IJPSR (2018), Volume 9, Issue 8 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 25 November, 2017; received in revised form, 19 June, 2018; accepted, 04 July, 2018; published 01 August, 2018

IN-VITRO AND *EX-VIVO* STUDIES ON THE ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTIARTHRITIC PROPERTIES OF *CAMELLIA SINENSIS*, *HIBISCUS ROSA SINENSIS*, *MATRICARIA CHAMOMILLA*, *ROSA SP.*, *ZINGIBER OFFICINALE* TEA EXTRACTS

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Keywords:

Antioxidant, Anti-inflammatory, Anti-arthritic, *Camellia sinensis* (green tea), *Hibiscus rosa sinensis* (hibiscus flower), *Matricaria chamomilla* (chamomile flower), *Rosa sp*.(rose flower), *Zingiber officinale* (ginger)

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ABSTRACT: The adverse effects of oxidative stress have been discussed widely in the present day. It is the main cause of protein denaturation, cardiovascular diseases, ageing, *etc*. Thus, the daily requirement of anti-oxidants has to be fulfilled through dietary sources. Inflammation is yet again another form of stress on the body. It is the major cause of multiple chronic disorders. It can lead to the formation of many other disorders such as arthritis, asthma, macular degeneration, *etc*. Arthritis is one such inflammatory disorder which is associated with swelling of joints, joint pain, *etc*. This research was based on the anti-oxidant, anti-inflammatory and anti-arthritic properties of tea extracts such as *Camellia sinensis*, *Hibiscus rosa sinensis*, Rosa, *Zingiber officinale*, *Matricaria chamomilla*. *Ex-vivo* tests for evaluating antioxidant property of samples using liver tissue by LPO assay was performed. In addition, tests such as inhibition of protein denaturation and RBC membrane stabilization were performed to determine anti-inflammatory effect of the sample. Collagen denaturation assay using chicken collagen to check anti-arthritic properties of samples were performed. All the tests showed positive results with the samples, which implied that the samples were potent in their anti-oxidant, anti-inflammatory and anti-arthritic activities. Thus, the use of natural sources to prevent disorders has become a more feasible option as there are no adverse effects. Although, there is further scope for research in this area and contribute to the ethnopharmacological field of medicine.

INTRODUCTION: Natural products are produced by plants or / and animals and have significance in the field of medicine and pharmacology [1]. Plants are a major source of structurally diverse molecules that are called phytochemicals.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(8).3543-51

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(8).3543-51

Some major classes of phytochemicals include phenols, polyphenols, alkaloids, terpenes, tannins, methyl xanthines, *etc*. which are majorly responsible for color, appearance, aroma and characteristic taste of brewed tea [2].

Camellia sinensis is an evergreen shrub, produces leaves that are used to prepare a popular beverage known as tea. The beverage is prepared by using variously processed leaves of the plant *Camellia sinensis*. The composition of tea also depends largely upon the climatic region in which it is grown, season, cultural practices of brewing, *etc*.[3]

Flavonols, flavanols, flavonol glycosides, polyphenolic acids put together can be called total polyphenols of tea and make up about 30% of the dry weight of tea shoot. Fresh tea leaf is unusually rich in the flavanol group of polyphenols known as catechins which may constitute up to 30% of the dry leaf weight [4]. Some of the major catechins present in tea are epigallo catechin (EGC) and epigallo catechin gallate (EGCG) [5]. Hibiscus is also known to have beneficial effects in reducing cardiovascular diseases [6, 7]. Hibiscus consists of many phytochemicals, which are known to have these ethnomedicinal properties [8, 9].

Some research suggests that anthocyanins, an anti-oxidant, may hold the key to hibiscus‘s anti-cancer properties [10, 11]. It is also known to reduce inflammation [12] within the body and thus, has a protective effect in maintenance of health [13, 14]. Chamomile are native in many countries throughout Europe, and are cultivated in such countries as Germany, Spain, Egypt, Morocco, France, Italy and parts of Eastern Europe [15]. The main constituents of the flowers include several phenolic compounds, primarily the flavonoids apigenin, quercetin, patuletin, luteolin and their glucosides and other therapeutic substances [15].

Chamomile has been used for centuries in many different cultures in the form of teas as a mild, relaxing sleep aid [16], treatment for fevers, colds, stomach ailments, and as an anti-inflammatory, to name only a few therapeutic uses [15, 16, 17]. The main components of rose was found to be citronellol, geraniol, nerol, phenyl ethyl alcohol, alpha-guaiene, nonadecane, eicosane, heneicosane, tricosane, geranyl acetate and eugenol have been reported from different parts of world [18].

