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Antioxidant and Anti-inflammatory Properties of a Nontoxic Herbal formulation – GSTC

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Abstract: Chronic inflammation is an etiological factor in most human degenerative diseases and oxidative stress, secondary to the inflammation is thought to contribute to the severity. Therefore, molecules having antioxidant and anti-inflammatory properties with lesser side effects are indeed essential. It has been recommended that combinations of different antioxidants may have a better biological efficacy than a single drug. Present study envisions a synergistic or additive effect of four medicinal plants, *Commiphora mukul* (Hook. ex Stocks.) Eng. (Burseraceae), *Salacia reticulata* Wight (Celastraceae),*Terminalia arjuna* (Roxb.) Wight & Arn (Combretaceae) and *Curcuma longa* Linn (Zingiberaceae) in a formulation (GSTC) at equimolar concentrations. In the preliminary analysis, GSTC was found to scavenge superoxide, hydroxyl, DPPH radicals and inhibited lipoxygenase mediated oxygenation of linoleic acid and peroxyl radical mediated peroxidation of human erythrocyte membrane lipids *in vitro*. In rats, acute oral dose of GSTC upto 2 g/kg bwt was nontoxic and at a concentration of 100 mg/kg bwt, reversed sodium fluoride mediated decrease in antioxidant molecules and enzymes activity. Further, GSTC at doses of 250 and 500 mg/kg bwt orally, reduced carrageenan induced paw oedema in mice.Overall, GSTC having considerably high antioxidant and anti-inflammatory potential together with its nontoxic nature finds promise as a therapeutic drug candidate in various chronic inflammatory diseases.
Keywords: Lipoxygenase, inflammation, oxidative stress, degenerative diseases.

Introduction

Cellular metabolic processes most often result in generation of free radicals. These free radicals are usually reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals and non-radicals like hydrogen peroxides and lipid hydroperoxides. Inflammatory reactions are considered a major source for oxidative radical generation [1]. At low or moderate concentrations, ROS can exert beneficial effects on cellular responses to infections and even gene expression. However, an overwhelming production of these species generates oxidative stress that can damage lipids, proteins and DNA [2].

Many degenerative diseases including cancer, atherosclerosis, Alzheimer's, Parkinsonism and arthritis have oxidative stress and inflammation as etiological factors. Current preventive and curative approaches of these ailments are hindered by wide range of side effects and toxicity of individual drugs. Although antioxidants are highly suggested mainly based on the results of animal experimentations, sufficient clinical success has not been achieved so far. Therefore, it is thought that compounds with antioxidant and anti-inflammatory effects having different biological spectrum properties, pertinent to the control of common risk factors, in combination

could be a choice for better preventive measures of chronic diseases. Plants are increasingly being used as a source of antioxidants because of their bioavailability and lesser side-effects. Population studies have indicated that frequent consumption of natural antioxidants, particularly in fruits and vegetables are associated with lower risk of cardiovascular disease and cancer [3,4]. It has been suggested that the synergistic/additive action of a wide spectrum of antioxidants may have better potential than a single antioxidant [5].

