e-planet **20** (1): 40-46 (June 2022)

Anti-oxidative effect of polyphenols of *Litsea glutinosa* leaf: An *in-vitro* assay

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Date of receipt: 17.05.2022 Date of acceptance: 29.05.2022

ABSTRACT

The study aims at extracting total phenolic content from leaf of *Litsea glutinosa* in different solvent medium under the microwave-assisted extraction system and assessment of its antioxidative activity. Solvents of extraction were classified into 7 groups such as Gr-A (100% methanol), Gr-B (100% de-ionized water), Gr-C (methanol: water: acetic acid in 70:25:05 v/v), Gr-D (ethyl acetate: methanol: water in 60:30:10 v/v), Gr-E (100% acetone), Gr-F (acetone: water: acetic acid in 90: 9.5: 0.5 v/v) and Gr-G (100% ethanol).Total polyphenols were quantitatively estimated following standard protocol. Organic and aqueous solvent mixture groups of Gr-C, D, and F exhibited significantly ($p<0.01$) higher concentrations of total polyphenols. Total and individual anti-oxidative activities were analyzed in the solvent group having the highest amount of total phenolics. The polyphenols extracted in Gr-D exhibited significantly higher antioxidant activities ($p<0.01$) in phospho- molybdenum and reducing power method than the solvents at Gr-C and F. The polyphenols extracted in the Gr-C solvent depicted significantly higher antioxidative activities ($p<0.01$) in the FRAP method followed by Gr-D and F. Also leaf extract in Gr-D solvent exhibited significantly higher superoxide (Ferric Reducing Antioxidant Power) and nitric oxide radical scavenging activities ($p<0.01$) followed by Gr-C and F. On the other hand, polyphenols extracted in Gr-F exhibited significantly ($p<0.01$) higher hydroxy radical scavenging activity and lipid peroxidation inhibition assay followed by Gr-C and D. The present study reflects that the plant derived phyto-chemicals can be better extracted through organic and aqueous mixture solvents than individual one with potent anti-oxidative activity.

Key words: Antioxidant, FRAP, free radical scavenging activity, microwave-assisted extraction, total phenolics

INTRODUCTION

Natural antioxidants have been associated with reduced risks of cancer, cardiovascular disease, diabetes, inflammation, bacterial disease and diseases associated with ageing (Kumar and Surh, 2008; Thatoi et al., 2008) owing to their free radical scavenging activities (Veerapur et al., 2009) by blocking the initiation or propagation step of oxidizing chain reactions or by scavenging various types of reactive species or chelating transition metal ions (Halliwell, 2007). Traditional knowledge of rural tribes of Indian natives plays a pivotal role in the discovery of new and safer lead molecules of plant origin with potential biological activities. Since, these are cheaper, locally available, easily consumable (raw) and simple, it serves as alternative medicine (Surveswaran et al., 2007) in most of the regions of India and the world also. Even though the scientific world has studied the efficacy of these plant-derived chemical constituents,

the mechanisms of their action have not yet been investigated systematically (Alasalvar et al., 2006). These components vary in chemical characteristics, polarities, and distribution in the plant matrix (Sultana et al., 2009). Therefore, suitable environment/ conditions to harvest the bio-active components of plants determine their efficacy/ potency for which extraction conditions viz nature of solvent and technique are the major concern to enhance the efficiency (Boateng et al., 2008).

The claims with regard to medicinal plants posing anti-oxidant, anti-inflammatory, anti-tumor, anti-mutagenic, anti-carcinogenic, anti-bacterial, hepato-protective or anti-viral activities are mainly attributed to polyphenols. Polyphenols are the secondary plant metabolites with one/ more phenolic hydroxyl groups attached to carbon-based aromatic phenyl-ring. There are over 8,000 structural variants of polyphenols distributed variedly in the plant kingdom.

