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Cytotoxicity, Total Phenolic Content and Antioxidant Activity of *Bergenia purpurascens* Rhizome

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Abstract

This study was aimed to evaluate the cytotoxicity of methanolic extract (ME) and aqueous extract (AE) of the rhizomes of *Bergenia purpurascens* through the Brine-shrimp bioassay, which revealed that ME possessed cytotoxicity with LC_{50} = 181.97 ìg/ml value. The plant extracts were also evaluated *in vitro* for antioxidant activity using different redox [Folin-Ciocalteu (FC) and Ferric Reducing Antioxidant Power (FRAP)] and radical scavenging [(2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)] assays. Both ME and AE possessed antioxidant activity and ME displayed comparatively a greater potential. The total phenolic contents in ME and AE was 1.27 and 0.06 g gallic acid equivalent (GAE)/100 g dry mass (DM), respectively. The FRAP values determined for ME and AE were 15.82 and 2.58 ìmol Fe(II)/100 g DM, respectively. The DPPH radical scavenging effect (IC_{50}) was for ME 19.86 ìg/ml and 295.91 ìg/ml for AE. At 100 ìg/ml concentration, up to 94% inhibition of $ABTS^{\bullet+}$ was observed in 6 min incubation.

Key words: antioxidant activity, *Bergenia purpurascens*, brine-shrimp bioassay, free radical, phenolic content

Introduction

Bioactive secondary metabolites of plants are often toxic at elevated dose; however, without analysis of minimum dose of the substances that are toxic to zoological systems is nothing in the process of drug discovery rather than pure phytochemistry. A wide variety of anticancer drugs exhibit cytotoxic effect by interfering with cell-cycle kinetics. Although not specific, the Brine-shrimp bioassay determines LC_{50} (Lethal concentration for 50% mortality) value of active compounds at ìg/ml to evaluate cytotoxicity thereby could correlate for antitumour effect (Meyer *et al.* 1982, McLaughlin 1991). Oxidative stress associated diseases such as aging, cancer, atherosclerosis and Alzheimer's can be prevented or suppressed by supplementation of antioxidants (Giasson *et al.* 2002). Antioxidants are the molecules that can neutralise reactive oxygen species (ROS) by accepting or donating an electron and delay the oxidation process of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions, thus could provide preventive effect against cancer (Gordon 1996). Reduction of molecular oxygen in mitochondria during cellular respiration, degradation of fatty acids in peroxisomes and phagocytosis of erythrocytes result in production of ROS in human body. Several studies revealed that natural phenolic compounds such as flavonoids, phenolic acids and tannins, etc. possess diverse biological functions and are more potent antioxidants than vitamin C and E (Vinson *et al.* 1995). Single electron transfer (SET) based assays are used to evaluate the reducing capacity of an antioxidant, in which the degree of colour change of the oxidant is proportional to the antioxidant concentration that measures spectrophotometrically at a specific wavelength.

Bergenia purpurascens (Hook. F. & Thomson) Engl. of family Saxifragaceae, locally known as Pakhanbed, is a winter perennial herb, 1 to 2 feet tall with thick stout, pink to purplish-red flowers and bears spirally arranged rosette of leathery, elliptic to ovate leaves, 6-35 cm long and 4-15 cm broad (Ghimire *et al.* 2008, CSIR 2000). The rhizome is considered as astringent,

styptic, tonic and effective in dissolution of renal stones. The local people in the Himalayan region also use rhizome paste in wounds, body ache and bone fracture. Recently, effectiveness of *B. purpurascens* for treating dysmenorrhea, cancer, rheumatoid disease, ankylosing spondylitis, skin rash and gout is documented in Chinese Patents (Chen 2009, Xie 2009, Ma 2010, Chen and Zhu 2011). The main chemical constituents identified in the rhizomes of *B. purpurascens* are bergenin, 4-O-galloylbergenin, 11-O-galloylbergenin, arbutine, 4,6-di-O-galloylarbutine, 6-O-galloylarbutine, 2,4,6-tri-O-galloyl-D-glucose, 1,2,4,6-tetra-O-galloyl-*â*-D-glucose, (+)-catechin, 7-O-galloyl-(+)-catechin, procyanidine *â*-3 and 3-O-galloylprocyanidine *â*-1 (Xin-Min *et al.* 1987) (Fig. 1).

