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EVALUATION OF VARIOUS PHYSIOLOGICAL PARAMETERS, TOTAL PHENOLIC AND FAVONOIDAL CONTENTS OF TWIGS OF *Camellia sinensis.*

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ABSTRACT
Camellia sinensis (CS) is an evergreen shrub commonly known as Teyaku belongs to the family *Theaceae*. It grows best in the moist, tropical climates found in Northeast India and many provinces in China. It is also well grown in Japan, Srilanka, Kenya and Turkey etc. Twigs of *Camellia sinensis* were collected from Kannan Devan Tea Plantation, Munnar. Authentication was done by Professor Ravi Prasad, Department of Botany, S.K University, Anantapur. Voucher specimens were preserved in the herbarium with voucher numbers SK871. In the present study twigs of the plant are defatted with petroleum ether and extracted by maceration for 72hrs with an optimized solvent, n-butanol. The Percentage yield was 45.66%. preliminary phytochemical screening is done and found various phytoconstituents like Alkaloids, Tannins, Flavonoids, Glycosides, Phenols and Terpenoids, various physiological parameters like Loss on Drying, Total ash, Acid insoluble ash, Water soluble ash, Swelling Index, Foaming index were performed. The total phenolic contents were estimated as 128.92±0.149% w/w equivalent of gallic acid respectively. Flavonoid contents were found as 41.13±0.672mg/g quercitin equivalent respectively. My further studies are aiming to structural elucidation and the anticancer activities of specific compounds in the extract.
Keywords: *Camellia sinensis, Theaceae, Teyaku.*

INTRODUCTION
Medicinal plants have been used in virtually all cultures as a source of medicine. The widespread use of herbal remedies and healthcare preparations, as those described in ancient texts and obtained from commonly used traditional herbs and medicinal plants has been traced to the occurrence of natural products with medicinal properties. The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has also been widely observed .
Camellia sinensis is an evergreen shrub with many stems arising from the base that grows not more than 2 m (6.6 ft). which is commonly known as 'Green Tea', grows best in the moist, tropical climates found Northeast India and the Szechuan and Yunnan provinces of China. The small leaf variety thrives in the cool, high mountain regions of central China and Japan. India

and China are the two major tea producers and exporters and followed by Sri Lanka, Kenya and Turkey, respectively belongs to the family *Theaceae*. *C. sinensis* is a rich source of polyphenolic components such as tannins that are proved effective in the management of stress. Its consumption is originated in China and famous throughout the world. Traditionally, tea is used as a health drink, to get rid of various physiological stresses. The leaves were used to treat infections, wounds and inflammations. The decoctions were also used to treat psychiatric disorders also. The decoction of *C. sinensis* along with other spices, it is used to treat gastric disturbances. The young twigs will be processed to increase their antioxidant content to improve its protective activities.

Breast cancer is the common cause of cancer death in women worldwide. Rates vary about five-fold around the world, but they are increasing in regions that until recently had low rates of the disease. Many of the established risk factors are linked to oestrogens. Risk is increased by early menarche, late menopause, and obesity in postmenopausal women, and prospective studies have shown that high concentrations of endogenous oestradiol are associated with an increase in risk. It is categorized into 3 major subtypes based on the presence or absence of molecular markers for estrogen or progesterone receptors and human epidermal growth factor 2. More than 90% of breast cancers are not metastatic at the time of diagnosis.

METHODOLOGY

All the plants collected were shade dried and powdered. The powdered material was initially extracted by Petroleum ether and then continued by maceration technique.

Plant Extraction:

The freshly collected leaves of ***Camellia sinensis*** was shade dried and coarsely powdered. The powder was passed through sieve no.40. The sieved powder was stored in airtight container for further use. Initially, 100g of each dried plant material powder was extracted with pet.ether, and macerated with with n-butanol for 72 hrs. It was then filtered and the solvent was evaporated and percentage yield was calculated and stored in desiccators until further use.

Phytochemical screening *Camellia sinensis* twigs:

Preliminary phytochemical screening was carried out for detection of phytoconstituents.

Test for Alkaloids:

A small portion of the solvent free extract was stirred separately with few drops of dilute hydrochloric acid and filtered. The filtrate was tested with various reagents for the presence of alkaloids.

