

Full Length Research Paper

Identification of phenolic compounds and assessment of *in vitro* antioxidants activity of 30% ethanolic extracts derived from two *Phyllanthus* species indigenous to Malaysia

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Phenolic compounds were identified in 30% ethanolic extracts derived from *Phyllanthus niruri* (*P. niruri*) and *Phyllanthus urinaria* (*P. urinaria*) using high performance liquid chromatography (HPLC) assay. *In vitro* antioxidants activity of the extracts was studied based on total phenolic contents (TPC) using Folin-Ciocalteu reagent and their scavenging activity towards radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The HPLC results show that gallic acid (GA), corilagin (Cor) and ellagic acid (EA) were the major components of the extracts and their quantifications in *P. niruri* were 11.867 ± 0.130 , 89.579 ± 0.602 and 37.309 ± 0.033 mg/g of extract respectively and in *P. urinaria* were 8.710 ± 0.091 , 56.382 ± 0.364 and 27.880 ± 0.263 mg/g of extract. The TPC of *P. niruri* and *P. urinaria* were 262.10 ± 1.04 and 277.98 ± 1.04 mg of gallic acid equivalent per gram of extract respectively and their scavenging based on IC₅₀ was 32.64 and 25.00 µg mass of the extract compared to the IC₅₀ of references standards GA (3.28 µg) and EA (2.99 µg). The results revealed the extracts as a potential source of natural antioxidants that can be utilized in cosmetics as skin antiaging, sun-blocking and whitening agents.

Key words: Corilagin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ellagic acid, Folin-Ciocalteu, gallic acid.

INTRODUCTION

Reactive oxygen species (ROS) and free radicals (FR) are small molecules naturally generated as by-product of cellular metabolism. Exposure to environmental hazards such as radiations, chemicals and gases increases their production in the body to toxicity levels (Govindarajan et al., 2005). Endogenous antioxidants in the body, such as glutathione and α-tocopherol can maintain and counteract the produced FR and ROS when they are within the physiological limit (Halliwell, 1996). Improper balance between the oxidants (FR and ROS) and antioxidants in

favour of the oxidants, is potentially leading to oxidative stress state (Sies, 1997). Oxidative stress is a fragmental state of DNA and cellular membrane damage, thus can ultimately lead to cells and tissues death due to proteins denaturation and lipids peroxidation (Ratnam et al., 2006). Consequently, make way for various human generative diseases like myocardial infarction, heart failure, hypertension, atherosclerosis, Parkinson's disease, Alzheimer's disease, muscular dystrophy, multiple sclerosis, diabetes, rheumatoid arthritis, chronic inflammatory diseases, sickle cell anaemia, acute renal failure, cancers and premature aging (Ferrari et al., 2004; Halliwell, 1987; Lefer and Granger, 2000; Nath and Norby, 2000; Pham-Huy et al., 2008; Praticò and Delanty,

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2000; Ratnam et al., 2006). Antioxidants are compounds capable of scavenging FR/ROS by terminating oxidative reaction chain in the biological tissues and hence can prevent cellular damage and oxidative stress associated with free radical induced generative diseases (Ratnam et al., 2006). Recognized dietary antioxidants such as ascorbic acid (vitamin C) and α -tocopherols (Vitamin E) can pick up and neutralize FR and ROS, prevent and reversed age related disorder and diseases (Halliwell, 1996). Plants secondary metabolites such as flavonoids and polyphenols compounds exhibited important commercial and biological role due to their antioxidants activity (Agati et al., 2007; Bendini et al., 2007; Di Mambro and Fonseca, 2005; Rice-Evans et al., 1997). They are good electron donors and having potentials redox pattern that can scavenge FR and ROS and prevent their harmful effects (Pietta et al., 1998). They are relatively stable due to resonance, delocalization and formation of side conjugated system with the hydroxyl group attached to the aromatic ring (Srinivasan et al., 2007). Therefore, they have been the major research issues for the last two decades (Gourine et al., 2010). Consequently, their commercial application as food supplements, food preservatives in nutraceuticals and skin anti-aging, sun-blocking and whitening agents in cosmeceuticals is highly increased (Peschel et al., 2006).

Phyllanthus urinaria and *Phyllanthus niruri* belong to widely distributed genus "*Phyllanthus*", family Phyllanthaceae (Samuel et al., 2005). The genus is found all over the world in the tropical and subtropical countries. More than 750 species of genus *Phyllanthus* have been described (Calixto et al., 1998; Wehtje et al., 1992). The genus "*Phyllanthus*" has been traditionally used internally to treat a broad spectrum of diseases such as diarrhoea, hepatitis, diabetes, abdominal pain, and kidney disorder (Chularojmontri et al., 2005; Mellinger et al., 2005). It is also used topically as a poultice to treat skin ulcers, sores, itching and wounds healing. The phytochemical compounds of many of *Phyllanthus* species such as tannins, ellagitannins flavonoids have been isolated and characterized (Ahmeda, 2005; Chang et al., 2003; Fang et al., 2008; Liu et al., 1999; Murugaiyah and Chan, 2007). The potential pharmacological effects of the many of these isolated compounds have been assessed (Ambali et al., 2010; Calixto et al., 1998; Krithika et al., 2009). Several studies have shown the antioxidants activity of various *Phyllanthus* species using different solvents and methods of extractions (Chularojmontri et al., 2005; Fang et al., 2008; Harish and Shivanandappa, 2006). Various phenolic compounds with antioxidants effect have been identified in *P. niruri* and *P. urinaria* (Harish and Shivanandappa, 2006; Markom et al., 2007; Murugaiyah and Chan, 2007). *P. urinaria* and *P. niruri*, being investigated in this study are found in Malaysia. The two species are closely-related in appearance and traditionally uses and locally known as "Dukung anak" which means carry baby; because the plants carry the

fruits on their backs and underneath the feathered-like leaves (Ahmeda, 2005; Markom et al., 2007; Ong and Norzalina, 1999). Our aim of this study was to identify and quantify the major components of the extracts chromatographically using high performance liquid chromatography (HPLC) and to assess an in vitro antioxidants activity in terms of total phenolic content and scavenging activity toward the radical 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) and to compare it to the scavenging activity of gallic and ellagic acids as control positive reference standards.