In this study, we evaluate the potential of a combination of four well-known medicinal plants, *Commiphora mukul* (Hook. ex Stocks.) Eng. (Burseraceae), *Salacia reticulata* Wight (Celastraceae), *Terminalia arjuna* (Roxb.) Wight & Arn (Combretaceae) and *Curcuma longa* Linn (Zingiberaceae) extensively used in the indigenous medicinal system, Ayurveda. The selection of these medicinal plants in combination is purely based on the reported efficacy of the individual plant extracts in scientific literatures. For instance, several experimental and clinical studies have reported that "guggulu' gum of *Commiphora mukul* lowers blood cholesterol level and decreases high blood pressure, reduce low-density lipoproteins while elevating the beneficial high-density lipoproteins [6]. Most of its biological efficacy is thought to be due to its reported chemical constituents such as phytosterols, guggul lipids and guggulsterones [7]. Further, it is very important to note that various medicinal preparations with guggul are used in the treatment of arthritis, skin diseases, obesity, digestive problems, infections in mouth and menstrual problem. *Salacia reticulata* extract is highly suggested to be antidiabetic by virtue of its inhibitory effect on intestinal maltase and glucosidase and prevents the digestion and absorption of carbohydrates. In chemical investigations, mangiferin (a xanthone), three catechins: (-)-epicatechin, (-)-epigallocatechin and (-)-4'-*O*- ethylepigallocatechin and two catechin dimmers were isolated [8]. *Terminalia arjuna* another component of the formulation under study is a locally available tree species in India, the bark extract of which has been shown to enhance cholesterol turnover in the liver and lowers β-lipoprotein lipids during hyperlipidemia[9]. Similarly *Curcuma longa*, a rhizome known for its active ingredient curcumin has traditional use in India and China, particularly, as an anti-inflammatory agent and for the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage and colic disease. Several other studies have proved the antioxidant and anti-inflammatory [10], hepatoprotective [11], anti-carcinogenic [12] and antimicrobial properties [13] of *Curcuma longa* rhizome. It is thus expected that a logical combination of these plant extracts may exert a more powerful effect and could be useful in many degenerative diseases in humans. Here, in a preliminary analysis, individual plant extracts at equimolar concentrations has been combined (GSTC), and its antioxidant and anti-inflammatory properties are studied in detail. The preparation of individual extract followed the same procedures as that described in several reported studies. The nontoxic nature of the individual plant extracts assure an overall safety for the herbal combination.

Materials and Methods

Plant materials:

Gum resin of *Commiphora mukul*, root of *Salacia reticulata*, bark of *Terminalia arjuna* and rhizome of *Curcuma longa* were obtained during the months of May–June 2012, from the Preparation Unit of Amala Ayurvedic Hospital and Research Centre, Thrissur, Kerala. They were authenticated by the Botanical Survey of India using herbarium sheets and voucher specimens were maintained with the BSI Sp. Nos: 77360, 61127, 43886 and 63180, respectively at the herbarium of Amala Ayurvedic Research Centre.

***Preparation of extracts*:**

The plant parts were air-dried separately in an oven at 40ºC, powdered using grinder and used for extraction. *C. mukul* was extracted using petroleum ether, *S. reticulata* using water, *T. arjuna* and *C. longa* using methanol. Approximately, 20 g of each powder was extracted with 150 mL of respective solvent using a magnetic stirrer for 72 h; changing solvent every 24 h. Each time the supernatants obtained by centrifugation were collected, pooled, concentrated and evaporated off the solvents in a water bath. Individual extracts were stored separately in air-tight bottles and kept below 4ºC. For preparation of GSTC, equal weight of individual plant extracts was mixed. *S.reticulata* root extract was dissolved in water while the remaining three were dissolved in minimum amount of dimethyl sulphoxide (DMSO). The equimolar solutions were mixed together and made up to a known concentration for various *in vitro* studies. For animal studies, *S. reticulata* was dissolved in water while the others were dissolved in minimum amount of propylene glycol. The formulation was then made up to the desired concentrations with water.

Animals:

Female Wistar rats weighing around 150 to 180 g and male Swiss Albino mice (25-30 g) were purchased from Small Animal Breeding Station, University of Veterinary Sciences, Mannuthy, Kerala,

maintained under standardized environmental conditions (22-28ºC, 60-70% relative humidity, 12 h dark/light cycle) and fed with standard rat feed (Sai Durga Feeds, Banglore, India) and water *ad libitum.* All the experiments conducted during the present study had prior permission from Institutional Animal Ethics Committee (IAEC) and strictly followed the guidelines of Animal Ethics Committee, Govt. of India.

DPPH radical scavenging activity:

Various concentrations of GSTC (10-100 µg/mL) was added to 1.5 mM, 2,2-diphenyl-1-1-picrylhydrazyl (DPPH) solution in a final volume 1 mL and incubated for 20 min [14].The absorbance of untreated and treated tubes were read at 517 nm against methanol as blank. Vitamin C was used as positive control.

Superoxide anion scavenging activity:

The superoxide radical scavenging activity of GSTC was determined by nitroblue tetrazolium (NBT) reduction method [15]. Vitamin C was used as positive control. Also, control tubes in which GSTC was added at the end of 15 min incubation period were simultaneously kept to confirm the results.

