Jurnal Teknologi

Full paper

The Effects of Solvents and Extraction Methods on the Antioxidant Activity of *P. niruri*

Hafizah Mohd Hadzri, Mohd Azizi Che Yunus*, Salman Zhari, Fahim Rithwan

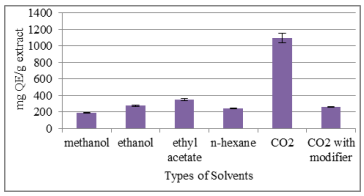
Centre of Lipid Engineering and Applied Research (CLEAR), c/o Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

*Corresponding author: azizi@cheme.utm.my

Article history

Received :4 October 2013
Received in revised form :
9 March 2014
Accepted :19 March 2014

Graphical abstract



Abstract

The effects of different types of solvents and extraction method were investigated to determine the presence of antioxidant contents and activity from the *P. niruri* plant. The aim of this study is to determine which extraction method will give higher natural antioxidant contents and antioxidant activity. The content of natural antioxidant and antioxidant activity were analysed by total phenolic content (TPC), total flavonoid content (TFC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay. The results showed that extracts from a supercritical fluid extraction (SFE) method without the addition of modifier showed the highest content of total phenolic (187.66 mg GAE/ g) and flavonoid (1100.93 mg QE/g) in *P. niruri* compared to the other methods of extraction with different type of solvents. The extract of *P. niruri* from different extraction methods showed antioxidant activity on DPPH radical scavenging assay. The soxhlet extraction method by methanol showed the lowest IC_{50} compared to the other methods of extraction. The results revealed that *P. niruri* extracts had different content of antioxidant and antioxidant activity. The solvent polarity and different methods of extraction play significant roles in determining the most suitable method for production of antioxidant contents and antioxidant activity from *P. niruri* extracts.

Keywords: Phyllantus niruri; antioxidant; total phenolic content; total flavonoid content; DPPH free radical scavenging activity

1.0 INTRODUCTION

The famous herbs from Phyllantus species are widely used in folk medicine like the Ayurvedic medical system for more than 3000 years are herbs which useful in the treatment of kidney problems, urinary bladder disturbances, diabetes, pain, jaundice, gonorrhoea, chronic dysentery, skin ulcers, and hepatitis B. [1] In Brazilian folk medicine, *P. niruri* from Euphorbiaceae is well known as 'quebra pedra' or 'stone breaker' have strong impact on kidney stones and gallstones. [2] The curing and relieving ability of *P. niruri* on certain diseases have been attributed to different classes of constituents including lignans [3], alkaloids (non polar) [4], flavonoids (medium polar) [5], terpenoids [6], phenylpropanoids [7] and the hydrolysable tannin, corilagin [8].

The traditionally used of *P. niruri* for the treatment of urolitic and as a diuretic in Paraguay was found to possess inhibitory activity against angiotensin converting enzyme (ACE) by 70% ethanol extracts of *P. niruri* from the screening test for biological activity. The presences of Corilagin are verified in several chromatographic studies in leaves and extracts of different species of the genus Phyllantus [9]. The biological activities of *P.niruri* have interesting relation with traditionally used as folk medicine. The whole parts of *P. niruri* by aqueous extract can inhibit the endogenous DNA polymerase of hepatitis B virus in vitro and in vivo [10]. In the ethanolic extracts of *P. niruri* from whole parts of the plant showed the presence of alkaloids, steroids, terpenes, coumarins, polyphenolic compounds such as phenolic acids and flavonoids by phytochemical analysis using TLC [11].

Antioxidants are the substances that can neutralize free radicals. Oxygen is a radical agent that can produces reactive oxygen species (ROS) which is highly toxic and causes various damages especially to human. ROS are considered to be the major causes of skin aging, cancer and certain skin disorder [12]. Although ROS usually have a short half-life, they can react with DNA, proteins and unsaturated fatty acids. Thus, as a defence systems against these free radicals, human need the substances that can neutralize and counter the free radicals. The harmful ROS can be defended by consuming the additional of antioxidants through herbs, foods or supplements [13]. The types of ROS such as superoxide radical anion, hydroperoxyl radical are produced in cells from byproducts of metabolism which are major contributor for a large number of degenerative diseases such as cardiovascular disease (CVD), diabetic, cirrhosis and several cancers [14]. The antioxidant activity and hepatoprotective potential [15] was found in *P. niruri*.