Litsea glutinosa belonging to Family Lauraceae is a medium-sized branched moist deciduous plant indigenous to India (Bhuniya et al., 2010). Pharmacological studies have confirmed that this plant exhibits a broad range of potential biological effects. However, the crude extract of the plant has been used as a traditional medicine for the treatment of various diseases that may pave pathway for future indigenous drug development. The study reveals the potentiality of various solvent extracts of different parts of this plant, particularly as an analgesic and wound healing (Devi and Meera, 2010), antidiarrhoeal (Bulbul et al., 2021), hepato-protective (Ghosh et al., 2016), anti-hyperglycemic and antihyperlipidemic (Zhang et al., 2018), anti-nociceptive (Rumzhum et al., 2012), anti-diabetic (Palanuvej et al., 2009), anti-inflammatory (Bhowmick et al., 2014) anti-bacterial and antioxidant (Arunodaya et al., 2016). Moreover, every part of this plant has great medicinal value and is being used internally as well as externally. It is applied externally on wounds to reduce inflammation, and also loss of appetite, abdominal pain in liver disorders, worm infestation, fever, and in general weakness. The seeds contain aromatic oils which have been used to make candles and soaps. The roots yield fibres used in Thailand for rope manufacture and paper pulp preparation. The young leaves are consumed by livestock as fodder. The pounded seeds are also applied medicinally against boils. The leaves and the mucilage in the gum from the bark have been used for poultices (Wang et al., 2010). The bark also acts as a demulcent and mild astringent in case of diarrhoea and dysentery (Devi and Meera, 2010). Indian tribes of Odisha province use aqueous paste and decoction of various parts of this plant along with ghee and honey traditionally for the treatment of rheumatoid arthritis, hepatic disorders, splenomegaly, fever, anorexia, obesity, diabetes, body ache, itches, wounds, stomach-ache, flatulent dyspepsia, helminthiasis, diarrhea, dysentery, urinary calculi, dysuria, and cystitis.

Oxidative stress of endogenous/exogenous origin can be overcome by chain-breaking antioxidants (CBAs) as 1st line of defense like tocopherol, ascorbic acid, glutathione (GSH), uric acid, carotenoids, ubiquinone, and polyphenols. The ongoing reaction can be terminated/ interrupted by autocatalytic reactions of antioxidant enzymes viz superoxide dismutase (SOD), catalase (CAT), and GPxs as 2nd line of defense. With the advancement of age and the decline of anti-oxidative defense natural and synthetic antioxidants are essential. Constraints of producing adverse effects on the liver restrict the use of synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Plant extracted polyphenols being safe in this regard are free to be used to overcome any such oxidative stress. Therefore, the present study aims to extract the polyphenols from *Litsea glutinosa* leaves in different solvent system and to assay the *in vitro* anti-oxidative effect by various chemical tests for advocating its efficacy through validation and development of potent lead molecules.

MATERIALS AND METHODS

Plant material

Litsea glutinosa, locally known as Jaysandha was collected from forest areas of Keonjhar district of Odisha, identified in the Department of Botany, O.U.A.T. following the description of Saxena and Brahman (1995). The plant leaves collected at pre-flowering stage were cleaned, dried under shade, and ground into fine structures for preparation of extracts.

Solvent and groupings

Solvents of extraction were classified into 7 groups such as Gr-A (100% methanol), Gr-B (100% de-ionized water), Gr-C (methanol: water: acetic acid in 70:25:05 v/v), Gr-D (ethyl acetate: methanol: water in 60:30:10 v/v), Gr-E (100% acetone), Gr-F (acetone: water: acetic acid in 90: 9.5: 0.5 v/v) and Gr-G (100% ethanol)

Extraction process

Extraction of phyto-constituents was done in Microwave-Assisted Extraction (MAE) method by Multiwave 3000-801V (Anton Paar) digestion system following the method of Senapati et al. (2013) where 2 g of ground mass in 20 ml of solvent was heated at 80°C for 25 minutes followed by 15 minutes cooling and filtration through what man No-1 filter paper to harvest the extract.

Removal of chlorophyll

Equal volumes of filtered crude extract and hexane were mixed and kept for 2 minutes. The supernatant was aspirated carefully to obtain chlorophyll free extract.

Estimation of total polyphenols and antioxidant activity assay

Total polyphenol content of different solvent extract was determined by the method of Singh et al. (2002). The anti-oxidant activities were assayed with use of the concerned extract obtained from 1 g of leaf powder. The total antioxidant activity of extracts under different solvents was evaluated by phospho-molybdenum (Prieto et al., 1999), FRAP (Benzie and Strain, 1996) and the reducing power method (Oyaizu, 1986). Individual antioxidant capacities of these extracts were estimated by scavenging of Superoxide, (SO) (Hyland et al., 1983), Nitric oxide, (NO) (Sreejayan Rao, 1997) and Hydroxyl radical, (OH^-) (Halliwell et al., 1997) whereas lipid per-oxidation inhibition assay (LPOIA) was estimated by the method of Ohkawa et al. (1979).

Statistical analysis : The data were subjected to analysis of variance to test the significance of difference of mean values between different groups according to Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The total polyphenol content of chlorophyll free extract of 1 g of powdered *L. glutinosa* leaf in different solvents was exhibited in Table 1. Significantly higher proportion of ($p<0.01$) total polyphenols were extracted in solvent mixtures of methanol with other solvents at Gr-D and C and in acetone at Gr-F as compared to other individual solvents at Gr-A, B, E and G. The values of total phenolics were in descending order from Gr-D > C > F > E > A > G > B.

