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PHYTOCHEMICALS AND *IN-VITRO* ANTIOXIDANT ACTIVITIES OF *ALOE VERA* GEL

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ABSTRACT

The phytochemicals (total phenolic, tannin, flavonoid, alkaloid, and saponin) contents in the Aloe vera gel derived from the leaf of *Aloe vera* (L.) Burm. f. (Synonym *Aloe barbadensismiller*) were extracted and their antioxidant capacity was studied by Ferric reducing antioxidant power assay (FRAP), by free radical-scavenging capability using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Three different solvents with three different polarities CH_3OH, CH_3OH: HCl, CH_3CH_3OH: H_2O were used at v/v ratio of 100, 98:2, 70:30, respectively. The acidified methanol solvent extracted the highest amounts of phytochemicals including total phenolic (4.64 mg gallic acid equivalent/g), tannin (3.84 mg tannic acid equivalent/g), alkaloid (662 mg piperine equivalent/g), and saponin (353 mg diosgenin equivalents/g) compared to the other two solvents. Similarly, in the extract with acidified methanol solvent, high level of total antioxidant activity (about 12 mg gallic acid equivalent/g) and scavenging effects expressed as 50% inhibition concentrations (IC_{50}) for DPPH and ABTS assay were determined to be about 61 µg/mL and 371 µg/mL, respectively, which are higher than those with the other two solvents. The gel extract could be used as a potent antioxidant in medicine and food industries.

Keywords: Acidified methanol, FRAP Method, DPPH, and ABTS inhibition.

INTRODUCTION

Aloe vera, the "wonder plant" (belonging to the Liliaceae family) has been used worldwide since ancient times for a variety of medicinal purposes (Akinyele and Odiyi 2007). It contains over 240 nutritional and medicinal ingredients, including vitamins (A, B_1, B_2, B3, B_6, B_{12}, C, E), minerals (Ca, Na, Fe, K, Mg, Cr, Mn, Cu, Zn, P), enzymes, sugars, lignin, saponins, sterols, amino acids (19 out of 20), salicylic acid, etc. No other plant contains so many ingredients beneficial to human health. Generally, the phytochemicals from the plants are nontoxic and have the capability to prevent chronic diseases. Aloe vera plants are also non-toxic, but a very few are extremely poisonous, due to the presence of hemlock-like substance (Atherton 1998). *Aloe barbadensis miller* is considered to be medicinally the most potent and, therefore, is the most popular.

The recovery of the phytochemicals from plants depends on the solubility of the chemicals on the solvents used for extraction. Mainly the solvent polarity plays a key role in increasing the solubility of the phytochemical (Naczk and Shahidi 2006). Most of the components in plants are largely polar; therefore methanol is effective to extract leaves and flower of Alphinia species (Wong 2006), young leaves of *Cameliasinensis* (Chan *et al.* 2007), Mulberry leaves (Yen *et al.* 1996) and young leaves of *Terminaliacatappa* (Chyau *et al.* 2002). Dehkharghanian *et al*. also reported in 2010 that differences in solvent

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polarity determine difference in extraction of phytochemical from plant and their antioxidant activity. Gel of aloe contains a large amount of bioactive compounds and show antioxidant activity (Hęś *et al.* 2019). Due to their antioxidant activity, they protect cells in human bodies against oxidative damage and reduce the risk of developing certain types of cancer.

Due to metabolic process and diseases in human body free radicals are frequently generated (Yeum *et al.* 2003) which can cause extensive damage to tissues and biomolecules leading to various other diseases, especially degenerative diseases and extensive lysis (Halliwell and Gutteridge 1999). Synthetic drugs can reduce the oxidative damage but they have adverse side effects. To avoid side effects, natural antioxidants from food supplements and traditional medicines are consumed (Bjelakovic and Gluud 2007, Chun *et al*. 2010, Jerome *et al*. 2011). Therefore, the demand of natural antioxidants contained in spices, herbs, and medicinal plants is increasing.

