Phytochemical composition, antioxidant and anti-bacterial activity of *Syzygium calophyllifolium* Walp. fruit

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Abstract *Syzygium calophyllifolium* fruits are among the important wild edibles used by the tribes of Western Ghats. However, this underutilized fruit remained unnoticed for its medicinal properties. Hence, the present study was undertaken to evaluate the antioxidant activity by DPPH[•], ABTS^{•+}, FRAP assays and antibacterial efficacy by well diffusion method. GC–MS and HPLC profiles of crude extract and column chromatographic fractions were also determined. The methanolic extract of fruit (MFE) showed high total phenolics, tannins and flavonoids. The fraction H (FH) displayed significant antioxidant property in DPPH[•] (IC₅₀ 2.1 µg/ml), ABTS^{•+} (19483.29 µM Trolox equivalents/g extract) and FRAP (65.5 mM Fe(II)/mg extract) assays over MFE. Moreover, FH also exhibited good antibacterial activity against *Escherichia coli* (32.0 mm), *Salmonella typhi* (27.0 mm), *Staphylococcus aureus* (27.3 mm) at 100 mg/ml concentration. GC–MS revealed 12 major compounds in MFE, HPLC analysis of MFE and FH depicted the presence of rutin and ellagic acid. This study suggested that FH could have high concentration of bioactive compounds like rutin and ellagic acid or its analogues compared to MFE which may be responsible for its strong antioxidant and antibacterial activity.

Keywords *Syzygium calophyllifolium* · Column fraction · Free radicals · Antibacterial · GC–MS · HPLC

Introduction

Fruits have a natural dietetic value through their significant content in minerals, alimentary fibers, pectic substances, essential amino acids, polyunsaturated fatty acids, vitamins, antioxidants (polyphenols, sulphur compounds, resveratrol), phytoncides (natural antibiotics), etc. and fruits are highly nutritious; it contains high quality minerals to supply a balanced diet. Many tropical fruits are labelled as underutilized fruits, that have been over looked by scientific research and development works despite the important role they can play in terms of food provision and food culture of rural people (Gruere et al. 2006). Therefore research on underutilised fruits explores the importance of it. These fruits are potentially rich source of many dietary secondary metabolites like phenolics. These secondary metabolites play a major role as antioxidants and are believed to play an important role in the prevention of many oxidative and inflammatory diseases (Arts and Hollman 2005). There is growing evidence that reactive oxygen species (ROS) are the cause of many diseases and that foods rich in antioxidants may contribute to overall health and disease prevention (Lotito and Frei 2006). The increasing frequency of antibiotic-resistant bacterial infections poses an alarming dilemma to health care today. Thus there is an urgent need to find effective drug against microbial pathogens (Debib et al. 2013). Search for natural antioxidant sources among plants used as food is necessary for health promotion. Aliakbarlu et al. (2014) reported that the compounds with antioxidant property also show high antimicrobial activity. Interest on naturally derived

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products has led to analyze compounds using modern techniques. Such an investigation has explored many useful wild plants from Western Ghats with many useful and disease preventing phyto-compounds in recent years. Hence, the wild edible fruit of *Syzygium calophyllifolium* is taken as a research interest in the present study. *S. calophyllifolium* Walp. [synonym: *Eugenia calophyllifolia* (Walp.) Wt.] is a wild edible (Binu et al. 2012) and underutilised fruit tree of the family Myrtaceae. Toda tribe of Nilgiris use stem bark of *S. calophyllifolium* against tooth ache (Rajan et al. 2005). The seed extract has potent anti-diabetic activity (Gurusamy et al. 2007). Recently, the plant was studied extensively for its physicochemical (Nagulan and Rajeshkumar 2016) and anti-diabetic activity (Chandran et al. 2015, 2016). The studies on the medicinal properties of this fruit are meagre. Moreover, extensive research on phytochemical and pharmacological properties of fruit has never been carried out till date. Therefore the purpose of the present study was to determine the total phenolic, tannin, flavonoid contents and elute fractions from the highly active extract of *S. calophyllifolium* fruit by thin layer and column chromatography techniques, for their comparative antioxidant and antibacterial study and to profile the bioactive phytochemicals responsible for these activity by gas chromatography–mass spectrometry and high performance liquid chromatography coupled with photo diode array detector (HPLC-PDA).