MATERIALS AND METHODS

Reagents

Folin-Denis' reagent, sodium carbonate 99%, 2,2-Diphenyl-1-picrylhydrazyl (DPPH 95%), gallic acid (GA, 99%) and ellagic acid (EA, 95%) were purchased from Sigma-Aldrich (St. Louis, MO., USA). Methanol was purchased from J. T. Baker (Philipsburg, USA), formic acid 98 to 100% assay from Merck (Darmstadt, Germany). Ethanol (99.7%) was purchased from Brightchem Sdn Bhd (Malaysia), sodium hydroxide from R & M Marketing (Essex, UK) and corilagin in-house prepared working standard was obtained as a gift from Nova Laboratories Sdn Bhd (Malaysia).

Extracts

The extracts of *P. urinaria* and *P. niruri* were prepared by Nova Laboratories Sdn. Bhd. (Malaysia) by dissolving 50 g of dried powdered materials from the aerial part of each plant (*P. niruri* and *P. urinaria*) in 500 ml of 30% ethanol at 60°C water bath for 1 h. The 30% ethanol liquid extract was filtered using Whatman No. 1 filter paper. The residue was re-extracted with another 500 ml of 30% ethanol. The two filtrates were combined and dried using rotatory evaporator at 60°C.

Identification of major phenolic compounds

GA external reference standard was prepared by dissolving 10 mg into 100 ml of distilled water. 10 mg EA of external reference standard was transferred into 100 ml volumetric flask and dissolved into 10 ml of sodium hydroxide 0.1 M and the volume was completed to 100 ml with water. Serial dilutions from the two references standards solutions of GA and EA were prepared in the range of concentration from 0.5.0 to 16 μ g/ml. Corilagin (Cor) working standard was prepared in concentration of 250 μ g /ml methanol. The solutions were ultra sonicated at ambient temperature for 10 min and filtered through nylon membrane filters 47 mm 0.45 μ m (Whatman, UK) before HPLC analysis was performed. The calibration curves were plotted with six concentrations each of the standard solution of GA and EA versus the areas under the peaks. The GA and EA standard curves equations were used to quantify in the extracts. Since corilagin is commercially unavailable and the small quantity available was enough only for identification purposes and triplicate runs was used to quantify corilagin in the extracts.

Samples solutions

10 mg of each extracts were weighed and transferred into 10 ml volumetric flasks. Each sample of *P. niruri* and *P. urinaria* was

Table 1. Liquid chromatography (LC) time programme of binary gradient pumps modules.

Time (min)	Module action	
	Pump A (% concentration)	Pump B (% concentration)
0.00	0	100
2.00	5	95
5.00	30	70
8.00	34	66
11.00	45	55
14.00	45	55
17.00	0	100
20.00	0	100

dissolved in formic acid 0.2%, vortexed for 20 s and then ultrasonicated at ambient temperature for 10 min at room temperature. The resulting samples solutions were filtered through nylon membrane filter 47 mm 0.45 µm diameter (Whatman, UK) before HPLC analysis were performed.

Detection and quantification limits (DL and QL)

The DL and QL were estimated based on the standard deviation of the response and slope (Guideline, 2005). The standard deviation (σ) of the responses of the lowest concentration in calibration curve of six runs ($n = 6$) and the slope of the calibration curves of GA and EA (S) were used to calculate DL and QL using Equations 1 and 2, respectively (Guideline, 2005). The sensitivity of the method was evaluated by the relative standard deviation (RSD %) of mean area under the peaks of the reference standards. The selectivity of the method and suitability of system were evaluated by comparing the retention time of the standards peaks to the samples peaks. The means of retention time of the GA and EA in the samples solutions were compared to external reference standards GA and EA mean retention time and were analyzed for null hypothesis using student's paired *t*-test.

$$DL = \frac{3.3}{S} \sigma \quad (1)$$

$$QL = \frac{10}{S} \sigma \quad (2)$$

Chromatographic condition

The HPLC analysis was performed using LC 20 AD Class LC-solution software, connected to SPD-20A UV/VIS detector, binary pump and temperature controlled column oven, (Shimadzu, Japan). Thermo Hypersil Gold™ (250 × 4.6 mm i.d., 5 µm) reversed phase column was used for all separations. The column oven temperature was set at 40°C and the external reference standards solutions and extracts were eluted with a binary gradient mode at a UV wavelength of 270 nm and 20 µl volume of injection (Rangkadilok et al., 2005). Methanol (solvent A) and formic acid 0.2% (solvent B) were used as mobile phase at flow rate of 1 ml/min. The liquid chromatography (LC) time programme of modules of the binary gradient pumps was set as in Table 1 and the retention time of the peaks was specified by band of 0.2 min.

Determination of total phenolic content (TPC)

The total phenolic content (TPC) of the extracts was studied spectrophotometrically by folin–ciocalteu reagent assay with slight modification (Bajpai et al., 2009). GA standard curve was plotted in the concentrations range of 50, 100, 150, 200, 250 and 500 mg/L in ethanol:water (10/90) solution and their corresponding absorbencies were measured at 765 nm visible wavelength. 10 mg of each extracts material were transferred in 25 ml volumetric flask and dissolved into ethanol:water (30:70). 100 µl from the samples solutions were placed in screw-capped test tubes, 500 µl of the Folin–Ciocalteu reagent and 1500 µl of distilled water (1/15 dilution) were added to the samples solutions respectively. The test tubes were properly shaken before incubated at room temperature for 1 min. After 1 min, 1000 µl of 20% sodium carbonate (Na₂CO₃) aqueous solution was added. The final mixture was vortexed for 10 s and then incubated for 2 h at room temperature. After 2 h, the absorbance was measured at 765 nm UV wavelength using Hitachi U-2000 spectrophotometer, Perkin Elmer Lambda, USA. The procedure was carried out in triplicate manner ($n = 3$) and the TPC the extracts was calculated using Equation 3. The results were expressed as milligram gallic acid equivalent per gram dry extract weight (mg GAE/g DW).

$$TPC = \frac{C}{DW} V \quad (3)$$

The experimental TPC were compared to the predicted TPC based on the quantified composes of the extracts. GA contains three hydroxyl group attached to aromatic ring as was used; the reference standard is equivalent to unity. The number of GA equivalent to each composes in the extracts was obtained by dividing the number of hydroxyl group in the composes by the number of hydroxyl group of GA (GAE). The predicted TPC were evaluated by summation of the total quantitative amount of each composes equivalent to GA multiplied by its quantified amount in the extract as shown by expression 4. In which, PTPC was the predicted TPC, COC was the content of composes in the extract and GAE was the GA equivalent of the composes.

$$PTPC = \sum COC \times GAE \quad (4)$$

Determination of radical scavenging activity

The powdered extracts and EA reference standard were dissolved in 99.7% ethanol in the concentration range of 0.1 to 2.0 mg/ml.