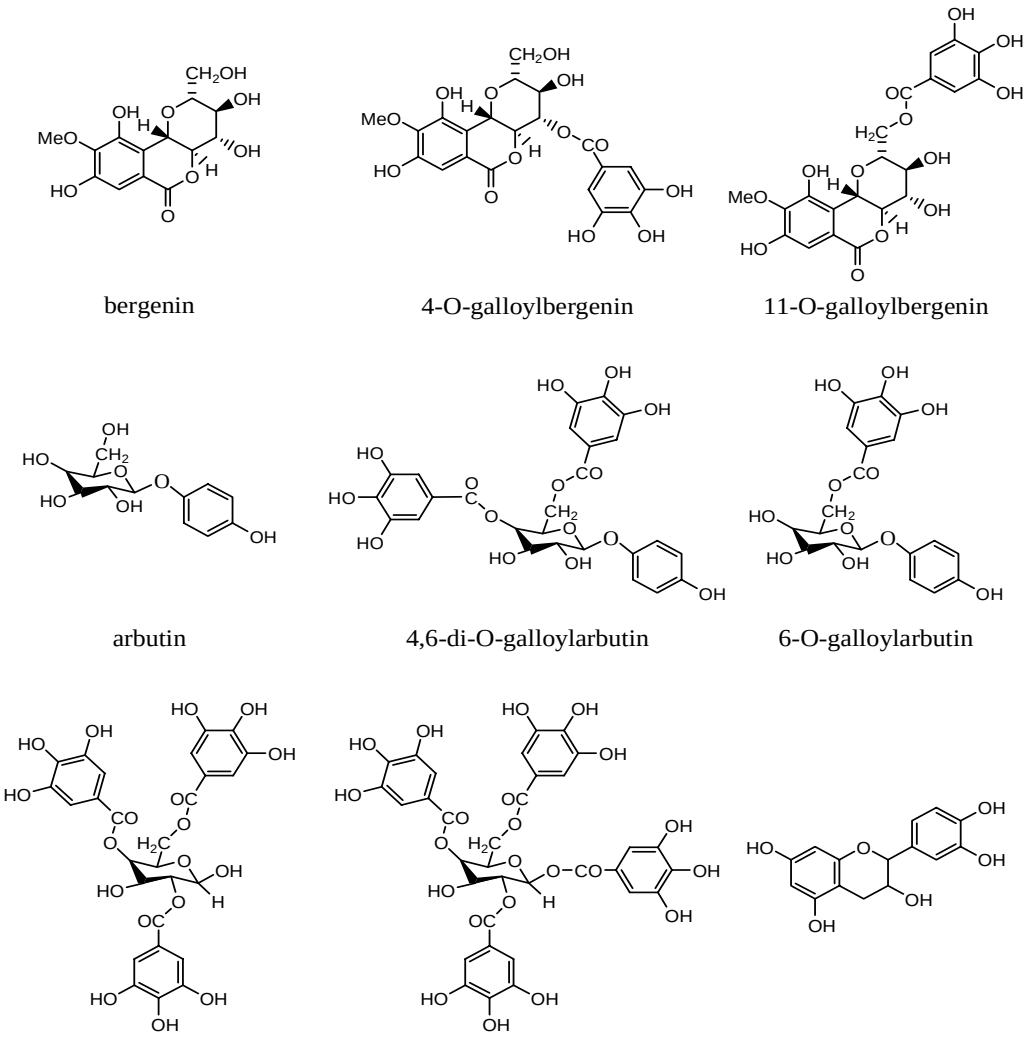


Fig. 1. Chemical constituents of *B. purpurascens*

Recently, we have reported antimicrobial property of the rhizomes of *B. purpurascens* (Bajracharya *et al.* 2011). To the best of our knowledge, no report on the cytotoxicity and antioxidant activity of *B. purpurascens* is available in the literature. Herein we report the cytotoxicity of the plant extracts employing the Brine-shrimp bioassay and evaluate their antioxidant activity.

