METABOLITE IDENTIFICATION OF SPONTANEOUSLY FERMENTED PAPAYA LEAF

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ABSTRACT

This study was conducted to identify the flavonoid component and phenolic acid in *C. papaya* fermented extract. *C. papaya* leaf typically has high amount of flavonoid compound such as, quercetin and kaempferol Here, The metabolite components of the fermented *C. papaya* leaf extract was resolved using chromatograpic analysis i.e. UHPLC followed by identification specific compounds using online database. Based on the database analysis, the compound detected i.e. Kaempferide 7-Methylkaempferol and 3-O-Methylkaempferol is a group of flavonoids kaempferol while 6-Acetylphenazine-1-carboxylic acid is a phenolic acid compound that are known to have great anti-oxidant and anti-cancer activity. From the result obtained, it can be conclude that the extracted *C. papaya* fermented contains flavonoid and phenolic compounds.

INTRODUCTION

The Carica papaya (*C. papaya*) is a tropical fruit belongs to *Caricaceae* family and well for its medicinal values. (Asha, R., 2014) Papaya plant is usually cultivated for their fruit, which is the main product that is being recognized especially in Asian for their good taste and contain nutritive value. The other parts of the tree: leaves and seed are beneficial to health (T. Julianti t al., 2014) Their leaves and other parts can traditionally be used in curing several disease such as dengue fever and malaria. Studies found that the young *C. papaya* leaves is more likely to be used compared to the older leaves. (Navdeep, G. et al., 2015)The phenolic compounds, phenolic acid and also flavonoid such as kaempferol and quercetin were being identified during this study and all these compound is known to have the therapeutic properties for cure the disease.

The fermentation process is one of the oldest method for food preservation and can help increase the shelf-life of the food. It is a non-harmful process and it also may increase the nutritional qualities of the food. (Lilis Nuraida, 2014) As mentioned before, *C. papaya* is known to have a good therapeutic property. Due to that, the *C. papaya* leave is being fermented as an alternative to enhance their taste, bioactivity and increase their functionality to be used as a medicine purposes.

Metabolite identification is not new, it is essential in many fields of sciences' life. As in this study, extract from *C. papaya* were known to have high contain of flavonoids group. Flavonoids is a group of the secondary metabolites characterized by diphenylpropane structure. This group can help in reducing the risk of chronic disease associates with plant-derived foods. (Montano et al., 2011) Beside that, evidence also told that some flavonoids is useful in curing several disease. The identifications of the flavonoid constituents is performed by using the method of UHPLC. Afzan et al. reported four flavonoids has been found in the C. papaya leaf extracted, including the quercetin and also kaempferol.

Several studies demonstrated fermentation to enhance the bioactivity of the medicinal plants. The lactic acid fermentation of Myrus communis berries using Lactobacillus plantarum strai and yeast extract were reportedly used to enhanced anti-oxidant activity of fruits in term of its DPPH scavenging activity, inhibition of linoleic acid peroxidation and increase the phenolics acids and flavonols content (Curiel et al., 2015). The same method was used in order to observe comparable outcomes during the fermentation of *C. papaya* leaves. Increase level of anti-oxidant and anti-inflammatory were reported from the fermentation of cactus pear (*Opuntia ficus-indica L.)* by lactic acid fermentation using several *Lactobacillus* strain. On the other hand, the increased level of its flavonoids bioactive compoundswere attributed to esterase enzyme activities which converted the flavonol glycoside into glycones (Filannino et al., 2016). In addition, fermentation of the mangosteen's α-mangostin by Colletotrichum gloeosporioides (EYL131) and Neosartorya spathulata (EYR042) fungi resulted to several novel metabolites with the expectation of α-mangostin's higher bioactivity (Arunrattiyakom et al., 2011).

In this study, the extraction of the fermented *C. papaya* has been done to determine the compound by using online database and their therapeutic compound has been identified from the metabolic compound based on the medicinal property.

METHODOLOGY

2.1 Preparation of samples

The *C. papaya* leaves (approximately about 10 kg) were collected from the papaya farm. The fresh leaves then being washed by using tap water and making sure the leaves were cleaned and no any physical dirt. The C. papaya leaves being grinded together with a little amount of distilled water by using kitchen blender. The grinded leaves were loaded into 50L storage tank. The fermentation was carried out anaerobically for 100 days at room temperature. There is no additional preservation or any starter culture.

2.2 Preparation of extract

The 500 mL samples at day 100 was collected and the solid debris is removed by using the centrifuge at a speed of 10,000g for 20 minutes at 4 °C. The supernatant was collected. Next, the samples was filtered by using rotary evaporator to remove the water content from the samples.

