Asian Journal of Biological and Life Sciences | Original Research

The brine shrimp lethality of the leaf extracts of *Piper baccatum* Blume and their antioxidant properties

Mylene M. Uy*, Maryjane P. Villanueva

Department of Chemistry, College of Science and Mathematics, MSU-Iligan Institute of Technology, A. Tibanga, Iligan City, Philippines.

E-mail : mylene603@yahoo.com Contact No. : +63-63-2215041 loc 123

Submitted : 12.08.2015 Accepted : 03.09.2015 Published : 31.12.2015

Abstract

Piper baccatum Blume was investigated for the possible presence of bioactive compounds. Ethanol (PbE), chloroform (PbC), hexane (PbH), aqueous (PbA) and decoction (PbD) extracts of *P. baccatum* were prepared and subjected to evaluation of bioactivities. The toxicological evaluation utilized the brine shrimp lethality test (BSLT) and the determination of antioxidant activities included the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay, the phosphomolybdenum assay for total antioxidant capacity and the Folin-Ciocalteu assay for total phenolics content. Among the extracts, the aqueous extract PbA exhibited the most toxic activity against the brine shrimp *Artemia salina* with an LC_{50} of < 10 ppm. Both the aqueous and decoction extracts (PbA and PbD) showed the same ability to scavenge the free radical DPPH (EC_{50}, 25.0 ppm). The hexane extract PbH possessed the highest total antioxidant capacity as expressed by its Ascorbic Acid Equivalents (AAE) value of 95.8 and Butylated Hydroxytoluene Equivalents (BHTE) value of 94.4. The highest total phenolics content was exhibited by the chloroform extract PbC having a Gallic Acid Equivalence (GAE) value of 99.2. The results indicate that bioactive compounds may be purified and isolated from the different extracts of *P. baccatum*.

Key words : medicinal plant, brine shrimp lethality, antioxidant activity, phytochemicals

INTRODUCTION

Plants have been used by man as drugs for more than a century and biologically active substances derived from plants have served as templates for the synthesis of pharmaceuticals[1-2]. Many researches have been involved in investigating the chemical constituents of species belonging to the genus Piper[3-10]. Aside from the traditional use of piper species as spices in food[10-11], studies have revealed that piper species also possess a number of pharmacological properties[12]like antifeeding[7], DNA-damaging[8], antibacterial[4,6], antifungal[3,5], antiplatelet[13-14], antioxidant[14], anti-inflammatory[9], antiamoebic[15], insecticidal[16-18], cytotoxic[19-22] and antiplasmodial[23].

P. baccatum is a dioecious vine that belongs to the family of Piperaceae[24]. The fruits of *P. baccatum* are used as spice and the decoction of its roots is traditionally used in the Philippines to treat venereal diseases. There are only few experimental studies that validate the therapeutic claims of the plant *P. baccatum*[25].

The brine shrimp lethality test is considered a useful tool for preliminary assessment of toxicity[26] and has been used for the detection of toxins[27-28], heavy metals[29], pesticides[30] and plant extract toxicity[31-34].

Plants have long been accepted to contain naturally occurring substances possessing antioxidant activity[35]. At present, a heightened interest exists in reaction oxygen species and their roles in many chronic disorders[36-37]. Accordingly, researches are being focused on the protective biochemical functions of naturally occurring antioxidants in plants. A large number of methods have been developed in order to evaluate antioxidant activity[38-41]. One of these methods is the DPPH radical scavenging activity assay which is considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties of pure compounds[42]. The DPPH assay has the advantage of having good stability, credible sensitivity, simplicity and feasibility[43]. Another *in vitro* model for the assessment of total antioxidant activity is the phosphomolybdenum method which is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH[44]. It evaluates both water-soluble and fat-soluble antioxidants[45]. Plant extracts with high phenolics content have been known to possess strong antioxidant activities due to intrinsic reducing capabilities[46]. Phenols in plant extracts react with specific redox reagents like the Folin-Ciocalteu reagent to form a blue complex that can be spectrophometrically quantified[47-48]. The Folin-Ciocalteu method is described in several pharmacopoeias[49]. The reaction forms a blue chromophore constituted by a phosphotungstic phosphomolybdenum complex[47,50] where the maximum absorption of the chromospheres depends on the alkaline solution and the concentration of phenolic compounds.