From the previous research found that the total phenolic compound (TPC), DPPH Scavenging Assay (DPPH) and FRAP Antioxidant Assay (FRAP) posses the highest content and activity

of antioxidants compared to the other species of phyllantus using water and methanol extract [16]. The methanol extracts produced the highest contents and activity of antioxidant compared to water extract. The leaves and fruits part of *P. niruri* by methanolic and aqueous extract also had inhibition of membrane lipid peroxidation (LPO), scavenging of 1, 1-diphenyl-2picrylhydrazyl (DPPH) radical and inhibition of reactive oxygen species (ROS) in vitro [15]. The effects towards anti-HIV using three different types of solvents (water, methanol, 50% ethanol) in pharmacology study of *P. niruri* was also reported [17]. Among the three types of solvents, 50% of ethanol extract can inhibit the most replication of the reverse transcriptase virus compare to the other solvents and the extracts also contains 1.10% geraniin and 2.28% (w/w) corilagin.

In recent years, the functional foods are widely preferred by consumers due to the functional ingredient from the traditional foods that possess ingredients which are able to provide a useful action for human health. These natural ingredients are chosen by consumers and usually extracted from natural sources such as plants, food byproducts or even algae and microalgae. Among many ingredients in natural sources, antioxidants compounds are being the most studied. In this study, the extraction of *P. niruri* by soxhlet is done using various solvents which are methanol, ethanol, ethyl acetate and n-hexane as extracting solvents because these might extract different compositions. However, this traditional extraction methods used to attain these sorts of products have several shortcoming thus they are time consuming, laborious and have low selectivity. Moreover, these techniques occupy large amounts of toxic solvents. To overcome these circumstances, a new extraction method which is supercritical fluid extraction (SFE) is being studied. These extraction methods give higher selectivity, shorter extraction times and do not utilize toxic solvents.

2.0 EXPERIMENTAL

2.1 Plant Material

P. niruri was purchased from Herbagus, Penang and pre treated using sun dried method. The sun dried *P. niruri* was grind to become powdered form. The particle size of *P. niruri* was 3 mm. The sample was divided into several parts for different extraction methods which are supercritical fluid extraction carbon dioxide with and without modifier and soxhlet.

2.2 Soxhlet Extraction (SE)

Twenty grams of *P. niruri* sample were weighed and placed in thimbles for four different solvent which are methanol, ethanol, ethyl acetate and n-hexane. Then, the thimbles were transferred into soxhlet extractors and cotton was inserted on the top of sample in thimbles to make sure that the sample will not spill out from the thimble during extraction. Four different types of solvents were added into each round flask, which is connected to the extractor and condenser. The extraction periods are 6 hours for each solvent. After the completion of extraction process, solvents were removed a rotary evaporator to obtain the crude extracts of *P. niruri*.

2.3 Supercritical Fluid Extraction (SFE)

P. niruri was extracted by supercritical carbon dioxide extractor with and without modifier (methanol) as a co-solvent using static-dynamic extraction. The pressure applied was 30 MPa and the temperature was 60ºC. *P. niruri* samples were taken from freezer and keep in room temperature for thawing purposes. After the thawing of samples, five grams of *P. niruri* samples were weighed by weighing scale and placed into a 1 L extraction vessel and the extraction vessel was sealed tightly. Then, the desired temperature was set (60ºC). Pressure within the extraction vessel was from a constant carbon dioxide flow rate and regulated by automated back pressure regulator. The SFE extraction was started after the desirable temperature (60ºC) and pressure (30 MPa) were achieved. The whole extraction process for *P. niruri* took around 90 minutes and the yield of *P. niruri* extraction was measured. After the completion of extraction, the extraction vessel was depressurized and the yield was collected.

