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Research Paper

Comparison of antioxidant and free radical scavenging activity of triterpenesα-amyrin, oleanolic acid and ursolic acid

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ABSTRACT

Triterpenes, α-amyrin, oleanolic acid and ursolic acid were previously described as the active components present in *Ficus pseudopalma*. It was also reported to have a strong scavenging activity against various free radicals, owing to aforementioned phytochemicals. In the present study, comparison of the antioxidant capacity of the standard triterpenes was performed using microtiter plate method. General antioxidant tests such as DPPH and FRAP assays and specific tests for the scavenging activity towards nitric oxide (NO•), superoxide ($•O_2^-$) and lipid peroxides (LOO•) were done. Thin layer chromatography of the crude ethanolic leaf extract of *F. pseudopalama* together with α-amyrin (0.831), oleanolic acid (0.769) and ursolic acid (0.769) revealed that two spots from the extract correspond to that of the standard compounds. Results showed that α-amyrin, oleanolic acid and ursolic acid are proton donors, all having an IC_{50} value of >333.33µM and possess a reducing power (RC_{50}>909.09µM). Ursolic acid (IC_{50}>166.67µM) gave the highest NO• scavenging activity followed by oleanolic acid then α-amyrin. Similarly, $•O_2^-$scavenging assay demonstrated that ursolic acid had the highest inhibitory activity against $•O_2^-$. Lastly, β-carotene bleaching assay, revealed that α-amyrin (IC_{50}<15µM), oleanolic acid (IC_{50}<15µM) and ursolic acid (IC_{50}<15µM) have prevented lipid peroxidation. Microtiter plate method unequivocally demonstrated that triterpenes are strong antioxidants and that these compounds are likely responsible for the antioxidant and free radical scavenging action of *F. pseudopalma*.

Keywords: *F. pseudopalma;* α-amyrin; Oleanolic acid; Ursolic acid; Antioxidant.

Abbreviations: Nitric oxide (NO•), superoxide ($•O_2^-$) and lipid peroxides (LOO•).

INTRODUCTION

The Philippines contains diverse natural resources wherein most of which remained to be underutilized and unstudied. The pharmacological effects of *F. pseudopalma* have been ascribed including antioxidant and antiurolithiatic activities (Santiago and Valerio, 2013; Acosta, et al., 2013). The said plant also demonstrated a cytotoxic

activity against human hepartocarcinoma (HepG2) cancer cell lines (Bueno, et al., 2013). These activities observed from *F. pseudopalma* may be attributed to triterpenes and sterols identified by Ragasa, et al. (2009).

Pentacyclic triterpenes are widely distributed throughout the plant kingdom. Of which about 4,000 known species were found to possess a wide spectrum of biological activities; they are bactericidal, fungicidal, antiviral, cytotoxic, analgesic, anticancer, spermicidal, anti-allergic, anti-diabetic, antiurolithic, and anti-inflammatory (Patocka, 2003., Vasquez, et al., 2012). Moreover, terpenoids modulate the activities of peroxisome proliferator-activated receptors (PPARs), a ligand-dependent transcription factor. These lipid sensors control the energy homeostasis inside the body and are usually related to the management for obesity-induced metabolic disorders (type 2 diabetes, hyperlipidemia, insulin resistance and cardiovascular diseases) (Goto, et al., 2010).

The triterpenoids, α-amyrin, oleanolic acid and ursolic acid, are among the identified phytochemicals in *F. pseudopalma* (Ragasa, et al., 2009). Some reports demonstrated the antioxidant activity of both oleanolic acid and ursolic acid (Tsai and Yin, 2008) but no reports yet for α-amyrin. In this study, the antioxidant activities of standard α-amyrin, oleanolic acid and ursolic acid were compared and establish the relevance of these compounds present in *F. pseudopalma.*

MATERIALS AND METHODS

Preparation of leaf extract: *Ficus pseudopalma* leaves were collected from the municipality of Baao in Camarines Sur, Philippines and were authenticated on the Botany Division of the Philippine National Museum. The leaves were air dried and were ground. One kilogram of the ground leaves was soaked with 95% ethyl alcohol in a 1:10 ratio. The filtrates after each solvent extraction were concentrated separately using the rotary evaporator (Eyela) at 40^{o}C until syrupy consistency was obtained (Santiago and Valerio, 2013). The crude extract was air-dried and was weighed obtaining a 4.57% yield.

Thin Layer Chromatography: Thin layer chromatography (TLC) was performed to show the presence of α-amyrin, oleanolic acid and ursolic acid in the crude ethanolic leaf extract of *F. pseudopalma*. Small spots of the standard compounds were introduced to a pre-activated TLC plate (8cmx5cm). The solvent system that was used consists of toluene, ethyl acetate and methanol in a ratio of 6:3:1. The TLC experiment was run on a closed chamber until the most of the components of the crude ethanolic leaf extract of *F. pseudopalma* had completely separated.

