Indian Journal of Traditional Knowledge
Vol 20(3), July 2021, pp 651-659

# Evaluation of free radical scavenging and anti-lipoxygenase activity in various fractions of ayurvedic polyherbal decoction, *Punarnavadi kashayam*

Bashi M B[a], Rahitha Devi S J[a] & Prakash Kumar B*[,b]

[a]Inflammation Research Lab, School of Biosciences, Mahatma Gandhi University, Priyadarshini Hills, Kottayam 686 560, Kerala, India
[b]School of Biosciences, Mahatma Gandhi University, Priyadarshini Hills P.O, Kottayam 686 560,
E-mail: prakashkumar@mgu.ac.in

*Received 11 October 2019; revised 11 December 2020*

*Punarnavadi kashayam* (PK), a classical ayurvedic polyherbal decoction prescribed for abating various inflammatory conditions, was analysed for its total phytochemical content, free radical scavenging, and anti-inflammatory activity in order to validate its traditional usage. PK was sequentially fractionated using solvents of different polarity and the preliminary phytochemical screenings were performed. Free radical scavenging activity was determined using various biochemical assays while the anti-inflammatory potential was evaluated by determining the anti-lipoxygenase activity using FOX assay. Ethyl acetate fraction showed better free radical scavenging activity as compared to the corresponding positive controls. The same fraction exhibited the better $IC_{50}$ for 5- and 12-lipoxygenase inhibition assay than the positive control, showing its anti-inflammatory property. The phytochemical analysis revealed a higher content of phytochemicals in ethyl acetate, dichloromethane and methanol fractions which displayed the correlation between phenolic content and the bioactivity. Overall, PK can effectively scavenge free radicals and inhibit lipoxygenase enzymes, thereby decreasing the production of free radicals and pro-inflammatory leukotrienes.

**Keywords**: Anti-inflammatory activity, Free radical scavenging activity, Lipoxygenases, Phenolic content, Phytochemicals, *Punarnavadi kashayam* (PK)

**IPC Code**: Int Cl.[21]: A61K 31/05, A61K 31/44, A61K 38/00, A61K 45/06, A61P 29/02

Free radicals are highly unstable atoms or group of atoms which tend to search for electrons from other lead molecules/atoms to become stable and make other molecules unstable which lead to a chain reaction in the body[1]. Different kinds of free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS) thus generated are the products of normal cellular metabolism[2]. However, the uncontrolled exposure of biological system to chemicals and other contaminants paves a way to an unbalanced production of free radicals which contributes to irreversible oxidative damage to biomolecules like lipids, nucleic acids, proteins and carbohydrates, which leads to the onset of ageing process and numerous chronic diseases like inflammatory arthropathies, cancer, Parkinson's disease, cardiovascular disease, etc., in humans.

Antioxidants are compounds which inhibit or trap free radicals generated in the body, thereby reducing the oxidative stress-related disorders[4]. Various synthetic antioxidants like propyl gallate, butylated hydroxyl toluene, butylated hydroxy anisole, etc., are available in the market, which are associated with adverse health effects[5,6]. Thus, there is a necessity for exploring the least toxic herbal alternatives against inflammatory diseases which are the rich source of natural antioxidants. Various studies emphasize the correlation of phytocompounds like phenolics-flavonoids present in the herbal plants with the antioxidant potential which helps in the free radical scavenging and thereby reduces degenerative diseases[7]. Oxidative stress and inflammation are closely related pathophysiological processes, one of which one can easily induce the other. Most of the phases of chronic inflammatory disorders are associated with free radical production and associated inflammation. Hence antioxidant therapy alone is unlikely to prevent inflammatory disorders[8].

Lipoxygenases are rate-limiting enzymes which catalyse the biosynthesis of leukotrienes from arachidonic acid released from the membrane phospholipids during inflammatory conditions and thus have an important role in the pathophysiology of inflammation. Therefore, inhibition of lipoxygenase enzymes attributes to the blockade of inflammatory complications. Mainly three types of LOX isoenzymes namely, 5-, 12- and 15-LOXs are associated with inflammatory disease conditions like asthma, cardiovascular diseases, atherosclerosis and

*Corresponding Author

even cancers[9]. Consequently, potential lead molecules with inhibitory effect on LOX enzymes could have a potential therapeutic role in reducing inflammation-related disorders[10]. Chemical compounds like zileuton, montelukast, pranlukast, etc., used as 5-LOX inhibitors for the treatment of inflammatory diseases, are associated with hepatic and gastrointestinal disorders[11]. In Ayurveda, the traditional system of Indian medicine, herbal formulations or combined plant extracts are used in the treatment of a wide variety of ailments and clinical application of this approach is found to be successful as conventional treatment[12]. *Punarnavadi kashayam* is a classical ayurvedic polyherbal decoction, mentioned in ayurvedic literature Sahasrayoga used to mitigate various proinflammatory disorders[13-15]. According to Sarangdhar Samhita, the decoctions with multiple herbs in a particular ratio contribute better therapeutic effects with less toxicity[16]. However, proper scientific confirmation is necessary for this combined herbal extract for validating its therapeutic effects. The present study focuses on the preliminary phytochemical screening and the antioxidant-anti-lipoxygenase activity of the *Punarnavadi kashayam* (PK) which comprises of eight herbs.

