

Investigation of *in vitro* Antioxidant and Anti-inflammatory Activities of Selected *Siddha* Polyherbal Formulations

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ABSTRACT

Objective: This study deals with the antioxidant and anti-inflammatory activities of selected *Siddha* polyherbal formulations such as *Amukkara choornam*, *Thirikadugu choornam*, *Eladi choornam* and *Thiripala choornam*. *Amukkara choornam* consist of seven herbals (withania, ginger, black pepper, long pepper, mesua, cardamom and clove). *Thirikadugu choornam* has three herbal ingredients ginger, black pepper and long pepper. *Eladi choornam* has seven raw drugs like cardamom, ginger, arrow root, yew leaves, Mesua, black pepper and clove. *Thiripala choornam* comprises of three dry fruits amla, chebulla and bellerica. **Methods:** Even though all these medicines have been used as analgesic for joint pain in *Siddha* system of medicine, their antioxidant and anti-inflammatory activities were not yet proved scientifically. In the present work, we have evaluated the antioxidant and anti-inflammatory properties of aqueous extract of the above-mentioned herbal formulations. **Results:** Among the investigated formulations, *Thiripala choornam* has six folds higher level of total phenolic content (1248 mf GAE/100 g) when compared to other formulations (190.42 – 199.78 mg GAE/100 g). Similarly, *Thiripala choornam* exhibited promising antioxidant activity in terms of radical scavenging potential against DPPH (IC-50: 156 mg/L), superoxide (IC-50: 2303 mg/L), hydrogen peroxide (IC-50: 294 mg/L) and hydroxyl radical (IC-50: 587 mg/L) and anti-inflammatory activity (IC-50: 24 mg/L). **Conclusion:** Among the investigated samples, *Thiripala choornam* was found to exhibit high antioxidant and anti-inflammatory potential and thus could be used as more effective analgesic formulation.

Key words: Aqueous extract, Polyphenols, Antioxidant, Anti-inflammatory, *Amukkara choornam*, *Thirikadugu choornam*, *Eladi choornam*, *Thiripala choornam*.

INTRODUCTION

Siddha system of medicine uses so many poly herbal formulations for the joint pain. The usage of Non-steroidal anti-inflammatory drugs (NSAID) in the treatment of painful musculo-skeletal conditions often results in adverse effects such as gastric irritation, renal damage etc. By the way, poly herbal medicines are not dangerous, efficient, time-tested and devoid of severe side-effects. *Siddha* medicine has many such herbal formulations for the treatment of joint pain (arthritis).¹ In this study we have selected four medicines, which are used for joint pain such as *Amukkara choornam*

(AMK), *Thirikadugu choornam* (TKC), *Eladi choornam* (ELC) and *Thiripala choornam* (TPC).² These *Choornams* are powdered form of a single drug or a mixture more drugs, which retain their medicinal value for three months.³

AMK therapeutically used as an analgesic, nervine tonic, gastric ulcer, hiccups, peripheral neuritis, and ashtma,⁴ rheumatic diseases, sexual insufficiency and insomnia.² It consists of several herbal ingredients like root of *Withania somnifera* (L.) Dunal, rhizome of *Zingiber officinale* Roscoe (ginger), dry seed of *Piper nigrum* L. (black pepper), dry

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seeds of *Piper longum* L. (long pepper), flower bud of *Mesua ferrea* L. (Indian rose chestnut), dry fruit of *Ellettaria cardamomum* L. (cardamom) and flower bud of *Syzygium aromaticum* L. (clove). All these ingredients are powdered and mixed in the ratio of clove – one part, Mesua – 2 parts, cardamom - 4 parts, long pepper – 8 parts, black pepper – 16 parts, dry ginger – 32 parts, withania root – 64 parts and sugar - 128 parts.^{4,5,6,7}

TKC medicinally used for respiratory problems like body pain, asthma, tuberculosis, pneumonia, sinusitis fever and digestive disorders like indigestion, loss of appetite, flatulence, constipation, nausea and vomiting, skin diseases and antidote for all poisons.² Previously research work proved that TKC has good anthelmintic activity (Devi et al., 2011). It has three ingredients such as rhizome of *Zingiber officinale* Roscoe, seeds of *Piper nigrum* L and *Piper longum* L, which are made into fine powder and mixed in equal quantity.^{5,6}