Materials

Collection and identification of plant material

Fresh fruits of *S. calophyllifolium* Walp. were collected from the Nilgiris in July 2013. The taxonomic identity of the plant material was confirmed with the Botanical Survey of India (BSI), Southern Circle, Coimbatore, Tamil Nadu, India and also plant name was conformed from the plant list. The fruits with seeds were washed under running tap water to remove surface pollutants and then air dried in the shade for 7 days. The dried fruits were homogenised into a fine powder using pulverizer and stored until used.

Chemicals

2,2-Diphenyl-1-picryl-hydrazyl (DPPH[•]), potassium persulphate, 2,2'-azinobis (3-ethyl-benzothiozoline)-6-sulfonic acid diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), butylated hydroxy toluene (BHT), rutin, gallic acid, tannic acid, ferrous chloride, ferric chloride, hydrogen peroxide, ferrous ammonium sulfate, ethylene diamine tetra-acetic acid (EDTA) disodium salt, *N*-(1-naphthyl) ethylene diamine

dihydrochloride, sodium nitroprusside, etc. were purchased from Himedia, Mumbai; Sisco Research Laboratories, Mumbai and Sigma Aldrich, USA. The TLC plates are purchased from Merk Company, Germany. All the other chemicals and solvents used were of analytical grade.

Preparation of plant extracts

The powdered fruit material (100 g) was packed in small thimbles and separately extracted with organic solvents (500 ml) such as petroleum ether, ethyl acetate and methanol in their increasing order of the polarity using a Soxhlet apparatus. Each time before extracting with the next solvent, the thimble was air dried. Finally, the material was macerated using hot water with constant stirring for 24 h. The water extract was also filtered using Whatman no. 1 filter paper. The different solvent extracts were concentrated by rotary vacuum evaporator and then air dried.

Extract recovery

The amount of extract recovered after successive extraction from dry plant sample was weighed and the percentage yield was calculated by the following formula,

$$\text{Extract recovery percent} = \left[\frac{\text{amount of extract (g)}}{\text{amount of dry plant sample (g)}} \right] \times 100.$$

Quantification assays

Total phenolics

The total phenolics of the plant extracts were determined according to the method described by Makkar (2003). The results were expressed as gallic acid equivalents (GAE).

Tannins

The total phenolics contain both tannin and non-tannin phenolics. The amount of tannins was calculated by subtracting the non-tannin phenolics from total phenolics. The method described by Makkar (2003) was used for determination of non-tannin phenolics. The analyses were also performed in triplicates and the results were expressed in tannic acid equivalents (TAE).

Flavonoids

The flavonoid contents of all the four extracts were quantified according to the method described by Zhishen et al. (1999). Rutin was used as the standard for the

quantification of flavonoids. The experiment was done in triplicate and the results are expressed as rutin equivalents (RE).

Thin layer and column chromatography

Fractionation of methanolic extract was done according to a slightly modified method of Tomer et al. (2009). The methanolic extract of the *S. calophyllifolium* fruit (15 g) with high phenol content was adsorbed onto silica gel (30 g) by triturating in a mortar. The activated silica gel (60–120 mesh) (150 g) was packed into a glass column of dimension 2 × 25 cm using chloroform as the solvent. A wet packing method was followed. Before packing, a cotton plug was kept at the bottom of the column to eliminate leaking of the solvent. The silica gel slurry was then stirred and quickly added without any air bubble formation till three-fourth of the column was filled. A substantial amount of mobile phase [chloroform: ethyl acetate (95:5–5:95) and ethyl acetate: methanol (95:5–5:95) in different ratios] was poured continuously into the column and allowed to drain without drying the column. The solvent collected initially was poured back into the column. Periodically, a piece of rubber tubing was used to agitate the column to release trapped air bubbles. Totally 645 fractions were eluted (10 ml each) and collected in dry test tubes. All the collected fractions were spotted and run on TLC plates and based on the TLC banding pattern; the fractions were pooled to get 20 pooled fractions. The recovery percentages of these pooled fractions were calculated and were subjected to in vitro antioxidant screening by DPPH[•] (Braca et al. 2001), ABTS^{•+} (Re et al. 1999) and FRAP (Pulido et al. 2000) assays for assessing the in vitro antioxidant potential.

