DENDROBENTHAMIDIA CAPITATA (WALL.) HUTCH. FRUIT (BHOMORA): AN OPULENT SOURCE OF POLYPHENOLS WITH ANTI-OXIDANT, ANTI-ELASTASE, ANTI-COLLAGENASE, AND ANTI-TYROSINASE ACTIVITIES FROM INDIAN HIMALAYAN REGION

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Abstract: Dendrobenthamidia capitata (Wall.) Hutch. fruit (Bhamora) is a popular yet underexplored wild edible fruit of Uttarakhand state of Indian Himalayan region. Therefore, the present study was aimed to analyze D. capitata fruit for its phenolic contents, antioxidant, anti-elastase, anti-collagenase, and anti-tyrosinase activities using in-vitro assays. Phenolics and flavonoids were extracted using 80 % aqueous acetone and acid acetone as solvents. Our analysis revealed high values of total phenolic contents of 4.38±0.08 mg gallic acid equivalents (GAE). g⁻¹ fruit weight and total flavonoid contents of 7.47±0.66 mg catechin equivalents (CE). g⁻¹ fruit weight in the acid acetone fruit extracts of D. capitata. D. capitata fruit extracts demonstrated remarkable antioxidant activities (ABTS+, 1,1-diphenyl-2-picrylhydrazyl, superoxide anion, linoleate peroxyl radicals scavenging, and ferric reducing activities) and notable anti-elastase, anti-collagenase, and anti-tyrosinase activities. High resolution liquid chromatography–mass spectroscopy analysis revealed presence of six phenolic compounds, namely, dihydrorobinetin, dihydromyricetin, retusin dimethyl ether, rotenone, bergenin (C-glycoside of 4-O-methyl gallic acid) and pеucenin in fruit extracts. This study recommends utilization of D.capitata fruit as functional food with prospective pharmaceutical, nutraceutical, and cosmecutical properties.

Keywords: Wild edible fruits, D. capitata, Antioxidant, Anti-elastase, Anti-collagenase, Anti-tyrosinase

Introduction

Increased consumption of vegetables and fruits has been acknowledged to be associated with lower risk of a number of chronic diseases, such as coronary heart diseases, cancers, immune dysfunction and diabetes owing to their unique combination of minerals, vitamins and polyphenols (Pandey and Rizvi, 2009). Recently, fruits have attracted tremendous attention among food scientists, nutritionists and consumers owing to their multiple health promoting properties including antioxidant, antimicrobial, anticancerous, antidiabetic, anti-inflammatory, anti-skin aging and anti-skin darkening activities associated with elevated polyphenol contents and delicious taste respectively (Wang et al., 1996; Sun et al., 2002; Mathur et al., 2011; Mueller et al., 2010; Dai and Mumper, 2010; Li et al., 2014; Mukherjee et al., 2011; Hu 2011; Chang 2009). Furthermore to cultivated fruits, wild edible fruits have also gained global concern due to their higher polyphenol contents and outstanding antioxidant activities (Meda et al., 2008; Chalise et al., 2010; Fu et al., 2010; Murillo et al. 2012; Siqueira et al., 2013). In continuation to the worldwide research, wild edible fruits of Indian Himalayan region (IHR) have also emerged as potential source of nutraceuticals including polyphenols with antioxidant, anticancer, anti-
inflammatory, anti-skin aging and skin lightening activities (Saini et al., 2012; Saini et al., 2013; Seal, 2011; Seal et al., 2013; Singh et al., 2014; Nayak and Chand, 2015; Singh 2015; Singh et al., 2017; Baral and Chand, 2017; Bhatt et al., 2017). *Dendrobenthamidia capitata* (Wall.) Hutch. is one of the underexplored fruits of IHR, locally known as Bhamora (commonly recognized as Himalayan strawberry) belongs to family *Cornaceae* is a subdeciduous tree commonly found in mixed Oak-Rhododendron forests of montane zones at 1500-2500 m altitude in the IHR (Gaur, 1999). Ripe fruits are pinkish-red, in globose heads, 2.5-3.0 cm across, succulent and each drupe one seeded. Recent preliminary studies on *D. capitata* fruits indicated presence of high nutritional composition along with moderate levels of polyphenols and associated bioactivities including antioxidant anti-inflammatory, anti-skin aging and skin-lightening activities (Singh et al., 2015; Rana et al., 2018). Present study was focused on systematic evaluation of total phenolic and flavonoid contents of *D capitata* fruit extracts. Study was also aimed to determine the antioxidant, anti-skin aging and skin lightening activities of the fruit extracts using in-vitro assays.