The decoction of the rose flowers was used for treatment of pain in abdomen and chest, menstrual bleeding and digestive ailments as it acts as a gentle laxative for constipation. It is also used as a cardiotonic agent for strengthening the heart [18, 19]. Ginger as a herbal medicinal product that shares pharmacological properties with nonsteroidal anti-inflammatory drugs [20]. Some researchers think that ginger may help prevent strokes, heart disease, and hardening of the arteries [20, 21]. A tea made of the root improves digestion, relieves gas and bloating, and stimulates appetite [22, 23]. It is known that gingerol, a substance in ginger, inhibits an enzyme that causes cells to clot [24].

Free radicals are involved in the development of various diseases which include cellular aging, coronary heart disease, mutagenesis, diabetes, carcinogenesis and neurodegeneration [6]. The biological properties of tea and tea polyphenols have an effect in cancer, cardiovascular disease, antioxidant, anti-inflammatory, antiobesity, and neurodegenerative disorders prevention [25, 26, 27]. Oxidative stress occurs when the production of reactive oxygen species reacts with biomolecules by donating oxygen atoms to different bio-molecules such as proteins, lipids, nucleic acids, *etc*., in the cell to cause many diseases and illnesses [7]. When the amount of reactive oxygen species [28] or free radicals is more than the body‘s ability to detoxify it [29], oxidative damage occurs [30, 31]. Consumption of dietary antioxidants prevents oxidation of DNA [32, 33], proteins and lipids within the body [34, 35, 36]. Enzymes such as superoxide dismutase [37, 38], catalase [39], glutathione non-enzymatic antioxidants like Vitamin C, tocopherols [40] and melatonin [41] prevents oxidative damage [42, 43].

Inflammation occurs in response to any kind of bodily injury and is accompanied by a non-specific immune response [44]. Inflammation is the body‘s way of responding to stress. Inflammation can cause many chronic diseases due to free circulation of high levels certain inflammatory mediators such as C-reactive proteins, interleukin-6, tumour necrosis factor-alpha [45]. It is found that the persistent increase in the circulatory levels of these inflammatory markers is an indication of cardiovascular disease [46]. Inflammatory disorders can be auto-inflammatory [52] in nature [47, 48]. Interleukins are found to be systemic biomarkers of inflammation [49, 50].

Arthritis is found to be one such auto-inflammatory disorder [51, 53, 54]. Arthritis is characterized by inflamed joints and can cause a very painful condition, restricting movement of the affected individual [53]. Some of the major symptoms of arthritis is pain, swelling, stiffness and decreased angular motion in the joints. Rheumatoid arthritis [60], gout, lupus, fibromyalgia, septic arthritis, juvenile idiopathic arthritis, psoriatic arthritis *etc*., are some of the common forms of arthritis.

Research has found that there are multiple biological markers that are found under circulation in case of prevalence of disease in the individual. C-reactive protein [59], acute phase serum amyloid A protein [54], cartilage oligomatrix protein (COMP), hyaluronic acid, chondroitin sulphates, keratan sulphates *etc*., [55, 56] are some of the biological markers [57, 58]. In this study, Tea is used as one such natural source of antioxidants [60], anti-inflammatory [61] and anti-arthritic agent that helps in the repair of damage caused due to free radical activity [6], inflammatory agents and mediators respectively.

Objective of Study:

- Antioxidant study was conducted on chicken liver tissue by *ex-vivo* method using inhibition of lipid peroxidation assay (LPO assay).
- Anti-inflammatory parameter was justified by performing assays such as inhibition of protein denaturation assay and HRBC membrane stabilization assay.
- Anti-arthritic assay was performed using *ex - vivo* method using chicken wing cartilage for checking the inhibition of collagen denaturation.

MATERIALS AND METHODS: Standards of gallic acid and quercitin, 10% Folin-Ciocalteu‘s reagent, 7.5% $NaHCO_3$, 10% aluminium chloride ($AlCl_3$) by Himedia, USV Ltd., 1M potassium acetate, methanol, 70% methanol, bovine serum albumin 1%, acetyl salicylic acid 1000 µg/ml and 100 µg/ml, diclofenac sodium 500 µg/ml and 10000 µg/ml, isosaline 0.85%, hyposaline 0.35%, phosphate buffer 0.15M, dextrose 2%, tri-sodium citrate 0.8%, citric acid 0.05%, sodium chloride 0.42%.

