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Research Article

EVALUATION OF *IN VITRO* ANTIOXIDANT ACTIVITY, ESTIMATION OF TOTAL PHENOLIC AND FLAVONOID CONTENT OF LEAF EXTRACT OF *EURYA JAPONICA* THUNB.

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ABSTRACT

The present study, we carried out to determine the antioxidant activity of the leaf extract of *Eurya japonica* Thunb. and to estimate its phenolic and flavonoid content. DPPH (2,2-diphenyl-1-picryl hydrazyl) radical scavenging capacity and reducing power assay (RPA) were carried out to evaluate *In vitro* antioxidant activity. Total phenolic content (TPC) was estimated using Folin-Ciocalteau's reagent and total flavonoid (TF) content by Aluminium chloride colorimetric method. It was observed that DPPH EC_{50} value of leaf extract of *Eurya japonica* Thunb. was 32.20 mg/ml and the reducing capacity of the extract was 173.70 ± 0.02. The phenolic and flavonoid content of the leaf extract was found to be 26.31 ± 0.13 mg gallic acid equivalents (GAE) /mg dry weight and 74.99 ± 1.83 mg GAE/mg dry weight respectively.The results suggested a positive relationship between total phenolic and flavonoid content with antioxidant activity of the leaf extract of *Eurya japonica* Thunb.

Keywords: Antioxidant activity, *Eurya japonica* Thunb. , DPPH (2,2-diphenyl-1-picryl hydrazyl), RPA (reducing power assay) , TF (Total flavonoid), TPC (Total phenolic content).

INTRODUCTION

Free radical is a chemical species which contains an unpaired electron spinning on the peripheral layer around the nucleus. Free radicals generated from the oxygen are called Reactive oxygen species (ROS) which cause damage to other molecules by extracting electrons from them in order to attain stability. Reactive oxygen species (ROS) ,which consist of free radicals such as superoxide(O_2^-) , hydroxyl (OH^-) , nitric oxide (NO), peroxyl (RO_2^-), lipid peroxyl (LOO^-) radicals and non free radical species such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2^{-1}) ,ozone (O_3), lipid peroxide (LOOH), are different forms of activated oxygen[1,2,3]. ROS are produced by all aerobic organisms and can easily react with most biological molecules including proteins, lipids, lipoproteins and DNA. This ROS can generate oxidative stress and produce many pathological diseases such as arthritis, diabetes, inflammation, cancer and genotoxicity[4,5]. Sources of free radicals include metabolism by-products, neutrophils, UV radiation, air and water pollutants, fatty foods, hazardous chemicals, and cigarette smoke.

Antioxidants are any substance that when present at low concentration compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate [6]. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols[7].

Living cells may generate free radicals and other reactive oxygen species by-products as a results of physiological and biochemical processes. Free radicals can cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in human beings[8]. Plants are endowed with free radical scavenging molecules, such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites, which are rich in antioxidant activity[9,10]. Studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, and antiviral activities[11,12]. The ingestion of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing[13,14] . In recent years, there has been a worldwide trend towards the use of natural phytochemicals since natural antioxidants are presumed to be safe as they occur in plants. Evidence suggests that compounds especially from natural sources are capable of providing protection against free radicals. This has attracted a great deal of research interest in natural antioxidants. It is necessary to screenout medicinal plants for their antioxidant potentials.

It has been suggested that the antioxidant activity of plants might be due to their phenolic compounds[15]. Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action[16]. Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity [17]. An easy, rapid and sensitive method for the antioxidant screening of plant extracts is to add free radical scavenging assay of 2,2-diphenyl-1-picryl hydrazyl (DPPH) stable radical. In the presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases[18].