**Materials and Methods**

General chemicals used in the study were more than 99% pure and of analytical grade procured from HiMedia Pvt. Ltd. (Mumbai, India). 2,2’-azinobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) was obtained from Calbiochem, Merck Pvt. Ltd. (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH), phenyl methosulfate (PMS), nitro blue tetrazolium (NBT), linoleic acid, β-carotene, catechin, ferrozine, catechin, kojic acid, epigallocatechingallate (EGCG), N-succinyl–ala–ala–ala–p-nitroanilide (AAAPVN), N-[3-(2-furyl)acryloyl]–leu–glu–pro–ala (FALGPA), 3,4-dihydroxy-L-phenylalanine (L-DOPA) and *Clostridium histolyticum* collagenase (EC 3.4.24.3), porcine pancreatic elastase (EC 3.4.21.36), and mushroom tyrosinase (EC 1.14.18.1) were purchased from Sigma-Aldrich (St Louis, MO, USA).

**Collection, identification, and authentication of fruit**

Fruit samples (along with small twig containing leaves) (half ripened as fully ripened fruits were unavailable) were harvested from various locations of Ramgarhtalla District Nainital in Uttarakhand state of India during the month of November. Fresh fruits were cleaned with running tap water and kept at −20°C until use within 1 month. The herbarium of fruit samples was authenticated by Botany division of Botanical Survey of India (BSI), Dehradun, Uttarakhand, India.

**Evaluation of total phenolic contents (TPCs) and total flavonoid contents (TFCs)**

Fruit extracts were prepared with 80% aqueous acetone and acid acetone as solvents (Saini et al., 2012). The TPCs were determined by Folin–Ciocalteau method (Singleton et al., 1999). Briefly, fruit extracts (1ml) was incubated with Folin–Ciocalteau reagent (0.2N, 2.5 ml) at room temperature (RT) for 5 min. Thereafter, sodium carbonate solution (75 g.l⁻¹in water, 2 ml) was added and further incubated for 2 h. Absorbance was measured using ultraviolet-visible (UV–Vis) spectrophotometer (Systronics, India, Model No. 119) at 760 nm against water control. A standard calibration curve was plotted using gallic acid (0.2 mg. ml⁻¹). The TPCs were expressed as mg gallic acid equivalent (GAE).g⁻¹ fruit weight (FW). TFCs of fruit extracts were determined according to Chang et al. (2002). For that, diluted extracts (0.6 ml) were incubated with sodium nitrite solution (5%, 0.3 ml) at RT for 5 min. Afterwards, aluminium trichloride solution (10%, 0.6 ml) was added and incubated further for 5 min. Absorbance was measured at 510 nm against water blank and TFC was expressed as mg catechin equivalents (CE).g⁻¹ FW using catechin (0.5 mg. ml⁻¹) as standard.

**Antioxidant activity**
Antioxidant activities of fruit extracts were evaluated as free radical scavenging activities such as DPPH radical scavenging activity (DPPH RSA), ABTS+ radical scavenging activity (ABTS RSA), linoleate hydroperoxide radical scavenging activity (LPRSA), superoxide radicals scavenging activity (SORSA), ferric reducing activity (FRA) using the protocols mentioned by Saini et al. (2014).

