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ANTIOXIDANT ACTIVITY OF PATHYASHADANGAM KASHAYAM : A CLASSICAL AYURVEDIC FORMULATION AND ITS INGREDIENTS

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Abstract

Pathyashadangam kashayam is a classical Ayurvedic formulation which finds therapeutic application in the treatment of diseases of eye, ear and tooth, migraine and cluster head ache. It is also prescribed by Ayurveda physicians for diabetes and neurodegenerative diseases. Since antioxidant potential of the kashayam might contribute to its therapeutic efficacy, an attempt has been made to evaluate the same. The correlation between antioxidant potential of the kashayam and its total phenol and flavonoid contents was also analyzed.

To evaluate the antioxidant potential of Pathyashadangam kashayam and its ingredients. The total phenol content of the kashayam was analyzed by Folin-Ciocalteau assay and the total flavonoid content by Aluminium chloride colorimetric assay. The antioxidant potential of the kashayam and its ingredients was evaluated by DPPH assay. Reducing power assay, total antioxidant capacity assay and NO radical scavenging assay were also employed for evaluation of antioxidant capacity of the kashayam. The correlation between the antioxidant potential and total phenol and flavonoid contents of the kashayam was analyzed using CORREL function of Microsoft Excel. The experimental data indicated that Pathyashadangam kashayam had remarkable potential to scavenge DPPH radicals comparable to standards Ascorbic acid and Trolox. Among the ingredients, *Terminalia chebula, Terminalia bellirica* and *Phyllanthus emblica* were found to be better scavengers of DPPH radicals than the kashayam, but all other ingredients exhibited decreased activity. The NO radical scavenging activity of the kashayam was also comparable to standard Ascorbic acid. Antioxidant potential of the kashayam was also confirmed from the reducing power and total antioxidant capacity assays. The kashayam had ample quantity of phenols and trivial quantity of flavonoids conferring it antioxidant potential. Pathyashadangam kashayam has significant antioxidant potential which contributes to its therapeutic ability. The total phenol and flavonoid contents of the kashayam were positively correlated to its antioxidant potential.

Keywords **:** Antioxidant activity, flavonoid, Pathyashadangam kashayam, Ayurvedic formulation.

Introduction

Food and oxygen are two fundamentals of human life, the energy needed for day to day activities being derived from oxidation of food items through respiration. Incomplete oxidation of food items leads to the generation of highly reactive and potentially dangerous reactive oxygen species (ROS). These include free radicals like superoxide and hydroxyl radicals and non-radicals like hydrogen peroxide, ozone and singlet oxygen (Liou and Storz, 2010). Free radicals like reactive nitrogen species, including nitric oxide are also produced in the body. Free radicals which contain one or more unpaired electrons in their outer orbitals are extremely reactive and cause oxidation or reduction. Even though, adequate amounts of free radicals are required for normal functioning of humans, their excessive generation leads to oxidative stress, which cause many disorders including ageing, cardiovascular diseases, cancer and Parkinson's disease (Pham-Huy *et al*., 2008)

An antioxidant is a substance which when present even at low concentrations in comparison to oxidisable substrate, delays or prevents oxidation of the substrate. They are widely used as ingredients of dietary supplements, in medicine and also as food preservatives. Antioxidants are believed to prevent neurological damage and hence considered as possible treatment for neurodegenerative diseases. Antioxidants produced by the body to combat oxidative damage are called endogenous antioxidants which include enzymatic antioxidants like superoxide dismutase, glutathione peroxidase, glutathione reductase, peroxiredoxins and catalase in addition to non-enzymatic antioxidants like metal binding proteins, polyamines and bilirubin (Mirończuk-Chodakowska *et al*., 2018). However, our endogenous antioxidant defense systems are sometimes incomplete and exogenous supply of antioxidants are essential in maintaining health.

Synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have toxic effects to some extents (Jayalakshmi and Sharma, 1986) and may promote tumour development (Kahl and Kappus, 1993). Therefore natural antioxidants are preferred over synthetic ones and Ayurvedic formulations made of natural ingredients with antioxidant potential can be of choice to combat oxidative damage.