Antibacterial activity

Microorganisms

The antibacterial activity of the extracts and fraction was tested individually on common gram-negative and gram-positive bacterial strains isolated from human pathogenic bacteria cases. All bacterial strains were obtained from ATCC, USA and Institute of Microbial Technology (IMTECH), Chandigarh, India. The organisms used for antibacterial study were, *Escherichia coli* (ATCC 25922), *Klebsiella* sp. (ATCC 15380), *Proteus* sp. (MTCC 1771), *Salmonella typhi* (MTCC 15691), *Staphylococcus aureus* (ATCC 25923). Inoculums of the respective organisms were prepared in Luria–Bertani (LB) broth medium and incubated for 24 h at 37 °C.

Well plate method

The MFE of *S. calophyllifolium* and its fractions were prepared at a concentration of 30, 60, 100 mg/ml. Kanamycin was used as the standard for antibacterial activity at the same concentrations. The LB agar medium was prepared and sterilized in an autoclave; the medium was then poured into sterile Petri dishes and allowed to cool at room temperature. The inoculums of the test organisms were spread onto the agar plates. The wells were prepared in the agar plates using an agar well puncher; in such a way that the wells were able to hold 50 µl of test samples. After the test solutions were added, the plates were incubated for 24 h at 37 °C. The antibacterial activity was evaluated by measuring the zone of inhibition against test each organism (Shinde et al. 2012).

Gas chromatography/mass spectrometry (GC/MS)

GC–MS analysis of methanol extract was carried out using a Clarus 600 gas chromatograph system equipped with Clarus 600 C mass spectrometer (PerkinElmer precisely, USA). An elite-5MS fused silica capillary column coated with a 5% diphenyl/95% dimethyl polysiloxane stationary phase (60 m × 0.25 mm, film thickness 0.10 µm; PerkinElmer precisely, USA) was used for GC/MS. The injector temperature was kept at 200 °C whereas the oven temperature was programmed from to gradually change 70–300 °C over a total run time of 35 min. Helium was used as carrier gas at a flow rate of 1.0 ml/min. An appropriate blank was run from which the solvent delay was fixed to 4 min. The electron ionization mode with ionization energy of 70 eV; ion source temperature of 200 °C; GC interface temperature of 240 °C; scan interval of 0.2 s and fragments range from 50 to 600 m/z were set for the MS analysis. About 1 µl of the methanol extract of *S. calophyllifolium* was injected manually in a splitless mode. The mass spectra of the respective peaks obtained in the GC/MS were compared with the mass fragmentation patterns of standards in the NIST (National Institute of Standard Technology) library using Turbomass software (5.4.0).

HPLC analysis

Quantification and identification of phytochemicals were carried out on a high performance liquid chromatographic system following the method of Ye et al. (2010) with slight modification. For quantification, the crude extract and column fraction ‘H’ were dissolved in 0.5 ml of 100% methanol at a concentration of 30 mg/ml. Waters 515 HPLC (Waters Corp., Milford, MA, USA) equipped with a photodiode array detector (PDA) (Model: Waters 2998,

Waters Corp.) was used for separation. Chromatographic separation was performed using a C18 column (Sigma Aldrich, USA) 250 mm × 4.6 mm; 5 µm. Precisely, 20 µl of sample was injected into the column by the auto sampler. The samples were eluted through the column with a gradient mobile phase consisting of A [0.2% (v/v) acetic acid in water] and B [0.2% (v/v) acetic acid in methanol] with a flow rate of 0.5 ml/min. Prior to injecting into the column, a 20 min linear gradient was programmed as follows: 0–4 min B (30%); 4–10 min B (50%); 10–20 min B (80%). The samples were monitored by detector at 254 nm at flow rate of 0.5 ml/min at 29 °C temperature. Rutin, ellagic acid, gallic acid and quercetin were used as reference compounds. The quantity of each compound was calculated using the following formula (Joseph and Thomas 1992):

$$\text{Quantity percent} = \frac{A_S \times W_{ST}}{A_{ST} \times W_S} \times \text{purity of standard}$$

were A_S , sample peak area; W_{ST} , weight of standard; A_{ST} , standard peak area; W_S , weight of sample.

Statistical analyses

All the experiments were done in triplicates and the results were expressed as mean ± standard deviation. The data was statistically analyzed using a one way ANOVA followed by Duncan's test for antioxidant studies. Mean values were considered statistically significant at $p < 0.05$.