All the ingredients of ELC such as cardamom (*Ellettaria cardamomum* L.), ginger (*Zingiber officinale* Roscoe), arrow root (*Maranta arundinacea* L.), yew leaves (*Abies webbiana* (D. Don) Spach), Indian rose chestnut (*Mesua ferrea* L.), pepper (*Piper nigrum* L) and clove (*Syzygium aromaticum* (L.) Merrill and Perry) are separately powdered and sieved through a fine mesh.⁵ then the required quantities of cardamom (64 parts), ginger (32 parts), arrow root (16 parts), yew leaves (8 parts), Indian rose chestnut (4 parts), pepper (2 parts), clove (1 part) and sugar (128 parts) are taken and thoroughly mixed (Kuppusamy and Uthamarayan, 1998). Therapeutically it is used for indigestion, gastritis, vatha diseases, pitha diseases, skin diseases, skin ulcer, and tuberculosis and over bleeding.⁵ this medicines standardization work was done by Thankamma et al, 1998.⁹

TPC consists of three dry fruits like *Terminalia chebula* Retz, *Terminalia bellirica* (Gaertn.) Roxb and *Phyllanthus emblica* L. All the fruits are crushed, removed the seeds and the peel was collected and made into fine powder and mixed in equal quantity.¹⁰ It act as a astringent and therapeutically it is used for gastric ulcer, mouth ulcer, skin disease, cough, anaemia, leucorrhoea, constipation, piles, wounds, gum bleeding, liver toxic symptoms and also used for analgesic, ascites and hypertension.^{2,11} Previously research study proved that TPC has anticancer activity in both cellular and *in vivo* models.¹² Another review article mentioned that TPC has effective antioxidant, antibacterial, anti-viral, and used to treat cataract and AIDS.¹³

Even though these medicines have several therapeutic uses and few research studies have been conducted on their therapeutic potential, the antioxidant and anti-inflammatory activities of aqueous extract of the

selected herbal formulations are not yet revealed. This research paper revealed with the *in vitro* antioxidant and anti-inflammatory properties of herbal medicines documented in *Siddha* classic text.

MATERIALS AND METHODS

Preparation of the drug

Raw ingredients of AMK, TKC, ELC, and TPC were procured from local herbal market, Thanjavur, Tamilnadu, India and identified and authenticated in the NABL accredited lab of CARISM, SASTRA University. All the ingredients were powdered (particle size 1 mm) individually using a lab mill and then mixed according formulation given in *Siddha* formulary of India, 1992.⁵

Extract preparation

Twenty five g of each formulation were taken separately in a conical flask and extracted with 250 ml of distilled water and kept for 24 h. Then the aqueous extract was filtered through filter paper. After filtration, the filtrates were dried in vacuo (freeze drier – Christ Alpha 2 – 4 LD Plus, Germany) and re-dissolved in water at 5000 mg / L ratio and used for further experiments.

Total phenol content

The total phenolic content of extract was estimated according to the method of Singleton et al., 1999.¹⁴ Sample (100 µl) was taken with 250 µl of Folin's-Ciocalteu reagent and 1000 µl of 5% of Na₂CO₃ were added and incubated for 30 min in dark place. Then the absorbance was measured at 720 nm. A calibration curve was prepared using standard gallic acid (16 – 100 mg/L; $y = 0.0094x - 0.0585$; $R^2 = 0.9939$) and used to express the results as gallic acid equivalents (mg GAE / 100 g sample).

DPPH radical scavenging activity

The DPPH radical scavenging assay was used to analyze the antioxidant property of aqueous extracts of the samples by following Sanchez-Moreno et al., 1998 method.¹⁵ Different concentrations (5000, 2500, 1250, 625, 312, 156, 78, 39, 19, 9.7 mg/L) of extract (100 µl) were added to 0.9 ml of methanolic solution of DPPH (2.5 mg/100 ml) and the reactants were incubated at room temperature for 30 min in dark. Solvent (distilled water) was used instead of extract in control. After 30 min, the absorbance was measured at 515 nm using a spectrophotometer and the radical scavenging activity of the extract was calculated and expressed on percentage basis.