This study establishes the extraction and antioxidant activities of the bioactive constituents present in the leaves of the medicinal plant *P. baccatum*.

MATERIALS AND METHOD

Plant collection and preparation of crude extracts

The whole plant of *P. baccatum* was collected from Brgy. Bonbon of Butuan City, Agusan del Norte, Philippines. About 1.2 kg of the plant's dried leaf sample were pulverized using a sterile electric blender, weighed and percolated with enough 95% ethanol for three days. The solution was filtered, concentrated in vacuo using a rotary evaporator at temperatures not exceeding 40°C and was weighed to give the crude ethanol extract (PbE). A portion of the crude ethanol extract (PbE) was sequentially partitioned in hexane:water and chloroform:water solutions. The hexane-soluble, chloroform-soluble and aqueous soluble portions were individually concentrated in vacuo and were

weighed to give the crude hexane (PbH), chloroform (PbC) and aqueous extracts (PbA), respectively. The decoction was prepared by boiling around 200 g of the fresh and clean samples of the plant's leaves which were cut into pieces, in sufficient amount of distilled water (1:2 ratio) for 5 minutes. The mixture was then filtered and freeze-dried to give PbD.

Brine shrimp lethality test

The crude extracts were evaluated for lethality to brine shrimp using standard methods with a slight modification[51]. Four concentrations of the extracts (1000-, 500-, 100-, and 10-ppm) were prepared in three replicates. The prepared test solutions were then subjected to lethality test against the brine shrimp *A. salina*. The number of dead and alive nauplii was counted after 24 hours. Using Reed-Muench method[52-53], LC_{50} values for all the crude extracts were determined.

DPPH radical scavenging activity

Using the method of Lee and Shibamoto[54], the DPPH radical scavenging activity of all test samples were examined by comparison with that of known antioxidant Ascorbic Acid (AA). The extracts were prepared at concentrations of 500-, 100-, 50- and 25 ppm. A 500-ppm stock solution was prepared by dissolving 1 mg of the extract with 2.0 mL methanol. Volumes of 200 µL, 100 µL and 50 µL from the 500-ppm stock solution were transferred in a 10-ml test tube and the remaining volumes (800 µL, 900 µL and 950 µL, respectively) were added with methanol to make 1-mL solution. The mixture was shaken vigorously and was allowed to stand at room temperature for one hour. Absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer. The percent of DPPH decoloration of the samples was then calculated according to the formula:

$$Antiradical\ activity = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of DPPH radical and methanol, A_{sample} is the absorbance of the DPPH radical and sample/extract. Each sample were assayed in triplicate and mean values were calculated.

Total antioxidant capacity assay by the phospho-molybdenum method

The total antioxidant capacity of the crude extracts was determined by the phosphomolybdenum method[44]. A 0.3 mL extract solution with 200-ppm concentration was dispensed into screw-capped test tubes and were added separately with 3.0 mL reagent solution of 6M H_2SO_4, 28mM sodium phosphate, and 4mM ammonium molybdate. The capped tubes were incubated at 95°C for 90 minutes. The absorbance was measured at 695 nm using a spectrophotometer after cooling it to room temperature. Methanol was used as the control. The antioxidant activity was expressed as ascorbic acid equivalents (AAE) and butylated hydroxytoluene equivalents (BHTE), determined from a linear equation established using ascorbic acid and BHT as reference standards. The results were reported as means of triplicate analysis.

Determination of total phenolic contents by the Folin-Ciocalteu method

Using the method of Makkar et al.[55], the total phenolics content of the crude extracts were determined. A volume of 0.1 mL (0.5mg/mL) of sample was combined with 2.8 mL of 10% Na_2CO_3 and 0.1 mL of 2N Folin-Ciocalteu reagent. Absorbance at 725 nm was measured after 40 minutes. Total phenolics were determined as milligrams of gallic acid equivalents per gram of sample by computing with standard calibration curve constructed for different concentrations of gallic acid (25-, 50-, 100-, 200 ppm). Results were reported in gallic acid equivalents (GAE).