The present study has been designed to evaluate the antioxidant potential of Pathyashadangam kashayam and its relation with phenol and flavonoid contents.

Materials and Methods

Chemicals and standards

All the chemicals and reagents used in the assays were of analytical grade. The ingredients of the kashayam viz. dried fruit pericarps of *Terminalia chebula* Retz., *Terminalia bellirica* (Gaertn.) Roxb. and *Phyllanthus emblica* L., aerial parts of *Andrographis paniculata* (Burm. f.) Wall. ex Nees., dried cured rhizome of *Curcuma longa* L., stem bark of *Azadirachta indica* A. Juss. and stem of *Tinospora cordifolia* (Willd.) Miers. were collected from the raw material division of Kerala Ayurveda Limited and the voucher specimens were deposited in the herbarium of KFRI, Peechi for future

reference (Accession numbers 13037-13043). Three batches of Pathyashadangam kashayam were prepared as per kwatha vidhi of Sarngadhara samhita (Gopalapilla, 1998) and analyzed for total phenol and flavonoid contents and antioxidant potential.

Estimation of Total phenol

The total phenol in methanol extract of the kashayam was measured by Folin Ciocalteu method (Singleton *et al*., 1999). 1 ml of the sample/ standard/ blank was added to 60 ml distilled water to which 5 ml Folin Ciocalteu reagent was added and mixed well. After 1 minute and before 8 minutes, 15 ml of 20 % sodium carbonate solution was added and the volume was made up to 100 ml. After 2 hours, the absorbance was measured at 760 nm at about 23° C, using a double beam spectrophotometer (Shimadzu, UV-1800). Standard solutions of 2-10 µg/ml of Gallic acid in methanol were prepared, five different concentrations of which were employed for the preparation of calibration curve wherein absorbance (y axis) was plotted against concentration (x axis). The linear equation was deduced from the calibration curve from which the concentration of the sample was calculated by substituting absorbance. Total phenolic content was expressed in mg Gallic acid equivalents (GAE)/ml of kashayam as mean ± standard deviation of three values.

Estimation of Total flavonoids

Total flavonoids in methanol extract of the kashayam was calculated by aluminium chloride method (Kosalec *et al*., 2004). 0.5 ml of the sample/standard/blank was mixed with 1.5 ml 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 molL-1 potassium acetate and 2.8 ml water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm using double beam spectrophotometer (Shimadzu, UV-1800). Standard solutions of 10-50 µg/ml of Quercetin in methanol were prepared and five different concentrations were employed for the preparation of calibration curve wherein absorbance (y axis) was plotted against concentration (x axis). The linear equation was deduced from the calibration curve and concentration of the sample was calculated from this linear equation by substituting absorbance. Total flavonoid content was expressed in mg Quercetin equivalents/ml of kashayam as mean ± standard deviation of three values.

DPPH radical scavenging assay

DPPH radical scavenging assay was based on the reduction of the stable free radical 2, 2-diphenyl - 1 - picrylhydrazyl (DPPH) which was measured by spectrophotometry (Aravind *et al*., 2016). Equal volumes of 0.2 mM methanolic DPPH solution and sample solutions in methanol were mixed, incubated for 15 minutes at room temperature in a dark room and the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percentage radical scavenging activity was calculated using the following formula

% scavenging activity = $[(A_{control} - A_{sample})/A_{control}] \times 100$.

Sample concentration required to scavenge 50% of DPPH radical, relative to the control (DPPH without antioxidant agents added) was reported as IC_{50} value. Ascorbic acid and Trolox were used as positive control. The experiments were repeated thrice and the results were expressed as mean ± standard deviation of three values.

