VALIDATION OF AQUEOUS ULTRASOUND ASSISTED EXTRACTION METHOD BY USING FRESH LEAVES OF *MORINGA OLEIFERA* WITH CONVENTIONAL EXTRACTION METHOD.

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Abstract
Aqueous extraction from fresh leaves of Moringa oleifera by Ultrasound Assisted Extraction (UAE) method was proposed in the present study. The proposed method was occupying most of the features of the green extraction method described in different literature. From successive five batches, the efficiency and efficacy of the proposed extraction method was compared with the conventional extraction method and found satisfactory performance. The average % yield (extraction efficiency) of the proposed method was observed 17.72± 1.67% statistically similar to the conventional methanol and ethanol extraction method. However, presence of tested phytochemicals were observed higher than the conventional method. Antimicrobial study (zone of inhibition or efficacy of the crude extracts) of the proposed UAE method was performed against Staphylococcus aureus (14.8 ± 2.20 mm), Streptococcus pyogenes (17.3 ± 3.20 mm), Escherichia coli (15.5 ± 2.55 mm) and Shigella boydii (19.4 ± 2.99 mm) and found statistically similar antimicrobial activities with the conventional methanol and ethanol extracts. Based on the experiment the Aqueous UAE method may be standardized as an effective green extraction procedure.
Key words: *Moringa oleifera*, Green extraction, Ultrasound Assisted Extraction (UAE).

1. Introduction:

Moringa oleifera is the cultivated species of the genus Moringa which is one of the 14 species of family Moringaceae (Malki and Rabey, 2015; Usman *et al*., 2018). It is commonly known as drumstick-tree or horse radish tree (Aja *et al*., 2014) which is native to sub-Himalayan tribes of North West India, Pakistan, Bangladesh and Afghanistan (Ogundele and Fadeyi, 2015). Locally this plant is known as Sojina in Bangladesh and widely distributed all over the country. The pod of the plants is used as a popular vegetable in Bangladesh. The plant is a short, slender, deciduous, perennial tree which is about 10 m tall. Feathery pale green leaves are accumulated in 30-60 cm long compound tripinnate. Its branches and stem are brittle in nature with corky bark. Pods (30-120 cm long) are pendulous, brown, triangular, splitting lengthwise into 3 parts when dry (Ajayi and Fadeyi, 2015). Moringa has diversified medicinal value and considered as one of the world's most useful trees, as almost every part of the tree, including leaf, fruit (pods), flowers, seed, root, bark, gum, and seed oil have been used for various ailments in the indigenous medicine as well as food (Ajayi and Fadeyi, 2015; Biswas *et al*., 2016). Moringa leaf has been purported to be a good source of nutrition which is referred to as 'superfood' because of its impressive nutritional profile and has been used successfully in some countries as a remedy for malnutrition (Onyagbodor and Aprioku, 2017; Usman *et al*., 2018). Phytochemically this plant is reach of flavonoids, tannins, anthraquinones, cardiac glycosides, alkaloids, triterpenoids, saponins, reducing sugars etc. (Tende *et al*., 2011; Nweze *et al*., 2014). In the present study the leaves of *M. oleifera* was chosen to validate the proposed green extraction method named "aqueous Ultrasound Assisted Extraction (UAE) from fresh leaves".

Ultrasonic extraction is very useful for the isolation and purification of bioactive principles (Ishtiaq *et al.,* 2009). The basic principle of this extraction is to application of high-intensity, high-frequency sound waves and their interaction with the plant's materials. Ultrasound eventually breakdown the cell wall for driving out all polar and non-polar compounds present in the plant's cell. As per the present study, a simple distill water was used as solvent to convert this extraction procedure as environment friendly and cost effective. The extraction method was further optimized by using fresh leaves juice instead of dried leaves powder to reduce the overall extraction process (Sadat *et al*., 2019). Water is a universal solvent which is less hazardous and cheaper than the organic solvents. The proposed method successfully comply several features of "six principles of green extraction of natural products" (Chemat *et al.,* 2012) and may be suitable for extraction both in laboratory and industrial purpose. As the literature survey did not yield any reference about earlier reports on the aqueous UAE extraction from *M. oleifera* fresh leaves. The objective of the present study was to standardize the aqueous UAE from fresh leaves of *M. oleifera* in comparison with the conventional methanol and ethanol extraction.