RESULTS

Brine shrimp lethality test

The results obtained for the mortality rate of the brine shrimp *A. salina* after 24-hour exposure and the LC_{50} values of the crude extracts of *P. baccatum* is summarized in Table 1.

DPPH radical scavenging test

Table 2 summarizes the averaged DPPH-radical scavenging activities of the crude extracts of *P. baccatum*.

Total antioxidant capacity by the phosphomolybdenum method

Depicted in Figure 1 is the total antioxidant capacity of the

Table 1: Brine shrimp mortality and LC_{50} values of the *P. baccatum* crude extracts.

Crude Extract	Percent Mortality after 24-h Exposure*,%				LC_{50},ppm
	10 ppm	100 ppm	500 ppm	1000 ppm	
PbE	3.0	100.0	100.0	100.0	28.84
PbH	0.0	100.0	100.0	100.0	30.90
PbC	0.0	100.0	100.0	100.0	30.90
PbA	63.0	84.0	88.0	94.0	<10.00
PbD	44.0	52.0	95.0	99.0	39.81

* - mean of triplicate analysis
PbE - *Piper baccatum* ethanol extract, PbH - hexane extract, PbC - chloroform extract, PbA - aqueous extract, PbD decoction.

Table 2: DPPH antiradical activities of *P. Baccatum* leaf extracts at various concentrations.

Samples	Antiradical Activity*, %				EC_{50}, ppm
	25 ppm	50 ppm	100 ppm	500 ppm	
AA**	28.6	63.4	97.1	96.6	43.5
PbE	2.7	5.3	13.6	72.2	349.1
PbH	1.6	13.3	13.2	21.4	>500.0
PbC	11.9	18.7	26.8	81.4	276.5
PbA	90.0	96.1	95.3	95.2	<25.0
PbD	61.1	89.8	83.1	81.9	<25.0

* - mean of triplicate analysis
** - Ascorbic acid standard
PbE - *Piper baccatum* ethanol extract, PbH - hexane extract, PbC chloroform extract
PbA - aqueous extract, PbD decoction.

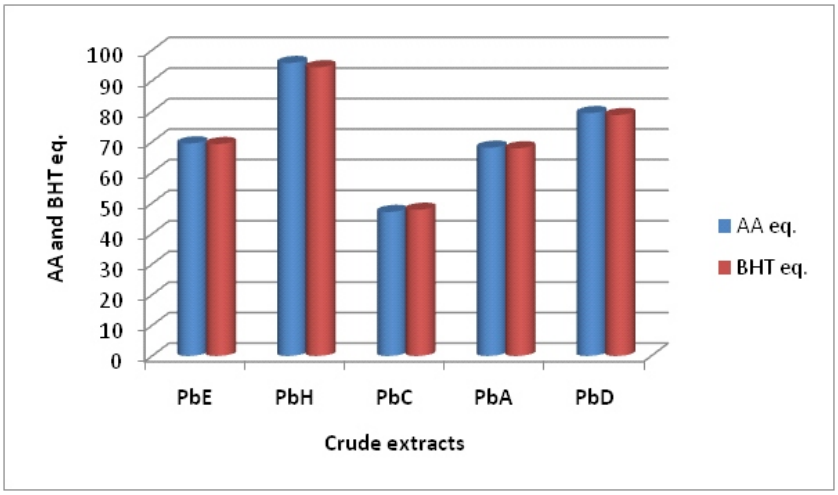

Figure 1: Total antioxidant capacities of *P. baccatum* leaf extracts at 200-ppm concentration expressed as ascorbic acid equivalents (AAE) and buty. lated hydroxytoluene equivalents (BHTE)