Nitric oxide radical scavenging assay

The nitric oxide radical scavenging activity of methanol extracts of three batches of kashayam was measured according to standard method (Hazra *et al*., 2008). 2 ml of 10 mM SNP was mixed with 0.5 ml phosphate buffered saline maintained at pH 7.4 to which 0.5 ml of test solution was added and the mixture was incubated at 25°C for 150 minutes. 1 ml sulfanilamide was added to 0.5 ml of the incubated solution and the mixture was allowed to stand for 5 minutes after which 1 ml of 0.1% w/v NED was added. The mixture was incubated at 25°C for 30 minutes and the absorbance of the pink chromophore was measured at 540 nm. The percentage radical scavenging activity was calculated using the formula $[(A_{control} - A_{sample}) /A_{control}] \times 100$. The sample concentration required to scavenge 50% of NO radical, relative to the control (without antioxidant agents added) was reported as IC_{50} value. Ascorbic acid was used as standard. The experiments were done in triplicate and the results were expressed as mean with standard deviation.

Evaluation of total antioxidant capacity

The total antioxidant capacity of three batches of kashayam was determined by a previously established method (Prieto *et al*., 1999). 0.1 ml of methanol extract of kashayam was mixed with 1 ml of the reagent solution (prepared from equal volumes of 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated at 95° C for 90 minutes after which the absorbance was measured at 695 nm using spectrophotometer against blank prepared with methanol and reagent mixture and incubated for the same duration. A standard curve was plotted using Ascorbic acid of known concentrations (6-30 µg/ml) and the results were expressed as mg Ascorbic acid equivalent (AAE)/g of the methanol extract of sample. The experiments were replicated thrice and the values were expressed as mean with standard deviation.

Reducing Power assay

Reducing power of the kashayam was calculated according to a previously proven method (Oyaizu, 1986). 1 ml of the sample/standard was mixed with 2.5 ml phosphate buffer (0.2 M at pH 6.6) and 2.5 ml of 1 % potassium ferricyanide and the mixture was incubated at 50° C for 20 minutes. 2.5 ml of 10% trichloroacetic acid was added to this mixture, centrifuged and 2.5 ml solution from the upper layer was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% $FeCl_3$.The absorbance of the resulting mixture at 700 nm was recorded. Increase in absorbance indicated increased reducing power. The assays were replicated thrice and the results were expressed as mean ± standard deviation of three values.

Results

Determination of total phenol and flavonoids

The total phenol content of Pathyashadangam kashayam was determined from linear regression equation of Gallic acid and expressed as mg GAE/ml of the kashayam ($y = 13.75x - 0.0179$, $R^2 = 0.9975$) (Fig 1). The total phenol content of PS 15, PS 23 and PS 25 was found to be 62.53 ± 3.1, 62.23 ± 2.4 and 58.86 ± 0.98 mg GAE/ml of kashayam respectively. The total phenol content of PS 15 and PS 23

showed great similarity, but PS 25 had marginally lower content of phenols.

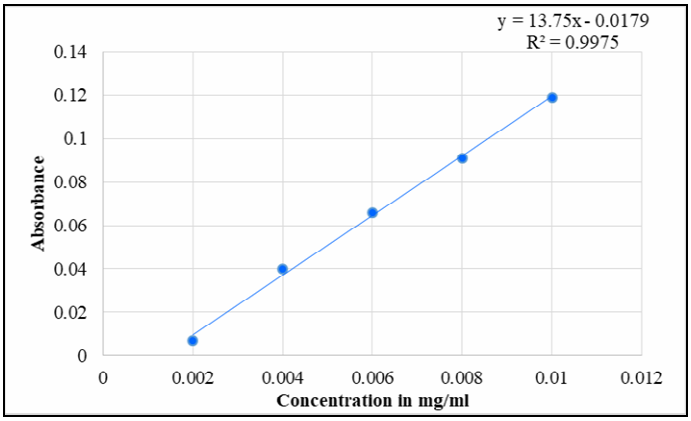

Fig. 1 : Calibration curve for standard Gallic acid.

The flavonoid content was determined from linear regression equation of Quercetin (y = 16.72 x-0.0148, R^2 = 0.9993) and expressed as mg Quercetin equivalent/ml of the kashayam (Fig 2). The total flavonoid content of PS 15, PS 23 and PS 25 was found to be 1.91 ± 0.11, 1.17 ± 0.10 and 1.31 ± 0.11 mg Quercetin equivalents/ml of the kashayam respectively. The total flavonoid content of three batches of kashayam were analogous.

