FATTY ACIDS IN AVOCADO OIL AND NUTRITIONAL, ANTIOXIDANT AND ANTIMICROBIAL ASSESSMENT OF THE PULP

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Abstract

Avocado (*Persea americana* Mill.) fruits contain many nutritional components which have medicinal importance as well. The oil (81%) from the fruit pulp was extracted by Soxhlet extraction with petroleum ether. The fatty acids in the oil were identified by GC-MS analysis by NIST spectral library. Eleven fatty acids were identified in the ester fraction: two unidentified acids; myristic acid; palmitoleic acid; palmitic acid; linoleic acid; petroselinic acid; oleic acid, as well as (11Z)-11-octadecenoic acid, stearic acid andisostearic acid, in the increasing order of retention time up to 23 min. One long chain alcohol and one free fatty acid were also suggested to be present in the pulp oil by FT IR. Avocado pulps were then analyzed for nutritional values present by using AOAC methods. The observed data revealed that avocado pulp had high energy value (344 kcal/100g). Moisture content (5.56 %) and ash content (5.23 %) were calculated in dried sample of avocado pulp. Protein, fat, fiber and carbohydrate contents were 6.55 %, 4.18 %, 8.14 % and 70.34 %, respectively. Agar well diffusion method was adapted to determine the antimicrobial activity of the avocado pulp extracts (PE, MeOH, EtOAc, EtOH and H_2O). The MeOH, EtOAc and EtOH extracts of avocado pulp were active against *Bacillus subtilis, Staphylococcus aureus, Bacillus pumilus, Candida albicans*and *Escherichia coli* while PE and H_2O extracts were inactive on all the tested microorganisms. All the extracts were inactive on *Pseudomonas aeruginosa.* In addition, the antioxidant activity of avocado pulp was slightly higher in the watery extract (IC_{50} = 19.32 µg/mL) than the ethanol extract (IC_{50} = 21.84 µg/mL) by DPPH assay method. The present study therefore confirms the nutritional and medicinal values of the avocado produced in Taunggyi.

Keywords: Avocado, fatty acids, nutritional values, antimicrobial activity, antioxidant activity

Introduction

There is currently great popular interest in natural products, which has motivated the search for a new sauce of bioactive compounds that can be beneficial for human consumption that can be beneficial for human consumption in place of synthetic compounds used in food industry as additives, and can contribute to the reduction of generated waste giving them a much more beneficial destination (Rodriguez-Carpena *et al.,* 2011). The avocado (*Persea americana* Mill.) is a fruit with antioxidant and antibacterial properties, produced in almost all tropical and subtropical regions of the world (Kate and Lucky, 2009). The avocado has an olive-green peel and thick pale yellow pulp that is rich in fatty acids such as linoleic, oleic, palmitic, stearic, linolenic, capric and myristic acids. This fruit is normally used for human consumption, but it also has been used as medicinal plant in Mexico and elsewhere in the world (Dreher and Davenport, 2013). The aim of this research was to study the fatty acid composition and nutritional values of avocado pulp oil and to evaluate the antioxidant and antimicrobial activity of avocado pulp.

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Materials and Methods

Plant Material

Fresh avocadopulps were collected from vegetable and fruit Myoma Market Taunggyi Township. The plant material was identified and authenticated at Department of Botany, Taunggyi University. The pulps were cut into small pieces and dried at room temperature. This sample was ground into powder in an electric blender and stored in airtight container.

Extraction of Avocado Pulp Oil

The powdered sample of pulp fruit (Avocado) (60 g) was weighed placed in a thimble made from Whatman paper No. 1 and then placed in a Soxhlet extractor. Petroleum ether was poured into the extractor until some of it overflowed into the flask. Petroleum ether was heated by means of a water bath. The extraction was assumed to be complete when a small amount of extract placed on a watch glass did not leave any residue on evaporation of solvent. Duration of about 5 h was required for the complete extraction during which time the petroleum ether was recycled about 30 times. The petroleum ether was removed by simple distillation until the stickey mass obtained. The above procedure was repeated with another two portions of the pulp fruit sample (180 g). The extracts were combined and then concentrated by distilled yielding brownish yellow colour crude extract (80.43 g).

Transesterification of Avocado Pulp Oil to Methyl Ester

The molecular weight of avocado pulp oil was taken as 880 g/mol, which was supposed to be a reasonable estimate (Watson, 2014). Avocado oil (11.2 g, or 25 mL) was placed in a 250 mL round-bottom flask, mixed with KOH (6 pellets, *i.e.*, *ca* 600 mg),about 5.4% of the mass of oil, and then with methanol (2.5 g, calculated mass corresponding to 6:1 molar ratio of methanol to oil, *i.e.*., twice the stoichiometric amount). The flask was then fitted with a reflux condenser and the mixture was stirred-heated in a water bath (bath temperature 65 - 70 °C) placed on a magnetic stirrer heater for 45 min with rapid stirring to ensure good mixing between the methanol and the avocado oil. At the end of the reaction, a clearer and less viscous mixture was obtained, indicating formation methyl esters from the original oil. This was also shown by the TLC (silica gel; hexane-diethyl ether-acetic acid, 85:15:1) using anisaldehyde-sulphuric acid spraying reagent. Methyl esters migrated a little higher (R_f 0.72) than the original oil (R_f 0.64) (Figure 1).

To the reaction product mixture the calculated volume (8 mL) of 1.25 M acetic acid was added slowly to neutralize the KOH. Then the mixture was stirred vigorously for a few minutes and then transferred to a 250 mL separatory funnel. Upon standing, separation of two distinct layers was observed. The lower layer of glycerol was drained out and the top layer was poured into an Erlenmeyer flask, dried over anhydrous sodium sulphate and filtered into a tared round-bottom flask. From the mass of the dried transesterified oil (8.0 g), the yield was calculated (yield 76.2 %). The crude reaction mixture (0.6 g) was fractionated on a silica gel column (40 g, diameter 1.7 cm) by eluting with hexane-diethyl ether-acetic acid (85 : 15 : 1). Three fractions A, B and C were obtained. The purified methyl ester mixture A was submitted for GC-MS analysis, as well as for recording the FT IR spectra. The fractions B and C were also submitted for recording FT IR spectra.

