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Anti-α-glucosidase and anti-inflammatory activities of fatty acid, fatty acid ester, and phytosterol compounds from papaya (*Carica papaya* L.) seed extracts

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Abstract

Papaya (*Carica papaya* L.) is a popular fruit in Thailand with numerous health benefits owing to its high content of vitamins and enzymes, which are associated with the prevention and treatment of gastrointestinal tract disorders. Papaya seeds are considered as agricultural waste, and the information about the bioactive compounds is limited by the currently available identification techniques. This study determined the chemical components present in the oil extracts of the Holland papaya seeds. The anti-α-glucosidase activity and nitric oxide accumulation, which are associated with the anti-inflammatory properties, were detected. Gas Chromatography-Mass Spectrometry (GC-MS) was used to identify the chemical components in the hexane and ethyl acetate seed extracts. As a result, abundant bioactive compounds, which could be categorised into three groups based on their structure, including phytosterol compounds, fatty acid esters and fatty acids, were detected in the papaya seed oil extracts. Additionally, the extracts produced good inhibitory effects on α-glucosidase, whereas the hexane extract exhibited anti-inflammatory properties without causing toxicity to cell lines. This study provides valuable information that could be potentially useful for increasing the value of agricultural and industrial papaya seed waste in the future.

Keywords: Agricultural residues, Gas Chromatography-mass Spectrometry (GC-MS), Natural extract, Papaya, Seed extract content

1. Introduction

Papaya (*Carica papaya* L.) is an economically important plant of the Caricaceae family, which is cultivated for its fresh fruit or vegetable in many countries, especially in the tropical areas. Keakdam and Holland are the most popular papaya cultivars in Thailand because of their superior taste and ease of cultivation. The pharmacological properties of papaya fruits have been well established, such as strengthening of the immune system and prevention of colon cancer. Ripe fruits are rich in vitamins A, B, C and minerals [1]. Apart from the fruits, extracts of papaya stems and leaves have also shown to reduce pain and swelling, increase blood clotting and enhance blood circulation. Papaya leaf extract is reported to exhibit anti-diabetic effect by reducing the activity of α-glucosidase [2], while the papain enzyme found in papaya's latex has anti-inflammatory properties, and can stimulate wound healing. Moreover, papaya roots have been used to increase diuresis [3].

Papaya fruits produce numerous small black seeds, which are a rich source of nutraceutical ingredients. Papaya seeds predominantly accumulate biochemical components, such as proteins, fibres, complex carbohydrates, oils, fatty acids, as well as bioactive chemicals, including terpenoids, polyphenols and phytosterols [4]. A previous review on *C. papaya* phytochemicals demonstrated that the seeds could counteract oxidative stresses and act as anti-inflammatory agents by limiting the production of inflammatory cytokines, which are the key molecules that trigger the inflammatory process [5]. The Sri Lankan papaya seeds were shown to predominantly accumulate terpenoids, polyphenols, and phytosterols, which are major antioxidant and anti-oxidative stress molecules [6]. Benzyl isothiocyanate is a key bioactive compound present in papaya seeds, which has been extensively

demonstrated to display anti-cancer and anthelmintic activities [7]. Similarly, the bioactive compounds in the methanolic extract of papaya fruit and seeds have been reported to demonstrate antibacterial and anti-oxidative properties [1,8-10]. For example, Zhou et al (2011) showed that vanillic acid and *p*-hydroxybenzoic acid from papaya seeds had significant antioxidant properties [10]. As a rich protein source, papaya seed has been considered as a functional feed in poultry nutrition [11].

The analyses of chemical bonding and mass characteristics using Fourier Transform Infrared Spectrometer (FT-IR) and Gas Chromatography-mass Spectrometry (GC-MS) have revealed that the phytochemicals present in the seeds are polar and hence, methanol and ethanol are mostly used for extraction due to the polar properties of the solvents [12-15]. However, the identification of non-polar phytochemicals inside the papaya seeds remains unreported, with studies extensively reporting only the predominant chemical groups in the papaya seed oil and not individual chemicals. Hence, this study aimed to characterise the chemical components in papaya seed oil extracted with hexane and ethyl acetate using GC-MS, and to perform chemical fingerprinting by comparing the peaks of identified compounds with their references in the National Institute of Standards and Technology (NIST) library. The crude extracts of both the solvents were then subjected to anti-α-glucosidase test to evaluate their ability to decrease or inhibit blood glucose level. In addition, the capacity of the extracts to reduce nitric oxide content in RAW264.7 cells was evaluated to determine their anti-inflammatory properties.