Superoxide radical scavenging activity

The capacity of extract to scavenge the superoxide anion radical was measured according to the method

described by Zhishen *et al.*, 1999.¹⁶ The reaction mixture was prepared using 3×10^{-6} M riboflavin, 1×10^{-2} M methionine, 1×10^{-4} M nitroblue tetrazolium chloride and 0.1 mM EDTA in phosphate buffered saline (pH 7.4). For the analysis, 3.0 ml of the reaction mixture was taken with 100 μ l of extract in closed tubes and illuminated for 40 min under fluorescent lamp (18 W). The absorbance was then read at 560 nm against the un-illuminated reaction mixture. Results are expressed as superoxide radical scavenging activity on percentage basis.

Hydrogen peroxide scavenging activity

The effect of extract on hydrogen peroxide was analyzed according to the method proposed by Ruch *et al.*¹⁷ The extract (100 microliter) was mixed with 5 ml of 45 mM hydrogen peroxide solution in 0.1 M phosphate buffer (pH 7.4). The reaction mixture was vortexed and incubated for 30 min at room temperature and then the absorbency was measured at 230 nm. The extract with phosphate buffer is used as a blank and the level of hydrogen peroxide remaining in the solution was calculated using a calibration curve. The hydrogen peroxide inhibition effect of extract was calculated and expressed on percentage basis.

Hydroxyl radical scavenging activity

The hydroxyl radical quenching activity of extracts was evaluated according to the method of Hagerman *et al.*, 1998.¹⁸ The reaction mixture consists of 10 mM phosphate buffer (pH 7.4), 2.8 mM Deoxyribose, 2.8 mM H₂O₂, 0.025 mM FeCl₃, 0.1 mM EDTA and 0.1 mM ascorbic acid in a total volume of 3 ml. With the reaction mixture, 100 microliter of extract was added and incubated at 37°C for 15 min. Then the reaction was terminated by the addition of 1 ml of 2.5% ice-cold TCA and 1% TBA. The reactants were mixed well and heated at 90 C for 15 min in a water bath and cooled to room temperature. The chromogen was extracted with 1-butanol and absorbency was measured at 530 nm. Based on absorbency value, the hydroxyl radical scavenging activity of extracts was calculated and expressed on percentage basis.

Anti-inflammatory activity

Anti-inflammatory activity of aqueous extracts of samples was evaluated using RBC membrane stabilization assay.¹⁹ Human blood (2 ml) was drawn from volunteer in a heparinized tube and centrifuged at 2000 rpm for 10 min. The pellet (RBC cells) was washed twice with PBS (9 ml) and finally the pellet was re-suspended in 10 ml of PBS. Different concentrations (5000, 2500, 1250, 625, 312, 156, 78, 39, 19, 9.7 mg/L) of extract (500 μ l) were added to 1 PBS, 1 ml of 3% H₂O₂ and

incubated for 30 min. In normal control, 1 ml PBS was added instead of extract and in negative control only H₂O₂ was added. After incubation, the contents were centrifuged at 2000 rpm for 10 min and the supernatant was used to measure the absorbance at 520 nm. Based on the absorbance, the percentage of RBC membrane damage and inhibition of membrane damage were calculated.

RESULTS AND DISCUSSION

Total phenol content

Plant phenols are the natural antioxidant compounds, have wide range of physiological properties such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardio-protective and vasodilatory effects.²⁰ the total phenolic content of selected polyherbal formulations was given in Figure 1. Among the studies formulations, TPC possesses the higher level of phenols when compared to other formulations of the study.