Methodology

Chemicals and equipment

2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Folin-Ciocalteu's Phenol (FCP) reagents were purchased from Sigma-Aldrich. 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka Chemie. Other chemicals used were laboratory grade and purchased from E. Merck, Glaxo, Qualigens and Ranbaxy chemical companies. Brine-shrimp (*Artemia salina*) eggs, Red Jungle Brand, were procured from Ocean Star International Inc., Snowville, UT, U.S.A. Spectrophotometric quantitation was carried out using 6715 UV/Vis Spectrophotometer JENWAY.

Collection and identification of plant material

Rhizomes of *B. purpurascens* were collected from the premises of Kalchuman Lake (altitude 3700-3800 m) in Manaslu Conservation Area, Nepal in June 2010. The plant material was shade dried and taken to the laboratory. The plant was authenticated from National Herbarium and Plant Research Lab, Godawori, Lalitpur, Nepal with a Voucher Specimen Number 23.

Extraction of the plant material

Air dried, ground rhizome of *B. purpurascens* (150 g) was successively extracted with hexane (700 ml, 6 h), chloroform (700 ml, 7 h), methanol (800 ml, 19 h) and distilled water (800 ml, 19 h) using a Soxhlet extractor. The extracts were concentrated under reduced pressure using a rotary evaporator. After further concentration over a warm water bath, the extracts were kept in a desiccator for a few days and then stored at 4 ^{o}C until further use.

Test for polyphenols

The plant extract (10 mg) was dissolved in methanol (1 ml) and then treated with three drops of 1% $FeCl_3$ solution.

Brine-shrimp bioassay

Cytotoxicity of the extracts was evaluated using the Brine-shrimp bioassay following the procedure developed by Meyer *et al.* (1982). Sample solution was prepared in a 10 ml volumetric flask by dissolving 100 mg of the plant extract in methanol. Calculated volume of the sample solution (5 ìl, 50 ìl and 500 ìl for 10, 100 and 1,000 µg/ml dose levels, respectively) was transferred in separate test tube containing 1.2 cm disc of filter paper (Whatman No. 1) in five replicates. A control tube for each dose level was also prepared. The sample tubes were carefully dried using a rotary evaporator and to this were introduced freshly hatched ten Brine-shrimp nauplii in artificial sea water (total volume 5 ml). After 24 h of illumination under a table lamp (60 Watt), the number of survivors was counted. No death was observed in the control tubes. The percentage death was calculated by comparing the mean of survivors in the test tubes. The LC_{50} values with 95% CI (Confidence Interval) were obtained by regression calculation.

Antioxidant capacity assays

Stock solution of the methanolic extract (ME) and aqueous extract (AE) of concentration 50 mg/ml was prepared by using 0.25 g of each extract dissolving in distilled water (5 ml). The working solutions were prepared by dilution of suitable aliquots of the stock solution with distilled water.

Folin-Ciocalteu (FC) assay

Working solutions of concentrations 25, 50 and 100 µg/ml were prepared. To 1 ml of each working solution taken in a test tube was added 2.0 M FCP reagent (0.5 ml) and vortexed for 3 min. Then 2 ml of aqueous Na_2CO_3 solution (75 g/l) was added and was kept in the dark at room temperature for 1 h. The absorbance at 760 nm against blank (distilled water) was recorded and the antioxidant concentration value was determined to gallic acid equivalent (GAE). A linear curve of standard gallic acid concentration versus absorbance was constructed using a series of 25, 50, 100, 150 and 200 µg/ml gallic acid solutions. The total phenolic content in the sample solutions was considered the slope of the linear curve derived from the constructed calibration graph and expressed in GAE/100 g dry mass (DM).

