



Comparative Assessment Of Antioxidant And Antiproliferative Activities Of Pulp And Seed Of Himalayan Bayberry Fruit

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Abstract: *Myrica esculenta* Buch-ham. Ex D. Don or “Himalayan bayberry” (family *Myricaceae*) is an extremely valuable wild edible plant whose root, bark, leaves and fruits possess therapeutic properties and are active ingredient in several ayurvedic formulations employed for the treatment of various ailments and disorders including asthma, chronic bronchitis, ulcers, anemia, fever, diarrhea etc. Polyphenols from Himalayan bayberry fruit pulp and seeds were extracted using 80% acetone. TPC and TFC in the pulp and seed extracts were determined according to the Folin-Ciocalteu method and aluminum chloride method respectively. Antioxidant potentials of pulp and seed extracts were determined as DPPHRSA, ABTSRSA and FRA. Anti-proliferative activities of the fruit pulp and seed extracts were analyzed against breast cancer cell line MDA-MB231. 80% acetone extracts of seed (MeSA) showed very high TPC (4143.02±29.3 mg Gallic acid equivalent/100 g FW and TFC (1397.72±28.44 mg catechin equivalent/100 FW) which were 11 and 5 times greater than that were found in pulp extracts (MePA). MeSA extracts showed exceptionally high antioxidant activities with respect to the DPPHRSA and ABTSRSA (IC₅₀, 0.1153 mg/ml and 0.0017 mg/ml respectively) and ferric reducing activities. In addition, MeSA extracts was highly toxic to the breast cancer cell line MDA-MB231 showing 92% inhibition at 10 mg/ml while remaining ineffective against normal transformed cell line HEK293. The inhibition was much higher than that showed by pulp extracts (64%) at the same concentrations. This is the first report demonstrating an excellent ability of *M. esculenta* seed aqueous acetone extract with superior antioxidant activity and antiproliferative potential against breast cancer cells. Therefore, this study recommends utilization of *M. esculenta* seed for the development of nutraceutical and pharmaceutical formulations.

Keywords: Antioxidant; Antiproliferative; Flavonoids; Himalayan bayberry; *Myrica esculenta*; Phenolics

Introduction

Myrica esculenta, Buch-ham. Ex D. Don (family *Myricaceae*), commonly known as “Kaphal”, or “Himalayan bayberry” is amongst highly valued wild edible plants growing between 900 and 2100 m above sea level distributed from Ravi eastward to Assam, Khasi, Jaintia, Naga and Lunshi hills in Indian Himalayan Region and extended to Malaya, Singapore, China and Japan (Gaur 2000). Himalayan bayberry is an evergreen tree, up to 14 m high, bark brownish-grey, rough, vertically wrinkled. Leaves alternate, crowded at

the end of branches, oblanceolate, 6-15x3-5 cm, entire, acute, glossy above, glaucous dotted below, petioles 7-14 mm long. *M. esculenta* flowering and fruiting season is August to October and April to June respectively (Gaur 2000). Different parts of the *M. esculenta* plant including root, bark, leaves and fruits possess therapeutic properties and are part of numerous ayurvedic formulations used for treatment of various ailments and disorders such as asthma, cough, chronic bronchitis, ulcers, inflammation, anemia, fever, diarrhea, and ear, nose and throat



disorders (Sood and Shri 2018; Kabra et al. 2019). *M. esculenta* is very popular amid the local folk for its delicious fruits and processed food products such as juices, syrups, jams and pickles (Dhyani and Dhar 1994). The fruits are of small size of around 2-7 mm with maximum area occupied with seed covered with a thin layer of juicy pulp of red to dark reddish brown color (Gupta 1989; Sood and Shri 2018, Bhatt et al. 2020). The previous methodical studies have established *M. esculenta* fruit as an opulent source of health promoting substances such as minerals, water soluble vitamins and polyphenols (Rawat et al. 2010; Seal et al. 2011; Saini et al. 2013; Mann et al. 2015; Kabra 2019). *In vitro* studies have demonstrated that *M. esculenta* fruit polyphenols are endowed with tremendous bioactivities including antioxidant, antimicrobial, antihelminthic, analgesic, antipyretic, anti-inflammatory and anticancer activities (Rawat et al. 2010; Saini et al. 2013; Pant et al. 2014; Mann et al. 2015; Bhatt et al. 2017; Belwal et al. 2019). The reverse phase-high performance liquid chromatography (RP-HPLC) analysis of the pulp and whole fruit extracts showed presence of elevated levels of phenolic acids and flavonoids including gallic acid, caffeic acid, chlorogenic acid, transcinnamic acid, p-coumaric acid, ellagic acid, anthocyanins, catechin and myricetin (Rawat et al. 2010; Saini et al. 2013; Bhatt et al. 2017; Belwal et al. 2019).