Antioxidant activity

Reactive Oxygen Species (ROS) are produced in both unstressed and stressed cells. Oxidation reactions generate free radicals that can start multiple chain reactions, which eventually cause damage or death to the cell. Free radicals cause many human diseases like cancer Alzheimer's disease, cardiac reperfusion abnormalities, kidney disease, fibrosis, etc. The free radicals formed in our body are combated by antioxidants that safely interact with free radicals and terminate the chain reaction

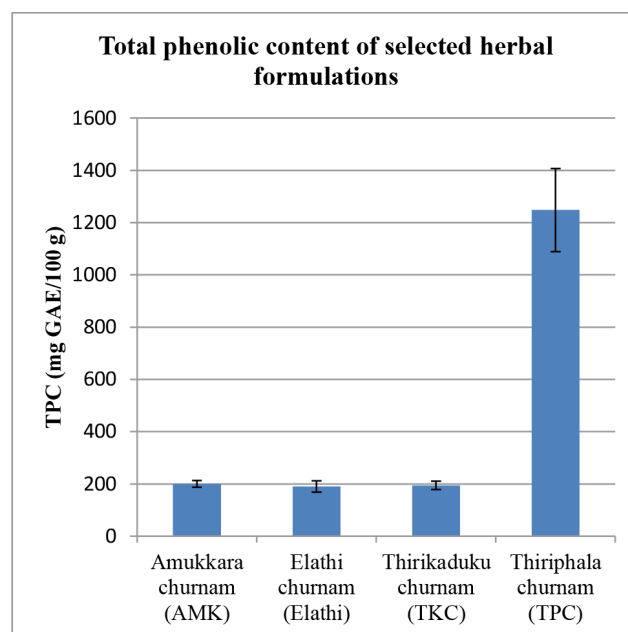


Figure 1: Total phenolic concentration of poly herbal formulations.

before vital molecules are damaged.²¹ Such oxidative process are dangerous for all living cells and oxidative stress is playing a significant part of many human diseases that can occur due to a lack of suitable nutrition and exercise, air pollution, smoking, artificial foods and more leading to lethal diseases such as cancer.

Plants have well developed defense system against ROS, involving both limiting the formation of ROS as well as instituting its removal. Under unstressed conditions the formation and removal of O₂ are in balance. However, the defense system, when presented with increased ROS formation under stress condition, can be overwhelmed. Plants respond to a rise in ROS that the defense system is unable to remove with increased enzymatic or non enzymatic antioxidant process, but the mechanisms underlying these processes is not well understood.²² Antioxidant compounds can eliminate these free-radical intermediates by being oxidized themselves and inhibit other oxidation reactions, thus preventing the harmful chain reactions. Therefore, it is very important to include antioxidants in our diets. In pharmaceutical industries antioxidants are added to enhance the stability of therapeutic agents that are susceptible to chemical degradation by oxidation. Unfortunately, recent research indicating that the synthetic antioxidants could have carcinogenic effects on human cells, thus fueling an intensive search for new, natural and efficient antioxidants.

DPPH radical scavenging activity

The DPPH radical scavenging activity of different *Siddha* poly herbal medicines were illustrated in the Figure 2. The evaluation of the antioxidant power by DPPH radical scavenging activity has been widely in use for different plant extracts. DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a stable radical, methanolic solution of which has dark purple colour with maximum absorption at 515 nm. Among the investigated herbal formulations, *Triphala churnam* exhibited highest DPPH radical scavenging power, which is followed by *Trikaduku churnam*, *Amukkara churnam* and *Eladi churnam*. All the herbal formulations exhibited dose-dependant antioxidant activity and the IC-50 value of 156, 1171, 2286 and 5067 µg / ml were recorded by *Triphala churnam*, *Trikaduku churnam*, *Amukkara churnam* and *Eladi churnam*.

Superoxide radical scavenging activity

The superoxide radical scavenging activity of aqueous extracts of selected poly herbal powders are exposed in Figure 3. Superoxide is produced by the addition of a single electron to oxygen, and several mechanisms exist by which superoxide can be produced *in vivo*. Several molecules, including adrenaline, flavine nucleotides, thiol

