Antioxidant, brine shrimp lethality and analgesic properties of propolis from Bangladesh

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Abstract

Propolis which is a natural resinous product from the honeybees, is becoming increasingly popular for its diverse therapeutic and industrial applications. In this study, we report for the first time the antioxidant, cytotoxic and antinociceptive activities of propolis from Bangladesh (BDP). The antioxidant activities were measured by biochemical analyses while cytotoxicity was evaluated by brine shrimp lethality bioassay. Analgesic activity was evaluated by tail immersion and acetic acid-induced writhing models in mice. BDP contained higher concentrations of polyphenols, flavonoids, tannins, ascorbic acid, vitamin E, reducing sugars and proteins, as compared to previous reports of propolis from several countries. BDP also exhibited higher free radical scavenging activities and a dose-dependent reducing power activity indicating its superior antioxidant potential. Moreover, BDP extract was most toxic to brine shrimp nauplii, with 50% lethal concentration (LC₅₀) of 57.99 µg/mL and exhibited considerable antinociceptive activities which may be mediated via central and peripheral inhibitory mechanisms.

Practical applications

BDP is a honeybee product, becoming increasingly popular due its potential contributions to human health. BDP is a rich source of natural antioxidants which has the potential to be used to ameliorate oxidative stress disorders and has useful antinociceptive activities. It may be useful as potential therapeutic agents against cancer, pesticidal agents and for other cytotoxic uses.

KEYWORDS

Propolis, Bangladesh, antioxidant activity, polyphenol, cytotoxicity, analgesics, DPPH

1 | INTRODUCTION

Propolis is the generic name for a complex resinous substance collected by *Apis mellifera* from various plant sources, including the flowers, leaves, buds and exudates (Toreti *et al.*, 2013). The natural product is used as sealant by honeybees to seal cracks and crevices in hives, smooth out the internal walls, and more importantly, to prevent the spread of microbial (bacterial and fungal) infections as well as the decomposition of dead creatures following hive invasion (Brumfitt *et al.*, 1990; Laskar *et al.*, 2010; Toreti *et al.*, 2013). Propolis is a popular folk medicine utilized since 300 BC. Propolis from different sources and countries have been reported to possess a wide array of pharmacological activities including antioxidant, antimicrobial, anticancer, anti-inflammatory, cytotoxic, immunomodulatory, as well as hepatoprotective properties (Ahmed *et al.*, 2017; Banskota *et al.*, 2000; Choi *et al.*, 2006; Silva *et al.*, 2017; Tan-No *et al.*, 2006). Due to its wide range of biological activities, it has recently been extensively used in food and beverage to promote good health and prevent oxidative stress disorders, including many chronic diseases such as, cardiovascular disease, diabetes and cancer (Ahmed *et al.*, 2017; Kumazawa *et al.*, 2004; Sforcin, 2016; Toreti *et al.*, 2013). However, the exact mechanisms for these activities have not been well elucidated. Most of the therapeutic effects of propolis, however, were proposed to be associated with its free radical scavenging activities (Laskar *et al.*, 2010).

Chemical analysis of propolis from across the world has pointed to the presence of at least 300 compounds. Propolis contains resin (50%), wax (30%), essential oils (10%), pollen (5%) and other organic compounds (5%) as well as different inorganic elements. Among these organic compounds, it contains a variety of bioactive compounds such as, phenolics and their esters, flavonoids of all forms, terpenoids, steroids, aromatic aldehydes and amino acids (Boufadi *et al.*, 2018; Debab *et al.*, 2016; Kumazawa *et al.*, 2004; Viuda-Martos *et al.*, 2008). Moreover, the composition of propolis is mostly depending on the vegetation at the site of collection, seasons, climate, environmental conditions and genetic diversity of the queen bees (Viuda-Martos *et al.*, 2008). In fact, propolis from Asia greatly differs from Europe, South America and Africa (Ahmed *et al.*, 2017; Kumazawa *et al.*, 2004).

Apiculture is one of the most promising agricultural sector in Bangladesh where honey is consumed on a large scale both commercially and non-commercially (Sarker *et al.*, 2015). Locally, honey is produced by two ways: naturally and cultured in wooden box by *Apis cerana*

or *Apis mellifera* (Figure 1). Additionally, there is a great prospect of beekeeping in Bangladesh due to its suitable geographical location, diverse plant species, agricultural crops and socio-economic context of the country (Moniruzzaman & Rahman, 2009). Furthermore, many government and non-government organizations (NGOs), such as Bangladesh Institute of Apiculture (BIA) and Proshikkhan Shikkha Karmo (PROSHIKA) provide technical supports to modernize apiculture practice and increase the production of bee products, primarily honey and secondarily propolis and others (Moniruzzaman & Rahman, 2009). To date, the composition and biological activities of propolis from various countries have been extensively reported by different researchers, but no single study has been conducted to investigate the chemical composition and/or biological properties of BDP though many references related to the biological properties and the application of Bangladeshi honey have been reported previously. Therefore, in this study, we aimed to investigate the antioxidant, cytotoxic and analgesic activities of BDP.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Gallic, tannic, L-ascorbic, catechin, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) standards were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Folin-Ciocalteu's phenol reagent, ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), potassium fericyanide [$\text{K}_3\text{Fe}(\text{CN})_6$], vincristine sulfate and tramadol were collected from Jayson Pharmaceuticals Ltd. Bangladesh. All of the chemicals and reagents used in this study were of analytical grade.