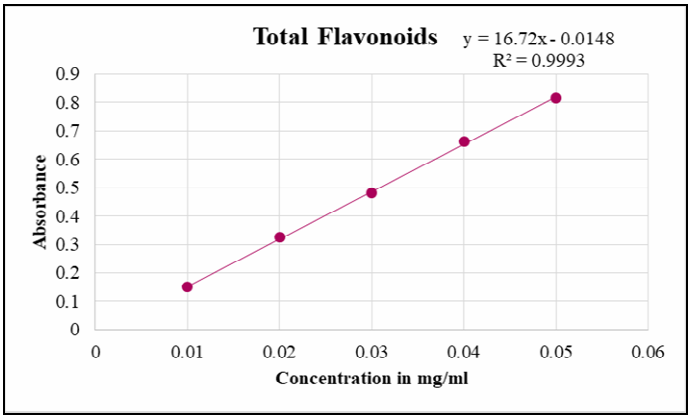

Fig. 2 : Calibration curve for standard Quercetin.

DPPH radical scavenging assay

All the three batches of Pathyashadangam kashayam showed remarkable ability to scavenge DPPH radicals, comparable to that of standards, Ascorbic acid and Trolox. It was also found that both the standards and samples scavenged DPPH radicals in a dose dependent manner (Fig 3).

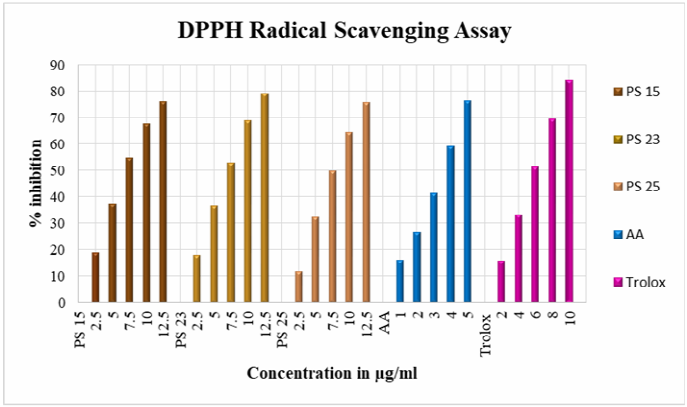

Fig. 3 : Comparison of DPPH radical scavenging activities of three batches of Pathyashadangam kashayam and standards Ascorbic acid and Trolox.

PS 15, PS 23 and PS 25 – Three batches of Pathyashadangam kashayam. AA – Ascorbic acid.

Among the ingredients of Pathyashadangam kashayam, *Terminalia bellirica* was found to be the best scavenger of DPPH radicals (Fig 4). *Terminalia chebula* also exhibited almost equal activity followed by *Phyllanthus emblica*. *Tinospora cordifolia* and *Andrographis paniculata* were found to have lesser potential to scavenge DPPH radicals than other ingredients.

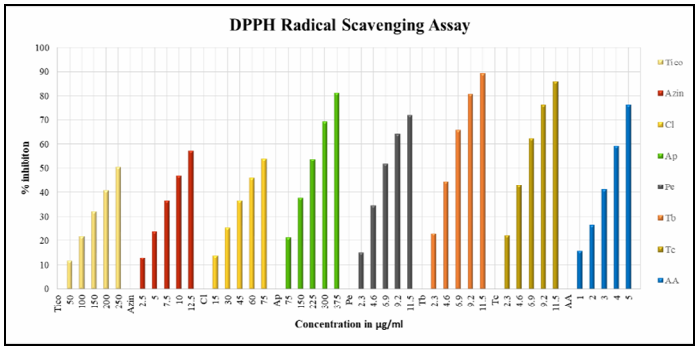

Fig. 4 : Comparison of DPPH radical scavenging activities of ingredients of Pathyashadangam kashayam and standard Ascorbic acid.