Ferric reducing activity power (FRAP) assay

The FRAP reagent was prepared freshly before the analysis by mixing 25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 10 mM TPTZ in 40 mM HCl

solution and 2.5 ml of 20 mM $FeCl_3.6H_2O$ solution. The acetate buffer was prepared by mixing 7.5 ml of 30 mM sodium acetate solution with 92.5 ml glacial acetic acid solution (1.7 ml/l). Thus prepared FRAP reagent was pre-warmed at 37 ÚC before using.

Working solutions of concentrations 4, 6 and 8 µg/ml were prepared. To each working solution (200 µl) taken in a test tube was added 2800 µl of the FRAP reagent, mixed well and incubated at 37 ^{O}C for 4 min. The change in absorbance due to formation of the coloured product ferrous tripyridyltriazine complex was determined at 593 nm against water as the blank solution. The FRAP value was determined by plotting a standard curve produced by the addition of aqueous solutions of $FeSO_4.7H_2O$ with known concentrations (25, 100, 200, 300, 400 and 500 µg/ml) to the FRAP reagent and expressed in µmol Fe(II)/100 g DM.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Working solutions of concentrations 4, 6 and 8 µg/ml were prepared. To each working solution prepared (0.5 ml) was added 0.1 mM methanolic $DPPH^{\bullet}$ solution (2.5 ml). The $DPPH^{\bullet}$ solution was prepared in prior by stirring 3.9 mg of DPPH in methanol (100 ml) for overnight at 4 ^{O}C. A control was prepared by mixing distilled water (0.5 ml) and 0.1 mM methanolic $DPPH^{\bullet}$ solution (2.5 ml). These solutions were vortexed well and then kept at room temperature in the dark for 30 min. The absorbance was measured at 517 nm against the blank solution consisting MeOH (2.5 ml) and distilled water (0.5ml).

The inhibition of initial absorbance of the $DPPH^{\bullet}$ solution was calculated by using Equation 1;

Scavenging activity (% of inhibition)

$$= (1 - A_{sample}/A_{control}) \times 100 \quad (1)$$

where, A_{sample} and $A_{control}$ are the absorbance values of the reaction mixture with and without sample, respectively.

Next, the obtained data of % inhibitions at different concentrations were computed to calculate IC_{50} (Concentration causing 50% inhibition). A standard linear curve was constructed using methanolic solutions of gallic acid with known concentrations of 5, 10, 15, 20 and 25 µg/ml *versus* absorbance. The DPPH value obtained was considered the slope of the linear curve derived from the constructed graph and expressed in g GAE/100 g DM.

2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay

The ABTS radical monocation ($ABTS^{\bullet+}$) was produced by reacting 10 ml of 7 mM aqueous ABTS solution with 175 µl of 140 mM $K_2S_2O_8$ solution and allowing the mixture to stand in the dark for 12-16 h at room temperature. Thus produced $ABTS^{\bullet+}$ solution was diluted with methanol until the initial absorbance value reached 0.70 ± 0.02 at 734 nm wavelength against water.

Working solutions of concentrations 25, 50 and 100 µg/ml were prepared. An aliquot of the $ABTS^{\bullet+}$ solution (4 ml) was mixed with 200 µl of each working solution or methanol (for control) and then vortexed. After 6 min of incubation at room temperature, the decrease in absorbance at 734 nm of each extract was recorded against water as a blank. The inhibition percentage of initial absorbance of the $ABTS^{\bullet+}$ solution at 734 nm was calculated using Equation 1. Graphs of fall in absorbance due to inhibition of the $ABTS^{\bullet+}$ at different interval of time (initially 6 min then at 30, 60, 90 and 120 min) *versus* concentration of the extract were then constructed to study the effect of incubation time.