2.2 | Propolis sample collection

Stingless bee propolis of the *tetratrigona* genus was collected from PROSHIKA Integrated Agricultural Farm Trust, Koitta, Saturia, Manikgonj, Bangladesh in April 2016 (Figure 1).

2.3 | Extract preparation

Propolis extract (10%) was prepared according to the method described by (Laskar et al., 2010; Mouhoubi-Tafnine et al., 2016). Briefly, solid propolis samples (50 g) were first cut into small

pieces using a sterile, smooth steel knife. The samples were soaked with pure ethanol (100%) for 24 hours. Then ethanolic sample solution was shaken (150 rpm) at 30°C for 48 hours. The extract was filtered with a Whatman no. 1 filter paper and the filtrate was collected. To maximize the phenolic extraction, the residual extract was further dissolved in ethanol (100%) and was shaken (150 rpm) at 30°C for 12 hours. The extract was filtered with a Whatman no. 1 filter paper and the filtrate was recollected. Finally, the collected filtrate was dried in a rotary evaporator (Buchi, Tokyo, Japan) under a reduced pressure (100 psi) and at a controlled temperature (40°C). The dried extract (yield 13.59 g) was collected and finally preserved at -20°C for subsequent studies.

2.4 | Phytochemical analysis

2.4.1 | Determination of total polyphenol content

The total polyphenol content (TPC) of the BDP extract was spectrometrically determined according to the Folin-Ciocalteu method (Singleton *et al.*, 1999) which was adopted by Afroz *et al.* (2014) with some slight modifications. Briefly, 0.4 mL of the extract (2 mg/mL) was mixed with 1.6 mL of 7.5% sodium carbonate (Na_2CO_3) solution. Then, 2 mL of 10-fold diluted Folin-Ciocalteu's reagent was added and the final reaction mixture was incubated for 1 h in the dark. The intensity of the blue-colored complex was measured at 765 nm using a PD-303S spectrophotometer (APEL, Japan). The TPC was determined as gallic acid equivalent (GAE) (12.50 - 200.00 $\mu\text{g/mL}$, $r^2 = 0.997$) and was expressed as mg of GAEs per g of BDP.

2.4.2 | Estimation of total flavonoid content

The total flavonoid content (TFC) of BDP was estimated using an aluminum chloride colorimetric assay (Chang *et al.*, 2002). First, 1 mL of extract solution (0.25 mg/mL) was mixed with 4 mL of distilled water. Then, 0.3 mL of 5% sodium nitrite (NaNO_2) was added to the reaction mixture and after approximately 5 min, 0.3 mL of 10% aluminum chloride (AlCl_3) was added. Six min later, another 2 mL of 1 M sodium hydroxide (NaOH) was added, followed by the immediate addition of 2.4 mL of distilled water to produce a total volume of 10 mL. The color intensity of the developed flavonoid-aluminum complex was measured at 510 nm against a

solvent blank. TFC was determined as catechin equivalent (CE) (5.00-80.00 $\mu\text{g/mL}$, $r^2 = 0.984$) and was expressed as mg of CEs per g of BDP.

2.4.3 | Estimation of the total tannin content

The total tannin content (TTC) in the BDP extract was estimated using the Folin-Ciocalteu method (Tambe & Bhambar, 2014) with tannic acid as a standard. Briefly, 0.1 mL of the solution (0.50 mg/mL) was mixed with 7.5 mL of distilled water and then, 0.5 mL of Folin-Ciocalteu reagent was added. To the above mixture, 1 mL of 35% sodium carbonate (Na_2CO_3) and 0.9 mL of distilled water were added. The solution was mixed followed by incubation for 30 min. The intensity of the developed blue-colored complex was measured at 725 nm. The results were expressed as g of tannic acid equivalent (TE) (6.25-100.00 $\mu\text{g/mL}$, $r^2 = 0.999$) per g of BDP.

