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In vitro evaluation of bio-protective properties of underutilized *Myrica esculenta* Buch.–Ham. ex D. Don fruit of Meghalaya

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Oxidative stress causes an imbalance between systemic manifestation of reactive free radicals and biological systems' ability to detoxify reactive intermediates, thus causing damage to all components of cells. Natural polyphenols with promising antioxidant and free radical scavenging activities can counter oxidative damage in cells. The present study focuses on the antioxidant, antimicrobial, anticancer activity of the MeOH extract of fresh soh-phie (*Myrica esculenta* Buch.-Ham. ex D. Don) fruits and their relation to phytoconstituents *in vitro*. The levels of phenolic, flavonoid and flavonol compounds were found to be 26.21±0.1 GAE µg/mg dry extract, 38.00±0.5 RE µg/mg dry extract and 122.75±0.1 RE µg/mg dry extract, respectively. MeOH extract showed DPPH (2,2-diphenyl-picrylhydrazyl hydrate) and ABTS (1,2,2′-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid]) radical scavenging activity in a dose-dependent manner with maximum inhibition of 91.91±0.2 % and 82.57±2.9 %, respectively. GC/MS screening revealed the presence of 4H-Pyran-4-one, pentadecanoic acid, 2-furancarboxaldehyde, phytol and hexadecanoic acid which may be responsible for its antimicrobial and antioxidant potential. LC-MS data also reveals presence of ferulic and gallic acid, which may have a significant role towards its anticancer activity. The data suggest that the MeOH extract of Soh-phie fruits has potential to be used as a source of natural antioxidants and preservative in the food industry.

Keywords: Anti-microbial, Anti-oxidant activity, Aromatic compounds, Phytochemicals, Secondary metabolites.

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Introduction

Natural products have inspired many developments which have led to advances in synthetic methodologies for developing various compounds possessing therapeutic prospects. Natural products and secondary metabolites mainly from plants have shown immense potential in treatment of several human diseases like diabetes, cancer, coronary heart diseases and infectious diseases[1]. Introduction and acceptance of aspirin, morphine, cinchona and digitalis for treating malaria in 17th century facilitated awareness among general people to believe in diverse flora and natural plant products which may offer huge structural diversity intended for pharmacological treatment of several disorders[2]. In addition to the conventional commercial fruits, underutilized fruits are also acquiring interest as potential food additive and affordable substitute of commercial fruits worldwide[3].

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Myrica esculenta Buch.-Ham. ex D. Don syn *M. nagi* Hook.f. non Thunb. belongs to the family Myricaceae and is commonly found in outer Himalayan region at altitude ranging from about 900 to 2100 m. In India, Soh-phie is found from Punjab to Assam, including Arunachal Pradesh, Meghalaya, Manipur, Nagaland, Mizoram, in khasia, Sylnet and Lushai hills. Apart from India the tree is also found in Nepal, China, Pakistan and Malaya islands. The Soh-phie tree can grow up to 10-15 m high and leaves are long and either pale or rust-colored. The tree has many hairy branches and the flowers that blossom on them are few and distant and rather small in size. The Soh-phie fruits are very perishable and eaten raw, and also used for making pickle[4]. Traditionally its bark in powdered form was used to treat dysentery, fever, asthma, liver diseases, anaemia and ulcer and has also been studied for its anti-inflammatory activity[4].

Hence the present study was carried out on its fruits to identify bioactive constituents via LC/MS, unknown organic and sensory compounds by Gas chromatography mass spectrometry and bio-

protective activities with the aim of studying its therapeutic potential.

Materials and Methods

Sample preparation and extraction

The fresh fruits were purchased from local markets in Meghalaya, India (Plate 1) and were authenticated at NISCAIR, Delhi (NISCAIR/RHMD/Consult/-2011-12/1744/44). Properly washed fruits were partially crushed and extracted with MeOH. Solvent removal yielded the extract which was stored at 4 °C for further analysis.

