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ORIGINAL ARTICLE

Antioxidant and Intracellular Reactive Oxygen Species/Reactive Nitrogen Species Scavenging Activities of Three Porcupine Bezoars from *Hystrix brachyura*

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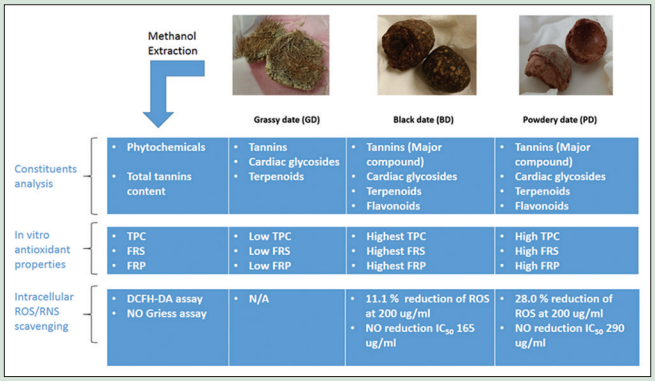
ABSTRACT

Background: Porcupine dates are phytobezoar stones that are used in Traditional Chinese Medicine (TCM) treatments against cancer, postsurgical recovery, dengue fever, etc. The medicinal values have not been scientifically investigated due to the availability and high pricing of the dates. **Objectives:** This paper represents the first report on the phytochemical content, *in vitro* antioxidant and intracellular reactive oxygen species (ROS)/reactive nitrogen species (RNS) scavenging properties of the extracts of three porcupine dates: grassy date (GD), black date (BD), and powdery date (PD). **Materials and Methods:** Dried samples were extracted with methanol and lyophilized. Samples were screened for phytochemical constituents, *in vitro* antioxidant assays based on total phenolic content (TPC), free radical scavenging, and ferric reducing power (FRP) as well as intracellular ROS and RNS scavenging properties. **Results:** Phytochemical screening and total tannins assay revealed that tannins, cardiac glycosides, and terpenoids were found in all porcupine dates with tannins forming the major portion of the TPC. In comparison to GD, BD and PD were found to contain significantly high TPC, radical scavenging activity, and FRP. At 200 μg/ml, BD and PD remarkably scavenged 2, 2-azobis (2-amidinopropane) dihydrochloride-induced ROS in RAW264.7 cells and significantly reduced nitric oxide in lipopolysaccharide-stimulated cells. **Conclusion:** Overall, BD and PD exhibited promising *in vitro* antioxidant as well as intracellular ROS/RNS scavenging properties.

Key words: Antioxidant activity, oxidative stress, phytochemical, porcupine date, Traditional Chinese Medicine

SUMMARY

- Tannins, cardiac glycoside, and terpenoids were found in all three types of porcupine dates with tannins being the major compounds
- Antioxidant contents and properties of three dates were in the order black date (BD) > powdery date (PD) > grassy date
- BD and PD extracts showed significant intracellular reactive oxygen species and reactive nitrogen species scavenging properties.



Abbreviations Used: TCM: Traditional Chinese Medicine, BD: Black date, GD: Grassy date, PD: Powdery date, TPC: Total phenolic content, FRS: Free radical scavenging, FRP: Ferric reducing power, NO: Nitric oxide, ROS: Reactive oxygen species, RNS: Reactive nitrogen species, GAE: Gallic acid equivalent, AAE: Ascorbic acid equivalent, PVPP: Polyvinylpolypyrrolidone, DCFH-DA: Dichloro-dihydro-fluorescein diacetate, AAPH: 2, 2-azobis (2-amidinopropane) dihydrochloride, LPS: Lipopolysaccharide

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INTRODUCTION

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated endogenously by enzymes and metabolic activities and exogenously through exposure to chemical and radiation.[1] Free radicals take part in facilitating various pathological conditions such as neurodegenerative diseases, chronic kidney diseases, and diabetes through damaging physiological macromolecules including DNA, proteins, and lipids.[2,3] Antioxidants have showed to potentially reduce or prevent the oxidative damage caused by free radicals which significantly impact the progression of these pathological condition.[2] However, endogenous antioxidants such as glutathione are sometimes insufficient to overcome the generation of free radicals in hectic metabolic condition, especially during the time of diseases and infections.[4] Hence, dietary intake of antioxidants is sometimes required to counter against excessive free radicals in the body.