Results and discussion

Yield recovery percentage of extract and fraction

Maximum yield was obtained from methanol extraction (MFE) of fruits, with a yield of 20.2% followed by hot water extract (HWFE) (8.1%). The yield percentage of petroleum ether (PEFE) (1.7%) and ethyl acetate (EAFE) (1.9%) extracts were found to be very low compared to the polar solvents. The methanol extract of *S. calophyllifolium* was fractionated by column chromatography for the identification of phytochemicals. The fraction recovered after chromatographic separation from MFE was weighed and the percentage yield was calculated. The fraction K showed highest yield percentage (9.3%). Whereas the low yield percentage was noted for Fraction F (0.05%). The order of yield percentage for different fractions was F (0.1%) > C, E (0.2%) > D (0.21%) > G (0.4%) > H (0.9%) > T (1.6%) > J (3.7%) > I (4.1%) > K (9.3%).

Quantification assays

Quantification of total phenolics content

The content of extractable phenolic compounds in the *S. calophyllifolium* fruit extracts were determined through a linear gallic acid standard curve ($y = 0.031x + 0.162$; $R^2 = 0.981$). The amount of total phenolics present in various extracts were statistically analysed and tabulated in Table 1. Among the four solvent extracts, the methanolic fruit extract (MFE) depicted the highest amount of phenolics (198.2 mg GAE/g extract) whereas the least in petroleum ether extract (1.6 mg GAE/g extract). The order of total phenolics present in different extracts of *S. calophyllifolium* fruit is methanol > hot water > ethyl acetate > petroleum ether. The lowest content of total phenolics in petroleum ether extract may be due to their low solubility in petroleum ether.

The total phenolic content was much higher than that reported previously (Reynertson et al. 2008) in *Syzygium cumini* fruits (9.9 mg GAE/g extract). The phenolic content in the plants are associated with their antioxidant activities (Quy et al. 2014), probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Chang et al. 2001). These high levels of total phenolics present in methanolic extract of *S. calophyllifolium* fruit confirms the free radical scavenging ability.

Quantification of tannins content

Tannins are water soluble polyphenols present in many fruits and have also been recognized as antioxidants. The tannins present in different extracts of *S. calophyllifolium* fruit are presented in Table 1. The higher amounts of tannins were observed in MFE (207.3 mg TAE/g extract). Whereas, moderate tannin content was noticed in hot water extract (115.7 mg TAE/g extract). Petroleum ether and ethyl acetate produced low amounts of tannins which may be due to the polarity of these solvents.

Many tannin components were suggested to be anti-carcinogenic and have been shown to reduce the mutagenic activity of a number of mutagens. Numbers of carcinogens and/or mutagens produce oxygen free radicals for interaction with cellular macromolecules (Chung et al. 1998). The anti-carcinogenic and anti-mutagenic potentials of tannins may be related to their anti-oxidative properties, which are important in protecting against cellular oxidative damage (Chang et al. 2001). The large amount of tannins in the fruit of *S. calophyllifolium* could also inhibit free radicals and diseases associated with them.

Table 1 Total phenolic, tannin and flavonoid contents of *S. calophyllifolium* fruit

Extracts	Total phenolics (mg GAE/g extract)	Tannins (mg TAE/g extract)	Flavonoids (mg RE/g extract)
Petroleum ether	1.6 ± 0.5 ^d	2.1 ± 3.8 ^d	38.6 ± 3.7 ^d
Ethyl acetate	29.4 ± 5.0 ^c	9.8 ± 9.7 ^c	102.3 ± 5.0 ^b
Methanol	198.2 ± 3.0 ^a	207.3 ± 11.3 ^a	116.6 ± 6.6 ^a
Hot water	85.5 ± 2.6 ^b	115.7 ± 12.4 ^b	90.6 ± 5.7 ^c

GAE gallic acid equivalents, TAE tannic acid equivalents, RE rutin equivalents

^{a-d}Values (mean ± standard deviation, n = 3) in the same column with different superscripted letters are significantly different ($p < 0.05$)

Quantification of flavonoids content

Table 1 represents the results of flavonoids present in the fruit. The results revealed higher amount of flavonoids in MFE (116.6 mg RE/g extract), followed by ethyl acetate (102.3 mg RE/g extract). Flavonoids are the most common and widely distributed group of plant phenolic compound, which usually are very effective antioxidant because of the scavenging ability conferred by their hydroxyl group (Yanishlieva-Maslarova 2001). These compounds from plants are known to be good natural antioxidant (Shyi et al. 2014). The flavonoids in *S. calophyllifolium* fruits suggest that it may possess great antioxidant potential with significant biological activities.