2.4.4 | Determination of total antioxidant capacity

The total antioxidant capacity (TAC) of the BDP extract was determined using the phosphomolybdenum method as described by Prieto et al. (1999). Briefly, 0.3 mL of the extract (0.20 mg/mL) was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and followed by incubation at 95°C for 90 min. After cooling at room temperature, the absorbance was measured at 695 nm. The TAC was determined as ascorbate equivalents (AEs) (6.25 - 200.00 $\mu\text{g/mL}$, $r^2 = 0.990$) and was expressed as mg of AEs per g of propolis.

2.4.5 | Determination of ascorbic acid content

The ascorbic acid content in the BDP extract was estimated by a method established by Omaye et al. (1979), with slight modifications (Ahmed et al., 2017). Briefly, 1 mL of extract (1 mg/mL) was mixed with 1 mL of 5% trichloroacetic acid (TCA) solution and centrifuged for 15 min at 3500 rpm. Then, 0.5 mL of the supernatant was mixed with 0.1 mL of DTC (2,4-dinitrophenylhydrazine/thiourea/copper) solution and incubated for 3 h at 37°C. To the mixture, 0.75 mL of ice-cold 65% H_2SO_4 was added. The solution was allowed to stand for an additional 30 min at room temperature. The developed colored complex was monitored at 520 nm. The ascorbic acid concentration was determined as ascorbate equivalent (AEs) (1.25-40.00 $\mu\text{g/mL}$, $r^2 = 0.986$) and was expressed as mg of AEs per g of sample.

2.4.6 | Estimation of reducing sugar content

The reducing sugar content in BDP was determined according to the Nelson-Somogyi method (Ahmed et al., 2017; Nelson, 1944). Briefly, 2 mL of BDP extract (1 mg/mL) and standards (made in 0.2% of benzoic acid) were transferred into two different test tubes, followed by the addition of 2 mL of copper reagent to each tube. The test tubes were heated for 15 min in a 100°C water bath and then cooled. Finally, 1 mL of arsenomolybdate color reagent was added to the reaction mixture. The absorbance was measured at 520 nm. Dextrose was used as a standard to prepare the calibration curve (6.25-100 µg/mL, $r^2 = 0.993$). The reducing sugar content was expressed as mg of D-glucose per g of BDP.

2.5 | Analysis of antioxidant properties

To investigate the antioxidant potential of the BDP extract, DPPH free radical and nitric oxide (NO) radical scavenging activities, reducing power capacity, cupric reducing antioxidant activity were performed.

2.5.1 | DPPH free radical-scavenging activity assay

The antioxidant potential of BDP was determined according to the DPPH radical-scavenging activity based on a method established by Braca et al. (2002). Briefly, 1 mL of the extract and ascorbic acid at varying concentrations (12.50 – 800.00 µg/mL) were mixed with 2 mL of 0.004% DPPH in methanol. The final mixtures were incubated for 30 min at room temperature in the dark. The absorbance was measured at 517 nm. The percentage of DPPH inhibition was calculated using the following equation:

$$\% \text{ of DPPH inhibition} = [(A_{\text{DPPH}} - A_s / A_{\text{DPPH}})] \times 100$$

where A_{DPPH} is the absorbance of DPPH in the absence of a sample and A_s is the absorbance of DPPH in the presence of either the sample or the standard.

DPPH scavenging activity is expressed as the concentration of sample that is required to decrease DPPH absorbance by 50% (IC_{50}). The value can be graphically determined by plotting the absorbance (the percentage of inhibition of DPPH radicals) against the log concentration of DPPH and determining the slope of the nonlinear regression.

2.5.2 | Nitric oxide radical scavenging assay

Sodium nitroprusside (SNP) can decompose to produce NO in aqueous solution at physiological pH (7.2). Under an aerobic condition, NO reacts with oxygen to form stable products: nitrate and nitrite. Based on this principle, the NO scavenging activity of BDP extract was investigated according to the method described by Alisi and Onyeze (2008).

Briefly, 4 mL of extract and ascorbic acid standard solution at varying concentrations (6.25 – 200.00 µg/mL) were mixed with 1 mL of SNP solution (5 mM) and were incubated at 30°C for 2 h. After that, an aliquot of 2 mL of the incubated solution was diluted with 1.2 mL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine-dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine-dihydrochloride was read immediately at 550 nm.

The percentage of NO inhibition was calculated using the following equation:

$$\% \text{ of NO inhibition} = [(A_{\text{NO}} - A_s / A_{\text{NO}})] \times 100$$

where A_{NO} is the absorbance of NO in the absence of a sample and A_s is the absorbance of NO in the presence of either the sample or the standard.

NO scavenging activity is expressed as the concentration of sample that is required to decrease NO absorbance by 50% (IC_{50}). The value can be graphically determined by plotting the absorbance (the percentage of inhibition of NO radicals) against the log concentration of NO and determining the slope of the nonlinear regression.