A. **Dragendorff's reagent** (Potassium bismuth iodide solution): Reddish brown precipitate.
B. **Mayer's reagent** (Potassium mercuric iodide solution): Cream colour precipitate.
C. **Wagner's reagent** (Iodine potassium iodide solution): Reddish brown precipitate.
D. **Hager's reagent** (Saturated solution of picric acid): Yellow precipitate.

ISSN 2515-8260 Volume 09, Issue 09, 2022

Test for Carbohydrates:

- **Molisch's test:** To the test solution add few drops of alcoholic a-naphthol, then add few drops of concentrated sulphuric acid through the sides of test tube, purple to violet colour ring appears at the junction. It indicates the presence of carbohydrates.
- **Fehling's test:** To 1ml of Fehling's solution A (aqueous solution of Copper sulphate- $CuSO_4$) add 1ml of Fehling's solution B (solution of potassium tartrate) and 2ml of test solution. Then mix well and boil. Formation of red precipitate of cuprous oxide indicates the presence of carbohydrates.
- **Benedict's test:** To 1ml of sample add 2ml of Benedict's reagent (a solution of sodium citrate and sodium carbonate mixed with a solution of copper sulphate) followed by heating in boiling water for 3 minutes and not more than 7 minutes. Formation of reddish precipitate within 3 minutes indicates the presence of complex sugars and brown / yellow precipitate indicates presence of simple sugars.
- **Barfoed's Test:** The extracts were treated with Barfoed's reagent and heated .Appearance of reddish orange colour precipitate indicates the presence of non- reducing sugars.

Test for Proteins:

- **Biuret's Test:** When the extracts were treated with copper sulphate solution, followed by addition of sodium hydroxide solution, appearance of violet colour indicates the presence of proteins.
- **Million's Test:** When the extracts were treated with Million's reagent, appearance of pink colour indicates the presence of proteins.

Test for Steroids:

- **Liebermann Burchard Test:** When the extracts were treated with concentrated sulphuric acid, few drops if glacial acetic acid, followed by the addition of acetic anhydride, there is a formation of violet ring in between the two layers, and the appearance of green colour in the aqueous upper layer indicates the presence of steroids.
- **Salkowski test:** Treat the extract with few drops of concentrated sulphuric acid, red colour at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of triterpenoids.
- **Sulfur powder test:** Add small amount of sulfur powder to the test solution, it sinks at the bottom. It indicates the presence of steroids.

Test for Sterols: When the extracts were treated with 5%potassium hydroxide solution, appearance of pink colour indicates the presence of sterols.

Test for Phenols:

When the extracts were treated with neutral ferric chloride solution, the appearance of violet colour indicates the presence of phenols.

When the extracts were treated with 10%sodium chloride solution, the appearance of cream colour indicates the presence of phenols.

ISSN 2515-8260 Volume 09, Issue 09, 2022

Test for Gums and Mucilage:

The extracts were treated with 25ml of absolute alcohol, and then solution was filtered. The filtrate was examined for its swelling properties.

Test for Glycosides:

General Test:

Test-A: Extract 200mg of drug with 5ml of dilute sulphuric acid by warming on a water bath. Filter it. Then neutralize the acid extract with 5% solution of sodium hydroxide. Add 0.1 ml of Fehling's solution A and B until it becomes alkaline (test with pH paper) and heat on a water bath for 2 minutes. Note the quantity of red precipitate formed and compare it with that of formed in test B.

Test-B: Extract 200mg of drug using 5ml of water instead of sulphuric acid. After boiling add equal amount of water as used for sodium hydroxide in the above test. Add 0.1ml Fehling's solution A and B until alkaline (test with pH paper) and heat on water bath for 2 minutes. Note the quantity of red precipitate formed. Compare the quantity of precipitate formed in the Test-B with that of formed in the Test-A. If the precipitate in Test-A is greater than in Test-B then glycosides are present.

Since, Test-B represents the amount of free reducing sugar already present in the crude drug, where as test A represents free reducing sugar plus those related on acid hydrolysis of any glycoside in the crude drug.

When a pinch of the extracts were dissolved in the glacial acetic acid and few drops of ferric chloride solution was added, followed by the addition of concentrated sulphuric acid, formation of red ring at the junction of two liquids indicates the presence of glycosides.

Test for Saponins:

- **Foam Test:** 1ml of extracts are diluted to 20 ml of distilled water and shaken well in a test tube. The formation of foam in the upper part of the test tube indicates the presence of saponins.