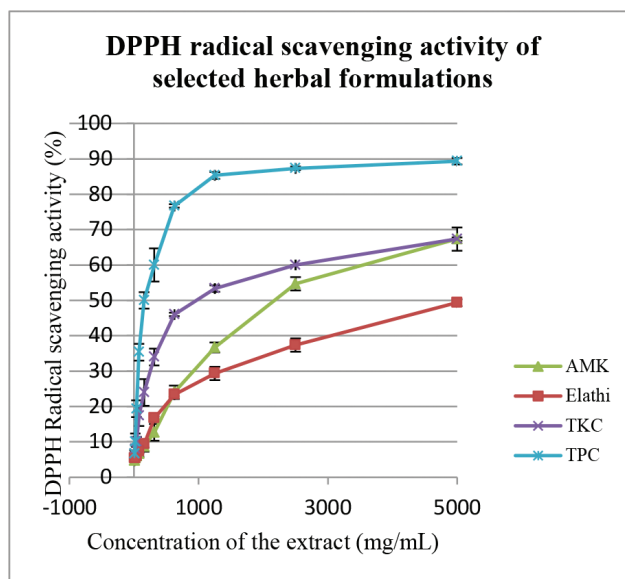


Figure 2: DPPH radical scavenging activity of selected herbal formulations.

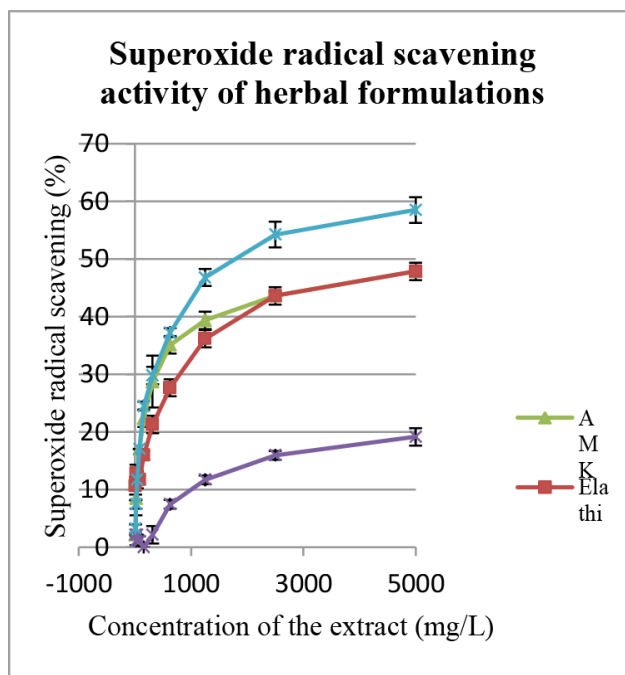


Figure 3: Superoxide radical scavenging activity of herbal formulations.

compounds, and glucose, can oxidize in the presence of oxygen to produce superoxide, and these reactions are greatly accelerated by the presence of transition metals such as iron or copper. The electron transport chain in the inner mitochondrial membrane performs the reduction of oxygen to water. During this process free radical intermediates are generated, which are generally tightly bound to the components of the transport chain. However, there is a constant leak of a few electrons into

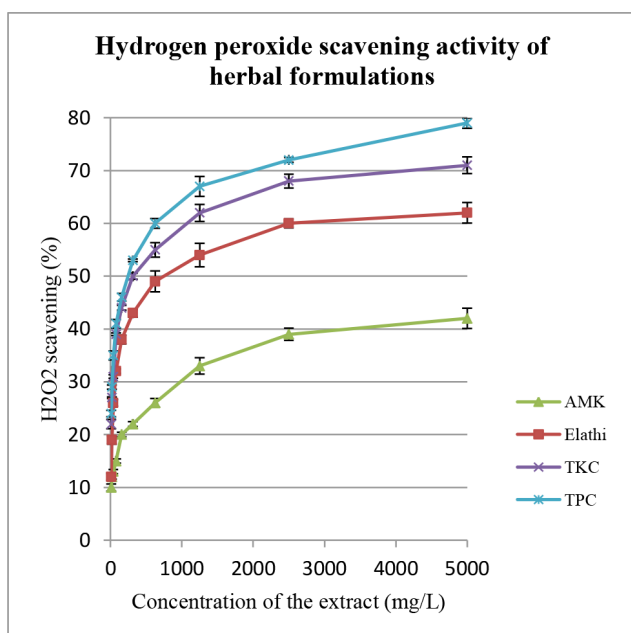


Figure 4: Hydrogen peroxide inhibition activity herbal formulations.

the mitochondrial matrix and it results in the formation of superoxide.²⁴ The superoxide radical scavenging activity of *Triphala churnam* is found to be very high (IC_{50} 2303 $\mu\text{g/ml}$), when compared to *Amukkara churnam* (IC_{50} 5222 $\mu\text{g/ml}$), *Eladi churnam* (IC_{50} 5222 $\mu\text{g/ml}$) and *Trikaduku churnam* (IC_{50} 13054 $\mu\text{g/ml}$).