2.4 Determination of Antioxidant Content

2.4.1 Total Phenolic Content (TPC)

Total phenolic content (TPC) of *P. niruri* was performed based on the method done by previous study with a slight modification [18]. 0.1 mL of sample or standard stock solution was transferred into test tube. Then, 1.0 mL of Folin-Ciocalteu reagent was mixed with the sample or standard stock solution and the mixture was left for three minutes. After three minutes, 300 µL of sodium carbonate solution was added into the test tube and the mixture was left for 90 minutes. After 90 minutes standing at room temperature, the absorbance was read at 725 nm by automated microplate reader, Multiskan GO. The analyses were performed in triplicate and the standard curve with serial gallic acid solution (ranging from 0.02-0.64 mg/mL) was used for calibration. The results of total phenolic content were expressed as mg of gallic acid equivalents (GAE) per 1g of extract.

2.4.2 Total Flavonoid Content (TFC)

Total flavonoid content (TFC) test of *P. niruri* was performed based on the previous study with a slight modification [19]. 250 µL of standard or samples solutions were added with 1.25 mL distilled water and mixed with 75 µL 5% $NaNO_2$ solution. The mixture was left at room temperature for 6 minutes. Then, 150 µL of 10% $AlCl_3$ was added into the mixture for five minutes. After five minutes, 0.5 mL 1M NaOH and 275 µL distilled water were added to the mixture. The analyses were performed in triplicate and the standard curve with serial quercetin solution (0.02, 0.04. 0.08, 0.16, 0.32, and 0.64 mg/mL) was used for calibration. The absorbance was read at 510 nm by automated microplate reader using Multiskan GO. The results of total flavonoid content were expressed as mg of quercetin equivalents (QE) per 1 g of extract.

2.4.3 DPPH Free Radical Scavenging Activity

The ability of *P. niruri* to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined by DPPH free radical scavenging activity assay. The scavenging effects of samples for DPPH were performed based on the method in the previous study with a slight modification [20]. Ascorbic acid was used as positive control. 1 mM of DPPH solution was prepared by diluting 3.94 mg DPPH in 10 mL of methanol. 200 µL of standard and *P. niruri* solution (800, 400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL) were added into microplate 96 well. Then, 50 µL of 1 mM DPPH solution was mixed into each well. The mixture of standard or *P. niruri* solution was left at room temperature in the dark place for 30 minutes. After 30 minutes of incubation period, the absorbances were read at 517 nm by automated microplate reader using Multiskan GO. The blank sample was 200 µL methanol and 50 µL of 1 mM DPPH. All samples were made in triplicate. The ability of *P. niruri* extracts and positive controls to scavenge the DPPH free radical was calculated using the following formula:

$$I\ \% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100\%$$

A_{blank} is the absorbance of 1 mM of DPPH solution with methanol while A_{sample} is the absorbance of the *P. niruri* extracts and positive controls solution. The lower absorbance indicates a higher scavenging activity followed by decreasing the intensity of purple to yellow colour. The radical scavenging activity of *P. niruri* extracts were interpreted via IC_{50} value. The IC_{50} is a concentration that can scavenge the 50% of DPPH free radical.

All experiments were done in triplicate. The results are given as mean standard deviation. T-test was used for comparison between the two means and a one-way analysis of variance (ANOVA) was used for comparison of more than two means. A difference was considered statistically significant when $p < 0.05$.

3.0 RESULTS AND DISCUSSION

3.1 Solvent Extraction Yield

The frequently used technique to attain antioxidants from plant materials is solvent extraction. Due to the variation in solubility and polarity, it has been suggested that there is no single solvent that can extract all the antioxidants from food [21].

Table 1 Various solvents extraction yields of *P. niruri* by different methods of extract.