The studies on whole fruit extracts showed much higher total phenols, flavonoids and antioxidant activities (Seal et al. 2011; Saini et al. 2013; Pant et al. 2014; Mann et al. 2015) than that showed by other researches in the fruit pulp extracts (Rawat et al. 2010; Belwal et al. 2019) and hence the contribution of seed bioactive in the enhanced polyphenol contents and antioxidant activities of whole fruit extracts could not be ignored. Seed of *M. esculenta* fruit which account for >15% of the total fruit weight is a byproduct generally discarded as waste (Bhatt et al. 2020). Till date no

literature is available on polyphenolic contents and any bioactivities present in the seeds of *M. esculenta*. However, the seeds of other species of *Myrica* i.e., *Myrica rubra* have been shown to be an affluent source of proteins, sugars and lipids (Cheng et al. 2008; 2009; Xia et al. 2013) although no information is available on the polyphenol contents and their associated bioactivities. Therefore, the present study focused on the determination of the total phenolics (TPC) and flavonoid contents (TFC) of Himalayan bayberry seeds and their antioxidant and anti-proliferative activities.

Materials and Methods

All the chemicals were of analytical grade and more than 99% pure. DPPH was procured from Sigma-Aldrich (Steinheim, Germany) while ABTS was obtained from Calbiochem, Merck (Darmstadt, Germany). Other chemicals and reagents were purchased from HiMedia Pvt., Ltd. (Mumbai, India).

Collection and authentication of *M. esculenta*

fruits: Fresh ripe Himalayan bayberry fruits (bright red to maroon in color) were harvested from nearby hilly locations of Agrakhal of Tehri Garhwal, Uttarakhand, India during April to May 2019. Fruits were cleaned under running tap water and kept frozen at -20°C till use. The herbarium of the bayberry fruit samples along with a small twig carrying leaves and flowers was prepared and further authenticated by Botanical Survey of India, Dehradun, Uttarakhand, India.

Preparation of fruit extracts

Polyphenols from Himalayan bayberry fruit pulp and seeds were extracted using 80% acetone (Saini et al. 2013). Briefly, Pulp of 25 g frozen fruits was carefully removed from the seeds and extracted three times with 25 ml of acetone (80% v/v) (MePA) for 30 min at room temperature (RT) with continuous stirring. The extraction



procedure was repeated two more times with the 25 ml of fresh acetone. For seed extracts preparation, the thoroughly washed seeds from 25 g of frozen fruits were homogenized in a mixer grinder for 5 min and extracted thrice with each of 25 ml of 80% acetone (MeSA). The extracts were filtered, pooled, and centrifuged separately (model no-C24; Remi Pvt Ltd., New Delhi, India) to obtain clear extracts. Extracts were stored - 20°C till further use.

Phytochemical analysis

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC): TPC and TFC in the pulp and seed extracts were determined according to the Folin-Ciocalteu (FC) method (Singleton 1999) and aluminum chloride method (Chang et al. 2002) using gallic acid and catechin as standard respectively.

Antioxidant activities: Antioxidant potentials of pulp and seed extracts were determined as DPPH radical scavenging activity (DPPHRSA), ABTS+ radical scavenging activity (ABTSRSA) and ferric reducing activity (FRA) using the protocols mentioned by Saini et al. (2013).

Anti-proliferative activities: Anti-proliferative activities of the extracts were analyzed against breast cancer cell line MDA-MB231 (American Type Culture Collection, ATCC, Pune, India) maintained on Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) with 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotic (ampicillin/streptomycin) in a humidified atmosphere with 5% CO₂ at 37°C. To

determine the cytotoxic effect of the fruit extracts, MDA-MB231 and normal transformed HEK293 cells (5x10³) were treated with the various dilutions of pulp and seed extracts (1.25-10 mg/ml) in the 96-well plate for 24 h and 48 h. Afterwards, the cell viability was determined using colorimetric assay in microplate reader (Fluostar Omega Spectrofluorometer; BMG Technologies, Offenburg, Germany) (Saini et al. 2013).

Statistical analysis

To avoid any ambiguity, experiments were performed in triplicates independently and each extract was analyzed at least 3 times for each parameter. The results were expressed as mean of 3 independent experiments (n=3) with standard error (SE). Statistical analysis of the data was performed using MS Excel and Prism 3 pad software (Microsoft, Redmond, WA, USA)

Results and Discussion:

TPC and TFC

Present study illustrated much higher TPC (>11 times) and TFC (>5 times) in seed extracts than that in pulp extracts of *M. esculenta* fruits (Table 1). The TPC (353.81±16.2 mg GAE/100 g FW) and TFC (263.83±17.04 mg CE/100 g FW) in pulp extracts are in accordance with the available literature on pulp extracts which showed TPC ranging from 1.78-5.51 mg GAE/ g FW and TFC ranging from 1.31- 4.73 mg QE / g FW (Rawat et al. 2011; Bhatt et al 2017; Bhatt et al 2019).