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity aqueous extracts of four *Siddha* poly herbal medicines were presented in the Figure 4. Any biological system generating superoxide will also produce hydrogen peroxide as a result of a spontaneous dismutation reaction. In addition, several enzymatic reactions, including those catalysed by glycolate oxidase and D-amino acid oxidase, might produce hydrogen peroxide directly. Hydrogen peroxide is not a free radical itself, but is usually included under the general heading of reactive oxygen species.²⁴ Among the investigated herbal formulations, *Triphala churnam* and *Trikaduku churnam* have exhibited strong inhibition effect against hydrogen peroxide with the IC_{50} value of 294 and 312 $\mu\text{g/ml}$, respectively, which are followed by *Eladi churnam* (IC_{50} 1157 $\mu\text{g/ml}$) and *Amukkara churnam* showed poor inhibition (IC_{50} value 5952 $\mu\text{g/ml}$).

The hydroxyl radical scavenging activity of *Siddha* medicines were presented in the Figure 5. The hydroxyl radical (OH), or a closely related species, is probably the final mediator of most free radical induced tissue damage. All the reactive oxygen species described above

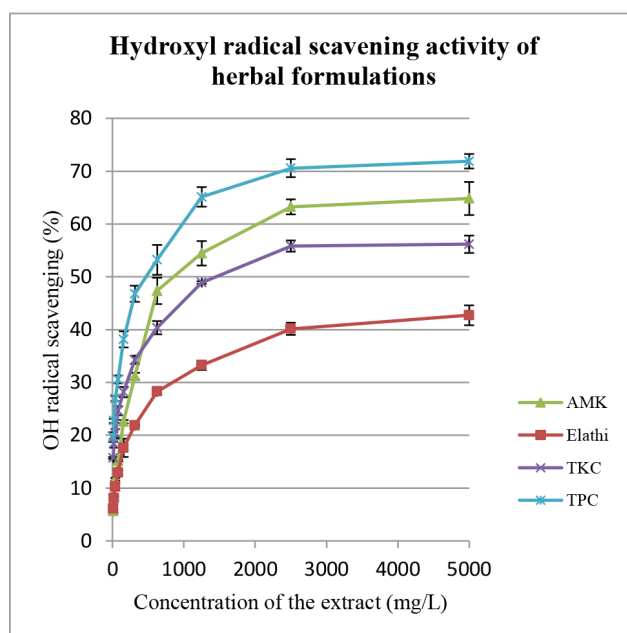


Figure 5: Hydroxyl radical inhibition.

exert most of their pathological effects by giving rise to hydroxyl radical formation. The reason for this is that the hydroxyl radical reacts, with extremely high rate constants, with almost every type of molecule found in living cells including sugars, amino acids, lipids, and nucleotides. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism *in vivo* is likely to be the transition metal catalysed decomposition of superoxide and hydrogen peroxide.²⁴ In agreement to other *in vitro* assays (DPPH, Superoxide and Hydrogen peroxide inhibition), *Triphala churnam* recorded prominent hydroxyl radical scavenging power (IC_{50} 587 $\mu\text{g/ml}$) among the investigated samples. But, in contrast the *Amukkara churnam* is also found to be effective against hydroxyl radicals (IC_{50} 1147 $\mu\text{g/ml}$) whereas *Trikaduku churnam* (IC_{50} 2238) and *Eladi churnam* (IC_{50} 5847 $\mu\text{g/ml}$) were found to be weak antioxidants.