Figure 1 Co- TLC chromatogram of transesterified and original avocado pulp oils

Determination of Nutrient Values of Avocado Pulp

The nutrient values (moisture, ash, fiber, fat, protein, carbohydrate and energy value) of avocado pulp were determined by AOAC methods at Union of Myanmar Federation of Chambers of Commerce and Industry (UMFCCI).

Preparation of Extracts

Sample of avocado pulp (20 g) was made into small pieces and subjected for homogenization for MeOH, EtOH, PE, and water. The obtained extracts were filtered with filter paper. The filtrates obtained were evaporated at rotary evaporator at 50 ˚C. The extracts were used to determine antioxidant and *in vitro* antimicrobial activities.

Preparation of Sample Solution

Sample (4 mg) and 10 mL of EtOH were thoroughly mixed by shaker. The mixture solution was filtered and the stock solution was obtained. The sample solutions (40, 20, 10, 5, 2.5, 1.25, 0.625 μg mL^{-1} concentration) were prepared from this stock solution by dilution with appropriate amount of EtOH.

Free Radical Scavenging Activity by DPPH Method

The effect on DPPH radical was determined using the method by Marinova and Batchvarov (2011). The control solution was prepared by mixing 1.5 mL of 60 M DPPH solution and 1.5 mL of EtOH using shaker. The test sample solution was also prepared by mixing thoroughly 1.5 mL of 60 M DPPH solution and 1.5 mL of sample solution. The mixture solutions were allowed to stand at room temperature for 30 min. Then, the absorbance of these solutions was measured at 517 nm by using UV-7504 spectrophotometer. Absorbance measurements were done in triplicate for each concentration and then mean values so obtained were used to calculate percent inhibition of oxidation by the following equation. The capability to scavenge the DPPH radical was calculate using the following equation:

$$\% \text{ Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where, $A_{control}$ = absorbance of control solution

A_{sample} = absorbance of tested sample solution.

IC_{50} value was calculated by linear regressive excel program. The results are described in Table 3 and Figures 9 and 10.

Screening of Antimicrobial Activity

Nutrient agar was prepared according to the method described by Cruickshank (1975). Nutrient agar was boiled and 20–25 mL of the medium was poured into the test tube and plugged with cotton wool and sterilized at 121°C for 15 min in an autoclave. After this, the tubes were cooled down to 30–35 °C and poured into sterilized petridishes and 0.1–0.2 mL of the test organisms were added into the dishes. The agar was allowed to set for 2–3 h; then 10 mm agar well was made by the help of sterilized agar well and cutter. After that, about 0.2 mL of the sample was introduced into the agar well and incubated at 37°C for 24 h. The inhibition zone which appeared around the agar well indicated the presence of antimicrobial activity.

Results and Discussion

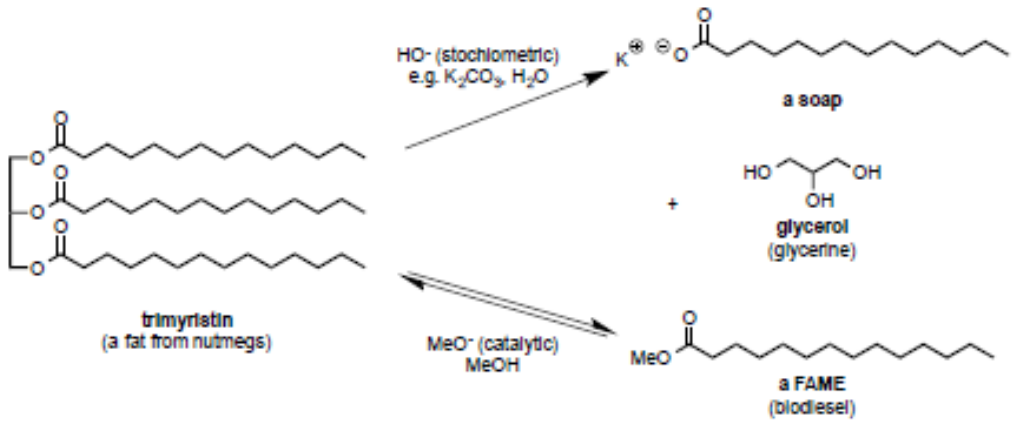
Extraction of Avocado Oil

Extraction of avocado pulp oil was carried out by transesterification method and yield percentage was determined to be 81 %.

Analysis of Fatty Acids in the Avocado Pulp Oil

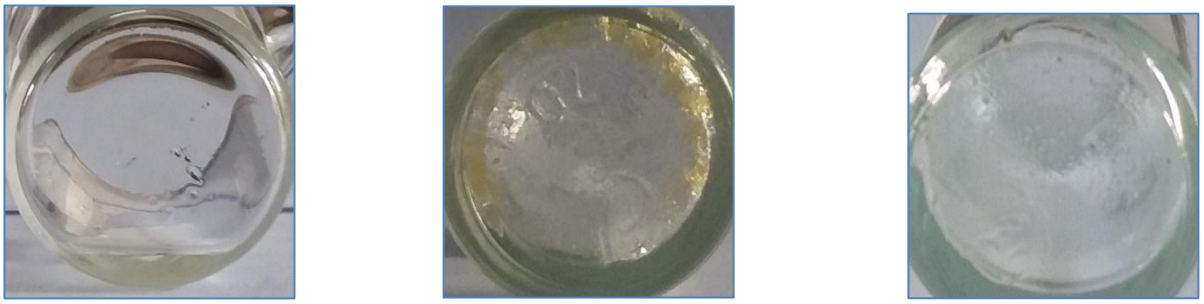
To investigate the fatty acids making up the glyceride of avocado pulp oil by GC-MS, they need to be converted to a more volatile compound. Thus in the present work methyl esters of the fatty acids were prepared by transesterification reaction.