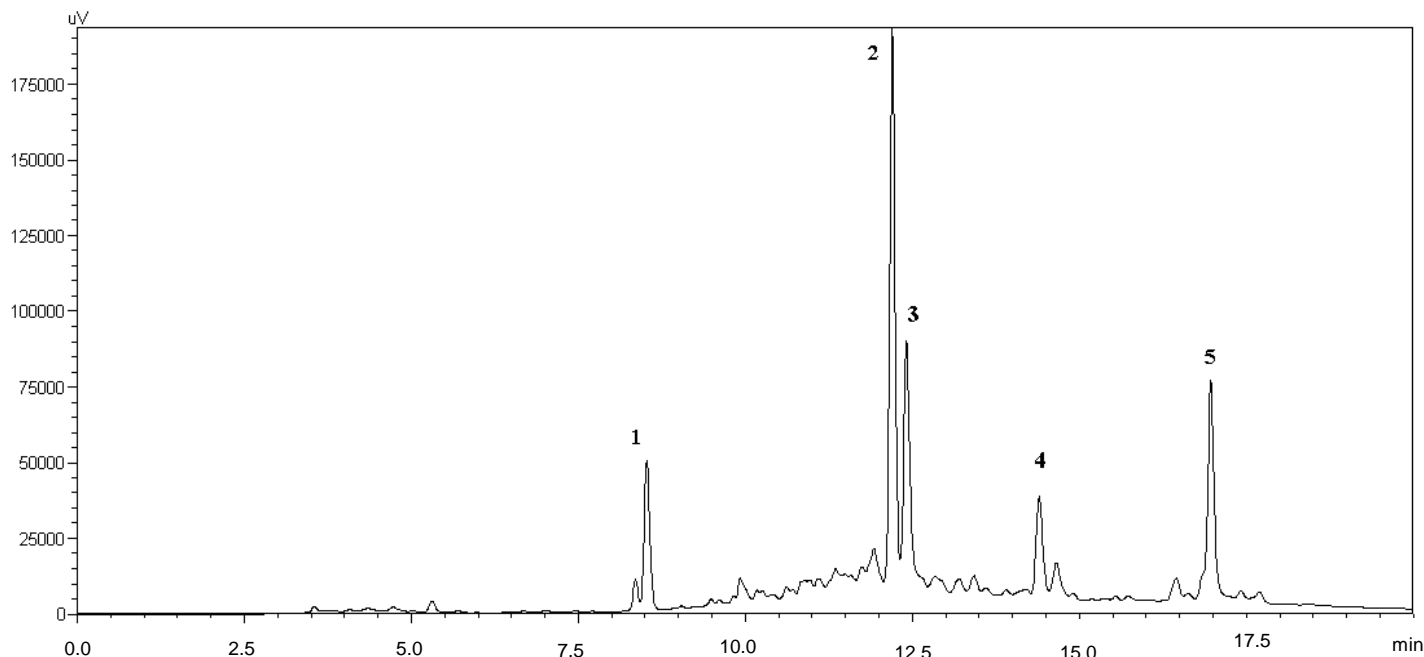


Figure 1. *P. niruri* chromatogram showing the peaks of Compound 1 gallic acid at a retention time 8.522 min, Compound 2, corilagin at 12.196 min, Compound 3 expected to be geraniin at 12.406 min and compound 5, ellagic acid at 16.963 min.

While GA reference standard was dissolved in distilled water in the range of 0.2 to 2.0 mg/ml. The extracts and the reference standards solutions of GA and EA were allowed to react with 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Fang et al., 2008; Krithika et al., 2009). 0.3 ml of 0.394 mg/ml DPPH and 2.4 ml of 99.7% ethanol were mixed in screw-capped test tubes. 0.1 ml of the extracts and the reference standards were added to a separate reaction mixture in the test tubes and allowed to stand for 30 min in the dark. The scavenging activity of the mixtures was measured at 517 nm visible wavelength. The experiment was carried in triplicates ($n = 3$) for each concentration of the reference standard and the extracts and the percentages of scavenging activity of the extracts were calculated using Equation 5, where Sc. A is the scavenging activity, A_o is the absorbance of the blank mixture (the absorbance of reaction mixture without extract or the reference standards), A_m was the absorbance of the reaction mixture with the extract or the reference standards. The results of scavenging activity of the extracts and the reference standards were plotted against their dose of the extracts and the standards in the mixtures (μg). The dose that inhibits 50% of DPPH radical activity (IC_{50}) were calculated from the equation of the scavenging activity plotted curves. The scavenging activities of the extracts were compared with GA and EA scavenging activity as positive control standards. The relative scavenging activity of the extracts to the reference standards GA and EA scavenging activity were analyzed for null hypothesis using student's *t*-test.

$$\text{Sc. A \%} = \frac{[(A_o - A_m)]}{A_o} 100 \quad (5)$$

Statistical analysis

Results were expressed as means \pm standard deviation (SD) of triplicates measurements. Student's *t*-test was used to analyse the

data and *p*-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Identification of major phenolic compounds

Phenolic compound in various *Phyllanthus* species extracts were identified using HPLC method (Colombo et al., 2009; Markom et al., 2007). The applied chromatographic conditions in this study show good resolution of five major compounds in the two extracts. Compound 1, gallic acid (GA), Compound 2, corilagin (Cor), Compound 3 expected to be geraniin, Compound 4 unknown phenolic compound and Compound 5 ellagic acid (EA) were the main active ingredients in the extracts (Figures 1 and 2). In addition *P. urinaria* contained other unknown compounds such as Compound 6 to 8 (Figure 2). The retention time of the phenolic compounds of the extracts were compared to the external reference standards solutions mixture chromatogram (Figure 3). It was cleared that the water soluble compounds eluted first such as GA and the last eluted was the lower water soluble EA due to the four fused aromatic rings (Figure 4). GA Compound 1, Cor Compound 2, and EA Compound 5 peaks were identified at a retention time of 8.548, 12.193 and 16.993 min, respectively in *P. niruri* chromatogram (Figure 1). The retention time of Compound 1 GA, Compound 2 Cor and Compound 5 EA were found to be located at 8.554, 12.197 and 16.996