Test for Terpenes: When the extracts were treated with tin and thionyl chloride, appearance of pink colour indicates the presence of terpenes.

Test for Tannins:

- **Ferric chloride test:** Treat the extract with ferric chloride solution, blue colour appears if hydrolysable tannins are present and green colour appears if condensed tannins are present.
- **Gelatin test:** To the test solution add 1% gelatin solution containing 10% sodium chloride. Precipitate is formed. It indicates the presence of tannins.

Test for Flavonoids:

- 5ml of extract solution was hydrolyzed with 10%v/v sulphuric acid and cooled. Then, it was extracted with diethyl ether and divided into three portions in three separate test tubes.1ml of

diluted sodium carbonate,1ml of 0.1N sodium hydroxide, and 1ml of strong ammonia solution were added to the first, second and third test tubes respectively. In each test tube, development of yellow colour demonstrated the presence of flavonoids.

- **Shinoda test:** To the test solution add few magnesium turnings and concentrated hydrochloric acid drop wise, pink scarlet, crimson red or occasionally green to blue colour appears after few minutes.
- **Zinc hydrochloride test:** To the test solution add a mixture of zinc dust and concentrated hydrochloric acid. It gives red colour after few minutes which indicate the presence of flavonoids.
- **Alkaline reagent test:** to the test solution add few drops of sodium hydroxide solution, intense yellow colour is formed which turns to colourless on addition of few drops of dilute acid indicates the presence of flavonoids.

Physicochemical Parameters

The ash values and extractives values were performed according to the official methods described in the Indian pharmacopeia and WHO guidelines on quality control methods -for medicinal plant materials (Kokate CK. et al., 2007; India Pharmacopoeia. 1996).

Determination of Total Ash

About 2 to 3 grams (accurately weighed) of plant powder was taken in a silica crucible previously ignited and weighed. It was incinerated by gradually increasing the heat not exceeding dull red heat (450^0C) until free from carbon, cooled and weighed. The percentage of ash was calculated with reference to the air dried powder

Determination of Acid Insoluble Ash

The total ash was boiled for 5 minutes with 10% w/v dilute hydrochloric acid and filtered through an ash less filter paper (Whatmann no. 41). The filter paper was ignited in the silica crucible, cooled and acid insoluble ash was weighed.

Determination of Alcohol Soluble Extractive

Plant powders were macerated with 100 ml of alcohol of the specified strength in a closed flask for 24 hrs, shaking frequently during 6 hrs and allowing it to stand for 18 hrs. It was filtered rapidly taking precautions against loss of alcohol and 25 ml of the filtrate was evaporated to dryness in a tarred bottomed shallow dish at 105^0C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air dried powder**.**

ISSN 2515-8260 Volume 09, Issue 09, 2022

Determination of Water Soluble Extractive

About 5 g of the each powder was added to 50 ml of water at 80^0C and to it 2 gm of Keiselghur was added and filtered. 5 ml of the filtrate was transferred to a tarred evaporating dish, the solvent was evaporated on a water bath, drying was continued for half an hour, finally it was dried in a hot air oven for 2 hours and weighed. The percentage of water soluble extractive was calculated with reference to air dried drug.

Loss on drying

The Loss on Drying Test is designed to measure the amount of water and volatile matters in a sample when the sample is dried under specified conditions.

The weighing bottle was dried for about 30 min and cooled in the desiccator and weighed it again accurately. 2gms of sample was taken into weighing bottle and the sample was spread as a thin layer less than 5mm and weighed it accurately and placed in oven dried at a temperature of about 10 degrees for 1/2hr and cooled and weighed until constant weight was obtained.

Swelling index

The swelling index is the volume in ml taken up by the swelling of 1gm of herbal material under specified conditions. Its determination is based on the addition of water or a swelling agent as specified. About 2gms of powdered drug was taken in a glass stoppered measuring cylinder and added water of about 25ml and shaken repeatedly for about 1hour and allowed to stand it for 24hours and volume in ml is read is measured**.** The procedure is repeated for two times.

