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# Physiochemical Investigation and Evaluation of Camellia Sinesis Leaves For in Vitro Antioxidant and Anti-Inflammatory Activity

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***Abstract***
*In terms of volume, teas surpass water as the most popular drink. Tea is reserved for infusions of the Camellia sinensis plant, which are caffeinated but not alcoholic drinks. Nutritional components, alkaloids (methylxanthines), phenolic compounds (phenolic acids, flavonoids, and tannins), and alkaloids (carbohydrates, proteins, and minerals) are abundant in the blossoms and leaves of the Camellia sinensis plant. Carbohydrates (glucose, fructose, sucrose, and polysaccharides), phenolic compounds (PCs), crude proteins, and saponins make up the flower's chemical makeup; white tea is derived from this plant. Moreover, it includes a diverse range of amino acids, including arginine, γ-aminobutyric acid, threonine, tyrosine, valine, methionine, leucine, phenylalanine, lysine, and theanine, among many others. Examining the antioxidant and anti-inflammatory properties of Camellia sinensis leaves using phytochemical and pharmacological means is the current goal of this study. The antioxidant activity of the extracts was evaluated using the DPPH (1,1-diphenyl-2-picryl hydrazyl) and nitric oxide scavenging free radical test technique, with ascorbic acid serving as the positive control. In vitro methods for assessing anti-inflammatory activity included the bovine serum albumin denaturation inhibition test and the hrbc membrane stabilization technique.*
***Keywords:*** *Camellia sinensis, Antioxidant activity, DPPH, in vitro anti-inflammatory activity*

## INTRODUCTION

Plants have been a model for medicinal practices since the dawn of mankind. Many human problems may be alleviated by the usage of plants, as mentioned in Ayurveda and other Indian literature [1]. Several thousands of the approximately 45,000 plant species found in India are believed to have therapeutic use. Numerous studies have shown the usefulness of traditional medical practices. A growing number of problems, including drug resistance, side effects, and the high expense of therapy, have plagued synthetic pharmaceuticals in recent years, prompting a shift in focus toward natural remedies derived from plants [2]. Herbals have also attracted attention due to their low cost. India is home to a wealth of traditional medicinal herbs and is considered by many to be one of the world's oldest civilizations [3].

The plant known as Camellia sinensis is the source of the aromatic tea. It belongs to the family Theaceae and is a member of the flowering plant genus Camellia. This species yields a variety of teas, including white, green, oolong, and black teas, each of which undergoes a unique processing to achieve a distinct degree of oxidation [4]. Although it employs stems and twigs instead of leaves, kukicha (twig tea) is similarly made from Camellia sinensis. The common terms for tea include shrub, tree, and plant [5]. Teas are categorized according to the degree of oxidation of its beneficial ingredients; the plant Camellia sinensis is the source of several types of teas used globally, including white, green, yellow, oolong, black, and pu-ehr. Methylxanthines (caffeine and theobromine), amino acids (L-theanine), and reducing sugars all have a role in how teas taste, smell, and feel in the mouth, as well as how they work in the body of the drink [6]. On average, teas contain 60% bioactive chemicals, the majority of which are flavan-3-ols, which are mostly epicatechins, catechins, and derivatives of these molecules. Antioxidant, anti-cancer, and anti-inflammatory are three of the most well-known benefits of these tea secondary metabolites [7]. As a result, the food sector has been making more and more use of Camellia sinensis extracts and the chemicals they contain. This study aims to investigate the antioxidant and anti-inflammatory properties of Camellia sinensis leaves using phytochemical and pharmacological methods.

## MATERIAL AND METHODS

### Macroscopic and Microscopic study

The Camellia Sinesis leaves were procured from local are of Assam India. The fresh leaves were separated and cleaned to remove unwanted materials. The fresh leaves were air-dried at room temperature for about one week. The dried leaves were coarsely powdered in a blender and were used for further analysis. Macroscopical characters were examined with the naked eyes to determine the color, shape, odor, margin, texture, base symmetry, etc. of the plant parts as per the requirement of Indian Herbal Pharmacopoeia**.** The Transverse sections were taken by placing the between the thumb and four finger of the left hand. Using sharp razor held in the right hand, thin section was made the razor across the object in quick successions. The sections were transferred in to watch glass containing water, added chloral hydrate to these sections, boiled, filtered and the sections were stained with phloroglucinol and hydrochloric acid (1:1) and the same was mounted in glycerin and observed under low power [8].