Solvents	Extraction yield (% w/w)	
	Soxhlet	SFE
Methanol	18.44	-
Ethanol	9.24	-
Ethyl acetate	5.15	-
N-Hexane	2.56	-
CO_2	-	5.28
CO_2 with 10 mL methanol	-	2.22

Table 1 shows the extraction yields of different solvents. The extraction yield was found to be reliant on extraction solvent. The extraction yields of *P. niruri* extracted by soxhlet using various types of solvents ranged from 2.56% to 18.44%, while SFE using CO_2 and CO_2 with addition of co-solvent had an extraction yield ranging from 2.22% to 5.28%. Extraction of *P. niruri* by soxhlet using methanol as solvent showed the highest yield of extract followed by ethanol, ethyl acetate and n-hexane, while SFE using carbon dioxide showed the highest yield of extract compared to SFE with addition of 10 mL methanol as co-solvent. With ethyl acetate or n-hexane as the solvent, the extraction yield found with soxhlet is close to that of the SFE using CO_2 and modifier.

3.2 Contens of Total Phenolics and Total Flavonoids

It has been proposed that the phenolic content of plant materials is related with their antioxidant activity [22]. The broadly used of TPC assay is usually to estimate relative amounts of phenolic compounds present in an extract. The simplest form of a phenolic compound which is gallic acid was used to express the TPC results as g gallic acid equivalents. A complex redox reaction with phosphotungstic and phosphomolybdic acids which is present in the TPC reagent is occurred in the extract with the presence of phenolic compounds [23]. The different reaction can be observed in conditions of the color changes due to oxidation of the TPC reagent depending on the number of phenolic groups present [16].

Table 2 Total phenolic content and total flavonoid content by different solvents and extraction method

Solvents	mg gallic acid equivalent / g extract		mg quercetin equivalent / g extract	
	Soxhlet	SFE	Soxhlet	SFE
Methanol	121.25 ± 7.31	-	186.8 ± 5.88	-
Ethanol	101.54 ± 5.16	-	274.8 ± 13.06	-
Ethyl acetate	52.7 ± 2.02	-	346.1 ± 21.34	-
N-Hexane	34.28 ± 0.25	-	241 ± 11.09	-
CO_2		187.66 ± 9.43	-	1101.8 ± 100.71
CO_2 with 10 mL methanol		57.41 ±0.65	-	262.1 ± 6.40

Values are presented as means ± SD (n=3)

