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Article

Antioxidant Activity of *Musa Paradisiaca* (Banana) Soft Pith and Its Cytotoxicity Against Oral Squamous Carcinoma Cell Lines

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***Abstract*— *Musa paradisiaca* also known as banana possess great medicinal value such as haematopoietic, immunomodulatory, antifungal, anti-diabetic and antioxidant effects. Antioxidant and antiproliferative activities between two different species, banana soft pith (BSP) Awak and Abu were conducted in this study. Briefly, BSP were collected (Baling, Kedah) and extracted by 70% ethanol forming crude extract. The crude extracts were then fractionated by *n*-hexane, ethyl acetate, butanol and water. All BSP extracts and fractions were subjected to total phenolic content (TPC), total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazil (DPPH) free radical scavenging assay, ferric reducing antioxidant potential (FRAP) assay and cytotoxic assay screening (MTT). Fraction ethyl acetate from BSP Abu showed the highest cytotoxic activity towards HSC-4 cell lines (26.95 ± 1.80 µg/mL) and the highest TPC (12.25 ±0.39 mg GAE/g) and FRAP assay (191.03 ± 0.83 mg FeSO4/g) values as compared to other fractions among BSP Abu. However, TPC (13.93 ± 0.28 mg GAE/g) and TFC (441.59 ± 19.31 mg QE/g) values of BSP Awak were found to be higher as compared to BSP Abu.**

***Keywords*— Total phenolics, total flavonoids, antioxidants, banana soft pith, radical scavenging activity, cytotoxicity.**

I. INTRODUCTION

Musa paradisiaca also known as banana and it is a tropical plant usually found in Malaysia. Banana plantation provides major annual income and a primary source of food I Malaysia [1]. In the north region of peninsular Malaysia, banana soft pith (BSP) had been used conventionally in local gastronomy. BSP is found in the innermost layer of the young banana stembark [2]. Banana also has a numerous therapeutic value such as haematopoietic and immunomodulatory activities [3], antidiabetic [4], antihyperglycemic and antihypertensive [5], anti-inflammatory [6], anti-leishmanial [7] and anthelmintic [8]. The use of BSP in conventional gastronomy and traditional treatments increased our interest to study pharmacological and physiological of BSP extract. Previous studies showed BSP is a potential chemopreventive plant [9]. Phytochemical screening of various parts of banana possess various kinds of phenolic compound such as tannins, glycosides and flavonoids [3,10]. However, to date, no study of antioxidant and antiproliferative effects of banana soft pith against oral squamous cancer cells have been reported. Therefore, the aim of this study is to determine antioxidant properties and the cytotoxicity of the BSP crude extract and fractions against oral squamous cancer cells (OSCC). Techniques of extraction influenced bioactivity of banana soft pith extract and fractions towards bioavailability of cancer cells. This has been proven by Sulaiman et al. (2011), whereby different extraction approaches will influence TPC and antioxidant activities of the extracts [11]. Few fractions were obtained from liquid-liquid extraction of BSP ethanol crude extract using different polarities of solvents which then tested for cytotoxic and antioxidant activities.

II. MATERIALS AND METHOD

A. Plant collection

Banana soft piths had been harvested in Kedah, Malaysia. The plant had been sent to Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia for identification and plant evaluation (SK2113/13).

B. Plant Extraction and Fractionation

Banana soft piths (BSPs) had been sliced into small pieces and dried it in an oven for 1 week. After that, BSPs was crushed into fine powder and saturated in ethanol (70%) for 4 days. After that, extraction of plant material had been done by separating it from solvent (ethanol) using a vacuum filtration. Crude extract was obtained by using a rotary evaporator under reduced pressure at 50˚Celcius.

Fractionation of crude extract had been done by using several solvents followed their polarity level, such as *n*-hexane, ethyl acetate, butanol and water. Crude extract was moved to funnel for separation, and it had been fractionation with *n*-hexane. The mixture had been shaking in separating funnel for 2 minutes. After that, two layers had been formed (aqueous layer and hexane layer) in the mixture. The bottom layer was removed into a flask. This step was repeated until the mixture not form two layers. Then, the residue was further continued with other solvents to acquire other fractions. Both of extract and fractions were stored at -20ºC.

