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Original Article

Validation of therapeutic anti-inflammatory potential of *Arjuna Ksheera Paka* – A traditional *Ayurvedic* formulation of *Terminalia arjuna*

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ABSTRACT

Arjuna Ksheera Paka (AKP), a traditional *Ayurvedic* formulation of *Terminalia arjuna* (*T. arjuna*) bark powder is used for its cardioprotective effects. However, its anti-inflammatory efficacy remained unexplored. In the present study, AKP was prepared in cow milk (as per standard *Ayurvedic* procedure) and compared with standard hydroalcoholic extract (HA) of *T. arjuna*. The extracts were analyzed for gross phytoconstituents levels, and their antioxidant activity was assayed by DPPH free radical scavenging activity and inhibition of lipid peroxidation. The *in vivo* anti-inflammatory activity of AKP and HA was studied in carrageenan-induced hind paw biphasic edema in C57BL/6 mice (at 200, 400 and 800 mg/kg BW).

The percentage extraction yield of AKP was two folds higher than HA implying that the phytoconstituents in AKP were diluted by a factor of 0.5. The total polyphenol content of HA was (3.8 times) higher than AKP and the antioxidant activity of HA was also higher compared to AKP. Both the extracts, however, showed significant ($p < 0.05$) anti-inflammatory activity in reducing paw edema in mice. The efficacy of HA was more than AKP at early phase of inflammation, whereas, in the late phase of inflammation AKP was more efficacious and equipotent to HA. Thus, regardless of low *in vitro* antioxidant activity, AKP exhibited potential *in vivo* anti-inflammatory activity. The higher efficacy of AKP could be due to the presence of milk solids. These milk solids may act as adjuvants to *T. arjuna*'s phytoconstituents, contributing to their sustained bioavailability, leading to higher *in vivo* anti-inflammatory efficacy at lower drug concentrations.

1. Introduction

India has a rich history of use of medicinal plants, herbs, and formulations containing herbs, both from traditional wisdom as well as cultural usage. The therapeutic uses of most of these plants and herbs have been mentioned in ancient *Ayurvedic* literature like *Charaka Samhita*, *Susruta Samhita* and various *Nighantus*. *Arjuna*, botanically identified as *Terminalia arjuna* (*T. arjuna*) Roxb. (Combretaceae), is one such important *Ayurvedic* medicinal plant. *T. arjuna* has been documented in *Charaka Samhita and Nighantus* to possess '*ama*' (free radicals) reducing properties (antioxidant[1]), *Shotha* (anti-inflammatory[2]), *Medohanti* (anti-hyperlipidemic[3]), *Udard prashamana* (treatment of urticaria[4]) *Kshatakshayavishastrajita* (treatment of respiratory diseases like tuberculosis[5]), *Kaphapittahruta* (treatment of vitiated conditions of *kapha* and *pitta*[6]) properties, which have also been scientifically validated. It has been characterized as a rich source of triterpenoids (arjungenine, arjunine, arjunic acid), tannins, phenolic acids (3, 4 dihydroxybenzoic acid, gallic acid) and flavonoids (quercetin-3-beta glycoside). Its role as cardiotonic has also been recognized by both *Ayurvedic* physicians as well as by the modern medical practitioners.[7,8]

Arjuna Ksheera Paka (AKP), a standard *Ayurvedic* preparation of *T. arjuna* described in *Chakradatta*,[9] is widely prescribed by *Ayurvedic* practitioners as a cardiotonic. The name *Ksheera Paka* is derived from the *Sanskrit* words '*ksheeram*' which means 'milk' and '*pakam*' which means 'to boil'. It consists of *T. arjuna* dry bark powder, cow milk and water in the ratio of 1:4:16. Other than the

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traditional preparations, several modern methods have also been tried for the extraction and isolation of the active components present in *T. arjuna*. These extraction methods employ the use of water (hydro-extraction), polar solvents like ethanol (alcoholic extraction) and water-alcohol (hydroalcoholic extraction), etc. as solvents (I.P., 2007).

The process of oxidation and inflammation are an integral part of physiology and a very delicate balance between oxidation and inflammation is necessary for the maintenance of homeostasis in the organism. Inflammation represents a highly coordinated set of events that allow tissues to respond to injury or infection. The inflammatory processes are regulated by a variety of cellular (mast cells, macrophages, etc.) and chemical elements (cytokines, leukotrienes, etc.). During the inflammation response, cytokines (e.g., tumour necrosis factor-α [TNF-α] and interleukin-1 [IL-1]) induce the production of many proinflammatory mediators, such as prostaglandins (PGs) and nitric oxide (NO).[10]

