Phytochemical investigation and evaluation of Anti-inflammatory activity of Camellia sinensis (Green Tea) leaves in Experimental Animals

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Abstract **- Present work to evaluate anti-inflammatory activity of Camellia sinensis (Green Tea). Pharmacological studies were carried out with methanol-water (1:1) extract of green tea (Camellia sinensis) leaves extract. Leaves extract was found to possess anti-inflammatory, analgesic and antipyretic activities at 1/10th of its LD50 dose of 40 mg/kg i.p. Phytochemical parameter also perform like extractive value, ash value, loss on drying etc. to check the standardization of plant extract. It was found that leaves extract inhibited the arachidonic acid-induced paw edema in rats which indicated that leaves extract produced the anti-inflammatory activity by inhibiting both the cyclooxygenase and lypooxygenase pathways of arachidonic acid metabolism. Leaves extract enhanced peritoneal cell count and the number of macrophages in normal mice. It is possible that the saponins present in leaves extract may be responsible for these activities of Green tea leaves extract.**

Index Terms **- Camellia sinensis; anti-inflammatory; Arachidonic acid; Standardization.**

1.INTRODUCTION

The International Association for Study of Pain (IASP) defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” [2]. It is to be noted that pain is not just a physical sensation. It is also an emotional experience. It varies from person to person and in the same person from time to time. Management of pain has to take this fact into consideration. The patient must be believed about the pain. It is the physician’s duty to relieve suffering. In addition, unrelieved pain can cause physical damage too. For one thing, it would worsen the pain experience by muscle spasm, peripheral and central sensitization and recruitment and by muscle spasm. Unrelieved acute pain can cause chronic pain, and long-standing pain can cause anatomical and even genetic changes in the nervous system. [3]

The Green tea is obtained from the tea plant Camellia sinensis belongs to the family Theaceae. Tea is the most consumed drink in the world after water. Green tea is a ‘non-fermented’ tea and contains more catechins than black tea or oolong tea. Catechins are in vitro and in vivo strong antioxidants. In addition, its content of certain minerals and vitamins increases the antioxidant potential of this type of tea. Presently, it is cultivated in at least 30 countries around the world. Tea beverage is an infusion of the dried leaves of Camellia sinesis. It is a widely used medicinal plant by the trials throughout India, China and popular in various indigenous system of medicine like Ayurveda, Unani and Homoeopathy Green tea has been consumed throughout the ages in India, China, Japan and Thailand.

Fig. 1: Camellia sinensis plant (leaves)

The chemical composition of green tea is complex: proteins (15-20 % dry weight), whose enzymes constitute an important fraction; amino acids (1-4 % dry weight) such as theanine or 5-N-ethylglutamine, glutamic acid, tryptophan, glycine, serine, aspartic acid, tyrosine, valine, leucine, threonine, arginine, and lysine; carbohydrates (5-7% dry weight) such as cellulose, pectins, glucose, fructose, and sucrose; minerals and trace elements (5% dry weight) such as calcium, magnesium, chromium, manganese, iron, copper, zinc, molybdenum, selenium, sodium, phosphorus, cobalt, strontium, nickel, potassium, fluorine, and aluminum; and trace amounts of lipids (linoleic and α-linolenic acids), sterols (stigmasterol), vitamins (B, C, E), xanthic bases (caffeine, theophylline), pigments (chlorophyll, carotenoids), and volatile compounds (aldehydes, alcohols, esters, lactones, hydrocarbons). Due to the great importance of the mineral presence in tea, many studies have determined their levels in tea leaves and their infusions [4]. Fresh leaves contain, on average, 3-4 % of alkaloids known as methylxanthines, such as caffeine, theobromine, and theophylline. In addition, there are phenolic acids such as gallic acids and characteristic amino acid such as theanine present [5].

2. MATERIALS & METHODS

Chemicals and drugs

CS preparation was obtained from Laxminarayan tea company Pvt. Ltd. Gwalior. The standard anti-analgesic drug Aspirin (Reckitt Benckiser, India) was purchased from retail pharmacy; Methanol, Chloroform, Ethanol, Molisch's reagent, copper sulfate, sodium hydroxide, Biuret reagent, Iodine solution, Sulfuric acid, Glacial acetic acid, Ferric chloride, Metal magnesium, Hydrochloride acid, Myer's reagent (Potassium Mercuric Iodide) was obtained from institutional store and was of analytical grade.

Phytochemical studies

Standardization of CS preparation

Determination of Ash Value

The determination of ash value is meant for detecting low grade products, exhausted drugs and sandy or earth matter. It can also be utilized as a means of detecting chemical constituents by making use of water soluble ash and acid insoluble ash [6].