Table 1. Total polyphenol content of *Litsea glutinosa* leaf extracts in different solvents (Mean ± SE) expressed as mg of Gallic acid equivalent per g of plant material (mg GAE / g of plant)

Gr-A	Gr-B	Gr-C	Gr-D	Gr-E	Gr-F	Gr-G
1.41^{a} ±0.05	1.27^{a} ±0.06	1.84^{c}±0.06	1.88^{c} ±0.05	1.48^{b}±0.06	1.63^{c} ±0.05	1.32^{a} ±0.05

Means with different superscripts within rows showed significant difference ($p<0.01$) between the groups.

Total antioxidant activities of the solvent extracts showing significantly higher contents of polyhenols were estimated for Gr-C, D and F by three different methods (Table-2). The polyphenols extracted in Gr-D exhibited significantly higher anti-oxidant activities ($p<0.01$) in phosphomolybdenum and reducing power method than the solvents at Gr-C and F. The polyphenols at Gr-C solvent depicted significantly higher activities ($p<0.01$) in FRAP method followed by Gr-D and F.

Table 2. Total antioxidant activity of *Litsea glutinosa* leaf extracts (Mean ± SE)

	Gr-C	Gr-D	Gr-F
Phosphomolybdenum (µM of AAE / g of plant)	349.51^{a}±4.29	484.57^{b}±4.34	298.10^{c}±2.96
FRAP (10^3 XµM of AAE / g of plant)	7.13^{a} ±0.12	5.46^{b} ±0.10	2.42^{c} ±0.06
Reducing power (µM of AAE / g of plant)	743.05^{a}±3.11	1059.29^{b}±3.29	379.01^{c} ±2.09

Means with different superscripts within the rows differ significantly ($p<0.01$).

Individual antioxidant activities of the extracts showing significantly higher contents of polyhenols were estimated for Gr-C, D and F by four different methods (Table 3). The polyphenols extracted in Gr-D exhibited significantly higher superoxide and nitric oxide radical scavenging activities ($p<0.01$) followed by Gr-C and F. On the other hand polyphenols extracted in Gr-F exhibited significantly ($p<0.01$) higher hydroxy radical scavenging activity and lipid peroxidation inhibition assay followed by Gr-C and D.

Table 3. Individual antioxidant (Radical Scavenging %) activity of *Litsea glutinosa* leaf extracts (Mean ± SE)

Parameters	Gr-C	Gr-D	Gr-F
Superoxide (SO)	29.76^{a} ±0.98	36.28^{a}±1.17	8.49^{b} ±0.22
Nitricoxide (NO)	19.24^{a}±0.88	27.21^{b}±1.11	16.96^{a}±0.77
Hydroxyl (OH)	24.96^{b}±0.96	20.55^{a}±0.92	26.64^{b}±0.92
Lipid Per-oxidation Inhibition (LPOI)	15.96^{a}±0.83	15.21^{a}±0.94	20.38^{b}±0.84

Means with different superscripts within the rows differ significantly ($p<0.01$).

Polyphenols are groups of compounds containing more than one hydroxyl groups directly attached to carbon-based aromatic phenyl-ring. Dietary polyphenols with over 8,000 structural variants are predominantly secondary metabolites of fruits, vegetables, wine, tea, leaf, chocolate and other cocoa products. These are mostly derivatives and/or isomers of flavones, isoflavones, flavonols, catechins and phenolic acids and possess similar biological properties of anti-oxidants. Total phenolic content, in the study, varied among the different solvents within the same plant. The concentration of total phenolic content was significantly lower ($p<0.01$) in individual water, methanol and ethanol extracts than combined Methanol + Water + Acetic acid mixture, Ethyl acetate + Methanol + Water mixture and Acetone + Water + Acetic acid mixture. It depicted that maximum components of poly phenols were more soluble in the mixtures of organic solvents than in aqueous and individual solvents. Due to the phytochemical diversity in polyphenol contents this variation between the experimental groups was recorded and it was corroborated with the reports of Siddhuraju et al. (2002).

