Extraction and isolation of important bioactive compounds from the fruit of *Physalis ixocarpa*

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Abstract: The current study investigates pharmaceutically important bioactive compounds in the fruits of *Physalis ixocarpa*. Two different extractions methods were used to study its effect on percent extract yield, recovery of bioactive compounds and antioxidant activity of the extracts. The data indicated that Soxhlet extraction had high efficiency of recovery than maceration method for extracting compounds; percent extract yield and antioxidant activity of the extracts. In maceration, the percent extract yield was found to be in order of water >methanol >ethyl acetate whereas in Soxhlet extraction, it was in order of methanol >water >ethyl acetate. Ethyl acetate extract produced by Soxhlet extraction showed strong antioxidant activity of 59.7% (250ppm) as compared to other extracts. Analysis of ethyl acetate extract showed the presence of Triglyceride. GC-MS study of triglyceride revealed the presence of trilinoleinic acid (9,12-Octadecdienoic acid), tripalmitin (hexadecanoic acid) and trioleinic acid (9-Octa decenoic acid). Four impure and three pure compounds were isolated from crude methanol extract of the fruit. The structure of pure compounds were identified by NMR and characterized as sugar, glucose and fructose.

Keywords: Bioactive compounds; antioxidant activity; GC-MS; Soxhlet extraction; Maceration, NMR.

INTRODUCTION

The plant materials obtained from medicinal plants are used in the pharmaceuticals, cosmetics and drug industries. It is reported that about eighty percent of the people in the developing countries depends on traditional herbal medicines for their health related problems (WHO, 1991). Several of currently available drugs are derived from the raw materials obtained from plants. These medicines are becoming popular among the people due to their easy availability, least side effects, low prices, environmental friendliness and lasting curative property. Different bioactive compounds present in medicinal plants work together to reach equilibrium in the body as they do in the plant and therefore produce gentle progressive healing effect within the body tissues. Extracts of many plants possess highly efficient antimicrobial and anti-oxidant activities (Bakht *et al*., 2018; Bilal *et al.,* 2018; Ayaz *et al.,* 2017; 2018; Wajid *et al.,* 2017). Extracts, infusion and dry powder from the leaves, seeds, stem, roots, fruits, foliage etc. of medicinal are used in the treatment of different health related issues in humans, plants and animals (Nostro *et al*., 2000). Different antimicrobial bioactive compounds of the medicinal plants were first documented in the late 19th century (Zaika, 1975). These bioactive compounds included tannins, terpenoides, alkaloids and flavonoid, which have been shown *in vitro* antimicrobial properties (Cowan, 1999).

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P. ixocarpa (tomatillo) is a close relative of the tomato and acclimatized to the tropical and subtropical humid condition. The plant is an annual herb with a Y shaped branches and weedy looking appearance. An unripe tomatillo is slightly sticky on the surface, 2.5-7.6cm in diameter, dried texture and a distinctive flavor (Hernandez SM and Rivera JRA, 1994). Ripe fruits are comparatively sweeter than unripe fruit and can be stored for one year in husk. Tomatillos are a good source of vitamin C, magnesium, phosphorus, potassium, beta-carotene and the antioxidant lutein. It contain large amount of ascorbic acid, nicotinic acid and solids when compared with tomato (Yamaguchi, 1983). Tomatillo is used in the preparation of sauces and other traditional dishes in Mexico (Zhang *et al.,* 2016). Tomatillo has more sodium and iron and less potassium and calcium as compared to tomato. Due to high total sugar content and high acid content leads to a better tasting fruit (Ostrzycka *et al.*, 1988). Tomatillo has also been used in folk medicine for the treatment of cough, fever and amygdalitis (Maldonado, Perez-Castorena, Garces and Martinez, 2011). Cytotoxic, antifungal and apoptotic activities has been reported from leaves, stems and fruits of *P. philadelphica* (Choi *et al.,* 2006; Maldonado *et al.,* 2011; Wajid *et al.,* 2017).