2. Materials and Methods

2.1. Collection of Plant Material

The leaves of *Moringa oleifera* was collected from Botanical Pesticide Garden of the Institute of Environmental Science (IES) of Rajshahi University (RU), Bangladesh. The plant was identified by the professional taxonomist of the Department of Botany, RU and a voucher specimen was deposited at the herbarium of the institute.

2.2. Extraction Procedure

Fresh and shade dried leaves was used in the study for comparison (Cheenickal and Mendez 2017). Fresh leaves of *M. oleifera* was washed properly by running tap water followed by distilled water and allowed for shade drying of the surface water (Francine *et al*., 2015). After 6 hours, 200 gm fresh leaves were taken and divided into four parts (Part A, B, C and D), 50 gm in each. Part-A: fresh leaves were used immediately for UAE aqueous extraction whereas Part-B, Part-C and Part-D: fresh leaves were allowed for week long drying. After grinding 50 gm fresh leaves of Part-B, Part-C and Part-D reduced to 21.9 gm, 23.3 gm and 22.6 gm respectively allowed for aqueous UAE, methanol and ethanol extraction.

Fresh leaves of Part-A of *M. oleifera* was blended in a conventional juice machine with 250 ml distilled water (material solvent ratio 1:5) for better extraction (Toma *et al.*, 2001). The juice was transferred to a 500 ml conical flask and placed in an ultrasonic bath for 30 minutes treatments at 40ºC bath temperature (Sadat *et al*., 2019). Fine powder of dried leaves (Part-B) was mixed with 109.5 ml distilled water (material solvent ratio 1:5) (Toma et al., 2001) and treated in ultrasonic bath as like Part-A. Power Sonic 405 (Microprocess Controlled Bench Top Ultrasonic Cleaner) was used for sonication. The plant extract was then filtered through three layers of polyester cloth and dried at 60ºC in a conventional water bath. Dried crude extract of aqueous UAE of Part-A and Part-B was then stored in an air tight bottle labeled as "MO-1-A" and "MO-1-B" respectively and preserved in cold chamber for further use.

Fine powder of dried leaves of Part-C and Part-D were soaked in 116.5 ml of methanol and 113 ml of ethanol in a conical flask in a ratio 1:5 for 72 hours with intermittent shaking as per standard method (Hussain and Hussain, 2012; Bashir *et al*., 2014; Latha *et al.,* 2015). The plant extract were then filtered by Whatman No.1 filter paper and concentrated by rotary evaporator under reduced pressure (in vacuum at 40ºC). The dried methanol (Part-C) and ethanol (Part-D) extract was then stored in an air tight bottle labeled as "MO-1-C" and "MO-1-D" and preserved in cold chamber for further use.

The whole procedure was repeated five times for using Mean ± S.D. for measuring statistical significance. SPSS 16.0 was used for statistical analysis.

2.3. Percentage of Yield Calculation

The percentage of yield indicate the efficiency of the extraction procedure which was calculated by using the following formula (Terblanche *et al*., 2017)

$$\%\, Yield = \frac{(W1\ x\ 100)}{W2} \text{ ---------- Eq. 1}$$

Where, $W1$: weight of dried crude extract and $W2$: weight of the plant starting material for extraction

2.4. Phytochemical Screening Test

Qualitative phytochemical test indicate the extraction efficiency of the crude extract. In the present study phytochemical tests were performed in the following way;

Alkaloids: (I) Dragendoff's test: Mother solution + 2% of H_2SO_4 + Heat + few drops Dragendoff's reagent → Orange red precipitate [Trease and Evans, 1989; Ajayi and Fadeyi, 2015]; (II) Mayer's test: Mother solution + 2% of HCl + Heat + few drops Mayer's reagent → turbidity or yellow precipitation [Ajayi and Fadeyi, 2015; Dash *et al*., 2017]

Anthraquinones: Mother solution + benzene or chloroform + 10% (v/v) ammonia solution → pinkish or color change [Ayoola *et al*., 2008; Ajayi and Fadeyi, 2015]

Flavonoids: (I) Mother solution + dilute ammonia Solution + Conc. H_2SO_4 → yellow coloration that disappear on standing [Ayoola *et al*., 2008]; (II) Mother solution + few drops of 1% aluminium solution → yellow coloration [Ayoola *et al*., 2008]