In a transesterification reaction, alcoholysis is done using a small amount of base such as KOH or K_2CO_3 as a catalyst. The reaction is reversible and excess amount of alcohol (methanol in the present work) is used to drive the reaction to completion to give another ester (fatty acid methyl esters or FAMEs in the present work). This is unlike hydrolysis of ester under basic condition, where the carboxylic formed is irreversibly deprotonated to a salt, namely a soap; the base is consumed as part of the reaction product. It may be noticed that transesterification requires a catalytic amount of base, whereas a stoichiometric amount of base is needed for saponification. Transesterification and saponification are illustrated below for trimyristin, which is a major constituent of nutmegs.



The crude transesterified oil (0.6 g) was fractionated on a silica gel (40 g) in a glass (diameter 1.7 cm) column by eluting with the solvent system,n-hexane-diethyl ether-acetic acid, (85:15:1), yielding a major fraction of a colourless clear oil **A** (R_f 0.72, purple on heating with anisaldehyde-sulphuric acid reagent) (0.2 g, yield 33 %, based on crude transesterified oil) supposed to be the pure mixture containing only methyl esters of fatty acids making up the glycerides of the avocado oil investigated.

This fraction was obtained by combining individually collected fractions to yield fraction B (yellow crystals, R_f 0.28 purple spot with anisaldehydesulphuric acid) (4 mg, yield 0.66 %, based on crude transesterified oil) and fraction C (oily, R_f 0.91, purple spot with anisaldehyde-sulphuric acid) (0.9 mg, yield 0.15 % based on crude transesterified oil (Figures 2 and 3).

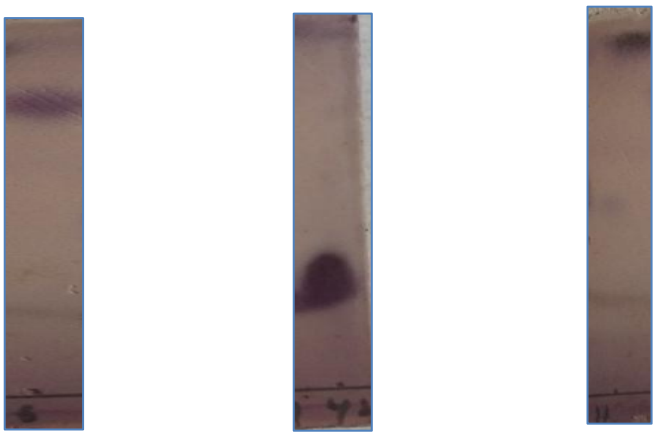


Fraction A
Methylated ester

Fraction B
Free acid

Fraction C
Long chain alcohol

Figure 2 Photographs of fractions A, B, C from avocado pulp oil



A B C

Solvent reagent - Anisaldehyde - conc: sulphuric acid
Solvent system - n-hexane : EtOAc : CH_3COOH
85 : 15 : 1
R_f value of A, B, C - 0.7201, 0.2972, 0.9189

Figure 3 Thin layer chromatograms of the fractions A, B, C

Analysis of Ester Fraction A

Fraction A is the methyl esters of the fatty acids making up the glycerides in the avocado pulp oil and GC-MS analysis showed the variety of these fatty acids.

First fraction A was first confirmed by its IR spectrum as esters (Figure 6 and Table 1). Next, the variety of methyl esters was indicated by GC-MS analysis of A. Thus, methyl ester of palmitic, palmitoleic and myristic acid are shown to be present in A (Figures 4 and 5a). Furthermore, two more fatty acids were also shown to be present by GC-MS (Figure 5b). Unfortunately, presence of the reported (Kadam and Salunkhe, 1995) major ester, methyl oleate could not be shown. This is supposedly caused by stopping too early the elution time, before methyl oleate could be eluted.

Analysis of fractions B and C

From their FT IR spectra, fraction B was shown to be fatty acid (Figure 7 and Table 1). As shown by TLC (Figure 3) they are present as free acids in the original avocado pulp oil. Similarly, fraction C was deduced as long chain alcohol (s) (Figure 8 and Table 1).