***Ex-vivo* Studies:** Mutton liver tissue followed by lipid peroxidation reagents using TBARS assay. Anti-arthritic assay: chicken collagen, saline (0.9%), phosphate buffer pH 7.6. All other solvents and chemicals used were of analytical grade.

Methodology:

Preparation of Plant Extract: 2.5g of powdered and dried green tea sample was boiled in distilled water to obtain 2.5% of aqueous green tea extract. The plant extract was boiled 10-20 min and cooled. This obtained extract was then filtered using a filter paper. The obtained plant extract was used for further tests and assays. Four different varieties of flavored teas were chosen for studies which are: Chamomile tea (100% chamomile extract), hibiscus tea (100% hibiscus extract), ginger tea (8%w/w) and rose green tea (10.22% w/w). These sample extracts were prepared the same way as the green tea sample. 2.5 gram of the sample was weighed and boiled with 100 ml of distilled water. The obtained solution was cooled and filtered. The extracts were then obtained was used for further assays.

Estimation of Total Phenolics: Colorimetric estimation method was employed for the determination of total phenolics present in the different samples of tea [62]. The reaction mixture was prepared by pipetting 0.1 ml of different sample extracts and made up to 1 ml with water. 5 ml of 10% Folin-Ciocalteu‘s reagent and 4 ml 7.5% $NaHCO_3$ was added to the tubes containing the extracts.

Blank was prepared concomitantly by pipetting 1ml of water, 5 ml 10% Folin-Ciocalteu‘s reagent and 4 ml 7.5% of $NaHCO_3$. The solution was incubated at room temperature for 45 min and cooled. The absorbance was read at 670 nm using spectrophotometer. The test was performed in duplicates to obtain concordant values. A standard gallic acid solution of the concentration 0.25 mg/ml was prepared. This solution served as a standard for phenolics estimation.

The concentration of phenolics in the samples was calculated based on the measured absorbance in correspondence with gallic acid standard absorbance values obtained. The amount of phenolics in the extracts was conveyed in terms of standard solution of gallic acid equivalent (mg of gallic acid/ gram of extract).

Determination of Flavonoid Content:

Pre-treatment of Plant Sample: 5 ml of plant extracts were taken in different test tubes and treated with methanol: water (8:2) and mixed well. 10 ml of ether diluted in 2 ml of water was added to each test tube and shaken well. The ether layer was aspirated out and was evaporated to half the volume from the initial volume. 10 ml of chloroform was added to the evaporated sample

and shaken well. The chloroform layer was then separated from the immiscible solution. This solution was then evaporated to dryness. The dried sample was dissolved in 20 ml of methanol. This was used for further estimation of total flavonoids in the sample.

Estimation of Flavonoids: The amount of flavonoids in the extracts was determined colorimetrically. Determination of flavonoids was performed using aluminium chloride colormetric method [63]. 0.1 ml of tea extracts of concentration 2.5% was pipetted with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Blank was prepared by using 1 ml of methanol followed by 0.1 ml of 10% aluminium chloride, 0.1ml of 1M Potassium acetate and 2.8 ml of distilled water. The above solution was incubated at room temperature for 30 min. Absorbance was read at 415 nm using colorimeter. The flavonoid content is tea extracts was expressed with respect to quercitin equivalent (mg of QE/g of tissues) from the calibration curve prepared by using standard solution of quercitin of concentrations varying from 10 to 100 µg/ml in methanol.

Antioxidant Activity:

Inhibition of Lipid Peroxidation Assay by *Ex-vivo* Method: Lipid peroxidation assay was performed by *ex-vivo* method using live liver tissue in *ex-vivo* plates. Control was prepared by weighing 0.5g of liver tissue and treating with 1ml of 0.9% saline, 0.5 ml of 2 ppm lead acetate and incubated at room temperature for 60 min in one *ex-vivo* well. In another well, tea extract was added after half an hour of incubation with lead acetate and allowed to stand for half an hour and in another well, lead acetate and tea extract were added simultaneously and incubated for 60 min.