Glycosides: Mother solution + 3 ml of glacial acetic acid + 1 drop of 5% ferric chloride Solution + 0.5 ml of Conc. H_2SO_4 →Brown or blue ring of the interface [Ayoola *et al*., 2008; Dash *et al*., 2017]

Saponins: Mother solution + equal volume water + vigorous shaken→ foam stable more than 10 minutes [Dash *et al*., 2017]

Tannins: Mother solution + 1% $FeCl_3$ solution → dark green color [Maxson and Rooney 1972; Hazali *et al*., 2015]

Terpenoids: Salkowski test: Mother solution+ 2ml Chloroform+ Carefully added 3 ml Conc. H_2SO_4 to form a layer → reddish brown color of the interface [Ayoola *et al*., 2008]

2.5. Antimicrobial Activity

Antimicrobial study is a well-known in-vitro method for measuring efficacy (pharmacological activity) of the crude extracts and may be used for comparison of extraction procedures. Antimicrobial study was done by the disc diffusion method (Baker *et al*., 1993; Mukhtar and Tukur, 2000; Bauer *et al*., 1966; Servan *et al*., 2011; Latha *et al*., 2015) on two Gram +ve bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* and two Gram –ve bactera *Escherichia coli* and *Shigella boydii*. Microorganisms were collected from the Microbiology Lab, Department of Biochemistry and Molecular Biology, Rajshahi University, Bangladesh. Nutrient agar media was used for sub-culturing bacteria at 37°C. The filter paper discs (sensitivity discs) impregnated with the 300 µg/disc of extracts was then placed on 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 diameter of visible zones of inhibition to the nearest millimeter. The observed result of clear zone in petridish was compared to the standard zone of inhibition: <8 mm = no sensitivity; <10 mm= insignificant sensitivity; 10-15mm = moderately sensitive; >16mm = highly sensitive (Mukhtar and Okafor, 2002). The procedure was repeated twice for each batch and average result was counted for statistical analysis.

3. Results and Discussion

Crude extract obtained from aqueous UAE from fresh leaves of *M. oleifera* (Part A) was observed greenish in color (Table-1) having yield value 17.72± 1.67% (average of 5 separate batch) presented in Table-2. Similar color was also observed in case of crude extracts obtained from Part-B but the yield value (19.92± 2.37%) was slightly higher than previous one. Methanol (Part-C) and ethanol (Part-D) crude extracts showed blackish in color having yield of 16.27 ± 1.22% and 17.97± 1.71% respectively. The difference between the % yields of four type of extraction was insignificant at 5% level of significance (Table-2). Mahdi *et al.* (2016) observed 25.022, 38.196 and 37.838%, yield from 95% ethanol, 50% ethanol and water extracts respectively by using 1:10 dried powdered leaves to solvent ratio at 45^{0}C for 48 hours. Compared to the above results the present ethanol and aqueous extraction was providing fewer yields. Okumu *et al.* (2017) found 14.23% yield from water and 17.51% yield from methanol (80%) extract which was similar to the present yield. On the basis of above results the present extraction method was proved almost similar to the conventional organic solvent extraction method.

Dissolution studies indicated that crude extract of Part-A was instantly soluble in water whereas freely soluble in methanol, ethanol and chloroform but sparingly soluble in DMSO, ethylacetate, dichloromethane (Table-3). Almost similar solubility profile was also observed in case of Part-B crude extract. Part-C and Part-D crude extracts were observed instantly soluble in methanol and ethanol whereas freely soluble in water and sparingly soluble in DMSO, ethylacetate, dichloromethane. Solubility profile of Part-A and Part-B indicated that the crude extracts contained both polar (hydrophilic) and non-polar (lipophilic) compounds, which justify the use of UAE for extraction of wide range of compounds from the plant materials.

Table 1: Physical observation of the dried Crude Extract

Crude Extracts	Optical Observation	Texture
A	Greenish color	Hard and sticky to the beaker
B	Greenish color	Hard and sticky to the beaker
C	Black	Soft and easy to withdraw
D	Black	Soft and easy to withdraw

Here, A: Aqueous UAE from Fresh leaves, B: Aqueous UAE from dried leaves, C: Methanol extraction from dried leaves and D: Ethanol extraction from dried leaves

