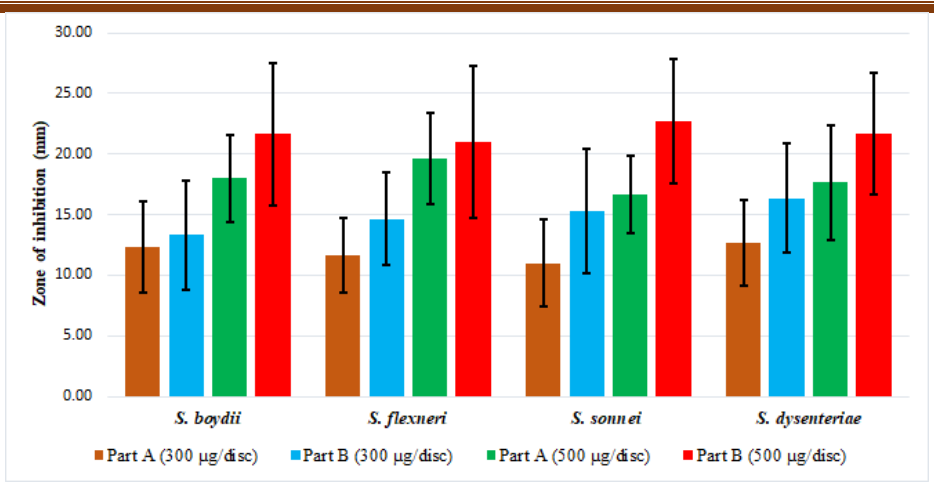

Chart 1: Comparison of antimicrobial sensitivity of aqueous and chloroform fraction obtained from ultrasound treated fresh leaves of *M. cordata*

Table 4: Thrombolytic effects of the chloroform fraction obtained from ultrasound treated fresh leaves of *M. cordata*

Sample	Dose	% of clot lysis Mean ± SD (n=4)	P
Control (C)	100 µl distil water/ml blood	3.5 ± 1.291	-
Streptokinase (Std.)	30000 I.U./ ml blood	71.5 ± 5.508	$P_{C=Std.}$ 0.000
Chloroform extract (Exp.)	30 µg/1 ml blood	37.25 ± 6.85	$P_{C=Exp..}$ 0.003 $P_{Std=Exp..}$ 0.002

$p \leq 0.05$ indicate significant difference

Conclusion:

Mikania cordata is generally recognised as an abundant weed which has an opportunity to become a lifesaving medicinal plant by its wise application for the treatment of different pharmacological emergencies. The present study observed antimicrobial effects of *M. cordata* leaves against the Shigella group of bacteria, and at the same time showed promising thrombolytic effects. The above findings justify the use of *M. cordata* leaves juice for the treatment of different bacterial infections especially in the stomach. The intended results also justify the regular consumption as green tea may be beneficial for the cardiac patients who have a previous record of heart attack or stroke and regularly medicated by blood thinning drugs such as aspirin. In the present study a new extraction approach was applied to facilitate the overall extraction procedure under a safe and green environment. In this study a very small amount of organic solvents were used for getting a considerably high amount of desired phytochemicals will make the process user friendly, less expensive and will be recognised as a green extraction method.

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