The isolation of bioactive compounds and antioxidant activity of the extracts mainly depends on the extraction method and extraction solvent (Arabshahi and Urooj 2007; Alothman *et al.,* 2009; Khan *et al.,* 2016b). The

current study was designed to determine the effect of different solvents and different extraction methods on extraction yield, recovery of bioactive compounds and bioactivity of the fruit of *P. ixocarpa.*

MATERIALS AND METHODS

Collection and identification of plant materials

P. ixocarpa was collected from the Baylybaba and Miaganu areas of District Shangla Khyber Puktunkhwa province of Pakistan. The plant specimen was identified by plant taxonomist, Department of Botany University of Peshawar, Pakistan and deposited in herbarium of Islamia College University Peshawar Pakistan and recorded with voucher number (WD1).

Extraction and isolation

ACS grade solvents were used in isolation and purification methods (Sigma–Aldrich Chemical Company, USA). Thin-layer chromatography (TLC) was performed on 250 and 500 lm silica gel plates (Analtech, Inc., Newark, DE, USA). Medium-pressure liquid chromatography (MPLC) was executed on Merck silica gel (60 mesh size, 35-70 lm, EMD chemicals, USA). Agilent Direct Drive 2500 MHz instrument and Agilent 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) were used for recording NMR and GCMS spectra respectively. MTT [3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide], Vitamin C and tert-butylhydraquinone (TBHQ) were purchased from Sigma–Aldrich Chemical Company. Microplate reader was used in MTT assay. All the solutions were stored in Bioactive Natural Products and Phytoceuticals Laboratory at Michigan State University (USA).

Maceration

Fresh fruits were collected from the plants. The husks were removed from the fruit and weighted (1014 g). The fruits were then cut into small pieces with sharp blades and blended with water (600 ml). The resulting puree was stirred for 3 hours and then centrifuged at 10000 rpm for 10minutes. The supernatant was removed and lyophilized. The weight of the lyophilized material was recorded. The residue was then stirred with 250ml of methanol, using hot plate magnetic stirrer for 3 hours and then centrifuged at 10000rpm for 10minutes. The supernatant was separated from the residues and again extracted with methanol. This process was repeated three times. All methanol extracts were then pooled and rotary evaporated to remove the solvents. The residues were then treated in ethyl acetate using same method of extraction (Seeram *et al*., 2001).

Soxhlet extraction

Fresh fruits of *P. ixocarpa* without husk (1014 gm) was cut into small pieces with sharp blades and lyophilized. The lyophilize material was converted into powder form and put it in to cellulose thimble and covered with cotton wool. The thimble was then placed in Soxhlet extraction chamber. Three hundred mL methanol was added into extraction chamber. The extract collected in the round bottom flask was then dried through rotary vapor and weight was noted. The same steps of extraction were also used for ethyl acetate, and water. The water extract was lyophilized in lypholizer.

Thin layer chromatography (TLC)

TLC was carried to separate different components of the extracts. The prepared extract was applied to pre-coated TLC plate in the form of small round spots by using capillary tube. The TLC plate was then placed in TLC chamber having suitable solvent system. The plate after development was taken out and observed under ultra violet light UV at both 254 nm and 366 nm. The UV active regions were marked and then sprayed with spraying reagent (10% Sulphuric acid), and placed in hot oven for few minutes for the observation of the UV inactive components of the extracts (Seeram *et al*., 2001).

Isolation of compound

Triglyceride

Ethyl acetate extract was run on TLC using hexane and acetone solvent system (5:1). TLC profile indicated the presence of seven spots. Out of the five spots only 2 spots were UV active.

MPLC of the ethyl acetate extract

MPLC was carried out to separate different compounds in ethyl acetate extract. For this purpose gradient solvent system of hexane and acetone (5:1) with bed volume of 100 ml was used. Twenty four fractions with volume of 5 ml was collected and finally two fractions (Wk/116/7= 470.9 mg, Wk/116/5B= 15.1mg) were obtained by combining the different fractions with the same TLC profiles. Fraction with more in quantity (332.4mg) was again passed through MPLC using gradient solvent system of hexane and acetone (30:1, 15:1, 5:1 and pure acetone) for further fractionation. Seventy four fractions with volume of 5ml were collected and finally four fractions were obtained by pooling together all the fractions with the same TLC profile. The fraction with large quantity was again run on TLC, showed single compound as shown in fig 4B. The structure of single compound was identified as triglyceride by NMR study.