Hydroxyl radical scavenging activity:

Hydroxyl radicals generated from Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction) degrade deoxyribose that resulted in thiobarbituric acid reactive substance (TBARS) which was then estimated [16]. The TBARS formed was estimated by TBA method [17]. The hydroxyl radical scavenging activity was determined by comparing absorbance of control tubes with that of treated tubes. Vitamin E was used as positive control.

Erythrocyte membrane damage assay:

Blood was obtained from blood bank donors of Amala Hospital. Following rigorous washing with PBS, the erythrocytes were re-suspended in PBS to get a hemocrit volume of 20%. Herbal formula at different concentrations (10-100 µg/mL) was added to erythrocyte suspension and incubated for 15 min at 37ºC before the addition of 200 mM of 2,2'-azobis (2-amidino propane) hydrochloride (AAPH). This mixture was incubated for 3 h at 37ºC in order to induce the free radical chain oxidation in erythrocytes, mediated by the peroxyl radicals generated by decomposition of AAPH [18]. Vitamin C was used as positive control.

Lipoxygenase assay:

This assay is based on 15-lipoxidase (15-LO) mediated reaction between oxygen and linoleic acid. The assay system consists of lipoxygenase 50 U and linoleic acid (100 nmole) in a total volume of 1 mL with phosphate buffered saline (PBS). The reaction was allowed to take place at room temperature for 1 h. The conjugated diene formed was monitored spectrophotometrically by an increase in absorption at 234 nm. Various concentrations (10-100 µg/mL) of GSTC were added to the assay system in 5 µL volume and IC_{50} values were determined from three consecutive experiments. Vitamin C was used as positive control.

Acute toxicity analysis:

Ten healthy Wistar rats were divided into two groups consisting of five animals each. One group served as vehicle control, while the other received a single 2 g/kg body weight dose of GSTC drug orally. The animals were observed for a period of two weeks for physiological abnormalities such as diarrhea, hair loss, mortality, abnormal behavior and changes in body weight.

Also blood parameters including hemoglobin (Drabkin's method: Agappe Diagnostics, Mumbai) and total WBC count were assessed during this period.

Protective effect against sodium flouride-induced oxidative stress:

The antioxidant activity *in vivo* was determined in a sodium fluoride induced model of oxidative stress [19]. Sprague-Dawley rats of average weight 170 to 200 g were divided into five groups of 6 animals each. Group I was kept as normal, without treatment. Group II, III and IV received 600 ppm sodium fluoride (NaF) in their drinking water for 7 days. Group II animals were kept as untreated control and GSTC at 100 and 500 mg/kg bwt 4 days prior and thereafter simulataneously with NaF were administered orally to groups III and IV animals. Group V served as positive control and was given ascorbic acid (3 mmol/L) in drinking water. All

animals were sacrificed on the 11th day following overnight fasting and various antioxidant enzymes activity and TBARS in the serum and liver tissues were evaluated.

Anti-inflammatory study:

Female Swiss albino mice were divided into 5 groups comprising of 6 animals each. In all groups, acute inflammation was induced by sub-plantar injection of 0.02 mL freshly prepared 1% suspension of carrageenan in 0.1% carboxymethyl cellulose in the right hind paw of mice [20, 21]. Group I with carrageenan alone served as untreated control. Group II was administered with diclofenac (10 mg/kg bwt) intra-peritoneally as reference drug. Group III served as vehicle control and received propylene glycol of the same concentration as that used for making the drug doses. Group IV and V were treated with 250 and 500 mg/kg bwt, respectively of GSTC orally for 5 consecutive days prior to carrageenan injection. The paw edema formed was measured using vernier calipers 1 h before and 6 h after carrageenan injection.

***Statistical analysis*:**

The *in vitro* assays were carried out at least three times and the values were expressed as mean ± SD. Statistical analysis of data was performed using ANOVA followed by student t-test to study the differences amongst the means. Values of $P < 0.05$ were considered as statistically significant.