Despite numerous studies conducted on the antioxidant activities of the leaf gel and pulp extract of this plant, there is no substantial information on the antioxidant properties of the leaf gel extract of this species in Bangladesh. Moreover, spatial and climatic conditions have impact on diversity of phytochemicals and *in vitro* antioxidant activity of *Aloe vera* (L.) Burm. f. (Kumar *et al.* 2017). Therefore, it was considered worthwhile to investigate phytochemicals and antioxidant activities of locally available A. vera plant in Bangladesh. Here we use three solvents with three different polarities, namely pure methanol, acidified methanol and aqueous ethanol to find out the effective solvent for the extraction of phytochemicals from A. leaf pulp gel.

Furthermore, the phytochemicals are complex in nature, and therefore a single method to evaluate the antioxidants activity is not satisfactory. Therefore, several standard methods were adopted to authenticate the nature of plant extract in terms of antioxidants (Gangwar *et al*. 2014).

HMATERIALS AND METHODS

Chemicals and reagents

Folin–Ciocalteu's (FC) reagent, methanol, ethanol, sulfuric acid, sodium carbonate, sodium nitrite, sodium hydroxide, sodium acetate, acetic acid, aluminium chloride, potassium peroxodisulphate, and iron (III) chloride were purchased from R & M Chemicals (Essex, UK). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylben-zothiazoline-6-sulfonicacid) (ABTS), 2,4,6-tris(1-pyridyl)-5-triazine (TPTZ), polyvinyl polypyrrolidone (PVPP) and 1,10-phenanthrolin, were purchased from Sigma–Aldrich. Gallic acid was supplied by Pleon partners GmbH (Turkey). All chemicals and reagents used in the study were analytical grade.

Collection and preparation of crude extract

The Aloe vera leaves were collected from a garden of Natore district, Bangladesh and transported to Dhaka within overnight. The voucher specimen number is 2017/05. The plant was authenticated by a botanist and the plant materials were kept in our laboratory scientifically for further documentation. The collected Aloe vera leaves were cleaned with flow of fresh water. From 10.10 kg of green aloe vera leaves 7.55 kg pulp gel was obtained which results 78.7g pulverized powder by heating at oven at 50 ºC followed by grinding.

The powder (25g) obtained from the gel was mixed with different types of solvents and shaken in an orbital shaker for 3 days in a flat bottomed container with occasional stirring, homogenization, and sonication (Pisoschi *et al.* 2016). Then the extracts were collected from the

mixture by filtering. This was done repeatedly until colorless extract was obtained. After collecting extract, the solvent was evaporated under reduced pressure at a temperature below 50ºC in a rotary evaporator (RE 200 Sterling, UK) and the final extract was obtained using the freeze-drying process. After removing solvent, three pure extracts were obtained corresponding to the three different solvent mixtures (see Table 1). Mother solution was prepared by dissolving the extracts in pure methanol to obtain solutions of concentration 10 mg/mL.

Table 1. Amount of *Aloe vera* gel extract obtained from 25g of dry powder using different solvents and preparation of mother solution.

Types of solvent/ratio (v/v)	Amount of extract (g)	Mother solution (extract, mg / methanol, mL)
CH_3OH /100	11.67	106.9/10.5
CH_3OH :HCl / 98: 2(32%)	11.72	111.0/11.0
CH_3CH_2OH : H_2O / 70 : 30	13.10	102.0/10.0

Preparation of stock (working) solution

Stock solution was prepared by mixing 1.0 mL of mother solution in 9.0 mL of methanol. Then the concentration of stock solution for each extract was 1 mg/mL.

Determination of total phenolic content

Total phenolic content was determined following the established method described by Chanda and Dave (2009) with some modifications. 1.0 mL of extract (concentration of extract is 1 mg/mL), 4.5 mL distilled water and 0.5 mL of Folin-Ciocalteu reagent (0.5 N) were mixed and incubated at room temperature for 10 min. Then 2.0 mL saturated sodium carbonate solution was added and further incubated for 1.30 hours at room temperature. Blank was also prepared following the same method with using water instead of extract and absorbance was measured at 765 nm.