Porcupine bezoars/dates are phytobezoars derived from plants materials where poorly metabolized phytochemicals are compacted with intertwined fibers in the gall bladder/stomach of Himalayan porcupine (*Hystrix brachyura*).[5] The *Hystrix* sp. porcupines are herbivore feeding on fallen fruits, medicinal plants, underground plant organs (roots and tubers), and tree bark in tropical rainforest rich in variety of herbs and medicinal plants.[5,6] Medicinal plants are known to be rich in antioxidant compounds, especially from their phenolic content, which had been shown to prevent deleterious effects caused by oxidative stress.[7] The usage of porcupine dates as alexipharmic was recorded in early medical

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text by hill tribes in Southern China and Southeast Asia.[8-10] In modern Traditional Chinese Medicine (TCM), it is used by Asian Chinese as a last resort medicine to treat various forms of cancer, dengue fever, meningitis, herpes, throat infection, pneumonia, diabetes, etc.[10-12]

In spite of its extensive usage among the Chinese community in Malaysia and other Southeast Asian countries, scientific verification of its efficacy has not been reported. In this study, we investigated three types of porcupine dates: grassy date (GD) with closely packed grassy and thread-like structure; black date (BD), a compact black solid with remnant of undigested shells; and powdery date (PD), a slightly reddish sphere composed of multiple layers and powdery on the outer layer [Figure 1]. These different types of porcupine dates are popular among Malaysian TCM users and type-dependent medicinal effects were illustrated from the users' informal testimonies. Through various chemical, biochemical, and cell-based assays, our study for the first time provided evidences on the phytochemical constituents, *in vitro* antioxidant and intracellular ROS/RNS scavenging properties which underlie the differential medicinal effects of these porcupine dates.

MATERIALS AND METHODS

Sample extraction

Methanol was used as solvent for extraction due to its ability to dissolve plant phenolics.[13] Approximately 5 g of different porcupine date samples supplied by a traditional Chinese herbs selling company, Soon Hing Cheong Ginseng, were ground into homogenous fine powder using laboratory pestle and mortar. Two hundred milligrams of each sample was extracted using 10 ml of methanol through continuous swirling on orbital shaker for 1 h at room temperature. Extracts were isolated from the undissolved samples by centrifuging at 3000 g for 5 min. Solvent of extract was initially reduced using stream of nitrogen gas and subsequently dried using lyophilization.

Phytochemicals test

The presence of alkaloids was tested using Dragendroff reagent test, Mayer's reagent test, and Wagner's reagent test according to previous methods with slight modification.[14,15] The flavonoids were tested using Shinoda test and flavonoids test according to Jones and Kinghorn.[14] Keller Killiani test and Kedde test were performed to investigate the presence of cardiac glycoside/deoxysugar.[14] The quinone was tested as described in Jones and Kinghorn.[14] Foam test was used to test for the presence of saponins according to Harborne.[16] Tests for terpenoids were done using Liebermann-Burchard test and Salkowski test.[14,17,18] Ferric chloride test, gelatin test, and vanillin test were performed to investigate the presence of condensed and hydrolysable tannins.[14,19,20]

Total phenolic content assay

Total phenolic content (TPC) in samples was determined using a modified Folin-Ciocalteu's reagent assay in sodium carbonate solution as earlier described.[21,22] One milligram of crude extract was weighed in an Eppendorf tube and dissolved in 1 ml of methanol. Using a 96 well-plate, 30 µL of dissolved extract (triplicate) was aliquoted into separate wells, followed by 150 µL of Folin-Ciocalteu's reagent (10 times dilution) and 120 µL sodium carbonate anhydrous (7.5% w/v). Test tubes were incubated at room temperature for 30 min before absorbance measurement at 765 nm using a Tecan Infinite M200 microplate reader. TPC activity was calculated based on a standard curve constructed using gallic acid, $y = 0.0093x$ ($R^2 = 0.9993$), where y is absorbance and x is the concentration of gallic acid in ppm. The TPC is expressed as mg gallic acid equivalent (GAE) per g of porcupine date.