Results and Discussion

Oxygen derived free radicals formed during metabolic reactions as well as secondary to inflammatory responses are thought to be involved in many disease pathologies. In the present study, GSTC was tested in comparison with known anti oxidants, vitamin C and E in the reductive, scavenging or inhibition of free radicals and radical mediated peroxidation using various *in vitro* assay systems. The stable free radical DPPH (2, 2-diphenyl-1-picryl hydroxyl) was effectively reduced by the addition of GSTC in a dose dependent manner (Figure-1a). The IC_{50} value calculated was 26 µg/mL. Vitamin C, a known reducing agent had an IC_{50} value of 2.5 µg/mL in the same system. The reduction of DPPH radical to DPPH-H indicated the hydrogen donating efficacy of GSTC. The reported IC_{50} values of individual extracts used in the formulation are 26.92 µg/mL (*C. mukul*), 29 µg/mL (*C. longa*), 8.3 µg/mL (*T. arjuna*) and <10 µg/mL (*S. reticulata*) [22, 23, 24, 25]

Superoxide generated in the photo reduction of riboflavin was effectively inhibited by the addition of varying concentrations of GSTC (Figure-1b). The concentration of the extract required to scavenge 50% superoxide anion was found to be 38 µg/mL. Vitamin C, which was used as a positive control had an IC_{50} value of 54 µg/mL. The inhibitory efficacy is therefore far better than vitamin C, a known antioxidant. This strongly suggests the potential of GSTC as radical scavenging agent.

Degradation of deoxyribose induced by hydroxyl radical generated from Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Figure-1c) was higher in the untreated control tubes as observed by the increased TBARs level. Addition of GSTC at concentrations ranging from 50-350 ng/mL reaction was found to significantly lower TBARs formation. The percentage inhibition was calculated with respect to control tubes which indicated a dose dependent inhibition. The efficacy of GSTC (IC_{50} value 250 ng/mL) was higher than that of vitamin E (IC_{50} value 7 µg /mL), a known chain breaking antioxidant.

AAPH release peroxyl radicals continuously in the assay system and induce membrane lysis of human erythrocytes. This is an indication of membrane lipid peroxidation. In the untreated control tubes, erythrocyte lysis was maximum at 3 h period following the addition of AAPH. The percentage decrease of membrane lysis with respect to control tubes was dose dependent. The concentration required for 50% inhibition was found to be 32 µg/mL (Figure-1d). Vitamin C used as positive control exhibited an IC_{50} value of 11 µg/mL. This clearly demonstrates the anti-lipid peroxidative effect of GSTC as well as its nontoxicity towards blood elements.

To confirm its anti-lipid peroxidative effect, another *in vitro* assay system was chosen wherein, Soybean 12/15 lipoxygenase introduce oxygen atom at unsaturated bonds of linoleic acid generating lipid hydroperoxide radicals and alcohols having conjugated diene structure. Lipoxygenase is an important enzyme in the production of leukotrienes in the arachidonate pathway. Therefore, this assay indicates the efficacy of additives as antioxidant, anti-lipidperoxidative and anti-inflammatory molecule. In the present study, the conjugated diene formation monitored at 234 nm (conjugated diene) in the untreated control tubes were high following one hour of incubation at room temperature. While in the treated tubes having various concentrations

of GSTC (0-100 µg/mL), conjugated diene content was reduced depending upon the dose. The IC_{50} value calculated was 45 µg/mL. The inhibitory effect of vitamin C (positive control) was equal in magnitude to that of the extract having an IC_{50} value of 50 µg/mL (Figure-2). Since in the combined form, GSTC, the percentage composition of individual extract is only 25% of total weight, it is assumed that GSTC possess a synergism of all four extracts.

Figure-1: GSTC shows free radical scavenging activity *in vitro*. Various concentrations of GSTC were added to (a) DPPH radical scavenging activity assay system. (b) Superoxide radical scavenging activity (c) Hydroxyl radical scavenging activity and (d) AAPH induced membrane damage. The percentage inhibition was calculated with respective control tubes. Values are ± SD of three different experiments.

