Evaluation of phytochemical constituents and antioxidant activity of selected actinorhizal fruits growing in the forests of Northeast India

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Hippophae salicifolia, *Elaeagnus pyriformis*, *Myrica esculenta* and *M. nagi* are actinorhizal plants growing in the sacred forests of Northeast India with multipurpose uses. The present investigation was undertaken to determine the phenol, flavonoid and flavonol contents of the fresh fruit juice of these plant species including the antioxidant potential by means of DPPH, H_2O_2 and NO scavenging activity and FRP. The total phenolic, flavonoid and flavonol contents of fruit juice ranged from 321.68±0.06 to 76.67±0.01 mg/g GAE, 272.92±0.07 to 20.12±0.02 mg/g QE and 258.92±0.08 to 18.72±0.02 mg/g QE, respectively. At 2.0 mg/mL concentration, DPPH scavenging activity was found to be the highest in *M. esculenta* (89.62%) and the lowest in *E. pyriformis* (17.58%). The reducing power activity was found significantly higher in *H. salicifolia* juice, which increased with increase in concentration. The H_2O_2 scavenging activity of *H. salicifolia* juice was found to be as high as 98.78%, while *Elaeagnus* juice was found to be less effective with just 48.90%. Juice of *H. salicifolia* showed the greatest NO scavenging effect of 75.24% as compared to juice of *E. pyriformis*, where only 37.54% scavenging was observed at the same concentration. Taking into account all the experimental data, it can be said that the fruits of *H. salicifolia* and both *M. nagi* and *M. esculenta* have good antioxidant activity compared to fruits of *E. pyriformis*.

[Goyal AK, Mishra T, Bhattacharya M, Kar P and Sen A 2013 Evaluation of phytochemical constituents and antioxidant activity of selected actinorhizal fruits growing in the forests of Northeast India. *J. Biosci.* **38** 797–803] **DOI** 10.1007/s12038-013-9363-2

1. Introduction

Actinobacteria belonging to the genus *Frankia* form symbiotic associations with woody dicotyledonous plants (popularly known as actinorhizal plants). *Hippophae salicifolia* D. Don, *Myrica esculenta* Buch.-Ham. Ex D. Don, *M. nagi* Thunb. and *Elaeagnus pyriformis* Hook. f. are few such plants found in the Himalayan region of India. *Hippophae* (sea-buckthorn) is a thorny, dioecious, deciduous, perennial, actinorhizal plant belonging to the family Elaeagnaceae. In India, *Hippophae* grows naturally in high-altitude areas of Jammu and Kashmir, Himachal Pradesh, Uttar Pradesh and Sikkim (Singh 1998). *H. rhamnoides* is the most commonly found species here, followed by *H. salicifolia*. *H. salicifolia* has a shrubby to tree-like habit, restricted to the Himalayan region, whereas *H. rhamnoides* is shrubby to bushy, growing at higher altitude and is widely distributed in Eurasia (Goyal *et al.* 2011a). On the other hand, *Elaeagnus* (silverberry or oleaster) is a deciduous, actinorhizal shrub belonging to the Elaeagnaceae family. In India only four species of *Elaeagnus* are recorded (Sharma and Kumar 2006). *E. pyriformis* is mainly encountered in the eastern Himalayas and Northeastern India (Asati and Yadav 2004). *Myrica* (Bayberry or box myrtle) varies between shrubs to trees and is mostly evergreen, dioecious belonging to Myricaceae. In India, it is distributed in the subtropical Himalaya from Ravi eastwards to Assam and in Khasi, Jaintia, Naga and Lushai Hills at an elevation of about 900–1200 m above mean sea level (Osmaston 1978). In Meghalaya, three different morphotypes of *Myrica* are encountered classified as *M. esculenta*, *M. nagi* and third intermediate between the two (Yanthan and Misra 2013). Besides being actinorhizal, these plants are also important medicinally and economically.