Results and Discussion

A successive soxhlet extraction of the ground rhizomes of *B. purpurascens* (150 g) yielded hexane extract (0.85 g, 0.56%, pale yellow), chloroform extract (0.50 g, 0.33%, yellowish brown), ME (58.25 g, 38.83%, dark reddish brown) and AE (11.21 g, 7.5%, dark brown). Upon $FeCl_3$ test, later two extracts displayed the presence of tannins and polyphenols producing violet-blue colouration and bluish-black precipitate, respectively. Thus, ME and AE were considered further for the biological assays.

The Brine-shrimp bioassay is a rapid, reliable and inexpensive tool available for testing bioactivity of a sample at concentrations of 10, 100 and 1,000 ìg/ml to evaluate cytotoxicity and antitumour properties. Table 1 shows the results of the Brine-shrimp bioassay after 24 h exposure of ME and AE to freshly hatched Brine-shrimp nauplii. The sample displaying LC_{50} value less than 1,000 is considered as pharmacologically active and is toxic. The ME showed 100% mortality at a concentration of 1,000 ìg/ml with LC_{50} value 181.97 ìg/ml, hence is cytotoxic. On the other hand, the AE ($LC_{50} = 4.46 \times 10^{21}$ ìg/ml) was found non-toxic.

Table 1. Brine-shrimp bioassay of *B. purupurascens* extracts

Samples	Percentage death at 24 h/dose			LC_{50} (µg/ml)	95% CI (µg/ml)
	10 (µg/ml)	100 (µg/ml)	1,000 (µg/ml)		
ME	2	12	100	181.97	181.97 ± 1.13
AE	0	0	2	4.46×10^{21}	$4.46 \times 10^{21} \pm 0.65$

The FC assay, although nonspecific to phenolic compounds as it can be reduced many non-phenolic compounds such as vitamin C, Fe(II), Cu(I) etc., is commonly known as the total phenol assay (Folin and Ciocalteu 1927). Since phenolic compounds contribute in antioxidant property, excellent linear correlations between the total phenolic profiles and SET based assays are often observed. The data of absorbance measurements in the FC assay displayed a linear variance between the concentration and the corresponding fall in absorbance (Fig. 2). The reducing capacity of the samples is expressed as the slope value of the linear curve. On the basis of calculated slope values, ME gave the highest slope value (1.68 GAE), showing the highest antioxidant capacity than the standard gallic acid, while AE exhibited lower reducing capacity (0.33 GAE). Furthermore, the total phenolic contents in ME and AE were 1.27 and 0.06 g GAE/100 g of DM, respectively. These observations might be correlated with the structure-antioxidant relationship (SAR) principles, where a higher number of available hydroxyl groups in the aromatic ring increase the reducing capacity. It is quite clear that the total phenolic content in the ME is high and hence ME exhibited more antioxidant activity.

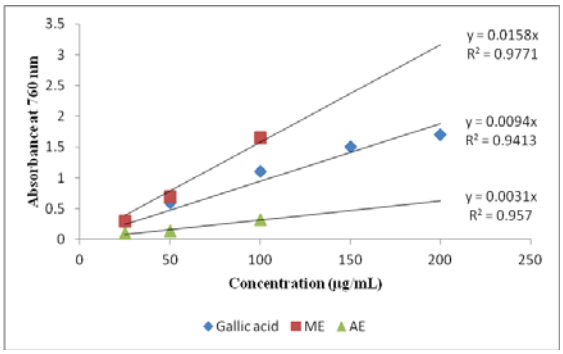


Fig. 2. Concentration-response curves in the FC assay showing reactions of the samples with the FCP reagent in linear concentration dependence at concentrations below 200 ìg/mL.

The FRAP assay measures the total antioxidant activity on the basis of the ability to reduce a ferric salt $Fe(III)(TPTZ)_2Cl_3$ to Fe(II) ions (Benzie and Strain 1996, Nenadis *et al.* 2003, Perez-Jimenez and Saura-Calixto 2006). The FRAP assay was carried out under acidic conditions (pH 3.6) in order to maintain the iron solubility. With reference to the calibration curve obtained at 593 nm for ferrous sulphate solution (R^2 = 0.9277) (Fig. 3), the FRAP values of ME and AE were calculated to be 15.82 and 2.58 ìmol Fe(II)/100 g of DM, respectively.

