Immunomodulatory Activity, GC-MS Analysis and Pharmacokinetic Potential of *Camellia Sinensis*

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Research Article

Keywords: C. sinensis, cellular immunity, humoral immunity, GC-MS analysis, SwissADME

Posted Date: July 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-708099/v1>

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Abstract

The immunomodulatory activities of medicinal plants are well-known. Medicinal plants found natural components enhance the immune system. However, both the *Camellia sinensis* is found a large number of bioactive compounds that make it strong to fight against ailments. It plays a significant role in cell-mediated and humoral immunity. The immunomodulatory activity of *C. sinensis* was carried out by neutrophil adhesion test, antibody titer and delayed-type hypersensitivity. The present study was aimed to investigate the immunomodulatory activity of methanolic extract of *Camellia sinensis* in Wistar albino rats. GC–MS analysis was carried out on the potent plant (*C. sinensis*) to recognize bioactive volatile compounds, for their therapeutic properties, respectively. *C. sinensis* treated animals showed a significant outcome at dose 200 mg. GC–MS analysis was carried out of the *C. sinensis* to recognize bioactive volatile compounds. Out of 20, five major compounds were found are 2-Pentanone, 4-hydroxy-4-methyl, Caffeine, 1-H Benzimidazole, 2-phenyl, Hexadecenoic acid, 15-methyl, methyl ester, Trans-13-Octadecenoic acid, methyl ester. In this study, *C. sinensis* has more potential to modulate the immune system in experimental animals. This study provides a substantial way to replace deleterious medicines and provides natural compounds that a part of the lifestyle to get rid of diseases. The highest % area was found is 1-H Benzimidazole 2-phenyl. The pharmacokinetic property of bioactive compound was carried out by SwissADME tool.

Introduction

The immune system plays a significant role to fight against foreign bodies (Rahayu *et al.*, 2018). Medicinal plants are an abundant source of bioactive substances that are obliging to enhance the immune system of our body. In Ayurveda, traditional medicinal plants create attention to build up a body defense mechanism against various threatening diseases (Shukla and Mehta, 2015). The notion of immunomodulation relates to the non-specific activation of the immune system. It is a non-antigen-dependent reaction and triggers the function and activation of macrophages, granulocytes, natural killer cells, lymphocytes, and also the production of various effectors molecules by activated cells (Sumalatha *et al.*, 2012). Immunomodulation is the process by which the immune system enhances towards the prevention and cure of many disorders in the human body such as respiratory disorder, inflammation in skin, cancer and some autoimmune diseases including rheumatoid arthritis and allergic manifestations (Tiwari *et al.*, 2011). Usually, allopathic drugs are used to modulate the immune system but these drugs are extravagant for underprivileged people. Therefore, herbal medicines are the best alternative to replace these medicines (Nfambi *et al.*, 2015). Tea is one of the most liked beverages procured from *Camellia sinensis* belongs to the family Theaceae. It contains many bioactive compounds such polyphenols, flavonoids, terpenoids, amino acids, etc. (Singh *et al.*, 2009) A major polyphenol found in tea is epigallocatechin (EGCG) which is popular for its antimicrobial, antioxidant and immunomodulatory, anticarcinogenic and antimutagenic properties (Monobe *et al.*, 2008). Wang *et al.* (2001) proved that tea bioactive compounds are responsible to strengthen the immune system.

Commonly, food polysaccharide found in algae, mushrooms, and plants is responsible to activate macrophage activity to engulf foreign particles which are effective in antitumor activity, immunomodulation, etc. (Schepetkin and Quinn, 2006). Tea extract consists of a broad range of biological activities, antioxidant, antiobesity, anticancer, and hepatoprotective activity (Leung *et al.*, 2001; Salminen *et al.*, 2012). The present study was aimed to evaluate the immunomodulatory parameters (neutrophil adhesion test, haemagglutination activity, and delayed-type hypersensitivity), identify the bioactive compound and phamakokinetic property of bioactive compound of *C. sinensis*.