Nuclear magnetic resonance (NMR)

Tested sample of 5-6 mg was dissolved in DMSO or D-methanol and filtered through cotton plug in to the NMR tubes. The sample was then analyzed by NMR for structure identification of different compounds (Seeram *et al*., 2001).

Isolation of triglyceride

Trilinolein/major, tripalmitin/minor, triolein/minor

^{1}H NMR (500 MHz, $CDCl_3$): δ 5.23−5.37 (m, H-2, -C*H*=C*H*-), 4.27 (dd, *J*=12.0, 4.0 Hz, H-1a, 3a), 4.12 (dd, *J*

=12.0, 6.0 Hz, H-1b, 3b), 2.75 (m, -CH=CH-CH_2-CH=CH-), 2.29 (m, -COCH_2-), 2.02 (m, -CH_2-CH=CH-CH_2-), 1.58 (m, -COCH$_2$-CH_2-), 1.23−1.35 (m, -CH_2-), 0.87 (m, -CH_3).

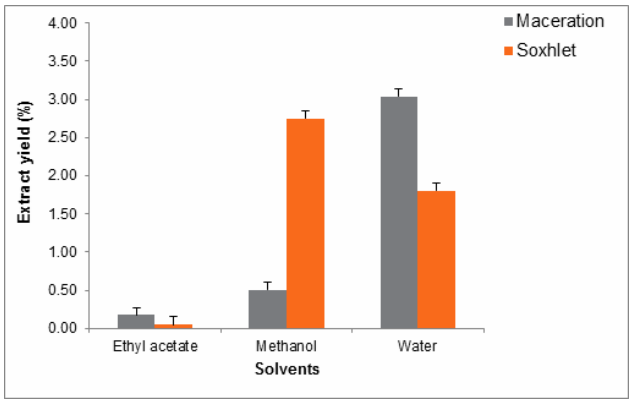

Fig. 1: Comparison of extract yield (%) of Soxhlet and Maceration extraction methods (Bar shows LSD at P<0.05).

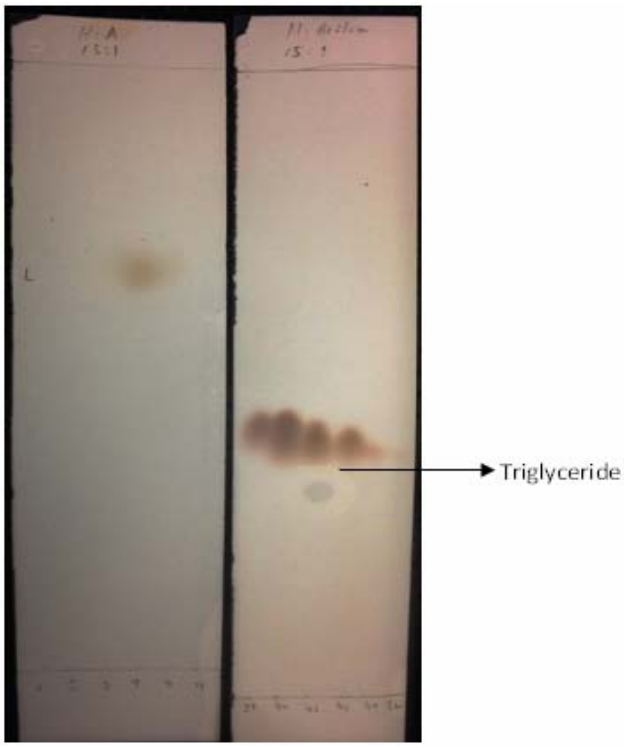

Fig. 2: TLC Profile of fractions obtained from ethyl acetate extract, A = TLC profile of impure compound from ethyl aceate extract, B= TLC of triglyceride in solvent system of hexane and acetone (15:1)

Isolation of sugar, glucose and fructose

Sugar, glucose and fructose were also isolated from the methanol extract of the fruit. For this purpose the methanol extract (1.6833gm) was precipitated with methanol by putting the water drop by drop, and then the precipitate was removed by centrifugation. The weight of precipitate thus obtained was 0.89% (15mg) of the total weight (1683.3mg) of the extract. The supernatant was dried through rotary evaporator. The dried sample was then used for preparative TLC. From the preparative TLC four impure and three pure compounds were obtained.