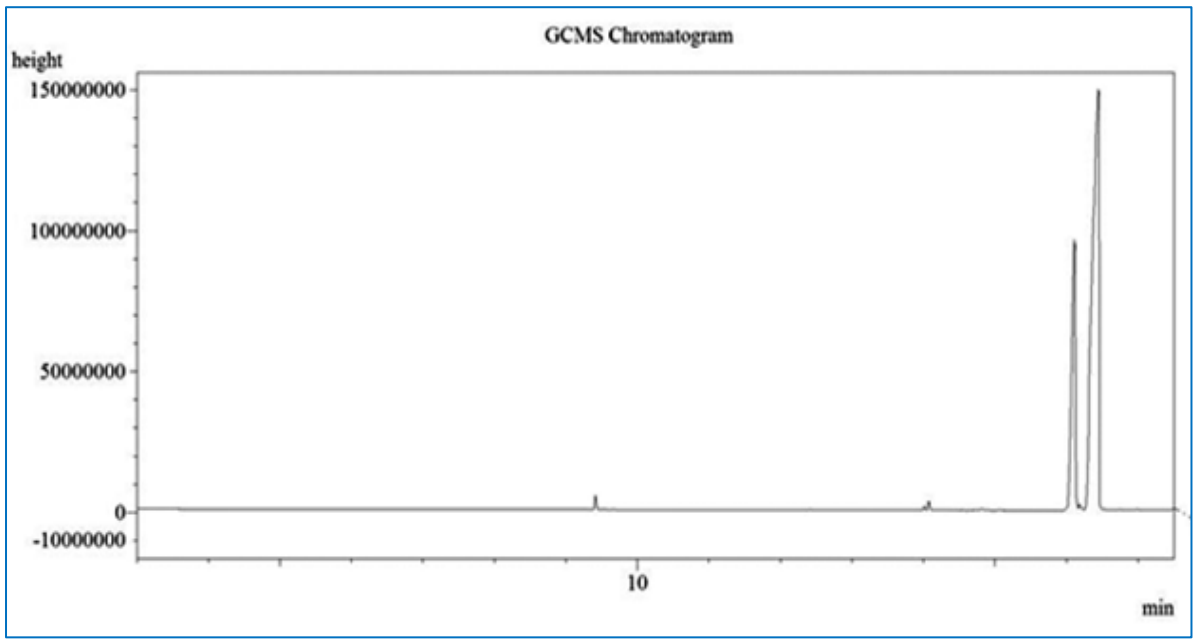


Figure 4 Gas chromatogram of separated methyl esters fraction A

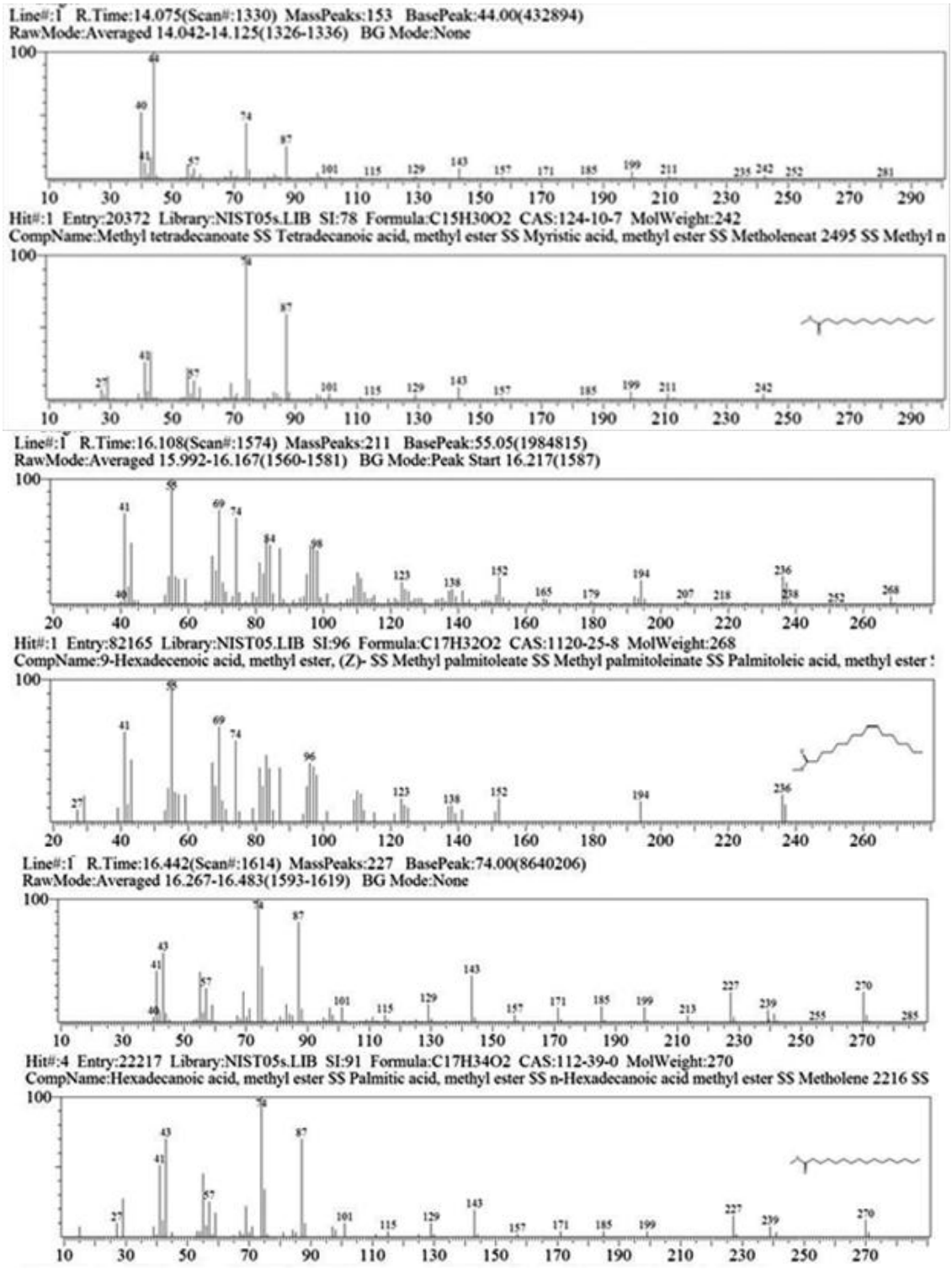


Figure 5a Identification of methyl esters of myristic (RT 14.075 min), palmitoleic (RT 16.108 min), and palmitic (RT 16.442 min) acids by comparison of the EI mass spectra of GC eluted compounds with respective library standard compounds

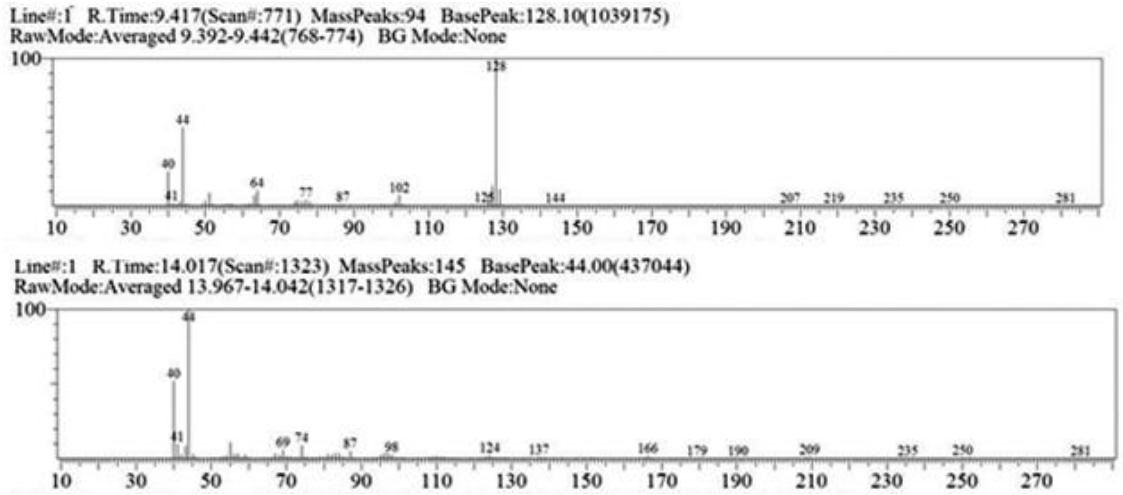


Figure 5b EI mass spectra of methyl esters of two unidentified fatty acids eluted with retention times 9.417 and 14.017 min from GC

