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Effectiveness of Solvent Extraction on Phytochemicals and Antioxidant Activities from Fresh and Dried Wheatgrass

Towkir Ahmed Ove[1*], Anwara Akter Khatun[1], Said Bin Saifullah[1,2] and Maruf Ahmed[3]

[1]Department of Food Science and Nutrition, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh.
[2]Department of Public Health, North South University, Dhaka, Bangladesh.
[3]Department of Food Processing and Preservation, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh.

Authors' contributions

This work was carried out in collaboration among all authors. Author TAO designed the research, wrote the manuscript and carried out most of the experiments. Author SBS participated in laboratory works and analysis of results. Author AAK was involved in the research design and literature collection. Author MA was involved in literature collection and manuscript development. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was intended to investigate and quantify the phytochemicals and the antioxidant activities of fresh and powdered wheatgrass.
Study Design: The experiment was performed by solvent extraction technique for determining the phytochemicals of wheatgrass samples.
Place and Duration of Study: The study was conducted at the Food engineering laboratory in Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh between June 2018 and December 2018.

**Corresponding author: Email: towkirahmedove@gmail.com;*

Methodology: Fresh and powdered wheatgrass were used for the experiment. Methanol and ethanol solutions were separately utilized, in an attempt to assume the efficient solvent for extraction. UV-Vis spectrophotometric methods were employed in general throughout the study. Total phenolic contents (TPC), total flavonoid contents (TFC), DPPH scavenging activity, ferric reducing antioxidant power (FRAP) and the total chlorophyll contents of both solvent extracts were analyzed.
Results: Phytochemicals and antioxidant activity were found lower in both solvent extracts of powdered wheatgrass than fresh leaves. Phenolic content and Chlorophyll were significantly ($P < 0.05$) higher in fresh samples (extracts). Due to heating, a significant decrease was observed in the DPPH radical scavenging activity of powdered extracts. Ferric Reducing Antioxidant Power (FRAP) was significantly ($P < 0.05$) higher in methanolic extracts than in ethanolic extracts. No significant ($P > 0.05$) difference was observed in total flavonoids contents.
Conclusion: These findings endorsed that both solvents demonstrate competitive potentials to extract bioactive compounds from wheatgrass and its derivatives and hence can be chosen specifically according to the components.

Keywords: Triticum aestivum; fresh; powder; ethanol; methanol; UV-vis spectrophotometer.

1. INTRODUCTION

Wheatgrass, the young leaves of wheat (*Triticum aestivum*) plant belongs to the Poaceae family. This therapeutic agent bags a part of ancient Ayurveda and still practiced broadly in India and Egypt [1]. It is a substantial source of vitamins, minerals, and alkaline earth metals. As yet, the popularity of wheatgrass is limited to the developed countries only. It is available in versatile forms, including juice, powders and health supplements. A supplement program commonly called “the wheatgrass diet” (juice or powder) is already in action, in a few handpicked communities [2]. Nevertheless, its universality is yet to be increased since only people with poor health conditions are keen to ingest these products [1].

Consumption of wheatgrass exerts insulating effects against quite a few chronic and short-term diseases. Several molecular studies and controlled animal trials confirmed that wheatgrass decreases the total cholesterol, low-density lipoprotein and triacylglycerol (TAG) level. Simultaneously it increases the high-density lipoprotein level to such a degree that it lowers the chance of cardiovascular diseases. Wheatgrass extracts can be administrated to improve metabolic profiles by increasing liver glycogen and decreasing the level of fasting blood glucose in diabetic patients [3].

Most of the phytochemicals present in wheatgrass are polyphenols, predominantly ferulic acid, pyrogallol, syringic acid, vanillic acid [4] and flavonoids, particularly apigenin which is known for its functions in anti-oxidation in anti-inflammation by inhibiting cytokine-induced leukocyte adhesion, anti-carcinogenesis, antiproliferative and proapoptotic activities [5]. It also contains numerous pharmacologically active enzymes like amylase, protease, lipase, cytochrome oxidase and trans-hydrogenase superoxide dismutase that decrease the impacts of radiation and overview poisons in the body [6]. The antioxidant activity counteracts inflammatory conditions, like arthritis and ulcerative colitis [7].