The solutions were taken in different test tubes and used for further assay. The supernatant solution was extracted and 2 ml of supernatant from each well of the ex-vivo plate was treated with 5 ml of glacial acetic acid and 0.5ml of 0.5% TBA. This solution was incubated in boiling water bath for 45 min and cooled. 0.05 ml of 5M HCl was added and absorbance was recorded at 535 nm using spectrophotometer. Percentage inhibition was calculated from the absorbance values.

Anti-Inflammatory Activity Assays:

Inhibition of Protein Denaturation: 0.5 ml of 1% BSA was used as control. Aspirin and diclofenac sodium were used as standard drugs that inhibit the protein denaturation process. 0.5 ml of BSA was treated with 0.1 ml of different tea samples and with drugs that were chosen for inhibition of denaturation. It was incubated at 51 ºC for 20 min. The solution was cooled and absorbance was read at 660 nm. Percentage inhibition was calculated [64, 65].

RBC Membrane Stabilisation Assay:

(a) Preparation of RBC Suspension: Anti-coagulated human blood (3 ml) was taken in equal volume of solution containing 2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% of sodium chloride was added to it. The solution was centrifuged at 10,000 rpm for 20 min. The pellet obtained was washed with isosaline and centrifuged as before for 10 min. 10% v/v suspension was treated with isosaline and the prepared RBC suspension was used for the membrane stabilization assay [66].

(b) Membrane Stabilisation Assay: Reference sample of different concentrations of aspirin and control was separately mixed with 1 ml of 0.15M phosphate buffer of pH 7.4, 2 ml of hyposaline and 0.5 ml of RBC suspension. Different concentrations of aspirin (0.1 mg/ml, 0.5 mg/ml and 1mg/ml) were used as standard drug concentrations. This solution was incubated at 37 ºC for 30 min followed by centrifugation at 3000 rpm. Supernatant was collected and estimated the amount of hemoglobin at 560 nm using spectrophotometer [66, 67]. The same was performed to determine activity of the samples under study by replacing aspirin with samples.

Inhibition of Collagen Denaturation Assay for the Evaluation of Anti-Arthritic Activity:

(a) Isolation of Collagen from Chicken Cartilage: Collagen was separated from the fore limb cartilages of chicken. Approximately, 0.2 g of collagen was weighed out and dissolved in 10ml of ice cold phosphate buffer (0.15M, pH 7.4) the collagen tissue was then macerated completely. The solution was kept under magnetic stirring for 10 min maintaining ice cold conditions at all times. This obtained solution was incubated along with phosphate buffer for 30 min. Centrifugation was

then performed for 10 min at 10,000 rpm. The supernatant was collected and inhibition of collagen denaturation assay was performed to determine the anti-arthritic activity of the samples.

(b) Inhibition of Collagen Denaturation Assay: 500 µl of collagen isolated from chicken cartilage was used. Aspirin was used as standard drug. The samples were added to collagen separately and incubated at 51 ºC for 20 min. Absorbance was read at 660nm using spectrophotometer. Percentage inhibition of collagen denaturation was calculated.

RESULTS AND DISCUSSION:

Total Phenolic Content in Tea Extracts:

TABLE 1: TOTAL PHENOLIC PRESENT IN TEA EXTRACTS

No. of samples	Tea samples	Total phenolic content (Mg GAE/G)
1	Chamomile	0.207 ± 0.0026
2	Ginger	1.022 ± 0.0098
3	Green tea	1.92 ± 0.0264
4	Hibiscus	0.362 ± 0.00608
5	Rose	0.524 ± 0.0015

