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Preliminary Phytochemical and Toxicity Determination of Ethyl Acetate Extract of *Cinnamomum Camphora*

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AIM- The aim of the present investigation is to study the preliminary study of dried leaves of *Cinnamomum Camphora*. **MATERIAL & METHODS-** Dried leaves of *Cinnamomum Camphora* were procured from the medicinal garden and campus of Pharmacy College in the month of September, Bhopal, Madhya Pradesh, India. Around 500 gm dried leaves of *Cinnamomum Camphora* were coarsely powdered weighed and filled in Soxhlet apparatus for extraction. First the powdered drug was defatted with petroleum ether (60°C-80°C); Defatted drug was then dried and again filled in soxhlet apparatus for successively extraction with dichloromethane, ethyl acetate, methanol and water as solvent. The extraction was carried out for a period of 72 hrs. The extract obtained was dried in vacuum to remove excess solvent and were weighed for the determination of % yields. Qualitative chemical tests of all extracts were subjected to various chemical tests to detect various Phytoconstituents. The acute oral toxicity studies were carried out according to the guidelines set by the Organization for Economic Co- operation and Development (OECD), revised draft guideline 423. **RESULTS-** Dried leaves of *Cinnamomum Camphora* were extracted using pet ether, dichloromethane, ethyl acetate, methanol and water and the percentage yield was found to be 5.2, 7.8, 8.5, 9.7, 9.4 %. The preliminary phytochemical analysis revealed that different active constituent present in different extracts such as carbohydrates, proteins, amino acids, fat, oils, steroids, terpenoids, glycosides, alkaloids, tannins and other phenolics compounds. **CONCLUSION-** No toxic effects were observed at a higher dose of 2000 mg/kg body weight of Wistar rats. Hence, 1/ 10th dose was selected as effective dose or therapeutic dose. The cut off value of 200 and 1/5 dose double of 400 mg/kg were selected for further activity.

KEYWORDS-

Preliminary Phytochemical, Acute Toxicity Study, Dried Leaves, *Cinnamomum Camphora*, OECD Guidelines

INTRODUCTION

Toxicity is the quantity to which a substance can damage humans or animals. It can be deliberate by its effects on the target organism, organ, tissue or cells. The toxic effects of a

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substance on animal physiology can variety from minor changes such as reduced weight gain, small physiological modification or change in the levels of circulating hormones, to severe effects in organ purposeful loss leading to death. Intermediate levels of toxicity may cause pain and suffering [1, 2]. These developments have been encouraged by discovery of teratogenic effects of drugs such as thalidomide, exposure of chemicals to the environment and employees and by conduct and assessment of toxicity studies as part of good manufacturing practice [3]. During toxicity studies, all the animals must be checkered for morbidity, mortality and specific signs of toxicological relevance. For example, neuro functional and neurobehavioral, ophthalmological observation, body-weight and food/water intake. The key haematological parameters investigated are mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), haemoglobin levels, haematocrit levels, packed cell volume (PCV) total and differential leukocytes, erythrocytes and platelet counts [4].

The acute toxic class method, a step-wise procedure, involves the use of three animals of a single sex per step. Depending on the mortality and/or moribund status of the animals, on average 2 to 4 steps may be necessary to allow decision on the acute toxicity of the material. The OECD Guideline 423 (2001) provides a reproducible method that uses a small number of animals as per appendices 2, 3 and 4 [5-7]. The aim of the present investigation is to study the preliminary phytochemical and toxicity study of *Cinnamomum Camphora* leaf.

Materials and Methods

Plant materials

The leaf part of *Cinnamomum Camphora* was collected from local area of Bhilwara and college campus of Sangam University in the month of August & September.

Authentification of Plant Materials

The plant materials i.e. leaf was taxonomically identified by Senior Scientist, College of Horticulture. The herbarium sheets were submitted in Department of Pharmacognosy, School of Pharmacy.

Preparation of Total Crude Extract

The plant material i.e. leaf was dried under shade and subjected to coarse powder for extraction process. Accurately weighed quantity of leaf powder of *Cinnamomum Camphora* was extracted using petroleum ether for the removal of fats and then different solvents were used according to polarity charts i.e. DCM, Ethyl acetate, methanol and finally water by

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soxhlet apparatus for 72 h. After drying, the respective extracts were weighed and percentage yield was determined [8].