Fig. 3: TLC profile of Soxhlet methanol extract using solvent system of Chloroform: Methanol: Acetic acid (3:1:1)

Compound 1

Fructose

^{1}H NMR (500 MHz, D$_2$O): δ 4.16 (m, fur H-3, 4), 4.09 (m, pyr H-5, 6a), 3.95 (m, pyrH-4), 3.88 (m, furH-5, 6a), 3.84 (m, pyrH-3), 3.76 (m, pyrH-1a, 6b), 3.72 (m, furH-6b), 3.64 (m, furH-1a), 3.61 (m, pyrH-1b), 3.60 (m, furH-1b); ^{13}C NMR (125 MHz, D$_2$O): δ 103.0 (fur C-2), 99.6 (pyr C-2), 82.2 (fur C-5), 76.9 (fur C-3), 76.0 (fur C-4), 71.2 (pyr C-4), 70.7 (pyr C-5), 69.1 (pyr C-3), 65.4 (pyr C-1), 64.9 (pyr C-6), 64.2 (fur C-1), 63.8 (fur C-6).According to the spectral data, the fructose was identified as the mixture of β-D-fructopyranose and β-D-fructofuranose (fig. 4).

Compound 2

Glucose

^{1}H NMR (500 MHz, D$_2$O): δ 5.28 (m, αH-1), 4.69 (m, β H-1), 3.86−3.94 (m, β H-4, 6a, αH-4, 6a), 3.74−3.84 (m, β H-6b, αH-3, 6b), 3.42−3.60 (m, β H-3, 5, αH-2, 5), 3.29 (m, β H-2); ^{13}C NMR (125 MHz, D$_2$O): δ 97.4 (β C-1), 93.6 (α C-1), 77.4 (β C-5), 77.3 (β C-3), 75.6 (β C-2), 74.3 (α C-3), 73.0 (α C-5), 72.9 (α C-2), 71.2 (α C-4), 71.1 (β C-4), 62.3 (β C-6), 62.1 (α C-6). According to the spectral data, the glucose was identified as the mixture of β-D-glucopyranose and α-D-glucopyranose (fig. 4).

Compound 3

Sucrose

^{1}H NMR (500 MHz, D_2O): δ 5.46 (d, J=3.7 Hz, H-1), 4.26 (d, J=8.8 Hz, H-3′), 4.10 (t, J=8.6 Hz, H-4′), 3.79−3.94 (m, H-3, 4, 6a, 6b, 5′, 6′a, 6′b), 3.72 (s, H-1′a, 1′b), 3.62 (dd, J=9.9, 3.7 Hz, H-2), 3.52 (t, J=9.4 Hz, H-5); ^{13}C NMR: δ 103.7 (C-2′), 92.2 (C-1), 81.4 (C-5′), 76.5 (C-3′), 74.0 (C-4′), 72.6 (C-3), 72.4 (C-5), 71.1 (C-2), 69.2 (C-4), 62.4 (6′), 61.4 (C-1′), 60.1 (C-6). According to the spectral data, the sucrose was identified as the α-D-glucopyranose, (1→2) β-D-fructofuranose (fig. 6).

GC-MS analysis of triglyceride

Hydrolysis of triglycerides and GCMS analyses of fatty acid methyl esters

An aliquot of triglycerides (20 mg) was stirred with KOH in MeOH (3M) (3 h), acidified with HCl and evaporated. The resulting fatty acid mixture was then methylated with CH_2N_2 to afford fatty acid methyl esters, according to the reported procedure (Ramsewak *et al*., 2001). The methyl esters thus obtained were analyzed on a GC capillary column, Agilent J&W VF-5ms GC Column, 30m x 0.25 mm, 0.25µm film thickness attached to a 10m EZ-Guard column with a 7inch cage. The conditions for the analyses were 1µL sample dissolved in hexane, helium carrier gas at a flow rate of 1.5mL/min with a split ratio of 10:1 and temperature gradient with an injector port temperature at 240ºC, held for 2 min, raised to 320ºC at a rate of 40ºC and held for another 10 min.