## Materials and Methods

*Punarnavadi kashayam* prepared according to Sahasrayoga was purchased from Kottakkal Arya Vaidya Sala, Kottayam, Kerala. Gallic acid monohydrate, caffeic acid, rutin trihydrate, butylated hydroxyl toluene, 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis-(3 ethyl) benzothiazoline)-6-sufonic acid) diammonium salt, potassium persulfate sulphanilamide, aluminium chloride, montelukast, EDTA, and xylenol orange were purchased from Himedia Laboratories, Mumbai. Sodium bicarbonate, sodium molybdate dihydrate, sodium nitrite, sodium hydroxide, ammonium iron II sulfate hexahydrate, potassium ferricyanide, 2,4,6-tri[2-pyridyl]-S-triazine (TPTZ), iron II sulfate heptahydrate, hydrogen peroxide solution 30% purified, ferrous sulfate, and triton X-100 were obtained from Merck Specialities Pvt. Ltd, India. 15-LOX pure enzyme was purchased from Cayman Chemicals, USA. Folin-Ciocalteu's phenol reagent and solvents like hexane, dichloromethane, ethyl acetate, methanol used for fractionation studies were of HPLC grade purchased from Himedia Laboratories, Mumbai.

### Fractionation of *Punarnavadi kashayam*

Sequential fractionation of *Punarnavadi kashayam* (PK) was carried out using five different solvents, viz., hexane, dichloromethane, ethyl acetate, methanol, and deionized water, based on their polarity. The fractions collected were evaporated to dryness using rotary evaporator and lyophilized. The percentage yield of each fraction was calculated and the fractions were named, PK I, PK II, PK III, PK IV and PK V, respectively. The dried extracts were stored at 4°C until further analysis.

### Qualitative phytochemical analysis

Qualitative phytochemical screening was done for the preliminary identification of various secondary metabolites such as flavonoids (Alkaline reagent test), phenols (Ferric chloride test), tannins (Braymer's test), saponins (Foam test), terpenoids (Salkowski's test), quinones, fatty acids, alkaloids (Wagner's test), carbohydrates (Molisch's test), cardiac glycosides (Keller Kelliani's test), amino acids and proteins, anthocyanins and coumarins present in *Punarnavadi kashayam* fractions according to standard protocols[17,18]. The stock solution was prepared for each fraction with methanol except for water fraction at concentration of 5 mg/ mL and was subjected to analysis. A control was maintained for all assays with corresponding reagent without sample.

### Quantitative phytochemical analysis

#### *Total phenolic content*

Total phenolic content in PK fractions was determined using Folin-Ciocalteu's method[19]. Briefly, the reaction mixture was prepared by mixing 0.1 mL of samples at concentration 1 mg/mL with 0.2 mL Folin-Ciocalteu's reagent and incubated for 4 min. After incubation, 1 mL of sodium carbonate solution (15%) was added to it. The mixture was incubated for 2 h under dark conditions and the absorbance was taken at 765 nm using UV spectrophotometer (Shimadzu, Japan). Total phenolics were calculated from the gallic acid (standard) calibration curve and was expressed as the gallic acid equivalent per gram (mg GAE/g) of extract.

#### *Total phenolic acid content*

The total phenolic acid content was quantified using a spectrophotometric method with Arnov's reagent[20]. The reaction mixture was prepared out of 0.1 mL of each fraction at a concentration of 1 mg/mL mixed with 0.6 mL deionized water, 0.1 mL 0.5% hydrochloric acid, 0.1 mL Arnov's reagent (10.0 g sodium molybdate and 10.0 g sodium nitrite) and 0.1 mL sodium hydroxide (4%). The pink colour chromophore obtained was spectrophotometrically measured at 490

nm and the concentration expressed as caffeic acid equivalent (mg CAE/g) on dry weight which was calculated from the caffeic acid calibration curve.

***Total flavonoid content***

The quantification of flavonoids was estimated by aluminium chloride colorimetric method[21]. Briefly, 0.5 mL of each sample / rutin standard solution was mixed with 1 mL deionized water, 0.3 mL sodium nitrite (5%) and kept for 5 min. After incubation, 0.3 mL aluminium chloride (10%) was added and incubated for 6 min at room temperature before 1 mL of 1M NaOH was added. The absorbance was recorded immediately at 510 nm against a blank using a UV spectrophotometer. Total flavonoid content was calculated from the rutin calibration curve as milligrams of rutin equivalent per gram of dry extract (mg RE/ g).