2.3 Chromatography method

The separation method is performed by using UHPLC system. The gradient elution has been performed as follows; The mobile phase consisted of the following 10 min sequence of linear gradient and isocratic solvents of solvent A (0.1% v/v of formic acid in water,) and solvent B (0.1% v/v of formic acid in acetonitrile) at a flow rate of 500 µL/min: 0–1.75 min, 5% B; 1.75–6.75 min, 5–30% B; 6.75–7.25 min, 30–95% B; 7.25–7.50 min, isocratic at 95% B; 7.50–8.0 min (washing step); and finally equilibrated under initial condition for 2 min (Afzan, 2012)

As for mass spectrometry analysis, the MicroTOF QII Bruker Daltonic and the ESI positive ionization is selected. Then, the accurate mass of molecular ions will appeared in TOF analyzer that will further process in data analysis software.

2.4 Identification of specific compunds

The highest peak obtained from the UHPLC analysis is used to identify the single compound at random retention time. The MetFrag database webpage is used for this identification (https://msbi.ipb-halle.de/MetFrag/).

RESULTS AND DISCUSSION

There were seven prominent compounds which belong to flavonoids and phenolic acids being highlighted by Canini et al. (2007) which became target for this study. The structure of the target compounds are shown in Figure 1:

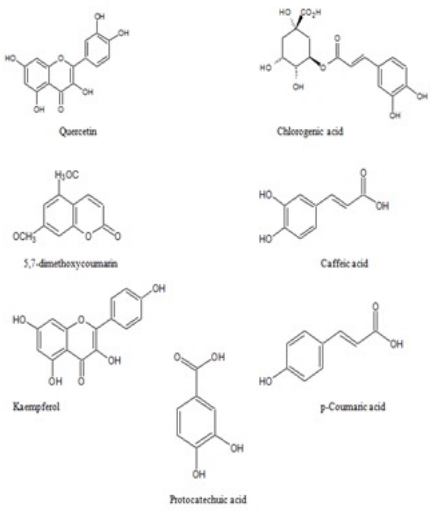


Fig 1 Compounds of C. papaya leaves extract (Senthivel et al., 2013)

The extraction of the fermented *C. papaya* leaves with MeOH enhance the capability of producing the flavonoid compounds. The BuOH is act as fractionate solvent because their ability to yield the flavonoid-rich compound. From the peak chosen randomly corresponding to a particular retention time (Fig.2), its MS and MS/MS spectra (Fig. 3) was analysed using online database (https://msbi.ipb-halle.de/MetFrag/).and found there are flavonoid content in the extracted samples (Table 1).. In this study, three compound of interest were the target; kaempferol, quercetin and phenolic acid compounds. During the analysis, there were many compounds detected, but were different from the targeted compounds. Some of the compounds detected are tabulated in Table 1.

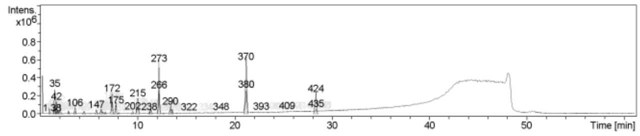
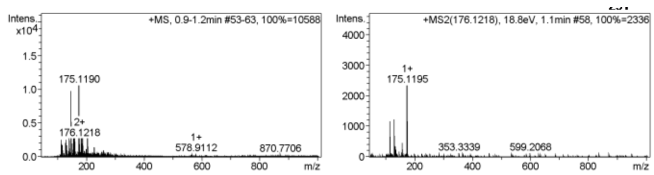


Fig. 2 Total ion chromatogram of metabolites from fermented *C. papaya* leaf in positive mode corresponding to Table 1



#	m/z	z	I	FWHM	S/N
1	141.9595		3557	0.0294	39.1
2	142.1589		2858	0.0259	31.4
3	147.1135		9756	0.0274	107.2
4	156.0675		2950	0.0308	32.4
5	158.9617		2709	0.0285	29.8
6	159.9678		4264	0.0263	46.8
7	175.1190		10588	0.0299	116.3
8	182.9845		4054	0.0312	44.5
9	186.9583		3395	0.0344	37.3
10	204.9648		3117	0.0338	34.2

#	m/z	z	I	FWHM	S/N
1	116.0709		1175	0.0229	465.6
2	130.0955		1223	0.0253	484.7
3	133.6161		374	0.1116	148.2
4	133.6530		338	0.1127	133.9
5	133.7921		142	0.0632	56.3
6	135.9484		255	0.0313	101.1
7	147.1120		181	0.0251	71.7
8	158.0926	1+	470	0.0298	186.3
9	175.1195	1+	2336	0.0306	925.7
10	176.1177	1+	298	0.0277	118.1

Fig. 3 Example MS and M/S spectra of metabolites from fermented C. papaya extract

4.1 Compound of Interests

i. The flavonoid kaempferol

The kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a compound that have a low molecular weight of 286.2 g/mol (This flavonoid has been identified in C. papaya leaves from the previous literature. In this study, the flavonoid kaempferol were identified from the highest peak results from the HPLC analysis. The peak from the UHPLC analysis at random retention time is being analysed using database webpage to identify the kaempferol content in the sample.