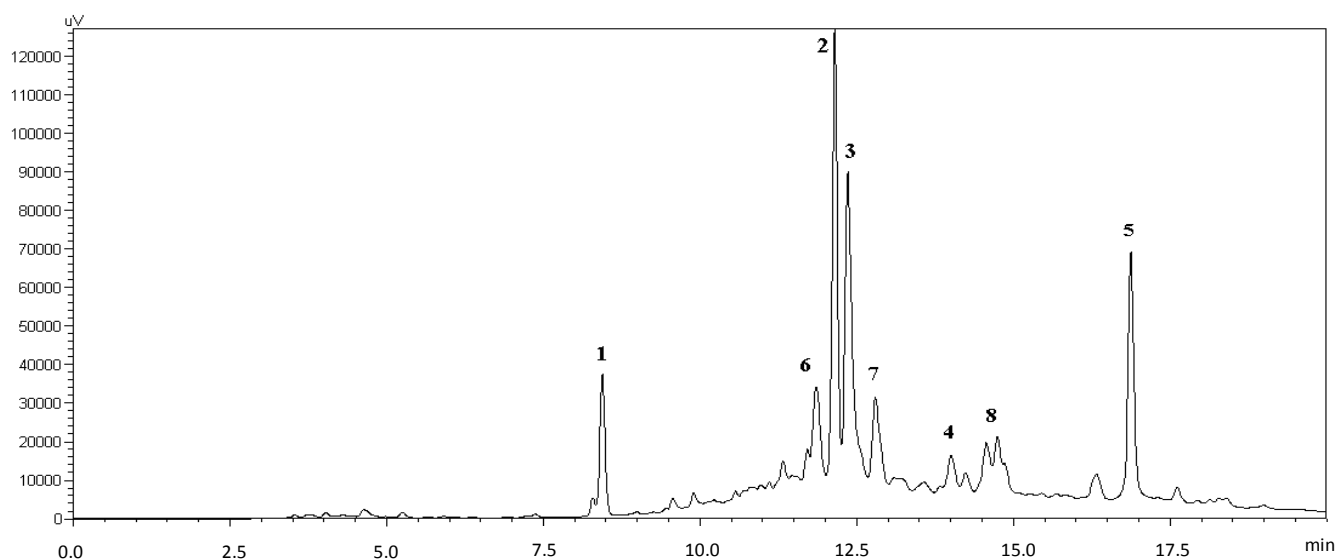


Figure 2. *P. urinaria* chromatogram shows the peaks of Compound 1, gallic acid at a retention time 8.436 min, Compound 2 corilagin at 12.144 min, Compound 3 expected to be geraniin at 12.353 min and Compound 5 ellagic acid at 16.868 min.

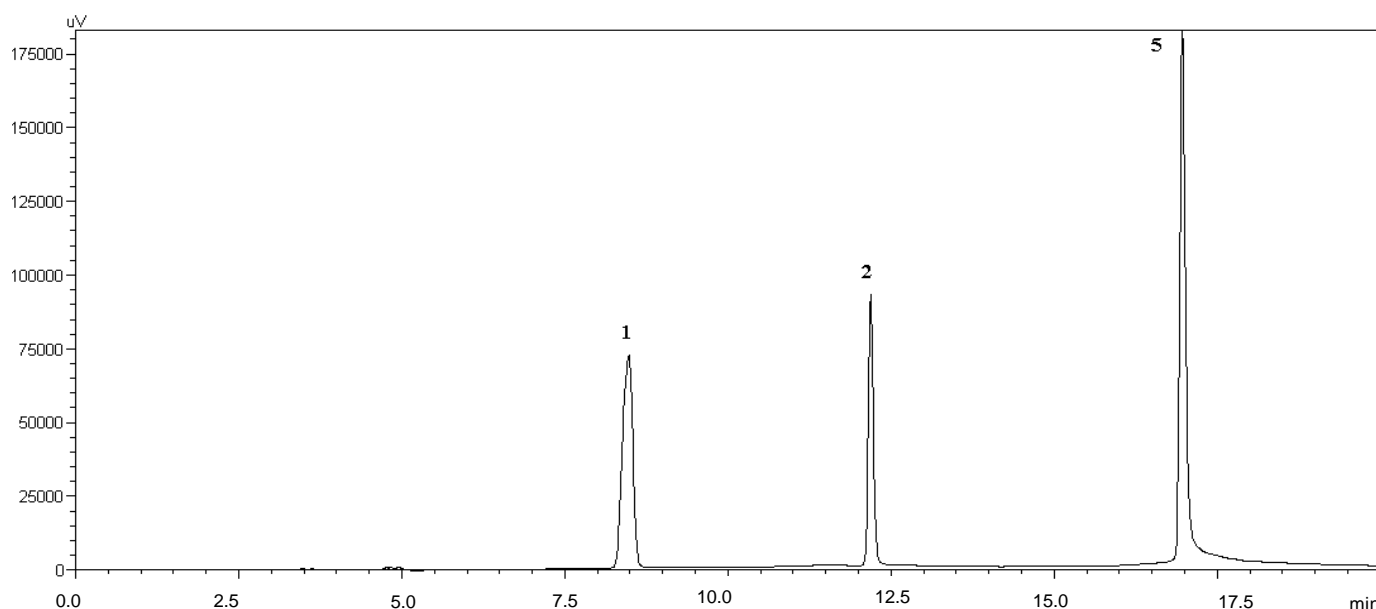


Figure 3. The chromatogram of external reference standards mixture of gallic acid Compound 1, corilagin Compound 2 and ellagic A Compound 5 peaks at 8.477, 12.183 and 16.961 min, respectively.

