ISSN No:-2456-2165

Validation of an Optimized Ultrasound Assisted Green Extraction Method by using Fresh Leaves of *Carica papaya*

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Abstract:- Green extraction generally addresses the discovery and design of extraction processes to reduce energy consumption, application of alternative solvents and renewable natural products, and ensure a safe and high quality extract. The proposed "aqueous ultrasound assisted extraction (UAE) from fresh plant material" have been designed based on the green extraction principles. In the present study, fresh leaves of *Carica papaya*was used for validating the process in comparison with some conventional methods. *C. papaya* is locally known as pappaiya and widely used as fruits and vegetable in Bangladesh. Traditionally the plant is rich of medicinal components and different parts of the plant are frequently prescribed for several household and folk remedies in different parts of the world. The proposed method was validated based on the extraction yield (%) value, presence of phytochemicals and the antimicrobial sensitivity compared with the reference extraction method. Aqueous UAE form fresh leaves of *C. papaya* was provide significantly high yield (31.80%) compared to the conventional method indicated the high efficiency of the extraction method. From phytochemical screening tests the presence of Alkaloid, Anthraquinones, Flavonoid, Glycoside, Saponin, Steroid, Tannin, Terpenoid and Vitamin C were observed in the crude extracts which was somewhat even better than the conventional method. The crude extract have observed promising sensitive against *Bacillus subtilis* and *Agrobacterium species*which was almost similar to the conventional crude extracts. Based on the present study, the proposed method may be recommended for an effective green extraction procedure suitable for both laboratory and industrial setup.

Keywords:- *Carica papaya, Bacillus subtilis, Agrobacterium species, Green extraction, Ultrasound Assisted Extraction (UAE), Validation.*

I. INTRODUCTION

Extraction of bioactive compounds and their precursors such as, antibiotics, chemo-preventive agents, alkaloids, etc. by the pharmaceutical industry are still largely dependents on conventional methods. A typical extraction process is normally started from the collection and authentication of plant material & drying which is subsequently included size reduction, extraction, filtration, concentration, drying & reconstitution [1]. Obviously, quality of an extract is largely depends on the starting material, selection of solvent for extraction, selection of extraction procedure etc.[2]. There is huge room of optimizing the existing extraction procedures by improving steps involving in the overall extraction process. Recent trends largely focused on finding solutions that occupy the principles of green chemistry connected to the 12 principles of green engineering[3]. Green chemistry mainly emphasizes the invention, design and application of chemical products and processes to reduce or to eliminate the use and generation of hazardous substances [4]. On the basis of "green chemistry", the "green extraction" may be defined as the process of discovery and design of extraction processes by reducing energy consumption, facilitating alternative solvents and renewable natural products to ensure a safe and high quality extract.This is of course not compromise the high quality of extracts enabling process intensification and a cost-effective production. In the present study, an optimized green extraction method named "aqueous UAE from fresh leaves of *Carica papaya*" waspropose as per the process established by Sadat and his coworkers [5-8]. The basic principle of this extraction is to application of high-intensity, high-frequency sound waves and their interaction with the plant's materials [9]. Since the ultrasound increases the rate of diffusion to allow more rapid penetration of solvent into the matrix of materials [10], UAE is known as the fast and efficient technique to extract chemicals from plant materials [11].

Carica papaya L. belongs to the family Caricaceae [12-14] is widely cultivated and a leading fruit in Bangladesh locally known as "pappaiya" or "Pepe" or "Papaya" which is consumed as its ripen and green state as a popular vegetable. This is a polygamous species and grow in three sexes: male (staminate), female (pistillate), and hermaphrodite (staminate) [15-16]. This plant is native to southern Mexico and neighboring Central America and is grown extensively all over the tropical regions [16-17]. Many parts of Bangladesh including Rajshahi, Bogra, Pabna, Chittagong Hill Districts, Rangpur, Jesssore, Ishurdi, Mymensingh and Dhaka produce good quality of papaya crop which is commercially marketed all over the country [18].From literature survey it was observed that leaves of the *C. papaya* is the most useful part among all used parts for the medicinal purpose including several household treatment. Evidence of using papaya leaves have been used as folk remedies to treat cancer in Australia, Brazil and

ISSN No:-2456-2165

Vietnam [19]. Aqueous leaves extract [20-21]and ethanolic extract [22] of *C. papaya* have proven anti-diabetic properties. The tea, prepared with the green papaya leaf, promotes digestion, prevent overweight and obesity, arteriosclerosis, high blood pressure and weakening of the heart [23]. Several ethno botanical studies describes number of medicinal uses including fracture healing [24], to treat constipation and indigestion [25], severe jaundice [26], to expel guinea worm [27], as a poultice [28], to treat oral candidosis, malaria, dengue, yellow fever [29] etc. However, pharmacological action depends mainly the presence of bioactive substances present in the plant extracts [30]. As per proposed green extraction method, the popularity of papaya leaves extracts for management of ailments will be possible to increase many fold. In the present study, the proposed green extraction method was validated based on the efficiency and efficacy parameters compared to the reference conventional extraction methods.