2. Materials and methods

2.1 Preparation of papaya seed extracts

Holland papaya seeds were collected during the rainy season from May to July 2020 in Nakhon Pathom province, Thailand. The seeds were gently washed with water without removing the outer coat and baked at 60°C for 20 h before grinding with a mortar and pestle. About 50 g of the ground seeds was mixed with 250 mL of hexane in a sealed Erlenmeyer flask, agitated overnight at room temperature and the hexane-extracted supernatant was separated from the papaya seed residue after 24 h. The remaining ground seed sample was mixed with 250 mL of ethyl acetate and then extracted following similar conditions during extraction with hexane. All extractions were filtered with a 25-mm Whatman No. 1 filter paper, and then evaporated with a vacuum evaporator. Samples were subsequently kept in tightly closed vials before GC-MS analysis and determination of biological properties.

2.2 Gas chromatography-mass spectrometry analysis

GC-MS identification of volatile compounds in different *C. papaya* extracts was carried out using Agilent Technologies GC systems with GC-7890B/MS-5977B model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS UI column (30 m × 0.25 mm, film thickness 0.25 µM). Helium was used as a gas carrier which was supplied at a flow rate of 1.0 mL/min. The initial temperature was set at 150°C and held for 2 min, then ramped at the rate 10°C/min to 240°C/min, and held for 1 min, followed by a final hold at 280°C (ramped at the rate 5°C/min) for 15 min, with a total run time of 35 min. The prepared extracts were solubilised in their respective solvents, filtered through a 0.45 µM membrane, then injected in a splitless mode. The compounds were identified by comparing their mass spectra with standards available in the installed NIST mass spectral library in the GC-MS instrument.

2.3 Anti-α-glucosidase activity assay

The anti α-glucosidase activity was determined according to Matsui et al [16]. Sample volumes of 50 µL were prepared by diluting hexane and ethyl acetate extracts in different concentrations of 0.1-1.0 mg/mL with 0.1 M phosphate solution and then incubated with 10 µL of one unit of α-glucosidase at 37°C for 15 min. Subsequently, 20 µL of 0.1 M p-Nitrophenyl glucopyranoside was added to each well and incubated at 37°C for 20 min. The reactions were stopped by adding 50 µL of 0.1 M sodium carbonate ($NaCO_3$), and the absorbance was measured at 405 nm. The percent inhibition was calculated using the following equation: percentage inhibition = [{(Absorbance of control - Absorbance of extract)/Absorbance of control} × 100] and half maximal inhibitory concentration (IC_{50}) was also determined and acarbose was used as a positive control [17].

2.4 Measurement of anti-inflammatory activity

The mouse RAW264.7 cell lines were seeded in the 24-well plates overnight. The cells were activated using 100 ng/mL lipopolysaccharide (LPS) for two hours before treatment with hexane and ethyl acetate seed extracts at IC_{10} concentration (the concentration that cause 10% cell death), and resveratrol was used as a positive control. Treated cells were then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. The Griess assay was

used to determine the anti-inflammatory properties [18], while the level of nitric oxide production was determined from the supernatants of the mouse RAW264.7 macrophage cell line.