**Free radical scavenging and reducing antioxidant activity**

***2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity***

DPPH, a stable free radical scavenging activity, was assessed using the method described by Sundarraju *et al.*,[22]. For the assay, 0.1 mL of 0.5 mM DPPH solution was mixed with 0.1 mL of PK fractions/BHT (positive control) at different concentrations (50 µg-1000 µg/mL). Then the reaction mixture was incubated for 30 min in dark at room temperature and the decrease in absorbance was recorded at 517 nm using the Thermo Scientific Varioskan multimode reader. The radical scavenging activity of each sample / positive control was calculated using the following equation. Percentage radical scavenging activity= $[(Abs_{control}–Abs_{test})/Abs_{control}] \times 100$, where $Abs_{control}$ is the absorbance of the control and $Abs_{test}$ is the absorbance of the sample.

***Azino-bis-(3 ethyl) benzothiazoline)-6-sulfonic acid) (ABTS*) cation scavenging activity***

In ABTS radical scavenging assay, the blue-green colour of $ABTS^+$ cation gets decolorised according to the extent of the antioxidant capability of the sample. ABTS cation radicals ($ABTS^+$) were prepared by mixing an equal volume of 7 mM aqueous solution of ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$) and the mixture was kept at room temperature in the dark for 16 h until the reaction was complete. After incubation, the mixture, blue/green chromophore was diluted with methanol to obtain an absorbance of 0.700±0.005 at 734 nm[23,24]. Then 0.9 mL $ABTS^+$ solution was added to 0.1 mL sample / positive control at different concentrations (10 µg-500 µg/mL) and mixed for 15 s. The decrease in absorbance taken immediately in Shimadzu UV1800 spectrophotometer at 734 nm. The percentage of $ABTS^+$ scavenging by the fractions/positive control was calculated using the following equation: percentage cation radical scavenging= $[(Abs_{control}–Abs_{test})/Abs_{control}] \times 100$, where $Abs_{control}$ is the absorbance of the control and $Abs_{test}$ is the absorbance of the sample.

***Ferric reducing antioxidant power (FRAP) assay***

FRAP assay was used to analyse the ability of PK fractions to reduce the ferric complex to ferrous form[25]. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6) with 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM ferric chloride hexahydrate (10:1:1) and warmed at 37°C immediately prior to the assay. In this assay, 140 µL of freshly prepared FRAP solution was added to 30 µL of each fraction / positive control, Butylated hydroxyl toluene (BHT) at concentration 1 mg/mL and incubated for 30 min at 37°C in dark. The absorbance of the coloured reactant was measured at 593 nm by the varioskan multimode plate reader. The net absorbance was interpolated on the calibration plot prepared using ferrous sulphate and the results were expressed in terms of mg ferrous / mg of extract.

***Hydrogen peroxide scavenging activity***

The hydrogen peroxide scavenging capability of PK fractions was measured by the standard method of Ruch *et al.*,[26,27]. Hydrogen peroxide solution (4 mM) were prepared in phosphate buffered saline at pH 7.4 and the reaction was initiated by adding 0.6 mL hydrogen peroxide to 0.1 mL samples / positive control at various concentrations (50-1000 µg/mL). The control was maintained with hydrogen peroxide and PBS without samples. The absorbance of the reaction mixture was read at 230 nm after 10 min incubation against the blank, PBS. BHT was used as the reference compound for the assay. The percentage of hydrogen peroxide scavenging by the fractions/ standard was calculated using the following equation: percentage cation radical scavenging= $[(Abs_{control}–Abs_{test})/Abs_{control}] \times 100$, where $Abs_{control}$ is the absorbance of the control and $Abs_{test}$ is the absorbance of the sample.

**Anti-inflammatory activity**

***Inhibition of 5-, 12- and 15- Lipoxygenases***

An enzyme-based 5-, 12- and 15-lipoxygenase assay was performed to evaluate the anti-inflammatory effect of PK fractions.

***5-LOX extraction from red potato tubers***

5-LOX was isolated from red potato tubers purchased from the local vendors. The extraction of 5-LOX was done as per the protocol described by Redanna *et al*.,[28]. Cleaned red potatoes were homogenised with 2 volumes of potassium phosphate buffer (100 mM) containing sodium bisulfite, ascorbic acid (2 mM), and EDTA (1 mM) at pH 6.3. The homogenate thus produced was filtered and the filtrate was subjected to centrifugation at 10,000 *g* for 20 min. The supernatant was 15% saturated with ammonium sulphate $(NH_4)_2SO_4$ with continuous stirring for 1 h for protein precipitation and the precipitate was removed by centrifugation at 15,000 *g* for 15 min followed by 45% $(NH_4)_2SO_4$ saturation. Centrifuge at 15,000 g for another 15 min to remove remaining proteins. The pellet was resuspended in 200 mL of 40 mM Potassium phosphate buffer (pH 6.3) and underwent dialysis against 2 L of the same buffer for 24 h. The resulting dialyzed solution was subjected to centrifugation and the supernatant was applied to DE 52-column chromatography (previously degassed and equilibrated with 40 mM potassium phosphate buffer). The column was washed with the same buffer at a flow rate of 1.5 mL / min until the absorbance of the eluent reached below 0.2 at wavelength 280 nm. Then the eluent was replaced with a linear KCl gradient by preparing 1 L buffer and 1 L buffer containing 0.25 M KCl following the fractions were collected and the protein level was measured. The fractions exhibiting 5-LOX activity were pooled and dialysed against the buffer overnight for further purification, which served as the enzyme source.