Eurya japonica is an evergreen broad-leaf understorey tree, in the family Theaceae. It is commonly known as *Uyanggal laba* in Manipuri language. This plant generally occurs in India, Southeast Asia, China, Taiwan, south part of Korea and Japan[19]. This is an economically important species in Korea, as the branches are widely used in floral tributes and wreaths. Leaves are used as poultice on skin eruption [20]. Preliminary phytochemical screening of the plant leaf extract of *Eurya japonica* revealed the presence of alkaloids, glycosides, flavonoids , terpenoids, saponins, tannins and reducing sugar. Studies have showed that the leaf of this plant has antibacterial properties[21]. Several tribals in North eastern India especially in Manipur use this plant traditionally for the treatment of diabetes. In the present study attempt has been made to establish a positive relationship between the antioxidant activity and the totoal phenolic & flavonoid content of the methanol leaf extract of the plant material.

MATERIALS AND METHODS

Sample preparation

The leaf of *Eurya japonica* was collected randomly from the Phayeng village of Imphal West district, Manipur, India. The plant material was botanically authenticated by Botanical Survey of India, Shillong. The leaves are dried and powdered. The powdered leaves (100 g) of *Eurya japonica* was weighed and subjected to soxhlet extraction with methanol for 72 hrs. The solvent was distilled off at lower temperature under reduced pressure in the rotary evaporator

and concentrated to dryness (crude extract). The dried extract was weighed and then stored in a freezer. The crude extract was used for the experiment.

Scavenging activity on DPPH radical

The radical scavenging activities of the plant extract against 2,2-Diphenyl-1-picryl hydrazyl (DPPH) radical was determined by a slightly modified method using UV spectrophotometry (electronic GEAESYD-20 thermo spectronic) at 517 nm[22,23]. Briefly, 1 ml of the crude extract solution at variable concentrations (0.02 - 0.05 mg/ml in methanol) was placed in a test tube, and 3 ml of methanol was added followed by 0.5 ml of 1 mM DPPH in methanol, kept for 35 mins at room temperature until to produce a stable colour and subsequently absorbance was measured at 517 nm. L- Ascorbic acid was used as the positive control. A blank solution was prepared containing the same amount of methanol and DPPH. The radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = {[Ab-Aa]/Ab} x 100

where Ab is the absorption of the blank and Aa is the absorption of the extract sample.

Different concentrations of the extract were selected in such a way that percentage of inhibition of colour of DPPH solution is above and below 50%. EC_{50} value was obtained from the linear regression equation. Correlation coefficient ($R^2 \geq 0.90$) was taken as highly correlated.

Reducing power assay

Reducing ability was performed by using potassium ferricyanide-ferric chloride system[24]. 1 ml of extract solution (0.2, 0.5, 0.8, and 1.0 mg/ml) was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The content was mixed and heated at 50 °C for 20 min and cooled; then trichloroacetic acid (2.5 ml, 10%) was added, and the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%), and the absorbance was measured at 700 nm against a blank. Increasing absorbance of the reaction mixture indicates increasing reducing power. All the tests were performed in triplicate.

Determination of total phenolic content (TP)

The total phenolic content was determined using Folin–Ciocalteu reagent with analytical grade gallic acid as the standard[25].1 ml of extract or standard solution (25 mg/ml) was added to deionized water (60 ml) and Folin–Ciocalteu phenol reagents (5.0 ml). After 5 minutes, 20% sodium carbonate (15.0 ml) was added to the mixture. After being kept in darkness for 2 h, the absorbance was measured at 760 nm using a spectrophotometer (GEAESYS-20, Thermospectronic). The same solution was used as blank without the extraction solution.

Amounts of TP were calculated based on gallic acid standard.

$$\text{Total Phenols \%} = \frac{A_{sample} \times W_{standard} \times 50}{A_{standard} \times W_{sample} \times 50} \times 100\%$$

The results were expressed as gallic acid equivalents (GAE) mg/mg of dry extract.

Determination of total flavonoid (TF) content

TF content was determined according to the procedure by Aluminium chloride colorimetric method[26]. Extract solution (1 ml, 0.1 mg/ml) was placed in a 10-ml volumetric flask, and then 5 ml of distilled water was added followed by $NaNO_2$ solution (0.3 ml, 5%). After 5 min, $AlCl_3$ solution (0.6 ml, 10%) was added. After another 5 min, NaOH solution (2 ml, 1 M) was added and volume was made up with distilled water. The solution was mixed thoroughly and absorbance was measured at 510 nm. TF amounts were expressed as milligrams of gallic acid equivalent per milligram of dry weight. All the tests were performed in triplicate.