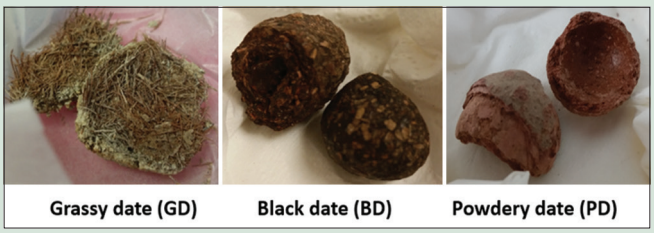

Figure 1: Physical classification of grassy date, black date, and powdery date

2, 2-diphenyl-1-picrylhydrazyl free radical scavenging activity assay

Free radical scavenging activity (FRS) utilizing 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined using a modified method as previously described.[23] Different dilutions of methanol extracts in 100 µL were added with 200 µL of DPPH (5.9 mg/100 mL 100% methanol). By monitoring absorbance at 517 nm after 30 min incubation in the dark, IC_{50}, concentration of extract required to destroy 50% of the radical was obtained. Ascorbic acid was used as the standard with IC_{50} scavenging activity of 0.00374 mg/ml. FRS activity expressed as ascorbic acid equivalent (AAE), was calculated as $IC_{50(ascorbate)}/IC_{50(sample)} \times 10^3$, and expressed in mg ascorbic acid/g sample.

Ferric reducing power assay

Different dilutions of sample in 100 µL were added with 250 µL phosphate buffer (0.2 M, pH 6.6) and 250 µL of potassium ferricyanide (1% w/v). Following 20 min incubation on a 50°C heating plate, 250 µL of trichloroacetic acid (10% w/v) was added. The solution was pipetted into aliquot of 250 µL and added with 250 µL of ddH_2O. Subsequently, 50 µL of ferric chloride solution (0.1% w/v) was added and mixture was left standing for 30 min at room temperature. One hundred microliter of each of the mixture was aliquoted into 96-well plate before measuring absorbance at 700 nm. Results are expressed as mg GAE/g based on calibration equation using gallic acid, $y = 0.0038x + 0.01$, where y is absorbance and x is concentration of gallic acid in ppm.

Total tannins content assay

Total tannins were determined using method as described in Makkar *et al.*[24] Tannins contents in the methanolic extract of BD and PD were isolated using a solid matrix binding agent polyvinylpolypyrrolidone (PVPP). The tannins-PVPP complex was removed using centrifugation, producing a tannins free extract. TPC assay was performed using Folin–Ciocalteau's reagent before and after PVPP treatment. The difference between the two TPC values gave the total tannins content in terms of GAE.

Intracellular reactive oxygen species scavenging activity assay

Intracellular free radicals scavenging was studied in RAW264.7 cells using dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay as described by Sittisart and Chitsomboon.[25] RAW264.7 cells (4×10^4 cells/well) were plated in a SPL 96-well black plate for 16-18 h. Cells were washed twice with phosphate-buffered saline (PBS) before pretreated with BD, PD (200 µg/mL) extracts, and curcumin (5 µM) for 24 h. After incubation, cells were washed twice with PBS before exposed to 20 µM DCFH-DA in Hank's balanced salts solution (+), followed by 45 min incubation in dark. On addition of ROS stimulator 2, 2-azobis (2-amidinopropane) dihydrochloride (AAPH), fluorescence intensity was measured

kinetically for 1 h with 5-min interval at excitation wavelength of 485 nm and emission wavelength of 535 nm using Tecan Infinite M200 microplate reader.

Reactive nitric oxide scavenging activity

To determine nitric oxide (NO) scavenging capacity, Griess reagent was used to measure the stable nitrite, NO_2^- which is an intermediate of NO reaction. Approximately 5×10^5 cells/well in 100 µL of RAW264.7 was plated into a 96-well plate for 24 h. Cells were costimulated with 1 µg/mL of lipopolysaccharide (LPS) at different concentration of sample extracts (0.2–0.0125 mg/mL) for 24 h. Medium supernatants were then collected and analyzed for nitrite amount using 10 µL/well of Griess reagent kit (Life Technologies, 1% sulfanilamide and 0.1% naphthylethylenediaminedihydochloride in 2% phosphoric acid). Absorbance was measured with microplate reader (Tecan Infinite M200) at 548 nm. The production of nitrite was quantified based on equation of standard curve generated using sodium nitrite ($NaNO_2$), $y = 0.0006x - 0.005$, where y is absorbance and x is concentration of nitrite (1–100 µM).