***12-LOX isolation from PRP***

Platelet 12-LOX was isolated from human platelets (Institutional human ethical clearance number: IEC/IRB No: 4/IHEC20082015) as described by Waslidge *et al*., with slight modifications[29]. Platelets were isolated from platelet-rich plasma (PRP) obtained from blood bank by centrifuging at 230 g for 5 min. Then the supernatant was collected and centrifuged at 500 g for 20 min and the pellets were resuspended in PBS (pH 7.2, 2 mM EDTA) and centrifuged for 5 min at 230 g to remove RBCs. The final pellet was resuspended in calcium-free Tyrodes (pH 7.4) and kept at room temperature for 30 min. The pellets (platelets) were lysed using 0.1% (v/v) Triton X-100 followed by freeze-thawing and the cell debris was removed by centrifugation. The total protein content was estimated using Bradford assay and the enzyme was stored at -80°C until use.

***Ferrous oxidation-xylenol orange assay***

FOX assay was performed as per the protocols described by John *et al*[30,31]. The assay was initiated by mixing 20 µL of the 5-, 12- and 15-LOX enzymes separately with different concentrations (0.01-100 µg / mL) of PK fractions / positive control (montelukast) and incubated for 20 min at dark condition followed by the addition of 50 µL linoleic acid substrate (140 µM). Incubated for 20 min in dark and finally the reaction was terminated by adding 130 µL FOX reagent. Then incubated the mixture for 20 min in dark and read the absorbance of the coloured complex at 560 nm in Varioskan multimode reader. A blank with methanol and a negative control containing the reaction system without sample were maintained for all experiments. Percentage of inhibition of 5/12/15-LOXs was calculated using the formula, $[(Abs_{control}-Abs_{test})/Abs_{control}]\times100$, where $Abs_{control}$ is the absorbance of the control, i.e., uninhibited reaction system and $Abs_{test}$ is the absorbance of enzyme activity in the presence of PK fractions/positive control. $IC_{50}$ values were calculated from percentage inhibition using Graph Pad Prism 5 and compared to that of positive control.

## Statistical analysis

Statistical analysis was performed by one-way analysis of variance using GraphPad Prism® version 5.03 and the significance of the test samples compared with the positive samples was determined by Dunnett's multiple comparison test at ($p\leq0.05$). All analyses were done in triplicates and the results were expressed in mean ± standard deviation.

## Results

**Yield of the fractions**

The percentage yield of each fraction is shown in Table 1.

***Phytochemical screening***

The preliminary phytochemical screening revealed the presence of a wide range of chemical entities in

Table 1 — Percentage yield of PK fractions

| *Sample id* | *Yield* | *Yield % (w/w)* |
|---|---|---|
| *PK I* | 4.076 | 1.1004 |
| *PK II* | 19.874 | 5.3656 |
| *PK III* | 40.800 | 11.0151 |
| *PK IV* | 111.146 | 30.0070 |
| *PK V* | 95.2000 | 25.7019 |

PK fractions, which were shown in Table 2. PK II and PK III exhibited the presence of secondary metabolites, flavonoids, phenols, tannins, terpenoids, quinones, alkaloids, carbohydrates, etc, where PK I found to have the least.

#### Quantitative phytochemical estimation

##### *Determination of total phenolics, phenolic acids and flavonoids content*

Total phenolics, phenolic acids and flavonoids content in PK fractions expressed in terms of gallic acid equivalence, caffeic acid equivalence and rutin equivalence respectively are as shown in Table 3.

#### Antioxidant potential of PK fractions

PK fractions were assessed for the antioxidant potential and ferric reducing ability using four different assays and compared with positive controls.

##### *DPPH and ABTS radical scavenging activity*

All fractions of *Punarnavadi kashayam* exhibited a concentration-dependent DPPH and ABTS radical scavenging activity compared to the positive control, BHT. Furthermore, fraction PK III showed the highest scavenging activity (Table 4).

##### *FRAP assay*

The antioxidants/reductants present in the fractions lead to the reduction of FeIII-TPTZ complex to FeII-TPTZ complex in the system to form purple colour depending upon the reducing power. Therefore, the reducing ability can be monitored by measuring the formation of a purple complex. The absorbance from the assay was compared with the ferrous sulphate calibration curve and the reducing capacity values were expressed as mg $Fe^{2+}$/mg extract, given was in Table 4. PK III (0.1162±0.0031 mg $Fe^{2+}$/mg extract) was found to exhibit a higher reducing capacity compared with standard BHT (0.1053 ±0.0092 mg $Fe^{2+}$/mg extract) and other fractions.

##### *Hydrogen peroxide scavenging assay*

A concentration-dependent hydrogen peroxide scavenging activity was exhibited by PK fractions and standard BHT. Results showed that the fraction PK III exhibited the lower $IC_{50}$ (1003±0.04104 µg/mL), when compared with other fractions and less compared to the positive control BHT (456.6±0.01384 µg/mL) which showed much lower $IC_{50}$ than PK fractions.