Material And Methods

Collection of Plant Sample

The young and healthy leaves of *C. sinensis* were collected from DTC, Dehradun, Uttarakhand. *C. sinensis* was identified and authenticated by Botanical Survey of India (BSI), Dehradun. The accession number given by BSI is 19a.

Extract Preparation

100 g of crushed leaves of *C. sinensis* was weighed and kept into the Soxhlet apparatus for about 6 hours for extraction. Petroleum ether, chloroform, acetone, methanol, ethanol, and water solvents were used for extraction. The methanolic of *C. sinensis* was selected based on their antimicrobial activity. The solvents of the extracts were evaporated by using Rota-evaporator and stored in a refrigerator for further use. According to our previous experiments, methanolic extract of *C. sinensis* was found more potent so, we use methanol extract of *C. sinensis* for the study.

Antigen Preparation:

Sheep red blood cells (SRBCs) were collected from the local slaughterhouse for antigen preparation. SRBCs and were washed three times with sterile pyrogen-free 0.9% saline. Cell concentration was adjusted 0.5×10^9 cell/ml for haemagglutination titer and delayed hypersensitivity reaction (Patel and Asdaq, 2010).

Animal Treatment

After assent from the institutional animal ethical committee and in (Ref. No. GKV/AHF/10/2018), laboratory breed of male Wistar albino rats weighing between 180 g and 200 g were procured from Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana. Husk (Paddy) was used as bedding and changed at regular interval of time. All animals were housed in polypropylene cages under standard environment maintained at 21 ± 2 °C and supplied with a standard pellet diet and water *ad libitum* in the Animal House (Reg. No. 1324/a/10/CPCSEA), Department of Pharmaceutical Sciences, Gurukula Kangri (Deemed to be University), Haridwar.

Experimental Design

The animals were divided into four groups containing six rats of each group, administered the following treatment. Group I: Normal control (saline). Group II: *Camellia sinensis* (50 mg/kg, p.o). Group III: *Camellia sinensis* (100 mg/kg, p.o). Group IV: *Camellia sinensis* (200 mg/kg, p.o).

Neutrophil adhesion test

A neutrophil adhesion test was carried out according to the method of Patel and Ashaq (2010). Rats were pre-treated orally with saline (Group I) as a control and the other 3 groups administered were methanolic extracts of *C. sinensis*. On the 14th day of treatment, blood samples were collected by puncturing the retro-orbital plexus into heparinized vials and examined for total leucocyte counts (TLC) and Differential leucocyte count (DLC). After initial counts, the same blood samples were incubated with 80 mg/ml of nylon fibers for 15 min at 37°C and again analyzed for TLC. The product of TLC and neutrophil (%) gives the neutrophil index (NI) of the blood sample. Neutrophil adhesion (%) was enumerated as follows:

$$\text{Neutrophil adhesion (\%)} = \frac{N_{lu} - N_{lt} \times 100}{N_{lu}}$$

Where,

N_{lu} = Neutrophil index (untreated blood sample)

N_{lt} = Neutrophil index (treated blood sample with nylon fiber)

Haemagglutination antibody (HA) titer

Rats of groups I, II, III, and IV were immunized with 0.5×10^9 SRBCs/rat by intraperitoneal route. Rats of groups II, III, and IV were pre-treated with methanolic *C. sinensis* extract for 14 days. The day of immunization with SRBCs was referred to as day 0. The animals were treated with plant extracts for 14 more days and blood samples were collected from each rat on day 15 for HA titer. The titer was determined by titrating serum dilutions with SRBC (0.025×10^9 cells). The microtitre plates were incubated at room temperature for 2 hours and examined visually for agglutination reaction. The minimum concentration of serum showing haemagglutination was expressed as HA titer (Fulzele *et al.*, 2003).

Delayed type hypersensitivity (DTH) response

The delayed-type hypersensitivity response was determined by following Bin-Hafeez *et al.*, 2003). Six animals per group were immunized with 1×10^9 SRBC/rat, subcutaneously. On the fifth day, all the animals were again immunized with 1×10^9 SRBC in the left hind foot. The right footpad injected with the same volume of saline served as control. Plant extract (methanolic extract of *C. sinensis*) was administered orally. DTH response was measured at 24 h after SRBC challenge on day 5 and expressed as a mean percent increase in paw volume.