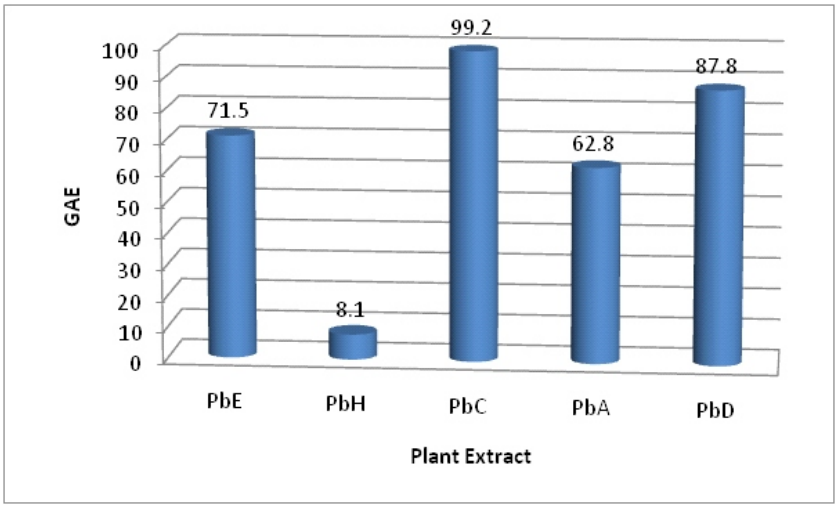

Figure 2: Total phenolics content of *P. baccatum* leaf extracts at 500-ppm expressed as gallic acid equivalence (GAE)

various crude extracts of *P. baccatum* expressed in terms of ascorbic acid equivalence (AAE) and butylated hydroxytoluene equivalence (BHTE).

Total phenolics content

Illustrated in Figure 2 are the total phenolics content of the crude extracts of *P. baccatum* determined using the Folin - Ciocalteu method.

DISCUSSION

Results show that the effects of the crude extracts of *P. baccatum* on the mortality of the brine shrimp *A. salina* depended on the type of crude extract and its concentration. For the ethanol, hexane and chloroform extracts, all of the brine shrimps died upon exposure to 100-ppm and higher concentrations of such extracts. Meanwhile, for the aqueous extract and decoction of *P. baccatum*, the effect to the mortality of brine shrimp was concentration dependent with the highest brine shrimp mortalities observed with the highest concentration tested (1000 ppm). Based on the LC_{50} values, the most lethal extract is the aqueous extract PbA since it only takes less than 10-ppm concentration of such extract to kill 50% of the brine shrimps.

According to Meyer et al.[56], a crude plant extract can be considered toxic (active) if it has an LC_{50} value of less than 1000 ppm while non-toxic (inactive) if the value is greater than 1000 ppm. The results in Table 1 indicated that all the crude extracts of *P. baccatum* have LC_{50} values of less than 1000 ppm which indicate that these extracts are considered to be toxic or active. Among the various extracts, the aqueous extract of *P. baccatum* (PbA) was the most active and the least active was the decoction (PbD).

The aqueous extract of *P. baccatum* exhibited the highest antiradical activity in all of the concentrations tested. It is followed by the decoction. The activities of the polar extracts PbA and PbD were relatively higher than those of the standard (Ascorbic Acid) at the 25- and 50-ppm concentrations. At the higher concentrations (100- and 500-ppm), the activities of PbA were comparable to those of ascorbic acid. Meanwhile, the hexane extract has the lowest antiradical activity among all the crude extracts. The results indicate that the polar extracts have greater ability to scavenge the radical DPPH than the medium-polar or nonpolar extracts.

The results indicate that among the crude extracts of *P. baccatum*, the hexane extract and the chloroform extract exhibited the highest and lowest antioxidant capacity respectively in terms of both Ascorbic Acid and Butylated Hydroxytoluene equivalents. Results also indicate that the ethanol and aqueous extracts have similar antioxidant capacities.

In terms of total phenolics content, the values ranged from 7.1 to 99.2 mg gallic acid/ g sample and the extracts can be arranged in the order PbH<PbA<PbE<PbC<PbD.

CONCLUSION

Results of the study have shown that all the crude extracts of *P. Baccatum* exhibited bioactivities in terms of toxicity to brine shrimp and antioxidant properties.The aqueous extract PbA was the most active in the brine shrimp lethality test with an LC_{50} value of < 10 ppm. The others extracts are active as well and these results warrant further investigations on the isolation and identification of the bioactive components present in the crude extracts. Over-all evaluation of results of the various *in vitro* antioxidant property methods, the decoction (PbD), aqueous (PbA) and chloroform (PbC) extracts of *P. baccatum* exhibited considerable results which make them good candidates for further investigation.