AA – Ascorbic Acid, Tc - *Terminalia chebula*, Tb - *Terminalia bellirica*, Pe - *Phyllanthus emblica*, Ap - *Andrographis paniculata*, Cl - *Curcuma longa*, Azin - *Azadirachta indica* and Tico - *Tinospora cordifolia*.

The concentration of extracts needed to scavenge 50% DPPH radicals relative to control was expressed as IC_{50}. Low IC_{50} values specified high antioxidant activity. The IC_{50} value of standard Ascorbic acid was found to be 3.4 ± 0.01 µg/ml and that of Trolox was 5.92 ± 0.1 µg/ml. The three batches of kashayam, PS 15, PS 23 and PS 25 showed IC_{50} values of 7.35 ± 0.12 µg/ml, 7.33 ± 0.21 µg/ml and 7.99 ± 0.49 µg/ml respectively. Thus the three batches of the kashayam exhibited great similarity in the IC_{50} values, which ascertained the ability of Pathyashadangam to scavenge DPPH radicals even at low concentrations, establishing its antioxidant potential. Among the ingredients, *Terminalia bellirica* with IC_{50} value of 5.36 ± 0.16 µg/ml, *Terminalia chebula* with IC_{50}, 5.77 ± 0.14 µg/ml and *Phyllanthus emblica* with IC_{50} 7.29 ± 0.04 µg/ml were found to be better scavengers of DPPH radicals than the kashayam. The DPPH radical scavenging capacity of all other ingredients were lower than the formulation, as was evident from their higher IC_{50} values. *Azadirachta indica* with IC_{50} value of 10.80 ± 0.87 µg/ml also showed significant activity, but *Curcuma longa* with IC_{50} value of 67.02 ± 1.08 µg/ml, *Andrographis paniculata* with IC_{50} value of 211.62 ± 0.80 µg/ml and *Tinospora cordifolia* with IC_{50} value of 246.95 ± 4.58 µg/ml showed less ability to scavenge DPPH radicals.

Reducing power assay

In all the three batches of Pathyashadangam kashayam and the standard Ascorbic acid, the reducing power was found to increase linearly with increase in concentration (Fig 5). There was remarkable similarity in the reducing power of three batches of kashayam. Since all the three batches of kashayam exhibited significant reducing power, the kashayam can act as a natural antioxidant.

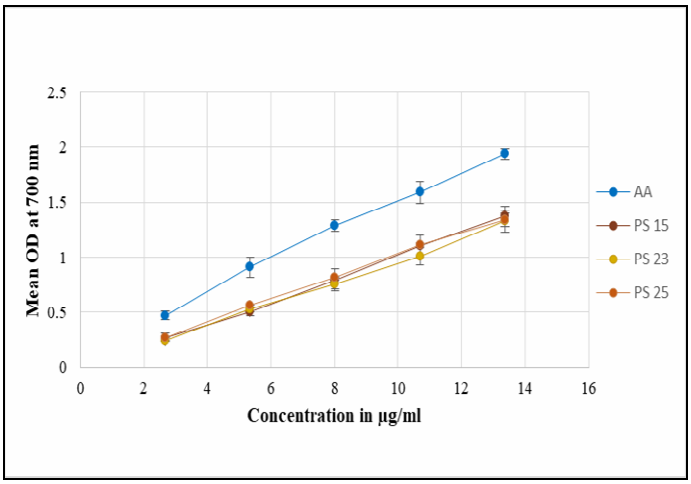

Fig. 5 : Comparison of Reducing powers of three batches of Pathyashadangam kashayam and standard Ascorbic acid.