In vitro antioxidant assays

DPPH scavenging activity

DPPH radical scavenging activity of the *S. calophyllifolium* fruit extracts and the 10 active fractions are presented in the Table 2. From the results, it is clear that fraction H (FH) from methanolic extract of *S. calophyllifolium* possess lowest IC₅₀ value of 2.1 µg/ml which is eight times higher than that of methanolic extract (IC₅₀ value 16.0 µg/ml). Moreover, the results are compared with that of natural (rutin) and synthetic antioxidants (BHT). This study confirms that the FH possess more antioxidant phytochemicals like phenolic and flavonoids compared to MFE. The petroleum ether extract found to have a minimal antioxidant activity (IC₅₀ value 240.7 µg/ml) as most of the phenolics were detected in high polar solvents. The significant IC₅₀ values for fractions H, C and D were 2.1, 11.5 and 4.5 µg/ml respectively. However, the order of IC₅₀ value in DPPH radical scavenging assay is H > C > D > F > MFE > E > G > HWFE > I > J > K > EAFE > T > PEFE.

This model of scavenging the stable free radical is widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability.

Banerjee et al. (2005) reported a lower IC₅₀ (168 µg/ml) for the fruit skin extract of *S. cumini* compared to *S. calophyllifolium*. This could be significantly correlated with total phenolic contents of these two plants. It has also been reported that the antioxidant activity of many compounds of botanical origin is proportional to their phenolics contents, suggesting a causative relationship between total phenolic content and antioxidant activity (Rice-Evans 2001). In the present study there exists a significant correlation between DPPH scavenging activity and total phenolics. Consistent with these results, we suggest that the extracts and fractions of *S. calophyllifolium* fruit possess a very potent antioxidant property which might be due to the phenolics and flavonoids in the extracts.

ABTS^{•+} scavenging activity

ABTS^{•+} assay is an excellent tool to determine the antioxidant activity of hydrogen donating antioxidants (scavenging aqueous phase radicals) and of chain breaking antioxidants (scavenging lipid peroxy radicals). The ABTS^{•+} scavenging activity of different extracts and the 10 best fractions of *S. calophyllifolium* fruit are presented in Table 2. The results clearly depict the high ABTS cation radical scavenging activity of fractions and methanolic extract compared to positive controls (rutin and BHT). The highest Trolox equivalent activity was shown by the FH (19483.29 µM TE/g extract) and activity is much higher than that of MFE (10334.27 µM TE/g extract). However fraction E and D exhibited significant ABTS scavenging activity. More than five fractions showed high Trolox equivalent antioxidant activity compared to that of crude methanolic extract of *S. calophyllifolium*. At the same time other crude extracts like ethyl acetate and hot water also showed effective antioxidant activity whereas petroleum ether extract noted a very low antioxidant activity. Highest ABTS^{•+} scavenging activity may be due to presence of flavonoids content in all extracts confirmed by correlation analysis ($r = 0.972$). Fraction H showed significantly higher scavenging activity than the crude MFE, which is in accordance with its higher concentration of active

Table 2 DPPH[•] scavenging activity, ABTS cation radical scavenging activities and ferric reducing antioxidant power assay of extract and methanolic fractions of *S. calophyllifolium* fruit