2.5.3 | Reducing power capacity assay

The reducing power capacity of the BDP extract was performed according to Oyaizu method (1986). In different test tubes, 2 mL of BDP extract or ascorbic acid standard were mixed at varying concentrations (6.25 – 200.00 µg/mL) with 1% potassium ferri-cyanide (2.5 mL) and followed by incubation for 10 min at 50°C to complete the reaction. Then, 2.5 mL of 10% trichloroacetic acid solution was added to the mixture. The final mixture was centrifuged at 3000 rpm for 10 min. After that, 2.5 mL of supernatant solution was withdrawn from each of the mixture tubes and subsequently mixed with distilled water (2.5 mL) and 0.1% ferriic chloride (0.5 mL). The absorbance of the solutions was measured at 700 nm against a blank. A typical

blank solution contained the same solution mixture without BDP extract or standard and it was incubated under the same conditions as the sample solution. Increased absorbance of the reaction mixture indicated the increase of reducing power of BDP.

2.5.4 | Cupric reducing antioxidant capacity assay

The cupric reducing antioxidant activity of organic extract of BDP was determined by the CUPRAC method as described by Rasat et al. (Apak *et al.*, 2004). An aliquot of 0.5 mL of extract or ascorbic acid standard at different concentrations (6.25 – 800.00 µg/mL) were mixed with 1 mL of cupric chloride (0.01 M). To the reaction mixtures, 1 mL of ammonium acetate buffer (pH 7.0), 1 mL of neocuproin (0.0075 M) and 600 µL of distilled water were added to adjust the final volume of 4.1 mL. The mixture solutions were incubated for 1 h at room temperature and the absorbance was read at 450 nm against the blank. The molar absorption of the CUPRAC method for each antioxidant was found from the slope of the calibration line concerned.

2.6 | Cytotoxicity test

Brine shrimp lethality bioassay was performed to investigate the probable cytotoxic action (Hossain *et al.*, 2017) of BDP extract. The eggs of brine shrimp, *Artemia saline* Leach, nauplii (Ocean 90, USA) are marine invertebrates (approximately 1 mm in size) and were collected and hatched in a tank at 37°C with a constant oxygen supply. Two days were lapsed to allow hatching and maturation the nauplii. Only mature nauplii were used in the experiment. According to the assay method, the stock solution of BDP extract was prepared by dissolving in dimethyl sulfoxide (DMSO). Eight different concentrations (500, 200, 100, 50, 20, 10, 5 and 1 µg/mL) were used in this assay. A similar volume of DMSO solution was used as negative control whereas vincristine sulfate (VS) was used as a positive control (Meyer *et al.*, 1982). About 10 living nauplii were taken to each of the vial containing extracts at specific concentrations. After 24 h, the vials were observed and the number of nauplii survived in each vial was counted. Subsequently, percentage mortality of brine shrimp nauplii was calculated for each concentration of the extracts. Larvae were considered dead if they do not show any external or internal movement following several minutes of observation. To ensure that the mortality observed in the bioassay could be attributed to bioactive compounds and not to starvation, the dead larvae were further compared with the control.

2.7 | Tail immersion test

The tail immersion method was used to evaluate the central mechanism of the analgesic activity of the sample. In this test, painful reactions in the animals were produced by a thermal stimulation, that is, by dipping the tip of the tail (1-2 cm) in hot water (Hasan *et al.*, 2014). Swiss albino mice were grouped and treated with 250 and 500 mg/kg body weight, respectively. The tramadol (100 mg/kg) was used as a standard drug while the control group received water only. All animals were fasted for 16 h with water *ad libitum*. After administration of standard and BDP extract, the basal reaction time was measured by immersing the tail tips of the mice in hot water heated at $55 \pm 1^\circ\text{C}$. The actual flick response of mice, that is, the time taken in seconds to withdraw the tail from hot water source was calculated and the results were compared with the control group. Mice with baseline latencies of more than 15 seconds were eliminated from the study since a latency period of 15 seconds was set as the cutoff point and the measurement was then stopped to avoid injury to mice. The latent period of the tail flick response was determined at 0, 1/2, 1, 2, and 3 h following the administration of BDP extract and standard drugs.

2.8 | Acetic acid induced writhing test

The method was based on Koster *et al.* (1959). Swiss albino mice were divided into four groups of five mice each and were pretreated with BDP extracts (250 and 500 mg/kg body weight), diclofenac (100 mg/kg) and distilled water (3 mL/kg). Forty five minutes later, each mouse was injected intraperitoneally with 0.7% acetic acid at a dose of 10 mL/kg body weight. The number of writhing responses was recorded for each animal during a subsequent 5 min period after 15 min of intraperitoneal administration of acetic acid. The mean abdominal writhing for each group was obtained. The percentage of inhibition of writhing was calculated using the following formula:

$$\% \text{ Inhibition} = (1 - \text{No. of Writhing (Drug/Standard)} / \text{No. of Writhing (Control)}) \times 100.$$

2.9 | Statistical analysis

All analyses were performed in triplicate, and the data are reported as the mean $\pm$ standard deviation (SD). Data were analyzed using SPSS (Statistical Packages for Social Science, version 16.0, IBM Corporation, New York, USA) and Microsoft Excel 2007 (Redmond, Washington, USA). Statistical analyses of the biochemical data were conducted using Bonferroni multiple comparisons test. $p < 0.05$ was considered as statistically significant.