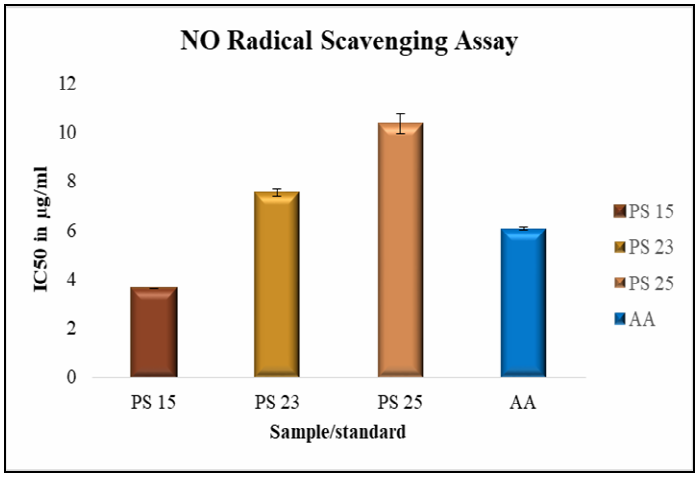

Fig. 6 : IC_{50} values of three batches of Pathyashadangam kashayam and standard Ascorbic acid against Nitric oxide.

Total antioxidant capacity assay

The total antioxidant capacity of PS 15, PS 23 and PS 25 were found to be 318.21 ± 29.89 mg AAE/g, 293.12 ± 18.89 mg AAE/g and 309.87 ± 25.13 mg AAE/g of methanol extract respectively. Thus the total antioxidant capacity was found to be similar for the three batches of kashayam.

NO radical scavenging assay

Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by methanol extract of the kashayam. The percentage of inhibition of NO radicals showed a dose dependent fashion in all the three batches (Fig 6). It was found PS 15 with IC_{50} value lower than that of the standard exhibited higher activity, but PS 23 and PS 25 with higher IC_{50} values than Ascorbic acid showed lesser activity. The assay revealed that Pathyashadangam kashayam had remarkable potential to scavenge NO radicals and the potency was comparable to that of Ascorbic acid. It was also noticed that unlike other assays, the three batches of kwath showed some variation in IC_{50} values.

Statistical analysis

The relationship between the total phenol and flavonoid contents of kashayam with the anti-oxidant activity was analyzed using the CORREL function of Microsoft Excel. It was observed that the Antioxidant activity (mg AAE/ml) of Pathyashadangam kashayam was positively correlated to its total phenol (mg GAE/ml) as well as flavonoid (mg QE/ml) contents (Table 1). A strong positive correlation was observed between DPPH radical scavenging activity and total phenol content of the kashayam whereas only moderate positive correlation was observed with total flavonoid content. The nitric oxide radical scavenging activity and total antioxidant capacity of the kashayam showed strong positive correlation with both total phenol and flavonoid contents. The reducing power of the kashayam was also positively correlated with total phenol as well as flavonoid contents, but stronger correlation was observed with total flavonoid contents of the kashayam.

Table 1 : Correlation between antioxidant activity of Pathyashadangam kashayam and total phenol and flavonoid contents.

Antioxidant activity (mg AAE/ ml of the kashayam)	Correlation coefficient	
	Total phenols (mg GAE/ml of kashayam)	Total flavonoids (mg QE/ml of kashayam)
DPPH radical scavenging activity	0.999	0.428
NO radical scavenging activity	0.760	0.890
Total antioxidant capacity	0.818	0.842
Reducing power	0.660	0.945

Discussion

Any atom or molecule that contains an unpaired electron in the outer orbit is called a free radical whose reaction with a non-radical, initiates a chain reaction resulting in the production of new radicals. Lipids, proteins, deoxyribonucleic acid and carbohydrates in the human body are susceptible to damage by free radicals (Halliwell, 2006). Reaction of free radicals with biological macromolecules which are non-radicals, damage them (Halliwell, 1994). Excessive generation of free radicals can cause damage to proteins resulting in loss of enzyme activity, lipid peroxidation which damage cell membranes and lipoproteins as well as DNA lesions which cause mutations.