### Physicochemical Evaluation

Physicochemical (Proximate) analysis helps to set up certain standard for dried crude drugs in order to avoid batch-to-batch variation and also to judge their quality. Their studies also give an idea regarding the nature of phytoconstituents present. Physicochemical studies of the air-dried powdered drug have been conducted to its high medicinal properties. The physic-chemical analysis such as ash value, foreign matter, loss on drying (LOD), foaming index, swelling index, extractive value and pH were performed by Indian Pharmacopoeia and the WHO-recommended parameters from Camellia Sinesis leaves powder. Fluorescence study was carried out [9].

### Determination of Total Phenolic content

The Total Phenolic content of plant extract was determined by using Folin's-Ciocalteu reagent and Gallic acid as a calibration standard by using Gallic acid equivalence (GAE). The dry extracts were diluted in methanol in the concentration of mg/ml of the samples and 1ml was transferred to 10 ml volumetric flasks, to which 0.5ml Folin's- Ciocalteu reagent was added. After one minute, 1.5ml of 20% (w/v) Na2CO3 was added and the volume made up to 10 ml with distilled water. The reaction mixture incubated at 25°C for one hour and the absorbance was measured at 760nm and compared with a prepared Gallic acid calibration curve. Quantification was done on the basis of a standard curve of gallic acid. A standard curve of absorbance against gallic acid concentration was prepared [10-11]. Results were expressed percentage w/w and calculated using following formula.

Total phenolic content (% w/w) = GAE×V×D×10–6×100/W,

GAE - Gallic acid equivalent (μg/ml),

V - Total volume of sample (ml),

D - Dilution factor,

W - Sample weight (g).

The Gallic acid equivalence standard curve was plotted between concentration and absorbance. Total phenolic contents in plant extracts were obtained by using the regression curve equation. The total phenolic content was determined as gallic acid equivalent per mg of extract.

### Determination of Total flavonoid content

The total flavonoid content was determined by aluminum chloride method. Quercetin was used as standard and calibration standard. Ten milligram of quercetin was dissolved in 100 ml of methanol (80%) (100 μg/ml) and then further diluted to 10, 20, 30, 40 or 50 μg/ml. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of methanol (95%), 0.1 ml of aluminium chloride (10%), 0.1 ml of 1 M potassium acetate and 2.8 ml of diluted water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with UV/VIS spectrophotometer. The amount of aluminium chloride (10%) was substituted by the same amount of distilled water in blank**.** Quantification was done on the basis of a standard curve of rutin [12]. A standard curve of absorbance against rutin concentration was prepared. Results were expressed as percentage w/w.

Flavonoids content (% w/w) = QE×V×D×10–6×100/W,

QE - Quercetin equivalent (μg/ml),

V - total volume of sample (ml),

D - dilution factor,

W - sample weight (g).

The Quercetin equivalence curve was obtained**.** Total flavonoid contents in plant extracts were obtained by using the regression curve equation.

### TLC Fingerprinting Analysis:

The studies were performed on size 5 x 20 cm and thickness 250 µm precoated silica gel 60 F254 plates. The prepared ethanol extracts (2 mg/ml in their respective solvents), and the standard quercetin and kaempferol solution (1mg/ml in methanol), respectively were applied to the TLC plates by using a capillary tube and the plates were allowed to air dry for 15-20 minutes after that the plates were kept inside Hot-air oven at 100-105°C for one hour, for activation of plates. The activated plates were immersed in a development chamber and TLC studies were carried out for different mobile phases with different ratios, covered with a proper lid, and then it was allowed to develop. After drying, then the TLC plates were kept inside the UV cabinet at short wavelength, 254 nm, and long-wavelength, 366 nm, for the visualization of the separated bands. Then, the Rf value of each different spot that was observed was calculated [13].

**Antioxidant activity**

**DPPH free radical scavenging activity**

The free radical scavenging activities of the plant extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) will be estimated. 2.0 ml of a methanol solution of the sample (extract/control) at different concentration (50–250µg/ml) will be mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). After 30 min reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer [14].

Inhibition of free radical DPPH in percent (I %) was calculated as follows:

I% = (1 – A sample/A blank) × 100

where A blank is the absorbance of the control reaction(containing all reagents except the test material). Extract concentration providing 50% inhibition (IC50) will be calculated from the graph plotted inhibition percentage against extract concentration.