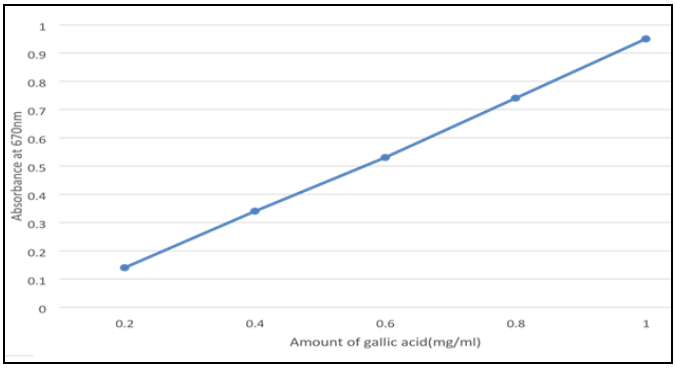

FIG. 1: CALIBRATION CURVE REPRESENTING THE STANDARD GALLIC ACID

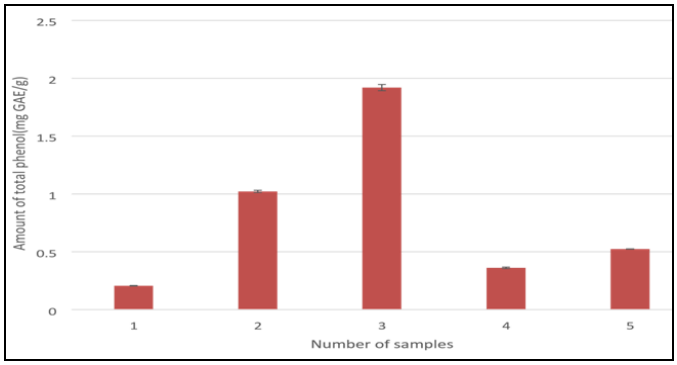

FIG. 2: TOTAL PHENOLIC CONTENT IN TEA EXTRACTS EXPRESSED IN TERMS OF GALLIC ACID EQUIVALENT. 1. CHAMOMILE TEA; 2. GINGER TEA; 3. GREEN TEA; 4. HIBISCUS TEA; 5. ROSE TEA. Represented in terms of mean ± SE (standard error).

Total Flavonoid Content in Tea Extract:

TABLE 2: TOTAL FLAVONOID CONTENT PRESENT IN TEA EXTRACTS

Tea Extracts	Total flavonoid Content (mg QE/g)
Chamomile tea	2.33 ± 0.0070
Ginger tea	10.9 ± 0.3
Green tea	24.7 ± 2.61
Hibiscus tea	9.33 ± 0.45
Rose tea	6.34 ± 0.48

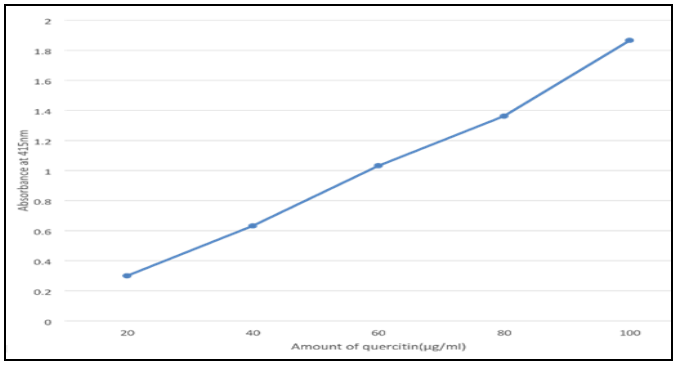

FIG. 3: CALIBRATION CURVE FOR FLAVONOID CONTENT

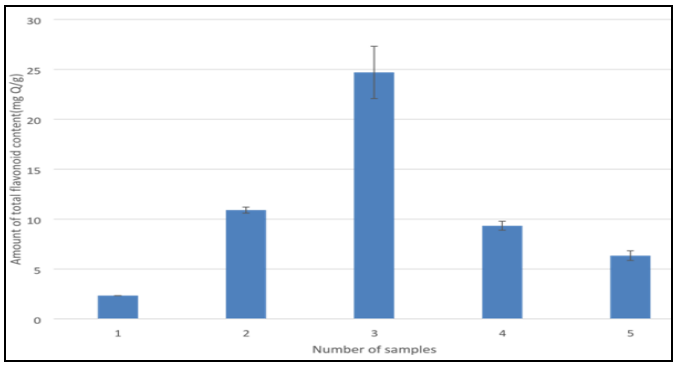

FIG. 4: TOTAL FLAVONOID CONTENT PRESENT IN TEA EXTRACTS. 1. CHAMOMILE TEA; 2. GINGER TEA; 3. GREEN TEA; 4. HIBISCUS TEA; 5. ROSE TEA. Represented in terms of mean ± SE (Standard error).