For standard curve determination we used gallic acid instead of samples at different concentrations (2, 4, 6, 8, 10 µg/mL), and the above method which was used for phenolic content was also used for the determination of standard curve.

Determination of Total Tannin

Quantitative estimation of tannin was carried out using the method described by Grubešić *et al*. (2005) with some modification. The total content of tannins adsorbed by PVPP was determined using Folin-Ciocalteu reagent (FC). About 1.0 mL (100 µg/mL) of extract solution was mixed with 100 mg of PVPP and then 9.0 mL methanol was also added with shaking for 2 hrs (adsorption of tannins). Blank was also prepared following the same method using water instead of extract and absorbance was measured at 765 nm. The total PVPP-adsorbed tannins are expressed as the number of equivalents of tannic acid (TAE) using the equation based on the calibration curve.

Determination of alkaloid

The total alkaloid contents were measured using 1,10-phenanthrolin described by Singh *et al.* (2004) with slight modifications. 1.0 mL of extract (1mg/mL) was taken for each test tube. Then 1.0 mL water, 1.0 mL 0.025 M iron (III) chloride in 0.5 M HCl and 1.0 mL 0.05 M of 1,10-phenanthrolin in ethanol were added. For blank, 4.0 mL water, 2.0 mL 0.025Miron (III) chloride in 0.5 M HCl and 2.0 mL 0.05 M of 1,10-Phenanthrolin in ethanol were added in a test tube. Then the absorbance was measured at 510nm. The values were expressed as piperine equivalents derived from a standard curve.

Estimation of total saponins content

Due to inhibitory effects, saponins are well known for anti-inflammatory on enzymes involved in the production of chemical mediator of inflammation (Gangwar*et al*. 2004). Total saponins content in A. gel was determined by the method described by Makkar *et al*. (2003). About 1.0 mL of plant extract was mixed with 2.0 mL 8% vanillin in ethanol solution. Then 2.0 mL of 72% sulfuric acid and 1.0 mL distilled water was added. This solution was kept in a water bath at 60 °C for 10 min. followed by cooling in ice cold water and the absorbance was measured at 544 nm. The values were expressed as diosgenin equivalents (mg DE/g extract) derived from a standard curve. For blank 4.0 mL water, 4.0 mL 8% vanillin in ethanol and 4.0 mL 72% sulfuric acid were added in a test tube.

Determination of flavonoid content

To protect human diseases like lipid peroxidation involved in thrombosis, carcinogenesis, atherogenesis, hepatotoxi-city etc. flavonoids play an important role (Tiwari 2001). Here total flavonoid content in A. gel was determined by Aluminium chloride method (Shwetha *et al*. 2012) using rutin solution as a standard. 1.0 mL of test sample and 4.0 mL of water were taken in 10.0 mL volumetric flask. After five min 0.3 mL of 5% sodium nitrite and 0.3 mL of 10% aluminum chloride was added. After 6 min incubation at room temperature, 2.0 mL of 1 M sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to the mark with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank as reference (UV-VIS Specord 205, Germany). For standard curve determination rutin hydrate solutions of different concentrations were used instead of sample solution.

Determination total Antioxidant capacity

(a) *FRAP Method*: In this study, the antioxidant activity was determined on the basis of the ability of antioxidant in the A. gel extracts to reduce ferric to ferrous in FRAP reagent (Alothman *et al.* 2009). FRAP reagent was prepared from acetate buffer (0.64 g sodium acetate and 3.2 mL acetic acid made up to 200 mL; pH 3.6), 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportion of 10:1:1 (v/v), respectively. The freshly made FRAP reagent (yellow in color) was warmed to 37 °C in an oven prior to use. A total of 0.5 mL of sample extract and 0.5mL distilled water were added to 3.0 mL of the FRAP reagent and mixed well. Successful reduction of ferric into ferrous in FRAP reagent results the formation of a blue-violet color of ferrous–TPTZ complex whose absorbance was measured at 593nm. Samples were measured in two replicates. Blank was prepared by using distilled water in place of extract. For standard curve gallic acid solutions of different concentrations were used in place of sample. The increase in absorbance due to the blue colored complex with Fe^{2+} ions at 593nm gives a measure of the antioxidant capacity.