Sustained or excessive inflammation, fuelled by oxidative stress can lead to numerous chronic diseases like atherosclerosis, obesity, diabetes, neurological, pulmonary diseases and cancer.[11] In order to prevent the progression of this inflammatory chain reaction, an effective treatment is required. The conventional line of treatment for inflammation includes the use of analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), steroidal anti-inflammatory drugs (SAIDs) and immune selective anti-inflammatory derivatives (ImSAIDs). Chronic and excessive use of these drugs poses great adverse health effects like gastrointestinal injury, cardiovascular and neurological side effects[12] necessitating a safer alternative treatment for inflammation. In recent years, search for antioxidant and anti-inflammatory activities of *Ayurvedic* formulations of herbs have been comprehensively investigated and have been found to be good sources of anti-inflammatory agents.[13] In this study, the *in vivo* anti-inflammatory and *in vitro* antioxidant efficacy of *Arjuna Ksheera Paka* were evaluated to validate the role of this ancient *Ayurvedic* preparation.

2. Materials and methods

2.1. Drugs & reagents

All the chemicals were of analytical grade and milliQ water (Milli-Q® Integral 3 Water Purification System) was used wherever required. 1, 1- diphenyl-2-picryl hydrazyl (DPPH) (Sigma–Aldrich), 2-Thiobarbituric acid (TBA) (Sigma–Aldrich), carrageenan (Himedia, Irish Moss; RM 1576), ascorbic acid (vitamin C) and α-tocopherol (vitamin E) (Sigma–Aldrich) as antioxidant standards, and aspirin (Disprin, Reckitt Benckiser India) as a standard anti-inflammatory drug were used in the experiments. Phosphate buffer saline (PBS) was prepared in milliQ water.

2.2. Extraction of test material

Dried test sample of *T. arjuna* was obtained from the *Dabur* Research and Development Centre, Ghaziabad as gratis, after mandatory ethnobotanical studies and quality assessment. The qualitative compliance of the herbal dry powder, in terms of color, odor, taste, ethanol soluble extract and water-soluble extract, the presence of foreign organic matter, total ash, acid insoluble ash, and moisture was confirmed according to the Indian Pharmacopoeia (IP) monographs.

AKP was prepared by a decoction in diluted cow milk in the following proportion; *T. arjuna* dry bark powder: cow milk: water (200 g: 800 mL: 3200 mL) at 65 °C for 8 h, till the total volume was reduced to 1/5th of the initial volume.[9] Hydroalcoholic extract (HA) of *T. arjuna* bark powder was prepared by maceration of 200 g of *T. arjuna* in 2 L of 50 % ethanol at room temperature for 20 h. The HA extract was centrifuged at 200 g for 10 min and the supernatant was collected. The extraction steps were repeated three times for HA and the collected supernatants were pooled.[14] HA and AKP extracts were filtered through Whatman grade 1 filter paper. The volume of the resulting filtrates was reduced to about 75 % in Buchi Rotavapor R205 at 45 °C. The AKP and HA extracts were finally dried by lyophilization. The residues were weighed and stored at−20 °C until further use. The percent yield of the extracts was calculated as follows-

$$\text{Extract yield } \% = (W1/W2) \times 100$$

where, W1 is net weight of the freeze-dried extract in grams and W2 is the total weight of the original sample in grams taken for extraction.

2.3. Qualitative primary phytochemical identification in AKP and HA

AKP and HA were screened for phytochemical constituents like terpenoids, carbohydrates, sterols, tannins, flavonoids, proteins, glycosides alkaloids and saponins by their specific respective tests-Liebermann-Burchardt test, Molisch's test, Salwoski's test, Libermann-Buchard test, Shinoda test, Ninhydrin test, Kellar–Kiliani test, Dragendorff's test and foam test.[15,16] The qualitative compliance of the extracts was confirmed according to Indian Pharmacopoeia (IP).[17]

2.4. Determination of phytochemical content of AKP and HA

The total polyphenol content in HA and AKP was determined by spectrophotometry according to International Organization for Standardization (ISO)14502-113,[18] using gallic acid as standard and expressed as mg gallic acid equivalents (GAE). The total flavonoid content and total anthocyanins content were estimated[19] and expressed as mg quercetin equivalents (QE) and mg cyanidin-3-glucoside equivalents (C-3-GE) per gram dry weight (mg equivalent/g DW). Catechins and proanthocyanidins reactive to vanillin were estimated[19] for determination of total tannin content and expressed as mg catechin equivalents (CE) per gram dry weight. The results were evaluated as per Indian Pharmacopoeia (IP).[17]