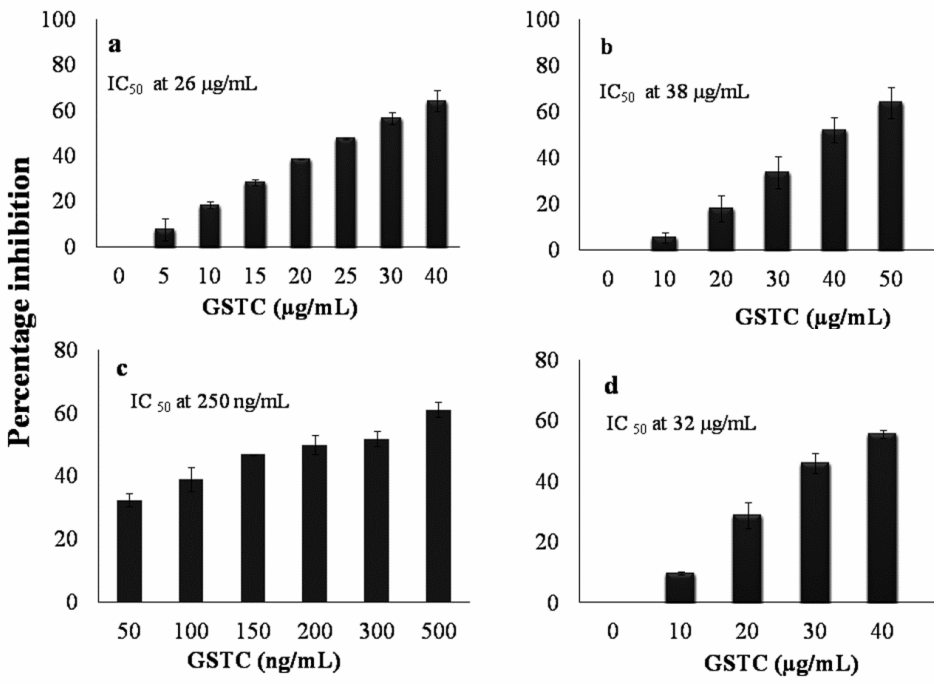
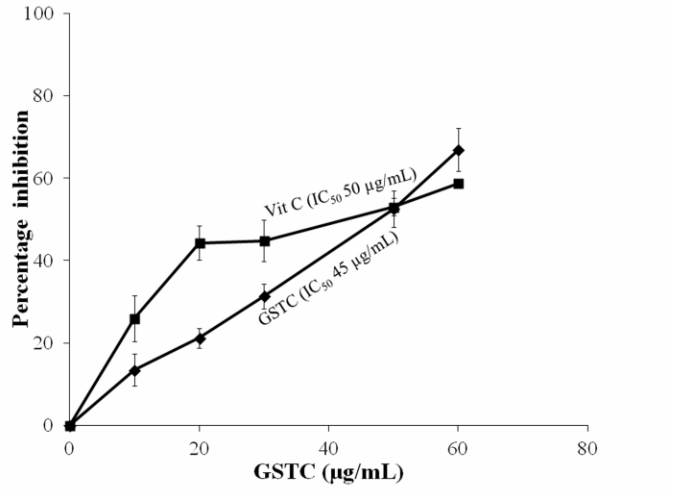


Figure-2: GSTC inhibits the activity of lipoxygenase. The reaction mixture contained linoleic acid (100 nmol/mL) and lipoxygenase at 50 U/mL in PBS, pH 7.4. GSTC at 10-60 µg/mL was added to the reaction mixture and incubated for 1 h at room temperature. Conjugated diene formed was measured based on the absorbtivity value at 234 nm. Percentage inhibition of conjugated diene formation was then calculated with respect to control tubes in which no drug was added. Vitamin C was the positive control. Values are ± SD of three independent experiments.



It is always essential to make sure that the chemicals/molecules are nontoxic in animals before undertaking any animal experiments. Therefore, an acute toxicity assessment was carried out using Wistar rats to better confirm the nontoxicity of the formulation. Administration of GSTC at a single dose of 2 g/kg bwt did not produce mortality, up to a period of two weeks. There were no unusual changes in behavior or locomotor activity of animals. The body weight was maintained without much variation (Figure-3) throughout the period and no diarrhea or hair loss was observed. No significant changes were produced in hematological parameters such as hemoglobin concentration and total count of leukocytes (Table-1).