Table 2 — Preliminary phytochemical analysis of PK fractions

| *Phytochemicals* | *Punarnavadi kashayam* | | | | |
|---|---|---|---|---|---|
| | ***PK I*** | ***PK II*** | ***PK III*** | ***PK IV*** | ***PK V*** |
| Flavonoids | - | + | ++ | + | ++ |
| Phenols | + | + | ++ | ++ | + |
| Tannins | - | + | + | ++ | - |
| Saponins | - | - | - | - | - |
| Terpenoids | - | + | ++ | ++ | ++ |
| Quinones | + | + | ++ | ++ | + |
| Fatty acids | - | - | - | - | - |
| Alkaloids | + | + | + | + | + |
| Carbohydrates | ++ | ++ | + | + | + |
| Cardiac glycosides | ++ | + | + | + | - |
| Amino acids and proteins | - | - | - | + | + |
| Anthocyanins | - | + | + | + | - |
| Coumarins | - | + | + | + | + |

'+' represents the slight positive reaction and '++' indicated for a strong positive reaction, whereas '- indicates the absence as compared to the corresponding negative controls which contained all reagents excluding the fractions.

Table 3 — Total phenolic, phenolic acid, flavonoid content in PK fractions

| *Sample ID* | *Total phenolic content (mg GAE/g residue)* | *Total phenolic acid content (mg CAE/g residue)* | *Total flavonoid content (mg RAE/g residue)* |
|---|---|---|---|
| *PK I* | 1.8167±0.764 | 0.513±0.444 | 1.2137±0.534 |
| *PK II* | 85.8167±1.893 | 2.333±0.444 | 7.6239±2.437 |
| *PK III* | 348.8167±4.646 | 109.205±7.895 | 36.000±0.678 |
| *PK IV* | 185.3167±9.802 | 1.026±0.444 | 2.9231±1.118 |
| *PK V* | 105.9833±2.887 | 2.538±0.769 | 26.5128±3.562 |

All values in the table were represented as mean ± SD (n=3)

Table 4 — $EC_{50}$ values of PK fractions to antioxidant assays compared with that of BHT

| *Sample* | *$EC_{50}$ (µg/mL)±SE* | | | *FRAP value (mg $Fe^{2+}$/ mg of residue)* |
|---|---|---|---|---|
| | *DPPH* | *ABTS** | *Hydrogen peroxide* | |
| *PK I* | 1771±0.009 | 5774±0.0262 | 2324±0.05677 | 0.0201±0.0004 |
| *PK II* | 84.70±0.010 | 40.85±0.1676*** | 1015±0.05684 | 0.0909±0.0034 |
| *PK III* | 10.06±0.016*** | 9.216±0.0105*** | 1003±0.04104 | 0.1162±0.0031* |
| *PK IV* | 250.1±0.009 | 50.46±0.0136*** | 2774±0.04417 | 0.1151±0.0013 |
| *PK V* | 133.2±0.028 | 700.7±0.0105 | 2095±0.1598 | 0.1079±0.0025 |
| *BHT* | 29.73 ± 0.008 | 81.06±0.0172 | 456.6±0.01384 | 0.1053±0.0092 |

***p-value <0.001 as compared to positive control BHT. All values in the table were represented as mean ± SE (n=3).

Table 5 — $IC_{50}$ values of PK fractions and standard montelukast against 5-, 12- and 15-LOX

| *Sample* | *$IC_{50}$ (µg/mL)±SE* | | |
|---|---|---|---|
| | *5-LOX* | *12-LOX* | *15-LOX* |
| *PK I* | 218.9±0.078 | 223264±0.3129 | 1810±0.2275 |
| *PK II* | 42.00±0.078 | 99.48±0.009312*** | 4313±0.2067 |
| *PK III* | 30.80±0.130*** | 88.18±0.05123*** | 266.4±0.0637 |
| *PK IV* | 99.10±0.078 | 99.35±0.01436*** | 3024±0.1723 |
| *PK V* | 39.30±0.230*** | 153.3±0.1533 | 1896±0.1944 |
| *Montelukast* | 42.00±0.048 | 141.8±0.08394 | 86.99±0.0515 |

***p-value <0.001 as compared to positive control montelukast. All values in the table were represented as mean±SE (n=3).

**Anti-inflammatory activity**

***5-,12- and 15-Lipoxygenase inhibition assay***

The effect of PK fractions in the production of leukotrienes by 5-, 12- and 15-lipoxygenase was assayed using FOX reagent. $IC_{50}$ of all fractions were calculated using statistical software GraphPad Prism® version 5.03 and compared with the positive control, montelukast (Table 5). Ethyl acetate fraction of PK (PK III) exhibited the lower $IC_{50}$ when compared with the positive control montelukast for both 5- and 12-LOXs. The same fraction showed the least $IC_{50}$ against 15-LOX compared with other fractions but higher than that of the positive control.