Keywords. Actinorhizal plants; antioxidants; *Hippophae salicifolia*; *Elaeagnus pyriformis*; *Myrica nagi*; *Myrica esculenta*; phenol

Published online: 1 October 2013

Traditionally the fruits of *H. salicifolia* are used as appetizer (Kunwar and Adhikari 2005), *E. pyriformis* for constipation (Kala 2005) and that of *M. esculenta* and *M. nagi* are used to prepare refreshing drinks; in addition they also possesses healing properties in case of ulcers, retention of placenta and bone fracture (Jeeva *et al.* 2011; Panthari *et al.* 2012). The seeds and flowers of *E. umbellata* Thunb. are said to be used as a stimulant in coughs and pulmonary infections (Parmar and Kaushal 1982a). We have previously reported antioxidant profiling of *H. salicifolia* growing in the sacred forests of Sikkim (Goyal *et al.* 2011a).

Antioxidants have the ability of protecting organisms from damage caused by free radical-induced oxidative stress (Goyal *et al.* 2010). Polyphenols, naturally occurring in many fruits, account for the majority of antioxidant activity (Li *et al.* 2012). However, polyphenols can undergo various reactions in the course of food processing and storage, which affect their stability (Cheynier 2005). At present, the probable toxicity of synthetic antioxidants has been condemned and thus there is a shift towards the use of natural antioxidants (Chtourou *et al*. 2011). It is strongly believed that regular consumption of plant-derived phytochemicals may drift the balance towards an adequate antioxidant status in the body (Mahomoodally *et al*. 2012). Thus, in recent years, interest on natural antioxidants, especially of plant origin, has increased manifolds. Literature showed that although some work has been done on different plant parts of the above-mentioned plant species (Seal 2011; Goyal *et al*. 2011a; Saikia and Handique 2013a,b), till date no comparative studies have been done on the fresh fruit juice of these symbiotic actinorhizal plants. Thus, keeping this in mind, this study aims to determine the total polyphenol contents (phenol, flavonoid and flavonol) and antioxidant activity with respect to DPPH (2,2-diphenyl-1-picryl-hydrazyl), H_2O_2 (hydrogen peroxide) and NO (nitric oxide) scavenging activity and FRP (ferric reducing power) assay of the fruits of *Hippophae*, *Elaeagnus* and *Myrica* plants growing in Northeast India.

2. Materials and methods

2.1 *Chemicals and reagents*

DPPH (2,2-diphenyl-1-picryl-hydrazyl), quercetin, sodium nitrite ($NaNO_2$), trichloroacetic acid (TCA), ascorbic acid, ferric chloride ($FeCl_3$), gallic acid sulphanilamide and naphthylethylene diamine dihydrochloride were obtained from Himedia Laboratories Pvt. Ltd, Mumbai, India. Potassium di-hydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), potassium hydroxide (KOH), sodium hydroxide (NaOH), potassium ferricyanide ($K_2Fe(CN)_6$), sodium carbonate (Na_2CO_3), hydrogen peroxide (H_2O_2), sodium acetate (CH_3COONa), methanol, sodium nitroprusside and glacial acetic acid were procured from Merck, Mumbai, India, and Folin-Ciocalteu reagent from Sisco research laboratory, Mumbai, India. Aluminium chloride ($AlCl_3$) and orthophosphoric acid were obtained from SD Fine Chemicals Limited, Mumbai, India. All chemicals and solvents were of analytical grade.

2.2 *Plant materials and extraction*

The fruits of *Myrica nagi*, *M. esculenta* and *Elaeagnus pyriformis* were collected from the local market of Shillong, Meghalaya, during April 2012 and that of *Hippophae salicifolia* was procured from north Sikkim during the same period. The plant materials were authenticated by Prof AP Das, Department of Botany, University of North Bengal. A voucher specimen has been preserved in the Botany Department, University of North Bengal, with voucher specimen numbers as 9657, 9658, 9659 and 9660 for *H. salicifolia*, *M. esculenta*, *M. nagi* and *E. pyriformis* respectively. The fruits were mechanically squeezed to separate the seed and the juice. The juice was stored at 4°C until required. The juice was diluted using double-distilled water (DDW) in desired concentrations just prior to use (Goyal *et al.* 2013).