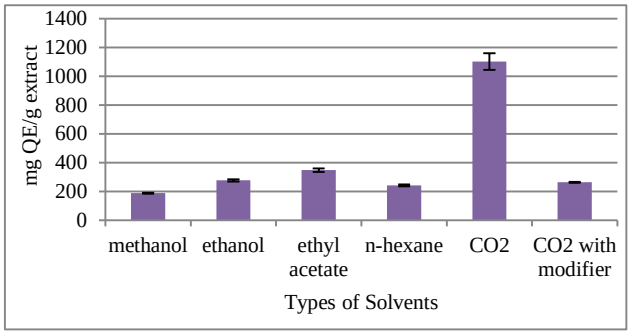


Figure 1 Total phenolic content by different solvents and extraction method

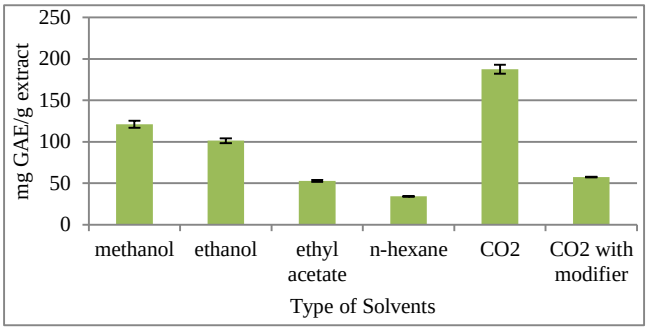


Figure 2 Total flavonoid content by different solvents and extraction method

The research found that the major components of *P. niruri* are active hydrolysable tannins which are semi polar compounds like ellagitannins and gallotannins that can be extracted using the mixture of ethanol and water [24]. The above results may be due to the fact that phenolics are frequently extracted in higher amounts using more polar solvents and the content of phenol is decreasing with semi or less polar solvents. The method of extract by SFE showed the contrary effects in TPC. The extract using CO_2 only possesses the highest contents of phenol compared to the soxhlet and SFE using addition of 10 mL of methanol. The higher yield may be due to low temperature used in SFE as compared to soxhlet. The high temperature and long period in processing conditions might result in the loss of natural antioxidants because heat may speed up their oxidation and other degenerative reactions. The reduction of antioxidant activity in the apple juice from 20-40% was occurred when an accelerated shelf-life test was performed at 80 °C for 4 days [25]. The other studies found that the heating at 100 °C for nine days can decrease the antioxidant activities up to 61% [26]. There was a statistically significant differences ($p<0.05$) in the mg gallic acid equivalents (GAE)/ g extracts among different solvents in soxhlet and SFE.

The other types of plant secondary metabolites are flavonoids such as flavonols, flavones and condensed tannins. The intake of foods like fruits and vegetables which possess flavonoid has been linked to protection against cancer and heart disease [27]. The total flavonoid content ranging from 186.8 mg to 346.1 mg and 262.1 mg to 1101.8 mg quercetin equivalent/g extract, respectively, was noted with the extracts of *P. niruri* by soxhlet and SFE. *P. niruri* extracted by soxhlet using ethyl acetate as solvent possessed the highest content of flavonoid followed by ethanol, n-hexane and methanol, respectively while SFE using CO_2 had highest content of flavonoid compared to CO_2 with addition of 10 mL methanol as co-solvent. Based on the result, SFE is the best method of extract for production of *P. niruri* compared to soxhlet. The above results might be due to the influence of temperature involved in extraction process. High temperature and long period of extract by soxhlet may degrade the compounds in *P. niruri* extracts.

In previous study they found that the using of heating extraction process produced lower flavonoid contents compared to maceration, due to the higher temperature can degrade the flavonoid compound [28]. The results proved that high temperature can lower the flavonoid compound in *P. niruri*. Thus, the method of SFE is more preferable compared to soxhlet in extracting of the flavonoid compound in *P. niruri*. There was a statistically significant differences ($p<0.05$) in the mg quercetin equivalents (QE)/ g extracts among different solvents in soxhlet and SFE.

3.3 DPPH Radical-Scavenging Effect

The dose in mass of the antioxidants resources which is extract or reference standard needed to inhibit the initial DPPH radical activity by 50% is known as IC_{50}. The smaller IC_{50} value indicates the high scavenging activity and strong antioxidants compound [29].

In the DDPH assay, 1,1-Diphenyl-2-picrylhydrazyl is the focal chemical broadly consumed for the determination of free radical scavenging activity of antioxidant compounds in extracts [30]. Diphenylpicrylhydrazl will be reduced to diphenylpicrylhydrazine when added to the plant extract which contain antioxidant compounds. The colour will be changed from purple to yellow during the process. One of the common ways to express the antioxidant activity is referring to a common reference standard. The common reference standard used for in this study is ascorbic acid.

Table 3 DPPH free radical scavenging activity effects by different solvents and methods of extract

Solvents	DPPH free radical scavenging activity effects			
	% of inhibition at 6.25 µg/mL	IC_{50} (µg/mL) by Soxhlet	% of inhibition at 6.25 µg/mL	IC_{50} (µg/mL) by SFE
Methanol	13.76	26	-	-
Ethanol	6.26	77	-	-
Ethyl acetate	3.077	500	-	-
N-Hexane	-	-	-	-
CO_2	-	-	7.07	-
CO_2 with 10 mL methanol	-	-	8.035	540