C. Total phenolic content (TPC)

TPC of banana soft pith extracts and fractions were evaluated using Follin-Ciocalteu's test, following the method from Ramli et al. (2011) with minor modifications [13]. About 80 µg of extracts and fractions (10 mg/mL) were added to 96-well plate and followed by 15% Follin-Ciocalteu (100 µL) and distilled water (20 µL). After 5 minutes, 100 µL of Na_2CO_2 aqueous (0.015 g/mL) were added in the mixtures. After that, the mixtures were incubated at 30ºC for 60 ˚minutes. Turbidity of samples was determined by spectrophotometer at absorbances 756 nm. The concentration of total phenolic compounds had been obtained by using Gallic acid as standard. TPC of testing samples were expressed in milligram gallic acid equivalent divided weight of sample in grams (mg GAE/g sample).

D. Total flavonoid content (TFC)

TFC had been determined by using the colorimetric assay method following from Khalil et al. (2012) with minor modification [14]. Briefly, a total of 0.3 mL sodium nitrate ($NaNO_3$, 5% w/v,) was added to 5 mL extracts and fractions, respectively. After 5 minutes, 10% of aluminium chloride (300 µL) was added to the concoction and accompanied by 1 M NaOH (2 mL) for 6 minutes. Subsequently, distilled water (2.4 mL) had been added to the mixture and the absorbance were observed at 510 nm using a spectrophotometer. TFC of the tested samples had been calculated using quercetin equivalent (QE) in milligram divide weight of sample in grams (mg QE/ g sample).

E. 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activities of the extracts and fractions were measured by following a method from Armania et al. (2013) with minor modification [15]. A total of 50 µL samples (10 mg/mL) and DPPH solution (195 µL) were mixed in 96-well plate. The concoction was left at dark room temperatures for 1 h. Turbidity of the concoction was observed at 540 nm using a microplate reader (Infinite M200). The percentage scavenging activity of the sample was determined by followed this equation:

DPPH radical scavenging activity (%) = [(Abs control – Abs sample)/ Abs control] x 100

F. Ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) assay

The FRAP assay was followed method reported by Sulaiman et al. (2011) [11]. The reagent of FRAP was prepared by mixing 300 mmol/L acetate buffer (2.0 mL), 20 mmol/L $FeCl_3$ solution (2.5 mL) in a 10:1:1 ratio. About 10 µL of samples were mixed with the FRAP reagent (190 µL). The mixtures were then incubated at 37ºC in the dark for 30 minutes and turbidity were measured at 593 nm using a microplate reader.

G. Cell culture

The human oral squamous cell carcinoma (OSCC) cell lines (HSC-4), were purchased from the RIKEN Cell Bank. RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin had been used to maintain the cell. The cells had been incubated at 37 ˚C in a humidified atmosphere of 5% CO_2.

H. Cytotoxicity Assay

Cytotoxicity study was evaluated by using MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as method followed from Salimi et al. (2014) [12]. The cells were cultured for 24 h in 96-well plates, after that, the cells were treated with extracts and fraction (50 -1000 µg/mL) which dissolved in 10 % DMSO. Then, MTT (5 mg/mL) was added to each well and incubated at 4 h. After that, media from the cells was removed and DMSO (0.2mL) was added to each well and the plate was shaken. Absorbances of the cells were observed at 545 nm. This step had been repeated to the cells, which incubated for 48 and 72 h.

I. Statistical analysis

Data were expressed as mean ± standard deviation (STD) from three independent experiments. The differences between means were analyzed by one-way ANOVA followed by Tukey's post hoc test. The p value less than 0.05 ($p<0.05$) was considered as statistically significant.

III. RESULT AND DISCUSSIONS

Yield determination is the first step to consider before commercialization of certain products [16]. As shown in Table 1, yield of ethanol crude extracts and 4 fractions from BSP Awak and BSP Abu were varied from 25.37% to 0.39%. Differences in yield percentage were affected according to different solvent used. Solvent usage for plant extraction has significant effects towards the content of bioactive compounds. The aqueous ethanol mixture is an organic polar solvent that was proven to extract the most bioactive compounds producing the crude extract. This is based on the capability of ethanolic solutions which increase permeability of cell walls, thus accommodating effective serial extraction to isolate bioactive compounds [13]. Then, subsequent liquid-liquid extractions were performed to separate different polarity compounds presence in the banana soft pith. Nonpolar solutions were used to isolate lipophilic bioactive compounds. Meanwhile, medium-polarity solvents were used to isolate intermediate polarity of bioactive compounds and polar bioactive compounds could be isolated by using polar solutions [11].