min, respectively in *P. urinaria* chromatogram (Figure 2). The chromatograms of GA, Cor and EA reference standards mixture shows peaks of retention's time at 8.477, 12.183 and 16.961 min, respectively (Figure 3). Figure 4 shows the chemical structure of the phenolic compounds identified in the extracts and geraniin which is structurally closed to corilagin, its peak could be much closed to the corilagin peak such as Compound 3 in the

chromatograms (Figures 1 and 2). The expectation was compared to previously identification of geraniin closed to corilagin in previous identification of phenolic compounds described previously (Thitilertdecha et al., 2010). It was cleared that these compounds are large molecules, have high molecular and their solubility are varied from water soluble compound such as GA to lower water soluble EA. It was also cleared from the chromatogram (Figure 1) and

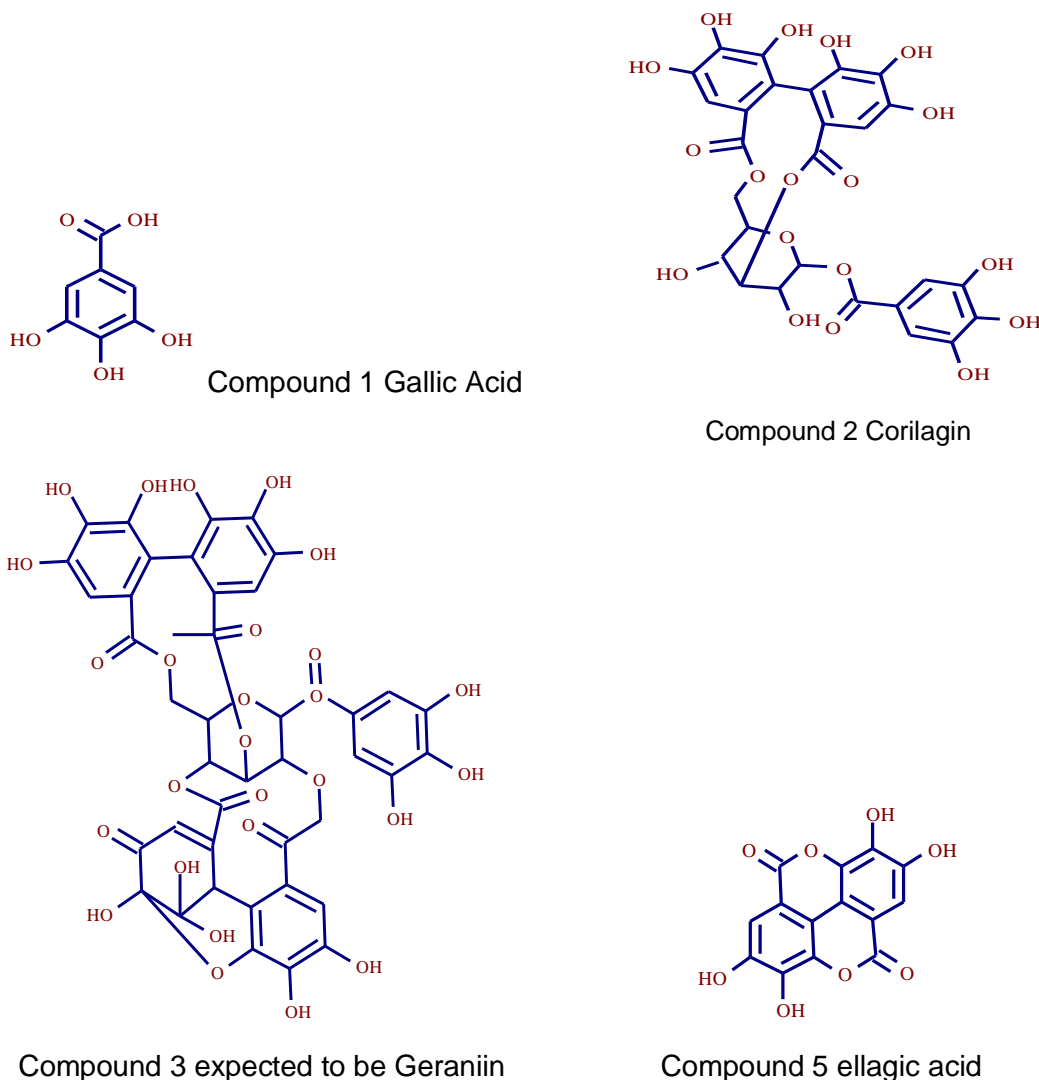


Figure 4. Chemical structures of the major compounds identified in the extracts.

that corilagin was the major compound in the extracts followed by Compound 3 which was expected to be geraniin, then EA and lastly GA. The important of polyphenols compounds is that they have amphiphilic properties which facilitate their antioxidants mechanism in both water and lipid phase (Sies and Stahl, 2004). Therefore, their application in cosmeceuticals will have important role in scavenging the ROS and FR results from excessive exposures to solar UV radiations. As it was known that the antioxidants properties of the polyphenolic compounds is because of the presence of hydroxyl group attached to aromatic ring as electron donating group (Ng et al., 2000). The more electron donating group available in the antioxidants compound, the more potent and stronger scavenging activity (Srinivasan et al., 2007). Based on this, geraniin which possess eleven hydroxyl groups attached to aromatic will be more potent compared to corilagin which has nine

hydroxyl groups and consequently, corilagin is a strong antioxidant compared to EA and GA which has four and three hydroxyl groups, respectively Figure 4. Furthermore, carboxylic acid group in GA can provide additional attack sites for free radicals and thus prevent them from attacking cell membrane and denaturation of protein (Srinivasan et al., 2007). Similarly, EA which contains two lactones' groups have contribution to its antioxidant activity.(Barch et al., 1996). Hence, the variation in content of these active ingredients in the extracts will affect the antioxidant activity. The quantification of major compounds in extracts was shown in Table 2. These compounds were also found in other plant extracts with content almost similar the *P. niruri* and *P. urinaria* and their uses as antioxidants material were explored (Rangkadilok et al., 2007). These polyphenolic compounds have been shown as antioxidants able to scavenge the free radicals reactive oxygen species (Fang

Table 2. Quantitative results of major active ingredients in the extracts (n = 3).

Dry weight of the extract (mg/g)	<i>P. niruri</i>	<i>P. urinaria</i>
Gallic acid (GA)	11.867±0.130	8.709±0.091
Corilagin (Cor)	89.579±0.602	56.382± 0.364
Ellagic acid (EA)	37.309±0.033	27.880±0.263

Table 3. Selectivity of the HPLC method based on the retention time of the external reference standards GA and EA to their retention in the extracts.