RESULT AND DISCUSSION

Radical scavenging activity by DPPH method

DPPH is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. DPPH method allows estimation of hydrogen radical donating ability of the extract[27]. This model represents the situation in metabolic system where an antioxidant will stabilise a free radical by reacting with the hydrogen radical. The results are expressed in EC_{50} (Effective concentration to reduce the initial concentration of DPPH to 50%). Lesser the EC_{50} value for an extract is considered to be associated with higher ability to donate hydrogen radical i.e antioxidant activity. In the present study EC_{50} values were found to be 32.20mg/ml and 4.86 mg/ml for the *Eurya japonica* and Ascorbic acid respectively. These data clearly indicates that *Eurya japonica* is a promising radical scavenger.

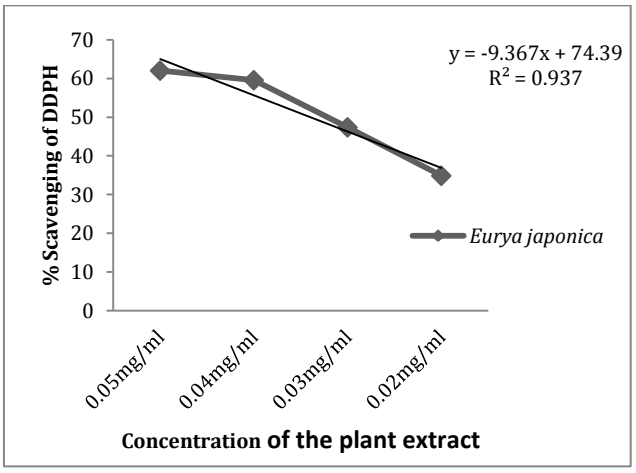

Fig 1 : Linear regression of %DPPH Scavenging Vs Concentration

Reducing power assay

The reducing capacity of the extract is another significant indicator of antioxidant activity. In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's blue at 700 nm. Increasing absorbance indicates an increase in reductive ability [28]. The results show that there was an increase in reducing power of the plant extract as the extract concentration increases. The results are expressed as absorbance X 100. The Reducing Power Assay of *Eurya japonica* was found to be 173.70.

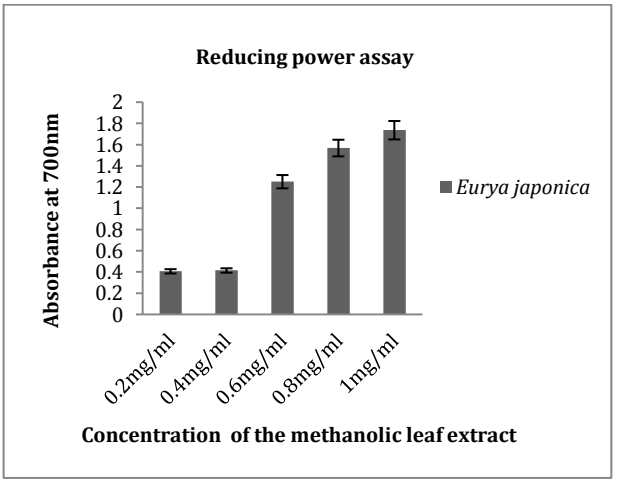

Fig 2: Antioxidant activity of the plant extract estimated by Reducing Power Assay.

Estimaton of total phenolic content

Phenolic compounds are having wide bioactivity including antioxidant properties/activity. The antioxidant activity of phenolic compound is due to hydroxyl functional group, however other factors eg., presence of electron withdrawing or releasing group in the aromatic ring having hydroxyl moiety will increase or decrease the activity. The phenols contain hydroxyls that are responsible for the radical scavenging effect mainly due to redox properties [29]. In the current study total phenolic content was found to be 26.31 mg GAE/mg dw for *Eurya japonica*. This shows that *Eurya japonica* has high content of phenolic compounds.