Taxonomic Hierarchy of Papaya [38,39]

Kingdom	Plantae
Sub kingdom	Viridiplantae
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Order	Brassicales
Family	Caricaceae
Genus	*Carica* L
Species	*Carica papaya* L.

Fig. 1: Upper part of a *Carica papaya* tree and Taxonomical classification

II. MATERIALS AND METHODS

A. Validation Protocol

The proposed extraction method was "Aqueous Ultrasound Assisted Extraction (UAE) from the fresh leaves of *Carica papaya*". During validation the process, both efficiency (quantity of extract) and efficacy (pharmacological activity) were assessed and compared with five reference extraction methods (Table 1). Crude extracts obtained from different extraction methods were compared on the basis of (i) Percentage of yield (quantitative efficiency of the extraction method) (ii) Presence of phytochemicals obtained from qualitative screening test (qualitative efficiency of the extraction method) and (iii) antimicrobial sensitivity (pharmacological efficacy of the extraction method). Each extraction methods were repeated three times and statistical data was used for validation process.

Method	Plant parts	Treatment	Method	Ref.
A	Fresh leaves (Juice)*	Ultrasound	Juice of 50gm fresh leaves was diluted to 250ml and send for 30 minutes ultrasound treatments at 40^{oC} bath temperature	[5-6,8]
B	Fresh leaves (Juice)*	Decoction	Juice of 50gm fresh leaves was diluted to 250ml and allowed to boil for 5 minutes before extraction	[31]
C	Dried leaves (powder)**	Ultrasound	Fine powder was mixed with distil water (1:5 ratio) and treated in ultrasonic bath for 30 minutes treatments at 40^{oC} bath temperature	[5-6,8]
D	Dried leaves (powder)**	Decoction	Fine powder was mixed with distil water (1:5 ratio) and allowed to boil for 5 minutes	[31]
E	Dried leaves (powder)**	Methanol cold extraction	Fine powder was mixed with methanol (1:5 ratio) and allowed for cold extraction up to 72 hours with intermittent shaking as per standard method	[32-34]
F	Dried leaves (powder)**	Ethanol cold extraction	Fine powder was mixed with ethanol (1:5 ratio) and allowed for cold extraction up to 72 hours with intermittent shaking as per standard method	[32-34]

Table 1: Extraction protocol of papaya leaves

* Juice of fresh leaves (50gm) was prepared by conventional blender by adding maximum 250ml distil water
** Powder was prepared by conventional blender and mixed with mix with distil water as a ratio 1:5.

B. Collection of Plant Material

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

C. Extraction Procedure

Material-solvent ratio is an important parameter for any type of extraction. Toma *et al.*, established the material-

ISSN No:-2456-2165

solvent ratio 1:5 for effective ultrasound extraction[35]. Both fresh and shade dried leaves were used in the present study[5-6,8,36].Fresh leaves of *Carica papaya* were washed properly by running tap water followed by distilled water and allowed for shade drying of the surface water [5-6,8, 37]. After 6 hours, the leaves were sliced in a small pieces and 300gm were isolated which were further divided into six parts (Part A, B, C, D, E and F), 50gm in each. As per the extraction protocol (Table 1), "Part-A" and "Part-B" of fresh leaves were used immediately for UAE aqueous extraction and decoction, whereas "Part-C", "Part-D", "Part-E" and "Part-F" of fresh leaves were allowed for week long drying. After grinding powdered leaves were collected and mixed with respective solvents in the ratio (1:5) respectively and allowed for aqueous UAE, decoction, methanol and ethanol extraction stated in Table 1.