## Discussion

Polyherbalism has been one of the main principles used in Ayurveda for curing various pathologic state and is little bit complex to explain in modern medical parameters[32]. *Punarnavadi kashayam*, an ayurvedic polyherbal decoction according to ancient Ayurvedic classical text Sahasrayoga, comprises of various parts of medicinal herbs viz., *Boerhavia diffusa* L. (Rt), *Azadirachta indica* A.Juss. (St Bk), *Trichosanthes lobata* Wall. (Pl), *Zingiber officinale* Roscoe (Rz), *Swertia chirayita* (Roxb.) Buch.-Ham. ex C.B.Clarke (Pl), *Tinospora cordifolia* (Willd.) Miers (St), *Berberis aristata* DC. (Rt), and *Terminalia chebula* Retz. (Fr.R) has been used in the treatment of chronic inflammatory diseases[33,13]. In order to evaluate the efficacy of kashayam as an anti-inflammatory medicine, its effect on leukotriene production by 5-, 12-and 15-lipoxygenase inhibition and antioxidant potential were given emphasis in this study. The phytochemical screening was carried out for the analysis of phytocompounds present in the decoction.

In this study, the kashayam was fractionated with solvents of increasing polarity in order to separate compounds present in the decoction and the results of phytochemical screening of PK fractions showed the presence of various phytocompounds like phenols, carbohydrates and quinones in all fractions, where PK II, PK III and PK IV exhibited the presence of almost all phytochemicals tested like flavonoids, phenols, tannins, terpenoids, quinones, alkaloids, carbohydrates, cardiac glycosides, anthocyanins and coumarins except fatty acids, saponins, amino acids, and proteins.

Prachee *et al*., mentioned that the phytochemicals are secondary metabolites of plants which are biologically active in nature. The phytochemicals like phenolics, flavonoids, terpenoids, etc., act as antioxidants which prevent oxidative cell damage caused by free radicals[34,35]. According to Hussain *et al*., 2016, the polyphenols including phenolics and flavonoids can directly interact with free radicals and terminate the chain reaction before causing serious damage to cells[36]. Since phenolics and flavonoids act as free radical scavengers, they prevent oxidative cell damage and help in reducing oxidative stress-related disorders. Hence the quantification of total phenolics, phenolic acids, and flavonoids considered to be

essential and evaluated. The result of quantitative estimation of total phenolics, phenolic acids and flavonoids are depicted in Table 3. PK III exhibited the highest content and PK I showed the least for all three phytochemicals quantified.

Free radicals generated by pro-inflammatory cells generally induce an alteration in cell membrane integrity which results in the release of arachidonic acid (AA) from membrane-bound phospholipids. AA so released is then metabolised *via* lipoxygenase pathway to produce leukotrienes which are potent pro-inflammatory molecules[37,38]. It is quite possible that the synergistic mechanism involving free radical scavenging activity and inhibition of lipoxygenase enzymes would probably exert its function as an anti-inflammatory agent. Free radical scavenging potential of kashayam fractions was evaluated using various assays like DPPH radical, ABTS* cation, FRAP and hydrogen peroxide scavenging assays. In all applied tests, PK fractions showed a better scavenging activity in a concentration-dependent pattern, where, ethyl acetate fraction of PK (PK III) presented the lower $EC_{50}$ compared with their positive control (BHT) except in hydrogen peroxide scavenging activity (Table 4). These results are in corroboration with the findings of Michel *et al*., that the antioxidant activity is directly proportional to the abundance of phytochemicals like phenolics and flavonoids[7]. The hexane fraction PK I presented the least $EC_{50}$ compared with standards and other fractions which may be due to the least presence of phytochemicals analysed.

5-lipoxygenase is the primary enzyme involved in the leukotriene synthesis, there emerge the requisite for developing 5-LOX inhibitors with least toxicity. Likewise, the inhibition of 12- and 15-LOX are equally important due to the role of them in the synthesis of leukotrienes which ameliorate the monocyte-endothelial interactions during inflammatory conditions and are therefore considered as one of the therapeutic strategies in the mitigation of inflammatory disorders[39,12]. In the present study, the ethyl acetate fraction showed a better inhibitory pattern against 5- and 12-LOX with lower $IC_{50}$ compared with the positive control montelukast, which is a synthetic LOX inhibitor. In the case of 15-LOX also, PK III showed the least $IC_{50}$ compared to the other fractions, but least comparable to the positive control (Table 5). All other fractions exhibited concentration-dependent inhibition of lipoxygenases. These results underpinned the findings of Mittal *et al*., that the secondary metabolites present in the herbs with high phenolic profile contribute to the inhibition of inflammatory disorders *via* molecular targets such as lipoxygenases and ROS pathways[40]. The results demonstrating the direct inhibition of lipoxygenases complemented with antioxidant activity can be considered as the preliminary steps underlying the popular use of *Punarnavadi kashayam* as an anti-inflammatory therapeutic.