Figure-3: Effect of administration of GSTC 2 g/kgbwt on body weight of rats. Propylene glycol as vehicle control and GSTC were administered and body weights of rats were recorded over a period of 14 days.

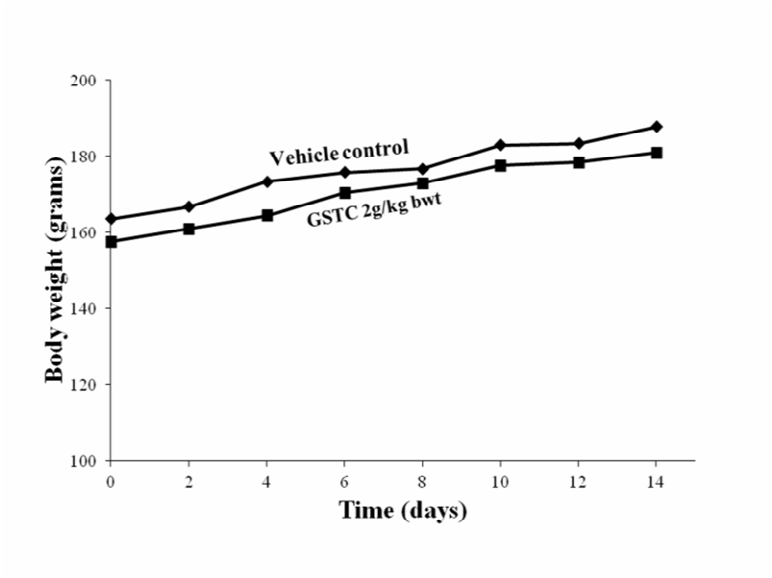


Table-1: Effect of administration of GSTC 2 g/kg bwt on haematological parameters of rats.

Group	Hb (g/dL)	WBC (cells/mm^3)
Vehicle control	13.26 ± 0.601	6832.5 ± 617.43
GSTC 2 g/kg bwt	13.66 ±1.87	6787.5 ± 532.87

Values are mean ±SD of 6 animals in each group.

In order to make sure that the observed antioxidant effect in *in vitro* studies are extended *in vivo*, sodium fluoride intoxication study was carried out. The results showed that the activities of catalase and superoxide dismutase and glutathione levels were moderately decreased in the serum of sodium fluoride treated rats. Administration of GSTC significantly improved the activities of these enzymes and maintained GSH level (**Table-2).** In liver tissue, the activity of catalase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase and level of glutathione were significantly increased in GSTC treated groups compared to untreated control where substantial decrease in the activities of these enzymes was observed as shown in Table-3.

In the *in vitro* assay system, the anti-lipoxygenase efficacy of GSTC was revealed which indicated the anti-inflammatory potential. To further confirm this, an acute anti-inflammatory model using carrageenan in mice was conducted. The intra-dermal administration of carrageenan in mice paw produced an acute local inflammatory response which created an edema of maximum intensity 2.76 ± mm at the third hour following administration. Animals that received 250 and 500 mg/kg bwt doses of GSTC had reduced edema during 3 h following carrageenan administration (1.6 ± mm). The percentage inhibition was 42% in both cases. Diclofenac used as reference drug exhibited an inhibition of 72% (Figure-4) Lipoxygenase pathway in arachidonate metabolism produce important inflammatory molecules such as leukotrienes. Carrageenan is thought to induce inflammatory response through the same arachidonate metabolic pathway[26]. Therefore, the present observations suggest that the lipoxygenase inhibitory activity of GSTC could be the reason for its anti-inflammatory activity.

Table-2: Serum level of various antioxidant enzyme activities in sodium fluoride ingested rats following treatment with GSTC.