Ex-vivo Studies on Inhibition of Lipid Peroxidation by Tea Extracts:

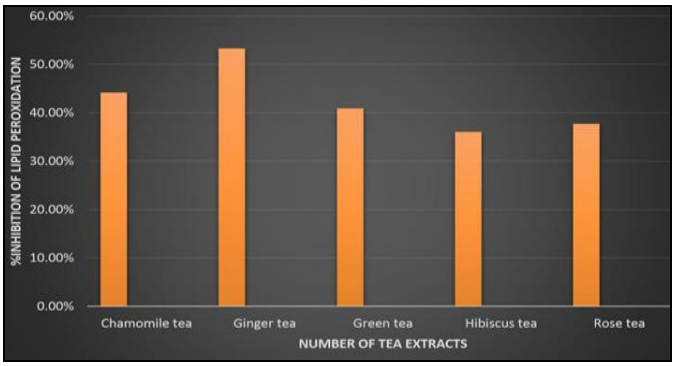

FIG. 5: INHIBITION OF LIPID PEROXIDATION IN CHICKEN LIVER TISSUE EXHIBITED BY TEA EXTRACTS STUDIED USING *EX-VIVO* METHOD

TABLE 3: PERCENTAGE OF INHIBITION OF LIPID PEROXIDATION BY TEA EXTRACTS USING *EX VIVO* METHOD

S. no.	Tea samples	% Inhibition of lipid peroxidation
1	Chamomile tea	44.15%
2	Ginger tea	53.24%
3	Green tea	40.90%
4	Hibiscus tea	36%
5	Rose tea	37.70%

Anti-Inflammatory Activity of Tea Extracts: Inhibition of Protein Denaturation by Tea Extracts:

TABLE 4: PERCENTAGE INHIBITION OF PROTEIN DENATURATION

Samples	% Inhibition of protein denaturation
Aspirin (0.1mg/ml)	68%
Aspirin (1mg/ml)	73%
Diclofenac sodium (0.5mg/ml)	88%
Diclofenac sodium (10mg/ml)	88%
Chamomile tea	88%
Ginger tea	91%
Green tea	91%
Hibiscus tea	56%
Rose tea	90%

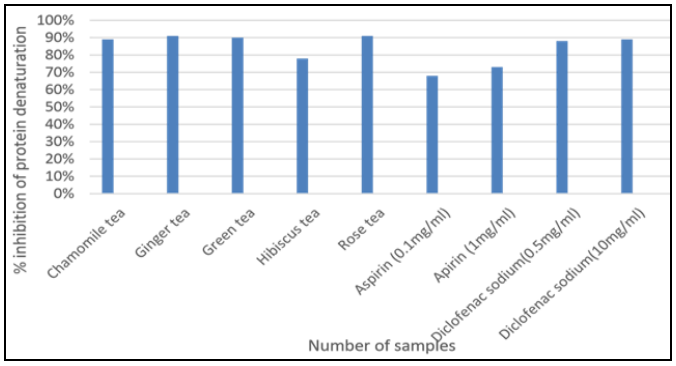

FIG. 6: PERCENTAGE INHIBITION OF PROTEIN DENATURATION EXHIBITED BY TEA EXTRACTS

HRBC Membrane Stabilisation Assay:

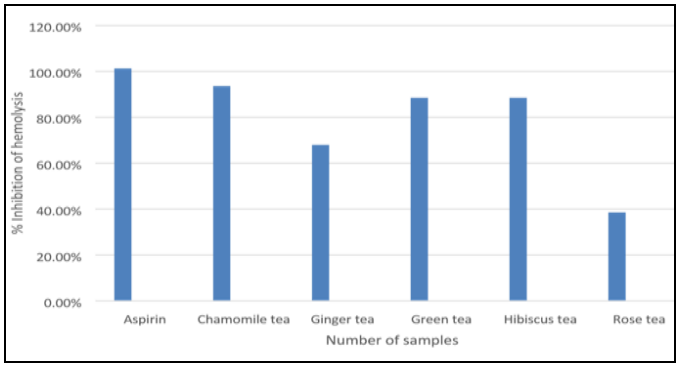

FIG. 7: GRAPHICAL REPRESENTATION OF PERCENTAGE INHIBITION OF HEMOLYSIS OF HRBC AND MEMBRANE STABILIZATION BY TEA EXTRACTS

TABLE 5: PERCENTAGE INHIBITION OF HEMOLYSIS OF HRBC FOLLOWED BY MEMBRANE STABILISATION BY TEA EXTRACTS COMPARED WITH STANDARD DRUG ASPIRIN

Samples	% Inhibition of hemolysis
Aspirin	101.28%
Chamomile tea	93.6%
Ginger tea	68%
Green tea	88.5%
Hibiscus tea	88.5%
Rose tea	38.5%

Anti-Arthritic Activity: Collagen Denaturation:

