

EVALUATION OF TOTAL POLYPHENOLS, FLAVONOIDS AND ANTIOXIDANT
ACTIVITY OF *MYRICA ESCULENTA* BUCH-HAM.EX D.DON FRUITSNishat Anjum^{1*} and Y.C. Tripathi²¹Science Department, Ras Bihari Bose Subharti University, Dehradun-248007, Uttarakhand, India.²Chemistry and Bioprospecting Division, Forest Research Institute, PO New Forest, Dehradun – 248006, Uttarakhand, India.***Corresponding Author: Nishat Anjum**

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ABSTRACT

Background: Wild fruits have been a source of nutrition and medicine for local people around the world. Such fruits often contain higher amount of nutrients and bioactive compounds than many cultivated counterparts.**Objective:** The study was to determine best conditions for optimal extraction of phenolic compounds from fruits of *Myrica esculenta*, for its superior antioxidant activity. **Methods:** Different solvents extracts of fruits were qualitatively examined for presence of various phytochemical groups by standard methods. Total phenolic and total flavonoid contents in the extracts were determined by modified Folin-Ciocalteu method and aluminium chloride colorimetric method respectively. The antioxidant efficacy was evaluated following DPPH radical scavenging protocol. **Results:** Maximum extractive value of *M. esculenta* fruits in solvents of higher polarity like methanol indicating the presence of polar compounds in greater quantity. Phytochemical screening of fruit extracts showed the presence of diverse range of phytochemicals in *M. esculenta* fruits. Methanol extract recorded the presence of maximum number of phytochemicals. The TPC of the chloroform, ethylacetate, acetone and methanol extracts was found as 1.99±0.014, 3.54±0.022, 5.26±0.05, and 7.12±0.042 mg GAE/g respectively whereas the TFC of the above extracts was recorded to be 0.52±0.031, 1.45±0.046, 4.54±0.017, and 5.23±0.014 mg QE/g respectively. Highest level of both TPC and TFC was found in methanol extract followed by acetone extract. Among all extracts, methanol extract showed the lowest value of IC₅₀ (55.00±0.341 µg/ml), indicating highest DPPH radical scavenging activity of the extract. Least radical scavenging was recorded in chloroform extract (IC₅₀, 154.23±0.243 µg/ml). **Conclusions:** The present study established fruits of *Myrica esculenta* as rich sources of phenolic compounds and natural antioxidants.**KEY WORDS:** Phytochemicals, Total phenolic, Total flavonoids, *Myrica esculenta*, Fruits, Antioxidant activity.

1. INTRODUCTION

Plants have been the basis of traditional medicines throughout the world for thousands of years and representing a rich source of bioactive agents.^[1] Recently, a great deal of interest has been directed towards the bioactivity of natural plants as sources of antioxidant.^[2] In fact, excessive production of the reactive oxygen species (ROS) during metabolic processes causes oxidative stress that result in irreversible chemical changes in macromolecules like proteins, lipids and DNA.^[3] Oxidative stress is an important contributor to the pathophysiology of a variety of human pathological conditions including cardiovascular dysfunction, atherosclerosis, inflammation, carcinogenesis, drug toxicity, reperfusion injury and neurodegenerative diseases.^[4] Though, some of the synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are available; they are suspected of being

carcinogenic.^[5,6] Therefore, great deals of research have been focused in recent years towards finding new and safe antioxidants from plant sources. A number of in vitro studies have indicated that phenolic compounds of plants like flavonoids, coumarines, phenolic acid, lignans, hydroxycinnamates and stilbenes have remarkable antioxidant activity.^[7] During the past two decades, a number of plants with traditional therapeutic usage have been investigated for their phenolic composition and antioxidant efficacy which can be considered as an excellent source of natural antioxidants.^[8-13]