3. Results

3.1 Comparison of bioactive compounds in the hexane and ethyl acetate papaya seed extracts

GC-MS analysis revealed that the bioactive compounds present in the hexane extract were predominantly non-polar compared with that of the ethyl acetate extract (Table 1 and Table 2). The percentage quality of compound peaks in both hexane and ethyl acetate papaya seed extracts ranged from 83% to 99% (Table 1 and Table 2). The chemical mass fingerprint of each GC-MS peak compared with the NIST library is shown in Figure 1. Phytosterols, such as γ-sitosterol, stigmast-4-en-3-one, stigmastanol, campesterol and squalene were the major group of compounds detected in the hexane extracts of papaya seeds. Additionally, fatty acid esters, including glycidyl oleate, glycidyl palmitate and glyceryl monooleate, as well as few fatty acids were identified in the hexane extracts (Table 1). In contrast, ethyl acetate extract contained fatty acid esters, especially glycidyl oleate as the major type of fatty acid ester. Notably, fatty acid content in the ethyl acetate extracts was substantially higher than that of hexane extract due the varying polarities, whereas both extracts contained oleic and hexadecanoic acids. The major fatty acid in the ethyl acetate extract was 9-octadecenoic acid, followed by octadecanoic acid, oleic acid, trans-13-octadecenoic acid, and hexadecanoic acid. High content of γ-sitosterol was detected in papaya seeds extracted with hexane than ethyl acetate solvent.

Table 1 GC-MS results of the bioactive compounds identified in the hexane extract of *Carica papaya*.

Peak	Retention time	Quantity (%)	Name of compounds	Formula	Molecular weight	Quality (%)
1	9.265	4.17	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	99
2	11.000	9.265	Oleic acid	$C_{18}H_{34}O_2$	282.5	99
3	12.580	1.42	Glycidyl palmitate	$C_{19}H_{36}O_3$	312.5	95
4	13.926	0.72	Pinostrobin chalcone	$C_{16}H_{14}O_4$	270.28	97
5	14.647	11.32	Glycidyl oleate	$C_{21}H_{38}O_3$	338.5	99
6	17.268	0.93	Glyceryl monooleate	$C_{21}H_{40}O_4$	356.5	95
7	18.981	2.76	Squalene	$C_{30}H_{50}$	410.7	99
8	25.192	4.16	Campesterol	$C_{28}H_{48}O$	400.7	99
9	25.384	1.25	Ergostanol	$C_{28}H_{50}O$	402.7	89
10	25.897	1.42	Stigmasterol	$C_{29}H_{48}O$	412.7	99
11	27.255	25.30	γ -Sitosterol	$C_{29}H_{50}O$	414.7	99
12	27.492	3.59	Stigmastanol	$C_{29}H_{52}O$	416.7	99
13	27.686	2.60	(Z)-Stigmasta-5,24(28)-dien-3β-ol	$C_{29}H_{48}O$	412.7	99
14	28.319	1.93	4-Campestene-3-one	$C_{28}H_{46}O$	398.7	93
15	29.170	4.32	(3β)-9,19-Cyclolanost-24-en-3-ol	$C_{30}H_{50}O$	426.7	99
16	30.967	10.67	Stigmast-4-en-3-one	$C_{29}H_{48}O$	412.7	98
17	31.496	1.14	(24E)-Stigmasta-4,24(28)-dien-3-one	$C_{29}H_{46}O$	410.7	95

Table 2 GC-MS results of the bioactive compounds identified in the ethyl acetate extract of *Carica papaya*.

Peak	Retention time	Quantity (%)	Name of compounds	Formula	Molecular weight	Quality (%)
1	3.538	0.24	(Isothiocyanatomethyl) benzene	C_8H_7NS	149.21	90
2	8.185	0.10	Methyl ferulate	$C_{11}H_{12}O_4$	208.21	97
3	9.270	2.69	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	99
4	9.378	0.75	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	90
5	9.937	0.17	Triphenylmethane	$C_{19}H_{16}$	244.3	98
6	10.395-10.487	6.16	Oleic acid	$C_{18}H_{34}O_2$	282.5	99
7	10.692	4.36	trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	282.5	99
8	10.964	13.45	9-Octadecenoic acid	$C_{18}H_{34}O_2$	282.5	99
9	11.153	6.16	Octadecanoic acid	$C_{18}H_{36}O_2$	284.5	93

Table 2 (continued) GC-MS results of the bioactive compounds identified in the ethyl acetate extract of *Carica papaya*.