**Nitric oxide (NO) scavenging activity**

Nitric oxide (NO) scavenging activity will be measured spectrophotometrically. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide, which interacts with oxygen to produce nitrite ions will determined by the use of Griess reagents. A volume of 2 ml of 10 mM sodium nitroprusside and 0.5 ml of phosphate buffer saline (pH 7.4) will be mixed with 0.5 ml of plant extract and ascorbic acid individually at various concentrations (50–250 µg/ml). The reaction mixture will be incubated at 25 °C for 150 min. After 150 min, 0.5 ml of incubation solution will be withdrawn and mixed with 1 ml of sulfanilic acid reagent (0.33 in 20% glacial acetic acid) and will be allowed to stand for 5 min at room temperature for completing diazotization. Then 1 ml of 0.1% w/v napthylethylenediamine dihydrochloride will be added, mixed well and the mixture will be incubated at room temperature for 30 min [15]. The absorbance will be taken at 540 nm. The amount of nitric oxide (NO·) radical inhibited by the extract will be calculated using the following equation:

I% = (1 – A sample/A blank) × 100

Where, A blank is the absorbance of the control reaction(containing all reagents except the test material).

**In vitro Anti-inflammatory Activity**

**In vitro Anti-inflammatory Activity by Human Red Blood Cell (HRBC) Membrane Stabilizing Activity**

Inflammation is caused by inflammatory mediators released due to rupture of lysosomes. Lysosomal membrane is structurally similar to HRBC membrane. Protective effect of an agent on heat/hypotonic saline induced erythrocyte lysis is known to be a very good index of anti-inflammatory activity of the agent. This is determined by measuring the intensity of red colour of the hemoglobin released due to rapture of RBC at 560 nm [16]. The percentage haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water as 100%. Percentage of protection was calculated using the following equation.

Atest % of membrane stabilization = 100 - x100 Acontrol

Where, Atest is the absorbance of the test solution and

Acontrol is the absorbance of the control solution.

**In vitro Bovine Serum Albumin Denaturation Inhibition Assay**

Denaturation inhibition study was performed by using Bovine Serum Albumin (BSA). 5% Bovine serum albumin prepared by 5g BSA was dissolved in 100ml phosphate buffer saline (pH 6.3). Phosphate buffer saline (pH6.3) prepared by dissolving 0.895g of disodium hydrogen phosphate and 0.68 g of potassium dihydrogen phosphate and 3.51g of NaCI in sufficient water to produce 1000ml.Adjust to pH 6.3 by adding HCI. The reaction mixture 3ml contained, 50 µl of the test solution (50-250µg/ml was prepared in methanol). 450µl of 5% w/v BSA was added to all the above test tubes. For control tests,50 µl of distilled water instead of test solution. The test tubes were incubated at 37°C for 20 minute and then

heated at 57°C for 3 minutes. After cooling the test tubes, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube [17]. The absorbance of these solutions was determined by using spectrophotometer at a wavelength of 660nm.

% Protein denaturation inhibition = [(Absorbance of control – Absorbance of test)/Absorbance of control] × 100

## RESULT AND DISCUSSION

### Macroscopic and microscopic study

Camellia sinensis is a species of evergreen shrub or small tree in the flowering plant family Theaceae. Its leaves, leaf buds, and stems can be used to produce tea. Common names include tea plant, tea shrub, and tea tree. The Camellia Sinesis leaves are typically alternate, simple, and lanceolate to obovate, ranging from 4 to 13 cm long and 2 to 5 cm broad. Young leaves are light green with short, white hairs on the underside, while older leaves are deeper green. The leaves can be shiny and often have a hairy underside. The edges of the leaves are serrated, meaning they have saw-tooth-like notches

**Figure 1: Camellia Sinesis leaves**

A transverse section of a Camellia sinensis (tea plant) leaf reveals a dorsiventral structure with distinct upper and lower epidermis layers, a heterogeneous mesophyll, and a midrib with vascular bundles. The mesophyll contains palisade and spongy parenchyma, and sclerenchymatous idioblasts (sclereids) are present, sometimes extending from the upper to lower epidermis. The lower epidermis also features stomata, typically surrounded by accessory cells.

Powder microscopy of Camellia sinensis (tea) leaves reveals characteristic features like epidermal cells, stomata, trichomes, and calcium oxalate crystals. Specifically, the upper epidermis exhibits straight-walled cells, while the lower epidermis shows sinuous walls and stomata. Trichomes (hairs) are often present on the lower surface, particularly in young leaves. Fragments of vascular tissue, fibers, and sclereids are also observed.