Total Ash value

Air dried powder of C. sinensis was weighed in a silica crucible and incinerated at a temperature not exceeding 450°C until free carbon, cooled and weighed and then the percentage of total ash with reference to the air dried powdered drugs was calculated.

Acid Insoluble Ash

The ash obtained in the above method was boiled for 5 minutes with 25 ml of dilute HCL. The residue was collected on ash less filter paper and washed with hot water, ignited and weighed. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

Water Soluble Ash

The ash obtained on total ash was be boiled for 5 minutes with 25ml of water. The insoluble matter was collected on ash less filter paper and washed with hot water and ignited to constant weight at a low temperature. The weight of insoluble matter was subtracted from the weight of ash. The % of water soluble ash with reference to the air dried drug was calculated.

Loss on Drying (moisture content)

Loss on drying is the loss in weight in % w/w resulting from water and volatile matter of any kind that can be driven off under specified conditions. A glass stoppered dried shallow bottle was weighed. To the bottle specific quantity of sample will be transferred, bottle and its contents were weighed. The sample was distributed as evenly as practicable by gentle sidewise shaking to a depth not exceeding 10 mm. The load bottle was placed in the oven and stopper was removed. The sample then dried to the constant weight for the specific time. The bottle and content were weighed.

Determination of Extractive Value
Extractive value is the amount of active constituents extracted with solvents from a given amount of medicinal plants material.

Alcohol (methanol) Soluble Extractive
4 g of coarsely air dried powdered of C. sinensis was macerated with 100 ml of alcohol in a closed flask for 24 hr, shaking frequently for 6hr and allowed to sand for 18 hr. It was then filtered rapidly taking precaution against loss of alcohol. 25 ml of the filtrate was evaporated to dryness in tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air dried drug.

Water (distilled) Soluble Extractive
4 g of coarsely air dried powdered of C. sinensis was macerated with 100 ml of chloroform water in a closed flask for 24hr, shaking frequently for 6hrand allowed to stand for 18hr. It was then filtered rapidly taking precaution against loss of water. 25 ml of the filtrate was evaporated to dryness in tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air dried drug.

Determination of pH Value
The pH of an aqueous liquid may be define as the common logarithm of reciprocal of the hydrogen ion concentration expressed in gram per litre. The pH of liquid was determined potentially by means of glass electrode and a suitable pH meter.

Preliminary Phytochemical Study
Preliminary phytochemical screening of formulation was carried out to identify the presence and absence of various phytoconstituents like flavonoid, alkaloid, carbohydrates etc in selected formulation [6]. This study was done by taking extraction of CS.

Preparation of extract
About 20gm of CS powder was soaked in 100 ml methanol and allowed to macerate for 48 hours and then filtered. Thus the filtered extract was stored at 4°C until used.

Test for different chemical group: The extracts of the plant material was subjected to various chemical tests for identification of its chemical constituents. Such as tests for alkaloids, carbohydrates, glycosides, fixed oils, tannins and phenolic compounds, saponins etc.

Test for Carbohydrate (Molish Test): To 1 ml test solution,1 drops of the Molish reagent (a solution of α-naphthol in 95% ethanol) was added. The concentrated sulphuricacid added from side of the test tube to observe the formation of violet ring at the interface of two liquid.

Test for Protein (Biuret test): It was used to determine the presence of peptide bonds in protein. To 3 ml of test sample, 3% NaOH and few drops of 1% CuSO4 solution were added. The formation of violet colour was observed for the presence of protein.
Test for Starch: 3 ml test solution was mixed with few drops of dilute iodine solution. Blue color appears. It disappears on boiling and reappears on cooling.

Test for Amino Acid: To 2.5 ml of test sample solution add few drop of 40 % NaOH and 10% lead acetate boiled the solution formation of black precipitate show the presence of amino acid.

Test for Steroid: To 1 ml of extract, 1 ml of chloroform and 1 ml conc. H2SO4were added. Shaken well, chloroform layer appears red and acid layer shows greenish yellow florescence.

Test for Glycoside: To the solution of the extract, glacial acetic acid few drops 5 % ferric chloride and concentrated sulphuric acid were added, and observed for a reddish brown coloration at the junction of two layers and the bluish green colour in the upper layer.

Test for Flavonoid: To 2 ml of extract, 0.75 ml of 50 % methanol solution was added. The solution was warmed and metal magnesium was added. To this solution, 2-3 drops of concentrated hydrochloric acid was added, red colour was observed for flavonoids and orange colour for flavones.