3| RESULTS AND DISCUSSION

3.1 | Phytochemicals

This is the first report on bioactive phytochemicals, antioxidant potentials, analgesic and cytotoxic activities of ethanol extracts of propolis from Bangladesh.

Polyphenols, flavonoids and tannins are the plant pigments that contribute to the functional quality, color and flavor of bee products and serve as powerful antioxidants due to the hydrogen-donating ability of their hydroxyl groups as well as their ability to donate electrons to arrest the production of free radicals [superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($O^{\bullet}$) and hydrogen peroxide (H_2O_2) and other pro-oxidants] due to oxidative stress (Afroz *et al.*, 2015). The non-enzymatic antioxidants (phenolics and flavonoids) react with the pro-oxidants, leading to inactivation of free radicals and other pro-oxidants. To counter the potential hazards of oxidative damage, the dietary consumption of antioxidant including phenolics and flavonoids may be regarded as the first line of defense against highly reactive toxicants (Denre, 2014; Tanvir *et al.*, 2017). The TPC of BDP was 80.16 ± 0.79 mg/g of sample (Table 1) or 295 ± 2.94 mg/g of extracts while that of TFC was 40.30 ± 2.72 mg/g of extracts respectively. Figure 2 represent the TPC and TFC in ethanol extracts of propolis reported from various countries, along with a comparison of the findings for BDP (Choi *et al.*, 2006; Kumazawa *et al.*, 2004; Laskar *et al.*, 2010). Most recently, Misir *et al.* (2018) reported the TPC, TFC and reducing power in ethanol extract of Turkish propolis at 114.07 ± 0.02 , 36.02 ± 0.08 and 246.80 ± 0.01 mg per g of sample, respectively. In other studies, Zehra *et al.* (2015) and Socha *et al.* (2015) investigated the antioxidant properties of propolis from Azerbaijan and Poland respectively. They reported that TPC in the samples ranged from 10.84 to 78.23 and 150.05 to 197.14 mg GAE/g, respectively. Further analysis of TTC indicated that BDP is a very good source of tannins (Table 1). Debab *et al.* (2016) estimated the tannin content in propolis from West Algeria which ranged between 4.59 and 0.81 mg per g of extract. Recently, we reported the TPC, TFC and TTC in Malaysian propolis (MP) to be of 15.93 ± 0.18 , 1.65 ± 0.10 and 5.81 ± 1.65 mg per g of sample, respectively (Ahmed *et al.*, 2017) which is significantly lower than that for BDP (Figure 3).

BDP ethanol extract also exhibited a significant amount of TAC at 200 μ g/mL (140.50 ± 3.54 mg/g of ascorbic acid equivalent). The phosphomolybdenum method used in this assay has previously been successfully adopted for specific determination of vitamin E in seeds. Because

of being simple and independent from other antioxidant measurement, the technique is routinely applied to determine vitamin E concentration in different extracts and to evaluate TAC (Prieto et al., 1999).

Ascorbic acid (vitamin C) is another essential nutrient that acts as a strong reductant via its direct radical scavenging activity as well as its involvement in the cyclic regeneration of oxidized vitamin E to its functional state by a non-enzymatic mechanism where it constitutes a strong line of defense against free radical induced cellular damage (Chan, 1993; Tanvir et al., 2017). Our findings indicated that BDP contains a significant amount of ascorbic acid (19.51 ± 0.47 mg/g of sample) compared to other antioxidants (Table 1) and the mean concentration is significantly higher than that for MP (0.91 ± 0.02 mg/g of sample) (Figure 3) (Ahmed et al., 2017). Likewise, propolis from Algeria has been reported to contain ascorbic acid ranging between 2.10 and 3.95 mg/100 g of propolis (Mouhoubi-Tafnine et al., 2016). It is plausible that the variation in vitamin C content observed in propolis from different countries may be influenced by the floral origin and climate condition as supported by Nagy (1980).

In addition to antioxidants composition, our study also confirmed that BDP is a rich source of reducing sugars (Table 1). Recently, Haghdoust et al. (2016) confirmed the presence of four reducing sugars in ethanol extract of propolis including D-fructose, glucofuranose, talose and treitol.