Microscale Antioxidant Assays

DPPH Scavenging Assay: The proton donating ability of the three standards were assed using the protocol as described by Santiago and Valerio (2013). α-amyrin, oleanolic acid and ursolic acid were dissolved in methanol and were diluted into different concentrations. Each of these solutionswas loaded into a 96-well microplate (10µl) and 140µl of 6.58x10^{-5}M DPPH solution was added. The microplate was incubated for 30min at 25^{O}C in the dark and the absorbance was measured at 517nm using Corona Microplate Reader SH-1000.

FRAP Assay: Ferric reducing antioxidant power assay was used to evaluate the reducing power of α-amyrin, oleanolic acid and ursolic acid. Forty microliter of the standard compounds, at different concentrations, were added with 100µl 1.0M hydrochloric acid, 20µl 1% sodium dodecylsulphate and 30µl 1% potassium ferricyanide. The resulting solutions were incubated at 50°C for 20 min. After which,

30µl of 0.1% ferric chloride was added to each solutions and aliquots (150µl) were transferred into a 96-well microplate. The absorbance was read at 750nm using Corona Microplate Reader SH-1000 (Santiago and Valerio, 2013).

Nitric Oxide Scavenging Assay: The nitric oxide scavenging ability of the standard compounds were determined by Griess reaction adapted from Santiago and Valerio (2013). α-amyrin, oleanolic acid and ursolic acid were dissolved in methanol and different concentrations of these compounds were prepared. A hundred microliters of each sample were added with 400µl 10mM sodium nitroprusside and 100µl phosphate buffered saline (PBS), pH 7.4. The solutions were incubated for 150 min at 25°C. After which, 100µl of each solution were transferred to a new tube and 200µl 0.33% sulfanilamide was added. The resulting solutions were incubated for 5 min at 25°C. Then, 200µl 0.1% napthyl ethylene diamine was added. Again, they were incubated for 30min 25°C. One hundred fifty microliters of the resulting mixture was transferred to a 96-well microplate in triplicate and was read at 540nm using Corona Microplate Reader SH-1000 (Hitachi, Japan).

Superoxide Scavenging Assay: α-amyrin, oleanolic acid and ursolic acid in methanol were diluted to different concentrations. Five microliter of each standard compounds were transferred in a 96-well microplate. Then, 50µl 73µM NADH, 50µl 156µmM nitro blue tetrazolium (NBT) and 50µl 60µM phena zine metho sulfate (PMS) were mixed to the standard compunds. A 5-minute incubation was done at 25°C before reading the absorbance at 560nm using Corona Microplate Reader SH-1000 (Hitachi, Japan) (Santiago and Valerio, 2013).

β-Carotene Bleaching Assay: The assay was conducted based on the protocol described by Lai and Lim (2011). An initial stock solution of β-carotene/linoleic acid was prepared by dissolving 5mg of β-carotene in 50ml of chloroform. Three milliliters of these solutions was added to 40mg of linoleic acid and 400mg of Tween 40. The resulting mixture was then evaporated using a rotary evaporator until the chloroform had evaporated. One hundred milliliters of distilled water was then added to the mixture and an initial absorbance of the final mixture was recorded at 470nm and 700nm.

In a small tube, 582µl of β-carotene/linoleic acid emulsion was mixed with 18µl of α-amyrin, oleanolic acid and ursolic acid at different concentrations. The tubes were incubated at 50°C for 60 min. Then the emulsion was loaded in a 96-well microplate in triplicates. The absorbance of the emulsion at 470nm and 700nm was determined using Corona Microplate Reader SH-1000 (Hitachi, Japan).

Statistical Analyses: Statistical tests were performed using SPSS version 17 and PHStat ver 3.2. Mean and its standard error (SEM) were used to summarize the data gathered from the experiment. Two way analysis of variance was used to determine if there is a significant difference in the mean percentage inhibition of DPPH, nitric oxide and superoxide radicals as well as mean percentage of reducing power according to concentrations and treatments. Tukey's HSD was used for post hoc analyses and *P* values less that 0.05 indicate significant difference.

RESULTS AND DISCUSSION

As shown in Figure 1, the TLC chromatogram of the crude ethanolic leaf extract of *F. pseudopalma* revealed the presence of α-amyrin, oleanolic acid and ursolic that corroborated the results obtained by Ragasa, et al. (2009). The chromatogram was visualized using iodine crystals. Rf values corresponding to each spots are summarized in Table 1.