Treatment	SOD (U/g Hb)	Catalase (U/g Hb)	Glutathione (nmol/mL)
Normal	97.71 ± 3.40	27.17 ± 2.15	38.94 ± 2.13
NaF 600 ppm	86.69 ± 12.47	11.22 ± 1.73	28.35 ± 1.92
NaF + GSTC (100 mg/kg bwt)	154.49 ± 15.54*	18.30 ± 3.121*	36.80 ± 6.59*
NaF + GSTC (500 mg/kg bwt)	159.71 ± 19.94*	17.42 ± 2.30*	30.70 ± 8.38*
NaF +Vit.C (3mmol/L)	161.54 ± 27.98*	33.18 ± 6.24**	47.02 ± 2.29**

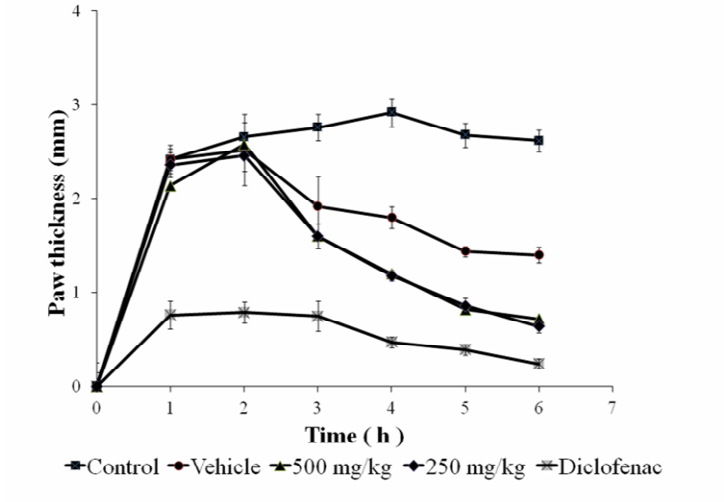
Values are mean ±SD of 6 animals in each group. * indicate P<0.05,** P<0.01, ^{ns} =not significant

Table-3: Liver tissue level of various antioxidant enzyme activity in sodium fluoride ingested rats following treatment with GSTC.

Treatment	SOD (U/mg protein)	TBARs (nmol/mg protein)	GSH (nmol/mg protein)	Glutathione peroxidase (U/mg protein)	Glutathione-S-transferase [a] (U/mg protein)
Normal	1.990 ± 0.31	1.54 ± 0.13	2.95 ± 1.05	9.26 ± 2.54	25.17 ± 8.02
NaF 600 ppm	1.137 ± 0.16	2.18 ± 0.87	2.53 ± 1.14	8.21 ± 1.05	22.23 ± 7.18
NaF + GSTC (100mg/kgbwt)	3.10 ± 0.03**	0.86 ± 0.11**	2.70 ±0.83*	13.99 ± 1.97*	108.83 ± 22.67**
NaF + GSTC (500mg/kgbwt)	1.79 ± 0.12**	1.27 ± 0.17***	2.12 ± 1.05*	9.20 ± 0.91^{ns}	66.27 ± 5.96*
NaF +Vit. C (3 mmol/L)	3.26 ± 0.60**	1.58 ± 0.40**	4.81 ± 0.47**	13.94 ± 1.92*	161.23 ± 31.83**

Values are mean ±SD from 6 animals in each group.* indicate P<0.05,**P<0.01, ^{ns}=not significant. [a] nmol of CDNB conjugate formed/min/mg protein.

Figure-4: Effect of GSTC on carrageenan-induced paw edema. Carrageenan was administered through dermal route at hind paw of mice following 5 days of GSTC administration (250 and 500 mg/kg bwt orally). Thickness of the paw edema was measured using vernier callipers following carrageenan administration. Diclofenac was used as positive control. Values are mean ± SD for 6 animals in each group.



Conclusion

Present study thus reveals the radical scavenging, anti-lipid peroxidative and anti-inflammatory potential of the herbal formulation, GSTC in *in vitro* assay systems. Further in animal models, sodium flouride induced oxidative stress and carrageenan induced inflammatory response was inhibited. The efficacy is likely to be better than the individual plant extracts used in the combination as well as the reported efficacy of other individual plant extracts. In addition, GSTC is found to be nontoxic in animals. Therefore it is suggested that GSTC exhibit a synergism of the efficacy of individual plant extracts. The antioxidant and anti-inflammatory property of GSTC may be promising in many chronic inflammatory human ailments. Different combinations of these four extracts apart from the present formulation need to be studied for further evaluation.

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