Wild fruits constituting an important nutritional and cultural resource for local people around the world often contain higher amount of nutrients and bioactive compounds than many cultivated species.^[14] *Myrica esculenta* Buch-Ham.ex D. Don commonly known as Kaphal or Box berry is one such wild species producing

edible fruits. It is an evergreen dioecious tree of the genus *Myrica* in the family Myricaceae comprising of 35-50 species of small trees and shrubs. Plant species of this genus are distributed in China, Taiwan, Japan, Western Highland of Cameroon, North America, Nepal and India. In India, it is found in the sub-tropical Himalayas tracks up to an altitude of 1200 to 2000m and also distributed from Ravi eastward to Assam, Arunachal Pradesh, Meghalaya, Nagaland, Manipur, Mizoram, Khasia, Sylhet, Himachal Pradesh, Jaintia, Simla, Bengal, Naga and Lushi hills. It usually grows between 900 to 2100m above sea level.^[15] All the parts of *M. esculenta* hold immense nutritional and therapeutic importance. Whole plant is an effective chemo preventive agent for skin. The bark is regarded as astringent, carminative and antiseptic. It is used in treatment of headache, fever, asthma, urinary discharges, piles, bronchitis, throat complaints, tumours, anaemia, chronic dysentery, ulcers, ophthalmia and other eye diseases.^[16,17] The oil from the flowers is useful in earache, diarrhea inflammation and paralysis.^[18] Fruits are known to have antimicrobial activity. The fruits yield a wax which is used externally for healing ulcers and juice extract from the unripe fruits is used as an anthelmintic.^[16,19] Owing its great therapeutic significance, the plant has been extensively studied for its phytochemical constituents and pharmacological activities of fruits.^[20-24] However, there is a lack of research investigating the most advantageous extraction solvent for greater antioxidant efficacy of *M. esculenta* fruits. It is important to note that both extraction yield and antioxidant activity of plant extracts are notably dependent on the solvent used.^[25-27] The present study was therefore aimed to investigate the different solvent extracts of *M. esculenta* fruits with regard to total phenolic and flavonoid contents and antioxidant properties.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All the organic solvents, chemicals and reagents used for the analytical works were of analytical grade and refer to Merck and Sd fine-chem Ltd. DPPH (1,1-diphenyl-2-picrylhydrazyl), Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), Ferric chloride (FeCl_3), aluminium chloride (AlCl_3), Ninhydrine, Dragendorff's reagent, Ehrlich reagent, etc. were purchased from Merck and HiMedia. Some of the detecting reagents were freshly prepared for qualitative phytochemical screening. UV/Vis double beam Spectrophotometer (Chemito 1700, Thermo Fisher) was used for spectrophotometric determination of Total phenolic content (TPC), Total flavonoid content (TFC) and DPPH radical scavenging activity.

2.2. Plant materials

Fresh fruits of *Myrica esculenta* free from diseases were collected from outskirts of Mandi, Himachal Pradesh, India. The collected fruits were identified and authenticated by Systematic Botany Section of Botany

Division, Forest Research Institute (FRI), Dehradun. A voucher specimen of the same has been preserved in the Chemistry & Bioprospecting Division, FRI for future reference. Fruits were cleaned properly under running tap water to make them free from dust and dried in shade at room temperature (25°C). Fruits were cut into small pieces, dried again in shade and then stored in airtight bags at 4°C till future use.

2.3. Preparation of extracts

Dried fruits of *M. esculenta* were successively extracted with organic solvents, petroleum ether ($60-80^\circ$), chloroform, ethylacetate, acetone, and methanol in the order of increasing polarity. Extracts so obtained were separately distilled under reduced pressure to obtain solvent free extracts and the extractive values were determined on dry weight basis. The resultant crude extracts were transferred into airtight sample bottles and kept at 4°C until they were used.^[28]

2.4. Qualitative Phytochemical screening

The petroleum ether, chloroform, ethylacetate, acetone and methanol extracts of *M. esculenta* fruits were subjected to qualitative phytochemical screening by reported methods to detect the presence and/or absence of different kinds of phytochemicals such as alkaloids, flavonoids, phenolics, tannins, steroids, saponins, carbohydrates, glycosides, proteins and free amino acids (FAA), etc. ^[29-31] All extracts were tested qualitatively using special reagents that produce characteristic colours changes with different categories of chemical constituents. All qualitative tests were replicated three times.

Since the phenolic content and antioxidant activity depends on the extraction solvents, comparative evaluation of different solvent extracts of *M. esculenta* fruits were done for selecting the optimal solvent offering maximum phenolic and flavonoid contents as well as antioxidant activity. Based on the results of qualitative phytochemical screening, chloroform, ethylacetate, acetone and methanol extracts of *M. esculenta* fruits were selected for the evaluation.