Antioxidant activity

The free radical scavenging activity was measured by MTT assay using TBHQ and vitamin C as positive control. MTT assay was measured according to standard procedure of Liu and Nair (2010). For testing the extract, an aliquot of 10µL of sample was taken from the stock solution ((10mg/mL) in a capped glass vial (2 ml) and added 190µL of MTT water solution (1mg/mL). The solution was then vertex for 1min and incubated at 37°C for 24h. At the end of incubation, DMSO (200µL) was added to this solution and vortexed again for 1 min. An aliquot (200µL) of the reaction mixture was transferred to a 96-well cell culture plate and the absorbance was measured at 570 nm on a universal microplate reader (Bio-Tek Elx800). Each tested sample was analyzed in duplicate. TBHQ and Vitamin C were prepared in DMSO and used as positive control.

STATISTICAL ANALYSIS

The results are presented as mean values of the triplicated data. MSTATC computer software was used for statistical analysis (Russel and Eisensmith, 1983).

RESULTS

Bioactive compounds in the fruit of Physalis ixocarpa

Effect of extraction procedure on total extract yield and recovery of compounds

Extraction is one of the most important steps in the study of medicinal plants, because it is necessary for the isolation and characterization of bioactive compounds. In the present study two methods of extraction was used to evaluate the effect of extraction on percent extract yield, recovery of bioactive compounds and antioxidant activity of the extracts. Maceration and Soxhlet extraction procedures were compared for the extraction of secondary metabolites from the fruits of *P. ixocarpa* in this study. Data regarding the percent extract yield produced by different extraction methods are shown in fig 1. Extraction from the fruits of tomatillo under ambient condition (Maceration) water extract was obtained in large quantity with extract yield of 3.04% followed by methanol extract (0.503%) and ethyl acetate extract (0.177%). Similarly, Soxhlet extraction method yielded different extracts in the order of methanol > water > ethyl acetate. However, the Soxhlet method was found to have high recovery of different extracts than maceration method of extraction.

Recovery of different compounds from the fruits of Tomatillo

Soxhlet extracts and the extracts produced through maceration showed different TLC profiles indicating that extraction method greatly affect the recovery of compounds from the plant material. Ethyl acetate extract produced through Soxhlet procedure showed the presence of seven compound spots while TLC profile of same extract produced through maceration indicated the presence of four compound spots. The TLC profile of methanol extract produced by maceration procedure has two spots. However, the TLC profile of the methanol extract obtained through Soxhlet assay revealed the presence of seven compound spots on TLC plate.

Ethyl acetate extract was passed through Medium pressure liquid chromatography (MPLC) for the separation of different compounds, and finally two fractions were obtained Wk/116/7 (470.9mg) and Wk/116/5B (15.1mg). MPLC purification of Wk/116/7 revealed the presence of one major and one minor impure compound (fig 2). The major compound was identified as triglyceride through spectroscopic techniques including 2D-NMR and GC-MS spectral analysis**.** Analysis of triglyceride through GCMS showed one major (Rt = 19.7 min) and two minor peaks at Rt = 17.2 and 19.2 min with m/z values of 294, 270 and 296 respectively for methyl esters of linoleic, palmitic and oleic acids (fig 4).

Analysis of the preparative TLC profile of the supernatant obtained from crystallization of methanol extract indicated the presence of seven spots as shown in fig. 3. Among 7 spots, three major spots were recovered as pure compounds and remaining spots were impure compounds. The pure compounds were identified as sugar, glucose and fructose by 2D-NMRspectral analysis.