GC-MS analysis (bioactive compounds)

The GC-MS analysis of methanolic extracts of *C. sinensis* was carried out by Pradhan and Dubey, 2021.

Interpretation of results of GC-MS was conducted using the database of the National Institute Standard and Technology (NIST 2.0).

Statistical analysis

The significance of data were analyzed statistically by using one-way ANOVA followed by a post hoc t-test with Bonferroni's comparison. All the value of results were expressed as mean $\pm$ SD and $p < 0.05$ was considered significant.

Evaluation of Pharmacokinetic Properties of bioactive compound (1-H Benzimidazole 2-phenyl)

Pharmacokinetic properties and drug-likeness evaluation of GC-MS compound (highest peak) were carried out using SwissADME a free online web tool used in evaluating ADME properties and drug-likeness of small molecules. Lipinski's rule of five is helpful at the pre-clinical evaluation discovery of drugs (Tripathi et al., 2019).

Results

Neutrophil adhesion test

A blood sample incubated with nylon fibers diminution in the neutrophil count ascribed to the adhesion of neutrophil to the nylon fibers. Dosages of 100 mg and 200 mg showed significant results ($p < 0.05$), but dose 200 mg produced more significant result ($p < 0.001$). Therefore, a 200 mg dose was found more effective than the other doses of both the *Camellia sinensis* (Tables 1).

Haemagglutination titre

The administration of an increased dose of extracts significant result was observed at dose 100 mg ($p < 0.01$) and 200 mg ($p < 0.001$) of *C. sinensis* compared with the control group. At dose, 50 mg did not show non-significant results when compared with the control (Figure 1).

Delayed type hypersensitivity reaction

The delayed hypersensitivity reaction was observed significantly at dose 100 mg ($p < 0.01$) and 200 mg ($p < 0.001$) of *C. sinensis* as compared to control. However, dose 50mg was found non-significant compared to control (Figure 2).

GC-MS analysis (bioactive compounds)

In the present study, there are five major peaks observed at 7.53, 32.45, 34.03, 35.41, and 38.77 RT are 2-Pentanone, 4-hydroxy-4-methyl, Caffeine, 1-H Benzimidazole 2-phenyl, Hexadecenoic acid, 15-methyl, methyl ester, Trans-13-Octadecenoic acid, methyl ester (Table 2).

Evaluation of Pharmacokinetic Properties of bioactive compound (1-H Benzimidazole 2-phenyl) by Swiss ADME

The bioactive compounds were identified through GC-MS analysis and the pharmacological properties were determined by the SwissADME tool. Molecule fulfills the standard criteria expressed by Lipinski's rule of five, meaning the activity of the compounds high potency. In a null shell, the property of the molecules are considered according to absorption, low toxicity level, and orally bioavailable. The Bioavailability Radar gives an outline of the drug-likeness of a molecule (Figure 4). The region denote with pink colour indicates the different properties of the compound (Table: 3; Figure: 4 and 5).

Discussion

The present work was done to assess the immunostimulatory activity of *C. sinensis* in an animal model by studying different parameters such as neutrophil adhesion test, haemagglutination titer, and delayed-type hypersensitivity test. In this study methanolic extract of *C. sinensis* possesses immunomodulatory properties which enhance both cellular and humoral immunity. The extract was found to be more efficacious at high concentrations (200 mg), but at 100 mg concentration, the extract was quite effective in modulating the immune system. The plant products aids to modulate the immune system either by stimulation or suppression (Wagner, 1984) and help to use an alternative drug for immune-compromised people. The drugs that stimulate the immune system are called immunostimulant drugs that help to enhance the non-specific and specific system such as macrophages, granulocytes, certain T-lymphocytes (Anarthe et al., 2014). Similarly, *Camellia sinensis* work as an immunostimulant to enhance the immune system (Monobe et al., 2008). *C. sinensis* showed more significant results at 50 mg ($p < 0.05$), 100 mg ($p < 0.01$) and 200 mg ($p < 0.001$) in neutrophil adhesion test. The percentage of neutrophil adhesion increase might be due to the upregulation of the $\beta 2$ integrins adhering firmly to the nylon fiber that is present on the surface of neutrophils (Srikumar et al., 2005). Adhesion of neutrophils to nylon fiber correlates the margination of polymorphonuclear lymphocyte present in the blood vessel and the number of macrophages reached in the inflammation site (Patel and Asdaq, 2010).