(b) *DPPH Free Radical Scavenging Activity*: The assay of the scavenging of DPPH radical was used to evaluate the antioxidant capacity of extracts from different plant materials. After successful scavenging of DPPH radical by the A.gel extract, the purple color of the reaction mixture was changed to yellow with a consequent decrease in absorbance at 517nm. DPPH free radical-scavenging activity was determined according to Katalinic *et al*. (2010) with some modifications. Briefly, the reaction mixture (4.0 mL), consisting of 3.0 mL of DPPH in methanol (0.004%) and 1.0 mL of various concentrations of the extract was incubated for 10 min in dark, then the absorbance was measured at 517nm against methanol as a blank and control was prepared by DPPH and methanol in place of sample extract. In this assay, the positive control was ascorbic acid and gallic acid. The percentage of inhibition was calculated using the formula:

DPPH inhibition (%)={($A_0 - A_1$)/A_0}×100 where A_0 is the absorbance of control and A_1 is the absorbance of test.

For standard test 1.0 mL of various ascorbic acid solutions (100 µg/mL) and gallic acid solutions (10 µg/mL) were used in place of sample extract.

In DPPH free radical scavenging method, IC_{50} (Half maximal Inhibitory Concentration) value is the concentration of the sample that could scavenge 50% of DPPH free radical.

(c) *ABTS free radical scavenging activity*: $ABTS^{\bullet+}$ free radical scavenging activity was carried out according to the method reported by Jiri *et al.* (2010) with some modification. 7 $mmol \cdot L^{-1}$ ABTS and 4.95 $mmol \cdot L^{-1}$ potassium peroxodisulphate were dissolved in distilled water to form green color of $ABTS^{\bullet+}$ radical. The solution was then diluted with distilled water in a 1:9 v/v ratio. Then again 50 mL was quantitatively transferred from 1:9 ratio's solution into 250 mL calibrated flask and diluted at 1:36 ratio. The solution was incubated for 12 hrs in the dark; the reagent is usable for 7 days if stored in the dark at 4 °C. Briefly, the reaction mixture (4.0 mL) consisting of 3.0 mL of ABTS and 1.0 mL of sample extract solution of various concentrations was incubated for10 min in the dark, then the absorbance was measured at 734nm against distilled water as a blank and the control was prepared by $ABTS^{\bullet+}$ reagents and distilled water in place of sample extract. In this assay, the positive control was gallic acid and ascorbic acid. A lower absorbance indicated greater antioxidant capacity of the extracts. The percentage of inhibition can be calculated using the formula:

ABTS inhibition (%)={($A_0 - A_1$)/A_0}×100 where, A_0 is the absorbance of control and A_1 is the absorbance of test. In ABTS free radical scavenging method, IC_{50} value is the concentration of the sample that could scavenge 50% of ABTS free radical.

RESULTS AND DISCUSSION

Table 2 shows the phytochemicals content (total phenolic compounds, tannin, alkaloid, saponin, and flavonoid) in the extract of A. leaf pulp gel using three different solvents-pure methanol, 2% (v/v) hydrochloric acid in methanol, and 30% water in ethanol. The acidified methanol solvent was found to extract higher amount of phytochemicals including phenolic, tannin, alkaloid, and saponin compared to other two solvents.