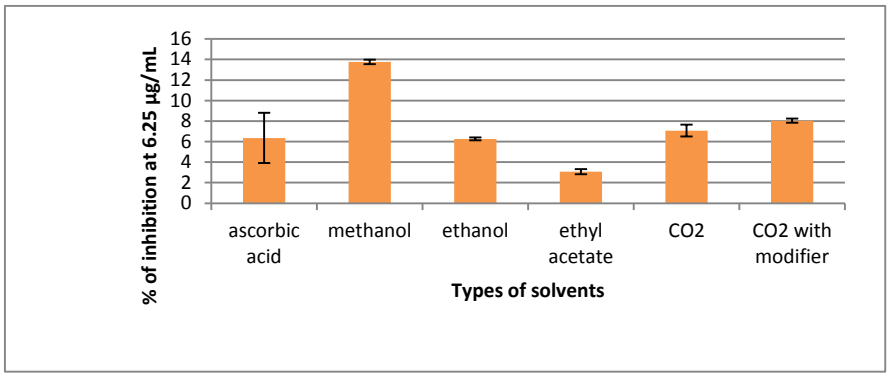


Figure 3 DPPH free radical scavenging activity effects by different solvents and methods of extract

The IC_{50} of *P.niruri* extracted by different technique and solvents presented in Table 3 ranged from 26-540 ug/mL. The extract of *P. niruri* by soxhlet using methanol as a solvent showed the lowest IC_{50} which is 26 µg/mL indicates high capacity of *P. niruri* to scavenge free radical of DPPH. There was no IC_{50} in soxhlet using n-hexane and SFE without modifier. The concentration of seriel dilution should be increased more than 1 mg/mL in order to achieve the IC_{50}. The structural factors like the number of phenolic, hydroxyl or methoxyl groups, flavones hydroxyl, keto group, free carboxylic groups and other structural features may influence the antioxidant activities of the individual compound which is present in the extracts [31,32]. The increase of sample or standard concentration can increase the scavenging effects on the DPPH radical [33]. There was a statistically significant differences ($p<0.05$) in the percent of inhibition among different solvents used in soxhlet while there was not statistically significant different ($p<0.05$) in the percent of inhibition among two groups of solvents in SFE.

■4.0 CONCLUSION

In conclusion, the natural antioxidants in *P. niruri* have different beneficial effects towards the human health. As this project attempted to study whether there is any influenced caused by different extraction method and solvents in extraction of *P. niruri* in determining the antioxidant content and activity, the objective is achieved because each extracts of *P. niruri* by different methods and solvents has different content of natural antioxidant and antioxidant activity. SFE extraction method was most effective for phenolic and flavonoid content in *P. niruri* while soxhlet extracted by methanol was the best method for DPPH free radical scavenging activity.

Acknowledgement

The research was funded by grant Q.J130000.2544.04h05: Extraction of Medicinal Compounds (Vitamin E and Beta Carotene) from Palm Mesocarp using Green Supercritical and Subcritical C02 Technology. Special thanks also to Universiti Teknologi Malaysia (UTM), Centre of Lipid Engineering and Applied Research (CLEAR) and Ministry of Higher Education (MOE).

References

[1] Calixto, J. B. 2000. Efficacy, Safety, Quality Control, Marketing and Regulatory Guidelines for Herbal Medicines (Phytotherapeutic Agents). *Brazilian Journal of Medical and Biological Research*. 33: 179–189.

[2] Micali, S., M. C. Sighinolfi., A. Célia., S. Stefani., M. Grande., A. F. Cicero and G. Bianchi. 2006. Can Phyllanthus Niruri Affect the Efficacy of Extracorporeal Shock Wave Lithotripsy for Renal Stones? A Randomized, Prospective, Long-Term Study. *The Journal of Urology*. 176: 1020–1022.

[3] Singh, B., P. K. Agrawal and R. S. Thakur. 1989. A New Lignan and a New Neolignan from Phyllanthus Niruri. *Journal of Natural Products*. 52: 48–51.