Retention time (min)	GA standard (n = 6)	EA standard (n = 6)	<i>P. niruri</i> (n = 3)		<i>P. urinaria</i> (n = 3)	
			GA	EA	GA	EA
Mean (min)	8.398	16.897	8.526	16.964	8.406	16.861
SD	0.013	0.042	0.033	0.031	0.012	0.010
RSD (%)	0.154	0.249	0.387	0.183	0.143	0.059
<i>P</i> -value	-	-	0.530	0.127	0.420	0.324

et al., 2008; Lin et al., 2008). They also can absorb the UV radiations and act as sun-blocking and whitening agents. It can penetrate the skin, pick up and neutralized the excessively generated ROS and FR due to the exposure to UV radiation and act as skin antiaging. To utilized the extracts as skin cosmetics, they were subjected to further treatment such as identification of TPC and scavenging activity towards stable radical DPPH.

Detection and quantification limits (DL and QL)

The HPLC analysis results in the present study show the linearity of the method by references standards at six levels of concentrations in the range of 0.5 to 16 µg/ml by the equations of the calibration curves of reference standards GA and EA. The calibration curve of reference standard GA equation was $y = 4978.79 + 60900.7 \times x$, $R^2 = 0.9994$. While the EA equation was $y = 54933.8 + 36348.1 \times x$ with regression factor $R^2 = 0.9981$. The DL of the two external reference standards GA and EA were 0.021 and 0.076 µg/ml, respectively. While the QL of the two external reference standards GA and EA were 0.070 and 0.252 µg/ml, respectively. The lower values of DL and QL reflect the sensitivity of the method. Furthermore, the sensitivity of the method was confirmed by the result of the relative standard deviation (RSD%) of the mean area under peak of both GA and EA reference standards 1.34 and 1.63%, respectively. It was also cleared that the DL and QL of GA is lower than EA. Hence the method was more sensitive to GA compared to EA.

Selectivity of the HPLC method

The selectivity of the HPLC method was evaluated by

comparing the retentions time of the external references standards GA and EA with retentions time of GA and EA active ingredients in the extracts Table 3. The small value of the relative standard deviation (RSD %) show the selectivity of the method. The SD of the means of the retention time is less than the retention time band set in the instrument which was 0.2 min. In addition, the statistical result also shows that the paired *t*-test tested satisfies the null hypothesis for two-sided with *p*-value > 0.05 and 95% confidence interval of the difference between the external reference standards retention time and the sample solution (Table 3). The proposed HPLC method was efficient, simple, rapid, cost effect and safe for the column on long uses since small amount of formic acid was utilized. Since the proposed HPLC method identified and quantified high content of phenolic compounds in the extracts, the extracts were subjected for further study to evaluate their antioxidants activities with respect to TPC and scavenging activity towards DPPH.

Total phenolic content

The quantification of the total phenolic content of *P. urinaria* and *P. niruri* were expressed as means ± SD and was found to be 277.98 ± 1.04 and 262.10 ± 1.04 mg of GAE/g dry weight of plant extract, respectively. The present study also revealed that *P. urinaria* and *P. niruri* extracts possessed high TPC compared to previous studies of extract from *P. niruri* (Harish and Shivanandappa, 2006; Kumaran and Joel, 2007). The TPC of the *P. niruri* was lower compared to the predict TPC based on the quantity of the major active ingredients in the extract; while the TPC of *P. urinaria* was higher compared to the predicted TPC which based on the

Table 4. Predicted TPC of the extracts based on the polyphenolic contents compared to the experimental TPC.

Compose	<i>P. niruri</i> (GAE/g DW)	<i>P. urinaria</i> (GAE/g DW)
GA	11.87±0.13	8.71±0.09
Corilagin	268.74±1.81	169.15±1.09
EA	48.50±0.04	36.24±0.34
Predicted TPC	329.11±1.98	214.10±1.53
Experimental TPC	262.10± 1.04	277.98±1.04

GA has 3 OH groups is equivalent to 1 GAE/g DW, corilagin has 9 OH groups. Therefore = 3GAE/g DW equivalent and EG has 4 OH groups = 1.33 GAE/g DW.

number of hydroxyl groups in each of the phenolic compounds to the number of hydroxyl groups in GA and its content in the extracts (Table 4). The variations in the predicted and experimental TPC of *P. urinaria* could be due to presence of much major and high content of unidentified compounds. Such compounds were 6, 7 and 8 which were found only in *P. urinaria* and Compound 4 which was expected to be geraniin with highest peak in *P. urinaria* chromatogram (Figure 2) compared to *P. niruri* chromatogram (Figure 1). Therefore, its high content in *P. urinaria* certainly will increase its TPC. This also could be reason of *P. urinaria* has high phenolic content despite it contain less amount of GA, Cor and EA compared to *P. niruri*. In case of *P. niruri*, the predicted and experimental TPC results (Table 4) were closely relevant since only Compound 4 was not quantified (Figure 1). The predicted TPC gives quick estimation to the TPC of the extracts despite that it required more study and broad application before been normalize as a measure.

Scavenging activity

The scavenging activity of *P. niruri* towards the stable radical DPPH was calculated as IC₅₀ from the plotted graph equation, $Y = -1684.82/X + 101.618$, with correlation coefficient factor $R^2 = 0.9938$ (Figure 5). The IC₅₀ is the dose in mass of the antioxidants materials (extract or reference standard) necessary to inhibit the initial DPPH radical activity by 50%. The smaller value of IC₅₀ means high scavenging activity and potent antioxidants compound. The IC₅₀ of *P. niruri* was 32.64 µg mass of the extract and its inhibition capacity to DPPH was 91.57% at dose of 200 µg. The IC₅₀ of *P. urinaria* was 25.00 µg mass of the extract and was inhibited 93.81% of DPPH scavenging activity at a dose of 200 µg based on the curve equation $Y = -1288.4/X + 101.528$ with regression coefficient $R^2 = 0.9982$ (Figure 6). The results of scavenging activities of *P. niruri* and *P. urinaria* were compared to the scavenging activity of the control positive reference standard GA and EA. The IC₅₀ of GA and EA were 3.28 µg and 2.99 µg mass of the reference standard, respectively. The maximum scavenging