2.5. Determination of total phenolic content

The concentration of phenolics in the extracts was determined using spectrophotometric method.^[32] Methanolic solution of extracts in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu reagent dissolved in water and 2.5 ml 7.5% sodium carbonate. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu reagent dissolved in water and 2.5 ml of 7.5% of Na_2CO_3 . The samples were thereafter incubated at room temperature for 45 min. The absorbance was determined using spectrophotometer at $\lambda_{\text{max}} = 765 \text{ nm}$. The observations were recorded in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was

repeated for the standard solution of gallic acid and the calibration line was constructed. Quantification was done on the basis of the standard curve of Gallic acid concentration range from 50 to 500 mg/ml ($r^2 = 0.998$). Total phenolic content calculated from the calibration curve was expressed as mg of gallic acid equivalent (GAE)/g dry weight.

2.6. Determination of total flavonoid content

Total flavonoid content of both crude extracts was determined using the aluminium chloride colorimetric method as described earlier.^[33] Briefly, 0.5ml of sample was mixed with 1.5ml of methanol and then, 0.1ml of 10% aluminium chloride was added followed by 0.1ml of potassium acetate and 2.8ml of distilled water. The mixture was incubated at room temperature for 30 min. The absorbance was measured by a spectrophotometer at $\lambda_{\text{max}} = 415\text{nm}$. The observations were recorded in triplicate for each analysis and their mean values were calculated. The same procedure was repeated for the standard solution of quercetin and the calibration line was constructed. Quantification was done on the basis of the standard curve of quercetin concentration ranging from 50 to 500 mg/ml ($r^2 = 0.999$). Total flavonoid content calculated from a calibration curve was expressed as mg of quercetin equivalent (QE)/g of dry weight.

2.7. Evaluation of DPPH radical scavenging activity

The antioxidant activity of the chloroform, ethylacetate, acetone and methanol extracts of *M. esculenta* fruits was determined as free radical scavenging capacity following modified 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging protocol.^[34] A stock solution of 1 mg/ml solution of each extract in methanol was prepared. Sixteen serial dilutions of the extract stock solution and one blank sample (control) was made in methanol. 2 ml of freshly prepared 1 mg/ml stock solution of DPPH was added to each test tube. The sample was thoroughly mixed and incubated in the dark at 28-30°C. The absorbance (A) was read at 517 nm after 25 minutes. The percentage DPPH radical scavenging was determined by the formula: -

$$\text{Inhibition (\%)} = [(A_{\text{Control}} - A_{\text{Sample}} / A_{\text{Control}})] \times 100$$

A_{control} is the absorbance of the control and A_{sample} is the absorbance of sample extracts^[28]. Ascorbic acid was used as a reference standard in different concentrations ($\mu\text{g/ml}$). The 50% inhibitory concentration value (IC_{50}), estimated from linear regression equations and expressed in $\mu\text{g/ml}$ is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radicals. Lower IC_{50} is indicative of better DPPH radical scavenging ability.

2.8. Statistical analysis

All experiments and measurements were replicated thrice, and the data were presented as Mean \pm Standard Deviation (SD). Statistical analysis was performed by SPSS 16.1. One-way analysis of variance (ANOVA) was utilized to evaluate differences. Correlation between the IC_{50} and total phenolic content were established using regression analysis at a 95% significance level. $P \leq 0.05$ was identified as a significant difference.

3. RESULTS AND DISCUSSION

3.1. Extractive value

The extracts of fruits obtained with petroleum ether, chloroform, ethylacetate, acetone and methanol were distilled under reduced pressure and finally dried over anhydrous sodium sulphate till constant weight. The percentage yield of extracts was calculated with reference to the dried plant material initially taken as presented in Table 1.

Table 1. Extractive value of *M. esculenta* fruits with different solvents.

Solvents	Extractive value (%)
Petroleum Ether	3.01
Chloroform	6.32
Ethyl acetate	6.01
Acetone	24.56
Methanol	26.45

The extract yield data as shown in Table-1 clearly indicated that *M. esculenta* fruits provide the highest yield with methanol followed by acetone and chloroform. Extraction with petroleum ether however furnished the lowest extract yield. In fact, different classes of phytochemicals have different degree of solubility in solvents of varying polarity. The extraction with solvents of increasing polarity involves of separating compounds of a plant according to their degree of solubility. The foregoing results suggested that the extraction yield increases with increasing polarity of the solvent used for extraction. The yields of extracts in different solvents provide information about the solubility of plant chemical constituents in different solvents thus suggesting the best solvent for extraction of phytochemicals. Maximum extractive value of *M. esculenta* fruits in solvents of higher polarity like methanol is indicative of the presence of polar compounds in greater quantity.