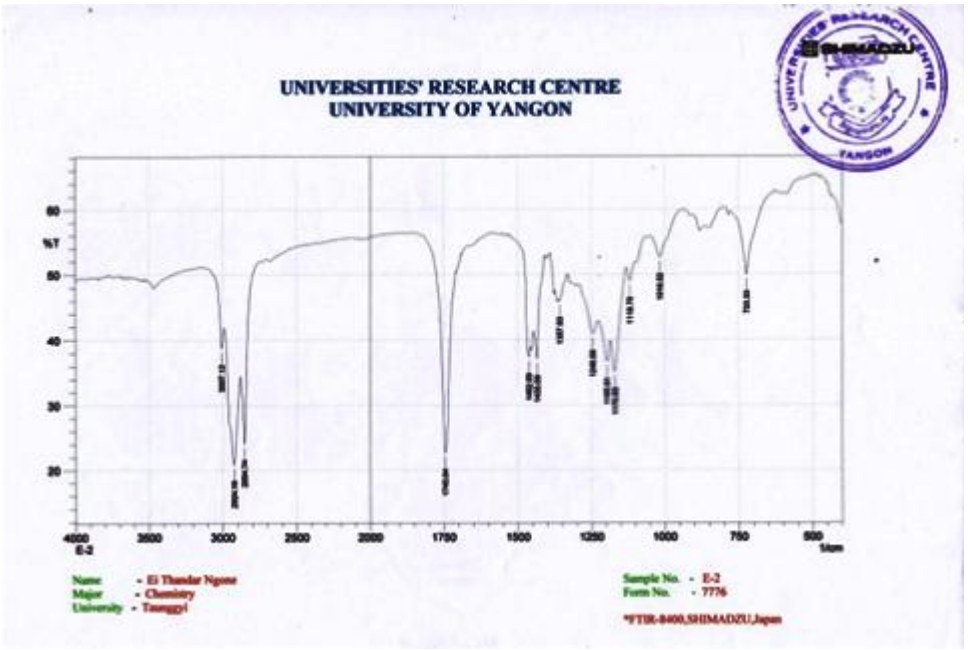


Figure 6 FT IR spectrum of isolated compound A from avocado pulp oil

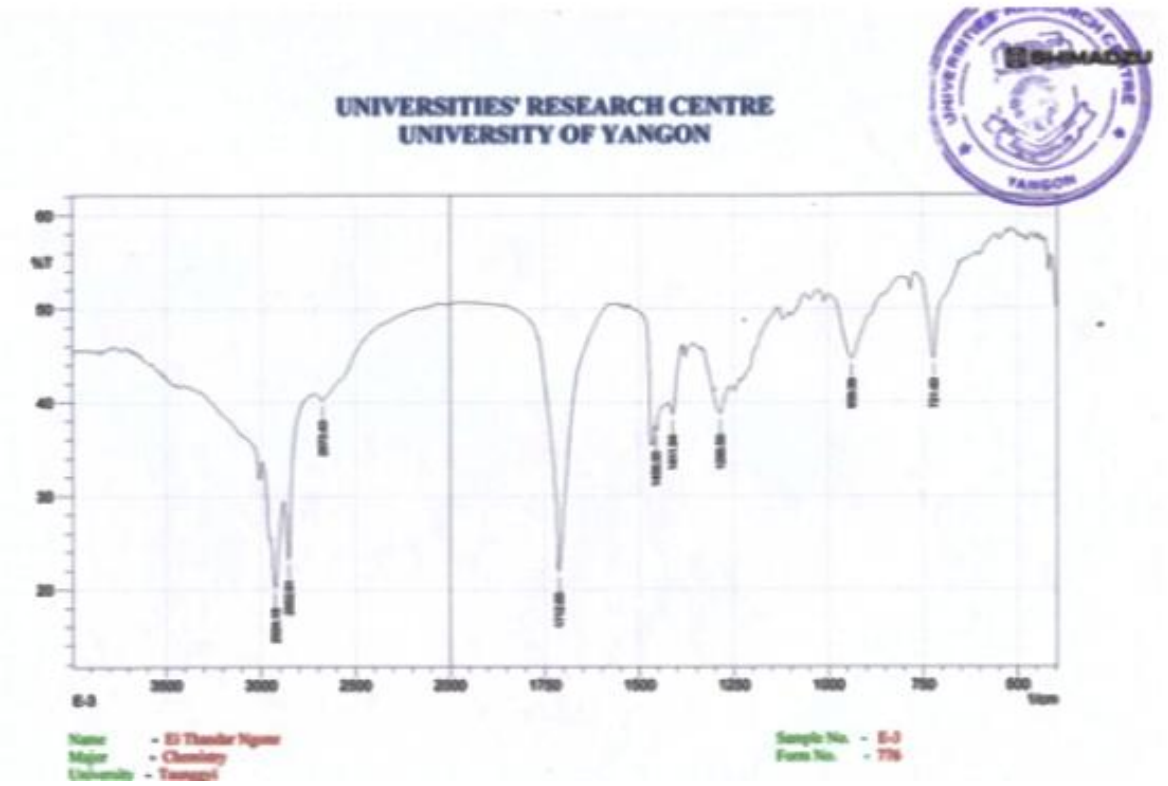


Figure 7 FT IR spectrum of isolated compound B from avocado pulp oil

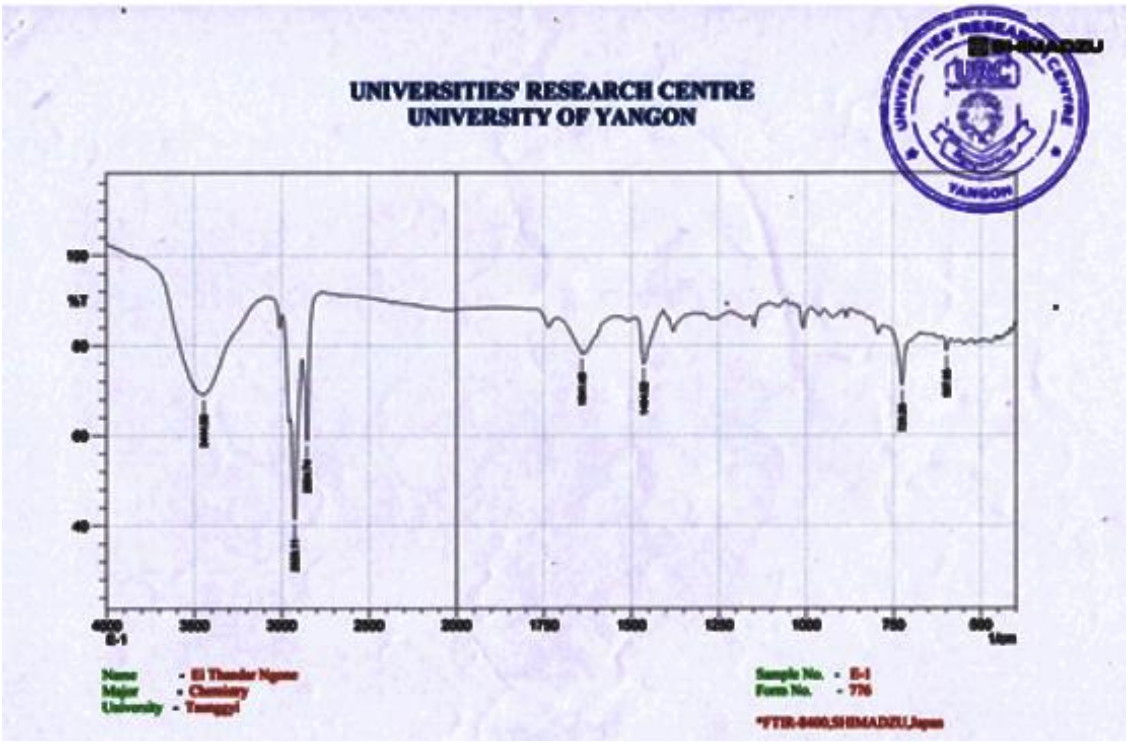


Figure 8 FT IR spectrum of isolated compound C from avocado pulp oil

Table 1 FT IR Spectral Data of the Fractions A, B and C from Avocado Pulp Oil

Sample code	Wavenumber (cm^{-1})								
	ν_{O-H}	$\nu_{=C-H}$	ν_{C-H}	$\nu_{C=O}$	$\nu_{C=C}$	$\delta_{as\ C-H}$	$\delta_{s\ C-H}$	ν_{C-O}	$\delta_{oop\ =C-H}$
A	-	3007	2924, 2854	1745	-	1462	1357	1195, 1170, 1016	723
B	3500-2500	3007	2924, 2852	1712	-	1456	1375	1286	939,721
C	3444	3007	2926, 2854	-	1641	1464	1375	1010	725

Nutrient Values of the Avocado Pulp

The nutritional values such as moisture, ash, crude fibre, fat, protein and carbohydrate in the avocado pulp fruit were estimated according to the AOAC methods conducted at UMFCCI. The results are shown in Table 2.

Table 2 Nutrient Values of Pulp of *Persea americana* Mill.

No.	Test Parameter	Test Method	Proximate Composition (%)
1	Moisture	AOAC	5.56
2	Ash	AOAC	5.23
3	Protein	AOAC (Kjeldahl Method)	6.55
4	Crude Fibre	AOAC (Fibre Cap Method)	8.14
5	Fat	AOAC(Buchi Soxhlet Method)	4.18
6	Total Carbohydrate	By difference	70.34
7	Energy value (kcal/100 g)		344

Antioxidant Activity of Crude Extracts of Avocado Pulp