Conferring on to Padalia et al. [6], wheatgrass is assumed as green blood for its saturated mass of chlorophyll. It demonstrates a similar structure to hemoglobin and makes up about 70% of cumulative chemical constituents [8]. The chlorophyll delivers a lucrative reaction on the blood transfusion of Thalassemia patients and reduces toxicity in cancer patients [9].

The use of different solvents plays a vital role in extraction efficiency and their successive health benefits [10]. The most universal solvents for bioactive compounds analysis are acetone, diethyl ether (DEE), ethanol and methanol. Likewise, we have found the repetitive practice of using methanol, ethanol, ethyl acetate and chloroform, etc. in the extraction of phytochemicals from wheatgrass and its derivatives. However, assessing some established optimization techniques we have found no suggestion relating to water as the best-suited extraction solvent, hence neglected [11-13]. Studies suggest that acetone and diethyl ether are volatile, inflammable, and narcotic. They also attack the spectrophotometer cuvettes made of polystyrene and polymethyl acrylates and consequently avoided. On the other hand,

ethanol and methanol are less volatile and considered suitable for laboratory practice [14].

About 40.5 million people die of Non-Communicable Diseases (NCD) each year which accumulates about 71% of the sum [15]. There is a thick chance for wheatgrass and its derivatives to perform like natural remedies against such unwanted phenomena. We hypothesized that the extraction efficiency is largely solvent-dependent. Due to less volatility and suitable for laboratory practice ethanol and methanol solutions were specifically used as extraction solvents in this study. The scope of the present study is to estimate the phytochemical quantities and antioxidant activities of fresh and powdered wheatgrass using different solvent extraction solvents.

2. MATERIALS AND METHODS

2.1 Collection of Reagents

Gallic acid, Folin-Ciocalteau reagent, Sodium carbonate (Na_2CO_3), Aluminium chloride ($AlCl_3$), Sodium nitrite ($NaNO_2$), Sodium hydroxide (NaOH), Standard quercetin, DPPH (1, 1-diphenyl-2-picrylhydrazyl), Disodium hydrogen phosphate dehydrate (Na_2PO_4), Sodium dihydrogen phosphate dehydrate ($NaH_2PO_4.2H_2O$), potassium ferricyanide, trichloroacetic acid, ferric chloride ($FeCl_3$), $FeSO_4.7H_2O$, methanol, ethanol, acetone and all other chemicals of analytical grade were procured from Merck, Darmstadt, Germany.

2.2 Cultivation of Wheatgrass

Wheat (BARI Gom-31) seeds were collected from local farmers of Dinajpur (25.6279° N, 88.6332° E), Bangladesh. Afterward, the seeds were flushed with groundwater and soaked overnight to germinate. Then they were sown over the soil free from fertilizers and pesticides in a plastic tray. A perpetual sprinkle of water was facilitated for the sprouted seeds covered with a wet cloth. On average, it took 9 to 10 days to reach 16 to 26 cm long [16]. Eventually, the wheatgrass was ready for reaping as the leaves grow, they eventually split. At this jointing stage, the blades can be snipped off, allowing for a second round of leaves to grow [17].

2.3 Preparation of Wheatgrass Powder

The wheatgrass was first chopped with a sharp knife. A cabinet dryer (CO-150, Human Lab Inc, Suwon-Si, Korea) was employed at 55 ± 5 ºC for 24 hours [16] to dry the grass until constant moisture content (10.67%) was achieved and afterward milled (Jaipan Mate 850W, Jaipan Industries Ltd, India) to powders. The powder was passed through meshes (0.5mm) and deposited in a labeled airtight receptacle for further investigations.

2.4 Preparation of Fresh and Wheatgrass Powder Extracts

The maceration technique [18] with modification was performed. One g of both fresh and dried grass was suspended in 100 ml of methanol and ethanol separately and kept on an orbital shaker (VS-8480SN, Vision Scientific Co. Ltd, Daejeon-Si, Korea) at 37 ºC for 48 hours for optimized extraction [18]. Subsequently, the supernatant was drained through Whatman no.1 filter paper and stored at -18 ºC (BDF-40V268, Biobase Biodustry Co. Ltd, Shandong, China) in an airtight receptacle for further investigations.