The swelling index was calculated by using the formula

$$\text{Swelling index} = \frac{\text{Final weight-initial weight}}{\text{Final weight}}$$

Foaming index

The foaming ability of an aqueous decoction of herbal materials and their extracts is measured in terms of a foaming index. About1gram of powdered drug was taken in to a 500ml conical flask and 100ml of boiling water was added to it and maintained at moderate boiling for 30 min collected and filtered into 100ml conical flask. The decoction was taken into 10 stoppered test tubes add it in successive portions of 1ml,2ml.....10ml and the volume is adjusted to 10ml and the test tubes was stoppered and shaken for 15 seconds lengthwise 2 shakes per second and allowed to stand for 15 min and the length of foam was measured.

Foaming index was calculated using the following formula: Foaming index =1000/a

Where, a = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1 cm is observed.

Quantitative estimation of Phytochemicals

Determination of Total Phenolics

The amount of total phenolic content of the extracts was determined by Folin-Ciocalteau reagent as oxidizing agent, gallic acid as standard. Exactly 0.1 ml of the extract was transferred to a 100 ml Erlenmeyer flask and the final volume was adjusted to 46 ml by addition of distilled water. 1 ml of Folin-Ciocalteau reagent diluted 10 times was added and incubated at room temperature for 3 min. 3 ml of 2% sodium carbonate solution was added and the mixture was shaken on a shaker for 2 h at room temperature. The absorbance was measured at 760 nm. Gallic acid was used as the standard (250-15.625 µg/ml) for a calibration curve. The phenolic compound content was expressed in terms of mg/ml (**Nagulendhran*et al.,* 2007**).

Estimation of Total Flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination. One milliliter of sample was mixed with 3 ml of methanol, 200 µl of 10% aluminum chloride, 200 µl ml of 1 M potassium acetate and 5.6 ml of distilled water and remains at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm with ultraviolet (UV) visible spectrophotometer. The content was determined from extrapolation of calibration curve which was prepared by using quercetin solution (6.25-100 µg/ml) in methanol. The concentration of flavonoid was expressed in terms of mg/ml (**Akinpelu*et al.,* 2010**).

Results and Discussion

Phytochemical screening of *Camellia sinensis* twigs:

Test	**n-butanolic extract of *Camellia sinensis***
Alkaloids	+
Carbohydrates	+
Tannins	+
Flavonoids	+
Steroids & Terpenoids	+
Glycosides	+

ISSN 2515-8260 Volume 09, Issue 09, 2022

Saponins	+
Phenols	+
Proteins	+
Fixed oils & Fats	+

+indicates Presence -- indicates Absence

Percentage yield of plants

The percentage yield of the extracts were found to be 45.66%, CS twigs extracts were semisolid in nature so, further shade drying is necessary and are light green in color.

Table: Percentage yield of plants

Plant Material	**Solvent used**	**Percentage yield**	**Nature**
Camellia sinensis	**n-butanol**	**45.66%**	semisolid

Physiological Parameters

The physiological characters for the both of the plants were shown in given table. All are found to within limits as per ayurvedic pharmacopeia

Table : Different physiological parameters of ***Camellia sinensis*** Twigs

Parameters	Plants powder
	Camellia sinensis (%)
Loss on drying	4.36
Total ash	15.21
Acid insoluble Ash	7.01
Water soluble Ash	8.20
Swelling Index	3.5
Foaming index	6

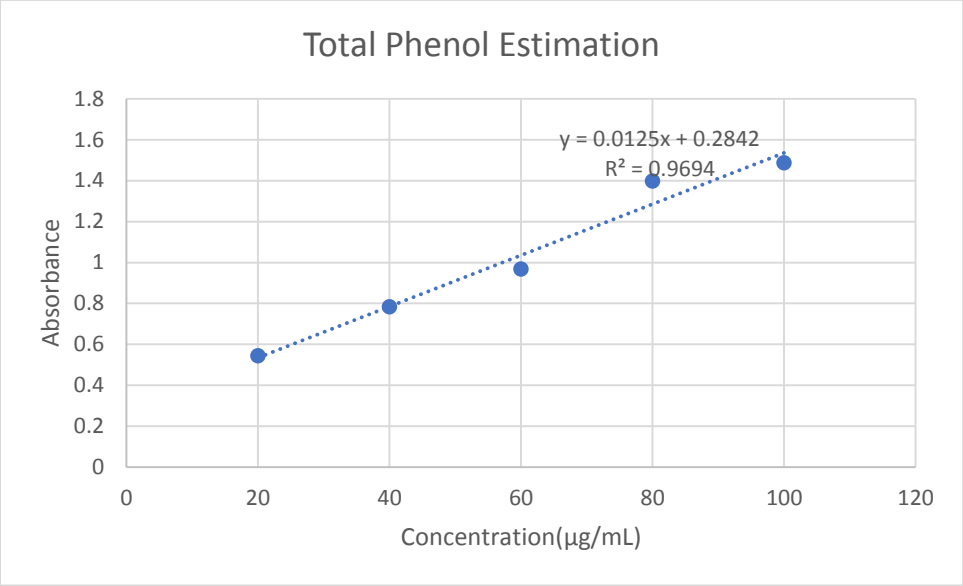
Total phenolic content: Total phenolic content in the *CS twigs* extract was showed to be 128.92±0.149 %w/w equivalent of gallic acid.