Statistical analysis

All data were plotted and analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, USA). Data analyses were done using one-way ANOVA with Tukey's *post hoc* test ($P < 0.05$).

RESULTS

Yield of extraction

The total yields of methanol extract were found to be 48.2% (BD), 39.0% (GD), and 35.5% (PD) based on one-time extraction with 1:50 sample: solvent ratio (w/v).

Phytochemical constituents

To determine the phytochemicals present in different kind of porcupine bezoars, we performed a series of phytochemical tests and results are summarized in Table 1. Hydrolysable tannins, cardiac glycosides and terpenoids were found in all three porcupine date extracts while flavonoids only present in BD and PD. It is important to note that phytochemical tests of BD and PD are much more positive (e.g., more intense color) as compared to GD, which give an approximate estimate of their relatively low phytochemical content.

Total phenolic content, free radical scavenging, and ferric reducing power

The TPC and radical scavenging activities of BD, PD, and GD were determined using TPC and FRS assays along with standard antioxidants, gallic acid, and ascorbic acid, respectively. FRS activity was determined by measuring the dose-dependent DPPH radical scavenging activity [Figure 2] and the IC_{50} values (6.61, 13.1, and 1467 µg/ml for BD, PD, and GD, respectively) obtained were converted to AAE [Table 2]. The antioxidant content and activities arranged in descending order are BD > PD > GD. BD extract has significantly highest antioxidant content with TPC (188.1 ± 6.2 mg GAE/g), followed by PD which is slightly lower with TPC (104.9 ± 2.9 mg GAE/g) [Table 2]. However, TPC of GD is significantly much lower as compared to BD and PD. Interestingly, TPC values are correlated to their FRS activity.

Ferric reducing power (FRP) assay demonstrating electron donating capacity of BD, PD, and GD was analyzed along with standard antioxidant gallic acid. Similarly, BD and PD show 6-fold higher FRP activity than GD [Table 2]. Due to relatively low antioxidant contents and properties, GD was excluded in subsequent cell-based assays.

Total tannins content assay

Preliminary phytochemicals screening showed high amounts of tannins in two types of porcupine dates. Total tannins content assay was performed to identify the amount of tannins present in porcupine dates relative to respective TPC. Table 3 summarizes the estimated percentage of tannins contents present in BD and PD by comparing the TPC before and after the removal of tannins using PVPP. It was observed that the major phenolic contents in BD and PD consisted of tannins (~ 93%).

Intracellular reactive oxygen species scavenging activity of black date and powdery date

DCFH-DA assay was used to study the direct scavenging effect of intracellular free radical in AAPH-stimulated RAW264.7. Intracellular cleavage of DCFH by oxidative radical produces a green fluorescence product (DCF) that was measured every 5 min for a duration of an hour. A time-dependent increase of fluorescence signal was observed in all treatments, which were normalized into percentage of fluorescence intensity based on APPH-stimulated control [Figure 3]. BD and PD at a concentration of 200 µg/ml demonstrated significant decrease in AAPH-induced fluorescence signal of 11.1% and 28.0%, respectively, at the 60-min endpoint. As a comparison, well-studied antioxidant curcumin (5 µM or 1.84 µg/mL) demonstrated 25.8% reduction of fluorescence at the 60 min endpoint of the experiment. Comparatively, PD-treated cells are more effective in reducing the AAPH-stimulated fluorescence intensity as compared to BD, suggesting better intracellular ROS scavenging capability.

Reactive nitric oxide scavenging assay

Dose-dependent decrease of LPS-induced NO was observed in those treated cells [Figure 4]. Significant reduction of NO was observed at concentration of 200 µg/ml with BD (66.9%) and PD (15.5%). Overall, IC_{50} of NO scavenging activity was 165 and 290 µg/ml for BD and PD, respectively.