For "ultrasonic treatment", both the juice of "Part-A" and the mixture of fine powder of "Part-C" were placed in the ultrasonic bath (Figure 2) for 30 minutes at 40ºC bath temperature [5-6,8]. For "decoction", both the juice of "Part-B" and the mixture of fine powder of "Part-D" were boiled 5 minutes and then allowed for cooling before filtration [31]. Conventional cold extraction method was conducted by soaking the fine powder of dried leaves of "Part-E" and "Part-F" in methanol and ethanol in a ratio 1:5 for 72 hours with intermittent shaking as per standard method [32-34]. After extraction, the plant extracts were first filtered by five layers of polyester cloth and dried at 60ºC in a conventional water bath. The dried crude extracts were then stored in an air tight bottle with identical label and preserved in cold chamber for further use.

The whole procedure was repeated three times for each method and calculated Mean ± SEM for measuring significance level. SPSS 16.0 was used for statistical analysis.

Fig. 2: Power sonica 405 model was used for ultrasound assisted extraction.

D. Percentage of Yield Calculation

The percentage of yield indicate the efficiency of the extraction procedure which was calculated by using the following formula [38]

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

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

E. Phytochemical Screening Test

Qualitative phytochemical test indicate the extraction efficiency of the crude extract. In the present study the extracts obtained from method A,B, C, D, E and F were subjected to phytochemical tests for plant secondary metabolites including alkaloids, anthraquinones, flavonoids, glycosides, saponins, steroids, tannins, terpenoids and vitamin C as per procedure of Allen and Harborne with slight modification and successfully applied in different previous publications [5,39-44] .

F. Antimicrobial Activity

Antimicrobial study was conducted by the disc diffusion method [45-48]ona Gram +ve bacteria *Bacillus subtilis* and a Gram –ve bacteria *Agrobacterium species*. Microorganisms were collected from the Microbiology Lab, Department of Biochemistry and Molecular Biology, Rajshahi University, Bangladesh. The filter paper discs impregnated with the 400µg/disc of extracts were placed on the surface of the inoculated nutrient agar media with the aid of sterilized pair of forceps. After allowing 30 minutes pre-diffusion, the petridish was then placed in incubator for 24 hours at 37^{0C}.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 procedure was repeated three time for each batch and all results were considered for statistical analysis.

III. RESULTS AND DISCUSSION

Crude extract obtained from aqueous UAE from fresh leaves of *Carica papaya* (extraction method "A") was observed green-yellowish in color having yield value 31.80±2.04% (Mean±SEM from three successive batches) presented in Table-2. Similar color was also observed in case of crude extracts obtained from method "C" i.e., crude extracts obtained through aqueous UAE from dried leaves method, however, the yield value (30.98±1.47%) was slightly smaller (yield difference statistically insignificant) than the previous one. Decoction technique was also applied on fresh and dried leaves in method "B" (Yield value 5.73±0.64%) and method "D" (Yield value 7.67±0.95%) respectively. Extraction efficiency of decoction techniques (applied in method B & D) were proved significantly poor than the UAE techniques (applied in method A & C). Conventional methanol cold extraction (method E) and ethanol extraction (method F) were performed to compare the proposed UAE extraction method. Yield value of methanol extract (13.48±0.88%) and ethanol extract (14.27±0.99%) were observed significantly poor than the UAE extract applied on method A and B. From previous similar study average methanol and ethanol extracts yield were observed 8.78% [48] and 6.25% [49] simultaneously. Compared to the previous study, slightly better methanol and ethanol yield values were observed in the present study, however, this may be due to the variation of filtration technique of the previous study. On the basis of above results the ultrasound assisted extraction was proved better than the conventional decoction and cold extraction method. It was also observed that ultrasound treatment provide similar extraction from both fresh and dried leaves, giving

ISSN No:-2456-2165

opportunity to avoid the drying stage (a time consuming or rate limiting stage) of the overall extraction method.

Phytochemical screening showed that crude extracts from method "A" and "C" of *C. papaya* contained most of the tested compounds tested in the present study (Table 3). Almost similar results were also observed in the crude extracts obtained from the conventional methanol and ethanol extraction method. However, fewer phytochemicals were observed in the extracts obtained from decoction method. Previous study was also justify the presence of alkaloids, glycosides, saponins, tannins etc. in the aqueous extract of leaves of *C. papaya*[50]. Another study reported that ethanolic and chloroform extract from fresh, green leaves contain alkaloids, flavonoids, tannins and saponins, while water extract contains tannins and saponins only [51-52]. From the present study it was observed that the proposed aqueous UAE from fresh leaves (Picture 3) showed almost similar phytochemical contents (Table 3) compared to the established methanol and ethanol extraction method. The above results indicated that the proposed method was capable enough to extract all possible compounds occasionally observed in the conventional methods of extraction.