Total flavonoid content: Estimation of total flavonoid content of *CS* has showed high total flavonol content 41.13±0.672mg/g of quercetin equivalent.

Calibration data for total phenolic contents

Gallic acid concentration (µg/mL)	Absorbance (Mean ±S.D)
20	0.543±0.008
40	0.782±0.001

60	0.967±0.007
80	1.398±0.003
100	1.487±0.002



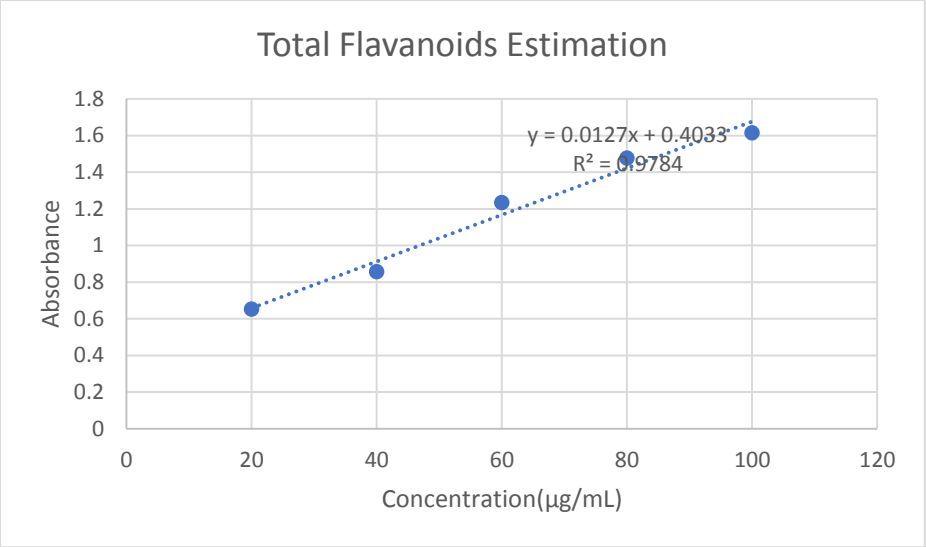
Estimation of total phenolic content of n-butanolic extract of *Camellia sinensis* twigs

Treatment	Concentration (µg/mL)	Total phenolic content (mg/g of gallic acid equivalent) (Mean ±S.D)
Camellia sinensis	1000	128.92±0.149

Results are mean of three values ± SD

Calibration data for total flavonoid contents

Quercetin concentration (µg/mL)	Absorbance (Mean ±S.D)
20	0.652±0.003
40	0.857±0.006
60	1.234±0.001
80	1.476±0.008
100	1.675±0.005



Estimation of total flavonoid content of n-butanolic extract of *Camellia sinensis* twigs

Treatment	Concentration (µg/mL)	Total flavonoid content (mg/g of quercetin equivalent) (Mean ±S.D)
CS	1000	41.13±0.672

Results are mean of three values ± SD

Conclusion:

Camellia sinensis twigs are taken and extracted with n-butanol by the process called Maceration. The preliminary phytochemical screening was done and found to be many secondary metabolites like Alkaloids, Glycosides, Flavonoids, Carbohydrates, Tannins, Steroids, Saponins and Tannins etc.The physicochemical parameters like LOD, Ash values like Total Ash, Acid insoluble ash, Water soluble ash, Extractive values, Swelling index and Foaming index etc., Total Phenolic and Flavonoidal studies are done. These plants are well known and have source of curing ailments for time immemorial. In future studies the isolated principles needs to be evaluated in scientific manner using specific animal models. Clinical studies are needed to confirm the actions.

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