Sample	DPPH [•] scavenging activity IC ₅₀ (μg/ml)	ABTS ^{•+} scavenging activity (μM TE/g extract)	Ferric reducing antioxidant power assay (mM Fe(II)/1 mg)
Petroleum ether extract	240.73	605.94 ± 147.24 ⁱ	0.43 ± 0.05 ^h
Ethyl acetate extract	41.13	7031.56 ± 510.07 ^g	33.33 ± 0.99 ^e
Methanol extract	16.07	10,334.27 ± 80.73 ^d	60.30 ± 1.30 ^c
Hot water extract	23.15	7937.14 ± 487.81 ^g	34.27 ± 0.83 ^e
Fraction C	11.55	5726.46 ± 870.12 ^h	11.71 ± 1.02 ^g
Fraction D	4.53	15,967.51 ± 545.36 ^b	64.64 ± 0.18 ^b
Fraction E	15.73	16,020.78 ± 802.03 ^b	62.84 ± 1.39 ^b
Fraction F	14.02	10,853.64 ± 234.10 ^d	60.46 ± 2.08 ^c
Fraction G	18.41	10,840.32 ± 440.08 ^d	60.57 ± 2.40 ^c
Fraction H	2.15	19,483.29 ± 838.36 ^a	65.56 ± 2.11 ^b
Fraction I	24.72	12,078.84 ± 234.10 ^c	60.36 ± 4.32 ^c
Fraction J	25.15	8989.21 ± 422.81 ^f	44.96 ± 5.70 ^d
Fraction K	39.72	5433.48 ± 892.46 ^h	27.50 ± 0.46 ^f
Fraction T	48.44	7471.03 ± 479.42 ^g	50.48 ± 0.21 ^c
BHT	7.93	8423.95 ± 96.32 ^f	112.56 ± 4.03 ^a
Rutin	6.35	9942.69 ± 109.84 ^e	99.19 ± 25.91 ^a
Vitamin E	9.57	–	21.24 ± 1.62 ^f

TE Trolox equivalents

^{a–i}Values (mean ± standard deviation, n = 3) in the same column with different superscripted letters are significantly different ($p < 0.05$)

components. All the fractions exhibited a wide range of radical scavenging activity in this assay. The presence of high concentration of biologically active compounds in *S. calophyllifolium* fruits could be the reason for the high antioxidant activity of these fractions. Reduction of ABTS^{•+} can be even more efficient than that of DPPH. The total antioxidant ability of all the studied samples seems to be sufficient for functioning as potential nutraceuticals. Most fruits tested with high antioxidant capacity in the DPPH assay, also showed a high antioxidant capacity in ABTS assay (Barreca et al. 2011).

Ferric reducing antioxidant power assay (FRAP)

FRAP is a simple inexpensive assay and may offer presumed index of antioxidant activity. The FRAP assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-*S*-triazine complex [Fe(III)-(TPTZ) 2]²⁺ to intensely blue coloured ferrous complex [Fe(II)-(TPTZ) 2]²⁺ in an acidic medium (Loganayaki and Manian 2010). The intensity of the blue colour produced by the samples were measured spectrophotometrically. Ferric reducing antioxidant power of all the extracts and 10 active fractions were statistically analysed and the results presented in Table 2. MFE exhibited lower ferric reducing activity [60.3 mM Fe(II)/mg extract] compared to FH [65.5 mM Fe(II)/mg extract]. Fractions E, F, G and I had similar

ferric reducing activity of 60 mM Fe(II)/mg extract. The ethyl acetate and hot water extracts also showed significant reducing power. From the results it was also clear that methanol, ethyl acetate, hot water extracts and fractions have higher reducing activity compared to that of Vitamin E.

The obtained results are in agreement with the findings from the previous assays and confirm that the antioxidant power of the *S. calophyllifolium* can be due to its strong electron transfer ability. Loots et al. (2006) reported that the ferric reducing antioxidant power of fruit juice correlated well with the polyphenol concentrations and they suggested that higher antioxidant activity of fruit juice might be due to the presence of phenolics. It is proved by correlation analysis of results, maximum positive correlation ($r = 0.911$) was observed between total phenolics content and ferric reducing antioxidant power. The preceding results clearly indicates that the fruit extracts can act as electron donors and react with free radicals, and convert them to more stable products, thus terminating the radical chain reaction.