## Conclusions

In conclusion, *Punarnavadi kashayam* exhibited better antioxidant and anti-inflammatory properties. This could be due to the presence of various bioactive phytochemicals present in the kashayam. The 5-, 12- and 15-LOX inhibitory activity of the fractions gives an insight into the anti-inflammatory potential of kashayam and provides a scientific backup for the use of *Punarnavadi kashayam* as an anti-inflammatory medication. Further work on identifying the active principles in the decoction that is responsible for the free radical scavenging and anti-inflammatory activities would help to develop lead compounds against inflammatory ailments.

## Acknowledgement

The Authors are grateful to DBT-MSUB-IPLSARE (BUILDER) programme (BT/ PR4800/INF/22/152/ 2012 dated 22/03/2012), School of Biosciences, Mahatma Gandhi University, Kerala, for the instrumentation facilities provided for the successful completion of this work. The authors like to thank Arya Vaidyasala Kottakal, Kerala, for their support.

## Conflict of interest

The authors have declared that there is no conflict of interest.

## Author(s) contribution

PKB conceived and designed the project. BMB and RDSJ conducted the experiments. BMB analysed the data and drafted the manuscript. All authors read and approved the final manuscript.

## References

1 Sari A C & Elya B, Antioxidant activity and lipoxygenase enzyme inhibition assay with total flavonoid assay of *Garcinia porrecta* Laness. stem bark extracts, *Pharmacogn J*, 9 (2) (2017) 257–266.

2 Singh N & Rajini P S, Free radical scavenging activity of an aqueous extract of potato peel, *Food Chem*, 85 (4) (2004) 611–616.

3 Venkatachalam U & Muthukrishnan S, Free radical scavenging activity of ethanolic extract of *Desmodium gangeticum*, *J Acute Med*, 2 (2) (2012) 36–42.

4 Aqil F, Ahmad I & Mehmood Z, Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants, *Turk J Biol*, 30 (2006) 177–183.

5 Dushing Y A & Laware S L, Antioxidant assessment of Ashokarishta—a fermented polyherbal Ayurvedic formulation. *J Pharm* Res, 5 (6) (2012) 3165–3168.

6 Husain A, Kaushik A, Awasthi H, Singh D P, Khan R, *et al*., Immunomodulatory andantioxidant activities of fresh juice extracts of Brahmi and Guduchi, *Indian J Tradit Know*, 16 (3) (2017) 498-505.

7 Michel P, Dobrowolska A, Kicel A, Owczarek A, Bazylko A, *et al.*, Polyphenolic profile, antioxidant and anti-inflammatory activity of Eastern Teaberry (*Gaultheria procumbens L.*) leaf extracts, *Molecules*, 19 (12) (2014) 20498–20520.

8 Biswas S K, Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? *Oxid Med Cell Longev*, 2016 (2015) 1–9.

9 Hu C & Ma S, Recent development of lipoxygenase inhibitors as anti-inflammatory agents, *Med Chem Comm*, 9 (2) (2018) 212–225.

10 Rackova L, Oblozinsky M, Kostalova D, Kettmann V & Bezakova L, Free radical scavenging activity and lipoxygenase inhibition of *Mahonia aquifolium* extract and isoquinoline alkaloids, *J Inflamm (Lond)*, 4 (2007) 1–7.

11 Steinhilber D & Hofmann B, Recent advances in the search for novel 5-lipoxygenase inhibitors, *Basic Clin Pharmacol Toxicol*, 114 (1) (2014) 70–77.

12 Mohan M C, Abhimannue A P & Kumar B P, Modulation of proinflammatory cytokines and enzymes by polyherbal formulation *Guggulutiktaka ghritam*, *J Ayurveda Integr Med*, 12 (1) (2021) 13-19.

13 I S A, Krishnan S, Peter J, Sabu V & Helen A, Scientific validation of anti-arthritic effect of kashayams – a polyherbal formulation in collagen induced arthritic rats, *J Ayurveda Integr Med*, 12 (1) (2021) 20-27.

14 Velayudhakurupp K, Sahasrayayogam, (Sreeramavilsam Publishers, Kollam, Kerala), 1950, p. 18.

15 Rao G P, Sahasrayogam: Compedium of 1000 Ayurvedic formulations, Sanskrit text with English translation and Prabhakara Vyakhyanam, (Chaukhambha publications), 2016, p. 124–125.

16 Parasuraman S, Thing G & Dhanaraj S, Polyherbal formulation: Concept of ayurveda, *Pharmacogn Rev*, 8(16) (2014) 73–80.

17 Ugochukwu S , Uche A & Ifeanyi O, Preliminary phytochemical screening of different solvent extracts of stems bark and roots of *Dennetia tripetala*, *Asian J Plant Sci Res*, 3 (2013) 10–13.

18 Madike L N, Takaidza S & Pillay M, Preliminary phytochemical screening of crude extracts from the leaves, stems, and roots of *Tulbaghia violacea*, *Int J Pharmacogn Phytochem Res*, 9 (10) (2017) 1300–1308.