Antioxidant benefits our health by cleaning free radicals out of our bloodstream. Different antioxidants benefit different parts of the body. Antioxidant activity of 95% ethanol, water extracts and standard ascorbic acid were estimated by measuring the DPPH radical scavenging activity of different concentrations of extracts. It is based on the reduction of colour of free radical DPPH in ethanolic solution by different concentration of samples. In this experiment, seven different concentrations 0.625, 1.25, 2.5, 5, 10, 20 and 40 μg/mL of each crude extract in ethanol and standard ascorbic acid in water were used. Determination the absorbance of each solution was measured at 517 nm using UV-visible spectrophotometer. The DPPH is a stable free radical to decolourize in the presence of antioxidants. The DPPH free radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the charges in absorbance. It was found that as the concentrations were increased, the absorbance values were decreased. The radical scavenging activity of crude extracts was expressed in term of percent inhibition and IC_{50} (50% inhibition concentration) values. The IC_{50} values were calculated after linear regression analysis

of the observed inhibition percentage Vs concentration of sample, where lower IC_{50} values indicated antioxidant activity.

The IC_{50} values for crude extracts and standard ascorbic acid are shown in Table 3, Figures 9 and 10. The IC_{50} values of water, ethanol and standard ascorbic acid were observed to be 19.32, 21.84 and 1.22 µg/mL, respectively. The IC_{50} value of water extract is lower than that of ethanol extract. All extracts showed very mild antioxidant activity when compared to standard ascorbic acid (1.22 µg/mL).