[4] Tempesta, M. S., D. G. Corley., J. A. Beutler., C. J. Metral., R. A. Yunes., C. A. Giacomozzi and J. B. Calixto. 1988. Phyllanthimide, A New Alkaloid from Phyllanthus Sellowianus. *Journal of Natural Products*. 51: 617–618.

[5] Than, N. N., S. Fotso., B. Poeggeler., R. Hardeland and H. Laatsch. 2006. Nirurifiavone, A New Antioxidant Flavone Sulfonic Acid from Phyllanthus Niruri. *Zeitschrift für Naturforschung*. 61: 57–60.

[6] Ueno, H., S. Horie., Y. Nishi., M. Kawasaki., S. Suzuki S., T. Hayashi., M. Arisawa., M. Shimizu., M. Yoshizaki., N. Morita., L. H. Berganza., E. Ferro and I. Basuald. 1988. Chemical and Pharmaceutical Studies on Medicinal Plants in Paraguay, Geraniin, an Angiotensin-converting Enzyme Inhibitor from 'paraparai mi', Phyllanthus niruri. *Journal of Natural Products*. 51: 357–359.

[7] Singh, B., P. K. Agrawal and R. S Thakur. 1986. Chemical constituents of Phyllanthus Niruri Linn. *Indian Journal of Chemistry*. 25B: 600–602.

[8] Duan, W., Y. Yu and L. Zhang. 2005. Antiatherogenic effects of Phyllanthus Emblica Associated with Corilagin and Its Analogue. *Yakugaku Zasshi*. 125: 587–591.

[9] Kumaran, A and R. J. Karunakaran. 2006. Nitric Oxide radical scavenging active components from Phyllanthus emblica L. *Plant Foods for Human Nutrition*. 61: 1–5.

[10] Venkateswaran, P. S., I. Millman and B. S. Blumberg. 1987. Effects of an Extract from *Phyllanthus Niruri* on Hepatitis B and Woodchuck Hepatitis Viruses: In Vitro And In Vivo Studies. *Proceedings of the National Academy of Sciences of the United States of America*. 84: 274–278.

[11] Cimangaa, R. K., L. Tonab, N. Luyindulac, K. Mesiab, M. Lusakibanzab, C.T. Musuambab, S. Apersa, T. De Bruynea, S. Van Mierta, N. Hermansa, J. Tott´ea, L. Pietersa and A.J. Vlietinck. 2004. In Vitro Antiplasmodial Activity of Callus Culture Extracts and Fractions from Fresh Apical Stems Of Phyllanthus Niruri L. (Euphorbiaceae): Part 2. *Journal of Ethnopharmacology*. 95: 399–404.

[12] Dreher, F. and H. Maibach. 2001. Protective Effects of Topical Antioxidants in Humans. *Oxidants and Antioxidants in Cutaneous Biology*. 29: 157.

[13] Shi, H. L., N. Noguchi and E. Niki. 2001. Introducing Natural Antioxidants. In J. Pokorny *et al.*, Antioxidants in Food: Practical Applications. (eds.). Woodhead Publishing Ltd. and CRC Press.

[14] Halliwell, B. 1996. Antioxidant in human health and disease. *Annual Review of Nutrition*. 16: 33–50.

[15] Harish, R. and T. Shivanandappa. 2004. Antioxidant Activity and Hepatoprotective Potential of Phyllanthus Niruri. *Food Chemistry*. 95: 180–185.