3.2 | Antioxidant activities

The free radical scavenging activities of BDP were evaluated by their potential to quench the synthetic DPPH free radicals as well as NO radicals. DPPH is a stable free radical that accepts an electron or hydrogen radical from a donor (usually the reducing agents present in the extracts) and become a stable diamagnetic molecule in methanol (Laskar et al., 2010). On the contrary, in NO scavenging assay NO is generated from sodium nitroprusside and is scavenged by BDP extract as well as ascorbic acid which is used as a standard. The findings are expressed as percentage of inhibition against the concentration where a dose-dependent inhibition was observed [Figures 4 (a) & (b)]. The inhibitory concentration 50% (IC_{50}) values are presented in table 2.

Debab et al. (2016) reported the IC_{50} values of propolis from West Algeria of 19.95 mg/mL, indicating its inferior antiradical activity than BDP (0.42 mg/mL). In addition, Al Naggar et al.

(2016) reported higher radical scavenging activities of propolis ethanolic extract from Canada (TPC: 410.81 to 429.61 mg GAE/g of extract). Our findings suggest that the free radical-scavenging activity of BDP extract may be attributed to the high contents of phenolic acids, flavonoids and other scavengers with a higher reducing capacity. Furthermore, to characterize the reducing power of BDP, three auxiliary tests were performed on the extracts. The reducing power capacity and cupric reducing antioxidant activity assessments were further evaluated to determine the reducing power features of BDP extract. In addition, the concentration dependent reducing activities of BDP extract as well as ascorbic acid (standard compound) have also been documented. According to variable trend in the concentration, it is concluded that BDP has a lower reducing power than that of standard [Figures 4 (c) & (d)]. Due to the fact that it is a strong electron or hydrogen donor, the phenolics, flavonoids and vitamins (including ascorbates and tocopherols) in BDP have the potential to neutralize free radicals, convert ferric to ferrous and cupric to cuprous forms hence showing a higher reducing activity. It is also hypothesized that BDP may have great relevance in the prevention and control of disorders where reactive nitrogen species (RNS) as well as reactive oxygen species (ROS) are thought to play key roles in its pathogenesis.

3.3 | Cytotoxic activity

The brine shrimp lethality assay has been established as a rapid, reliable, economic and practical bioassay tool for the determination of bioactive cytotoxic compounds in plant extracts. The National Cancer Institute (NCI, USA) demonstrates that the correlation between brine shrimp lethality assay and *in vitro* growth inhibition of human solid tumor cell lines is significant as a prescreening tool for anticancer drug research (Hasan et al., 2016; Meyer et al., 1982).

In our bioassay, BDP was most toxic to brine shrimp nauplii, with LC_{50} of 57.99 $\mu\text{g/mL}$ ($X^2 = 2.72$, $p < 0.05$) which was significantly higher than the standard anticancer drug vincristine sulphate with LC_{50} of 1.52 $\mu\text{g/mL}$ ($X^2 = 100.57$, $p < 0.05$) (Table 3). According to Meyer et al. (1982), using this bioassay tool, the extracts derived from natural sources with LC_{50} value ≤ 1000 $\mu\text{g/mL}$ were claimed to contain bioactive principles. In our study, the BDP extract showed strong toxicity against nauplii, which might be attributed to the presence of active secondary metabolites including alkaloids, organic acids, phenolic compounds, flavonoids, tannins that are

present in BDP and may be useful as potential therapeutic agents against cancer, pesticidal agents and for other cytotoxic uses.

3.4 | Tail immersion test

The tail immersion model is established to investigate the centrally acting analgesics. In this test, the sensory neurons sensitize the nociceptors, thus minimizing the involvements of endogenous compounds, such as prostaglandins (PG) (Woolfe & MacDonald, 1944). The test is particularly useful for identification of the involvement of opioid receptors in the activity of narcotic drugs or other analgesic agents that produce effect in the central mechanism (Hasan et al., 2014). In this thermal nociception model, the ethanolic extract of BDP at both doses (250 mg/kg and 500 mg/kg) significantly prolonged the latency period ($p < 0.0001$) compared to control. Additionally, the BDP extract at 500 mg/kg dose exerted a gradual lengthening of latency reaction over time, suggesting the antinociceptive effects of the extract through the stimulation of opioid receptors (Table 4). The observed analgesic activity of BDP maybe partially explained by their robust antioxidant properties (Table 2 and Figure 4) similar to that of morphine. Morphine is an opioid drug and has been reported to show effective reducing power, free radical scavenging and metal chelating activities that may contribute to some of its central analgesic activity (Gülçin *et al.*, 2004) and in turn, the central analgesic effects of BDP. The phytochemicals determined in BDP extracts such as flavonoids and polyphenols (particularly tannins and other alkaloids) were found to have antinociceptive activity and inhibited the pain perception (Hasan et al., 2014).