2.3 *Determination of biochemical constituents*

Total soluble phenols were determined by the Singleton and Rossi (1965) method with slight modifications. Briefly, the fruit juice in desired concentration (0.5 mL) was mixed with 0.5 mL of Folin Ciocalteu reagent (previously diluted 1:1 with distilled water) and incubated for 5 min at room temperature (RT), and then 1 mL of 2% Na_2CO_3 solution was added. After incubation at RT for 10 min, the absorbance was measured at 730 nm with a UV-Vis spectrophotometer (Thermo Scientific make). The total flavonoid content was determined according to Zhishen *et al*. (1999) with minor modifications (Goyal *et al.* 2010) using quercetin as a standard. Briefly, the juice (0.25 mL) was added to 1.25 mL double-distilled water (DDW) followed by 75 µL of 5% $NaNO_2$. After 5 min of incubation at room temperature, $AlCl_3$ (0.15 mL, 10%) was added. After a further incubation for 6 min at room temperature (RT), the reaction mixture was treated with 0.5 mL of 1 mM NaOH. Finally, the reaction mixture was diluted with 275 µL of DDW. The absorbance was measured at 510 nm after incubating for 20 minutes at RT. The method developed by Kumaran and Karunakaran (2007) was used to estimate the total flavonols using quercetin as a standard. To 2 mL of fruit juice, 2 mL $AlCl_3$ (2%) along with CH_3COONa was added and incubate at RT for 2.5 h and optical density (OD) was taken at 440 nm.

2.4 *In vitro antioxidant properties of the extracts*

Four different *in vitro* test systems viz. DPPH scavenging activity, ferric reducing power assay, hydrogen peroxide scavenging activity and nitric oxide scavenging activity were used to access the antioxidant potential of the different fruit juice.

2.4.1 *Free radical scavenging activity (DPPH method):* The antioxidant activity of the extracts along with the standard was assessed on the basis of the radical scavenging effect of the stable DPPH free radical as per the modified protocol by Goyal *et al.* (2010). DPPH solution (0.006% w/v) was prepared in 95% methanol. Fruit juices (200 µL) in different concentration (0.2–2.0 mg/mL) were mixed with DPPH solution, so that the final volume was 2 mL and discoloration was measured at 517 nm (with Themo UV1 spectrophotometer) after incubation for 30 min in dark. In case of control, methanol was taken instead of the plant sample. Ascorbic acid was used as a reference standard. Percentage scavenging of the DPPH free radical was measured using the following equation:

$$(\%)\,\text{Scavenging activity} = \frac{(A_o - A_1)}{A_o} \times (100) \qquad (1)$$

where A_o is the absorbance of the control and A_1 the absorbance in the presence of the sample.

2.4.2 *Ferric reducing power assay:* The reducing power of the extracts was determined according to the method of Oyaizu (1986). Different concentrations of juice (0.2–2.0 mg/mL) in 1 mL of DDW was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with DDW (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6) was used as blank solution.

2.4.3 *Hydrogen peroxide scavenging activity:* The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.* (1989) with minor modification (Goyal *et al.* 2010). An aliquot of H_2O_2 (2 mM) and sample at various concentrations (0.2–2.0 mg/mL) were mixed (1:0.6 v/v) and incubated for 10 min at room temperature. After incubation, absorbance was read at 230 nm was determined against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity of hydrogen peroxide was calculated using the equation 1.

2.4.4 *Nitric oxide scavenging activity:* Nitric oxide scavenging activity was measured as per Mishra *et al.* (2012). Sodium nitroprusside solution (1 mL, 10mM) was added to the fruit juice (1 mL) in PO_4 buffer (pH - 7.4) followed by incubation at 25°C for 2.5 h. After incubation, to the solution (2.5 mL) Griess reagent (1 mL) was added and incubated at 25°C for 30 min. OD was noted at 540 nm. Percentage scavenging was measured as per equation 1.

2.5 *Statistical analysis*

All samples were tested and analysed in triplicates. Results were calculated as the mean ± SD (standard deviation) for each sample. Statistical analysis was done with one-way analysis of variance using Graph pad Prism, Version 4.0 (Graph Pad Software, San Diego, CA, USA).