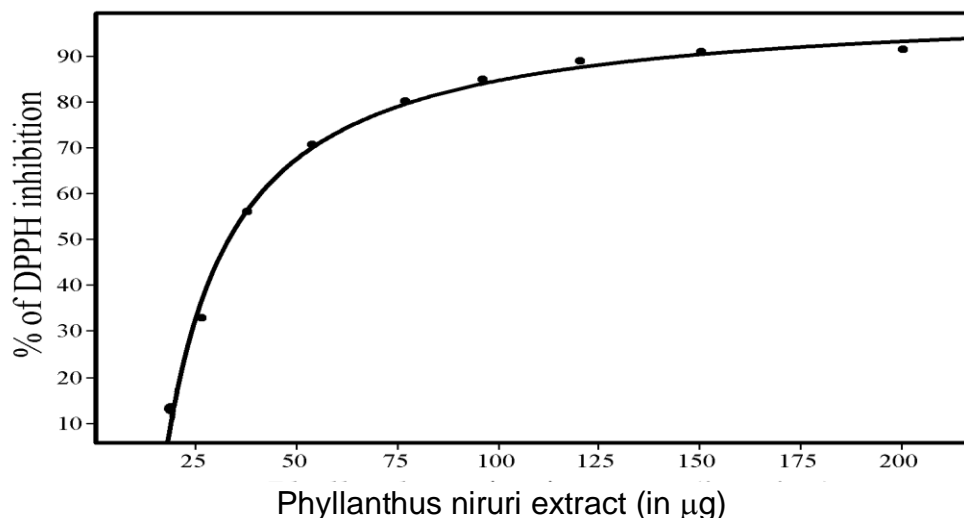
capacity of the positive control external references standards GA and EA against DPPH was 95.95% and 94.01% at a dose of 200 µg and 100 µg respectively (Figures 7 and 8). The scavenging activity curves of the extracts and the reference standards were non-linear with increasing mass of the extract or the reference standards in the form $Y = A/X + B$. These curves show the maximum scavenging activity of the extracts and the control reference standards and behave as biological curves. The inhibition capacity was the maximum percentage of scavenging activity of the extract/reference standard towards DPPH. The maximum inhibition capacity never reaches 100% due to the plateau feature of the scavenging activity profile of both the extracts and the control positive standards which is the characteristic of biological activity. The profiles were typical biological activity curve and closely resemble enzyme kinetic curve. The results of scavenging activity of the extracts and the references standards reported in Table 5 show that the scavenging activity of both extracts were much lower compared to the control reference standards compare to the TPC of the extracts. According to the TPC of the extracts, they must have lower IC₅₀ than actually obtained. This might be because the high TPC phenolic content of the extracts was due to high molecular weight and bulky molecule corilagin and geraniin compared to the planar GA and EA used as reference standards. The bulky molecules, corilagin and geraniin might have some steric hindrance to donate the phenolic hydrogen of the hydroxyl group to DPPH compared to the relatively small molecules GA and EA (Figure 4). Hence corilagin and geraniin might be slower in scavenger DPPH. While in case of GA and EA which were small planar compounds geometry enable them to participate easily in the reaction with DPPH. Therefore, the scavenging activity of the extracts was mainly due to GA and EA in the extracts. This can be predicted also from the relative scavenging activity of the extracts to the reference standards. The relative scavenging activity (R_{SCA}) was the ratio of the scavenging activities of the extracts to the scavenging activity of the control positive standard. From the relative scavenging activity (R_{SCA}) of the extract to GA and EA.

Table 5. Antioxidants activity based on IC₅₀ (μg) and the capacity of inhibition to DPPH (%) of the extracts compared to the reference standards.

Extract/standard	IC ₅₀	Capacity of DPPH inhibition (%)
<i>P. niruri</i>	32.64	91.57
<i>P. urinaria</i>	25.00	93.81
Gallic acid	3.28	95.95
Ellagic acid	2.99	94.01
<i>p</i> -value	-	0.00

Table 6. Relative scavenging activity (R_{ScA}) of extracts to GA and EA scavenging activity.

R _{ScA}	<i>P. niruri</i>	<i>P. urinaria</i>
GA	9.96	7.63
EA	10.90	8.35
Mean±SD	10.43±0.67	7.99±0.51
<i>P</i> -value of R _{ScA}	0.029	0.029

**Figure 5.** *P. niruri* scavenging activity profile against 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), the curve equation $Y = -1684.82/X + 101.618$, correlation coefficient $R^2 = 0.9938$ and IC₅₀ = 32.64 μg.

standards (Table 6), the R_{ScA} of *P. urinaria* was 7.630 GA and contained 8.709 μg GA /mg dry weight of the extract. While in *P. niruri*, the R_{ScA} was 9.960 and the content of GA was 11.867 μg /mg dry weight of *P. niruri*. The percentage ratio of the R_{ScA} of GA to its contents in *P. niruri* and *P. urinaria* extracts were approximately 83.9 and 87.6%, respectively. This result shows that the scavenging activity of the extracts was mainly due to GA. Similarly, the contribution of EA in the scavenging activity

of the *P. niruri* and *P. urinaria* extracts was 22.5 and 23.1%, respectively. Therefore, the scavenging activity of both extracts towards DPPH was due to the GA and EA in the extracts. Hence DPPH was not suitable indicator for scavenging activity of the extracts contains such bulky molecules like corilagin and geraniin. The inhibition capacity and the relative scavenging activity were significantly different and the null hypothesis was rejected for both *p*-value <0.05 as shown in Table 5 and 6.

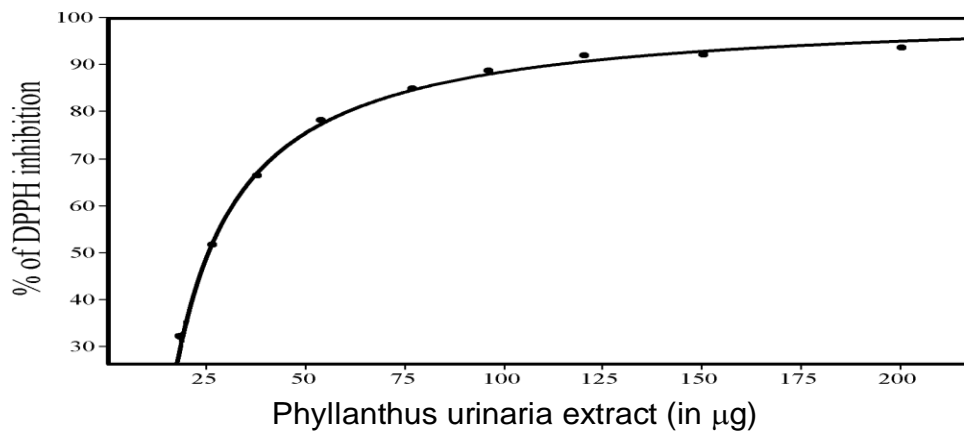


Figure 6. *P. urinaria* scavenging activity profile against 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), the curve equation $Y = -1288.4/X + 101.528$, correlation coefficient $R^2 = 0.9982$ and $IC_{50} = 25.00 \mu\text{g}$.