Table 1: Showing data of *in vitro* antioxidant activity , Total Phenolic and Flavanoid content in the methanolic Leaf extract of *Eurya japonica*

Plant	DPPH EC_{50} (mg/ml)	RPA #	TP* (mg GAE/mg dw)	TF* (mg GAE/mg dw)
Eurya japonica	32.20	173.70 ± 0.02	26.31 ± 0.13	74.99± 1.83

EC_{50} in mg/ml. The significance difference was analysed by one-way Anova followed by Tukey's post hoc test. P<0.05 was considered significant.
***Result are expressed as mean ± S.D (n=3) from 3 independent observation, gallic acid as standard.**
#Absorbance given by 1000 ppm (1mg/ml) solution of extract X 100.

Estimation of total flavonoid content

Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2[30]. Flavonoids serve as health promoting compound as a results of its presence as anion radicals[31].

Total flavonoid contents were found to be 74.99 mg GAE/mg dw for *Eurya japonica*. The compounds such as flavonoids, which hold hydroxyl groups, are responsible for the radical scavenging activity in the plants[32,33]. It has been acknowledged that flavonoids show significant antioxidant action on human health and fitness. It is known that flavonoids act through scavenging or chelating process[34,35].

The crude methanol extract of *Eurya japonica* leaf have indicated strong antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress induce diseases such as diabetes, which would be beneficial to the human health. This may be related to the high amount of phenolic and flavonoid compounds present in this plant extract. We have also established the relationship of total phenolic , flavonoid contents and the free radical scavenging activity. Further studies are needed to clarify the *in vivo* potential of this plant in the management of human diseases resulting from oxidative stress.