Table 1: Phytochemical tests on methanol extracts of grassy date, black date, and powdery date

Phytochemical test	GD	BD	PD
Alkaloids			
Wagner's reagent test	-	-	-
Mayer's reagent test	-	-	-
Dragendorff's reagent test	-	-	-
Flavonoids			
Shinoda test (flavanols, flavones)	-	-	-
Flavonoid test	-	++	++
Cardiac glycosides/deoxysugar			
Keller–Killiani test	+	++	++
Kedde test	+	++	++
Quinones			
Quinone test	-	-	-
Tannins			
Ferric chloride test (condensed and hydrolysable tannins)	+	++	++
Gelatin test	-	-	-
Vanillin test (condensed tannins)	-	-	-
Terpenoids			
Salkowski test	+	+	+
Liebermann–Burchard test	+	++	++
Saponins			
Foam test	-	-	-

-: Absent; +: Present in small amount; ++: Present in significant amount as judged by the intensity of the colored solution; GD: Grassy date; BD: Black date; PD: Powdery date

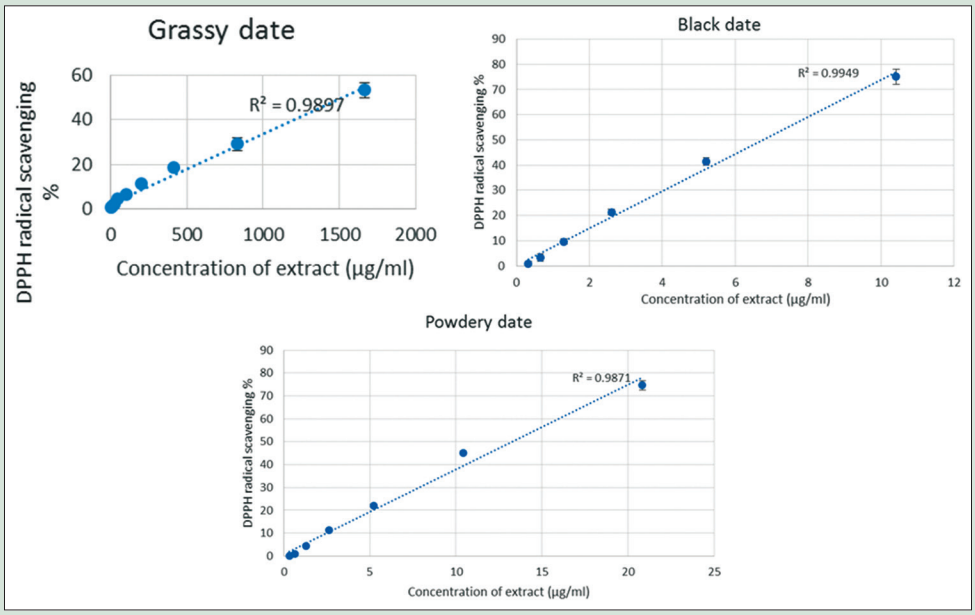

Figure 2: Dose-dependent 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity of methanol extracts of grassy date, black date and powdery date

Table 2: Total phenolic content, free radical scavenging, and ferric reducing power antioxidant activity of methanolic extracts of black date, grassy date, and powdery date

Methanol extracts	TPC (mg GAE/g)	FRS (mg AAE/g)	FRP (mg GAE/g)
BD	188.1±6.2^{a} (90.7±3.0)	565.5±24.8^{a} (272.6±11.9)	97.95±1.69^{a} (47.23±0.81)
GD	27.9±4.6^{b} (9.9±1.6)	2.55±0.12^{b} (0.99±0.05)	15.94±0.04^{b} (6.23±0.01)
PD	104.9±2.9^{c} (40.9±1.1)	284.7±1.33^{c} (101.1±0.5)	97.53±0.99^{a} (34.64±0.35)

Different letters indicate significant difference at $P<0.05$ determined using one-way ANOVA. The bracketed values are in mg/g of porcupine date sample and the nonbracketed values are in mg/g of extract. TPC: Total phenolic content; FRS: Free radical scavenging, FRP: Ferric reducing power; GD: Grassy date; BD: Black date; PD: Powdery date; GAE: Gallic acid equivalent; AAE: Ascorbic acid equivalent