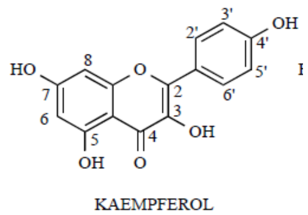
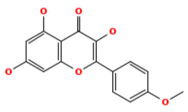


Fig. 4 Structure of kaempferol

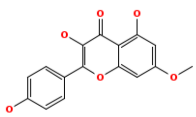
Table 1 Compound found from the extracted *C. papaya* leaves

Compound Name	Formula	Mass	Positive	PPM	M-H]$^+$ (m/z)
Phenmetrazine, Valerianine	$C_{11}H_{15}N_1O_1$	177.1154	177.099	100	178.1068 [M+H]+
N'-Nitrosonornicotine	$C_9H_{11}N_3O_1$	177.0902			[M+H]+
Anthopleurine	$C_7H_{15}N_1O_4$	177.1001			[M+H]+
Dihomomethionine	$C_7H_{15}N_1O_2S_1$	177.0823			[M+H]+
Heritonin, 3'-O-Methylbatatasin III, Dihydroechinofuran	$C_{16}H_{18}O_3$	258.1256	258.134	100	259.1414 [M+H]+
Cyclo[(Z)-alpha,beta-didehydrophenylalanyl-L-leucyl]	$C_{15}H_{18}N_2O_2$	258.1368			[M+H]+
sn-Glycero-3-phosphocholine, Glycerophosphocholine	$C_8H_{21}N_1O_6P_1$	258.1101			[M+H]+
Coixinden B, 1'-Acetoxychavicol acetate	$C_{13}H_{14}O_4$	234.0892		100	235.1176 [M+H]+
Strigolactone ABC-rings	$C_{14}H_{18}O_3$	234.1256			[M+H]+
2,6-Diamino-7-hydroxy-azelaic acid	$C_9H_{18}N_2O_5$	234.1216			[M+H]+
Geranyl phosphate	$C_{10}H_{19}O_4P_1$	234.1021			[M+H]+
Dibenz[a,h]acridine	$C_{21}H_{13}N_1$	279.1048	279.094	100	280.1015[M+H]+
Graveoline	$C_{17}H_{13}N_1O_3$	279.0895			[M+H]+
Thiamine acetic acid	$C_{12}H_{15}N_4O_2S_1$	279.091			[M+H]+
3-O-Methylkaempferol, Scutellarein 6-methyl ether, Hispidulin, Kaempferol 4'-methyl ether, Kaempferide, Tectorigenin, 7-Methylkaempferol, +)-6a-Hydroxymaackiain, (+)-Sophorol, Cajanin, Chrysoeriol, Leptosidin, Luteolin 4'-methyl ether, 1,6-Dihydroxy-3-(hydroxymethyl)-8-methoxy-9,10-anthracenedione	$C_{16}H_{12}O_6$	300.0634	300.077	100	301.0845 [M+H]+
Farrerol	$C_{17}H_{16}O_5$	300.0998			[M+H]+
1-Amidinostreptamine 6-phosphate, 1D-1-Guanidino-3-amino-1,3-dideoxy-scyllo-inositol 4-phosphate	$C_7H_{17}N_4O_7P_1$	300.0835			[M+H]+
Uric acid ribonucleoside	$C_{10}H_{12}N_4O_7$	300.0706			[M+H]+
4-(beta-D-Glucosyloxy)benzoate	$C_{13}H_{16}O_8$				[M+H]+
Tazobactam	$C_{10}H_{12}N_4O_5S_1$	300.0528			[M+H]+
Coelogin, 4-Hydroxyhomopterocarpin, Pratensein, (-)-Sparticarpin, (-)-Variabilin, Phellopterin, Isophellopterin	$C_{17}H_{16}O_5$	300.0998			[M+H]+
Methoxybrassinin	$C_{12}H_{14}N_2O_1S_2$	266.0548	266.0455	100	267.0528 [M+H]+
5'-Dehydroinosine	$C_{10}H_{10}N_4O_5$	266.0651			[M+H]+
6-Acetylphenazine-1-carboxylic acid, Carbamazepine-o-quinone	$C_{15}H_{10}N_2O_3$	266.0691			[M+H]+

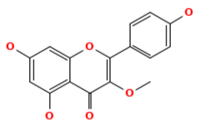
Referring to the exact structure of kampferol, the database compound obtained has identified and compared in order to identify the metabolite. Some of the kaempferol derivatives found in samples are shown below:



(a)



(b)



(c)

Fig. 3 (a) Kaempferide (b) 7-Methylkaempferol and (c) 3-O-Methylkaempferol

ii. The flavonoid quercetin

The quercetin is a bioflavonoid that has been studied by many researcher for over 30 years. The IUPAC nomenclature for the quercetin is 3,3',4',5,7-pentahydroxyflavanone: in which the OH group is attached at positions 3, 5, 7, 3' and 4. The main difference between kaempferol and the quercetin is the lacking of OH group at the position 3' (Palak et al. 2016). From the studies, found that the quercetin is the most powerful flavonoids found usually in vegetables and fruit. According to Palak et al., the quercetin is best described because of their ability to act as anti-oxidant.