3.5 | Acetic acid induced writhing test

Abdominal contraction response induced by acetic acid is very a sensitive method to screen peripheral analgesic effects which is sensitized by PG (Bentley et al., 1983; Hasan et al., 2014). In mice, acetic acid contributes to the release of arachidonic acids from the phospholipid membrane and synthesize PG via the cyclo-oxygenase (COX) dependent pathway (Davies *et al.*, 1984). Acute inflammation induced by acetic acid is directly related to increased levels of prostaglandin E2 (PGE2) and prostaglandin F2 α (PGF2 α), the products of lipoxygenase (LOX) pathway in peritoneal fluids and the release of endogenous vasodilators including bradykinin, serotonin and histamine which are involved for the stimulation of the central nociceptive neurons (Dey et al., 2010; Hasan et al., 2014; Levine et al., 1984; Verma et al., 2005). Any substance causing inhibition of acetic acid-induced writhing may have anti-inflammatory and

analgesic effects, preferably via the inhibition of PG biosynthesis, which is also known as a peripheral mechanism of pain inhibition. In our study, the ethanol extract of BDP showed significant analgesic effects induced by acetic acid in the mice model. In fact, BDP extracts at 250 mg/kg and 500 mg/kg produced significant 41.72% and 46.68% inhibition of writhing in test animals compared to the control mice, respectively (Figure 5). The number of writhing responses of BDP extracts at 250 mg/kg and 500 mg/kg were 5.98 ± 0.34 and 5.47 ± 1.01 , respectively as compared with the number of writhing responses (3.26 ± 0.86) and percent inhibition of the responses (68.23%) shown by diclofenac (standard drug) at 100 mg/kg, thus indicating the high potency of the BDP extract as a analgesic agent as shown by its antinociceptive activity. Similar findings also claimed that propolis extracts exert its anti-inflammatory effects through the inhibition of PG biosynthesis and platelet aggregation (Khayyal *et al.*, 1993). Moreover, propolis suppressed the generation of COX and LOX during acute peritonitis induced by zimosan and inhibits high production of LTB₄ and LTC₄ *in vivo* as well as NO generation besides inducing hydrogen peroxide release via activation of macrophages in a dose-dependent manner (Mirzoeva & Calder, 1996; Orsi *et al.*, 2000). Such anti-inflammatory action of BDP extracts may be attributed to active phenolics that have the potential to inhibit PG biosynthesis (Randjelović *et al.*, 2015) and may also be contribute to other compounds such as ascorbic acids, as well as tannins in BDP (De Almeida & Menezes, 2002). Furthermore, the free radical scavenging activity of the aforementioned compounds in BDP extract may also be effective against pathological alterations, such as edematous conditions. Therefore, this study confirmed the *in vitro* antioxidant and anti-inflammatory activities of BDP extract which is proposed to occur by either protecting against oxidative stress or by inhibition of enzymatic pathways (COX and/or LOX) of PG biosynthesis of inflammatory process.

The study has some limitations. The study lacks information on fat soluble vitamins and other bioactive hydrophobic secondary metabolites. However, further research is underway to isolate the bioactive constituents and to elucidate the underlying molecular mechanisms of their antitumor, anti-inflammatory and analgesic effects.

4 | CONCLUSIONS

The study highlights the antioxidant, cytotoxicity and analgesic properties of propolis from Bangladesh which confirms the fact that it is a rich source of polyphenols, flavonoids, tannins,

vitamins C and E, and reducing sugars, all of which are strong antioxidants. The active constituents have strong free radical scavenging activity and therefore may confer effective protection against oxidative stress-induced cellular damages. The findings from the brine shrimp lethality and antinociceptive activities further suggest the therapeutic application of propolis in cancer treatment as well as in agriculture, cosmetic and other industrial applications. In conclusion, propolis from Bangladesh has the potential to ameliorate oxidative stress and has good cytotoxicity and antinociceptive activities.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Figure Legends

Figure 1: Bangladeshi propolis production (a) maintenance of honey bee colonies in bee hives (b) manual collection of propolis from bee hives and (c) raw propolis.

Figure 2: TPC and TFC in BDP when compared with propolis samples from different countries. Data are presented as mean $\pm$ SD.

Figure 3: Phytochemicals present in pure (100%) ethanolic extract of propolis from Bangladesh and 70% ethanolic extract of Malaysian propolis (Ahmed et al., 2017).

n= 3. Data are presented as mean $\pm$ SD. BDP: Propolis from Bangladesh, MP: Malaysian propolis

Figure 4: (a) DPPH free radical scavenging activity, (b) NO free radical scavenging activity, (c) reducing power capacity, and (d) cupric reducing antioxidant activity of ethanolic extract of BDP along with its ascorbic acid concentration.