Table 3 % RSA (Radical Scavenging Activity) and IC_{50} Values of Crude Extracts of Avocado Pulp

No.	Extract	% RSA (mean ± SD) in different concentrations (µg/mL)							IC_{50} (µg/mL)
		0.625	1.25	2.5	5	10	20	40	
1	Ethanol	26.61 ±0.75	29.94 ±0.21	31.67 ±0.52	35.97 ±0.21	43.10 ±0.98	49.41 ±0.84	56.06 ±0.32	21.84
2	Watery	25.99 ±0.55	28.55 ±0.48	32.85 ±0.21	35.00 ±2.12	41.03 ±0.43	50.66 ±0.24	63.13 ±0.79	19.32
3	Ascorbic acid	25.92 ±0.79	51.14 ±0.75	60.43 ±0.32	72.07 ±0.60	77.20 ±1.15	83.09 ±0.12	90.23 ±0.72	1.22

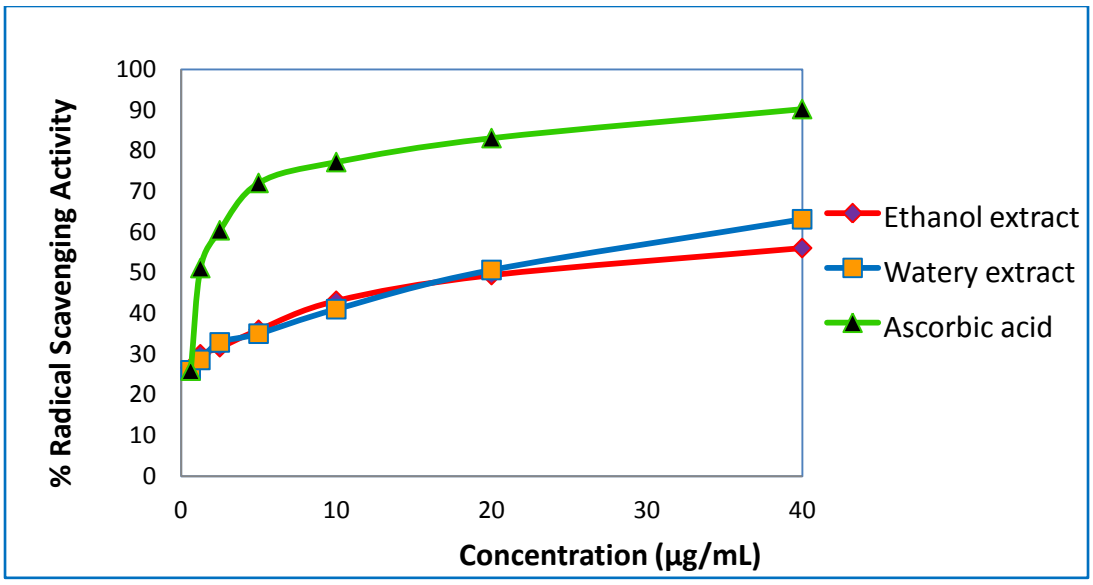


Figure 9 Radical scavenging activity of different concentrations of crude extracts of avocado pulp

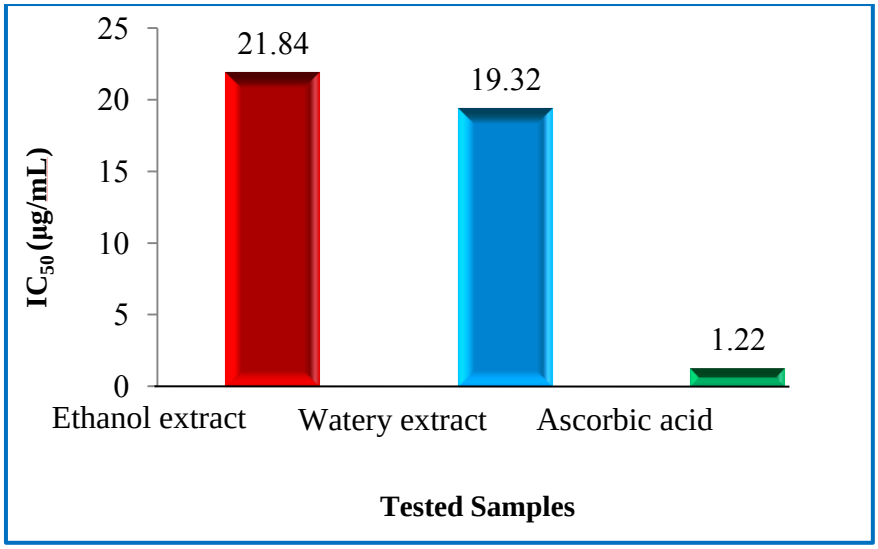


Figure 10 A bar graph of IC_{50} values of crude extracts of avocado pulp

Antimicrobial Activity of Crude Extracts by Agar Well Diffusion Method

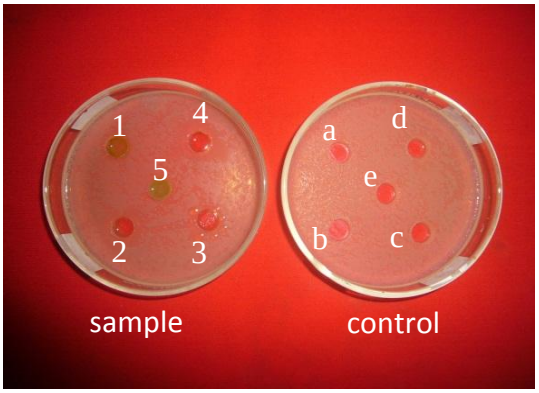
The crude extracts were tested against six pathogenic microorganisms by using agar well diffusion method. MeOH, EtOAc and EtOH extracts of the pulp were active by agar well diffusion method on the five microbial species tested namely *Bacillus subtilis*, *Staphylococcus aureus, Bacillus pumilus, Candida albicans* and *Escherichia coli* but inactive on *Pseudomonas aeruginosa*. Non-polar extract PE and H_2O extracts were inactive by agar well diffusion method on the six microbial species. The photograph of inhibition zone diameters of different crude extracts against the six species of organisms tested are shown in Figure 11, and the results are summarized in Table 4.