Table 1 Total polyphenol contents and antioxidant activities of the Himalayan bayberry fruit extracts

| Activities | Pulp | Seeds |
|----------------------------|--------------|---------------|
| | MePA | MeSA |
| TPC (mg GAE/100 g FW) | 353.81±16.2 | 4143.02±29.3 |
| TFC (mg CE/100 g FW) | 263.83±17.04 | 1397.72±28.44 |
| DPPHRSA (mg CE/100 g FW) | 465.14±8.2 | 2658.74±54.93 |
| ABTSRSA (mg BHTE/100 g FW) | 682.19±17.01 | 3463.27±35.05 |
| FRA (mg AAE/100 g FW) | 965.15±68.56 | 9500.3±88.08 |



1 MePA, *Myrica esculenta* pulp aqueous acetone extract; MeSA. *Myrica esculenta* seed aqueous acetone extract, GAE, gallic acid equivalents; CE, catechin equivalents; BHTE, BHT equivalents; AAE, ascorbic acid equivalents, TPC, total phenolic content; TFC, total flavonoid content; DPPHRSA, DPPH radical scavenging activity; ABTSRSA, ABTS radical scavenging activity; FRA, ferric reducing activity. 2 Each value is expressed as mean \pm SE (n=3) The seed extracts in the present study showed much higher TPC (4143.02 \pm 29.3 mg GAE/100 g FW) and TFC (1397.72 \pm 28.44 mg CE/100 g FW) than that in the whole fruit extracts of *M. esculenta* demonstrated by previous studies (Seal 2011; Saini et al. 2013; Pant et al. 2014; Mann et al. 2015). Seal et al (2011) showed TPC of 28.56 \pm 0.78 mg GAE and TFC of 2.25 \pm 0.08 QE/g dried fruit weight (DFW) of *M. esculenta*. Saini et al. (2013) demonstrated 1309.0 \pm 13.9 mg GAE TPC and 468.0 \pm 22.3 mg CE TFC per 100 g of FW. Pant et al (2014) studied TPC and TFC of methanol extracted dried extracts of *M. esculenta* fruit and showed 30-44 mg TPC and 4.9-5.4 mg TFC in 1 g of dried methanol extracts. In another study, Mann et al (2015) demonstrated TPC 26.21 \pm 0.1 μ g GAE and TFC 38.0 \pm 0.5 μ g rutin equivalents per mg dried fruit extracts. Nevertheless, there is no study available on the TPC and TFC in the seeds of *M. esculenta* or any of the *Myrica* species. Earlier, a single study by Fang et al. (2011) isolated flavanones and dihydrochalcones from the *M. gale* seed showing their presence in the seed.

Antioxidant activity

Antioxidant activities of the *M. esculenta* fruit pulp and seed extracts were studied with respect to free radical scavenging activities (DPPHRSA and ABTSRSA) and ferric reducing activity (Table 1). Observation showed very high

DPPHRSA, ABTSRSA and FRA in seed extracts as compared to that in pulp extracts (Table 1). DPPHRSA, ABTRSA and FRA were more than five, and nine times higher in seed extracts as compared to that in pulp extracts (Table 1). Higher antioxidant activities of seed extracts were further confirmed by their lower IC₅₀ values for DPPHRSA (0.1153 mg/ml) and ABTSRSA (0.017 mg/ml) as compared to the pulp extracts which showed higher IC₅₀ values (DPPHRSA, 3.770 mg/ml and ABTSRSA, 0.2834 mg/ml). Antioxidant activities in seed extracts in present study is much higher than that which were earlier reported in whole fruit extracts or pulp extracts (Seal 2011; Saini et al. 2013; Pant 2014, Mann et al. 2015; Rawat et al. 2011; Belwal et al. 2019). The higher antioxidant activities in seed extracts as compared to pulp extracts might be attributed to the greater TPC and TFC levels in seeds.