Anti-inflammatory activity

Cyclooxygenase is the important enzymes in the production of prostaglandins, prostacyclins and thromboxanes which are involved in inflammation, pain and platelet aggregation. Steroidal and non-steroidal anti-inflammatory drugs are currently the most commonly used drugs in the treatment of acute inflammatory disorders. Its adverse effect damaged to the renal and gastric systems. These drugs obstruct cyclooxygenase enzyme activity results to prevent prostaglandin production. These type of SAID and NSAID drugs chronic usage gives

Table 1: Anti-inflammatory activity.

S. No	Herbal formulation	Anti-inflammatory activity (IC-50 values, µg/ml)
1.	<i>Amukkara choornam</i>	35.19
2.	<i>Thirikadugu choornam</i>	83.93
3.	<i>Eladi choornam</i>	58.96
4.	<i>Thiripala choornam</i>	24.00

harmfulness to human biological system such as liver, gastrointestinal tract, cardiovascular, renal failure.²⁵

Along with the presently studies herbal formulations of *Amukkara churnam* (35.19), *Thirikadugu choornam* (83.93), *Eladi choornam* (58.96) and *Thiripala choornam* (24.00) were displayed that *Thirikadugu choornam* has higher level of anti-inflammatory activities in terms of RBC membrane stabilization. Table 1

CONCLUSION

In the present work, we have evaluated the total phenolic content, antioxidant and anti-inflammatory activities of four common herbal formulations such as *Amukkara churnam*, *Eladi churnam*, *Trikaduku churnam* and *Triphala churnam*. Even though herbal ingredients of the selected formulations are different, all are useful in treating arthritis (Joint pain). A good relationship was noticed between high content of phenolic compounds and higher level antioxidant activity of *Triphala churnam* in terms of radical scavenging activity against DPPH, superoxide, hydrogen peroxide and hydroxyl radicals and also anti-inflammatory property through RBC membrane stabilization. Such remarkable antioxidant and anti-inflammatory properties of *Triphala churnam* might be due to its herbal ingredients like *Embelica officinalis*, *Terminalia chebula* and *Terminalia bellerica*, which all are known as rich source of polyphenols. Among the investigated herbal formulations, *Triphala churnam* could be effective against oxidative-stress related diseases including arthritis.

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CONFLICT OF INTEREST

There is no conflict of interest.

ABBREVIATIONS USED

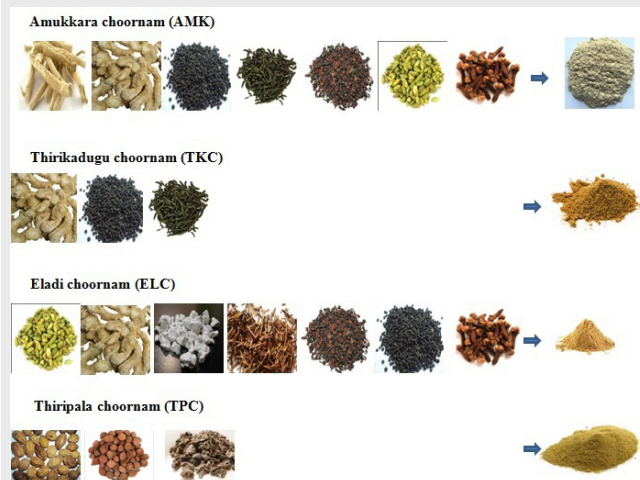
NSAID: Non-steroidal anti-inflammatory Drugs; **AMK:** Amukkara choornam; **TKC:** Thirikadugu choornam; **ELC:** Eladi choornam; **TPC:** Thiripala choornam; **AIDS:** Acquired immune deficiency syndrome; **NABL:** National Accreditation Board for Testing and Calibration Laboratories; **GAE:** Gallic acid equivalents; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **H₂O₂:** Hydrogen peroxide; **FeCl₃:** Ferric chloride; **EDTA:** Ethylenediamine tetra acetic acid; **TCA:** Trichloroacetic acid; **TBA:** Thio barbituric acid; **RBC:** Red blood cells; **PBS:** Phosphate buffered saline; **ROS:** Reactive oxygen species; **IC-50:** Inhibitory concentration-50; **OH[•]:** Hydroxyl radical.

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PICTORIAL ABSTRACT



SUMMARY

- Among the investigated herbal formulations, Triphala churnam is found to be effective antioxidant and anti-inflammatory drug.

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