Table 3: Percentage of tannins and nontannins phenolic in methanol extract of black date and powdery date in relative to its total phenolic content determined using Folin-Ciocalteau's reagent

	BD	PD
Percentage tannins of TPC	93.6±0.7	93.0±0.4
Percentage nontannins of TPC	6.43±0.72	7.03±0.37

TPC: Total phenolic content; BD: Black date; PD: Powdery date

DISCUSSION

Based on the phytochemical tests, we have identified various phytochemicals content in porcupine dates, including but not limited to cardiac glycosides, terpenoids, flavonoids, and tannins. We found all three types of porcupine dates tested in this study contain similar types of phytochemicals except that flavonoids were absent in GD. Among all, cardiac glycosides, often found in plants such as *Digitalis*, *Strophanthus,* and *Scilla*, possess antiarrhythmic properties and are used in treatment of congestive heart diseases.[26,27] The presence of cardiac glycosides in the extract of porcupine dates suggests their potential action on the heart functionality. In addition, terpenoids are the largest class of natural products with more than 40,000 known compounds.[28] Being abundant in plants, terpenoids together with flavonoids and tannins have been well known as free radical scavengers.[29,30] In concordance with the rich phenolic content, we observed both BD and PD showed comparable *in vitro* antioxidant properties to that of *Camellia sinensis* tea leaf extract,[23] which is known for its high antioxidant property.

When antioxidant properties are expressed as per gram of porcupine dates, our study showed the antioxidant properties of BD sample (TPC: 90.7 ± 3.0 mg GAE/g; FRS: 272.6 ± 11.9 mg AAE/g; FRP: 47.23 ± 0.81 mg GAE/g) [Table 2] and PD sample (TPC: 40.9 ± 1.1 mg GAE/g; FRS: 101.1 ± 0.5 mg AAE/g; FRP: 34.64 ± 0.35 mg GAE/g) [Table 2] were comparable to those reported in oolong tea extract. Hot water extract of the tested oolong teas was reported to contain high TPC (TPC range: 75.0 ± 4.6 to 90.9 ± 4.6 mg GAE/g of dried leaves) and possessed high FRS activities (FRS range: 144.5 ± 30.5 to 161.7 ± 24.8 mg AAE/g of dried leaves).[23] Notably, BD contains two times more active in FRS activity when compared to oolong teas. In contrast, GD was found to contain the least amount of total phenolic compounds (TPC: 9.9 ± 1.6 mg GAE/g). In the antioxidant assays, the lowest FRS activity (FRS: 0.99 ± 0.05 mg AAE/g) and the weakest FRP were also observed for GD (FRP: 6.23 ± 0.01 mg GAE/g) in parallel with its low phenolic content [Table 2]. These findings imply that antioxidant properties of porcupine dates could possibly be contributed by varied amount of phenolic compounds in different porcupine dates.

Our study provided explicit evidences to show that BD contains the highest amount of TPC (~7 times higher than GD) followed by PD (~4 times higher than GD) together with significant higher radical scavenging activity and FRP. Through total tannins content test, we revealed that tannins are the major phenolic compounds consisting of 93.6% ± 0.7 and 93.0% ± 0.4 of the TPC in BD and PD, respectively. Tannins are macromolecule abundantly found in oak tree bark[31,32] which is a common diet of porcupine (*Hystrix* sp.). In tropical and subtropical regions of Southeast Asia, oak trees from subgenus *Cyclobalanopsis* are