Anti-proliferative activities

The cytotoxic effects of pulp and seed extracts of Himalayan bayberry fruit on the breast cancer cell line MDA-MB-231 and normal transformed cell line (HEK293) were analyzed by employing MTT assay. Cells were cultured with an extracts concentration equivalent to 1.25, 2.5, 5.0, 7.5 and 10 mg FW/ml. Both the pulp and seed extracts significantly inhibited the proliferation of MDA-MB-231 while exhibiting no cytotoxicity towards HEK293. Inhibition was dose dependent, and the degree of inhibition was different for pulp and seed extracts. Pulp extracts (MePA) decreased viability of MDA-MB-231 cells to 37% at the extracts concentration of 10 mg/ml showing 63% inhibition (Fig). However, the seed extracts (MeSA) extracts were much more effective showing similar decrease in viability to 34% (64% inhibition) at the much lower extracts concentration of 5.0 mg/ml while showing maximum inhibition up to 92% (8.0% viability) at 10 mg/ml (Figure 1).

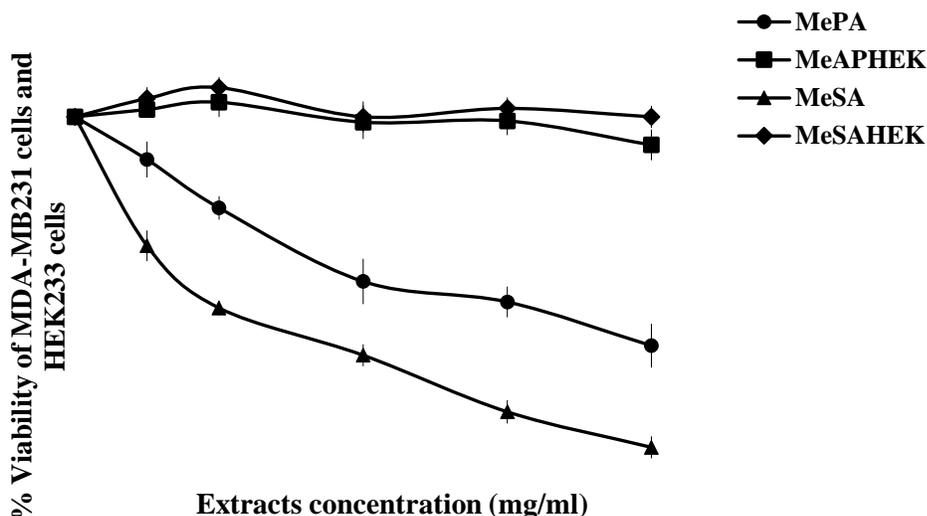


Figure 1. Effects of Himalayan bayberry fruit extracts on breast cancer cell lines MDA-MB-231 and normal transform cell line HEK293. Extracts concentration was equivalent to the mg FW/ml. Each value is expressed as mean±SE (n=3).

The median inhibitory concentration (IC_{50}) was also lower for seed extracts (3.8 mg FW/ml) compared to that of pulp extracts (6.98 mg FW/ml). The observations are in accordance with the earlier studies on anticancer activities of *M. esculenta* whole fruit extracts (Saini et al. 2013; Mann et al. 2015). Saini et al. (2013) showed high cytotoxicity of *M. esculenta* whole fruit extracts on cervical cancer cell lines i.e., C33A (80%), HeLa cells (80%), and SiHa cells (78%) while displaying no effect on normal transformed cell line HEK293 and PBMCs. Mann (2015) showed moderate cytotoxic effects of methanolic extracts at concentration of 5 mg/ml on viability of MDA-MBA-231 (46.19%), HepG2 (50%) and HeLa cell (48.29%). The likely anticancer effects of the whole fruit bayberry extracts were strongly supported by the presence of gallic acid, caffeic acid, chlorogenic acid, p-coumaric acid, trans-cinnamic acid, ellagic acid, catechin, chlorogenic acid, and myricetin (Rawat 2010; Saini 2013, Belwal 2019) which are known to have

tremendous cytotoxicity against variety of cancer cells (Losso et al. 2004; You et al. 2003; Ko et al. 2005; Chang et al. 2010; Amal et al. 2011). Seed extracts showed reasonably better anticancer effects against MDA-MB-231 than pulp extracts. This might be due to the presence of higher TPC and TFC in general or seed specific phenolics or flavonoids (or their increased levels) with anticancer activities. Further study involving complete identification of polyphenols in the seed extracts is in progress which would provide the information regarding the major contributor of anticancer effects in Himalayan bayberry seed extracts.

To the best of our knowledge, the present report is the first investigation showing the tremendous ability of *M. esculenta* seed acetone extract to scavenge a variety of free radicals, and to selectively inhibit the proliferation of breast cancer cells. This indicate that the *M. esculenta* seed extract could be employed to develop the newer, safer, effective, and selective anticancer drugs against cervical cancer or may be



consumed to maintain the antioxidant levels of the body which may in turn prevent the free radicals mediated pathological diseases and inhibit cancer growth.

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