3. Results

The total phenolic, flavonoid and flavonol contents of fruit juice ranged from 321.68±0.06 to 76.67±0.01 mg/g GAE, 272.92 ±0.07 to 20.12±0.02 mg/g QE and 258.92±0.08 to 18.72±0.02 mg/g QE respectively (table 1). Among the various fruits under study the highest phenolic content was recorded in *Myrica esculenta*, whereas *Hippophae salicifolia* showed higher flavonoid and flavonol contents compared to others.

The DPPH scavenging activity of different fruit juice compared to the standard ascorbic acid is shown in figure 1. Fruit juice from different plants showed comparable level of scavenging activity as measured by DPPH, across concentration

Table 1. Polyphenol contents of the different fresh fruit juices (n=6, X±SEM)

Plant	Phenol (mg/g GAE)	Flavonoid (mg/g QE)	Flavonol (mg/g QE)
Hippophae salicifolia	150.2±0.03	272.92±0.07	258.92±0.08
Elaeagnus pyriformis	76.67±0.01	20.12±0.02	18.72±0.02
Myrica nagi	260.17±0.03	119.2±0.05	111.2±0.01
Myrica esculenta	321.68±0.06	187.2±0.04	155.2±0.02

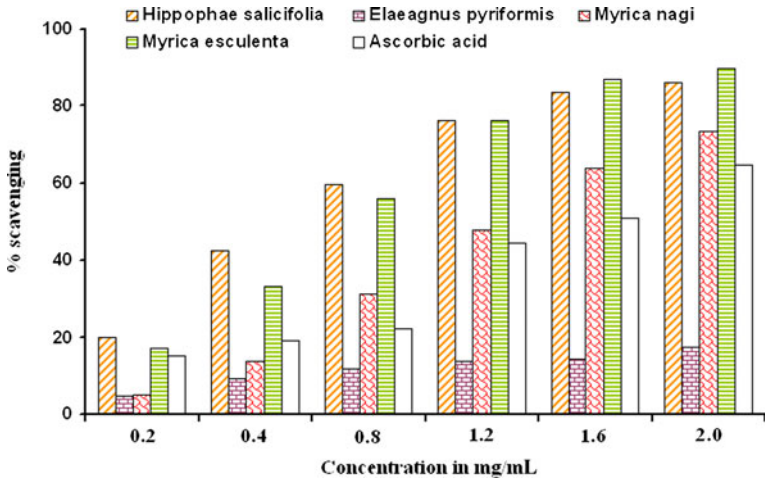

Figure 1. DPPH scavenging activity of different fruit juices compared to standard ascorbic acid.

ranging in between 0.2 to 2.0 mg/mL. At the highest concentration of 2.0 mg/mL *M. esculenta* had the highest scavenging activity of 89.62% followed by *H. salicifolia* (86.15%), whereas *Elaeagnus pyriformis* with 17.58% recorded the lowest and at the same concentration the standard ascorbic acid exhibited 64.76% scavenging activity.

Figure 2 represents the reductive capability of the fruit juice compared to ascorbic acid. The reducing power of the juice was found to be notable, which increased gradually with a rise in concentration as compared to standard. As illustrated in figure 2, Fe^{3+} was reduced to Fe^{2+} in the presence of extract and ascorbic acid, which is a measure of reductive capability. From the figure it is evident that *H. salicifolia,* even at a low dose of the extract, had maximum reducing power, when compared with other fruits under study.

The H_2O_2 scavenging activity of different fruit juices is depicted in figure 3. High H_2O_2 scavenging activity was found irrespective of the fruit types, across the concentration gradient ranging from 0.2 to 2.0 mg/mL. However, *H. salicifolia* had the highest activity irrespective of the fruit juice concentration used and was considerably uniform ranging between 95.38% and 98.79%. In the case of other fruit juices, activity increased with the increase of juice concentration.