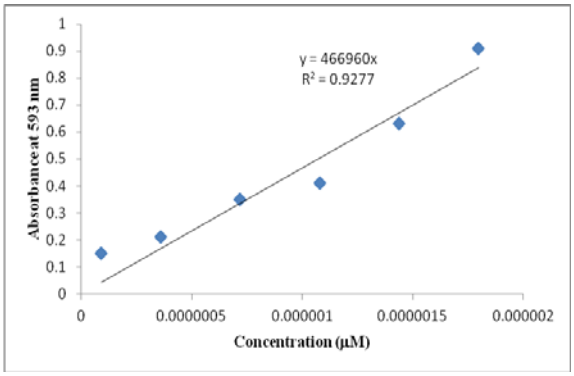
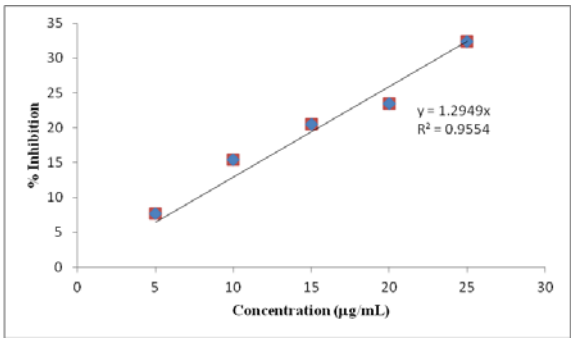


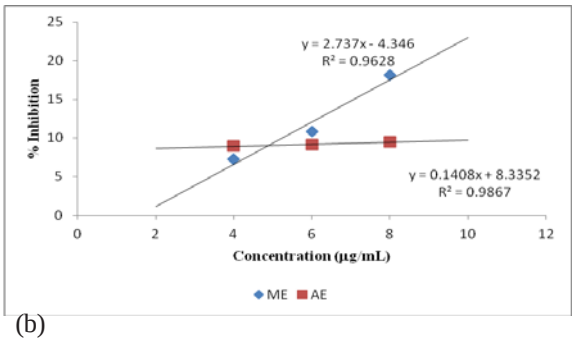
Fig. 3. Concentration-response ferrous sulphate standard curve in the FRAP assay.

The DPPH assay method is based on the capability of an antioxidant to donate a hydrogen radical or an electron to DPPH•, which is a stable free radical with deep violet colour (Brand-Williams *et al.* 1995). When an odd electron become paired with DPPH•, the absorbance reduces and the solution gets decolourisation from its initial deep violet to light yellow. In the DPPH assay, we found that the % inhibition of the ME at 8 ìg/ml concentration resulted in 18.2%, while for similar extent of inhibition, about 12.5 ìg/ml concentration of the standard gallic acid was essential. Next, the curves plotted between % inhibition and concentration for the standard gallic acid (R^2 = 0.9554), ME (R^2 = 0.9628) and AE (R^2 = 0.9867) showed a linear variance (Fig. 4). The IC_{50} values calculated for the standard gallic acid, ME and AE

were 38.61, 19.86 and 295.91 ìg/ml, respectively. Since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value would reflect a greater antioxidant activity of the sample. Therefore, the result clearly indicated that ME contains more potential antioxidant constituent than the standard gallic acid and the pronounced effect was not only due to the phenolic content, instead the presence of potent free radical scavengers is envisaged. To evaluate the antioxidant activities of ME and AE in comparison with gallic acid (Fig. 5), the DPPH values were calculated to be 99.59 and 15.03 g GAE/100 g of DM, respectively.



(a)



(b)

Fig. 4. Scavenging capacity of the samples on DPPH$^{\bullet}$: (a) standard gallic acid, (b) ME and AE.