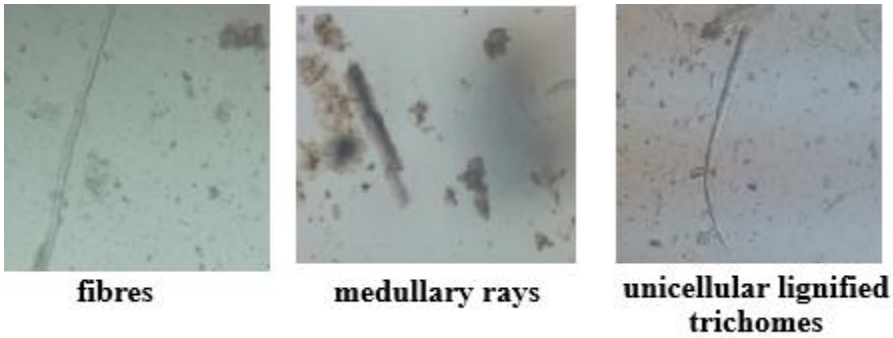



**Figure 5: Powder microscopy of Camellia sinensis**

### Physico-chemical Investigations

Physicochemical parameters of crude drugs are physical and chemical properties used to evaluate their quality, purity, and potential for biochemical variations. These parameters include moisture content, ash values (total, acid-insoluble, water-soluble), extractive values (using different solvents), and melting point, among others. Analyzing these parameters helps in identification, quality control, and ensuring the stability and efficacy of crude drugs. The study of various physicochemical parameters of Camellia Sinesis leaf powder was performed and the results were listed in Tables 3 and 4.

**Table 5.2: Physicochemical parameters of Camellia Sinesis leaves**

| S. No. | Physicochemical parameter values | (% w/w) |
|---|---|---|
| 1 | Total ash | 6.31±0.24 |
| 2 | Acid insoluble ash | 1.87± 0.11 |
| 3 | Water soluble ash | 0.96±0.09 |
| 4 | Foreign organic matter determination | 7.34 |
| 5 | Loss on drying | 8.13 ± 0.49 |

**Preliminary Phytochemical Screening**

The qualitative phytochemical screening of the tubers for the presence of alkaloids, carbohydrate ,reducing sugars, glycosides like anthraquinones, flavanoids, saponins, tannins, phenolic compounds, fixed oils, fats, proteins, amino acids and sterols in extracts of the this plant were carried out.

Qualitative phytochemical screening of the various extracts of Camellia Sinesis showed the presence of various secondary phytoconstituents. The ethanolic extract was found to have a high content of alkaloids, flavonoids, saponins, and saponin glycosides. This shows that high polar secondary phytoconstituents were extracted with ethanol when compared to petroleum ether.

**Table 5.4: Qualitative phytochemical screening of various extracts of fresh leaves and stems of Camellia Sinesis**

| S.No. | Constituents | Pet. ether | Ethanol |
|---|---|---|---|
| 1. | Alkaloids | + | + |
| 2. | Flavonoids | + | + |
| 3. | Proteins andamino acids | - | - |
| 4. | Carbohydrates | - | + |
| 5. | Cardiac glycosides | + | + |
| 6 | Saponin glycosides | - | - |
| 7 | Phenolic compounds | _ | + |
| 8 | Tannins | + | + |
| 9 | Terpenoids | - | + |
| 10 | Steroids | - | - |
| 11 | Fixed oils and fats | + | - |
| 12 | Gum & mucilage | - | - |

**+ present -absent**

**Chromatographic Analysis:**

**Thin Layer Chromatography (TLC)**

Various mobile phases with varying ratios were tried for the ethanol extract of Camellia Sinesis. In which, the most suitable mobile phase for the separation of secondary metabolite was found to be chloroform : ethyl acetate : methanol (7 : 2 : 1). TLC profile of ethanolic extract of fresh leaves and stems of Camellia Sinesis were shown in Figure 1.

**Figure 1: TLC of Camellia Sinesis leaves ethanol extract**

**8.2.2.8. Determination of Total Phenolic content**

The Total Phenolic content of plant extract was determined by using Folin's-Ciocalteu reagent and Gallic acid as a calibration standard by using Gallic acid equivalence. The Gallic acid equivalence standard curve was plotted between concentration and absorbance. Total phenolic contents in plant extracts were obtained by using the regression curve equation. The total phenolic content was determined as gallic acid equivalent per mg of extract.

Petroleum ether possess 4.87 mg GAE/g dry extract whereas Ethanol extract found to have 18.71 mg GAE/g dry extract wt.