2.5. Quantification of polyphenol content in AKP and HA by HPLC

Polyphenols from HA and AKP were quantified using Dionex PA2 RSLC (Ultimate 3000) HPLC.[20] HA and AKP were dissolved in 90 % methanol containing 0.5 % acetic acid. HA and AKP samples were filtered through 0.2 μm filter and aliquots of 2 μl were injected into Dionex Acclaim PA2 RSLC C18 column (2.2 μ, 100 mm × 2.1 mm) at 35 °C. Elution was done using solution A containing 50 mM sodium phosphate buffer: methanol (9:1) and solution B containing 50 mM sodium phosphate buffer: 70% methanol (3:7) in a gradient manner at a flow rate of 0.47 mL/min for 20 min. Compounds present in the eluting sample were monitored at 280 nm using a PDA detector. Peaks were identified by their retention time and absorption spectra were compared to authentic standards and the results were evaluated as per Indian Pharmacopoeia (IP).[17]

2.6. In vitro antioxidant activity of AKP and HA

2.6.1. DPPH free radical scavenging assay

The free radical scavenging activity of both extracts was estimated by the decoloration of a methanol solution of DPPH.[21] The extent of loss in color indicated the free radical scavenging efficacy

as measured by the decrease in DPPH absorbance in a spectrophotometer (UNICAM UV 300) using vitamin C as standard. The reaction mixture comprising of 1.0 mL of DPPH in methanol (0.15 mM), 1.0 mL of methanol and 1.0 mL of the extracts in milliQ water (1–100 μg/mL) were incubated in dark for 30 min, and the absorbance was measured at 517 nm.

2.6.2. TBARS assay

A non-enzymatic lipid peroxidation assay involving the use of rat liver mitochondria as lipid source and Fe^{2+}–H_2O_2 as peroxidation inducers was used for estimation of antioxidant activity. Liver mitochondria were prepared in 1.15 % KCl medium at 4 °C in ultracentrifuge (Optima L-90 k).[22] The antiperoxidative activity of the extracts on Fe^{2+}–H_2O_2 induced lipid peroxidation was determined by the reduction in malondialdehyde (MDA) formation in the system as compared to vitamin E standard and expressed as thiobarbituric acid reactive substances (TBARS). Mitochondria (500 μg of mitochondrial protein/mL incubation mixture) were incubated with Fe^{2+}–H_2O_2 system (200 mM $FeSO_4$, 100 nM H_2O_2 and 0.15 mM NaCl) for a period of 60 min at 37 °C. *T. arjuna* extracts in milliQ water at various concentrations (10–800 μg/mL) or milliQ water as a control were added 2 min prior to the addition of peroxidation inducer Fe^{+2}–H_2O_2. Peroxidation was terminated by adding twofold volume of cold 20 % trichloroacetic acid. After adding an equal volume of TBA (1% w/v), the mixture was heated at 90 °C for 30 min and then cooled to room temperature followed by centrifugation.[23] The absorbance of the supernatant was measured at 532 nm. Acid hydrolyzed 1, 1, 3, 3 tetraethoxypropane was used as an authentic standard for MDA.

2.7. Experimental animals

7-8 weeks old C57BL/6 mice (n = 54) weighing 30–35 g were obtained from National Centre for Laboratory Animal Science, NIN, India, with approval from Institutional Animal Ethics Committee (IAEC No. 53/IAEC/NIN/2011/12/BDK/C57BL6 Mice) and housed in accordance with guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Autoclaved food and water were provided ad libitum.

2.8. In vivo anti-inflammatory activity of AKP and HA

Anti-inflammatory activity was investigated by measuring carrageenan-induced paw edema in completely randomized design. As per study design, 54 C57BL/6 mice were randomly divided into nine groups (n = 6; 3 male+3 female). Group I (negative control) and group II (positive control) animals received distilled water 10 mL/kg P.O. Group III (standard) animals received anti-inflammatory drug aspirin (91 mg/kg). Groups IV, V and VI received HA, respectively at concentrations of 200, 400 and 800 mg/kg P.O. Groups VII, VIII and IX received AKP, respectively at concentrations of 200, 400 and 800 mg/kg P.O. Aspirin, HA and AKP were administered to the respective groups 60 min prior to administration of carrageenan. Animals of group II received 50 μL of PBS subcutaneously in right hind paw. The animals of all other groups received 50 μL subcutaneous injection of carrageenan (300 μg/paw) dissolved in PBS, to right hind paw. Paw edema was measured by digital Plethysmometer LE7500 (Panlab S.I.), at several time points, i.e., before administration of carrageenan and 0, 1, 2, 3, 4, 5, 6, 7, 24, 48, 72 and 96 h after the injection of carrageenan. The difference between the initial and subsequent readings gave the actual edema volume, which was compared with positive control. Percent inhibition was calculated by

$$\text{Percent inhibition} = [1 - (V_t/V_c) \times 100]$$

where: V_t = Paw volume after carrageenan injection and V_c = Paw volume before administration of carrageenan injection.