The amount of total phenolic compounds extracted using acidified methanol solvent was 4.64±0.06 mg/g which is 4.7 and 3.7-fold higher than pure methanol and aqueous ethanol, respectively. Similarly, Tannin content was also found 2 and 3 times higher with acidified methanol solvent compared to pure methanol and aqueous ethanol solvents, respectively. The alkaloid extracted from the A. leaf pulp gel would be amphiphilic in nature since the extracted amount of alkaloid in all three solvents was not so different from each other.

The recovery of saponin in pure methanolic extraction was 30 mg/g (on the basis of dried pulp) however it is 12 and 8-fold higher in acidified methanol and aqueous ethanol. This suggested that the extractable saponin from A. leaf pulp is hydrophilic in nature. Most of the extracted phytochemicals are polar in nature, therefore they have the highest solubility in the most polar solvents, viz. acidified methanol. On the other hand, the flavonoid of aqueous ethanolic extract was the highest in concentration compared with pure methanolic and acidified methanolic extract. This might be due to the presence of higher amount of hydrophilic flavonoid in A. gel.

Quispe*et al.* (2018) found phenolic compounds and flavonoids in Aloe vera gel in Chile. The contents of total phenolics and flavonoids were identified by Ray *et al.* (2013) in the extracts of *Aloe vera* L. gel from different growth periods of plants. Wintola and Afolayan (2011) investigated the total phenols, flavonoids, tannins, alkaloids and saponins in the whole leaf extract of *Aloe vera by* using standard methods. Some of our results are consistent with those of previous authors.

Table 2. Phytochmicals content and antioxidant capacity in the extract of A. gel obtained using three different solvents*.

Types of solvents	Phytochemicals (mg/g)					Antioxidant capacity		
	Phenol	Tanin	Alkaloid	saponin	Flavonoid	FRAP (mg/g)	DPPH (IC_{50} mg/mL)	ABTS (IC_{50} mg/mL)
CH_3OH	0.99±0.03	2.168± .047	517.3 ± 6.6	29.43± 2.5	24.99± 2.42	1.262 ±0.109	3509.6 ± 2.1	1136.7 ± 23.0
CH_3OH : HCl	4.64 ±0.06	3.848 ± 0.077	662.1± 48.2	353.6 ± 2.2	17.55 ± 1.69	11.987 ±0.716	60.9 ± 0.3	370.9 ± 11.3
CH_3CH_2OH :H_2O	1.19 ±0.04	1.736±0.043	410.3 ± 6.0	252.9 ± 18.7	36.91 ± 2.25	1.933 ±0.029	4685.3 ±244.5	1156.4± 16.1

*each parameter shown in this Table was measured three times

Antioxidant capacity

Usually, scavenging specific radicals, inhibiting lipid peroxidation or chelating metal ions by plant material is an indicator of antioxidants capacity of that material. In this study, three different methods were used to estimate the antioxidant capacity of the A. gel extracts; they are ferric reducing/antioxidant power assay (FRAP assay), DPPH and ABTS free radical-scavenging assay.

FRAP Assay

At 1000 µg/mL the total antioxidant capacities of the A. gel extract were 11.98±0.72, 1.26±0.11, and 1.93±0.03 mg/g for acidified methanol, pure methanol and aq. ethanol solvents, respectively (see Table 2). This result indicates that maximum antioxidant activity of A. gel extract is obtained with acidified methanolic solvent. These results also support the findings in this study that the amount of total phenolic compounds extracted with acidified methanolic solvent was the highest compared to other solvents.

ABTS Radical Scavenging Activity

The result showed that different concentrations of A. gel extract having various grade of scavenging potential for $ABTS^{\bullet+}$. Based on IC_{50} value acidic methanolic solvent was found to be most active compared with other two solvents (Table 2). Maximum %inhibition was 96.39 ± 2.90at 1000 μg/mL among different concentrations of 100, 200, 400, 600, 800, and 1000 μg/mL (see Figure 1) with IC_{50} 370.9±11.3. For standard, gallic acidIC_{50} value is 2.52 µg/mL.