ACKNOWLEDGMENTS

The authors are thankful for the financial and technical assistance provided by the Department of Science and Technology-Science Education Institute and Department of Science and Technology-Philippine Council for Health Research and Development of the Philippine government.

REFERENCES

1. Farnsworth NR, Akerele O, Pingel AS. Medicinal plants in therapy. Bull. World Health Organization. 1985:63:965-998.

2. FarnsworthNR, Soejavtu DD. Global importance of medicinal plants. In: Conservation of medicinal plants.Akerele O, Heywood V and Synge H (eds).: Cambridge University Press, Cambridge, 1991. pp.25-51.

3. Evans PH, Bowers WS, Funk EJ. Identification of fungicidal and nematocidalcomponents in the leaves of *Piper betle* (Piperaceae). J. Agri. Food Chem.1984:32:1254-1256.

4. Orjala J, Wright AD, Behrends H, FolkersG,Sticher O. Cytotoxic and antibacterial dihydrochalcones from *Piper aduncum*. J. Nat. Prod.1994:57(1):18-26.

5. Alecio AC, Bolzani VS, Young M, Kato M, Furlan M. Antifungal amide from leaves of *Piper hispidum*. J. Nat. Prod.1998:61:637-639.

6. Lugar P, Weber M, Dung NX, Luu VT, Rang DD, Tuong DT, Ngoc PH. The crystal structure of3-(4'- methoxyphenyl) propanoylpyrrole of *Piper lolot* C.DC from Vietnam. Cryst. Res. Technol. 2002:37(6): 627-633

7. Srivastava S, Gupta MM, Tripathi KA, Kumar S. 1,3-Benzodiaoxole-5-(2,4,8-triene-methyl nanoate) & 1,3-benzodiaoxole-5-(2,4,8-triene-isobutyl nonoate) from *Piper mullesua*. Indian J. Chem. 2000:39B:946-949.

8. Ma J, Jones SH, Marshall R, Johnson RK, Hecht SM. A DNA damaging oxoaporphinealkaloid from *Piper caninum*. J. Nat. Prod.2004:67:1162-1164.

9. Lin LC, Shen CC, Shen YC, Tsai TH. Antiinflammatoryneolignans from *Piper kadsura*. J. Nat. Prod 2006:69:842-844.

10. ChahalJ ,Ohlyan R, KandaleA,Walia A, Puri S. Introduction, phytochemistry, traditional uses and biological activity of genus Piper: A review. Int. J. Cur.Pharma. Rev. Res.2011:2(2):130-144.

11. Prasad AK, Kumar V, Arya P, Kumar S, Dabur R, Singh N, Chhillar AK, Sharma GL, Ghosh B, Wengel J, Olsen CE, Parmar VS. Investigations toward new lead compounds from medicinally important plants. Pure and Applied Chemistry. 2005:77(1): 25-40.

12. Ghosh R, Darin K, Nath P, Deb P. An overview of various Piper species for their biological activities. Int. J.Pharma. Res. Rev.2014:3(1):67-75.

13. Li CY, Tsai WJ, Damu AG, Lee EJ, Wu T, DungNX, Thang TD, Thanh L. Isolation and identification of antiplatelet aggregatoryprinciples fromleaves of the *Piper lolot*. J. Agri. Food

Chem.007:55:94369442.

14. Lei D, Chan CP, Wang YJ, Wang TM, Lin BR,Huang CH, Lee JJ, Chen HM, Jeng JH, Chang MC.Antioxidative and antiplatelet effects of aqueous inflorescence *Piper betle* extract. J. Agri. Food Chem.2003:51(7):2083-2088.

15. Joshi N, Garg HS, Bhakuni DS. Chemical constituents of *Piper schimidtii*: structure of a new neolignanschimiditin. J.Nat. Prod.1990:53(2):479-482.

16. Jensen HR, Scott IM, Sims S, Trudeau VL, Arnason JT. Gene expression profiles of *Drosophila melanogaster* exposed to an insecticidal extract of *Piper nigrum*. J. Agri. Food Chem.2006:54:1289-1295.

17. Chauret DC, Bernard CB, Arnason JT, Durst T, Krishnamurthy HG, Vindas PS, Moreno N, San Roman L, Poveda L.Insecticidal neolignans from *Piper decurrens*. J. Nat. Prod.1996:59(2):152-155.