Crude extracts obtained from method A, C, E & F of *Carica papaya* leaves were found promising antimicrobial activity observed from three successive antimicrobial study from each batch of extract's on *Bacillus subtilis* (Gram +ve) and *Agrobacterium species* (Gram –ve)presented in the Chart-1. Statistically no significant differences were observed on the studied bacteria indicated the same efficacy of those four extracts (Table 4). However, extracts obtained from decoction (method B & D) showed comparatively poor sensitivity.

Extraction Method	Starting materials (gm)	Quantity of material used for extraction		Solvent (1:5 ratio) used (ml)	Crude extract (gm)	Yield	
		(gm)	Mean±SEM			%	Mean±SEM
A*	50	50	-	250	17.8	35.6	31.80±2.04
	50	50		250	15.6	31.2	
	50	50		250	14.3	28.6	
B*	50	50	-	250	2.3	4.6	5.73±0.64^{x}
	50	50		250	3.4	6.8	
	50	50		250	2.9	5.8	
C*	50	22.2	21.23±0.73	111	7.4	33.33	30.98±1.47^{b}
	50	19.8		99	5.6	28.28	
	50	21.7		108.5	6.8	31.34	
D*	50	19.6	19.67±0.46^{a}	98	1.4	7.14	7.67±0.95^{x,y}
	50	18.9		94.5	1.8	9.52	
	50	20.5		102.5	1.3	6.34	
E*	50	19.3	21.13±0.99^{a}	96.5	2.3	11.92	13.48±0.88^{x,y}
	50	22.7		113.5	3.4	14.98	
	50	21.4		107	2.9	13.55	
F*	50	22.8	20.57±1.22^{a}	114	3.4	14.91	14.27±0.99^{x,y}
	50	20.3		101.5	2.5	12.32	
	50	18.6		93	2.9	15.59	

Table 2: Yield variation of Papaya leaves extracts

* As per extraction protocol of papaya leaves (Table 1)
[a] Insignificant ($p>0.5$) weight variation after drying compared to the "C"
[b] Insignificant yield variation ($p>0.5$) compared to the "A"
[x] Significant yield variation ($p<0.5$) compared to the "A"
[y] Significant yield variation ($p<0.5$) compared to the "C"

Phytochemical Tests	Crude extract					
	A*	B*	C*	D*	E*	F*
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	+	-	+	-	+	+
9. Vitamin C	+	+	+	-	-	-

Table 3: Phytochemical screening of UAE and conventional crude extracts

* As per extraction protocol of papaya leaves (Table 1)
(+) indicated presence of compound, and (–) indicated absence of compound

Mother solution

The color of the mother solution was slightly yellowish

Tannin (+ve)

2 ml mother solution + few drop 0.1% ferric chloride. observed blue-black coloration (65-66)

Saponin (+ve)

After shaken vigorously a stable persistent froth was formed (63)

Flavonoid (i) (+ve)

Mother solution + Conc. H_2SO_4. Yellow coloration observed which was disappeared on standing (64)

Flavonoid (II) (+ve)

Mother solution + Few drops of 1% aluminium solution. A yellow coloration was observed (64)

Steroid (+ve)

Mother solution + 2 ml of acetic anhydride + 2 ml concentrated H_2SO_4. Color change from violet to blue was observed (46)

Terpinoid(+ve)

Mother solution + 2 ml of $CHCl_3$ + 3 ml Conc. H_2SO_4 was added to from a layer. A reddish brown coloration of the inter face was formed (64)

Glycoside (+ve)

Mother solution + 2 ml of glacial acetic acid + one drop of ferric chloride solution. This was underlayed with 1 ml Conc. H_2SO_4. Brown ring of the interface was observed (63, 64)

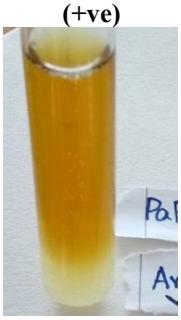
Alkaloid /Dragendroff test (+ve)

0.5ml of mother solution + 2ml of HCl + 1ml of Dragendroffs reagent. Orange precipitate found (61, 62)

Alkaloid: Mayer test (+ve)

1.2 ml mother solution + 0.2 ml dilute HCl + 0.1 ml of Mayer's reagent. A yellowish Puff colored precipitate was developed (62, 63)

Anthraquinone (+ve)



5 ml of $CHCl_3$ + 2ml mother solution + 10% ammonia solution. A bright pink color in the aqueous layer was not observed (62, 64)

Vitamin C (+ve)