In addition to the obtained results, lupeol, another triterpene, was also detected on the TLC chromatogram of the crude ethanolic leaf extrat of *F. pseudopalma* as shown in the latest report of Santiago and Mayor (2014).

Assessment of the Antioxidant Activity of α-Amyrin, Oleanolic Acid and Ursolic Acid: α-amyrin, oleanolic acid and ursolic acid are pentacyclic triterpenes that were identified by Ragasa, et al. (2009) to be present on the leaf extract of *F. pseudopalma.* According to the study of Santiago and Valerio (2013), the crude ethanolic leaf extract of *F. pseudopalma* possessed a good scavenging activity against several free radicals including DPPH, NO• and LOO•. In this regard, the study was done to know whether this elicited antioxidant function of the said plant was contributed by its active phytochemicals.

Standard α-amyrin, oleanolic acid and ursolic acid were used for the antioxidant tests. As shown by the DPPH scavenging assay, oleanolic acid had the highest scavenging activity at 333.33µM (P<0.05) followed by oleanolic acid and α-amyrin (P<0.05). This assay measures the ability of sample to act as a proton donor and eventually help stop the damaging effect of reactive free radicals.

On the other hand, the ferric reducing ability of the standard compounds showed that α-amyrin had the highest reducing power at 909.09µM (P<0.05) followed by oleanolic acid and ursolic acid (P<0.05). Reducing power of these compounds were evaluated by their ability to reduced Fe^{3+} ions to Fe^{2+} as shown by the formation of a Prussian blue solution.

Both the proton donating ability and the reducing power of the compounds are good markers for the antioxidant capacity. As demonstrated, the three compounds can act as potent antioxidants that may have been contributors to the antioxidant activity that was elicited by *F. pseudopalma.*

Specific antioxidant tests were also performed to elaborate the scavenging ability of the standard compounds towards biologically active free radicals. Nitric oxide (NO•) is one of the reactive nitrogen species present in the body that acts for several functions (Hofseth, et al., 2003). All three standard compounds have a concentration-dependent inhibition of NO•, where ursolic acid had the highest scavenging activity at 66.67µM concentration (P<0.05) among the three followed by oleanolic acid and α-amyrin having the lowest activity (P<0.05).

On the other hand, superoxide ($\bullet O_2^-$) radical scavenging assay showed that ursolic acid, oleanolic acid and α-amyrin demonstrated similar inhibitory activity towards $\bullet O_2^-$ (P>0.05). Although oleanolic acid had an evident concentration-dependent $\bullet O_2^-$ scavenging, the activity of ursolic acid and α-amyrin showed that they are more effective scavengers at low concentrations (P>0.05) compared to oleanolic acid.

The exhibited inhibitory activity of the standard chemical compounds towards NO• and $\bullet O_2^-$ shows that these compounds are helpful in maintaining the ROS levels inside the body and avert the occurrence of oxidative stress. Inside the body, NO• and $\bullet O_2^-$ react with one another to form a more reactive specie called peroxynitrite ($ONOO^-$), which in effect causes a more severe damage to the cells (Mayer, et al., 2006). In relation to that, NO• is usually associated to inflammation, rheumatoid arthritis and osteoarthritis (Murrell, et al., 1996) and carcinogenesis (Hofseth, et al., 2003). Furthermore, reaction of $\bullet O_2^-$ with H_2O_2 also produces another highly reactive ROS, which is the hydroxyl radical (•OH). By inhibiting the production of NO• and $\bullet O_2^-$, oxidative stress-related diseases such as diabetes, cardiovascular diseases as well as neurodegenerative disorders may be prevented from occurring.

Inhibition of lipid peroxidation was measured by using β-carotene bleaching assay (Lai and Lim, 2011). In this assay, oxidation of β-carotene together with linoleic acid produced free radicals through the abstraction of a hydrogen atom from the diallylic methylene group in linoleic that subsequently attack the highly unsaturated structure of β-carotene. Antioxidants take part in this reaction by neutralizing the active linoleic free radical in order to abstract its action towards β-carotene (Jayaprakasha, et al., 2006). All of the three standard compounds have similar inhibiting activity towards lipid peroxidation even at low concentrations (P>0.05).