Phenolic compounds have been known to possess antioxidant activity and proven to act as prevention to many diseases [17]. TPC was estimated as mg GAE/g sample. As shown in Table 1, BSP Awak butanol fraction showed the highest TPC value (13.93 ± 0.28) when compared to other samples from the same cultivar. Whereas Ethyl acetate

fraction of BSP Abu possessed the highest TPC value (12.25 ± 0.39). However, both species exhibited similar TPC values ($p>0.05$). Therefore, ethyl acetate and butanol were seemed as better solvents to extract phenolic content in BSP. Flavonoids are known to contribute greatly in antioxidant activity or free radical scavenging activity of various plant extracts generally attributed to their structural features [18]. TFC value was estimated as mg QE/g sample. For TFC value, the highest values were found in *n*-hexane fractions of both BSP Awak (441.59 ± 19.31) and Abu (316.78 ± 9.27). It was speculated *n*-hexane fraction was the best solvent to extract flavonoids content in BSP Awak and Abu.

TABLE I
YIELD PERCENTAGE TOTAL PHENOLIC CONTENT (TPC) AND TOTAL FLAVONOID CONTENT (TFC) OF BSP CRUDE ETHANOL EXTRACTS AND FARCTIONS FROM TWO DIFFERENT SPECIES (ABU AND AWAK)

Species	Sample	Yield (%)	TPC (mg GAE/ g extract)	TFC (mg QE/ g extract)
Awak	Crude Ethanol	14.19	$9.90^{a} \pm 0.38$	$33.16^{a} \pm 1.85$
	Hexane	1.55	$11.55^{cd} \pm 0.17$	**$441.59^{f} \pm 19.31$**
	Ethyl Acetate	1.49	$13.27^{ef} \pm 0.22$	$238.24^{d} \pm 6.06$
	Butanol	0.70	**$13.93^{f} \pm 0.28$**	$107.67^{c} \pm 5.17$
	Water	5.91	$10.65^{bc} \pm 0.28$	$25.82^{a} \pm 0.85$
Abu	Crude Ethanol	25.37	$10.57^{bc} \pm 0.62$	$82.48^{bc} \pm 2.89$
	Hexane	2.71	$8.82^{a} \pm 0.07$	**$316.78^{e} \pm 9.27$**
	Ethyl Acetate	0.56	**$12.25^{de} \pm 0.39$**	$270.76^{d} \pm 4.91$
	Butanol	0.66	$11.66^{cd} \pm 0.12$	$100.00^{c} \pm 1.64$
	Water	0.39	$10.65^{bc} \pm 0.28$	$45.03^{ab} \pm 1.57$

The highest value for TFC and TPC between different extracts and fractions were written in bold. Values are mean ± standard deviation of triplicate analyses. Values from the same column followed by the same letter are not statistically different ($p<0.05$) as measured by Tukey's HSD test.

The antioxidant activity of banana soft pith was related to the plant species and the different extraction methods employed. From Table 2, butanol fraction of BSP Awak showed the highest antioxidant properties ($p<0.05$) when compared with other samples. This fraction showed the highest FRAP activity (208.73 ± 1.85) and DPPH inhibition values (92.42 ± 0.65). Meanwhile, for BSP Abu, butanol fraction also showed the highest percentage of free radical inhibition (DPPH analysis) at 10 mg/mL extract concentration (56.40 ± 0.39). However, for FRAP analysis, ethyl acetate fraction has the highest antioxidant activity (191.03 ± 0.83). As the results mentioned before, butanol and ethyl acetate fractions had better DPPH free radical scavenging ability than the corresponding extract and fractions for both species, this could be attributed to their higher phenolic compounds [19]-[22]. In addition, the presence of phenolic compounds contributes to the antioxidant properties of the tested fractions. Previous study done by Zhang et al. (2015) showed the highest antioxidant extracts corresponds to the highest total phenolics and flavonoids content [23].