A dose-dependent increase in nitric oxide scavenging activity of various fruit juice is illustrated in figure 4. Both *H. salicifolia* and the standard ascorbic acid showed comparable NO scavenging activity of 75.24% and 75.11% at the concentration of 2.0 mg/mL. Between the fruits of two *Myrica* sp., *M. esculenta* showed higher activity with 60.75% compared to *M.*

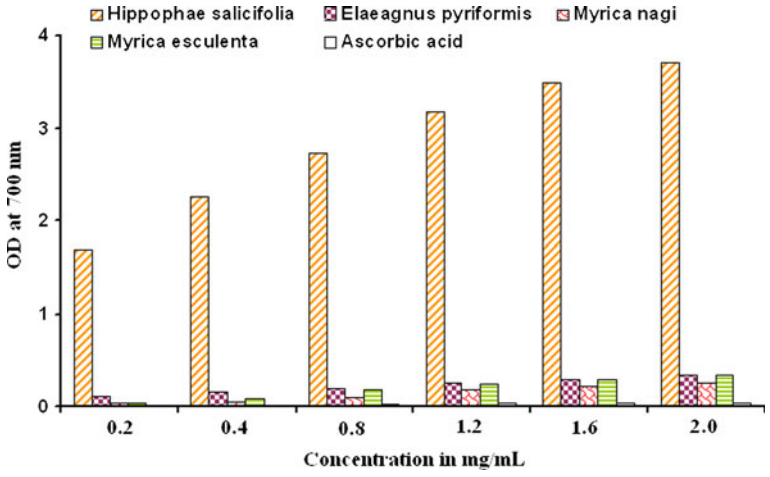

Figure 2. Reducing power assay of different fruit juices compared to standard ascorbic acid at 700 nm.

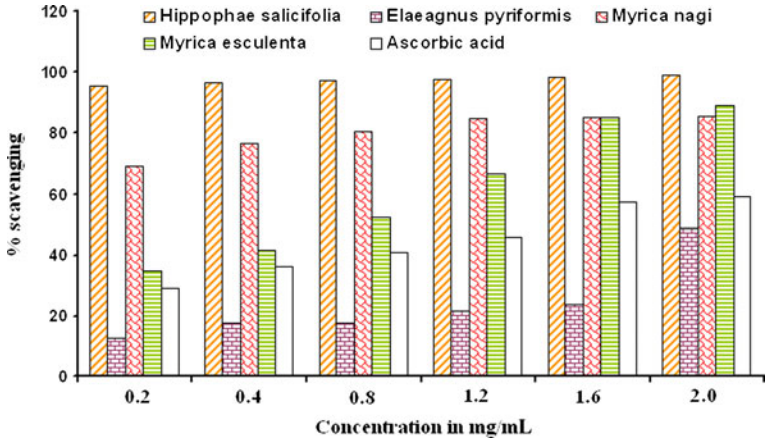

Figure 3. H_2O_2 scavenging activity of different fruit juices compared to standard ascorbic acid.

nagi with 51.71%. *E. pyriformis*, however, had much reduced nitric oxide scavenging activity of only 37.54%.

4. Discussion

The present study showed that nutrition values are different with respect to the plant type. The various methods employed to estimate the poylphenolic contents and antioxidant potential of different fruits of actinorhizal plants clearly demonstrated differences in the chemical constitutes.

The difference in the polyphenolic contents as evident in the current study among the various fruits of actinorhizal plants may be due to the genetic, physiological and ecological status. The presence of phenolics in plants has been well established as the major components responsible for their antioxidant potential due to their redox properties. They have the capacity to adsorb and neutralize the free radicals generated during oxidative stress (Florence *et al*. 2011). Apart from this the flavonoids and flavonols are also widespread in plants, naturally contributing in the free radical scavenging activity together with phenolics (Pourmorad *et al*. 2006). In the present study the results indicate that the flavonoids and flavonols contents contributed significantly in enhancing the antioxidant property in comparison with the phenolic content. Other studies also supported this fact (Seal 2011; Goyal *et al.* 2011b).

The results of DPPH scavenging activity reveals that the fruit of *Myrica esculenta* contains powerful inhibitor compounds compared to *Hippophae, M. nagi* and *Elaeagnus*, which acts as potential antioxidants and thus scavenges the DPPH radicals to form stable reduced DPPH molecules.