Phytochemical analysis and determination of secondary metabolites

Total phenolic[5], flavonoid[6], flavonol[7], crude saponins[8] and crude alkaloids[9] were determined. GC-MS (Agilent Technologies) was used for identification of secondary metabolites[10]. 1 µL of sample solution was injected in split injection (1:20) at 280 °C. The oven was programmed from 65 °C (5 min) at 15 °C/min to 180 °C (10 min), at 5 °C/min to 280 °C (15 min). Helium was used as carrier gas. The mass spectrometric detector (MSD) was used in the scan mode (m/z 35-1050) for the samples. The MS transfer line temperature was set to 280 °C, solvent delay was 3 min, and ion source and Quadrupole temperature were 230 °C and 150 °C, respectively. Screening of volatiles and semi-volatiles were performed using NIST'05 library.

Plate 1—Fruits of *Myrica esculenta* Buch.-Ham. ex D. Don

Identification and quantification of phytochemicals

The polyphenols in MeOH Soh-phie fruit extract were analyzed by the chromatographic system consisting of an Agilent 1100 series HPLC instrument equipped with 6460 triple quad MS detector. Analytical separations of the extracts were carried out on a C18 column (4.6 mm×100 mm×5 µm, Agilent Technology) at a flow rate of 0.8 /min, with a two solvent mobile phase (eluent A=10 mM ammonium acetate and 1 % acetic acid in water; eluent B=1 % acetic acid in methanol). The gradient elution was carried out as follows: 0-3 min, 15-50 % A; 3-5.5 min, 50-90 % A; 5.5-9 min, 90 % A; 9-9.5 min, 90-15 % A; 9.5-10 min, 15 % A. The sample injection volume was 20 µL. The fragmentation was done in ESI-MS/MS (Agilent jet stream) in negative ionization mode. Data was acquired and quantified by Agilent triple quad LC-MS Mass Hunter work station based on external standardization by employing calibration curves in the range of 1-50 ng/ based on the peak area calculated from selected ion chromatograms of the corresponding $[M-H]^-$ ion.

Identification of aroma active compounds

Volatile compounds were isolated from air dried fruits sample using cryo-focusing (cold trap) TDS (Markes International Limited, UK) at 150 °C. Isolated volatiles were adsorbed on a Tenax trap at -10 °C (up to 10 min) and then the trap was desorbed at 300 °C (up to 3 min). The desorbed compounds were separated by HP-5 ms (0.25 mm×30 m× 0.25 µm) and identified by an Agilent 6890 GC equipped with 5973 MSD (Agilent Technologies, USA). The oven was programmed from 60 °C (0 min) at 3 °C/min to 240 °C (0 min), total run time 60 min (Analysis of essential oil compounds using retention time locked methods and retention time databases, 5988-6530EN, Agilent Technologies). The compounds were identified by matching the mass spectra (quality match > 90 %) and retention indices (RI) with the NIST and the Flavor 2 library (Agilent Technologies, USA) of standard compounds. The odor descriptions have been matched with the literature[11-13].

Antioxidant activity

Free radicals scavenging activity of the MeOH extract was determined through DPPH, ABTS and FRAP assay[14].

Anti-microbial activity

Test strains

The antimicrobial activity of the MeOH extract was assessed on several food borne pathogens. The pathogenic strains were procured from IMTECH, Chandigarh, India. Antibacterial activity of MeOH extract was screened on three Gram-positive (*Staphylococcus aureus* MTCC96, *Staphylococcus epidermis* MTCC106, *Bacillus subtilis* MTCC121) and three Gram negative bacteria (*Proteus mirabilis* MTCC425, *Escherichia coli* MTCC739 and *Salmonella enterica* MTCC3219).

Agar well diffusion method

Agar disc diffusion method was used to determine the antimicrobial activity of MeOH extract of Soh-phie fruit[15] against test microorganisms. The test microorganisms fresh culture (1×10^8 CFU/mL) (100 µL) was spread on media agar plates. Initially for screening, sterile, 6 mm diameter hole was impregnated in agar plate with 50 µL of extract. Inoculated plates were kept for 24 h under optimum conditions (37 °C). Around the bored hole clear zone of inhibition indicated presence of antibacterial activity. Tetracycline antibiotic was used as standard.