TABLE II
ANTIOXIDANT ACTIVITIES OF BSP CRUDE EXTRACTS AND FRACTIONS OBTAINED FROM TWO DIFFERENT SPECIES AS DETERMINED BY DPPH FREE RADICAL SCAVENING ASSAY AND FRAP REDUCING ASSAY

Species	Sample	DPPH (% inhibition of 10 mg/mL sample)	FRAP (mg $FESO_4$/g sample)
Awak	**Crude Ethanol**	$26.09^{b} \pm 0.61$	$73.25^{a} \pm 1.34$
	Hexane	$70.13^{g} \pm 1.34$	$157.75^{d} \pm 4.07$
	Ethyl Acetate	$31.62^{c} \pm 0.23$	$207.83^{f} \pm 1.59$
	Butanol	**$92.42^{h} \pm 0.65$**	**$208.73^{f} \pm 1.85$**
	Water	$45.27^{e} \pm 0.48$	$86.43^{b} \pm 1.89$
Abu	**Crude Ethanol**	$20.99^{a} \pm 0.53$	$89.62^{b} \pm 0.82$
	Hexane	$28.83^{bc} \pm 0.24$	$93.09^{b} \pm 0.93$
	Ethyl Acetate	$35.01^{d} \pm 0.52$	**$191.03^{e} \pm 0.83$**
	Butanol	**$56.40^{f} \pm 0.39$**	$140.33^{c} \pm 1.48$
	Water	$22.76^{a} \pm 0.59$	$92.04^{b} \pm 2.03$

Bold value shown was the highest antioxidant activities when compared to other samples. Values are mean ± standard deviation of triplicate analyses. Values in the same column followed by the same letter are not statistically different ($p<0.05$) as measured by Tukey's HSD test.

The ethanol crude extract and four BSP fractions from 2 cultivars (Abu and Awak) were evaluated for cytotoxicity using MTT assay against oral squamous cell carcinoma cell lines, HSC-4 at 24, 48 and 72 h (Table 3).

TABLE III
CYTOTOXICITY OF BSP ETHANOL CRUDE EXTRACTS AND FRACTIONS FROM 2 CULTIVARS AGAINST HSC 4 CELL LINES

Species	Sample	24h	48h	72h
Awak	**Crude Ethanol**	> 1000	> 1000	739.09±3.8
	Hexane	171.00±1.0	172.47±0.5	166.67±1.1
	Ethyl Acetate	397.16±7.4	256.75±1.1	213.45±30.2
	Butanol	> 1000	> 1000	847.94±16.7
	Water	> 1000	> 1000	> 1000
Abu	**Crude Ethanol**	930.00±2.0	698.00±9.0	691.00±7.0
	Hexane	97.75±0.4	58.13±0.2	44.30±6.0
	Ethyl Acetate	> 100	38.60±0.9	**26.95±1.8**
	Butanol	> 1000	> 1000	> 1000
	Water	> 1000	> 1000	> 1000

Sample with lowest IC_{50} value (µg/mL) compared with other extracts and fractions against oral squamous cancer cell lines, HSC-4 measured by MTT assay was written in bold. Values are mean ± standard deviation of triplicate analyses.

Results demonstrated fraction ethyl acetate of BSP Abu was the most cytotoxic with the lowest IC_{50} value (26.95 ± 1.80 µg/mL) compared to other tested extracts and fractions from both cultivars. This plant extract can be considered as a potential anticancer agent due to its cytotoxicity effects towards oral cancer cell lines. Ethyl acetate with the lowest IC_{50} value is the most active fraction that inhibits the growth of the oral squamous cancer cell. One factor that contributes to these properties was the presence of certain phenolic

compounds in the fraction which also contributes to antioxidant properties as noted earlier. This is also supported by a previous study, it showed that the accumulation of gallic acid, quarcetin and epicatechin in *Musa paradisiaca* inflorescence (flower) ethyl acetate fraction reduced the oxidative stress and inflammatory effects in diabetic rats [6]. Phenolic and flavonoid in plants are natural bioactive compounds that possess anticancer and antioxidant properties which is crucial in curing various degenerative diseases. Therefore, further study on identification of the bioactive compounds and mechanism underlying the active fraction in inducing cell death is very much needed for deeper understanding.

IV. CONCLUSIONS

Overall, it can be concluded two species of banana soft pith used in this comparative evaluation varied according to their antioxidant activities, TPC and TFC. Butanol, hexane and ethyl acetate are identified as the most efficient solvent in extracting flavonoid, phenolic and antioxidant compounds from BSP Awak and Abu species. Meanwhile, the ethyl acetate fraction is the best solvent to extract bioactive compounds which induced cytotoxicity in oral cancer cell line. This may indicate some contribution from phenolic and flavonoid compounds presence in ethyl acetate fraction. Further research is needed to understand antiproliferative effects of the ethyl acetate fraction in HSC-4 oral cancer cell lines and identification of responsible bioactive compounds in this fraction.

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