In case of reducing power assay, the transformation of Fe^{3+} to Fe^{2+} in the presence of either the extract or the standard (ascorbic acid) is a measure of reducing capability (Singh *et al.* 2012). It is evident from the result that *H. salicifolia* showed higher reducing capability compared to other fruit types which

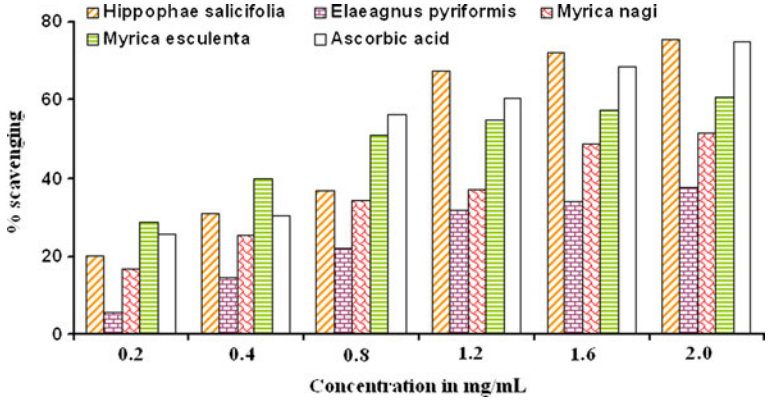

Figure 4. Nitric oxide scavenging activity of different fruit juices compared to standard ascorbic acid.

might be probably due to the presence of these polyphenols in the plants that are responsible for their antioxidant activity along with some of their pharmacological effects (Nakbi *et al.* 2010; Khabade *et al*. 2012). The higher inhibitory action is directly proportional to the potential to break the free radical chain by donating hydrogen atom, which elicits the antioxidant activity (Ajith 2010; Kilicgun and Dehen 2009).

Hydrogen peroxide although being a weak oxidizing agent has the potential to inactivate a few enzymes directly (Hazra *et al.* 2010). In the presence of redox active transition metals Fe^{2+} and Cu^{2+}, H_2O_2 is transformed into hydroxyl radical which might be the key to its toxic effect (Lapidot *et al.* 2002). Thus, the amount of H_2O_2 that accumulate in the cells should be monitored. *H. salicifolia* fruits scavenged H_2O_2 at a considerable rate in comparison to *Elaeagnus* and *Myrica.* This may be attributed to the higher content of flavonoids and flavonols in *Hippophae*.

Nitric oxide (NO) is a powerful intermediary of several physiological processes like smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. NO, a diffusible free radical, has diverse functions as an effector molecule in several biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Acharya *et al.* 2010). From figure 4, it is evident that at lower concentration, *M. esculenta* shows higher NO scavenging activity than others, while with increase in concentration *H. salicfolia* fruits replaces *M. esculenta* in terms of NO scavenging activity. *M. nagi* was found to have moderate antioxidant activity.

The difference in the phytochemical content between the two species of the same genus *Myrica* viz. *M. nagi* and *M. esculenta*, previously thought to be synonymous (Parmar and Kaushal 1982a, b; Balakrishnan 1983), are different in phytochemical activity in our study. This strongly supports the molecular studies on *Myrica* carried out by Yanthan *et al.* (2011) and Yanthan and Misra (2013) to establish the two morphotyes as two different species.

5. Conclusion

Actinorhizal plants are major partners in biological nitrogen fixing symbiosis with an actinobacteria, *Frankia*. Because of symbiosis, these globally distributed actinorhizal plants are able to colonize harsh environmental terrains under diverse ecological niches and are some of the early successional species to inhabit ecologically threatened areas (Benson and Silvester 1993). However, besides being eco-friendly, some of these plants have food and medicinal values. The present investigation established that all the fruits of studied actinorhizal plants are a good source of natural antioxidants. Among the four fruits, *Elaeagnus pyriformis* was found to have lower polyphenolic contents and thus had lower antioxidant activity. *Hippophae salicifolia*, with low phenolics but high flavonoids and flavonols content, possessed higher antioxidant activity. Moreover, this study may be an additional justification to the molecular study for considering *Myrica nagi* and *M. esculenta* as two different species (Yanthan *et al.* 2011; Yanthan and Misra 2013). However, further in-depth taxonomical study is required to solve the taxonomic dispute of *Myrica*.

Acknowledgements

This work was partially funded by a UGC project on *Frankia* and actinorhizal plants. AKG acknowledges the UGC for providing the BSR fellowship. TM is grateful to DBT, India, for providing the studentship. AS acknowledges the receipt of the DBT-CREST award.

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