2.5 Determination of Total Phenolic Contents

The procedure described by Majhenič et al. [19] was done with a few modifications. We used Folin-Ciocalteu reagent as the oxidizing agent and Gallic acid as standard. In 0.5 ml of extract solution, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with distilled water) and 2.5 ml of Na_2CO_3 (7.5% w/v) solution were mixed. The mixture was incubated at room temperature for 20 minutes. Subsequently, the absorbance (UV-1800, Shimadzu Corp, Kyoto, Japan) was measured at 760 nm. TPC was calculated as mg of GAE/g (Gallic acid Equivalent per gram) of the extractive.

2.6 Determination of Total Flavonoids Contents

The procedure described by Zhishen et al. [20] with Quercetin as standard was performed. One ml of each sample extract was diluted with 200 µl of distilled water separately followed by the addition of 150 µl of sodium nitrite (5%) solution. This mixture was incubated for 5 minutes and then 150 µl of aluminum chloride (10%) solution was added and allowed to stand for 6 minutes. Then 2 ml of sodium hydroxide (4%) solution was added and made up to 5 ml with distilled water. The mixture was centrifuged (BKC-TH16RII, Biobase Biodustry Co. Ltd, Shandong, China) at

4,000 rpm (for 5 minutes and left for 15 minutes at room temperature. Subsequently, the absorbance was measured at 510 nm. TFC was calculated as mg QE/g (Quercetin Equivalent per gram) of the extractive.

2.7 Determination of DPPH Radical Scavenging Activity

The procedure described by Blois [21] with modified volume was performed. A volume of 100 µl of both fresh and powder extracts was mixed with 1.9 ml of the methanolic solution of DPPH (0.3 mM). An equal amount of methanol and DPPH without the sample was served as the control. Solutions were incubated at room temperature in the dark for 20 minutes. Subsequently, the absorbance was measured at 517 nm against methanol as blank.

The free radical scavenging activity percentage was calculated using the following formula.

$$\text{Inhibition \%} = (Ac-As)/Ac\times100$$

Where, Ac = absorbance of the control and As = absorbance of the sample

2.8 Determination of Ferric Reducing Antioxidant Power (FRAP)

The procedure described by Oyaizu [22] with $FeSO_4.7H_2O$ (Ferrous sulphate heptahydrate) as standard was performed. In a test tube, 2.5 ml of each extract was taken and mixed with 2.5 ml sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide solution (1%). The mixture was incubated in the water bath (VS-1205SW1, Vision Scientific Co. Ltd, Daejeon-Si, Korea) at 50 ºC for 20 minutes. After incubation, 2.5 ml trichloroacetic acid (10%) was added and the solution was centrifuged at 650 rpm for 10 minutes. Later, 2.5 ml of supernatant was mixed with 2.5 ml distilled water and 0.5 ml ferric chloride solution (0.1%). The absorbance was measured at 700 nm against methanol as blank.

2.9 Total Chlorophyll Content

A combined procedure described by Anand et al. [23] and Shengqi et al. [24] was performed. One ml of each extract was transferred to a test tube containing 4 ml of 80 % acetone. The contents were centrifuged at 4,000 rpm for 15 minutes. The absorbance of the supernatant was measured at both 645 and 663 respectively [23].

Total chlorophyll, chlorophyll a, and chlorophyll b were calculated using the following equations given by Shengqi et al. [24].

$$\text{Chlorophyll a (µg/ml)} = 12.72\ A_{663} - 2.59\ A_{645}$$
$$\text{Chlorophyll b (µg/ml)} = 22.9\ A_{645} - 4.67\ A_{663}$$
$$\text{Total chlorophyll (µg/ml)} = 20.31\ A_{645} + 8.05\ A_{663}$$
$$\text{Total chlorophyll (mg/g)} = (20.31\ A_{645} + 8.05\ A_{663}) \times V/ (W\times1000)$$

Where, A_{645} = Absorbance at 645 nm, A_{663} = Absorbance at 663 nm, V= volume of the extraction solvent in each sample (ml), W= weight (g) of the sample.