Table 2 Some of therapeutic properties of quercetin

Property	Explaination
Anti-oxidant	Protect the body against reactive oxygen species that produced during the normal oxygen diffuse.
Protection for heart	Reducing cardiovascular disease.
Inflammation, Injury and Pain	Inhibits the formation of the prostaglandins, leukotrienes and release histamine
Cancer	Contain anti-proliferative effects in most of cancer cells and enhance the effectiveness chemotherapeutic agents.

Based on the database findings, there were no quercetin compound detected among fermented *C. papaya* leaf metabolites. However, according to Ayodele et al., the quercetin compound were obtained in the fresh leaves of *C. papaya*. In addition, as mentioned by Agung Nughoro et al in their studies stated that kaempferol is found to have highest component in the sample, followed by quercetin. On the other hand, some limitations might be done in order to yield the quercetin compound in the extracted fermented sample for *C. papaya.* The further study about quercetin and its derivative need to be fully understood in order to determining the specific compound since many possibilities of compound might appeared during screening the sample.

iii. Phenolic acid

Phenolic acid also is a secondary metabolites that has same group of metabolites with kaempferol and quercetin but differ in term of structures. Flavonoids have polyphenolic structure (Ali et al. 2011) while phenolic acid were not. The phenolic acids can be determine from their structure that usually found through the linked of ester, acetal bonds or ether. Differ with the quercetin and kaempferol, it is the subgroup of phenolic acid under subgroup of flavonol. (Ali et al. 2011) It has several derivatives that exist especially in plant. The compound is being absorbed by UHPLC in UV region that typically using variable-wavelength detector. The compound composed of complex group of the secondary products in the plant.

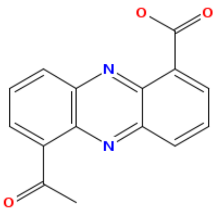


Fig. 4 6-Acetylphenazine-1-carboxylic acid

Fig. 4 shows one of the phenolic acid component found in extracted sample. Phenolic acids also known to have medicinal value just like the flavonoid compound. Recent study stated that the phenolic acid and flavonoid contain great antioxidants and has been proved to be more effective compared to vitamin C and carotenoids (Dai et al. 2010)

4.3 Medicinal property

As mentioned earlier, the flavonoids and phenolic compound are well known to have the medicinal property. However, the specific compound were not listed. The targeted flavonoids compounds in this research study which are kaempferol, quercetin and phenolic acid were found to have the medicinal property that beneficial to human. This theory is supported by many researcher mentioning about the benefits and therapeutic property of flavonoids and aphenolic acids. All the green plants have the potential sources of anti-oxidant activity. As in *C. papaya* extract, it has high possibility to act as anti-cancer, cure dengue fever and prevention HIV-1 activity. Apart of that, the phenolic acids and flavonoids plays many roles but the most specific roles has not been completely studied yet.

4.3 Unsual component

Despite having all the good medicinal property compound, an unusual component is found in the sample of fermented extracted *C. papaya* leaves, which is dihydroechinofuran. It is the monosubtituted of benzoquinone, the component that also found in the sample studied. In addition, the pattern is similar to the dihydroshikonofuran. As mentioned by Hiroshi et al., the dihyroechinofuran is a benzoquinone derivative from the dihydroshikonofuran.

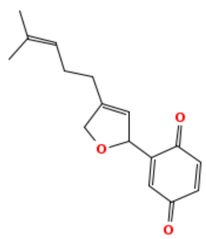


Fig. 5 Dihydroechinofuran component

CONCLUSION

Fermented *C. papaya* is abundant with compounds which mostly belong to the flavonoid such as kaempferol and phenolic acids. However, quercetin being one of the prominent compound on *C. papaya* leaf was undetected. However, there still have higher chances to identify the compound with limitation that can be made by modifications of the experimental method. The derivatives of phenolic acid were successfully obtained during the database screening. It is important to know the structure and derivatives of the component while UHPLC is one of the best method in providing the evidences in screening the specific component of flavonoids. This present study concluded that the fermented *C. papaya* leaves extract contained the flavonoids which consisted of kaempferol and the phenolic acids.

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