2.9. Statistical analysis

The free radical scavenging activity, mitochondrial lipid peroxidation was analyzed using linear regression analysis followed by the percent inhibition. The level of statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test using the Graph Pad Prism5 software.

3. Results

3.1. Test material quality compliance, extract yield and qualitative primary phytochemicals in extracts

T. arjuna bark powder was reddish brown in color, had a characteristic aromatic odor and acrid bitter taste. The ethanol soluble extract was 25 % (IP-not less than 20 %) while water-soluble extract was 27 % (IP-not less than 20 %). No foreign organic matter was found (IP-not more than 2 %), while total ash, acid insoluble ash, and moisture were 24 % (IP-not more than 30 %), 1.84 (IP-not more than 2 %) and 1.24 % respectively. A TLC spot with Rf value 0.5 corresponding to gallic acid[24] was obtained in developing solvent toluene: acetic acid: ethyl acetate (1:0.1:1).

The recovery of *T. arjuna* dry extract was 26.01 % in HA and 52.43 % in AKP (Table 1). Terpenoids, carbohydrates, sterols, tannins, flavonoids, glycosides, alkaloids, organic acids and saponins were present in HA, while in AKP all the phytoconstituents, except sterols, were detected in addition to proteins (Table 2).

3.2. Total polyphenol, flavonoid, tannin and anthocyanin content

The amount of total phenol, flavonoids, tannins and anthocyanins in HA and AKP of are shown in Table 3a. It is evident that all the phytochemicals were higher in HA than AKP. The levels of theses constituents in AKP as a percent of HA were 26, 10, 1, 10 for phenols, flavonoids, tannins and anthocyanins respectively. Thus, the least difference was observed in polyphenols while the maximum difference was observed in the case of anthocyanins. Though the difference in total polyphenol content was 74 %, this was not reflected in the case of the identified polyphenols, as shown in Table 3b.

3.3. Antioxidant capacity

3.3.1. Free radical scavenging activity

The *in vitro* free radical scavenging activity was tested by DPPH free radical activity assay. The standard (vitamin C) as well as both the extracts of *T. arjuna* showed antioxidant activity by scavenging

Table 1
Percent yield of freeze dried *T. arjuna* stem bark extracts *Arjuna Ksheera Paka* (AKP) and hydroalcoholic extract (HA).

S. No.	*T. arjuna* extracts	Extraction solvent	Solute-solvent proportion	Yield (%)
1	*Ksheera Paka*	Milk: water (1:4)	1:4:16	52.43
2	Hydroalcoholic	Ethanol: water (1:1)	1:5:5	26.01

Table 2
Qualitative detection of primary phytochemicals in *T. arjuna* stem bark extracts *Arjuna Ksheera Paka* (AKP) and hydroalcoholic extract (HA).

S. No.	Phytochemical	AKP	HA
1	Terpenoids	+	+
2	Carbohydrate	+	+
3	Sterols	−	+
4	Tannins	+	+
5	Flavonoids	+	+
6	Protein	+	−
8	Glycosides	+	+
9	Alkaloids	+	+
10	Organic Acids	+	+
11	Saponins	+	+

DPPH free radicals in a linear manner (Fig. 1A). The IC_{50} value for standard antioxidant vitamin C was 4.25 μg/mL, while it was 7.51 and 70.52 μg/mL for HA and AKP, respectively. The free radical scavenging activity of HA was comparable to the standard, vitamin C and the free radical scavenging activity of AKP was ten folds less than that of HA.

3.3.2. TBARS assay

TBARS assay was used for the estimation of peroxide inhibitory activity of the test compounds. Vitamin E was used as a standard, which inhibited 50% peroxide production at a concentration of 33.57 μg/mL. The IC_{50} values for HA and AKP were 31.63 and 2630.25 μg/mL, respectively (Fig. 1B). Thus, the peroxide inhibitory activity of HA was comparable to vitamin E and the activity of HA was approximately 150 times higher than AKP.

3.4. Anti-inflammatory activity

The efficacy of the test extracts to reduce mice paw inflammation was determined by the percent inhibition showed at different intervals. The sub-plantar injection of carrageenan given into the mouse paw produced a biphasic paw edema response, which peaked respectively, at 8 and 72 h, followed by a decrease in edema. The edema induced by carrageenan was observed in all the groups of experimental C57BL/6 mice.

Both the extracts of *T. arjuna* displayed dose-dependent anti-inflammatory activity at various time points by decreasing edema as compared to positive control (Fig. 2A and B). The edematogenic response was significantly reduced in the group treated with 800 mg/kg of AKP both in the first and second phase of inflammation at 6, 8, 24, 48, 72 and 96 h (Fig. 2A) followed by 400 mg/kg. At a lower dose of 200 mg/kg the activity was observed only in the second phase of inflammation i.e. at 48, 72 and 96 h post carrageenan administration. All the *T. arjuna* treatment groups showed significant percent inhibition of inflammation in the late phase of inflammation at 48, 72 and 96 h. The HA, on the other hand, was significantly active at a concentration of 200 mg/kg in the first phase of inflammation, while at 400 and 800 mg/kg it was highly active in both phases of inflammation.