2.10 Statistical Analysis

Statistical analyses were done through IBM SPSS Statistics 25 (IBM Corporation, New York, USA) and RStudio, Version 1.2.5042 (RStudio PBC, Massachusetts, USA) at the significant level of $P < 0.05$ with a 95% confidence interval. All the observations were done in triplicates and reported as mean ± SD (standard deviation) calculated using one-way ANOVA. Comparisons between fresh and powdered wheatgrass upon extraction were calculated using Tukey's HSD (Honestly Significant Difference) test. Paired sample t-test was used to compare the differences between extraction solvents ($P < 0.05$), either in fresh or powdered conditions. Estimated phytochemicals and antioxidant activities with statistical data were tabulated in Table 1, and quantified Chlorophyll was graphically presented in Fig. 1.

3. RESULTS AND DISCUSSION

3.1 Total Phenolic Contents

The present study interpreted that there was a significant difference in TPC between fresh and powdered wheatgrass. TPC of fresh and powdered wheatgrass for both solvent extracts was ranged from 9.15 ± 0.18 to 42.09 ± 2.73 mg GAE/g (Table 1). FWG (fresh wheatgrass) showed slightly higher TPC in ethanolic extract than in methanolic extract. Additionally, from the statistical analysis, it was shown in Table 1 that methanolic extract had significantly ($P < 0.05$) higher TPC than ethanolic extract for WGP (wheatgrass powder). Previous researches informed that methanol and ethanol can dissolve polar compounds such as phenolic compounds with low and medium molecular-weights and

medium polarity [25]. From this point of view, the differences among the extraction values can be explained by the variation in polarities of the solvents and differences in composition, which selectively extract various hydrophobic or hydrophilic phenolic compounds from the sample [26,27]. The obtained results of WGP were similar to the findings of Shukla et al. [28] that was 10.7 ± 1.0 mg GAE/g of powder in methanolic extract and higher than the findings of Elif et al. [29] who had determined TPC for encapsulated powder in methanolic extract. The notable differences between the literature and our findings could be due to the genotype of the cultivated wheat, harvesting and processing conditions and environmental factors [29]. The obtained results of the present study also showed that the drying treatment to powder formation had caused a significant decrease in total phenolics due to their thermosensitivity [30]. According to Réblová [31] and Chomchan et al. [4], wheatgrass contains various thermosensitive phenolic compounds like pyrogallol, vanillic acid, syringic acid and ferulic acid. Various published reports propounded that irreversible chemical changes cause a significant decrease in phenol contents due to heat treatment [30,32]. This might be caused by the binding of polyphenols with other compounds or by alterations in the chemical structure of polyphenols which cannot be extracted and determined by convenient methods [33]. As demonstrated, it can be elaborated that previous studies also concede our study. These findings further confirmed that extraction solvents carry out a significant role in the extraction of phenolic compounds from the sample.

3.2 Total Flavonoid Contents

The obtained values are given in (Table 1). Both extracts for FWG showed (5.71 ± 0.90) and (6.60 ± 0.30) mg QE/g respectively. Furthermore, WGP contained (4.97 ± 1.24) and (4.97 ± 0.82) mg QE/g for both solvent extracts respectively. The present study indicated that there was no significant difference ($P > 0.05$) of TFC between both solvents for the samples (FWG and WGP). In literature, it was described that methanol and ethanol can dissolve polar compounds such as aglycon flavonoids and the major flavonol aglycons, are quercetin, kaempferol, myricetin and two flavone aglycons luteolin and apigenin [26]. Moreover, during a thermal treatment, these selected flavonoids are heat sensitive especially rutin and quercetin [27]. From this perspective present study also had found marginally lower flavonoid content of powdered wheatgrass than the fresh sample in both extracts. Previous articles reasonably appreciated that thermal processes are able to reduce the flavonoid content of herbaceous plants [27] that concedes our study. Besides, Afshar et al. [34] described in their study that there is a positive relation between phenolic and flavonoid composition, and antioxidant activity. Accordingly, the lower content of TPC led to a decrease in TFC of powdered wheatgrass than the fresh sample, although the value was not significantly conflicting as our study also depicted the same.