Table 2: Percent (%) Yield of different types of extraction method

Batch No.	Weight (gm) of leaves				Weight (gm) of Crude Extract				% Yield			
	A	B	C	D	A	B	C	D	A	B	C	D
1st	50	21.9	23.3	22.6	8.8	3.8	3.9	3.9	17.60	17.35	17.26	16.74
2nd	50	23.2	21.6	20.9	9.6	4.2	4.2	3.2	19.20	18.10	15.31	19.44
3rd	50	25.8	24.5	24.6	7.9	5.2	4.8	4.4	15.80	20.16	17.89	19.59
4th	50	20.1	20.4	21.7	9.8	4.7	3.2	3.3	19.60	23.38	15.21	15.69
5th	50	22.3	22.3	23.6	8.2	4.6	4.1	3.7	16.40	20.63	15.68	18.39
Mean ± S.D.	50 ± 0	22.6 ± 2.09	22.42 ± 1.57	22.6 ± 1.47	N/A	N/A	N/A	N/A	17.72 ± 1.67	19.92 ± 2.37	16.27 ± 1.22	17.97 ± 1.71
P	N/A	N/A	0.68[a]	0.98[a]	N/A	N/A	N/A	N/A	N/A	0.14[b]	0.24[b]	0.86[b]

Here, A: Aqueous UAE from Fresh leaves, B: Aqueous UAE from dried leaves, C: Methanol extraction from dried leaves and D: Ethanol extraction from dried leaves

[a] Significance level compared to B ($p>0.05$, indicated insignificant difference)

[b] Significance level compared to A ($p>0.05$, indicated insignificant difference)

Table 3: Dissolution pattern of the dried crude extract

Solvent	Crude Extracts			
	A	B	C	D
Distilled water	+++	+++	++	++
Methanol	++	++	+++	+++
Ethanol	++	+	+++	+++
Chloroform	+	+	++	++
DMSO	+	+	++	++
Ethylacetate	+	+	+	+
Dichloromethane	+	+	+	+

Here, A: Aqueous UAE from Fresh leaves, B: Aqueous UAE from dried leaves, C: Methanol extraction from dried leaves and D: Ethanol extraction from dried leaves

+++ Instantly soluble; ++ Freely soluble (1-3 minutes); + Sparingly soluble (> 5 minutes and vigorous shaking to dissolve)

Phytochemical screening showed that crude extract from Part-A and Part-B of *Moringa oleifera* contained most of the tested compounds including alkaloid, anthraquinones, flavonoid, glycoside, saponin, tannin, terpenoid except steroid (Table-4). Observation also showed that saponin and steroid were absent in methanolic extract (Part-C) whereas saponin, steroid and terpenoid were absent in ethanolic extract (Part-D). Previous study of Ajayi and Fadeyi (2015) reported the absence of steroid in their aqueous leaf extract. Castillo *et al.* (2013) reported absence of tannin and saponin in aqueous and ethanolic leaves extract. Fahal *et al.* (2018) observed that absence of saponin in aqueous extract, tannin in ethanolic extract. Through phytochemical screening it was observed that ultrasound assisted extraction may be a suitable extraction procedure instead of conventional extraction procedure. Findings also indicated that drying stages of plants may be overlooked as similar compounds extracted both from the fresh and dried plant's parts during ultrasound treatment.

Crude extracts of Part-A, Part-B, Part-C and Part-D of *M. oleifera* leaves was found promising antimicrobial agent observed from two successive antimicrobial study from each batch of extract on *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli* and *Shigella boydii* (Chart-1). Statistically no significant differences were observed on the studied bacteria indicated the same efficacy of those four extracts (Table-5).

Table 4: Phytochemical screening of UAE and conventional crude extracts

Phytochemical Tests	Crude extract			
	A	B	C	D
1.Alkaloid,(i) Dragendroffs' test	+	+	+	+
(ii) Mayer's test	+	+	+	+
2. Anthraquinones	+	+	+	+
3. Flavonoid (i): by H_2SO_4	+	+	+	+
(ii): by aluminum	+	+	+	+
4. Glycoside	+	+	+	+
5. Saponin	+	+	-	-
6. Steroid	-	-	-	-
7. Tannin	+	+	+	-
8. Terpenoid	+	+	+	+

Here, E1: Aqueous UAE from Fresh leaves, E2: Aqueous UAE from dried leaves, E3: Methanol extract from dried leaves and E4: Ethanol extract from dried leaves