Table 3a
Quantitative status (mg equivalent/g DW) of some of the active phytochemicals in *T. arjuna* stem bark extracts *Arjuna Ksheera Paka* (AKP) and hydroalcoholic extract (HA); Gallic acid equivalent (GAE), quercetin equivalent (QE), cyanidin-3-glucoside equivalents (C-3-GE) and catechin equivalents (CE).

S. No.	Phytochemical	AKP	HA
1	Total polyphenol content GAE/g DW	98.01 ± 14.75	380.8 ± 18.67
2	Total flavonoids content QE/g DW	11.7 ± 0.91	114.1 ± 11.07
3	Total anthocyanin content C-3-GE/g DW	0.1 ± 0.06	7.4 ± 0.4
4	Total tannin content CE/g DW	4.8 ± 0.26	49.3 ± 8.21

Table 3b
Quantitative status (mg/100 g DW) of some of the polyphenol in *T. arjuna* stem bark extracts *Arjuna Ksheera Paka* (AKP) and hydroalcoholic extract (HA).

S. No.	Polyphenols	AKP	HA
Phenolic Acids			
1	Gallic acid (trihydroxybenzoic acid)	159.45	275.42
2	Protocatechuic acid (3,4 dihydroxybenzoic acid)	1.06	74.39
3	p-Coumaric acid (4-hydroxycinnamic acid)	20.63	26.25
Flavonols & Flavon-3-ols			
1	Quercetin (3-β-D-glucoside)	359.40	939.33
2	Catechin	50.32	23.80
Others			
1	Ellagic acid	26.08	16.10

The *T. arjuna* extracts exhibited higher single dose potency than aspirin, which was comparable to the action of HA at 200 mg/kg concentration (Fig. 2B). HA at 800 mg/kg exhibited the highest inhibition of 95.35 % at 96 h, followed closely by AKP 800 mg/kg with inhibitory activity of 90.73 %. The overall dose-dependent anti-inflammatory activity of all test substances was HA (800 mg/kg) > AKP (800 mg/kg) ≥ HA (400 mg/kg) > AKP (400 mg/kg) > HA (200 mg/kg) > AKP (200 mg/kg), in that order. The inhibitory activity of the standard drug, aspirin (91 mg/kg) was significant in the early phase of inflammation (6 and 8 h), while it did not exhibit significant anti-inflammatory activity in the late phase of inflammation. The HA 800 mg/kg treated group showed maximum percent inhibition (73.80) in the early phase of inflammation at 8 h post carrageenan administration, while AKP 200 mg/kg was least effective.

4. Discussion

Chakrapani Dutta, in the late eleventh century, in his book *Chakradatta*[9] described the use of *T. arjuna* in the form of AKP, a traditional extraction method using cow milk and water. Cow milk is widely used as an *Anupana* (the carrier with which the herbal medicines are prescribed) in *Ayurvedic* system of medicine. The therapeutic potential of *T. arjuna* is well defined in the traditional literature and also validated as an adjunct therapy to prevent and ameliorate coronary artery disorder via its anti-inflammatory and cardioprotective effects,[25] however, very limited information is available with regard to the therapeutic activity of its traditional preparations including AKP. Recent findings on bovine milk (proteins and lipids)[26–28] have also prompted validation of the anti-inflammatory effect of AKP, a widely used traditional preparation of *T. arjuna*.

As an extraction solvent for AKP dilute milk (milk: water) could solubilize water and lipid soluble active components of *T. arjuna* bark. The percentage extraction yield of AKP was two folds higher than HA implying that the phytoconstituents in AKP were diluted by a factor of 0.5 as the increase in weight of AKP was due to milk solids (proteins and lipids),[29] also confirmed by the presence of proteins in AKP which were absent in HA. Preliminary phytochemical analysis of HA and AKP of *T. arjuna* revealed the presence of flavonoids, phenolic compounds, alkaloids, glycosides, steroids, and tannins. The total polyphenol content in HA was approximately four times higher than AKP. However, these results may not reflect the actual status of the active phytoconstituents in AKP, because of their binding to milk solids, and would require (gut) digestion for their release.[28] The presence of milk solids (mainly casein) may lower the detection level but increase the bioavailability of bioactive components of *T. arjuna* by protecting them against oxidation and digestive alteration by entrapment in casein molecules, as the binding of drugs to casein increases their solubility.[29] The open structure of casein, due to high proline content, makes it readily