**Anti-elastase and anti-collagenase activity**

Anti-elastase and anti-collagenase activities were determined according to Kim et al. (2004) for anti-collagenase assay; fruit extracts were pre-incubated with the collagenase enzyme (stock 0.8 U.ml\(^{-1}\)) for 15 min before adding substrate to start the reaction. The final reaction mixture (150 μl) contained 50mM Tricine buffer, 0.8mM synthetic substrate FALGPA, 0.1 unit collagenase and various dilutions of fruit extracts. Absorbance at 335 nm was measured immediately after adding substrate and then continuously for 20 min using microplate reader (Fluostar Optima, BMG Labtech, Germany). Anti-collagenase activity of fruit extracts was determined as mg EGCG equivalents.g\(^{-1}\) FW. EGCG (250 μM) was used as standard inhibitor. Anti-elastase assay involved incubation of fruit extracts with the porcine pancreatic elastase (stock solution 3.33 mg.ml\(^{-1}\) in sterile water) for 15 min before adding the substrate. The final reaction mixture (250 μl) contained 0.2mM Tris-HCl buffer (pH 8.0), substrate 0.8mMAAPVN, 1 μg.ml\(^{-1}\) elastase and 25 μl fruit extracts. Absorbance values between 381 and 402 nm (following pre-screen scans) were measured continuously for 20 min after adding substrate. EGCG (250 μM) was used as a standard and anti-elastase activity of the fruit extracts was represented as mg EGCG equivalents.g\(^{-1}\) FW.

**Anti-tyrosinase activity**

Anti-tyrosinase activity was evaluated with respect to fruit extracts ability to inhibit mushroom tyrosinase enzyme (Masamota et al., 1980). Reaction mixture included phosphate buffers (0.1M, 20 μl, pH 6.8), L-DOPA(0.85mM, 20 μl) as the substrate, and 20 μl diluted fruit extracts was incubated at 25°C for 10 min. Afterwards, mushroom tyrosinase (1000 U.ml\(^{-1}\), 20 μl) was added to initiate the reaction and incubated for 25 min. The absorbance was measured at 492 nm. Kojic acid (0.5 mg.ml\(^{-1}\)) was used as positive control and anti-tyrosinase activity was measured as milligrams of kojic acid equivalents (KAE).g\(^{-1}\) FW.

**HR-LC-MS**

HR-LC-MS analysis of *D. capitata* fruit extracts was performed at Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology, Mumbai, Maharashtra, India on commercial basis (Singh et al., 2016). A small quantity of dried sample was dissolved with 80% acetone, and filtered through a glass syringe using 0.2 μm filter and then analyzed in UHPLC (1290 Infinity Binary pump, Agilent Technologies) coupled with 6550 i-Funnel QTOF mass spectrometer (the mobile phase was a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN) at a flow rate of 0.3 ml.min\(^{-1}\) for 30min. The column used was Zorbax SBC18 Rapid Resolution HD, 2.1 × 100 mm, 1.8 μm (Agilent Technologies, USA). The nitrogen gas flow was 13 ml.min\(^{-1}\) at 25°C, and the sheath gas flow was 11 ml.min\(^{-1}\) at 30°C with a nebulizer pressure at 35 psi. The capillary voltage was 3500 V with a nozzle voltage of 1000 V. Fragmentation energy was kept at 175 V. The analysis was done in both +ESI and –ESI mode, the data was acquired in Agilent Masshunter Data Acquisition software (Version B.05.00) and the data were analyzed in Agilent Masshunter Qualitative Software (Version B.06.00).

**Statistical analysis**

To rule out any inconsistencies, three independent extractions were performed and each extract was analyzed at least three times for each parameter. The results were expressed as mean of three independent experiments (n = 3) with calculation of standard error (SE). The statistical analysis of the data was performed using MS Excel and Prism 3 pad software (Microsoft, Redmond, WA, USA).