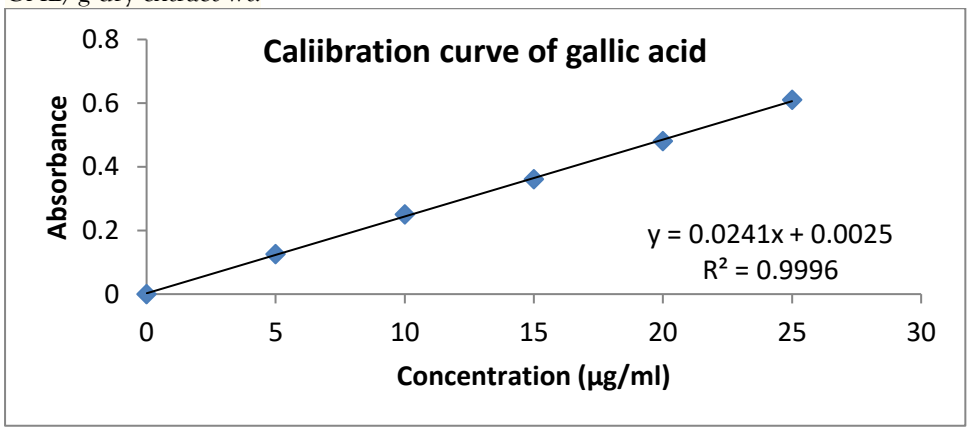



**Figure 8.1: Standard curve of gallic acid by UV spectroscopy (260 nm)**

**8.2.2.9. Determination of Total flavonoid content**

The total flavonoid content was determined by aluminum chloride method. Quercetin was used as standard and calibration standard. The Quercetin equivalence curve was obtained**.** Total flavonoid contents in plant extracts and formulation were obtained by using the regression curve equation.