18. Miranda RP, Bernard CB, Durst T, Arnason JT, Vindas PS, Poveda L, San Roman L.Methyl 4-hydroxy-3-(3'-methyl-2'-butenyl)benzoate,major insecticidal principle from *Piper guanacastensis*. J. Nat. Prod.1997: 60(3):282-284.

19. Duh YC, Wu YC. Cytotoxic pyridonealkaloids from the leaves of *Piper aborescens*. J. Nat. Prod.1990:53(6):1575-1577.

20. Orjala J, Mian P, Rali T, Sticher O. Gibbilimbols A-D, cytotoxic and antibacterial alkenylphenols from *Piper gibbilimbum*. J. Nat. Prod.1998:61:939-941.

21. Tang GH, Chen DM, Qiu BY, Sheng L, Wang YH, Hu GW, Zhao FW, Ma LJ, Wang H, Huang QQ, Xu JJ, Long CL, Li J. Cytotoxic amide alkaloids from *Piper boehmeriaefolium*. J. Nat. Prod.2011:74(1):45-49.

22. Pan L, Matthew S, Lantvit DD, Zhang X, Ninh TN, Chai H, de Blanco EJC, Soejarto DD, Swanson SM, Kinghorn AD. Bioassay-guided isolation of constituents of *Piper sarmentosum* using a mitochondrial trans membrane potential assay. J. Nat. Prod.2011:74(10):21932199.

23. Flores N, Jimenez IA, Gimenez A, Ruiz G, Gutierrez D, Bourdy G, Bazzocchin IL. Benzoic acid derivatives from Piper species and their antiparasiticactivity. J. Nat. Prod. 2008:71(9): 1538-1543.

24. Singh A, Deep A, Sharma P. Black pepper: king of spices. Int. J Pharma. Res. Tech..2011:1(2):01-07.

25. Nakatani N, Inatani R, Ohta H, Nishioka A. Chemical constituents of peppers (Piper spp.) and application to food preservation: aaturally occuring antioxidative compounds. Environmental Health Perspectives. 1986:67:135-142.

26. Solis PN, Wright CW, Anderson MM, Gupta MP,Phillipson JD.A microwell cytotoxicity assay using *Artemia salina*. Plant Med. 1993:59:250-252.

27. Harwig J, Scott P. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. Appl.Microbiol. 1971:21:1011-1016.

28. Jaki B, Orjala J, Bürji HR, Sticher O. Biological screening of cyanobacteria for antimicrobial and molluscicidal activity, brine shrimp lethality, and cytotoxicity. Pharm. Biol. 1999:37:138-143.

29. Martínez M, Del ramo J, Torreblanca A, Díaz-Mayans J. Effect of cadmium exposure on zinc levels in the brine shrimp *Artemia partenogenética*. Aquaculture. 1998:172:315-325.

30. Barahona MV, Sánchez-Fortún S.Toxicity of carbamates to the brine shrimp *Artemia salina* and the effect of atropine, BW284c51, iso-OMPA and 2-PAM on carbaryl toxicity. Env.Pollut. 1999:104:469-476.

31. Lalisan JA, Nuñeza OM, Uy MM. Brine shrimp (*Artemiasalina*) bioassay of the medicinal plant *Pseudelephantopus spicatus* from Iligan City, Philippines. Int. Res. J. Bio. Sci. 2014: 3(9):47-50.

32. Elias NU, Nuñeza OM, Uy MM. Evaluating the potential cytotoxic activity of *Acmella grandifolia* flower and whole plant using bring shrimp lethality test. Int. J. Bio. Sci. 2014:3(10):90-92.

33. Juzavil J, Mondejar E, Nuñeza O, Uy M. Effect of *Phyllanthus niruri* and *Passiflora foetida* extracts on the mortality and survival rate of the brine shrimp *Artemia salina* Res. J. Rec. Sci. 2015:4(2):61-67.

34. Espinosa JB, Uy MM. Evaluation of toxicity and antioxidant activities of the crude leaf extracts of *Cnidoscoluschayamansa*. AAB Bioflux. 2015:7(2) :109-114.