[16] Poh-Hwa, T., C. Yoke-Kqueen., J. Indu Bala and R. Son. 2011. Bioprotective Properties of Three Malaysia Phyllanthus Species: An Investigation of the Antioxidant and Antimicrobial Activities. *International Food Research Journal*. 18(3): 887–893.
[17] Notka, F., G. R. Meier and R. Wagne. 2003. Inhibition of Wild-type Human Immunodeficiency Virus and Reverse Transcriptase Inhibitor-Resistant Variants by Phyllanthus Amarus. *Antiviral Research*. 58: 175–186.
[18] Singleton, V. L., R. Orthofer and R. M. Lamuela-Raventos. 1999. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin Ciocalteau Reagent. *Method Enzymol*. 299: 152–178.
[19] Zhishen, J., T. Mengcheng and W. Jianmin. 1999. The Determination of Flavonoid Contents in Mulberry and Their Scavenging Effects on Superoxide Radicals. *Food Chem*. 64: 555–559.
[20] Lee, C. H., L. Yang., J. Z. Xu., S. Y. V. Yeung., Y. Huang and Z. Y. Chen. 2005. Relative Antioxidant Activity of Soybean Isoflavones and Their Glycosides. *FoodChem*. 90: 735–741.
[21] Garcia-Alonso, M., S. dePascual-Teresa., C. Santos-Buelga and J.C. Rivas-Gonzalo. 2004. Evaluation of the Antioxidant Properties of Fruits. *Food Chem*. 84: 13–18.
[22] Velioglu, Y. S., G. Mazza., L. Gao, and B. D. Oomah. 1998. Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, Andgrainproducts. *J.Agric.FoodChem*. 46: 4113–4117.
[23] Wong, S. P., L. P. Leong, and J. H. W. Koh. 2006. Antioxidant Activities of Aqueous Extracts Selected Plants. *Food Chemistry*. 99: 775–783.
[24] Masturah, M., M. Masitah, W. D. Wan Ramli, S. Harcharan, and M. J. Jamaliah. 2006. Extraction of Hydrosable Tannins from Phyllanthus Niruri Linn: Effects of Solvents and Extraction Method. *Separation and Purification Technology*. 52: 487–498.
[25] Van der Sluis, A. A., M. Dekker and M. A .J. S. Boekel. 2005. Activity and Concentration of Polyphenolic Antioxidants in Apple Juicesta Bility During Storage. *J. Agric. Food Chem*. 53: 1073–1080.
[26] Cheng, Z., L. Su., J. Moore., K. Zhou., M. Luther., J.J. Yin and L.L. Yu. 2006. Effect of Postharvest Treatment and Heat Stress on Availability of Wheat Antioxidants. *J. Agric. Food Chem*. 54: 5623–5629.
[27] Hertog, M. G. L., P. C. H. Hollman and D. P. Venema. 1992. Optimization of a Quantitative HPLC Determination of Potentially Anti Carcinogenic Flavonoids in Vegetables And Fruits. *J. Agric. Food Chem*. 40: 1591–1598.
[28] Rafael, C. D., N. L. Magda and R. B. Nadia. 2008. Quantification of Phenolic Constituents and Antioxidant Activity of Pterodon Emarginatus Vogel Seeds. *Int. J. M. Sci*. 9: 606–614.
[29] Elrashid, S. M., M. N. Azmin., H. S. Mohamed., Z. A. Ghassan., A. K. Muthanna and A. S. Munavvar. 2011. Identification of Phenolic Compounds and Assessment of in Vitro Antioxidants Activity of 30% Ethanolic Extracts Derived from Two Phyllanthus Species Indigenous to Malaysia. *African Journal of Pharmacy and Pharmacology*. 5(17): 1967–1978.
[30] Aziz, T., E. D. Mehmet., M. Nazime., K. Ibrahim and G. Kudret. 2007. Antioxidant and Antimicrobial Activities of Laetiporus Sulphureus (Bull.) Murrill. *Food Chemistry*. 101: 267–273.
[31] Hasan, S. M. R., M. Hossain., R. Akter., M. Jamila., E. H. Mazumder and S. Rahman. 2009. DPPH Free Radical Scavenging Activity of Some Bangladeshi Medicinal Plants. *J of Med Plants Res*. 3(11): 875–879.
[32] Patt, D. E. and B. J. F. Hudson. 1990. *Natural Antioxidants Not Exploited Commercially*. Elsevior Applied Science, London: U. K.
[33] Patel, V. R., P. R. Patel and S. S. Kajal. 2010. Antioxidant Activity of Some Selected Medicinal Plants in Western Region of India. *Adv in Bio Res*. 4 (1): 23–26.