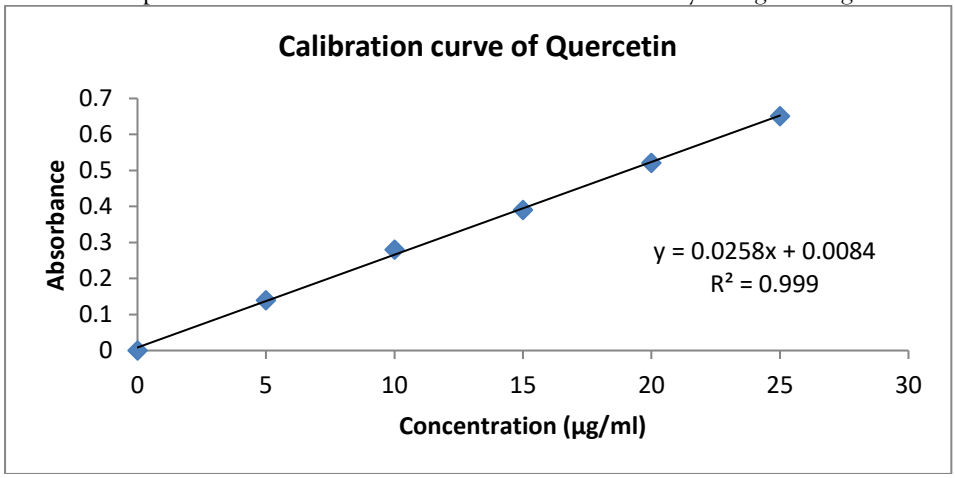


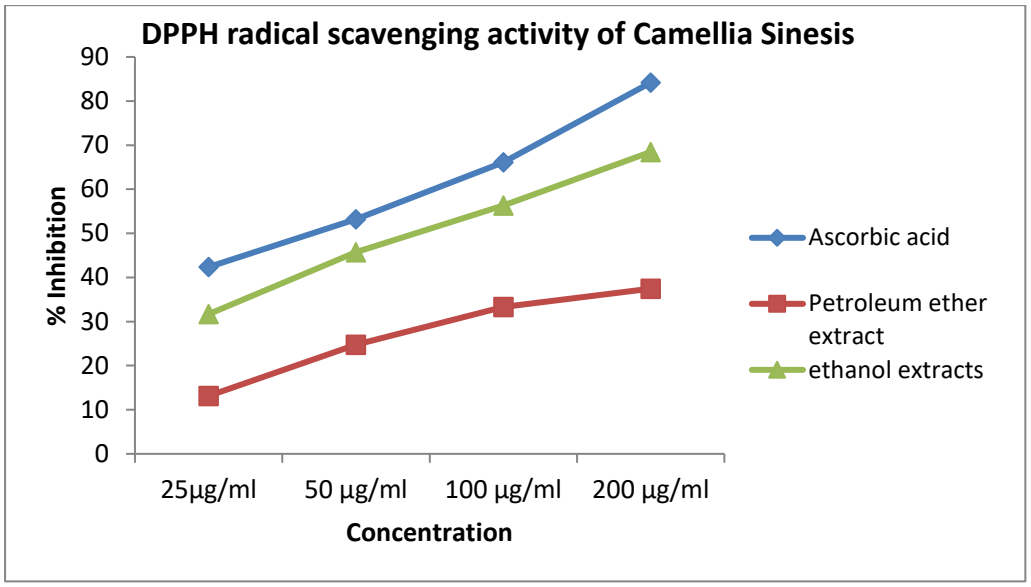

**Figure 8.2: Calibration curve of Quercetin at λ max 415 nm**

Total flavonoid content of Petroleum ether extract found to have 0.87 mg QE/g dry extract whereas Ethanol extract found to have 14.91 mg QE/g dry extract.

**Anti-oxidant Activity:** Analysis of the free radical scavenging activities of the selected Camellia Sinesis extracts revealed a concentration dependent free radical scavenging activity resulting from reduction of DPPH, NO radical to non-radical form. The scavenging activity of Ascorbic acid, a known antioxidant used as positive control, was however higher.

**DPPH radical scavenging model**

DPPH radical is considered to be a model for a lipophilic radical. A chain in lipophilic radicals was intiated by the lipid autoxidation. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capacity of DPPH was determined by the decrease in its absorbance at 517nm, which is induced by antioxidant. Positive DPPH test suggests that the samples were free radical scavengers. The scavenging effect of l-Ascorbic acid, and plant extracts increased gradually with increase in concentration.




**DPPH radical scavenging activity of Camellia Sinesis extracts**

**Effect of Camellia Sinesis extract on Nitric oxide scavenging model**

Nitric oxide plays an important role in various types of inflammatory processes in the body. In the present study Camellia Sinesis ethanol extracts checked for its inhibitory effect on Nitric oxide production.

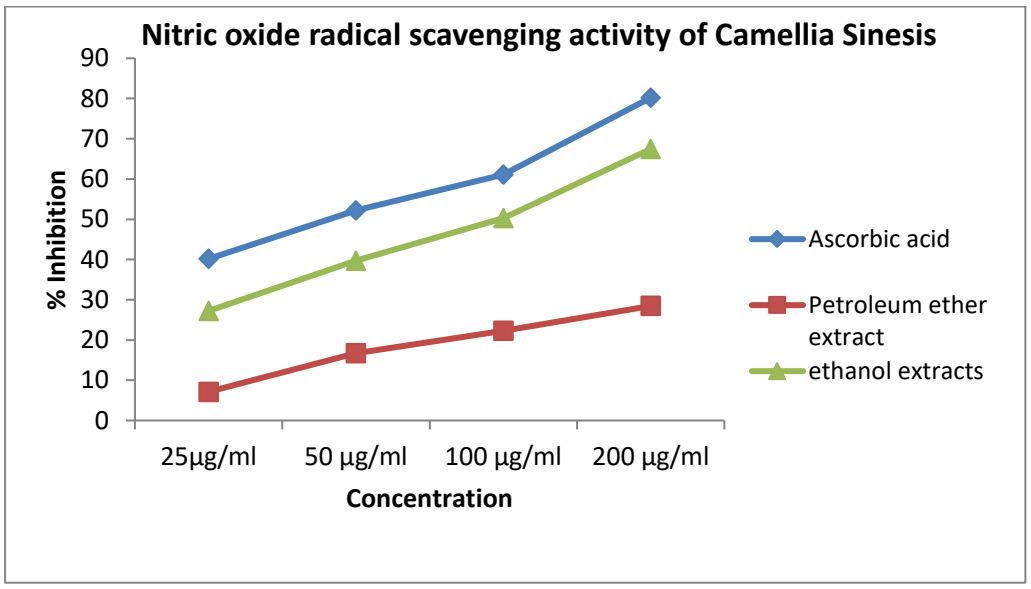



**Table 6.13: Nitric oxide radical scavenging activity of Camellia Sinesis extracts**

Nitric oxide radical generated for sodium nitroprusside at physiological pH was found to be inhibited by the extracts. The ethanol extract of Camellia Sinesis at varied concentrations showed remarkable