Here, (+) indicated presence of compound, and (–) indicated absence of compound

Table 5: Antimicrobial activity of UAE and conventional crude extracts

Batch and test no	**Zone of Inhibition (mm)**															
	Staphylococcus aureus				***Streptococcus pyogenes***				***Escherichia coli***				***Shigella boydii***			
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
1.1	14	16	17	15	15	14	22	16	16	17	14	14	22	24	17	20
1.2	12	14	15	17	13	16	19	14	12	13	12	18	18	19	16	22
2.1	16	19	16	16	21	15	16	19	19	17	13	15	25	17	22	18
2.2	16	18	14	18	20	14	13	16	15	16	14	17	19	16	24	24
3.1	11	13	14	13	16	22	20	21	13	12	20	16	18	24	19	15
3.2	16	14	20	17	13	18	17	20	14	20	18	18	16	26	25	19
4.1	17	12	14	13	22	24	19	18	14	14	14	14	19	16	24	17
4.2	14	14	17	14	19	22	21	18	15	12	12	16	23	18	18	21
5.1	18	18	22	15	18	17	14	16	20	22	19	13	18	15	19	22
5.2	14	16	15	13	16	15	17	17	17	14	17	17	16	21	22	19
Mean ± SD	14.8 ± 2.20	15.4 ± 2.37	16.4 ± 2.72	15.1 ± 1.85	17.3 ± 3.20	17.7 ± 3.68	17.8 ± 2.94	17.5 ± 2.12	15.5 ± 2.55	15.7 ± 3.37	15.3 ± 2.95	15.8 ± 1.75	19.4 ± 2.99	19.6 ± 3.92	20.6 ± 3.20	19.7 ± 2.67
p	N/A	0.46	0.07	0.72	N/A	0.77	0.76	0.87	N/A	0.82	0.86	0.81	N/A	0.91	0.46	0.82

Here, A: Aqueous UAE extract from Fresh leaves, B: Aqueous UAE extract from dried leaves, C: Methanol extract from dried leaves and D: Ethanol extract from dried leaves

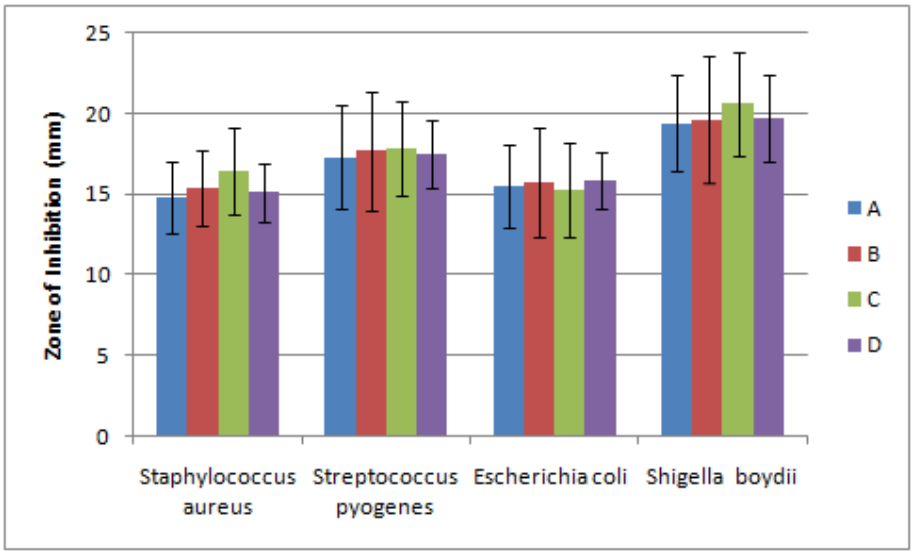

Chart 1: Comparison of antimicrobial study of the crude extracts of *M. oleifera* extracted by using different extraction method (Here, A: Aqueous UAE from Fresh leaves, B: Aqueous UAE from dried leaves, C: Methanol extract from dried leaves and D: Ethanol extract from dried leaves)

Conclusion:

The proposed "aqueous ultrasound assisted extraction from fresh leaves of *M. oleifera*" was observed the similar efficiency compared with the conventional extraction method without compromising the efficiency and efficacy of the crude extracts. Additionally the proposed method successfully reduced the overall extraction process by using simple instruments. The cost effective, environment friendly, time efficient, operation simplicity nature of this method obeyed most of the principles of green extraction method. However, more studies with different plants may be recommended for establishing and popularizing this method in the extraction world of the natural products.

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