Table 1. Phytochemicals and antioxidant quantities of fresh and dried wheatgrass

	Fresh Wheatgrass			Wheatgrass Powder		
	Methanol	**Ethanol**	**P**	**Methanol**	**Ethanol**	**P**
TPC (mg GAE/g)	37.56 ± 1.41^{a}	42.09 ± 2.73^{a}	0.237	10.89 ± 0.03^{b}	9.15 ± 0.18^{b}	0.009
TFC (mg QE/g)	5.71 ± 0.90^{a}	6.60 ± 0.30^{a}	0.43	4.97 ± 1.24^{a}	4.97 ±0.82^{a}	0.47
DPPH (%)	31.78 ± 0.93^{a}	23.86 ± 0.90^{b}	0.003	25.66 ± 2.36^{ab}	20.10 ± 1.04^{b}	0.127
FRAP (mg $FeSO_4.7H_2O$/g)	112.28 ± 4.02^{a}	79.86 ± 3.18^{b}	0.003	30.69 ± 0.64^{c}	21.39 ± 0.74^{c}	0.0007

Note: Effects of solvent (methanolic and ethanolic) extracts on total phenolic contents (TPC); total flavonoid contents (TFC); DPPH scavenging activity; ferric reducing antioxidant power (FRAP) of fresh and wheatgrass powder. a-c: Different superscript letters indicate statistically significant differences ($P < 0.05$) among fresh and powdered samples. Paired t-test (P) indicates the comparison of the extraction values within groups, either in fresh or powdered conditions

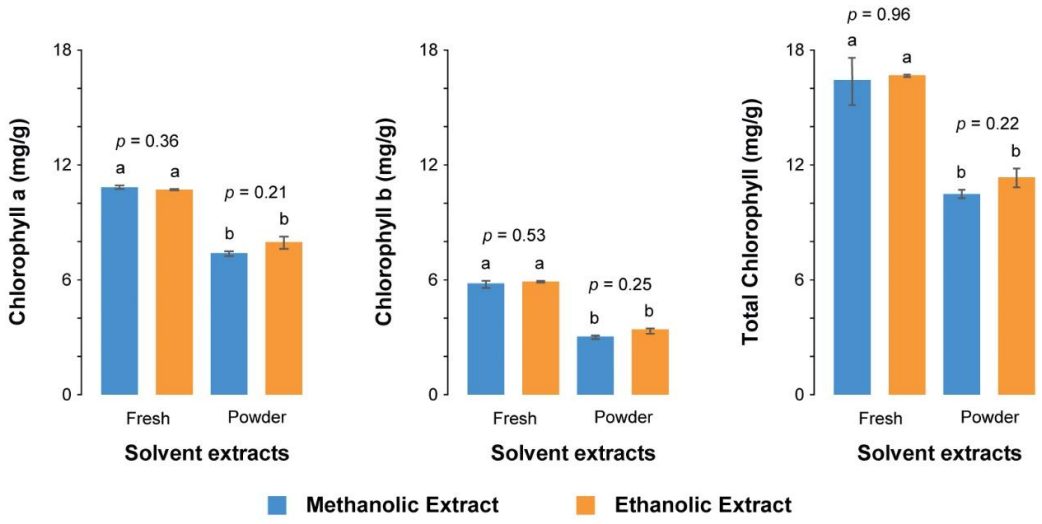

Fig. 1. Effects of solvent (methanolic and ethanolic) extracts on Chlorophyll-a, Chlorophyll-b and total chlorophyll content of fresh and wheatgrass powder

**a-b: different superscript letters indicate statistically significant differences (P < 0.05) among samples*

3.3 DPPH Radical Scavenging Activity

The extracts of FWG and WGP are screened (Table 1) for DPPH radical scavenging activity assay. FWG in methanolic extract showed a significantly (P < 0.05) higher scavenging activity than ethanolic extract. Additionally, methanolic extract displayed higher scavenging activity compare to ethanolic extract in WGP. It had been reported that an enormously constructive relationship between total phenols and antioxidant activity appears in numerous plant species [35]. Usually, DPPH radical scavenging activity is higher with higher TPC. Various investigations have agreed with the cause that is presumably the combined effect of the phenolic compounds in various concentrations and their high hydrogen atom-donating abilities [36]. This study also identified that WGP in both extracts has a lower % inhibition than the fresh extracts due to heating, causing a decrease in TPC and TFC. The influence of thermal treatment on foods either it is vegetables or fruits often causes changes in antioxidant activity: plant extracts from fresh material show a higher radical scavenging activity than heat-treated material [27]. Furthermore, the methanolic extract showed higher inhibition activity than the ethanolic extract in WGP. Parit et al. [37] revealed that among all extracts tested for WGP, DPPH scavenging activity comprises 30% which concedes this study. In that manner, the results depicted that wheatgrass is very strong in polyphenol activity and radical scavenging activity. However, more investigations are necessary for a more satisfactory understanding of their mechanism of action as antioxidants.