Peak	Retention time	Quantity (%)	Name of compounds	Formula	Molecular weight	Quality (%)
10	12.588	0.54	Glycidyl palmitate	$C_{19}H_{36}O_3$	312.5	95
11	14.076	0.67	1,21-Docosadiene	$C_{22}H_{42}$	306.6	99
12	14.655	15.22	Glycidyl oleate	$C_{21}H_{38}O_3$	338.5	99
13	15.032	0.90	2-Hydroxy-1-(hydroxymethyl)ethyl palmitate	$C_{19}H_{38}O_4$	330.5	89
14	15.282	0.62	Tricosanal	$C_{22}H_{46}O$	338.63	97
15	15.539	0.34	Di (2-Propylpentyl) phthalate	$C_{24}H_{38}O_4$	390.6	83
16	17.285	1.60	Glyceryl monooleate	$C_{21}H_{40}O_4$	356.5	93
17	18.989	1.61	Squalene	$C_{30}H_{50}$	410.7	99
18	23.148	0.29	Cholesterol	$C_{27}H_{46}O$	386.7	99
19	25.220	3.29	Campesterol	$C_{28}H_{48}O$	400.7	99
20	25.414	1.09	Ergostanol	$C_{28}H_{50}O$	402.7	86
21	25.916	0.97	Stigmasterol	$C_{29}H_{48}O$	412.7	99
22	27.288	15.89	γ -Sitosterol	$C_{29}H_{50}O$	414.7	99
23	27.519	2.62	Stigmastanol	$C_{29}H_{52}O$	416.7	93
24	27.722	2.48	(Z)-Stigmasta-5,24(28)-dien-3β-ol	$C_{29}H_{48}O$	412.7	99
25	28.349	1.45	4-Campestene-3-one	$C_{28}H_{46}O$	398.7	98
26	29.203	2.59	(3β)-9,19-Cyclolanost-24-en-3-ol	$C_{28}H_{50}O$	426.7	99
27	31.001	6.63	Stigmast-4-en-3-one	$C_{29}H_{48}O$	412.7	99
28	31.533	0.80	(24E)-Stigmasta-4,24(28)-dien-3-one	$C_{29}H_{46}O$	410.7	95

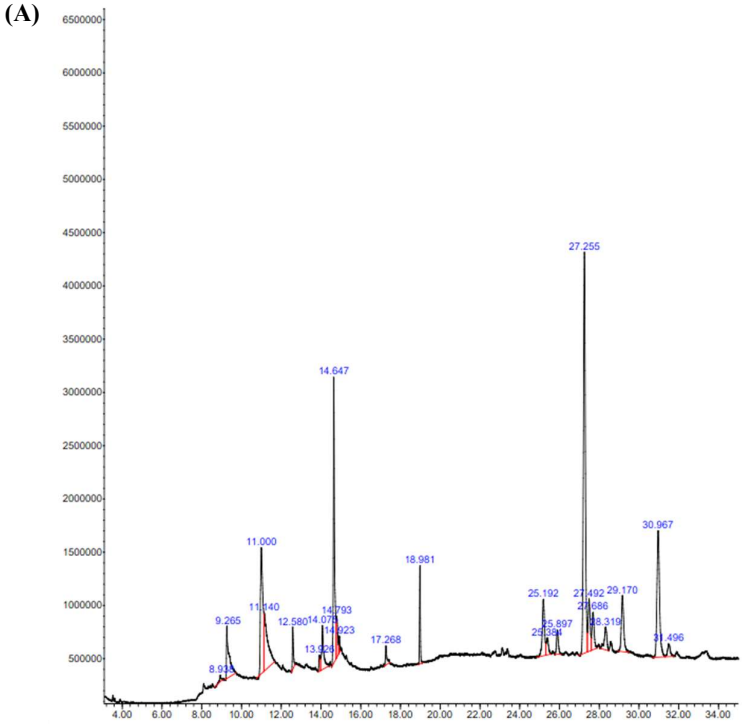

Figure 1 GC-MS chromatogram of papaya seeds extracted with (A) hexane.