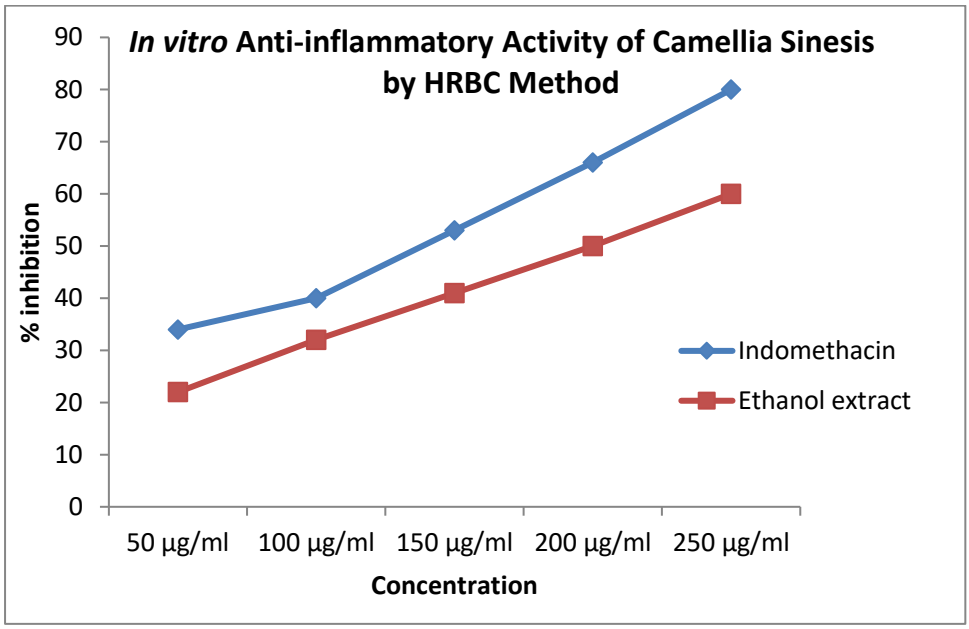
inhibitory effect of nitric oxide radical scavenging activity compared to other extract. Results revealed that all the tested extract showed the percentage of inhibition in a dose dependent manner. The ethanol extract of Camellia Sinesis at varied concentrations showed remarkable inhibitory effect of nitric oxide radical scavenging activity.

**Table 6.19: In vitro 50% inhibition concentration ($IC_{50}$) of Camellia Sinesis extracts**

| Extract /compound | $IC_{50}$ (µg/ml) DPPH model | $IC_{50}$ (µg/ml) NO model |
|---|---|---|
| Ascorbic acid | 67 | 98 |
| ethanol extracts | 92 | 127 |

**In vitro Anti-inflammatory Activity by HRBC Membrane Stabilization Method**

The ethanol extract of Camellia Sinesis at different concentrations (50,100,150,200 &250µg/ml) showed significant ($p<0.001$) stabilization towards HRBC membrane. In this study the ethanol extract of Camellia Sinesis (250µg/ml) displayed potent action