3.4 Ferric Reducing Antioxidant Power

Results showed that the methanolic solvent (112.28 ± 4.02 mg $FeSO_4.7H_2O$/g for FWG and 30.69 ± 0.64 mg $FeSO_4.7H_2O$/g for WGP) had higher FRAP than ethanolic solvent extracts (79.86 ± 3.18 mg $FeSO_4.7H_2O$/g for FWG and 21.39 ± 0.73 mg $FeSO_4.7H_2O$/g for WGP) respectively (Table 1). According to the obtained results, ethanol was less efficient in the extraction of antioxidant compounds than methanol, even though their polarities were similar. The longer chained ethyl radical results in lower solvation of antioxidant molecules than the methyl radical [38], which were responsible for the reduction of ferric ion. Therefore, the increasing order of reducing ability was found as ethanolic (powder) < methanolic (powder) < ethanolic (fresh) < methanolic (fresh). It was also observed that heating had caused lower FRAP values of WGP than FWG as our study depicted a positive co-relationship between TPC and FRAP.

3.5 Total Chlorophyll Content

It was observed that there was no significant difference (P < 0.05) in total chlorophyll content in both extracts of FWG samples. Total chlorophyll content of WGP in methanolic extract

(10.51 ± 0.23 mg/g) [out of which chlorophyll-a and chlorophyll-b were (7.42 ± 0.14 mg/g) and (3.04 ± 0.1 mg/g) respectively] was found lower than ethanolic extract (11.36 ± 0.48 mg/g) [out of which chlorophyll-a and chlorophyll-b were (8.02 ± 0.33 mg/g) and (3.35 ± 0.14 mg/g) respectively] (Fig. 1). According to Padalia et al. [6], chlorophyll comprises the most substantial active component that is known to have an active role in the inhibition of the metabolic activation of carcinogens. Based on Fig. 1, total chlorophyll content was found in increasing order as methanolic (powder) < ethanolic (powder) < methanolic (fresh) < ethanolic (fresh). The results showed that chlorophyll content was significantly lower after powder formation in both extracts. Researchers explained that during thermal processing, chlorophyll undergoes isomerization. While isomerization, the central magnesium atom in the porphyrin ring of chlorophyll is substituted by two hydrogen atoms, consequently leading to pheophytin and pheophorbide formation [39,40]. Investigators claimed that porphyrin is a responsible pigment for imparting colors to vegetables and fruits, which is available as Chlorophyll-a and b [23]. The degradation of both chlorophyll-a and chlorophyll-b causing the breakdown of total chlorophyll in WGP extracts [40]. The present study also recorded similarly. The appreciable amount of chlorophyll found in this study can detoxify the body and strengthen immunity [17].

4. CONCLUSION

Worldwide, various researches are ongoing about wheatgrass and its associated products. Our study was attempted to contribute necessary knowledge about the importance of wheatgrass as an important source of phytochemicals and antioxidants. A perceptible amount of phytochemicals and antioxidant activity was identified and determined from fresh and powdered wheatgrass using methanol and ethanol. Regarding phytochemicals, their amount was significantly high in most of the cases in methanolic extracts. As an exception, the phenolic and flavonoid contents of fresh wheatgrass showed the opposite results but those were nonsignificant. Besides, the overall flavonoid and chlorophyll contents differed nonsignificantly in both solvents. Similar results were observed regarding the antioxidant activities. Antioxidant activities were found to be higher either significantly or nonsignificantly in methanolic extracts. It seems methanol was apparently better in our study but ethanol was not far behind. Before we conclude our findings we suggest that more work should be done to find out the optimized extraction solution incorporating both solvents. It also clearly indicates that a whole lot of scientific exploration needs to be done on this herb which could provide the lead to a natural remedy against synthetic drugs for diverse diseases.

DISCLAIMER

The authors Towkir Ahmed Ove, Anwara Akter Khatun Said Bin Saifullah and Maruf Ahmed declare that they have no conflict of interest and have no affiliations with any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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