Antibacterial activity of methanol extract and its fraction

Antibacterial activity of *S. calophyllifolium* fruit methanolic extract and its fraction (H) were assessed based

Table 3 Antibacterial activity of *S. calophyllifolium* fruit methanolic extract and its fractions H

Organisms	Zone of inhibition (diameter in mm)								
	Methanolic extract (mg/ml)			Fraction H (mg/ml)			Kanamycin (mg/ml)		
	30	60	100	30	60	100	30	60	100
<i>E. coli</i>	11.0 ± 1.0 ^h	12.3 ± 1.5 ^g	18.0 ± 1.0 ^f	12.6 ± 1.5 ^g	21.3 ± 1.1 ^e	32.0 ± 1.0 ^c	27.3 ± 2.1 ^d	41.0 ± 1.5 ^b	46.3 ± 2.5 ^a
<i>Klebsiella</i> sp.	11.3 ± 1.5 ^h	16.3 ± 1.5 ^f	18.0 ± 1.0 ^e	14.0 ± 1.0 ^g	19.6 ± 1.5 ^d	25.6 ± 2.5 ^c	23.6 ± 2.5 ^c	36.0 ± 3.0 ^b	45.0 ± 1.0 ^a
<i>S. aureus</i>	06.0 ± 1.0 ^h	13.0 ± 1.0 ^f	18.0 ± 1.0 ^e	11.0 ± 1.0 ^g	17.6 ± 1.5 ^e	27.3 ± 2.0 ^c	21.3 ± 1.5 ^d	34.6 ± 1.5 ^b	45.0 ± 2.0 ^a
<i>S. typhi</i>	09.0 ± 1.0 ⁱ	14.0 ± 1.0 ^g	19.6 ± 1.5 ^e	12.6 ± 1.5 ^h	17.6 ± 1.5 ^f	27.0 ± 1.0 ^c	21.0 ± 1.0 ^d	37.0 ± 1.0 ^b	43.0 ± 1.5 ^a
<i>Proteus</i> sp.	06.0 ± 1.0 ^h	12.3 ± 0.5 ^g	20.0 ± 1.0 ^d	15.0 ± 1.0 ^f	20.0 ± 1.0 ^d	26.0 ± 1.0 ^c	18.0 ± 1.7 ^e	35.0 ± 1.0 ^b	44.0 ± 1.0 ^a

^{a-i}Values (mean ± standard deviation, n = 3) in the same row with different superscripted letters are significantly different ($p < 0.05$)

Fig. 1 Chromatogram of GC–MS analysis of *S. calophyllifolium* fruit methanolic extract

on its phytochemical constituents and antioxidant activity. The zone of inhibition was measured and the results were statistically analysed and tabulated (Table 3). From the results it is clear that samples exhibited moderate antibacterial activity against all the five bacterial pathogens in a concentration dependent manner. Compared to ME, a better zone of inhibition was noted for the fraction H against *E. coli* (32 mm at 100 mg/ml concentration). On the other hand, the growth of *S. aureus* was also effectively inhibited by Fraction H (27.3 mm at 100 mg/ml concentration). But the methanolic extract showed very low level of antibacterial activity compare to fraction. Moreover, *Klebsiella* sp. was inhibited by both fraction H and methanolic extract at the 100 mg/ml concentration and the inhibition zones were 25.6 and 18 mm respectively.

The results suggest that fruit extract can be used for preventing food contaminants like *E. coli*. *S. aureus* is the most common species of *Staphylococcus* to cause Staph infections. Moreover, *Klebsiella* sp. has become important pathogens in nosocomial infections (Podschun and Ullman 1998). It can be assumed that the phenolics, flavonoids and tannins present in the fruit extracts might be a responsible factor for the activity. Compared to methanol extract high activity of fractions presumes the presence of active phytochemicals at high concentration contrast to crude extract. These results are in agreement with the results of antioxidant assays and this might be used as a lead to continue the search of active substance in the extract of *S. calophyllifolium* fruit.