**Results**
TPC and TFC
The TPC was evaluated using gallic acid as standard \((R^2 = 0.999)\). The result showed significantly higher (\(p<0.001\)) TPC in acid acetone (\(3.57\pm0.12 \text{ mg GAE.g}^{-1} \text{ FW}\)) than in acetone fruit extracts (\(4.38\pm0.08 \text{ mg GAE.g}^{-1} \text{ FW}\)). The TFC was determined using catechin as standard \((R^2 = 0.9996)\). The acid acetone fruit extract showed significantly higher TFC (\(7.47\pm0.66 \text{ mg CE.g}^{-1} \text{ FW}\)) than that of acetone extracts (\(3.38\pm0.2 \text{ mg CE/g FW}\)).

| Extracts         | TPC mg GAE. g\(^{-1}\) FW | TFC mg CE. g\(^{-1}\) FW |
|------------------|---------------------------|--------------------------|
| 80% acetone      | 3.67\pm0.12               | 3.78\pm0.2               |
| 80% acid acetone | 4.38\pm0.08               | 7.47\pm0.66              |

Each value was expressed as mean ± SE (\(n = 3\)).

Antioxidant activity
The antioxidant activities of \(D. \text{ capitata}\) fruit extracts were determined using various parameters such as free radical scavenging activities against four different types of free radicals (ABTS\(^{+}\), DPPH, linoleate peroxide, ferric and superoxide) and ferric reducing activities (Table 2). The ABTS assay measures the ability of fruit extracts to scavenge the cationic radical ABTS\(^{+}\) produced by the oxidation of ABTS. The blue green colored ABTS\(^{+}\) cation radicals are reduced to its non-radical, colorless ABTS form by the fruit antioxidants. We observed high levels of ABTS scavenging activity in both the extracts. It was slightly higher in acid acetone extract (\(6.83\pm0.23 \text{ mg BHAЕ.g}^{-1} \text{ FW}\)) than that in acetone extracts (Table 2). This was further confirmed by lower concentration of acid acetone extracts required to inhibit 50% of ABTS radical inhibition (IC50) (Table 3). The DPPH scavenging activity of extracts is based on the ability of fruit phenols to neutralize the stable and neutral free radical DPPH (Gulcin et al., 2010). The DPPH free radical scavenging activity was found to be marginally higher in acid acetone extracts (\(9.53\pm0.11 \text{ mg CE.g}^{-1} \text{ FW}\)) as compared to that in acetone extracts. This higher recovery of DPPH scavenging phenols in acid acetone fruit extracts was further established by its lower IC50 values which were even much lower than that was shown by ascorbic acid standard (Table 3). Linoleate hydroperoxide radical scavenging activity (LPRSA) of fruit extracts were determined through β-carotene bleaching test which involved production of linoleate hydroperoxide as free radicals at 50 °C. These free radicals interact with β-carotene, leading to its bleaching. Thus, the β-carotene bleaching rate is inversely proportional to the antioxidant activity of extracts that neutralize free radical formed in the system. Linoleate peroxide scavenging activities was faintly higher in acid acetone extracts than that in acetone fruit extracts which was also evident by its lower IC50 values of acetone fruit extracts (Table 3). Superoxide (\(O_2^{-}\)) is the reactive oxygen radical produced by one-electron reduction of molecular oxygen in metabolic processes and is the major source of other radicals (Liu et al., 1997). Therefore, it was very important to examine the superoxide anion radical scavenging ability of \(D. \text{ capitata}\) fruit extracts. This assay determines the ability of fruit extracts to neutralize the superoxide anion radicals generated in the PMS/NADH system which subsequently leads to a decrease in the reduction of nitroblue tetrazolium to a chromogenic product. The higher superoxide scavenging activity was seen in the acid acetone extract (\(78.7\pm1.25 \text{ mg AAE.g}^{-1} \text{ FW}\)) in compared to acetone fruit extracts (Table 2). The IC50 values of the acid acetone fruit was also lower than acetone fruit extracts (Table 3). The ferric reducing assay measures the ability of phenolics to reduce Fe\(^{3+}\) to Fe\(^{2+}\). The ferric reducing activity of acid acetone fruit extracts was 15.84\pm1.25 mg AAE.g\(^{-1}\) FW higher than the acetone fruit extracts (Table 2).
Extracts | IC50 values of Free radical scavenging activities
---|---
| ABTS\(^+\)RSA | DPPHRSA | LPRSA | SORSA |
| mg BHAЕ.g\(^{-1}\)FW | mg AAE.g\(^{-1}\)FW | mg BHAЕ.g\(^{-1}\)FW | mg CE.g\(^{-1}\)FW |
| Acetone | 1.482 | 1.008 | 1.830 | 1.641 |
| Acid acetone (mg.ml\(^{-1}\)) | 1.376 | 0.936 | 1.787 | 1.237 |
| BHAЕ (μg.ml\(^{-1}\)) | 8.71 | | 6.807 | |
| AAE (mg.ml\(^{-1}\)) | | 4.258 | | |
| CE (μg.ml\(^{-1}\)) | | | | 12.815 |