Table 4 Antimicrobial Activity of PE, EtOAc and H_2O Extracts from Pulp of Avocado Pulp by Agar Well Diffusion Method

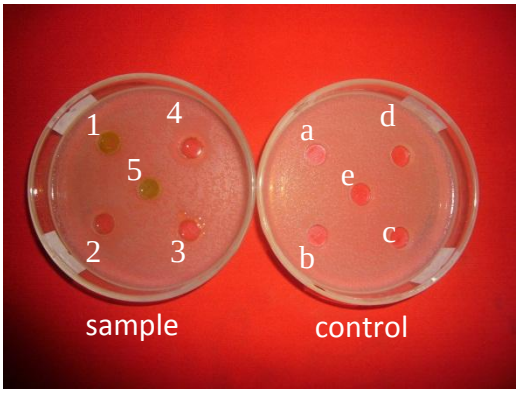
No.	Extracts	Diameter of Zone of Inhibition against different microorganisms (mm)					
		I	II	III	IV	V	VI
1	Pet-Ether	–	–	–	–	–	–
2	MeOH	13 (+)	13 (+)	–	12 (+)	13 (+)	13 (+)
3	EtOAc	11 (+)	12 (+)	–	13 (+)	11 (+)	12 (+)
4	EtOH	12 (+)	13 (+)	–	12 (+)	12 (+)	12 (+)
5	H_2O	–	–	–	–	–	–

Agar well – 10 mm
10 mm ~ 14 mm (+)
15 mm ~ 19 mm (++)
20 mm above (+++)

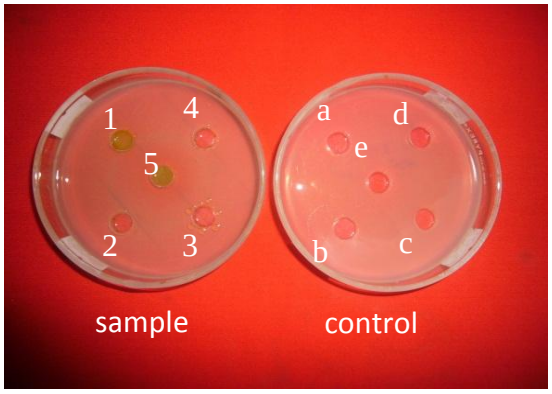
Organisms
I – *Bacillus subtilis*(N.C.T.C-8236)
II – *Staphylococcus aureus*(N.C.P.C-6371
III – *Pseudomonas aeruginosa*(6749)
IV – *Bacillus pumilus*(N.C.I.B-8982)
V – *Candida albicans*
VI – *Escherichia coli* (N.C.I.B-8134)



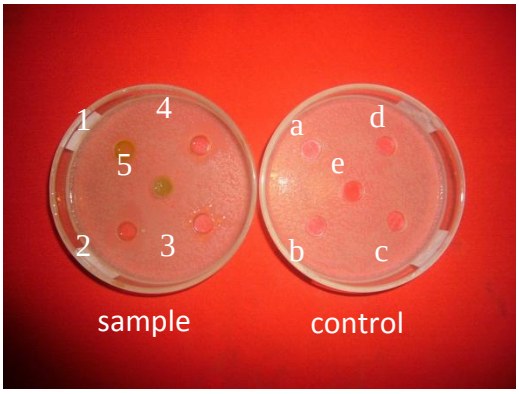
Bacillus subtilis



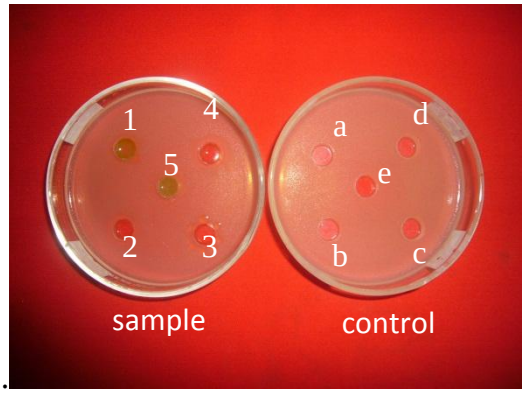
Staphylococcus aureus



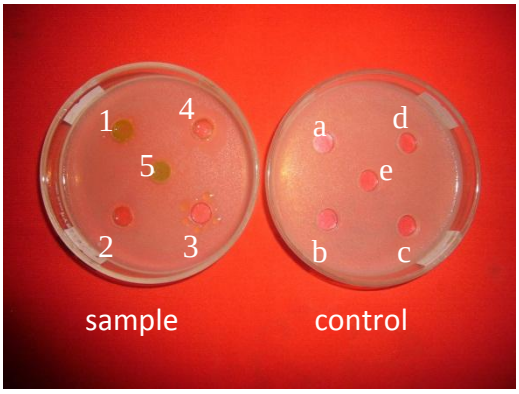
Pseudomonas aeruginosa



Bacillus pumilus



Candida albicans



Escherichia coli

1 – PE extract

2 – MeOHextract

3– EtOAcextract

4 – EtOHextract

5 – H_2O extract

a – PE control

b – MeOH control

c – EtOAc control

d – EtOH control

e – H_2O control

Figure 11 Antimicrobial screening of PE, MeOH, EtOAc, EtOH and H_2O extracts from the avocado pulp by agar well diffusion method

Conclusion

Avocado pulp oil (81 %) was extracted by Soxhlet extraction with petroleum ether. GC-MS analysis of avocado oil after transesterification to fatty acid methyl esters, showed the presence of eleven fatty acids namely, myristic, palmitoleic, palmitic, linoleic, petroselinic, oleic, [11z]-11-octadecenoic, stearic, isostearic acids and two unidentified acids. Moreover, a long chain alcohol and a free fatty acid were also identified by FT IR in the avocado pulp oil. The nutritional values of avocado pulp were moisture (5.56%), ash (5.23 %), protein (6.55 %), fiber (8.14 %), fat (4.18%), carbohydrate (70.34 %) and energy value (344 kcal/100g). The antioxidant activity activity of the avocado pulp by DPPH assay was slightly higher in the water extract (IC_{50} = 19.32 µg/mL) than the ethanol extract (IC_{50} = 21.84 µg/mL). In the screening of antimicrobial activity, against *B. subtilis,S. aureus, B. pumilus, C. albicans* and *E. coli*, the ethanol, methanol and ethyl acetate extracts of Avocado pulp were active, whereas petroleum ether and water extracts were inactive on all the tested microorganisms. Therefore, avocado species can be taken as a good source of nutritious food.

Acknowledgements

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