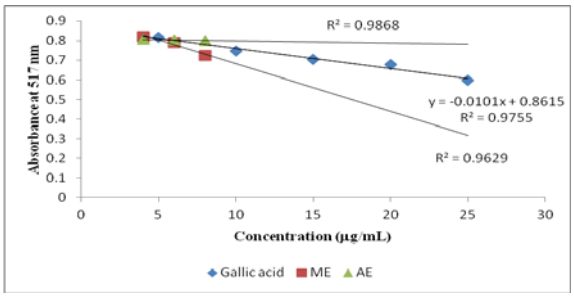
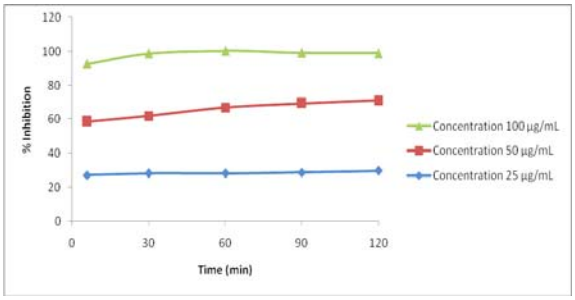
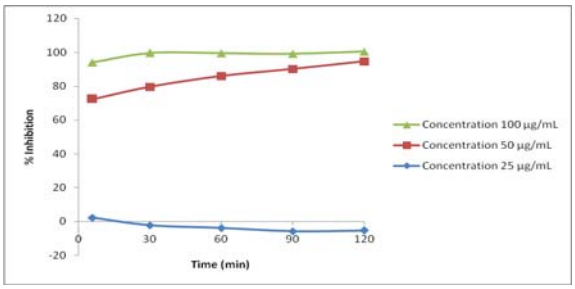


Fig. 5. Scavenging capacity of standard gallic acid, ME and AE on DPPH$^{\bullet}$ determined at 517 nm after 30 min incubation.

The ABTS assay was carried out following the method described by Re and co-workers (Re *et al.* 1999). The ABTS assay measures the ability of an antioxidant to neutralise ABTS$^{\bullet+}$. It has been reported that the reaction of many phenolics with ABTS$^{\bullet+}$ occurs rather slowly (Stratil *et al.* 2006). In such a case, the results are dependent on the time of incubation and lead to final values underestimated. We decided to know the affect of incubation time in the ABTS assay and therefore recorded the absorbance of the samples tested as well as control at different intervals of time. At 100 ìg/ml concentration, comparably a very high % of inhibition (92.54-94.00%) was recorded for both ME and AE in 6 min of incubation (Fig. 6). At 50 ìg/ml concentration, AE displayed high % of inhibition compared to ME. The % inhibition was gradually increased with time and found that the reaction with ABTS$^{\bullet+}$ completed apparently within 60 min. It can be seen from Figure 6 that % inhibition increased from 58.7 to 71.2% (for ME) and from 72.4 to 94.6% (for AE). On the other hand at 25 ìg/ml concentration, the depletion was not remarkable. These results indicated that the rhizomes of *B. purpurascens* must contain some water soluble phenolics, which react slowly with ABTS$^{\bullet+}$ and possess free radical scavenging activity. It is noteworthy that the absorbance values were gradually decreased for the control indicating instability of the ABTS$^{\bullet+}$.



(a)



(b)

Fig. 6. ABTS$^{\bullet+}$ scavenging capacity of the samples: (a) ME, (b) AE.

In conclusion, the rhizome of *B. purpurascens* displayed cytotoxicity effect against Brine-shrimp nauplii hence is potential for antitumour activity. Due to the presence of phenolic contents, both ME and AE were found to possess antioxidant activity, including reducing power and free radical scavenging properties. Compared to AE, ME was found promising for antioxidant activity. We believe that *B. purpurascens* rhizome bears promising therapeutic potential and could be considered as a potential source for drug discovery, therefore warrants further investigation.