Three different chemical methods were used to estimate the total anti-oxidant activity of the plant derived polyphenol extracts to study the comparative and absolute effects. The total anti-oxidant activity by phospho-molybdenum assay is based on the reduction of Mo^{VI} to Mo^{V} and the formation of a green Mo^{V} complex with a maximal absorption at 695 nm. It was observed that antioxidant activity was higher in polyphenol extracted from solvent consisting of Ethyl acetate + Methanol + Water mixture and than those extracted in Acetone + Water + Acetic acid mixture. The result was in accordance with the findings of Rumzhum et al. (2012). The reducing property of bioactive phenolic compounds was associated with antioxidant activity (Siddhuraju et al., 2002). The study of reducing power of different solvent extract of leaf samples revealed increased anti-oxidative activity of the extracts with increase concentration of polyphenol content. It was concluded that the amount of phenolic constituents were more in extracts with solvent components Ethyl acetate + Methanol + Water, Methanol + Water + Acetic acid and Acetone + Water + Acetic acid. Because phenolics present in these leaf extracts are good electron donors and could terminate the radical chain reaction by converting free radicals to more stable products. Our findings were in good agreement with earlier reports where it was stated that the antioxidant properties were concomitant with the development of reducing power (Hu et al., 2003). The relevant chemical reaction of the

FRAP method involves a single electron reaction between Fe $(TPTZ)_2$ (III) and a single electron donor ArOH. The polyphenols like caffeic acid, tannic acid, ferulic acid, ascorbic acid, and quercetin, etc. react with Fe $(TPTZ)_2$ (III) slowly. So the reducing power cannot be correctly measured and there is possible interference due to the UV-Vis absorption at 593 nm by compounds other than Fe $(TPTZ)_2$ (II). Therefore, the FRAP assay cannot be used in biological samples as many vegetable extracts being colored may have similar interference. The result corroborates with the reports of Sharma et al. (2019) on ethanol extract only.

The topography of soil, climatic condition, season and stage of leaf collection might be the reason for variable contents of polyphenols in different plants. Besides, the organic solvents/ their mixtures and method of extraction also added to the aforesaid attributed reason. But the variation of total antioxidant properties of the plants might be due to difference in the constituents of plants responsible for extending protection against oxidative stress and ROS generation. In our study it might be due to different concentrations of polyphenols in various plants which showed variable antioxidant properties.

Individual Antioxidant Activity was determined by superoxide, nitric oxide and hydroxyl radical scavenging activity along with lipid peroxidation inhibition assay. The superoxide radicals were generated by illuminating the solution. The relative scavenging effect of total phenolics of different solvent extracts showed that the scavenging activity on superoxide radicals with the highest by the phenolics from Ethyl acetate + Methanol + Water and Methanol + Water + Acetic acid mixture extracts. Sodium nitroprusside in standard phosphate buffer produces nitric oxide and the plant extracts scavenge it to show their protective effects. The relative scavenging effect nitric oxide radicals of total phenolics of different solvent extracts showed that the scavenging activity on superoxide radicals with the highest by the phenolics from Ethyl acetate + Methanol + Water and Methanol + Water + Acetic acid mixture extracts. The variations in per cent of scavenging nitric oxide between different solvents and their mixtures were significant owing to their significant different concentrations of total polyphenols (Gil et al., 2000). The leaf extracts also exhibited a potent scavenging activity for hydroxyl radical. Deoxyribose is degraded into malonaldehyde on exposure to hydroxyl radicals generated by Fenton systems. On heating the mixture under acid conditions, malonaldehyde was detected spectrophotometrically by reaction with thiobarbituric acid to form a pink chromogen (Smith et al., 1992). The relative scavenging effect of total phenolics of different solvent extracts of leaf sample showed that the highest scavenging was exhibited by the phenolics extracted from Acetone + Water + Acetic acid and Methanol + Water + Acetic acid mixture. Thiobarbituric acid reactive substances are produced as by-products of lipid peroxidation when induced by the ferrous sulfate in poultry brain homogenate. The plant extracts in different organic solvents were exposed to inhibit the production of MDA to assay their antioxidant activities. The relative scavenging effect of total phenolics of different solvent extracts of leaf sample showed that the highest scavenging was exhibited by the phenolics extracted from Acetone + Water + Acetic acid and Methanol + Water + Acetic acid mixture.

CONCLUSION

The scavenging of superoxide, nitric oxide, hydroxyl radical and inhibition of MDA production were the avenues of chemical methods origin to measure the anti-oxidative effects of plant extracts. The plant extracts in different solvents showed different scavenging activities based on the solvent used for extraction. The solvents as well as methods of extraction played a significant role in contributing different polyphenolic components derived from plant leaves. The wide diversity in these components in the plant extracts was responsible to exhibit difference in antioxidant activity through scavenging various ROS radicals. It might be the reason of obtaining a variable per cent of scavenging different ROS radicals in various methods by the plant extracts of different solvent origin.

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