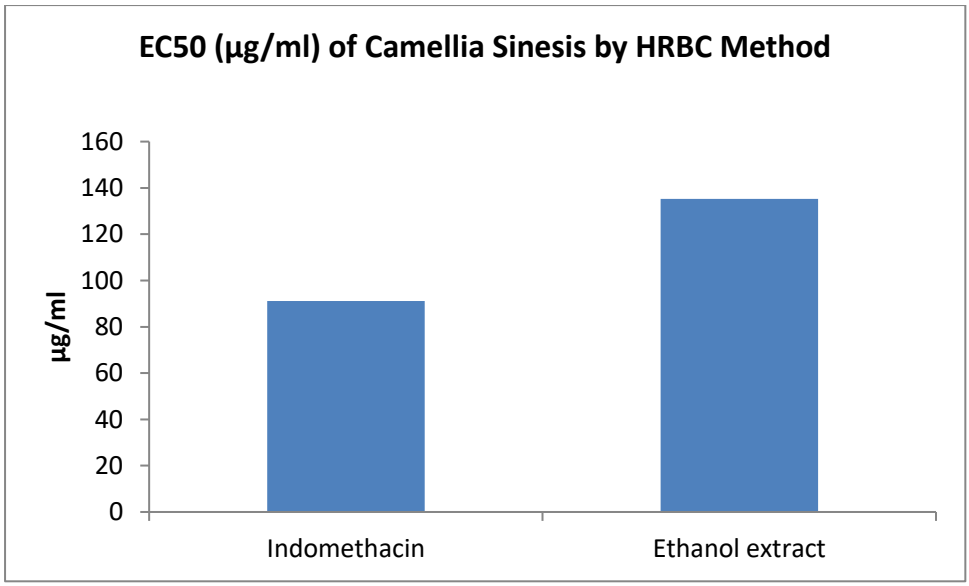
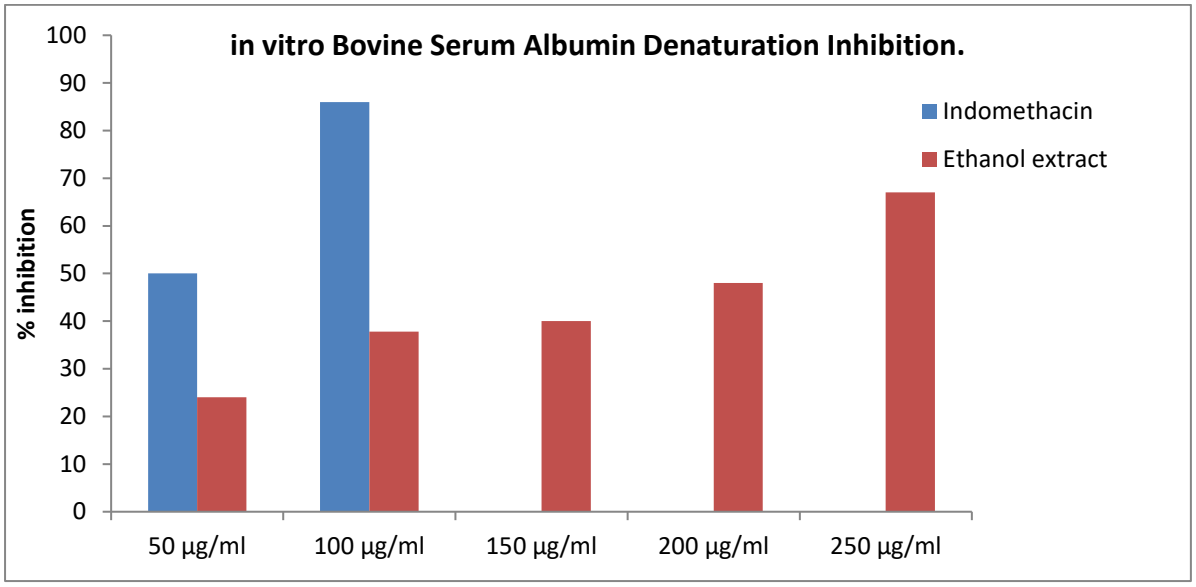




**Table 5.13: In vitro Anti-inflammatory Activity of ethanol extract of Camellia Sinesis by HRBC**

METHOD

**In vitro Bovine Serum Albumin Denaturation Inhibition Assay:**

The Bovine Serum Albumin (BSA) Denaturation Inhibition Assay is an in vitro method to screen for anti-inflammatory compounds by measuring their ability to prevent heat-induced denaturation of BSA, a model protein for inflammation. Samples with test compounds are mixed with BSA solution, incubated under heat to cause denaturation, and then the degree of denaturation is measured, typically by absorbance at 660 nm after cooling. Higher absorbance readings indicate more denaturation, while higher sample concentrations that lead to lower absorbance readings demonstrate stronger anti-inflammatory potential.




In the Bovine Serum Albumin Denaturation Inhibition Assay ethanol extract of Camellia Sinesis at different concentrations (50,100,150,200&250µg/ml) displayed activity. In this study the ethanol extract of Camellia Sinesis at 250µg/ml concentration displayed, maximum activity.

## CONCLUSION

Plants possess a wide array of bioactive compounds that exhibit significant and remarkable biological activity. Bioactive compounds, sometimes referred to as secondary metabolites, are naturally produced as a result of the normal metabolic pathways in medicinal plants. Analysis of the free radical scavenging activities of the selected Camellia Sinesis extracts revealed a concentration dependent free radical scavenging activity resulting from reduction of DPPH, NO radical to non-radical form. The scavenging activity of Ascorbic acid, a known antioxidant used as positive control, was however higher. Moreover, its anti-inflammatory and antioxidant properties suggest its potential for managing inflammatory conditions and oxidative stress-related disorders. These characteristics used as a promising candidate for future biomedical applications, paving the way for further exploration and development in the field of medicine.

Tea leaves, particularly from the Camellia sinensis Linn plant, are known for their antioxidant properties, which helps to neutralize harmful free radicals in the body. This antioxidant activity is widely studied due to its potential health benefits, including reducing the risk of diseases like cancer and cardiovascular issues. The main goal of this study was to examine the potential of utilizing matured tea leaves,

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