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References

Bajracharya, G. B., R. Maharjan and B. L. Maharjan. 2011. Potentially antibacterial activity of *Bergenia purpurascens*. *Nepal J. Sci. Technol.* ***12***:157-162.

Benzie, I. F. F. and J. J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry* ***239***:70-76.

Brand-Williams, W., M. E. Cuvelier and C. Berset. 1995. Use of a free radical method to evaluate antioxidant activity. *LBT – Food Sci. Technol.* ***28***:25-30.

Chen, L. and D. Zhu. 2011. Traditional Chinese medicine preparation for treating lung cancer. *Faming Zhuanli Shenqing*, CN 102000263 A 20110406.

Chen, R. 2009. A Chinese medicinal preparation for the treatment of cancers such as lung cancer, gastric cancer, breast cancer, cervical cancer and pancreatic cancer. *Faming Zhuanli Shenqing*, CN 101455702 A 20090617.

CSIR. 2000. *The wealth of India: A dictionary of Indian raw materials and industrial products. First Supplement Series, Vol I.* Council of Scientific and Industrial Research, New Delhi, India.

Folin, O. and V. Ciocalteu. 1927. Tyrosine and tryptophan determination in protein. *J. Biol. Chem.* ***73***:627-650.

Ghimire, S. K., I. B. Sapkota, B. R. Oli and R. R. Parajuli. 2008. *Non-timber forest products of Nepal Himalaya: Database of some important species found in the mountain protected areas and surrounding regions.* WWF Nepal, Kathmandu, Nepal.

Giasson, B. I., H. Ischiropoulos, V. M. Y. Lee and J. Q. Trojanowski. 2002. The relationship between oxidative/nitosative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. *Free Radical Biology and Medicine* ***32***: 1264-1275.

Gordon, M.H. 1996. Dietary antioxidants in disease prevention. *Nat. Prod. Rep.* ***13***: 265-273.

Ma, J. 2010. Method for manufacturing traditional Chinese medicine decoction for treating lung cancer, gastric cancer, bone marrow cancer and uterine cancer. *Faming Zhuanli Shenqing*, CN 101829283 A 20100915.

McLaughlin, J. L. 1991. Assays for bioactivity. In *Methods in plant biochemistry* (Ed. K. Hostettmann). Academic Press, London. Vol. 6, p: 1-33.

Meyer, B. N., N. R. Ferrigni, J. E. Putnam, L. B. Jacobsen, D. E. Nichols and J. L. McLaughlin. 1982. Brine shrimp: A convenient general bioassay for active plant constituents. *Planta Med.* ***45***: 31-34.

Nenadis, N., I. Zafiropoulou and M. Tsimidou. 2003. Commonly used food antioxidants: A comparative study in dispersed systems. *Food Chemistry* ***82***:403-407.

Perez-Jimenez, J. and F. Saura-Calixto. 2006. Effect of solvent and certain food constituents on different antioxidant capacity assays. *Food Research International* ***39***:791-800.

Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans. 1999. Antioxidant activity applying an improved ABTS radical cation decolourization assay. *Free Radical Biol. Med.* ***26***:1231-1237.

Stratil, P., B. Klejdus and V. Kuban. 2006. Determination of total content of phenolic compounds and their antioxidant activity in vegetables – Evaluation of spectrophotometric methods. *J. Agric. Food Chem.* ***54***:607-616.

Vinson, J. A., Y. A. Dabbagh, M. M. Serry and J. Jang. 1995. Plant flavonoids, especially tea flavonols, are powerful antioxidants using an *in vitro* oxidation model for heart disease. *J. Agric. Food Chem.* ***43***:2800-2802.

Xie, Y. 2009. A method for treating arthromyodynia such as rheumatism and rheumatoid disease. *Faming Zhuanli Shenqing*, CN 101480469 A 20090715.

Xin-Min, C., T. Yoshida, T. Hatano, M. Fukushima and T. Okuda. 1987. Galloylarbutin and other polyphenols from *Bergenia purpurascens*. *Phytochemistry* ***26***:515-517.