3.2. Preliminary phytochemical screening

Phytochemical screening was carried out to qualitatively assess the chemical composition of *M. esculenta* fruits using commonly employed precipitation and coloration reaction to identify the major natural chemical groups. Results showing the presence or absence of various phytochemical constituents in different extracts as presented in Table 2.

Table 2: Qualitative phytochemical analysis of *Myrica esculenta* fruits Extracts.

Phytochemical Groups	Extracts				
	Petroleum Ether	Chloroform	Ethyl acetate	Acetone	Methanol
Alkaloids	-	+	+	+	+
Steroids	+	+	+	+	+
Terpenoids	+	+	+	+	+
Flavonoids	-	+	+	+	+
Phenolics	-	+	+	+	+
Anthocyanins	-	-	-	+	+
Tannins	-	-	+	+	+
Saponins	+	-	-	+	+
Glycosides	-	+	-	-	+
Carbohydrates	-	-	-	+	+
Proteins	-	-	-	-	+
Amino acid	-	-	-	-	+

(+) Present, (-) Absent

Different extracts of *M. esculenta* on qualitative phytochemical screening (Table -2) showed the presence of steroids, terpenoids and saponins in petroleum ether extract; alkaloids, steroids, terpenoids, phenolics, flavonoids, and glycosides in chloroform extract; alkaloids, steroids, terpenoids, phenolics, flavonoids, anthocyanins, and tannins in ethylacetate extract; alkaloids, steroids, terpenoids, phenolics, flavonoids, anthocyanins, tannins, and carbohydrates in acetone extract and alkaloids, steroids, terpenoids, phenolics, flavonoids, anthocyanins, tannins, saponins, glycosides, carbohydrates, proteins, amino acids in methanol extract. Methanol extract recorded the highest number of different phytochemical groups. Phytochemicals detected in the fruit extracts are important bioactive agents which might be involved in the therapeutic action of this plant part. Phenolics, flavonoids and related compounds were detected in all extract except petroleum ether extract.

Plant phenolics constituting one of the major groups of compounds act as primary antioxidants or free radical scavengers whereas flavonoids, the most important natural phenolics are regarded as one of the most diverse and widespread groups of natural compounds. These compounds possess a broad spectrum of biological activities including radical scavenging properties.^[35] It was thus logical to determine their total amount in the *M. esculenta* fruit extract.

3.3. Total phenolic and flavonoid contents

The total phenolic and flavonoid contents in the different extracts of *M. esculenta* fruits are presented in Table 3.

Table 3. Total phenolic and flavonoid contents in *M. esculenta* fruits extracts.

Extracts	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QE/g extract)
Chloroform	1.99±0.014	0.52±0.031
Ethyl acetate	3.54±0.022	1.45±0.046
Acetone	5.26±0.051	4.54±0.017
Methanol	7.12±0.042	5.23±0.014

The TPC in the chloroform, ethylacetate, acetone and methanol extracts calculated from the calibration curve ($R^2 = 0.998$) was found as 1.99±0.014, 3.54±0.022, 5.26±0.05, and 7.12±0.042 mg GAE/g of extracts respectively whereas the TFC calculated from the calibration curve ($R^2 = 0.999$) of the above extracts was recorded to be 0.52±0.031, 1.45±0.046, 4.54±0.017, and 5.23±0.014 mg QE/g of extract respectively. Highest level of both TPC and TFC was found in methanol extract followed by acetone and ethyl acetate extracts. Variations in the extract yields and phenolic contents of various extracts are attributed to polarities of different compounds present in the plant parts as previously reported.^[36,37] Phenolic compounds act as antioxidants

owing to their redox properties.^[38] Flavonoids, the largest group of naturally occurring phenolic compounds also exhibit antioxidant activity due to the presence of free hydroxyl (-OH) groups.^[39] As such, the total phenolic concentration could be considered as the basis for antioxidant activity.^[40]