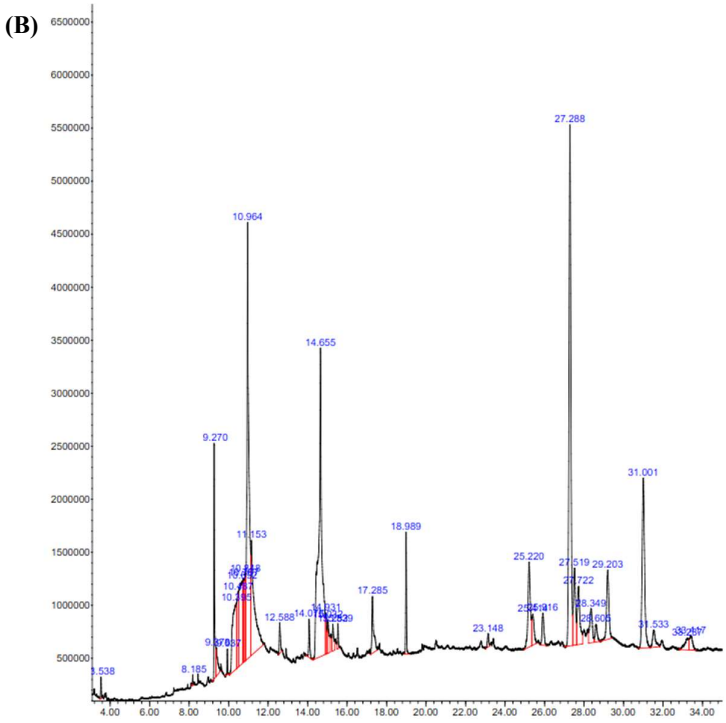

Figure 1 (continued) GC-MS chromatogram of papaya seeds extracted with (B) ethyl acetate.

3.2 Evaluation of anti-α-glucosidase activities of different solvent extracts

The anti-α-glucosidase property of hexane and ethyl acetate extract were determined by performing α-glucosidase enzymatic assay. The results showed that both extracts inhibited half of the enzyme activity at the concentration (IC_{50}) of 0.393 mg/mL and 0.127 mg/mL in hexane and ethyl acetate extract respectively (Table 3). These results were considerably lower that of acarbose (IC_{50} = 0.887 mg/mL) suggesting that the two extracts could effectively inhibit α-glucosidase activity when compared to acarbose.

Table 3 The inhibitory concentration (IC_{50}) of hexane and ethyl acetate extracts.

Extract	IC_{50} of anti-α-glucosidase activity (mg/mL) (mg/mL)	IC_{50} of survivability (mg/mL)	IC_{10} of survivability (mg/mL)
Hexane	0.393 ± 0.03	2.40 ± 0.01	0.83 ± 0.05
Ethyl acetate	0.127 ± 0.05	3.10 ± 0.01	0.91 ± 0.03
Acarbose	0.887 ± 0.01	N.D.	N.D.

N.D. was not determined.

3.3 Determination of the IC_{50} values of the extracts against RAW264.7 cells using the MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out to evaluate the cytotoxicity of the extracts towards the RAW264.7 cells. The results revealed that IC_{50} values were 2.40 and 3.10 mg/mL for hexane and ethyl acetate extracts, respectively, which suggested the potential non-toxicity of the extracts towards RAW264.7 cells as the concentration to kill the cell is not in µg/mL range (Table 3).

3.4 Evaluation of anti-inflammatory activities using the griess assay

The Griess assay was used to determine the level of nitric oxide produced by the cells when triggered with the LPS ligand synthesised from *Escherichia coli*. The anti-inflammatory effect of the hexane extract decreased the nitric oxide concentration from 16.0 µM to 12.4 µM, which was comparable to the nitric oxide concentration of the resveratrol positive control (12.0 µM) (Figure 2). The obtained results suggested that the hexane extract can reduce the inflammatory effect while the ethyl acetate extracts slightly induce inflammation.

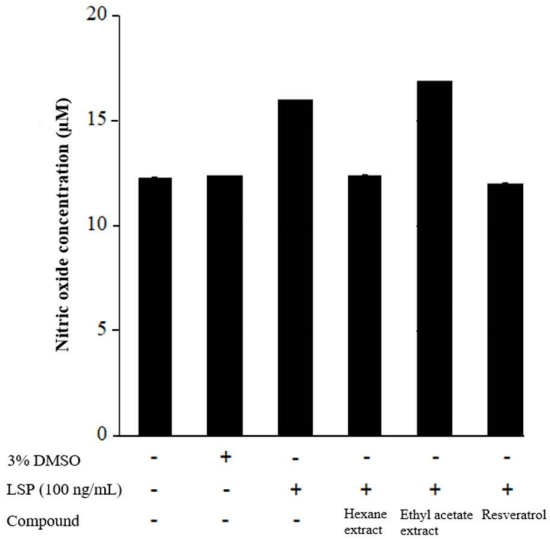

Figure 2 Nitric oxide concentration in mouse macrophage cell line (RAW264.7). The solvent used was 3% dimethyl sulfoxide (DMSO). LPS, lipopolysaccharide. Resveratrol is a bioactive compound with anti-inflammatory properties. The experiment was performed twice with three replications.