The haemagglutination test correlates with humoral immunity. The *C. sinensis* extract showed effects on the humoral immune system. The results of *C. sinensis* were found to be most effective at 200 mg with a value of 469.33 ($p < 0.001$). Likewise, at 100 mg also *C. sinensis* was found to be significant with a value of 213.33 ($p < 0.01$). The humoral immune response is composed of B cells with antigens and eventually proliferating into plasma cells that secrete antibodies. Antibodies producing cells bind to antigens, and neutralizes it, and accelerates the elimination of antigens by readily ingesting phagocytic cells (Fulzele et al., 2003). This was indicated by the mean value of the haemagglutination titer that depicted a dose-dependent elevation for the production of antibodies (Nfambi et al., 2015). According to the

Coombs and Gell classification (1975), the delayed-type hypersensitivity is also known as type IV cell-mediated immune response. This test directly correlates with the cell-mediated response and was found to be significant with *C. sinensis* at doses 100 mg ($p < 0.01$) and 200 mg ($p < 0.001$). In DTH, when challenged by the antigen, the T-lymphocytes were converted into lymphoblasts and producing lymphokines that attract more macrophages at the site of reaction. Due to the presence of flavonoids, the humoral immune response amplifies by proliferating macrophages and B-lymphocytes for the production of antibodies (Makare *et al.*, 2001). It is used to analyze the skin response after inoculation of the antigen intradermal which depends on antigen-specific memory T-cells and the results were due to the augmentation of neutrophils and mononuclear cells. T-cell activation recruits the accumulation of macrophages which induces vasodilation, inflammation, and increases vascular permeability (Janeway *et al.*, 2001; Goronzy and Weyand, 2007). It also uplifts the phagocytic activity and increases the production of lytic enzymes for the more efficient killing of microorganisms (Janeway *et al.*, 2001). An increase in the DTH response indicates that plant extracts have a stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction (Mitra *et al.*, 1999). By findings of immunomodulatory activity, *C. sinensis* has great potential to enhance the immune system. Whereas with the avail of GC-MS for finding the bioactive compounds that are amenable for boosting the immune system. The possible compounds present in the methanolic extract of *C. sinensis* by GC-MS analysis. About 20 peaks are observed in the GC-MS study of *C. sinensis*. The major and minor peaks were discussed (Table 2). It is explicit that these peaks are for the phytoconstituents (bioactive compounds) present in the plant which are responsible to possess many biological and therapeutic potential. The current study confirmed that the methanolic extract of *C. sinensis* possesses potent antimicrobial, antioxidant, anti-inflammatory, anti-cancerous properties.

The pharmacokinetic property and drug-likeness were carried out by Swiss ADME (Table: 3; Figure: 4 & 5). The bioactive compound extracted from our study somehow fulfills the standard requirement of Lipinski's rule. The inbuilt BOILED-Egg model finds out that showed 1-H Benzimidazole 2-phenyl) the ability of BBB penetration as well as GI absorption. The phytocompound was found PGP positive as substrate in the present predictive model. There is a possibility that 1-H Benzimidazole 2-phenyl) may not create impediment glycoprotein activity. The prophecy of the bioactive molecule may be from natural product through the computational study of pharmacokinetics, drug-likeness, bioavailability and medicinal chemistry friendliness are the potent research province for new drug development from the natural available compound by using the SwissADME online tool (Daina *et al.*, 2017; Chakravarty *et al.*, 2019). The present predictive study was done on phytocompounds of *C. sinensis*. It was well-known that the physicochemical properties such as solubility and lipophilicity prediction are also detected the small molecule whether progressing a successful drug candidate (Daina *et al.*, 2017; Tripathi *et al.*, 2019). The graphical representation of Blood-brain barrier and Gastrointestinal absorption predicted method (BOILED-Egg) has already been proposed as an accurate predictive model, which supports the computational prediction of the lipophilicity and polarity of studied small molecules (Karmakar *et al.*, 2019). Furthermore, the leaf extract of *C. sinensis* has been used for the anti-inflammation, antimicrobial and immune enhancement drug in the experimental study done by the researchers (Shaharyar and Mazumder, 2017; Pradhan and Dubey, 2021).