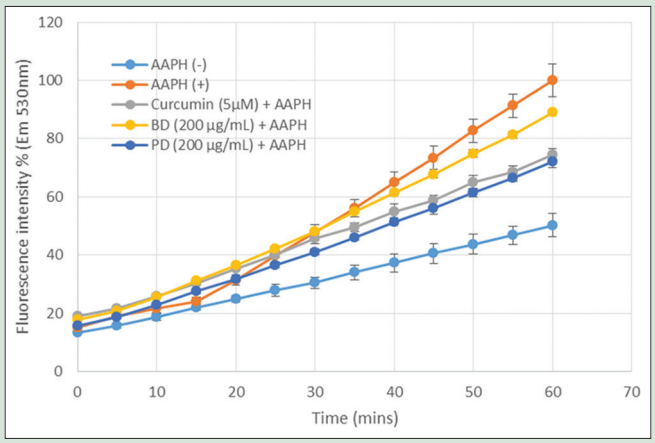

Figure 3: Intracellular reactive oxygen species of 2, 2-azobis (2-amidinopropane) dihydrochloride-stimulated RAW264.7 cells measured by the dichloro-dihydro-fluorescein diacetate. RAW264.7 cells were pretreated with indicated concentrations of curcumin, black date, or powdery date for 24 h before reactive oxygen species stimulation by 2, 2-azobis (2-amidinopropane) dihydrochloride. Unstimulated cells were used as negative control. Results are represented by mean ± standard error of mean of three independent experiments

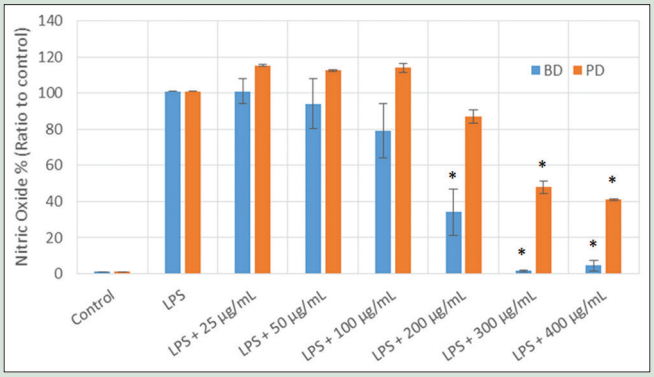

Figure 4: Nitric oxide scavenging activity of lipopolysaccharide-stimulated RAW264.7 cells measured using Griess reagent. RAW264.7 cells were cotreated with indicated concentration of black date and powdery date for 24 h. Results are represented by mean ± standard error of mean of three independent experiments (n = 3) (*represent significant reduction as compared to lipopolysaccharide treated group with $P < 0.05$)

commonly found.[33,34] We believe those tannins contained in porcupine diet which are undigested might have been highly concentrated and incorporated into the dates during its formation in the stomach. Chemically, tannins have a structure with numerous hydrolysable moieties such as gallic and ellagic acids which contribute to the high antioxidant properties.[35,36] Therefore, the high content of tannins maybe the major factor that underlies the remarkable antioxidant activities of BD and PD. Intriguingly, these dates also showed significant intracellular ROS scavenging activity in RAW264.7 murine macrophage cells.

On the other hand, we observed apparent effects of BD and PD in suppression of NO induced by LPS in RAW264.7 cells. NO is a key RNS that serves as intermediary in several physiological processes including immune regulation in the body. Deregulation of NO could lead to various inflammatory diseases.[37-41] Antioxidants that are able to scavenge NO were found to suppress inflammatory responses through preventing release of pro-inflammatory mediators bradykinin and histamine[42-44] as well as preventing improper infiltration of leukocytes, mast cells, and macrophages.[42,45,46] Many local medicinal plants which show antioxidant activities are also found to inhibit reactive NO, for instance *Clinacanthus nutans*, which is commonly known as snake grass.[47,48] In our laboratory, methanol extract from snake grass was found to significantly inhibit LPS-induced NO production (IC_{50} 230 μg/ml) in RAW264.7 cells (unpublished data). For porcupine dates, we observed comparable NO scavenging activities in the macrophage cells treated with extracts from BD and PD (IC_{50} 165 and 290 μg/ml) suggesting their potential anti-inflammatory properties. Notably, higher NO scavenging activity observed in BD-treated cells was in concordance with its high content of TPC and remarkable antioxidant properties.

CONCLUSION

In conclusion, phytochemicals such as tannins, terpenoids, and cardiac glycosides were found in all three types of porcupine dates. In comparison to GD, the BD and PD contain highest amount of TPC and show remarkable activities in scavenging free radical and reducing iron (III) ions. At the same time, intracellular ROS/RNS induced in murine macrophage cells was also found to be significantly suppressed by BD and PD. The ability in scavenging NO suggests the potential anti-inflammatory properties of these porcupine dates. Further work is in progress to determine the antiproliferative and antiviral activities of the porcupine dates.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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