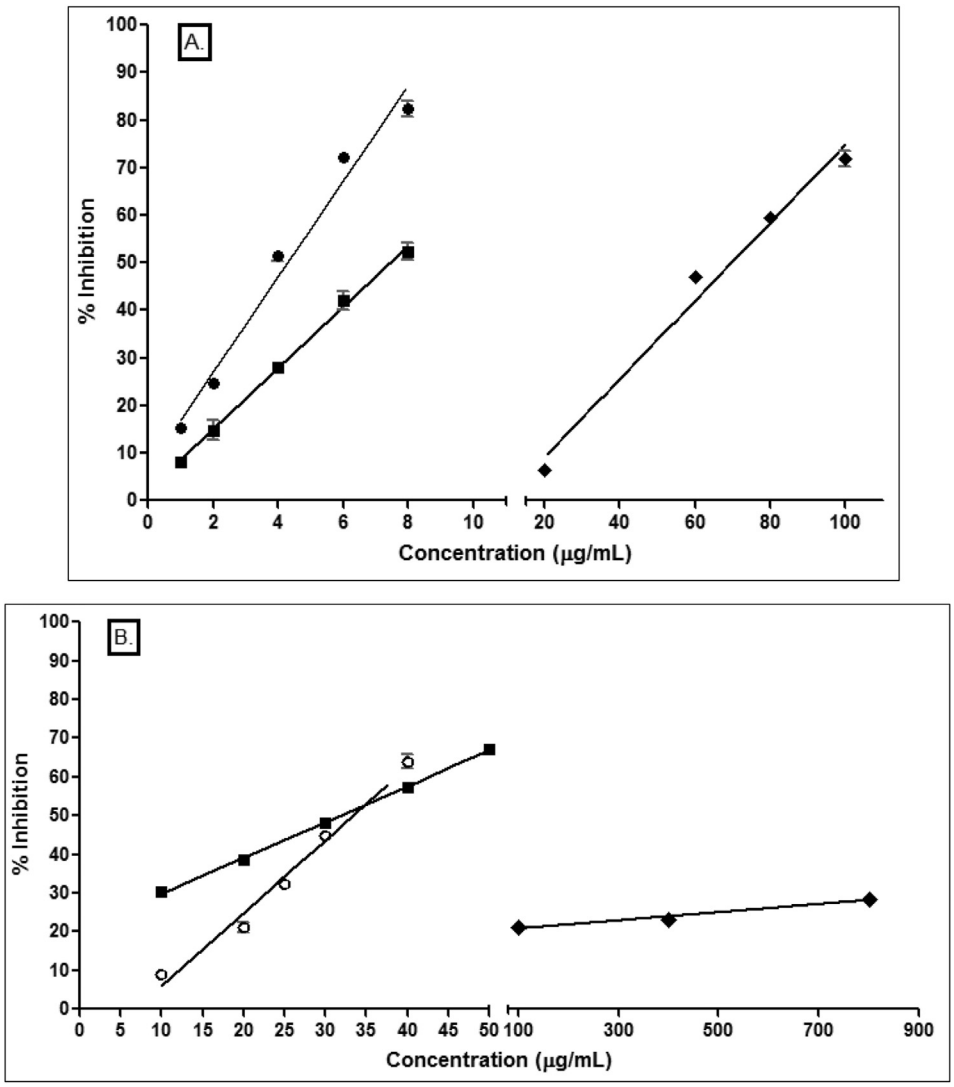

Fig. 1. Antioxidant activity in *T. arjuna* stem bark extracts *Arjuna Ksheera Paka* (◆) and hydroalcoholic extract (■). (A) DPPH free radical scavenging activity with vitamin C (●) as the standard and (B) free radical inhibition activity with vitamin E (o) as the standard.

accessible for proteolytic cleavage. This characteristic of casein along with the acid-soluble calcium–phosphate bridging, promises an excellent target-activated release mechanism for unloading drugs in the stomach.[26] Therefore, casein-based formulations are promising for controlled drug delivery.

Polyphenols like phenolic acids (gallic acid) and hydrophilic flavonoids (quercetin) possess free radical scavenging properties and/or possess anti-inflammatory properties. Studies indicate the inhibitory action of these natural antioxidants on reactive oxygen species implicated in inflammation, lead to down regulation of down-regulating the formation of proinflammatory eicosanoids derived from the cyclooxygenase (COX) pathway of arachidonic acid metabolism.[30] The free radical scavenging activity of HA was found to be equipotent to the standard vitamin C which was 10 times more potent than AKP formulation in scavenging free radicals, and similar trend was also observed in free radical inhibiting activity. The free radical inhibition activity of HA was comparable to the standard vitamin E in their IC_{50} values. The significantly high antioxidant activity in HA may be due to a higher detectable amount of polyphenols.