Based on the study, the results prove that the leaves of *Camellia sinensis* has the potential to enhance the cell-mediated immunity and it may be effective in several immunocompromised clinical conditions.

Conclusions

The immunostimulatory effect produced by *Camellia* species helps in the stimulation of both cellular and humoral immune system and also increases the total leucocyte counts. This study proves the use of tea beverages shall be helpful to immunosuppressed people by enhancing the immune system. The GC-MS (Gas chromatography-Mass spectrometry analysis depicted the biologically active compounds that are accountable for enhancing the immune system. The prediction of pharmacokinetics potential, bioavailability, drug-likeness and medicinal chemistry friendliness revealed that the bioactive compound present in *C. sinensis* can be a lead compound for new drug discovery for antimicrobial, immune enhancement and anti-inflammatory phytomedicine. However, it is suggested further *in vivo* and computational assay for toxicology, pharmacology and experimental bioavailability study for reliving phytocompound to validate the present augury.

Declarations

Acknowledgment

The authors acknowledge the Head of the Department of Botany and Microbiology for providing laboratory facility, Head of, Department of Pharmaceutical Sciences for availing Animal house facility and the Director of Tea Estate, Dehradun (Uttarakhand) for providing tea leaves.

Ethical statement

All the experimentals were followed under the guidelines of Organisation for Economic Co-operation and Development (OECD) for the supervision and use of Laboratory Animals at Gurukula Kangri (Deemed to be University).

Consent to Participate

Not Applicable

Consent to Publish

Yes

Author Contribution

Surbhi Pradhan experimented and prepared the manuscript. R.C Dubey corrected and finalized the manuscript.

Funding

No funding source.

Competing Interest

The authors declare that they have no conflict of interest.

Availability of Data and Material

All data have been provided.

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Tables

Table 1: Neutrophil adhesion test of *C. sinensis*.

Treatment	TLC		Neutrophil %		Neutrophil Index (NI)		Neutrophil adhesion %
	UTB	NFTB	UTB	NFTB	UTB	NFTB	
Control	7.56 ± 0.23	6.9 ± 0.19	25.15 ± 0.18	24.71 ± 0.39	190.31 ± 6.41	170.57 ± 5.69	10.35 ± 1.83
Extract (50 mg)	7.88 ± 0.15	7.33 ± 0.26	27.81 ± 0.63	25.4 ± 0.93	219.34 ± 8.33	186.15 ± 7.08	15.09 ± 2.50*
Extract (100 mg)	8.11 ± 0.24	7.81 ± 0.21	30.08 ± 1.30	25.75 ± 1.40	244.26 ± 14.35	201.5 ± 15.57	17.50 ± 3.95*
Extract (200 mg)	8.23 ± 0.24	7.9 ± 0.19	36.5 ± 3.09	28.33 ± 2.13	300.96 ± 31.72	224.01 ± 20.10	25.22 ± 5.64***

All values are expressed as mean±Standard deviation of six observations. UTB, untreated blood; NFTB, nylon fiber treated blood. Values are statistically significant at P < 0.05*, P < 0.001*** when compared with control group.

Table 2: Activity of Pyto-components (bioactive compounds) identified in the methanolic extract of *Camellia sinensis*.