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REFERENCES

1. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford University Press.1999.
2. Yildirim A, Mavi A. Comparision of antioxidant and antimicrobial activities of Tilia (*Tilia argenta* Desf Ex DC) ,saga (*Salvia triloba* L.) and black tea (*Camelia Sinesis*) extracts. J. Agric Food Chem. 2000; 48: 5030 -5034.
3. Gulcin I, Oktay MO. Determination of antioxidant activity of Lichen *Cetraria islandica*(L.) Ach. J. Ethanopharmacol. 2002a; 79: 325-329.
4. Kourornakis AP, Galanakis DK. Synthesis and pharmacological evaluation of novel derivatives of antiinflamatory drugs with increased antioxidant and antiinflamatory activities. Drug Development Research. 1999; 47: 9-16.
5. Gulcin I , Buyukokuroghee ME. On the in vitro antioxidant properties of melantonin. J. Pineal Research. 2002b; 33: 167-171.
6. Halliwell B, Gutteridge JMC. Free radicals,other reactive species and disease. In: Free radical in biology and medicine.Clarendron press.Oxford, 1999; 617-783.
7. Hyoung LS. Antioxidant activity of browing reaction product isolated from storage-aged orange juice. J Agric Food Chem. 1992; 40 (4): 550-552.
8. Harman D. Aging: phenomena and theories. Ann NY Acad Sci. 1998; 854: 1-7.
9. Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs, J Agric Food Chem. 2001; 49(11): 5165-5170.
10. Cai YZ, Sun M, Corke H..Antioxidant activity of betalains from plants of the Amaranthaceae. J Agric Food Chem. 2003; 51(8): 2288-2294.
11. Sala A, Recio MD, Giner RM, Manez S, Tournier H, Schinella G, Rios JL. Antiinflammatory and antioxidant properties of *Helichrysum italicum*. J Pharm Pharmacol. 2002; 54(3): 365-371.
12. Rice ECA, Miller NJ, Bolwell PG, Bramley PM , Pridham JB. The relative activities of Plant-derived polyphenolic flavonoid. Free radical Res, 1995; 22: 375-383.
13. Ashokkumar D, Mazumder UK, Gupta M, Senthilkumar GP, Selvan VT. Evaluation of Antioxidant and Free Radical Scavenging Activities of *Oxystelma esculentum* in various *in vitro* Models, J Comp Integ Med, 2008. 5(1), Article 9.
14. Veerapur V P, Prabhakar K R, Parihar V P, Kandadi M R, Ramakrishana S, Mishra B, Satish R B S, Srinivasan K K, Priyadarsini K I, Unnikrishnan M K .*Ficus racemosa* Stem Bark Extract: A Potent Antioxidant and a Probable Natural Radioprotector. Evid Based Complement Alternat Med, 2009; 6(3) : 317-324.
15. Cook NC, Samman S. Flavonoids- chemistry, metabolism, cardioprotective effects and dietary sources. Nutr. Biochem. 1996; 7: 66-76.
16. Frankel E. Nutritional benefits of flavonoids. International conference on food factors: Chemistry and Cancer Prevention, Hamamatsu, Japan. [Abstracts]. 1995. C6- 2.
17. Gryglewski RJ, Korbut R, Robak J. On the mechanism of antithrombotic action of flavonoids. Biochemical Pharmacol. 1987;36: 317- 321.
18. Koleva II, Van Beek TA, Linssen JPH, de Groot A, Evstatieva L. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochemical Analysis. 2002; 13: 8-17.
19. Ohwi J, Kitagawa M. New flora of Japan. Shibundo, Tokyo (in Japanese) (revised). 1992; 1716.
20. Senapati District Unit:Flora & Fauna [http://senapati.nic.in/FloraandFauna.html.] dated10-11.2012.
21. Thingbaijam R, Dutta BK, Paul SB. Phytochemical screening and antibacterial studies of the leaf extract of E*urya japonica* and *Ficus auriculata* , J. Pure and Applied Microbio. 2011; 5(2): 909-916.
22. Ayoola GA, Sofidiya T, Odukoya O, Coker HAB. Phytochemical screening and free radical scavenging activity of some Nigerian medicinal plants, J. Pharm. Sci. & Pharm, Pract. 2008; 8: 133-136.
23. Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity, Lebensmittel Wissenschaft and Technologie. 1995; 28: 25-30.
24. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Jpn J Nutr . 1986; 44: 307–315.
25. Waterman PG, Mole S. Analysis of Phenolic Plant Metabolites, Method was modified from References. Blackwell Scientific Publication. 1994; 83-85.
26. Dewanto V, Wu X, Adom KK, Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem . 2002; 50: 3010–3014.

27. Sreejayan N , Rao MNA. Free radical scavenging activity of Curcuminoids. Drug Res. 1996; 4:61-69.
28. Olayinka A, Aiyegoro AI, Okoh. Preliminary phytochemical screening and *In vitro* antioxidant activities of the aqueous extract of *Helichrysum longifolium* DC. BMC Complementary and Alternative Medicine, Oxford University Press. 2010; 10:21.
29. Rice-Evans CA, Miller NJ, Paganga G. Antioxidant properties of phenolic compounds, Trend Plant Sci. 1997; 4: 152-159.
30. Li H, Wang Z, Liu Y. Review in the studies on tannins activity of cancer prevention and anticancer, Zhong-Yao-Cai. 2003; 26(6): 444-448.
31. Hausteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharm. 1983; 32 : 1141-1148.
32. Das NP ,Pereira TA. Effects of flavonoids on thermal autooxidation of Palm oil: structure- activity relationship. J. Am. Oil Chem. Soc. 1990; 67: 255-258.
33. Younes M. Inhibitory action of some flavonoids on enhanced spontaneous lipid peroxidation following glutathione depletion, Planta Medica. 1981; 43: 240-245.
34. Kessler M, Ubeaud G, Jung L. Anti- and pro-oxidant activity of rutin and quercetin derivatives. J. Pharm. Pharmacol. 2003; 55: 131-142.
35. Cook NC, Samman S. Flavonoids- chemistry, metabolism, cardioprotective effects and dietary sources. Nutr. Biochem. 1996; 7: 66-76.