Minimal inhibitory concentration (MIC)

Broth micro-dilution method was used to determine MIC of MeOH extract against various tested microbes[16]. MICs were determined through a standard two-fold micro-dilution technique. Tests were performed in sterile flat-bottom 96-well microplates by maintaining a constant volume (200 µL/tube) for serial dilutions of extracts. Control and test growth was inoculated with 5 µL (10^8 CFU/mL) of microbial culture suspension. After 24 h, growth was detected by addition of 40 µL of INT (0.5 mg/mL) to each well. The INT color changed from yellow to purple where microbial growth occurred. The MICs were expressed in mg/mL and were defined as the lowest extract concentration for which the optical density of a well was null. Tetracycline was used as positive control.

Anticancer activity

MTT colorimetric assay was used to evaluate sensitivity of HepG2, Hela and MDA-MB-231 cells to MeOH extract. Cells were seeded in a flat bottom 96 well plate and incubated at 37 °C, 5 % CO_2 for 24 h. All the cell lines were exposed to MeOH extract at various concentrations of Soh-phie fruit. DMSO cells were served as control. After 24 h cells were treated with MTT reagent (20 µL in each well) and further incubated for 3-4 h at 37 °C at 5 % CO_2. 100 µL solubilisation buffer was added to dissolve formazan crystals to give purple colour. Optical density was recorded at 570 nm in micro plate reader (Spectra max plus 384). Cell viability percentage was determined as [1-(OD of treated cells/OD of control cells)]*100.

Results and Discussion

Phytochemical screening and secondary metabolites

The study revealed the presence of phytochemicals namely phenolics (26.21±0.1 GAE µg/mg dry extract), flavonoids (38.00±0.5 RE µg/mg dry extract), flavonols (122.75±0.1 RE µg/mg dry extract), crude saponins (8.27 %) and alkaloids (7.48 %). Studies on phenolic compounds have revealed them to contribute towards chemoprevention (e.g., antioxidant, anti-carcinogenic or anti-mutagenic and anti-inflammatory effects) and to be helpful in inducing apoptosis by arresting cell cycle. Phenolic compounds also helps in regulating carcinogen metabolism and ontogenesis expression thus inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation and blocking signaling pathways[17].

Plants with alkaloids have been determined to exhibit analgesic and antibacterial properties and are used in medicines for reducing headache and fever[18]. Studies have reported that saponins possess antidiarrheal, anticancer and anthelmintic properties[19].

A total of 31 compounds were identified in the methanol extract of Soh-phie fruit demonstrating various phytochemical and antibacterial activities. Retention time and percent area are represented in Table 1. Major constituents present in extract were characterized as 2-furancarboxaldehyde (46.87 %), oxirane (9.95 %), 1-ethyl-4-methylcyclohexane (4.35 %), myo-inositol (3.52 %), methyl d-lyxofuranoside (3.49 %) and furfural (3.37 %).

Various compounds having bioactivity have been characterized in different medicinal plants. 4H-Pyran-4-one is found to be a potent anti-inflammatory compound possessing antibacterial activity. Dodecanol, long chain fatty alcohol, was studied for its antibacterial activity against *S. aureus*[20]. Saturated fatty acid, aldehydes and fatty acid methyl esters such as pentadecanoic acid, 2-furancarboxaldehyde and hexadecanoic acid which possess antimicrobial and