Mother solution + 1 drop 5% w/v sodium nitroprusside + 1 ml of diluted NaOH + 0.4 ml HCl. The yellow color turns blue (46)

Fig. 3: Pictorial representation of phytochemical tests of *Carica papaya*

ISSN No:-2456-2165

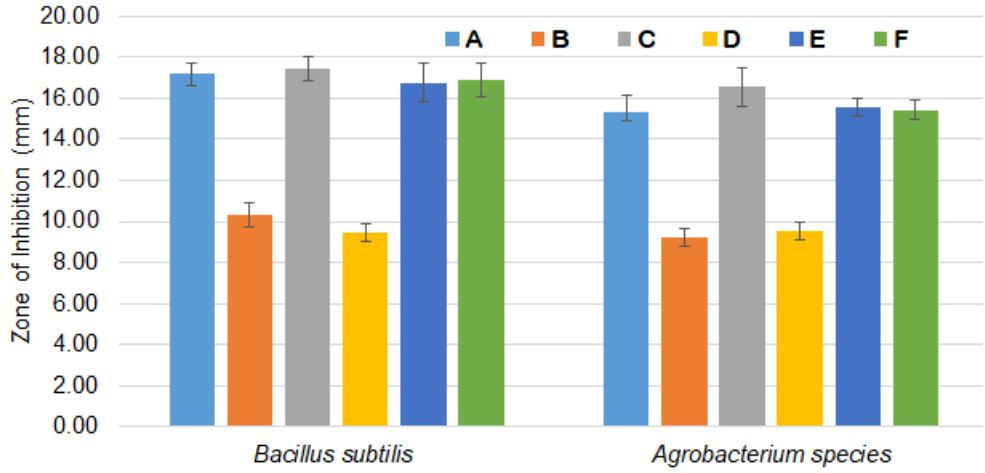


Chart 1: Comparison of antimicrobial sensitivity (efficacy) of the crude extracts of *C. papaya* leavesextracted by using different extraction method (Here, A: Aqueous UAE from fresh leaves, B: Aqueous decoction from fresh leaves, C: Aqueous UAE from dried leaves, D: Aqueous decoction from dried leaves, E: Methanol cold extract from dried leaves, F: Ethanol cold extract from dried leaves)

Batch and test no	Zone of Inhibition (mm)											
	Bacillus subtilis (Gram +ve)						*Agrobacterium species* (Gram –ve)					
	A*	**B***	**C***	**D***	**E***	**F***	**A***	**B***	**C***	**D***	**E***	**F***
1.1	17	12	18	10	15	18	16	8	17	10	15	14
1.2	20	9	17	8	13	16	14	9	15	9	16	16
1.3	15	10	17	8	21	15	19	10	16	9	17	15
2.1	17	11	18	10	20	14	18	11	14	8	14	14
2.2	18	8	19	10	16	16	13	8	14	10	16	14
2.3	16	8	14	8	13	14	14	8	20	11	14	18
3.1	19	10	16	11	16	18	12	9	14	9	15	15
3.2	17	12	20	11	19	21	14	11	17	8	15	16
3.3	16	13	18	9	18	20	18	9	22	12	18	17
Mean ± SD	17.22 ± 0.52	10.33 ± 0.60	17.44 ± 0.58	9.44 ± 0.41	16.78 ± 0.97	16.89 ± 0.84	15.33 ± 0.83	9.22 ± 0.4	16.56 ± 0.94	9.56 ± 0.44	15.56 ± 0.44	15.44 ± 0.47
p	N/A	0.00	0.777	0.00	0.746	0.737	N/A	0.000	0.278	0.000	0.782	0.914

Table 4: Antimicrobial activity of UAE and conventional crude extracts of *C. papaya*

* As per extraction protocol of papaya leaves (Table 1)

IV. CONCLUSION

The proposed "aqueous ultrasound assisted extraction from fresh leaves of *Carica papaya*" was found better from many points of the conventional extraction methods without compromising the efficiency and efficacy of the crude extracts. The proposed method successfully avoided the time and energy consuming drying process normally practiced in the conventional extraction method. At the same time the proposed method was successfully avoided the uses of hazardous organic solvents during the extraction procedure of crude extracts which will also be reduced the overall cost of the extraction process. The proposed method was proved cost effective, environment friendly, time efficient, operation simplicity nature of this method obeyed most of the principles of green extraction method and duly validated compared with the conventional extraction methods. The method may be suitable for industrial setup as its easy operational, cost effective and hazardous free green nature of extraction. Further study is recommended for scaling up the method for industrial purpose.

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