3.4. Antioxidant activity

The chloroform, ethylacetate, acetone and methanol extracts of *M. esculenta* fruits exhibited concentration-dependent DPPH radical scavenging activity expressed in terms of IC_{50} value (Fig. 1). Ascorbic acid was used as standard antioxidant compound. The IC_{50} ($\mu\text{g/ml}$) values stand for the concentration of the extracts causing 50%

inhibition of absorbance, a lower value would reflect greater antioxidant activity of the sample. Among all extracts, methanol extract showed the lowest value of IC_{50} ($55.00 \pm 0.341 \mu\text{g/ml}$), which is less than $100 \mu\text{g/ml}$ thus indicated the highest DPPH radical scavenging activity of the extract. Lowest radical scavenging was recorded in chloroform extract as reflected from higher IC_{50} value of $154.23 \pm 0.243 \mu\text{g/ml}$. Ascorbic acid showed IC_{50} value of $31.12 \pm 0.212 \mu\text{g/ml}$ with DPPH radical scavenging assay (Table-4).

Table 4. DPPH radical scavenging capacity (IC_{50}) of *Myrica esculenta* fruits extracts.

Extracts	DPPH IC_{50} ($\mu\text{g/ml}$)
Chloroform	154.23 ± 0.243
Ethyl acetate	110.22 ± 0.291
Acetone	80.11 ± 0.412
Methanol	55.00 ± 0.341
Vitamin C	31.12 ± 0.212

Mean \pm SD (n=3)

Methanol extract of *M. esculenta* fruits showed the highest antioxidant activity among all other solvent extracts which may be due to higher amount of TPC and TFC as compared to other extracts (Table 3). The relationship between DPPH radical scavenging activity and TPC was established by correlation analysis (Fig. 1). The results exhibited a positive linear correlation ($R^2 = 0.8963$) between them suggesting that antioxidant components in *M. esculenta* fruits could scavenge free radicals. The results are in agreement with the previous findings corroborating a strong correlation between the phenolic content and antioxidant activity of plants.^[41]

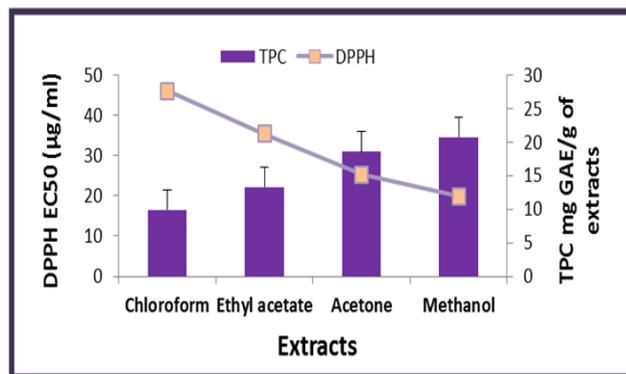


Fig.1. Correlation between TPC and DPPH-RS of *Myrica esculenta* fruits extracts.

Natural anti-oxidants have the ability to protect organisms from damage caused by free radical-induced oxidative stress and therefore can be used to fight a variety of ailments and diseases. The utility of antioxidant therapies in many diseases is well recognized. Phenolic compounds have capacity of reducing oxidative cellular damage caused by free radical.^[42,43] The results suggested that the fruits of *M. esculenta* can be good source natural antioxidant.

4. CONCLUSION

Results of the studies have led to the conclusion that for optimal extraction of phenolic compounds from *M. esculenta* fruits and its enhanced antioxidant activity, methanol is the most advantageous solvent. The results of the study showed highest antioxidant activity in methanol extract of fruits, and a strong linear correlation between phenolic content and antioxidant capacity determined by the DPPH assay thereby validating the traditional use of *M. esculenta* as source of natural antioxidants. The results suggest that *M. esculenta* fruits can be a potential source of antioxidant agents and could be used as a natural antioxidant and preservative in food and non-food systems. It is therefore, investigations to elucidate the chemical composition of phenolic and flavonoid constituents, in vivo studies to better establish the functionality of the studied plant and potential applications as natural antioxidants are needed. In addition, further analysis is also required to isolate and characterize the bioactive chemical compounds of the plant responsible for its broad range of pharmacological activity.

ABBREVIATIONS

DPPH - 1, 1-Diphenyl-2-Picrylhydrazyl
TPC - Total Phenolic Content
TFC - Total Flavonoid Content

CONFLICT OF INTEREST

Conflict of interest declared none.

AUTHORS CONTRIBUTION STATEMENT

Ms. Nishat Anjum carried out the experimental work, recorded data, evaluated the results and drafted the manuscript. Dr. Y.C. Tripathi conceptualized the work, designed experimental elements and guided data analysis and interpretation and drafting the manuscript.

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