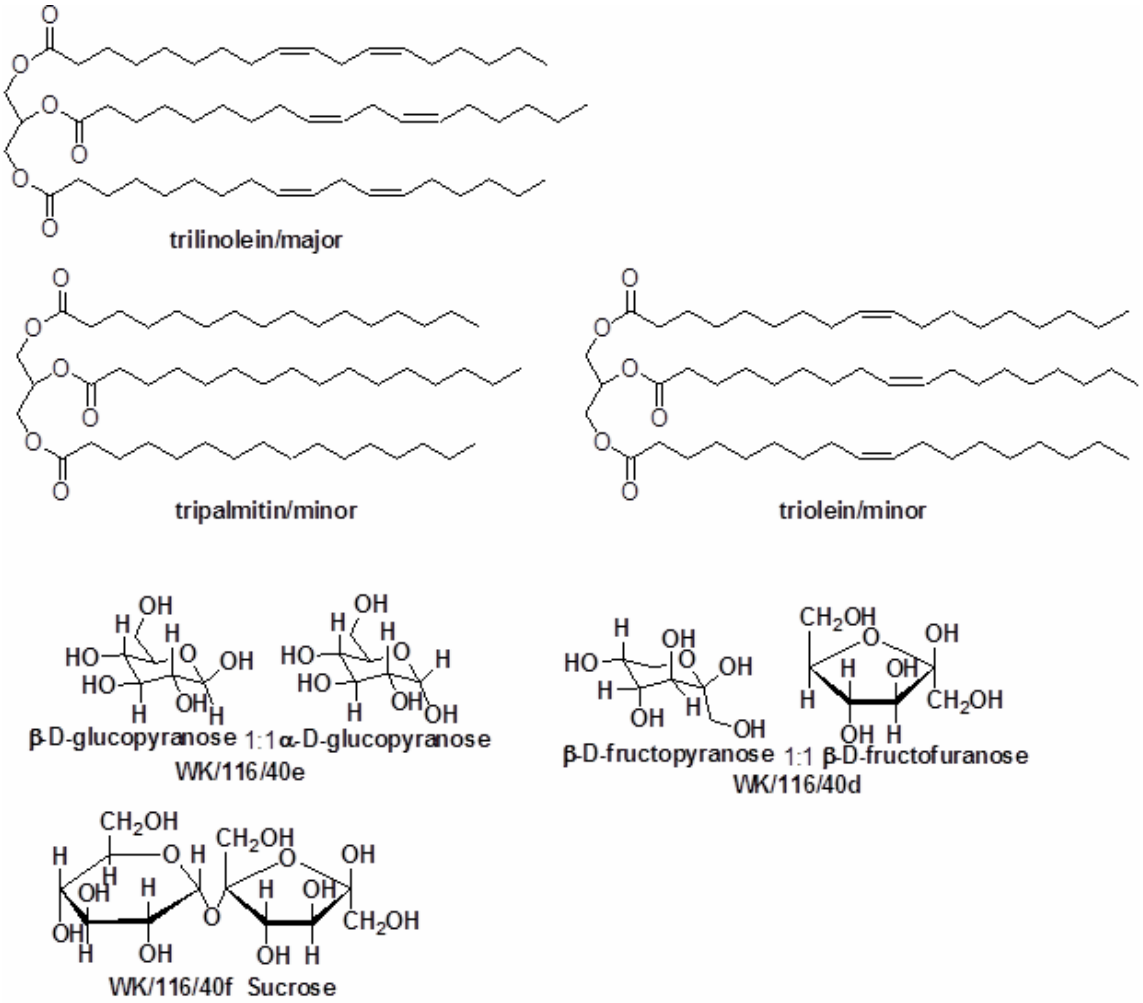

Fig. 4: Structures of fatty acids, glucose, fructose and sucrose detected in the fruit of *Physalis ixocarpa*

Table 1: Comparison of antioxidant activity of the Soxhlet extracts with the extracts produced through Maceration

Extract Sample	Concentration (ppm)	Extraction method	Absorption	%Antioxidant activity
Methanol	250 ppm	Soxhlet	0.155±0.0098	25.4
Ethyl acetate	250 ppm	Soxhlet	0.364±0.0056	59.7
Water	250 ppm	Soxhlet	0.261±0.0282	42.7
Methanol	250 ppm	Maceration	0.122±0.044	20.0
Ethyl acetate	250 ppm	Maceration	0.199±0.0067	32.0
Water	250 ppm	Maceration	0.155±0.0077	25.4
TBHQ			0.64±0.0424	-
Vitanin C			0.61± 0.0424	-
MTT			0.73 ± 0.0014	-

Antioxidant activity of different extracts from fruits of Tomatillo

The total antioxidant activity of different extracts prepared by two extractions methods were measured (table 1). The data indicated that Soxhlet extracts possess strong radical scavenging activity as compared to other extracts. Among the Soxhlet extracts, ethyl acetate extract possess 59.7% of radical scavenging activity followed by water (42.7%) and methanol (25.4 %) extract. Similarly in maceration method of extraction, ethyl acetate extract showed 32.0% of antioxidant activity followed by water (25.4 %) and methanol extract (20.0%).