Carrageenan-induced mice paw edema was selected over conventional rat paw model as it has been reported to exhibit a biphasic inflammatory response and thus can be used to obtain statistically differentiable inflammatory phases.[31] The carrageenan-induced paw edema is sensitive to COX inhibitors, and commonly used for screening the anti-inflammatory activities of NSAID and natural products. COX enzymes are one of the crucial enzymes involved in the production of prostaglandins (PGs) by the metabolism of arachidonic acid, leading to the pathogenesis of acute inflammation. The interplay between different inflammatory mediators, under the influence of pro-inflammatory enzymes COX-1 and COX-2 have been identified to be responsible for the expression of two distinct phases of acute inflammation.[32] Studies indicate that discernible reduction of the first phase of carrageenan-induced mouse paw edema is brought about by preferential inhibition of COX-1, whereas it significantly increases edema in the second phase. While COX-2 is upregulated only in the second phase of the paw edema caused by carrageenan.[33] NO (derived from both the endothelial NO synthase [eNOS] and inducible NO synthase [iNOS] isoforms) on the other hand is associated with both the phases of inflammation.[33,34]

Oral administration of *T. arjuna* extracts and standard drug aspirin inhibited carrageenan-induced paw edema in C57BL/6 mice assessed at 0, 1, 2, 4, 6, 8, 24, 48, 72 and 96 h after sub-plantar