35. Gupta VK, Sharma SK. Plants as natural antioxidants. Nat. Prod. Radiance. 2006:5(4):326-334.

36. Larson RA. The antioxidants of higher plants. Phytochem. 1988:27:969-978.

37. Mensor LL, Menezes FS, Leitao GG, Reis AS, dos Santos TC, Coube CS, Leita˜o SG. Screening of Brazilian plant extracts for antioxidant activity by the Use of DPPH free radical method. Phytother. Res. 2001:15:127-130.

38. Khal R, Hilderbrand AG. Methodology for studying antioxidant activity and mechanism of action of antioxidants. Food Chem. Toxicol. 1986:24:1007-1014.

39. Frankel EN.n search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. Trends Food Sci Technol. 1993:4:220-225.

40. Robards K, Prenzier PD, Tucker G, Swatsitang P, Glover W. Phenolic compounds and their role in oxidative processes in fruits. Food Chem. 1999:66:401-436.

41. Moure A, Cruz JM, Franco D, Dominguez JM, Sineiro J, Dominguez H, NunezMJ, Parajo JC. Natural antioxidants from residual sources. Food Chem. 2001:72:145-171.

42. Huang DJ, Chen HJ, Lin CD, Lin YH. Antioxidant and antiproliferative activities of water spinach (*Ipomoea aquatica* Forsk) constituents. Bot. Bull. Acad. Sin. 2005:46:99-106.

43. Jin J, Li Z, Zhang F.Scavenging function of mulberry vinegar extractives for 1,1diphenyl-2-picrylhydrazyl (DPPH). J. Northwest Sci-Tech. Univ. Agri. Forestry 2006:34(3):135137.

44. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Ana.Biochem. 1999:269:337341.

45. Aliyu AB, Ibrahim MA, Musa AM, Musa AO, Kiplimo JJ, Oyewale AO. Free radical scavenging and total antioxidant capacity of root extracts of *Anchomanes difformis* Engl. (Araceae). ActaPoloniaePharmaceutica - Drug Research.

2013:70(1):115-121.

46. Han X, Shen T, Lou H. Dietary polyphenols and their biological significance Int. J. Mole. Sci., 8:950988.

47. Schofield P, Mbugua DM, Pell AN. 2001 Analysis of condensed tannins: A review. Anim. Feed Sci. Tech. 2007:91:2140.

48. Blainski A, Lopes GC, Palazzo de Mello JC. Application and analysis of the FolinCiucalteu method for the determination of the total phenolic content from *Limonium brasiliense* L. Molecules. 2013:18:6852-6865.

49. Council of Europe. Determination of tannins in herbal drugs. In: European Pharmacopoeia 6th Ed.: European Directorate for the Quality of Medicines, Strasbourg, France, 2007.p.A286.

50. Gülçin I, Sat IG, Beydemir S, Elmastas M, Küfrevioğlu, ÖI. Comparison of antioxidant activity of clove (*Eugenia caryophylata* Thunb) buds and lavender (*Lavandulastoechas* L.). Food Chem. 2004:87:393400.

51. Krishnaraju AV, Rao TVN, Sundararaju D, Vanisree M, Tsay H, Subbaraju GV. Assessment of bioactivity of Indian medicinal plants using Brine shrimp (*Artemia salina*) lethality assay. Int. J. Applied Sci. Eng. 2005:3:125-134.

52. Reed, LJ Muench H. A simple method of estimating fifty percent endpoints. The American Journal of Hygiene. 1938:27:493497.

53. Pizzi M. Sampling variation of the fifty percent end-point, determined by the ReedMuench (Behrens) method. Human Biology. 1950:22(3):151-190.

54. Lee KG, Shibamoto T, Antioxidant activities of volatile components Isolated from Eucalyptus species. J. Sci. Food Agric. 2001:81:1573-1579.

55. Makkar, H.P.S., Bluemmel, M.mBorowy, N.K., Becker, K.Gravimetric Determination of Tannins and their Correlations with Chemical and Protein Precipitation Methods. J. Sci. Food Agric. 1993:61:161-165.

56. Meyer BN, Ferrigni NR, PutnamJE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: aconvenient general bioassay for active plant constituents, Planta Med. 1982:45:31-34.