BDP: Propolis from Bangladesh

Figure 5: Effects of ethanolic extract of BDP on acetic acid-induced writhing test (a) number of writhing observed between different groups (b) percentage of inhibition observed between different groups.

n = 5. Data are presented as mean $\pm$ SD. The bars with different values (a, b) indicate significant difference when compared between different doses of the extract and standard at $p < 0.05$. One way ANOVA followed by Tukey multiple comparisons was performed to analyze this relationship. The bars with different value (x) indicate significant difference when compared against control at $p < 0.01$. A one way ANOVA followed by Dunnet's multiple comparison was performed to analyze this data set.

Table 1: Total polyphenols, flavonoids, tannins, protein, ascorbic acid and reducing sugar contents in BDP.

Phytochemicals	Amount present in propolis (mg/g)
Total polyphenols (GAEs)	80.16 $\pm$ 0.79 ^a
Total flavonoids (CEs)	10.95 $\pm$ 0.74 ^b
Total tannins (TEs)	51.69 $\pm$ 2.30 ^c
Ascorbic acid (AEs)	19.51 $\pm$ 0.47 ^e
Reducing sugar (D-glucose)	32.14 $\pm$ 0.94 ^f

n = 3. Data are presented as mean $\pm$ SD, Values in the same column that do not share superscript letters indicate significant difference at p<0.05. A one way ANOVA followed by Tukey multiple comparisons was performed to analyze the relationship.

GAE, gallic acid equivalents; CE, catechin equivalent; TE, tannic acid equivalent; AE, ascorbate equivalent. BSA, Bovine serum albumin.

Table 2: DPPH and NO radical scavenging activities of BDP

Sample	DPPH radical scavenging activity, IC ₅₀ (μg/mL)	NO radical scavenging activity, IC ₅₀ (μg/mL)
BDP	419.07 $\pm$ 0.98 ^a	88.33 $\pm$ 0.15 ^a
Ascorbic acid	19.85 $\pm$ 0.28 ^b	25.82 $\pm$ 0.05 ^b

n = 3. Data are presented as mean $\pm$ SD, ^{a, b} Values in the same column that do not share superscript letters (a, b) indicate significant difference at p<0.05. DPPH: 1,1-diphenyl-2-picrylhydrazyl, NO: nitric oxide, IC₅₀: inhibitory concentration 50%, BDP: Propolis from Bangladesh

Table 3: Brine shrimp lethality activity of ethanolic extract of BDP

Test sample	LC ₅₀ (μg/mL)	CI	χ ²	p value
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BDP	57.99 ^a	12.94 - 165.06	2.72	<0.05
VS	1.52 ^b	1.19 - 1.99	100.57	<0.05

n = 3. Data are presented as mean $\pm$ SD, ^{a, b}. Values in the same column that do not share superscript letters (a, b) indicate significant difference at $p < 0.05$. Fenny probit analysis was performed to determine LC₅₀ values, confidence interval (CI) limit, Chi square (χ^2) and p value. LC₅₀: lethal concentration 50%., BDP: Propolis from Bangladesh, VS: Vincristine sulfate

Table 4: Effects of BDP extract on the latency time in tail immersion test.

Group	Doses (mg/kg)	Latency Time (h)				
		0	½	1	2	3
Control		3.13 $\pm$ 0.06	3.48 $\pm$ 0.06	3.59 $\pm$ 0.33	4.21 $\pm$ 0.33	4.76 $\pm$ 0.08
Tramadol	100	6.14 $\pm$ 0.13	10.30 $\pm$ 0.09 ^{y,a,b}	12.49 $\pm$ 0.13 ^{y,a,b}	14.71 $\pm$ 0.20 ^{y,b}	14.90 $\pm$ 0.20 ^{y,a,b}
BDP	250	4.24 $\pm$ 0.08	5.32 $\pm$ 0.07 ^{y,a}	5.74 $\pm$ 0.21 ^{y,a}	8.35 $\pm$ 0.48 ^{y,a}	5.14 $\pm$ 0.35 ^{y,a}
	500	6.37 $\pm$ 0.11	7.82 $\pm$ 0.03 ^{y,b}	8.17 $\pm$ 0.15 ^{y,b}	8.55 $\pm$ 0.19 ^{y,a}	9.92 $\pm$ 0.14 ^{y,b}

n = 5. Data are presented mean $\pm$ SD, Overall time effect [F (4, 96) = 125.16, $p < 0.0001$] is considered very highly significant. ^{a, b}. Values in the same column that do not share superscript letters (a, b) indicate significant difference from each other at each specific hour when compared between different doses of the extract and standard at $p < 0.05$. A pnc way ANOVA followed by Bonferroni multiple comparisons was performed to analyze this relationship. ^y value in the same column that do not share superscript letters (y) indicate significant difference at $p < 0.0001$ when compared against control. A one way ANOVA followed by Dunnett's multiple comparisons was performed to analyze this relationship.

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