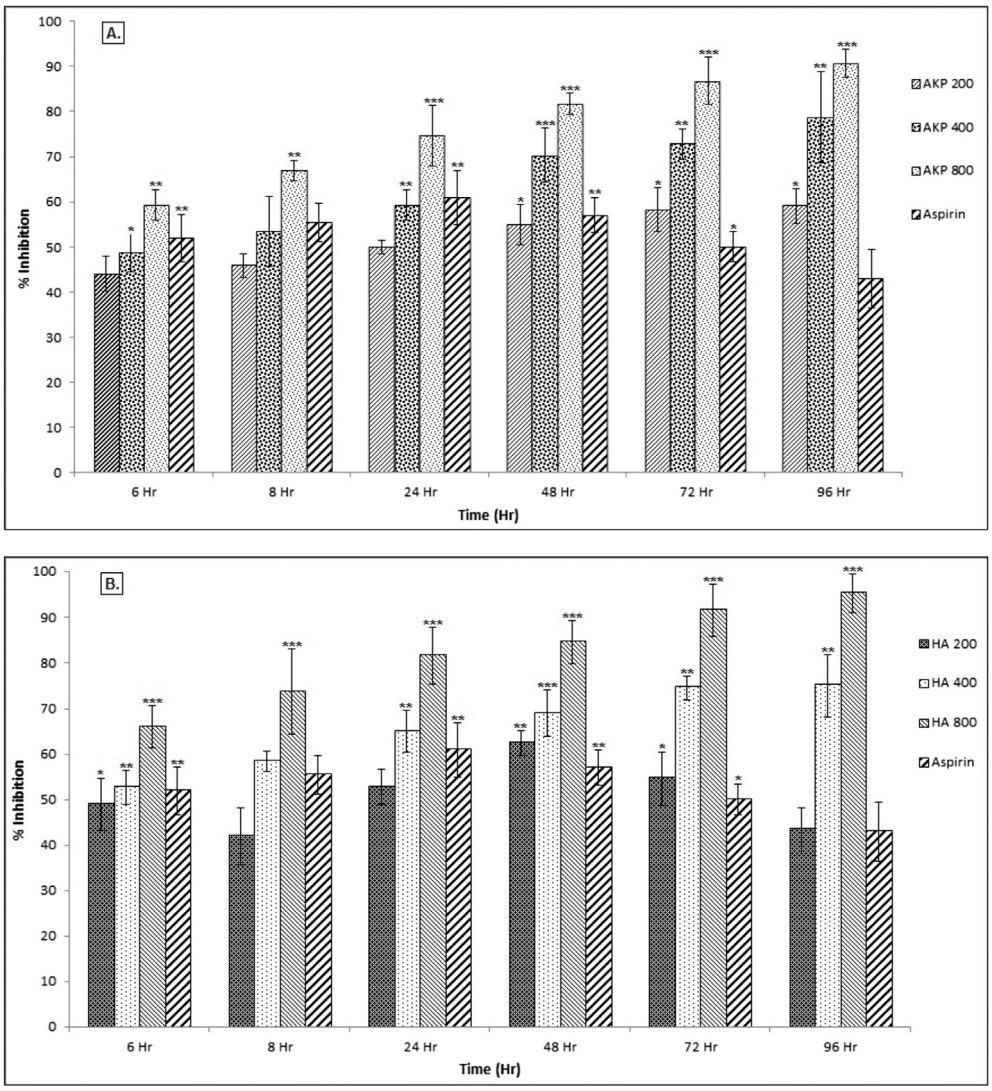

Fig. 2. Effect of *T arjuna* bark powder extracts *Arjuna Ksheera Paka* (AKP) and hydroalcoholic extract (HA) at 200, 400, 800 mg/kg on carrageenan (300 μg/paw) induced paw inflammation in C57BL/6 mice at different time intervals (6 h–96 h). Aspirin (91 mg/kg) was used as the standard anti-inflammatory drug. Values are Mean ± SE (n = 6); ANOVA: 0.0001, Bonferroni multiple comparison posthoc test: *p < 0.05, **p < 0.01, ***p < 0.001 significantly different from positive control. A. AKP versus aspirin, B. HA versus aspirin.

injection of carrageenan. A biphasic inflammatory response to carrageenan that developed in the first 8 h followed by a second phase that started from 24 h and continued up to 96 h, which conforms to earlier reports.[31] AKP, as well as HA of *T. arjuna*, showed a dose-dependent anti-inflammatory activity. Both *T. arjuna* extracts were found to be active in the first phase of inflammation at concentrations of 800 mg/kg, though higher inhibitory activity was found with HA. Their response in the second phase was higher at all the three concentrations of 200, 400 and 800 mg/kg, compared to aspirin which was found to be most active in the early phase of inflammation possibly due to its inhibition of COX-1.[35] Our observations indicate that the plant extracts were active only at a higher concentration in the first phase of inflammation, which would imply that the phytoconstituents are required at a higher concentration to inhibit COX-1, eNOS and iNOS pathways which are active in the first phase. The relatively higher anti-inflammatory effect of AKP in the later phase of inflammation might be through inhibition of the COX-2 pathway, leading to suppression of the synthesis and release of prostaglandins from the carrageenan-injected paw. On the whole, the potency of AKP in *in vivo* anti-inflammatory response was better than HA, as the response was observed at least half the concentration of active *Arjuna* phytochemicals. Thus, the high *in vivo* activity of AKP at lower drug concentration would also possibly reduce chances of any overdose related toxicity.

Our observation on AKP showing low antioxidant activity *in vitro* but exhibiting potent anti-inflammatory activity *in vivo* also finds support from observations on tea.[36] Studies suggest that interactions between tea polyphenols and proteins found in milk diminish total antioxidant capacity *in vitro*,[37] but the addition of milk to black tea did not significantly alter the plasma catechins or flavonols in human volunteers, suggesting that adding milk to tea does not substantially affect the bioavailability of tea catechins or flavonols.[36] Furthermore, some studies have indicated that milk solids (proteins and lipids) possess anti-inflammatory activity. Whey protein, lactoferrin (LF) has been documented to reduce inflammation in various animal models by inhibiting the production of the inflammatory cytokines TNF-α, IL-1b and IL-6 in monocytes.[38] The oral administration of another whey protein, α-lactalbumin (α-LA) before carrageenan injection in rat footpads inhibited the increased formation of IL-6 and PGE2 in paw exudates. α-LA also showed selective inhibition of COX-2 as compared

to COX-1.[27] While saturated fatty acids (capric and lauric acid) and unsaturated fatty acids (conjugated linoleic acid) in milk have the potential to down-regulate production of COX-I and COX-II pro-inflammatory enzymes.[28] Thus, the overall effect of AKP at a relatively lower concentration of *Arjuna* is due to sustained *in vivo* bioavailability of the drug, initiated by the hydrolysis of the phytoconstituents-casein complex in the gut. The anti-inflammatory effect of *Arjuna* in AKP, especially in the later phase, may also be accentuated by the added effect of some milk peptides and proteins.

5. Conclusion

The levels of *Arjuna* active phytoconstituents were higher in HA as compared to AKP due to the presence of milk solids in AKP. AKP has potential anti-inflammatory properties even though it exhibited low *in vitro* antioxidant activity. The milk protein, casein present in AKP may contribute to sustained bioavailability of active phytoconstituents *in vivo*, while whey proteins like α-LA and LF and lipids like capric, lauric and conjugated linoleic acid might also enhance its anti-inflammatory potential post-digestion. Thus the overall *in vivo* anti-inflammatory activity of AKP is an outcome of several factors which may have contributed to the potency of AKP at relatively lower detectable drug concentrations than HA. The ease of preparation of AKP over HA also gives it an edge for adopting AKP as a home remedy. Development of standardized, synergistic, safe and effective traditional herbal formulations like AKP with scientific evidence may offer faster and more economical alternatives. It would be useful to compare HA and AKP under prolonged uninterrupted use.

Conflict of interest

The authors declare that there is no conflict of interest.

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