IC\(_{50}\): concentration of extracts required to inhibit 50% free radicals.

**Anti-elastase and anti-collagenase activities**
Elastase and collagenase are the enzymes located in extracellular matrix. They are responsible for breaking elastin and collagen fibers, the main components of extracellular matrix and thus leading to loosening of skin resulting in skin aging. Anti-elastase and anti-collagenase activities of the fruit extracts were determined by using EGCG as standard inhibitor \((R^2 = 0.984\) and \(R^2 = 0.971\), respectively). The observations showed overall low anti-elastase activity in both the extracts of *D. capitata* fruit though it was significantly higher in acid acetone extracts \((5.7\pm1.26\) mg EGCЕ.g\(^{-1}\)FW). In contrary to low anti-elastase activity, both the *D. capitata* fruit extracts showed admirable anti-collagenase activities. The activity was better in acid acetone fruit extracts \((25.68\pm1.77\) mg EGCЕ.g\(^{-1}\)FW) than in acetone extracts.

**Table 4: Anti-elastase, anti collagenase and anti-tyrosinase activities of *D.capitata* fruit extracts**

| Extracts | Anti-elastase assay | Anti-collagenase assay | Anti-Tyrosinase assay |
|---|---|---|---|
| | mg EGCЕ.g\(^{-1}\)FW | mg EGCЕ.g\(^{-1}\)FW | mg KJЕ.g\(^{-1}\)FW |
| Acetone | 1.09\pm0.033 | 21.89\pm0.36 | 72.46\pm2.96 |
| Acid acetone | 5.7\pm0.17 | 25.68\pm1.77 | 65.56\pm1.37 |

Each value was expressed as mean ± SE \((n = 3)\).

**Anti-tyrosinase activity**
Tyrosinase enzyme is one of the key enzymes involved in the melanin biosynthetic pathway responsible for darkening of skin color. Therefore, any tyrosinase inhibiting activity is considered as skin lightening activity. Anti-tyrosinase activity of the *D. capitata* fruit extracts was determined using kojic acid as standard inhibitor of tyrosinase enzyme \((R^2 = 0.969)\). The observation showed noteworthy anti-tyrosinase activity in both the
extracts (Table 5). It was higher in acetone fruit extract (72.46±2.96 mg KAE. g⁻¹ FW).

**Polyphenolic Composition**

Composition of phenolic compounds in aqueous acetone fruit extracts of *B. capitata* was determined qualitatively by HR-LC-MS analysis. The identification of compounds was performed by comparing their mass spectra and fragmentation pattern with polyphenol database (supplied by Agilent Technologies) using Agilent Masshunter Qualitative Software (Version B.06.00). The analysis revealed presence of four flavonoid compounds (dihydrorobinetin, dihydromyricetin, retusin dimethyl ether, rotenone) while two phenolic acid derivatives (bergenin or C-glycoside of 4-O-methyl gallic acid and peucenin) in *D. capitata* fruit extracts (Table 6; Fig. 1)