19 Maurya S & Singh D, Quantitative analysis of total phenolic content in *Adhatoda vasica Nees* extracts, *Int J PharmTech Res*, 2 (4) (2010) 2403–2406.

20 Złotek U, Szymanowska U, Baraniak B & Karaś M, Antioxidant activity of polyphenols of adzuki bean (*Vigna angularis*) germinated in abiotic stress conditions, *Acta Sci Pol Technol Aliment*, 14 (1) (2015) 55–62.

21 Khan R A, Khan M R, Sahreen S & Ahmed M, Evaluation of phenolic contents and antioxidant activity of various solvent extracts of *Sonchus asper* (L.) Hill, *Chem Cent J*, 6 (1) (2012) 12.

22 Sundarraju D, Anbu J, Ravichandran V & Senthil K L, *In-vitro* free radical scavenging potential of polyherbal extract, *Int J Phytopharm*, 5 (2) (2014) 71–75.

23 Chinchu J U & Prakash K B, *In-vitro* anti-lipase and antioxidant activity of polyherbal ayurvedic medicine Varanadi kashayam, *Int J Pharm Sci Res*, 9 (12) (2018) 5373–5381.

24 Merlin T & Kumar B P, Free radical scavenging, antioxidant potential and nitric oxide inhibition in human THP1 derived macrophages by Kokilaksham kashayam, *Asian J Pharm Clin Res,* 12 (7) (2019) 82-86.

25 Karthivashan G, Tangestani F M, Arulselvan P, Abas F & Fakurazi S, Identification of bioactive candidate compounds responsible for oxidative challenge from hydro-ethanolic extract of *Moringa oleifera* leaves, *J Food Sci*, 78 (9) (2013) C1368–1375.

26 Ruch R J, Cheng S & Klaunig J E, Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea, *Carcinogenesis*, 10 (6) (1989) 1003–1008.

27 Keser S, Celik S, Turkoglu S, Yilmaz O & Turkoglu I, Hydrogen peroxide radical scavenging and total antioxidant activity of Hawthorn, *Chem J*, 02 (01) (2012) 9–12.

28 Reddanna P, Whelan J, Maddipati K R & Channa R C, Purification of arachidonate 5-lipoxygenase from potato tubers, *Methods Enzymol*, 187 (1990) 268–277.

29 Waslidge N B & Hayes D J, A colorimetric method for the determination of lipoxygenase activity suitable for use in a high throughput assay format, *Anal Biochem*, 231 (2) (1995) 354–358.

30 Mohan M C, Abhimannue A P & Kumar B P. Identification and characterization of berberine in *Tinospora cordifolia* by liquid chromatography quadrupole time of flight mass spectrometry (LC MS/MS Q-tof) and evaluation of its anti inflammatory potential, *Pharmacogn J*, 9 (3) (2017) 350–355.

31 Jacob J, Prakash K B, Abhimannue A P, Mohan M & Babu B M, Inhibition of lipoxygenase enzymes by extracts of *Tinospora cordifolia*: A study of enzyme kinetics, *J Nat Prod*, 7 (2014) 203–209.

32 Karole S, Shrivastava S, Thomas S, Soni B, Khan S, Dubey J, *et al*., Polyherbal formulation concept for synergic action: a review, *J Drug Deliv Ther*, 9(1-s) (2019) 453–466.

33 The Ayurvedic Pharmacopoeia, Pharmacopoeia commission for Indian medicine & Homoeopathy, Govt. of India, Ministry of AYUSH, Vol IX (2016) 67.

34 Prachee D, Ayushi T, Praveen T, Shikha A, Rai A K & Geeta W, Phytochemical and phytoelemental profile of *J. officinale*, *Int J Pharmacogn Phytochem Res*, 11(1) (2019) 5–9.

35 Pratap H, Ram H N, Chandrashekar K S, Pai V, Khan S & Kamath V, Total phenolic and flavonoid content, antioxidant effects and antidiarrheal activity of balacaturbhadrika churna-an Ayurvedic preparation, *Indian J Tradit Know*, 18 (3) (2019) 485-492.

36 Hussain T, Tan B, Yin Y, Blachier F, Tossou M C & Rahu N, Oxidative stress and inflammation: what polyphenols can do for us?, *Oxid Med Cell Longev*, 2016 (2016) 1–9.

37 Lambeu G & Gelb M H, Biochemistry and physiology of mammalian secreted phospholipases A2, *Ann Rev Biochem*, 77 (2008) 495-520.

38 Natarajan R & Nadler J L, Lipid inflammatory mediators in diabetic vascular disease, *Arterioscler Thromb Vasc Biol*, 24 (9) (2004) 1542-1548.

39 Adebayo S A, Dzoyem J P, Shai L J & Eloff J N, The anti-inflammatory and antioxidant activity of 25 plant species used traditionally to treat pain in southern African, *BMC Complement Altern Med*, 15 (2015) 159.

40 Mittal M, Siddiqui M R, Tran K, Reddy S P & Malik A B, Reactive oxygen species in inflammation and tissue injury, *Antioxid Redox Signal*, 20 (7) (2014) 1126–1167.