The demonstrated antioxidant activities of oleanolic acid and ursolic acid in this study corroborated the findings that were reported on other articles. In the study of Yin and Chan (2007), they determined the antioxidant activity of oleanolic acid and ursolic acid at different temperature and pH in comparison to the activity of α-tocopherol. It was shown that these two compounds have a high antioxidant activity towards 2, 2'-azobis-(2-amidinopropane) and a good scavenging activity against $\bullet O_2^-$. Furthermore, in the study of Somova, et. al. (2003), they showed that pure compounds of oleanolic acid and ursolic acid increased glutathione peroxidase and superoxide dismutase (SOD) level in Dahl salt-sensitive (DSS), insulin resistant rat model of genetic hypertension, which demonstrate their antioxidant activity. Lastly, pretreatment of oleanolic acid and ursolic acid in H_2O_2 and MPP^+-induced PC12 cells increased SOD activity and decreased malonyldialdehyde (MDA) formation (Tsai and Yin, 2008). SOD is an enzyme that regulates the production of $\bullet O_2^-$ inside the body to maintain its required biological level and in order to prevent oxidative damage in the cells. MDA is an endogenous product of both enzymatic and oxygen radical-induced lipid peroxidation (Niedernhofer, et al., 2003). The decreased level of MDA implicates that the pure compounds of oleanolic acid and ursolic acid can inhibit lipid peroxidation as well.

CONCLUSION

In summary, the findings showed that pure compounds of α-amyrin, oleanolic acid and ursolic acid exhibited antioxidant activity and showed a good scavenging action towards biologically present free radicals. In this regard, this activity elicited by the three compounds may actually been responsible to the observed antioxidant activity of *F. pseudopalma.*

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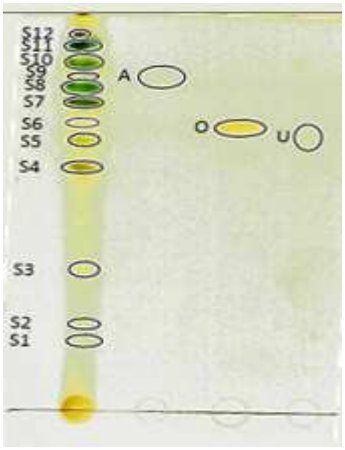

Figure-1: TLC chromatogram of crude ethanolic leaf extract of *F. pseudopalma* together with standard (A) α-amyrin, (O) oleanolic acid and (U) ursolic acid.

- The Solvent system used were toluene: ethyl acetate: methanol (6:3:1) and chromatogram was viewed using iodine crystals.

Table-1: Rf values of the spots obtained from the TLC chromatogram of the crude ethanolic leaf extract of *F. pseudopalma* together with α-amyrin, oleanolic acid and ursolic.

Spots of crude ethanolic leaf extract of *F. pseudopalma*	Rf Value	Standard Chemical Compounds	Rf Value
S1	0.215	α-Amyrin	0.831
S2	0.292	Oleanolic Acid	0.723
S3	0.354	Ursolic Acid	0.723
S4	0.600		
S5	0.677		
S6	0.723		
S7	0.769		
S8	0.800		
S9	0.831		
S10	0.610		
S11	0.908		
S12	0.938		

Table-2: Proton-donating activity of α-amyrin, oleanolic acid and ursolic acid as measured by DPPH assay.

Concentration (µM)	% Inhibition		
	α-amyrin	oleanolic acid	ursolic acid
33.33	-3.93	5.77	3.70
46.67	-6.93	4.39	3.46
133.33	-3.46	8.78	7.16
333.33	1.62	10.16	10.62

Table-3: Ferric reducing property of α-amyrin, oleanolic acid and ursolic acid measured by FRAP assay.

Concentration (µM)	% Reducing Power		
	α-amyrin	oleanolic acid	ursolic acid
90.91	17.80	13.58	14.19
127.27	17.00	15.37	13.67
181.82	27.45	17.88	13.58
909.09	34.33	22.68	14.01

Table-4: Nitric oxide radical scavenging activity of α-amyrin, oleanolic acid & ursolic acid measured by Griess assay.

Concentration (µM)	% Inhibition		
	α-amyrin	oleanolic acid	ursolic acid
16.67	8.61	27.11	33.64
23.33	7.52	35.81	36.90
66.67	11.88	41.25	39.08
166.67	17.32	44.51	37.99

Table-5: Superoxide radical scavenging activity of α-amyrin, oleanolic acid and ursolic acid as measured by $\bullet O_2^-$ assay.

Concentration (µM)	% Inhibition		
	α-amyrin	oleanolic acid	ursolic acid
25.8	12.58	10.67	22.06
32.3	15.63	11.10	22.49
64.5	12.72	12.63	13.82
161.3	16.53	15.91	13.67

Table-6: Inhibition of lipid peroxidation of α-amyrin, oleanolic acid and ursolic acid as measured by β-carotene bleaching assay.

Concentration (µM)	% Inhibition		
	α-amyrin	oleanolic acid	ursolic acid
15	66.35	77.98	66.77
21	88.24	91.14	76.17
30	92.87	87.85	93.44
150	87.85	89.98	80.38