| S.No. | Compounds                               | Retention time (min.) | MS (m.z⁻¹)          | Mass       | Formula        |
|-------|-----------------------------------------|-----------------------|---------------------|------------|----------------|
| 1     | Dihydrorobinetin                        | 9.66                  | 287.0526            | 304.0559   | C₁₅H₁₂O₇      |
| 2     | Dihydromyricetin                         | 12.008                | 303.0469            | 320.0503   | C₁₅H₁₂O₈      |
| 3     | Bergenin (C-Glycoside of 4-O-methyl gallic acid) | 0.898             | 351.0651            | 328.0758   | C₁₄H₁₆O₉      |
| 4     | Retusin dimethyl ether                   | 0.916                 | 335.0898            | 312.0994   | C₁₈H₁₆O₈      |
| 5     | Peucenin                                 | 20.864                | 243.0996            | 260.1029   | C₁₅H₁₆O₄      |
| 6     | Rotenone                                 | 5.186                 | 415.0957            | 394.1429   | C₂₃H₂₂O₆      |

**Table 5.** Polyphenolic composition of the acetone fruit extracts of *D. capitata*.

**Fig. 1:** Polyphenolic composition by HR-LS-MS analysis in *D. capitata*

Present study is the first report demonstrating *D. capitata* fruit as the abundant source of phenolics and flavonoids endowed with excellent antioxidant, strong anti-aging and skin lightening activities. Previous studies in our laboratory and others demonstrated 80% acetone and acid acetone as the best solvent for maximum recovery of polyphenols and associated bioactivities (Meda et al., 2008; Seal, 2011; Saini et al., 2012; Saini et al., 2013; Saini et al., 2014). Therefore, present study used 80% aqueous acetone and acid acetone as solvent for the extraction of phenolics and
flavonoids from *D. capitata* fruits. The present study demonstrated significantly higher level of TPC and TFC flavonoid contents in acid acetone extracts of *D. capitata* fruit Himalayan. This is due to the fact that acidic conditions leads to enhanced hydrolysis of bound polyphenol glucosides (phenolic acid and flavonoid glycosides) aiding into elevated recoveries of the same (Rice-Evans, 2002; Meda et al., 2008; Gorinstein et al., 2011). Our result of high phenolics and flavonoids in *D. capitata* fruit is supported by the previous study by Singh et al. (2015) who reported 356.66±12.00 mg GAE.100 g⁻¹ FW and 337.75 CE.100 g⁻¹ FW TPC and TFC respectively in acetone fruit extracts. Our study evidently demonstrated presence of significant free radical scavenging activities against a diverse range of free radicals (DPPH neutral, ABTS cationic, superoxide anionic, and linoleate hydroperoxide free radicals) and noteworthy ferric reducing activity in acid acetone extracts. The phenolics and flavonoid contents of various extracts were very well correlated with their free radical scavenging and ferric reducing activities suggesting their equal contribution in the antioxidant activities. Our observations on antioxidant activities of *D. capitata* fruits are in consonance with the previous studies by Singh et al. (2015) in the same fruit and other in wild edible fruit (Meda et al., 2008) which showed high polyphenol contents and their high antioxidant capacities. *D. capitata* fruit extracts also showed excellent anti-collagenase and anti-tyrosinase activities which was much better than that demonstrated by Singh et al. (2016) in the acetone fruit extracts of *D. capitata*. Our findings are also supported by the presence of dihydromyricetin which was earlier shown to inhibit MMP-9 protease. In addition, till date, many phenolic and flavonoid compounds have been shown to possess anti-skin aging and skin-lightening activities by inhibiting elastase, collagenase and tyrosinase enzymes respectively in in vitro assays (Mukherjee et al., 2011; Orhan et al., 2014; Jennifer et al., 2012; Binic et al., 2013; Zhang et al., 2014; Li et al., 2014; Kim et al., 2015).

**Conclusion**

The ability of phytochemicals of Himalayan strawberry i.e., *D. capitata* acetone and acid acetone fruit extract to scavenge a variety of free radicals, and to selectively inhibit collagenase and tyrosinase enzymes are highly appreciable; this implies that the *D. capitata* fruit or fruit extract may be consumed to maintain the antioxidant levels of the body which may in turn prevent the free radicals mediated pathological diseases and slow down the premature skin aging and sun-light induced darkening of skin. Alternatively, fruit extracts could be employed to develop the newer, safer and effective skin cosmetic creams that fight premature skin aging and tanning.

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