DISCUSSION

In the present study, two extractions methods were used to evaluate their effect on the percent extract yield, compound recovery and antioxidant activity of the extracts. By comparing the two methods, the Soxhlet extraction could provide better results than maceration

method for extracting compounds; percent extract yield and antioxidant activity of the extracts. Moreover, the Soxhlet extraction showed significant advantage in solvent consumption over maceration. Percent extract yield measures the solvent and extraction method efficiency to extract specific components from the original material, and expressed in percentage. Data regarding the percent extract yield indicated that Soxhlet extraction had high efficiency of recovery over maceration. In maceration, the percent extract yield was found to be in order of water > methanol > ethyl acetate whereas in Soxhlet extraction, it was in order of methanol > water > hexane > ethyl acetate. The percent extract yield of Soxhlet ethyl acetate extract was found to be smaller than same extract produced by maceration, due to the solubility of some compounds in both hexane and ethyl acetate. This might be leaching of compounds to hexane extract which was done before ethyl acetate extract. In Soxhlet extraction the percent extract yield of methanol extract was greater than water extract, which is contrary to maceration method of extraction. This could be due the polarity of the solvent running in opposite direction to each other in the two different method of extraction. However, the overall percent extract yield and recovery of Soxhlet extraction was found to be more effective than maceration method of extraction. Our results are in line with the results of Murugan and Parimelazhagan (2013), who reported that Soxhlet extraction produce high percent extract yield as compared to maceration and fractionation.

Antioxidant property of the different extracts was measured by MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bro-mide] assay. The present investigation indicated that Soxhlet extracts showed strong radical scavenging activity as compared to extracts produced by maceration, suggesting that Soxhlet extraction extract high concentration of antioxidants from the plant material than maceration method of extraction. This study showed that the selection of extraction method is very essential in natural product isolation. Overall, successive Soxhlet extraction was found to be best in obtaining the isolation of compounds; percent extract yield and antioxidant activity than the maceration. The current study also pinpointed the suitable solvent for the optimum radical scavenging in the two different methods of extractions. In both methods, the ethyl acetate was proved as best solvent for the recovery of antioxidant compounds. Similar findings were also reported by Allouche *et al*. (2001), Kalogerakis *et al.* (2013) and Khan *et al*. (2017). For the further study ethyl acetate and methanol extract were selected for the isolation of bioactive compounds based on their antioxidant potential and TLC profile. Seven compounds from methanol extract and two compounds from ethyl acetate fraction were isolated and chemically identified through spectroscopic techniques including 2D-NMR and GC-MS spectral analysis. Among the isolated compounds, four compounds were pure and the remaining needs further purification. The pure compounds include three major compounds from crude methanol extract such as sucrose, fructose, glucose and triglyceride from ethyl acetate extracted fraction. The fructose and glucose purified from methanolic extract were confirmed as mixtures of β-D-fructopyranose and β-D-fructofuranose, β-D- and α-D-glucopyranose respectively, by proton and carbon NMR spectral experiments (Zhang *et al*., 2013; Horton and Walaszek, 1982). Similarly, sucrose was identified as the α-D-glucopyranose and (1→2) β-D-fructofuranose (Horton and Walaszek, 1982).

Analysis of triglycerides through GC-MS revealed the presence of one major and two minor fatty acids in their molecules. The major fatty acid was trilinoleinic acid (9,12-Octadecadienoic acid) and the minor was tripalmitin (hexadecanoic acid) and trioleinic acid (9-Octa decenoic acid). The presence of 9,12-Octadecadienoic acid in ethyl acetate extract may be the possible reason for its antioxidant activity. The strong antioxidant and antimicrobial potential of this fatty acid was already reported in previous studies (Murugan and Parimelazhagan, 2013; Gobalakrishnan *et al*., 2014).

CONCLUSION

From the current study it is concluded that Soxhlet extraction had high efficiency of recovery than maceration method for extracting compounds; percent extract yield and antioxidant activity of the extract. Moreover ethyl acetate was selected as best solvent for the optimum radical scavenging in the two different methods of extractions.

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