Table 4 Compounds identified from GC–MS analysis

S. no.	RT (min)	Compound name	M. formula	Area percentage
01	6.374	3-Oxabicyclo[3.3.0]octan-2-one,7-methylene	C ₈ H ₁₀ O ₂	0.2
02	9.02	Carbamic acid, hydroxy-, ethyl ester	C ₃ H ₇ O ₃ N	0.2
03	12.037	1-Silacyclo-3-pentene	C ₄ H ₈ Si	0.1
04	14.468	[1-(Diethylamino)ethylidenimino]sulfur pentafluoride]	C ₆ H ₁₃ N ₂ F ₅ S	0.1
05	15.63	Valeric acid, 3,5-dihydroxy-2,4-dimethyl	C ₇ H ₁₂ O ₃	0.1
06	16.61	Z-28-heptatriaconten-2-one	C ₃₇ H ₇₂ O	0.1
07	17.946	L-Valine, 3-mercapto	C ₅ H ₁₁ O ₂ NS	0.2
08	19.56	2h-azepin-2-one, hexahydro-1-(2-propenyl)	C ₉ H ₁₅ ON	0.2
09	21.20	Acetamide, <i>n</i> -[3(3dimethylaminopropylamino propylamino)propyl]-2hydroxyimino-2phynyl	C ₁₆ H ₂₆ O ₂ N ₄	0.1
10	22.74	1,3-Propanediamine, <i>n</i> '-[3-(dimethylamino)- <i>n</i> - <i>n</i> -dimethyl	C ₁₀ H ₂₅ N ₃	0.3
11	24.17	3-Piperidinamine, 1-ethyl-	C ₇ H ₁₆ N ₂	0.8
12	25.52	<i>N</i> -[3-[<i>n</i> -aziridyl]propylidene]-3-methylaminopropylamine	C ₁₀ H ₂₁ N ₃	0.8
			Total area percentage	3.1

RT retention time (minutes), M. formula molecular formula

Gas Chromatography/Mass Spectrometry (GC/MS)

The results obtained for various antioxidant assays revealed that methanolic extract of *S. calophyllifolium* fruit has higher antioxidant activity compared to other solvent extracts. To identify the active phytochemicals in the methanolic extract, GC/MS analysis was carried out and the chromatogram is shown in Fig. 1. Twenty major peaks were observed from the chromatogram. The mass spectrum of each peak was compared with that of compounds in the NIST (National Institute of Standard Technology) library and 12 compounds were identified and tabulated in Table 4. Among these 12 compounds L-valine, 3-mercapto found to have poison neutralizing properties. This compound is also used as a drug to treat rheumatoid arthritis. These chemical compounds have also shown the property to bind and remove ions from solutions (Mc Clements and Callender 1990).

HPLC analyses of methanolic extract (ME) and its fraction (H)

The antioxidant and antibacterial activity of the MFE and FH led to the quantitative HPLC analysis with authentic standards. Figure 2 shows HPLC chromatograms of standards, MFE and FH. Five peaks are observed from the MFE chromatogram, peak no 3 (RT 10.208 min) and 4 (RT 10.892 min) match the relative retention time of rutin (RT 10.014 min) and ellagic acid (RT 10.854 min). Among the 8 peaks obtained in the chromatogram of FH two peaks had the same relative retention time as rutin and ellagic acid. These results are the evidence for the presence of rutin and

ellagic acid or their analogues in *S. calophyllifolium* fruit methanolic extract and its fraction. However FH showed higher concentration of both the compounds (1.3% rutin and 13.0% ellagic acid) compared to MFE (2.8% ellagic acid and 1.1% rutin).

These results were in agreement with findings of Reynertson et al. (2008) where five different *Syzygium* species were shown to possess rutin and ellagic acid as its major phytoconstituents. There is a particular interest in the amounts of ellagic acid, a dimeric derivative of gallic acid, in these fruits because of the increasing evidence of its anti-carcinogenic and antioxidant effects (Maas and Galletta 1991). Moreover, ellagic acid represented 88% of the total phenolic content analyzed in fruits (Hakkinen et al. 1999). Rutin (quercetin-3-rutinoside) is one of the major flavonoids found in a variety of plants. It is receiving increasing attention due to its various health beneficial biological activities, including anti-oxidative, anti-inflammatory and anti-hypertensive effects (Sun et al. 2011).

Conclusion

The MFE of *S. calophyllifolium* had high amounts of total phenolics, tannins and flavonoids. The crude extract and fractions demonstrated high in vitro antioxidant and antibacterial activities. These results explain the extensive bioactivity of *S. calophyllifolium* fruit. The higher antioxidant and antibacterial activity of methanolic extract and its fractions may be due to the presence of active principles such as ellagic acid and rutin or its analogues in greater

Fig. 2 HPLC Chromatograms of standards, methanolic extract and its fraction H of *S. calophyllifolium* fruit

concentration compared to other extracts of *S. calophyllifolium* fruit. This study appeals the need for better characterization of phytochemicals present in the wild fruits which would increase their commercial value. In conclusion, the antioxidant properties and phytochemical investigation of the *S. calophyllifolium* fruits are expected to increase the use of this fruits in food industry especially in new human health-oriented products.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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