Test for Alkaloid: To 0.25 g of each extract 2.5 ml of 1 % aqueous hydrochloric acid was added and kept in water bath. To 0.5 ml of filtrates Mayer's reagent (Potassium Mercuric Iodide) was added. Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Test for Tannin: To 0.5 ml of extract solution, 1 ml of water and 1-2 drops of ferric chloride solution was added. Blue colour was observed for gallic tannins and green black for catecholic tannins.

Test for Saponin: To 1 ml extract 2 ml distilled water was added. Persistent foam was observed.

Quantitative Estimation of Phytoconstituents

Thin layer chromatography

Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose (blotter paper). This layer of adsorbent is known as the stationary phase.

Principle: It is used on the principle of adsorption chromatography or partition chromatography or combination of both, depending on adsorbent, its treatment and nature of solvents employed. The components with more affinity towards stationary phase travels slower. Components with less affinity towards stationary phase travel faster.

Pharmacological investigations

Animals

Swiss albino male mice (20-25 gm) were obtained from the animal house of IPS College of Pharmacy, Gwalior. The animals were maintained in a well-ventilated room with 12:12 hour light/dark cycle in polypropylene cages. Standard pellet feed and drinking water was provided ad libitum. Animals were acclimatized to laboratory conditions one week prior to initiation of experiments.

Dose Determination

The dose of C. sinensis for mice used in present study was calculated by using dose conversion table [Paget and Barner, 1964]. The available therapeutic dose of human was converted into dose for experimental animals, thus the calculated therapeutic dose was 40 mg/kg body weight for mice. Hence the dose of 40 mg/kg was selected for the study.

Acute Toxicity Study

Acute toxicity study was carried out to determine the safe dose by acute toxic class method of oral toxicity as per Organization for Economic Co-operation and Development (OECD) 423 guidelines. The overnight fasted mice (n=3) were orally administered C. Sinensis the limit test dose of 2000 mg/kg and observed continuously for behavioural, neurological and autonomic profiles for 4 h and after a period of 24, 72 h and thereafter up to 14 days for any lethality, moribund state or death [1].

Experimental Design: Animals were divided randomly into five groups of six animals in each.

For acetic acid induced writhing test

Group I: Received saline (10 ml/kg, p.o) and served as control group.

Group II: Received Aspirin (100 mg/kg,p.o.)

Group III: Received CS (40 mg/kg, p.o., for 8 days)

For hot plate test

Group IV: Received saline (10 ml/kg, p.o) and served as control group.

Group V: Received CS (40 mg/kg, p.o., for 8 days)

Assessment of peripheral analgesic activity

Acetic acid induced writhing test: 30 min after receiving dose as per the experimental design for every group, each mouse was given intra peritonea 1 % aqueous solution of acetic acid (10 ml/kg body weight) [Yi et al., 2003]. Immediately after administration of acetic acid, the number of writhesor stretches (a syndrome, characterized by a wave of contraction of the abdominal musculature followed by extension of hind limbs) was counted for 20 min. A reduction in the number of writhes as compared to the control group was considered as evidence for the presence of analgesia, expressed as percent inhibition of writhing.

3. RESULT AND DISCUSSION

Phytochemicals studies

Standardization of CS

Physico-chemical constants: The physico-chemical constants of CS were found as mentioned in the Table

Table 1: Physico-chemical evaluation of CS

Standardization parameters	Value(% w/w)
Ash analysis	
❖ Ash Content (Total Ash)	11.6
❖ Acid In-Soluble Ash	2.6
❖ Water Soluble Ash	2.6
Extractive value (Maceration Process)	
❖ Methanolic soluble	12
Moisture content (Loss on Drying)	5.5

pH (1% aqueous solution)	4.5

Table 2: Preliminary Phytochemical evaluation of CS

Phytochemical Test	CS
Carbohydrate	Positive (+)
Protein	Negative (-)
Starch	Negative (-)
Steroid	Positive (+)
Glycoside	Positive (+)
Flavanoid	Positive (+)
Alkaloid	Positive (+)
Tannin	Partial Positive (++)
Saponin	Positive (+)
Amino Acid	Positive (+)

Preliminary Phytochemical Screening

The preliminary phytochemical analysis of CS showed that the plant contains alkaloids, carbohydrates, sterols, saponins, phenolic compounds and tannins.

Acute Toxicity Study

Acute oral toxicity studies revealed the non-toxic nature of CS preparation. There was no morbidity observed or any profound toxic reactions found at a dose of 2000 mg/kg p.o. which indirectly pronouns the safety profile of CS preparation.

Quantitative Evaluation

Thin layer Chromatography

A (sample) = CS, B (standard) = CS extract

Table 3: Rf value of sample and standard.