Table 1—Secondary metabolites profiling of Soh-phie fruit MeOH extract

Compound Name	RT	Cas#	*% Area	Compound Name	RT	Cas#	*% Area
Furfural	3.342	98-01-1	3.37	Oxirane	13.362	38954-75-5	9.95
1H-Imidazole, 2, 4-dimethyl	3.442	930-62-1	0.64	2-Fluorobenzyl alcohol	13.585	446-51-5	0.47
2,5-Furandione, dihydro-3-methylene	5.042	2170-03-8	44.98	1,6-Anhydro-.beta.-D-glucopyranose (levoglucosan)	13.751	498-07-7	2.78
2-Furan carboxaldehyde, 5-methyl-	5.286	620-02-0	3.52	2-Deoxy-D-galactose	14.618	1949-89-9	0.98
Thymine	7.286	65-71-4	2.14	Methyl d-lyxofuranoside	15.129	1000129-06-4	3.49
Methyl 2-furoate	7.364	611-13-2	1.32	Myo-Inositol	15.962	472-96-8	3.52
Thiopivalic acid	8.230	55561-02-9	0.58	2-Dodecanol	17.317	10203-28-8	0.32
2-Propanamine	8.352	30533-08-5	0.51	Phthalic acid	18.184	1000314-93-8	0.29
4H-Pyran-4-one	8.508	28564-83-2	2.58	Pentadecanoic acid	18.695	5129-60-2	0.46
2-Furancarboxaldehyde	10.052	67-47-0	46.87	n-Hexadecanoic acid	19.017	57-10-3	0.76
4-Mercaptophenol	10.374	637-89-8	1.69	3-Chloropropionic acid	20.339	1000281-77-2	0.25
5(Hydroxymethy)-2 (dimethoxymethyl)furan	11.096	90200-14-9	0.61	9,17-Octadecadienal	20.394	56554-35-9	0.50
5-Acetoxymethyl-2-furaldehyde	11.285	10551-58-3	0.94	2-Chloroethyl linoleate	20.661	25525-76-2	0.26
2-Methoxy-4-vinylphenol	11.385	7786-61-0	0.63	9,12,15-Octadecatrien-1-ol	20.727	506-44-5	1.31
1-Ethyl-4-methylcyclohexane	12.174	3728-56-1	4.35	Squalene	26.393	7683-64-9	1.72
Phytol	12.307	150-86-7	3.72	1,1,1,3,5,5, 5-Heptamethyltrisiloxane	33.758	1873-88-7	1.88

* % Matching with NIST library (mean 'Q value' is 91± 5.12 %, n=3); RT Retention time of the compound, in a minute; "area (%)" the percentages of the area of the total ion chromatogram represented by the peaks of each of the compounds identified.

antioxidant properties were also identified. Phytol identified in fruit extract showed antinociceptive activity which may be linked with antioxidant activity of phytol in mice, suggestive of central and peripheral effect without altering the motor functions[21]. Studies have also confirmed phytol to have anti-inflammatory, antimicrobial, diuretic and anticancer activities[22]. Identified myo-inositol known as B complex vitamin helps in enhancement of ovulary function and also improves oocytes quality[23]. Furfural and their derivatives reported to possess antimicrobial properties[24]. Squalene, a 30 carbon organic compound is being used as an adjuvant in vaccines and also serves as chemo preventive compound due to which it is included in Mediterranean diet[25,26].

Aroma active compounds identification

Compounds detected were identified through AMDIS target library of volatile aromatics (Flavor 2) and NIST'05 mass spectrum library. Six compounds have been identified in fresh fruits of soh-phie (Table 2) which were of sensory quality and confirmed as sweet, floral, fruity mushroom, creamy and bready. Benzyl alcohol was found to contribute the most towards the aroma profile of the fruits and provide it with a sweet, floral, fruity odor post dilution. Benzyl alcohol at low concentrations is used as a bacteriostatic preservative in topical drugs, cosmetics and intravenous medication (Prescribing Information for Ulesfia Lotion). Other compounds have found uses in cosmetic, flavor and fragrance industries (Table 2).

Antioxidant, antimicrobial and anticancer activities

Antioxidants play vital roles in cellular function and have significant role in biological processes which are related with cancer, aging, including vascular and inflammatory damage[27]. MeOH extract free radical scavenging activity was studied by its ability to reduce the DPPH, ABTS and FRAP stable free radicals. IC_{50} values of MeOH extract were found to be 4.14±0.23 mg/mL and 2.39±0.78 mg/mL for DPPH and ABTS respectively. FRAP activity was 0.15±0.8 µg TE/mg extract. Antioxidant activity may be attributed to the presence of phenolics, flavonoids, secondary metabolites and bioactive compounds