TABLE 6: PERCENTAGE INHIBITION OF COLLAGEN DENATURATION BY TEA EXTRACTS

S. no.	Tea samples	% Inhibition of collagen denaturation
1	Chamomile tea	5.1%
2	Ginger tea	8.5%
3	Green tea	14.9%
4	Hibiscus tea	6.9%
5	Rose tea	11.33%
6	Aspirin	36%

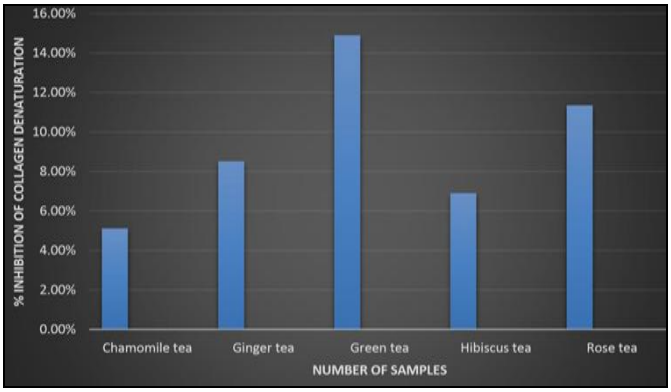

FIG. 8: INHIBITION OF COLLAGEN DENATURATION EXHIBITED BY TEA SAMPLES

SUMMARY: From this comparative study of five different tea extracts, variation in the content of phenols and flavonoids reflecting their greater activity of antioxidant, anti-inflammatory and anti-arthritic properties were determined. The live liver tissue was found to be protected from lipid peroxidation by the green tea extracts performed using the *ex-vivo* method.

The percentage inhibition of lipid peroxidation was shown to be highest in ginger tea exhibiting greater antioxidant property that is concerned with the scavenging of free radicals which are the sole reason for various cardio-vascular ailments causing direct damage to the cardiovascular tissues. Inflammation leads to the release of lysosomal enzymes, which have a damaging activity on

tissues leading to lipid peroxidation in macromolecular biological membranes. When these biological membranes containing polyunsaturated fatty acids are damaged by lipid peroxidation, they proceed through symptomatically, without indicating the patho-logical conditions such as heart attacks, septic shocks and arthritis.

The percentage inhibition of protein denaturation was found to be directly proportional to prevent inflammation. Maximum inhibition of protein denaturation was shown by ginger tea, green tea and rose tea comparatively. Lysosomal membrane stabilisation plays a prominent role in preventing inflammation caused by activated neutrophils which releases inflammatory mediators such as proteases. On release of these enzymes to the extracellular environment causes greater tissue damage. The maximum percentage stabilisation of HRBC membrane was found to be exhibited by chamomile tea reflecting the anti-inflammatory property.

Arthritis is one of the major disorders manifested by inflammation. Arthritis is characterized by events; primarily inflammation, secondly cartilage destruction. Reduction of inflammation can be brought about by daily consumption of green tea in turn reducing the cartilage destruction which is further responsible for decreasing the joint pain. Several studies have shown that the presence of large amount of collagen in the cartilage renders the cartilaginous properties such as flexibility, elasticity, rigidity and helps in easy movement of hands and legs. Heat induced protein denaturation would cause the collagen to lose its optimum functionality structure leading to cartilage destruction. Inhibition of collagen denaturation was observed by the tea extracts proving that the extracts chosen have anti-arthritic property and maximum percentage inhibition was exhibited by green tea.

CONCLUSION: The antioxidant, anti-inflammatory and anti-arthritic property of tea extract samples were evaluated efficiently using *in-vitro* and *ex-vivo* methods. The aimed parameters are very essential to maintain homeostatic conditions of the body. Each parameter is interconnected for beneficial management of cardiovascular system.

ACKNOWLEDGEMENT: We thank the institution for providing us all facility

CONFLICT OF INTEREST: There is no conflict of interest in this work.

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How to cite this article:
Singh KG, Sonia S and Konsoor N: *In-vitro* and *ex-vivo* studies on the antioxidant, anti-inflammatory and antiarthritic properties of *Camellia sinensis*, *Hibiscus rosa sinensis*, *Matricaria chamomilla*, *Rosa sp*., *Zingiber officinale* tea extracts. Int J Pharm Sci & Res 2018; 9(8): 3543-51. doi: 10.13040/IJPSR.0975-8232.9(8).3543-51.