S. no.	Sample	R_f Value
1	A	0.87
2	B	0.90

Effect of CS on acetic acid-induced writhing test in mice

As shown in Table 2, indicate that there is a significant reduction in writhing in mice treated with the CS preparation and standard. The CS preparation (40 mg/kg, p.o. for 8 days) reduced writhing count significantly, inhibiting pain by 40.1% compared to control whereas aspirin showed 56.5 % inhibition at the dose of 100 mg/kg as compared to the control, and the results were statistically significant (Table 2 and Fig. 2).

Table 4: Effect of CS on acetic acid-induced writhing test in mice

Group	Treatment	No. of Wriths in 20 min	% Inhibition
I	Control(saline, 10 ml/kg, p.o)	28 ± 4.16	
II	Aspirin(100 mg/kg, p.o.)	12 ± 1.15**	56.5 ± 2.32**
III	CS(40 mg/kg p.o., 8 days)	16.3 ± 0.88*	40.1 ± 5.31*

Values are Mean ± S.E.M. (n=6), *p<0.01, **p<0.001 as compared to control

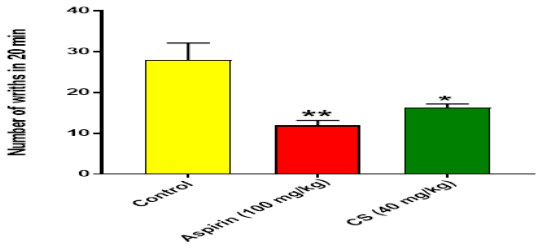

Fig. 2: Effect of CS on acetic acid-induced writhing test in mice

Effect of CS on hot plate test in mice

No significant difference was observed by CS treated group when compared to control group in latency time in hot plate test. The physiochemical parameters viz. ash content, extractive value, moisture content, pH indicated that the CS preparation intended for study was of requisite pharmacopoeial standards and up to the mark.

Acute toxicity study of CS revealed that there was no toxicity of any nature or moribund stage during the observation period. This illustrated that the non observable adverse effect dose level (NOAEL) of CS is more than 2000mg/kg. Human dose was converted to mice therapeutic dose based surface area calculation [10]. Thus CS was administered at a dose of 40 mg/kg for present study.

The present study has shown that oral administration of the CS was effective in producing significant peripheral analgesic effects in mice. The analgesic effect of CS was evaluated indifferent models of pain viz central pain model of hot plate and peripheral pain model of acetic acid induced writhing test. The acetic acid induced writhing has been associated with increased level of prostaglandines E2 and prostaglandins F2α in peritoneal fluids [7]. By increasing capillary permeability the rise in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain [9]. It was found that CS significantly inhibited acetic acid induced responses.

The mechanism of analgesic effect of CS could probably be due to blockade of the effect of endogenous substances that excite pain nerve endings. While no significant effects were observed by CS in hot plate test. This suggests that the analgesic property of CS is peripherally mediated.

4. CONCLUSION

Pain is an unpleasant sensation that can range from mild, localized discomfort to agony. The treatment of pain includes opioids and non-opioids drug like steroidal and non-steroidal anti-inflammatory drugs (NSAIDS) and neuropathic agents. Different types of adverse effects are confronted with the regular use of these drugs [8]. Plants can be used as a great source of medicinal agents in pain management for safety and comfort of the patients.

There are few reports found that describe the analgesic activity of C. Sinensis. analgesic activity of the C. Sinensis in mice. Therefore in present study analgesic activity of CS preparation was evaluated in mice. The study was carried out using acetic acid induced writhing model and hot plate test. The CS preparation was given orally for 7days to mice at the dose of 40 mg/kg. In acetic acid induced writhing mode, Acetic acid causes inflammatory pain by increasing capillary permeability [9]. The Writhes induced by acetic acid injected intraperitoneally is due to sensitization of nociceptors by prostaglandins. This test is useful for evaluation of mild analgesic non-steroidalanti-inflammatory compounds [10]. The CS preparation caused a significant inhibition of writhes in mice. The effect of the CS was comparable to that of aspirin, a cyclooxygenase inhibitor. This suggests that the CS may have a peripheral analgesic action. However, CS did not show any significant effect in hot plate model of pain, indicating its analgesic effect devoid of central pain pathway.

In conclusion, the data indicated that CS (green tea) possesses peripheral analgesic activity. This provides evidence for its use locally in human medicine to relief pain in the treatment of ailments accompanied with pain. Further studies are needed to confirm the activity, isolate the active compounds and explain the mechanisms related to this pharmacological effect.

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