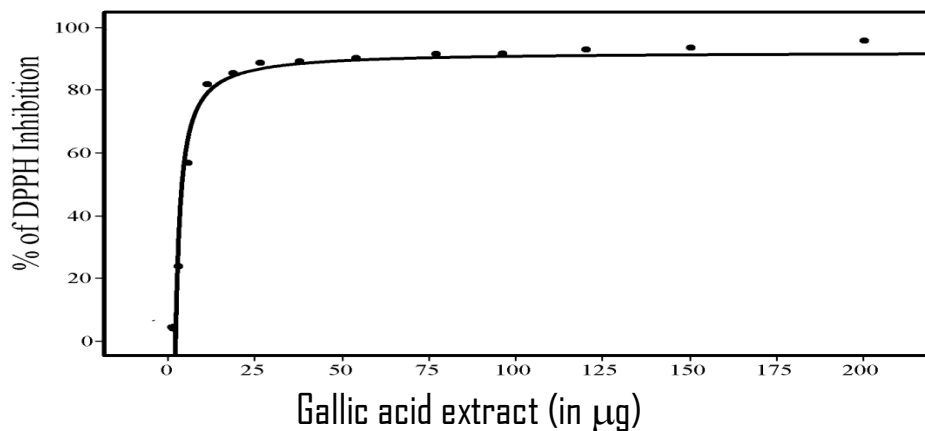


Figure 7. Gallic acid scavenging activity profile against 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), the curve equation $Y = -138.615/X + 92.3058$, correlation coefficient $R^2 = 0.9395$ and $IC_{50} = 3.28 \mu\text{g}$.

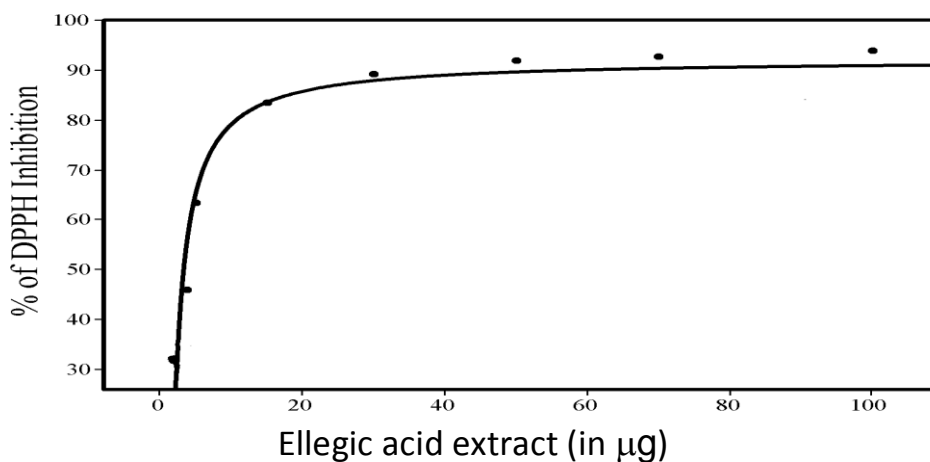


Figure 8. Ellagic acid scavenging activity profile against 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), the curve equation $Y = -126.56/X + 92.27$, correlation coefficient $R^2 = 0.9394$ and $IC_{50} = 2.99 \mu\text{g}$.

The results of scavenging activity (Table 5) consequently show that *P. urinaria* was more potent compared to *P. niruri* based on IC₅₀ value and capacity of scavenging activity. The result was consistent with the TPC finding which showed *P. urinaria* with high TPC compared to *P. niruri*. Therefore *P. urinaria* was strong inhibitor to DPPH activity compared to *P. niruri* as well as it has high total phenolic content (TPC). This result was also consistent with the previous finding which is, the higher the TPC, the stronger scavenging to DPPH (Tawaha et al., 2007; Zheng and Wang, 2001). Similarly, it was also noticed that EA IC₅₀ was lower compared to GA IC₅₀, hence it was more potent than GA. EA also returned high scavenging capacity towards DPPH since it inhibited 94.01% of DPPH activity at a dose of 100 µg compare to GA which inhibited 95.95% of DPPH activity but at a dose of 200 µg (Figure 7 and 8). This might be because of differences in the number of hydroxyl group attached to the aromatic ring in GA and EA chemical structures and hence more availability of donating the phenolic hydrogen of hydroxyl group as scavenger in the reaction (Figure 4). The results of scavenging activity of GA and EA from this study and the scavenging activity of *P. niruri* and *P. urinaria* were comparable to the previous study of plants material of similar content of our extracts (Rangkadilok et al., 2007). The scavenging capacity of the extracts from this study was higher at higher dose as compared to previous (Kumaran and Joel, 2007).

Conclusions

The proposed HPLC method successfully identified and quantified the phenolic compounds of the extracts. The method was quite simple, rapid, sensitive, selective, cost effective procedure, friendly and safe to the column and instrument on long uses. The HPLC analysis, TPC and the scavenging activities results show that the extracts contain high phenolic materials comparable to the references standards gallic acid and ellagic acid. DPPH was not a good indicator of scavenging activity of the extract because it mainly composes bulky molecules such as corilagin and geraniin. The result of antioxidants evaluation is revealed to the extracts as a potential natural source of antioxidants. The high antioxidants properties of the extracts might make them versatile in various fields of nutraceuticals, pharmaceuticals and cosmeceuticals applications. Specifically, our interest in this time is formulation of these extracts as skin antiaging, sun-blocking and whitening agents.

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