Table 2—Aroma profiling of Soh-phie fruit

RT	Cas#	Compound Name	*% Area	Molecular Formula	**odor
3.353	98-01-1	Furfural	4.87	$C_5H_4O_2$	Sweet, brown, woody, bready, caramellic, with a slight phenolic nuance
5.297	620-02-0	2-Furancarboxaldehyde	3.44	$C_5H_4O_2$	fatty musty waxy caramellic
7.375	611-13-2	Methyl 2-furoate	4.50	$C_6H_6O_3$	fruity mushroom fungus tobacco sweet
10.019	456-47-3	Benzyl alcohol	29.86	C_7H_8O	Sweet, floral, fruity with chemical nuances
19.028	57-10-3	n-Hexadecanoic acid	1.79	$C_{16}H_{32}O_2$	Low heavy waxy, with a creamy, candle waxy nuance
20.727	506-44-5	Linolenyl alcohol	2.44	$C_{18}H_{32}O$	Cosmetic uses

* Values for "peak area (%)" are the percentages of the area of the total ion chromatogram represented by the peaks of each of the compounds identified. ** The odor characteristics of the compounds identified are: Chung et al., 1993; Cullere et al., 2004b; Suvimol and Pranee, 2008; and www.thegoodscentscompany.com.

Table 3—Antimicrobial activity of Soh-phie fruit MeOH extract

	Gram positive bacteria						Gram negative bacteria					
	Staphylococcus aureus		*Bacillus subtilis*		*Staphylococcus epidermis*		*Escherichia coli*		*Salmonella enterica*		*Proteus mirabilis*	
	ZI±SD (mm)	MIC*	ZI±SD (mm)	MIC	ZI±SD (mm)	MIC	ZI±SD (mm)	MIC	ZI±SD (mm)	MIC	ZI±SD (mm)	MIC
MeOH SP) extract	16±0.5	2.5	12±0.2	2.5	18±0.5	1.25	15.5±0.5	2.5	10±0.1	2.5	12±0.2	2.5
Tetracycline	20.9±0.1	6.0±0.1	25.3±0.5	6.2±0.2	15±0.2	5.9±0.5	20.2±0.1	6.0±0.32	14.6±0.5	6.9±0.1	16.6±0.1	6.2±0.2

*MIC = Minimum inhibitory concentration in mg/ml; ZI = Zone of inhibition in mm.

present in the fruit. LC/MS screening also revealed presence of gallic and ascorbic acid which are known to have tremendous antioxidant activity due to strong reducing power ability[28].

Antimicrobial activity was studied against 3 Gram positive and 3 Gram negative bacteria as summarized in Table 3. *S. epidermis* and *S. aureus* were found to be more sensitive towards methanolic extract. Antimicrobial activity may be due to the presence of antimicrobial compounds such as dodecanol, phytol, furfurals, and 4H-Pyran-4-one in the extract.

MeOH extract screening showed moderate anticancer activity for HepG2, Hela and MDA-MB-231 cell lines. All the three cell line grow in DMEM media with high glucose and when subjected to different concentration of MeOH extract resulted in 46.19 %, 50 % and 48.29 % inhibition of MDA-MB-231, HepG2 and Hela cells at 5 mg/mL, respectively. It was observed that there was a gradual increase in % inhibition with increase in dose of MeOH extract which may be due to presence of bioactive compounds which render the plant with anticancer proliferative activities. LC/MS analysis revealed the presence of ferulic acid (4-hydroxy-3-methoxycinnamic acid), gallic acid and ascorbic acid. Ferulic acid is a ubiquitous phenolic acid and an effective component of Chinese medicinal herbs. It is known to have anticancer, antioxidant, antimicrobial, anti-inflammatory, antithrombotic and antihypercholesterolemic bioactivity[29]. Studies on gallic acid have revealed that it inhibited A549 (human lung adenocarcinoma cell line) cell growth and viability[30]. Thus presence of ferulic and gallic acid may contribute towards anticancer potential of the fruit.

Conclusion

Present study revealed that methanol extract of Soh-phie fruit demonstrated high phenolic content and potent antioxidant activity achieved by free radical scavenging and FRAP assays. The extract demonstrated good inhibitory activity against bacterial food pathogens. Thus, MeOH extract of fruit could be a potential source of food preservative. GC-MS also revealed presence of some bioactive compounds, which may also be used as therapeutic agents. Further activity guided isolation and characterization of bioactive compounds may lead to development of food preservative agents.

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