3.5 Morphological validation of RAW264.7 cells after treatment with the extracts

To confirm the toxicity effects of the extracts towards RAW264.7, morphological characteristics were investigated in the cells stimulated with LPS after treatment with the extract for 24 h. The results revealed that cells treated with hexane extract had normal features and were less differentiated compared with those treated with ethyl acetate extract (Figure 3). In contrast, the cells treated with resveratrol extract caused the cells to shrink and clump together, suggesting cytotoxic effects. Overall, these results suggested that the hexane extracts were not only non-toxic to cells but could also show anti-inflammatory properties.

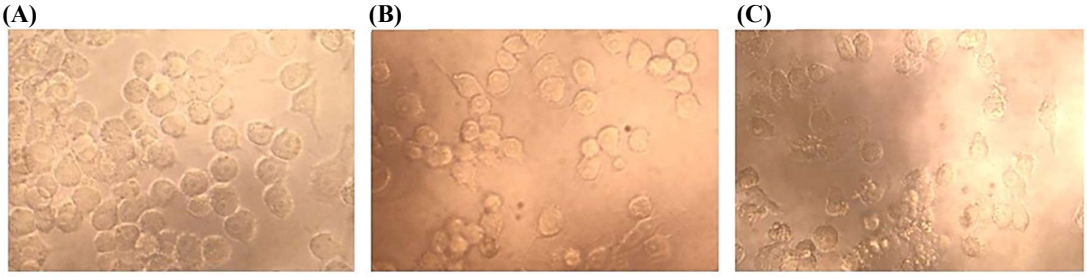

Figure 3 LPS stimulated RAW264.7 treated with (A) hexane extract, (B) ethyl acetate extract, (C) resveratrol.

4. Discussion

C. papaya is a famous tropical plant in Thailand because of its fruits and seeds, which exhibit numerous nutritional and medicinal properties. Although papaya seeds are categorised as agricultural waste, studies have been conducted to profile their bioactive compounds, with an aim to use the seeds more efficiently. This study presents the first effort to elucidate the content of bioactive compounds using GC-MS in papaya seed extracted with hexane and ethyl acetate solvents. Previous studies have reported that benzyl isothiocyanate is the key bioactive compound in papaya seed [3-4,7,19,20]. However, our results did not reveal the presence of this compound in the seed extracts (Table 1 and Table 2), whereas fatty acids, fatty acid esters, and phytosterols were found in abundance. This result suggests that the content of bioactive compounds obtained depends on the type of extraction solvent used. For example, the quantities of various fatty acid-types containing the carboxylic groups were more distributed in the ethyl acetate fraction than in the hexane fraction. The fatty acid ester and phytosterol groups, which were less polar than fatty acids, were abundantly detected in the hexane fraction.

Phytosterols are the predominant antioxidant agents previously reported in the papaya seed extracts [10,21]. This study revealed that phytosterols, such as γ-sitosterol, stigmast-4-en-3-one, stigmastanol, campesterol, and

squalene were present in hexane and ethyl acetate extracts, with their higher accumulation profiles being detected in the hexane extract. Notably, γ-sitosterol was highly accumulated in the Holland papaya seeds, with 25.30% and 15.89% concentrations in hexane and ethyl acetate extract, respectively (Table 1 and Table 2). Additionally, fatty acid esters and fatty acids were detected in the papaya seed extracts, with 9-octadecenoic acid at a concentration of 13.45%, as the main type of monounsaturated fatty acid in the ethyl acetate extract (Table 2). This finding was consistent with a previous report that the seed extracts contain long chain fatty acids, including stearic acid (saturated fatty acid, C18:0) and oleic acid (monounsaturated fatty acid, $18:1^{\Delta 9}$) due to its relatively low saponification value and the level of free fatty acids, which lowers the oil spoilage [1].