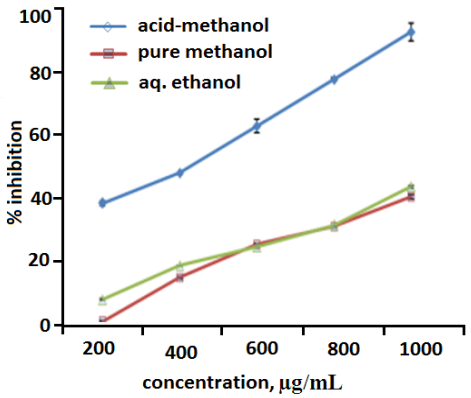

Fig.1. Inhibition (%) of $ABTS^{\bullet+}$ by different concentration of A. gel extract using three different solvents. Absorbance of only ABTS solution at 734 nm was 0.61 (experimental control).

DPPH Radical Scavenging Activity

Potential antioxidant activity of A. gel extract was observed with DPPH radical. Based on IC_{50} value the acidified methanolic solvent extracts most of the phytochemicals which is 60.9±0.3µg/mL (see Table 2) whereas for standard gallic acid it is 5.11 µg/mL. With A. gel extract obtained with acidified methanolic solvent, 81.24±0.93(%) inhibition of DPPH was obtained at 1000 μg/mL. This is the maximum inhibition among six different concentrations:100, 200, 400, 600, 800, and 1000 μg/mL.The percentage of DPPH inhibition by A. gel extracts obtained with pure methanolic and aq. ethanolic solvents was found to be15.26±0.07 and 12.27±0.37, respectively (see Fig. 2) at a concentration of 1000 μg/mL.

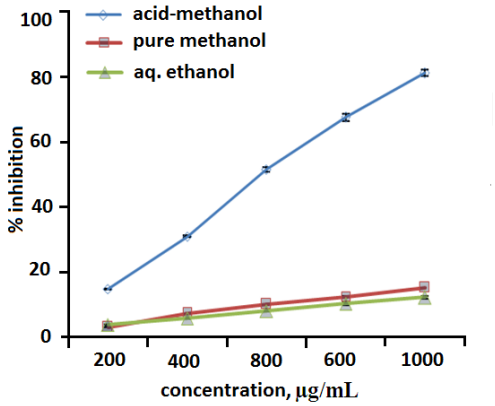

Fig. 2. Inhibition (%) of DPPH by different concentration of A. gel extract at three different solvents.

Muñoz *et al.* (2015) identified the DPPH scavenging activity was greater in freeze-dried gel of aloe vera in Chile. Quispe *et al.* (2018) also identified the antioxidant activity (DPPH, ABTS and FRAP) of chilian *aloe vera*. In addition, Ray *et al.* (2013) observed that aloe gel scavenged the DPPH and ABTS free radical in a dose dependent manner. Our results indicate that this A. vera species from Bangladesh is also associated with antioxidant activities.

CONCLUSIONS

The results indicate that A. leaf pulp gel extract contains significant amounts of phenolic, tannin, alkaloid, saponin, and flavonoid compounds. Except flavonoid the amounts of the other phytochemicals extracted in acidified methanolicsolvents were the highest compared to pure methanolic and aq. Ethanolic solvents. Therefore, it can be said that most of the phytochemicals in A. gel are polar in nature. The *in-vitro* antioxidant activity of these phytochemicals was evaluated by FRAP assay, DPPH, and ABTS methods in this study. All these three methods show that the extracts of A. gel obtained by acidified methanolic solvent exhibit the highest antioxidant activity compared to the extracts with the other two solvents.

Significant correlation was observed between the efficiency of solvents to extract phytochemicals and antioxidant capacity of A. gel. These correlations confirm that the phenolic, tannin, alkaloid, saponin compounds are the main micro-constituents contributing to the antioxidant activities of A. gel and acidified-methanolic solvents is the potential solvent for extraction of the micro-constituents.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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