Compound name	RT	Molecular Formula	Molecular weight	Area %	Biological Activity
2-Pentanone, 4-hydroxy-4-methyl	7.53	C ₆ H ₁₂ O ₂	116.16	23.90	Antimicrobial potential (Sheibani et al., 2016; Garcia et al., 2016)
Cyclohexasiloxane, dodecamethyl	20.45	C ₁₂ H ₃₆ O ₆ Si ₆	444.92	1.03	Antimicrobial and Antioxidant (Gong et al., 2012; Jasim et al., 2015)
2',6' Dihydroxyacetophenone, bis(trimethylsilyl)ether	24.96	C ₁₄ H ₂₄ O ₃ Si ₂	296.51	1.88	Antineoplastic (colorectal and colon cancer), Stimulant of erythropoiesis, Antiosteoporosis, Treatment of bone diseases, Antifungal, Carminative (Kokila et al., 2020).
Caffeine	32.45	C ₈ H ₁₀ N ₄ O ₂	194.19	5.33	Antimicrobial property (inhibit synthesis of proteins and DNA by inhibiting the incorporation of adenine and thymidine) (Gupta and Kumar, 2017)
1-H Benzimidazole, 2-phenyl	34.03	C ₁₃ H ₁₀ N ₂	194.23	33.46	Antimicrobial, Antiviral, Anticancer, Anti-inflammatory, Analgesic (Shaharyar and Mazumder, 2017)
7-Hexadecenoic acid, methyl ester, (Z)	35.27	C ₁₇ H ₃₂ O ₂	268.4	1.02	Antibacterial (Hagr, 2019)
Hexadecanoic acid, 15-methyl, methyl ester	35.41	C ₁₈ H ₃₆ O ₂	284.5	7.19	Antieczematic, Macrophage colony stimulating factor, Flavor, Antioxidant, Hypocholesterolemic, 5-alpha reductase inhibitor ((Kokila et al., 2020; Patil and Jadhov, 2016)
7-Octadecenoic acid, methyl ester	35.76	C ₁₉ H ₃₆ O ₄	296.5	6.37	Antibacterial (Elizabeth and Arumugam, 2014)
Heptane, 1-bromo-6-methyl	35.98	C ₈ H ₁₇ Br	193.12	1.73	-
Oxacyclotetradecan-2-one	37.40	C ₁₃ H ₂₄ O ₂	212.33	0.73	Antibacterial (Peng et al., 2013; Jing et al., 2002)
N(Trifluoroacetyl)N,O,O',O''tetrakis(trimethylsilyl)norepinephrine	38.43	C ₂₂ H ₄₂ F ₃ NO ₄ Si ₄	553.9	0.39	Antibacterial (Soliman et al., 2016)
Trans-13-Octadecenoic acid, methyl ester	38.77	C ₁₉ H ₃₆ O ₂	296.5	8.32	Cancer preventive, Anti-inflammatory activity, mood and sleep disorders and also in cannabinoid regulated depression, Anti-fungal activity (Gideon et al., 2015; Joshi et al., 2018;

Table 3: Pharmacokinetics of bioactive GC-MS compound by SwissADME.	
Physicochemical Properties	
1-H Benzimidazole, 2-phenyl	C ₂₀ H ₁₆ N ₂
Mol. wt.	284.35 g/mol
Lipophilicity	
Log P _{o/w} (WLOGP)	4.75
Water Solubility	
Class	Moderately soluble
Pharmacokinetics	
Gastrointestinal absorption	High
BBB permeant	Yes
Druglikeness	
Lipinski	Yes
Ghose	Yes
Veber	Yes
Egan	Yes
Bioavailability	0.55
Medicinal Chemistry	
PAINS	0 alert
Synthetic accessibility	2.18
XLOGP3	>3.5
Abbreviations: GI: Gastrointestinal absorption; BBB: Blood-brain barrier permeation	

Figures

Figure 1

Haemagglutination titre of *Camellia sinensis*.

Figure 2

Delayed type hypersensitivity of *Camellia sinensis*.

Figure 3

GC-MS analysis of *Camellia sinensis*.

Figure 4

The Bioavailability Radar of 1-H Benzimidazole, 2-phenyl by SwissADME.

Figure 5

The BOILED-Egg represents for intuitive evaluation of passive gastrointestinal absorption (HIA) white part and brain penetration (BBB) yellow part as well as blue and red points PGP positive and negative in function of the position of the small molecules in the WLOGP-versus-TPSA graph (Molecule 1: 1-H Benzimidazole, 2-phenyl).