The anti-α-glucosidase activity of Holland papaya seed extracts revealed that ethyl acetate extracts had lower IC_{50} values than those of hexane extract (Table 3), suggesting that the content of α-glucosidase inhibitor in ethyl acetate extract was greater than that in hexane. Phytosterols, such as stigmastanol and ergosterol exhibit the anti-α-glucosidase activity [22], which was consistent with the observed anti-α-glucosidase activity of papaya seed extracts with the two solvents used in this study. However, phytosterol content was lower than that of fatty acids in ethyl acetate extracts, which indicated that the phytosterol is not the only compound with anti-α-glucosidase activity in papaya seed extracts. Moreover, this finding revealed that the ethyl acetate extract was composed of 9-octadecenoic acid (13.45%), oleic acid (6.16%) octadecanoic acid (6.16%) and trans-13-octadecenoic acid (4.36%). Interestingly, 9-octadecenoic acids in hexane extract, which are the *trans*-isomer of oleic acid, were previously reported to inhibit the α-glucosidase activity in the sea cucumber [23]. In addition, oleic acid demonstrated the strongest activity (K_i =23.1 μM) against α-glucosidase enzyme with reversible inhibition mechanism compared to other unsaturated fatty acids [24]. These observations supported our results that showed higher fatty acid content in ethyl acetate fraction than in the hexane fraction, which was mainly contained hexadecanoic acid (4.17%) and oleic acid (9.265%).

The anti-inflammation properties of papaya seed extracts were analysed with the Griess assay to determine the extent of nitric oxide reduction in RAW264.7 cell lines after stimulation with *E. coli* derived LPS. The results showed that the hexane fraction had greater nitric oxide reduction capacity than ethyl acetate fraction (Figure 2), indicating that the hexane fraction had potentially higher anti-inflammatory compounds. Previous studies have shown the capacity of palmitic acid (hexadecanoic acid) to decrease prostaglandin E2 content by inhibiting the activity of prostaglandin E2-9 reductase [25, 26]. Prostaglandin E2 is a modulating agent for the cell inflammatory metabolism that regulates nitric oxide synthase activity after injury or infection to produce nitric oxide (NO) [27]. Oleic acid also showed a better capacity to reduce the nitric oxide level in RAW264.7 than palmitic acid [28]. Interestingly, ethyl acetate fraction also contained 6.16% oleic acid, which was less than observed in the hexane fraction (9.265%) or other isomer molecules. The anti-inflammatory property is not only affected by oleic acid content, but also by the stereoisomer of the molecule. Moreover, studies have reported the anti-inflammatory properties of numerous phytosterols, which occurred in high amounts in the hexane fraction in this study (Table 1 and Table 2). Overall, these results emphasises that palmitic acid, oleic acid, and phytosterols, such as sitosterol, campesterol, and stigmasterol are potential anti-inflammatory agents that act by down-regulating the expression of inflammatory related gene, including tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Moreover, the results of this study elucidate the biological properties of the active ingredients in papaya, which could also support the utilisation of the oil extracted from its seed as an additive or emulsifier in the cosmetic industry. Despite previous evaluations of chemical content in the papaya seed extracts using GC-MS, most studies used methanol or ethanol extracts, which are not suitable for some chemical compounds that exhibit low polarity. Therefore, the characterisation of chemical content in the papaya seed extracts in this study will potentially bridge the gap of information with previous research.

5. Conclusion

The bioactive compounds in papaya seeds were successfully determined in the extracts obtained using hexane and ethyl acetate solvents. GC-MS analysis identified some bioactive compounds with pharmacological potential in the papaya extracts. The ethyl acetate extract demonstrated the anti α-glucosidase activity, while the hexane extracts showed anti-inflammatory activity with no cell toxicity. Our study is the first one to evaluate the anti α-glucosidase and anti-inflammatory activities of the papaya seed extracts, and the results also showed their potential anti-diabetic activity. Overall, the identification of bioactive compounds in the Holland papaya seed extracts and the verification of their biological activities reported in this study could support the pharmacological studies and use of papaya seeds, which are still considered as an agricultural waste. Further studies are therefore needed to isolate the bioactive compounds and elucidate their underlying modes of action towards various disorders.

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