Even though ROS and RNS exert beneficial effects on immune response and cellular function at low or moderate levels and are vital to human health, they are deleterious at high concentrations. Disparity between formation and neutralization of ROS/RNS causes oxidative stress. The bio-molecular damage caused by attack of reactive species upon the constituents of living organisms is called oxidative damage. Many diseases like cancer, diabetes, Alzheimer's, atherosclerosis and neurodegenerative diseases can be caused by oxidative damage (Halliwell and Whiteman, 2004). Living organisms produce substances that are capable of revitalizing or impeding oxidative damage to fight free radicals (Alves *et al.*, 2010). Molecules of biological origin which remove the free radical intermediates and thereby

prevent or delay oxidation of substrates even at low concentrations are called natural antioxidants. Plant secondary metabolites, particularly flavonoids (Miller, 1996) and polyphenols have remarkable antioxidant potential (Pandey and Rizvi, 2009). Natural antioxidants, preclude the development of disease by averting oxidative stress by excess free radicals and by checking the development of secondary radicals. On account of harmful effects reported for synthetic antioxidants like BHT, natural antioxidants are gaining momentum to counteract the free radicals associated diseases.

Scavenging of DPPH free radical is the basis of a common antioxidant assay which is a reliable method to determine the antioxidant capacity of biological substrates. The measurement of depletion of DPPH specifies the intrinsic ability of a substance to donate electrons or hydrogen atoms to this reactive species in a homogenous system (Paixao *et al*., 2007). The activity is generally measured as inhibition percentage of the pre-formed free radical by antioxidants, and usually expressed as the concentration required to obtain a 50% antioxidant effect denoted as IC_{50} or EC_{50} (Chen *et al*., 2013). DPPH radical scavenging assay of Pathyashadangam kashayam revealed that all the three batches of kashayam showed remarkable ability to scavenge DPPH radicals and the activity was comparable to standards Ascorbic acid and Trolox, which suggested significant antioxidant potential of the kashayam. Among the ingredients of the kashayam, *Terminalia chebula, Terminalia bellirica* and *Phyllanthus emblica* were better scavengers of DPPH radicals than the kashayam, but all other ingredients showed decreased activity. The results also suggested the possibility of using *Terminalia chebula, Terminalia bellirica* and *Phyllanthus emblica* as natural antioxidants.

Compounds with reducing power have the ability to donate electrons and reduce the oxidized intermediates of lipid peroxidation processes and hence can act as primary and secondary antioxidants (Soni and Sosa, 2013). In the reducing power assay, the colour of the test solution changes from yellow to various shades of blue, according to the reducing power of antioxidant samples. The increase in absorbance of reaction mixture at 700 nm specifies increase in reducing power, which is an indication of its potent antioxidant capacity (Gulcin, 2015). The reducing power of Pathyashadangam kashayam was found to increase with increase in concentration, which suggested that the electron donating ability of the kashayam is concentration dependent.

The total antioxidant capacity of the formulation was analyzed by Phosphomolybdenum assay which is grounded on the reduction of Phosphate-Mo (VI) to Phosphate Mo (V) and consequent formation of bluish green colored phosphate/Mo (V) complex at acidic pH. The total antioxidant capacity of Pathyashadangam kashayam was found to be 318.21±29.89 mg AAE/g for PS 15, 293.12±18.89 for PS 23 and 309±25.13 mg AAE/g for PS 25 respectively.

Nitric oxide (NO), an important chemical mediator generated by endothelial cells, macrophages and neurons is involved in the regulation of various physiological processes (Lata and Ahuja, 2003). NO radical scavenging assay is based on the measurement of nitrite ions, generated by the reaction of NO (produced by decomposition of sodium nitroprusside in aqueous solution at physiological pH) with oxygen under aerobic conditions, using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to decreased production of nitrite ions (Parul *et al*., 2013). NO radical scavenging assay of Pathyashadangam kashayam revealed that the formulation is an excellent scavenger of NO radicals and the activity was comparable to standard Ascorbic acid.

Conclusion

From all these assays it could be inferred that Pathyashadangam kashayam has significant antioxidant potential which explains its therapeutic ability. It could explain its indication in the management of migraine and also its use in treatment of diabetes and neurodegenerative diseases. The results also suggest that *Terminalia chebula, Terminalia bellirica* and *Phyllanthus emblica* could be used as natural antioxidants to combat free radical induced damages. From the correlation studies it could be concluded that the antioxidant capacity of Pathyashadangam kashayam might be attributed to the phenols and flavonoids present therein.

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