Preliminary Phytochemical Tests

Qualitative chemical tests of different extracts were subjected to various chemical tests to detect various phytoconstituents [9, 10].

Selection of animals

Wistar albino rats of either sex between 2 and 3 months of age weighing 150-200 g were used which were procured from the central animal house of College of Pharmacy, India. All animals were housed in an animal room under normal condition of 25±1^{0}C, 12-h light and dark cycle. The animals were allowed free to access commercial rat pallet diet (Lipton India Ltd, Mumbai, India) and water *ad libitum*. The bedding materials of the cages were changed every day. All the experimental procedures were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The study designs were approved by the Institutional Animal Ethical Committee of College of Pharmacy, (MP), India.

TOXICITY STUDIES

Acute toxicity studies after oral administration of different extracts in rats

The ethyl acetate extract was suspended in 2.5 % Tween 80 in normal saline. The test dose was administered in a single dose by gavages using appropriate intubation canula. Food was withheld for an additional 3-4 hours after dosing.

The Globally Harmonized Classification System (GHS) in acute toxicity category (ATC) method of the OECD was used to conclude the LD50 range (OECD Guidelines, 2001) as per appendix 2. Since in attendance was no prior information on toxicity of *Cinnamomum Camphora* extract, for animal wellbeing reasons, the starting dose was selected to be 200 mg/kg body weight (Appendix 3). Since no death occurred at this dosage level, then the next higher dose, 2000 mg/kg was used. In 2000 mg/kg category, all the three rats were treated with 2000 mg of extract per kg body weight of the rat. The volume of the extract given was designed according to the weight of each rat, ensuring that the volume fed to the rat did not exceed 2 mL.

Mortality and other clinical signs were recorded. The test was considered in such a way that if one or no animal died after 24 hour, the procedure was frequent by using three rats at the same dose level. Absence of death or death of only one animal during the repeat dose

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roundabout that the LD50 range is more than 2000 mg/kg but lies between 2000 mg/kg and 5000 mg/kg. Moribund rats, those obviously in pain or showing signs of severe and continuing distress were humanely killed and considered in the understanding of the test results in the same way as animals that died on test.

The rats were observed independently after dosing at least once within the first 30 minutes, then periodically during the first 24 hour. The parameters of interest were changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems for signs of toxicity that may include; tremors, convulsions, salivation, diarrhea, lethargy, somnolence, or coma or death. The mortality rates for each dose group were recorded for the first 24 hours. Standard pathology events were followed to examine the dead or sacrificed animals and the pathological changes recorded [11].

Acute toxicity after intraperitoneal administration

The procedure used conformed to the OECD guideline that is used in acute toxicity testing (OECD 423 guidelines, 2001) and as per Appendices 2 to 4. The ethyl acetate extract was dissolved in phosphate buffered saline with 3 % DMSO. The extracts were first filtered through filter paper, and then all the way through 0.2 µm Millipore filters to ensure sterility of the solution for intraperitoneal administration. The concentration of the solution was adjusted to ensure that the volume delivered per animal based on individual body weight was between 1 ml and 2 ml, the volume recommended for rats. The solution was then injected via intraperitoneal route starting with the 2000 mg/kg body weight dosage level [12].

The GHS/ATC method was then used to estimate the LD50 range. For example, for a starting dose of 2000 mg/kg body weight (Appendix 2), three animals were injected with 2000 mg/kg each. If 0-1 death occurred within 24 hours, the experiment was frequent with 3 more animals. If during the repeat exercise 0-1 death occurred, it was accomplished that the LD50 range for the extract falls between 2000 and 5000 mg/kg body weight. On the other hand, if 2-3 deaths occurred during the first or repeat exercise it was assumed that the LD50 range was below 2000 mg/kg and therefore the experiment was shifted to test procedure with a starting dose of 300 mg/kg body weight.

Sub-acute toxicity

Feed and water were provided *ad libitum* and the Wistar rats were allowed 7 days for becoming accustomed. The rats aged 6-8 weeks, were randomly owed and housed in cages, each containing 5 rats; males and females separately with wood shaving bedding changed

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twice a week to uphold hygiene. The animals were assigned at unsystematic to three treatment groups of 5 animals per sex and a control group. A total of 40 animals were used. Each treatment group received a different concentration of the plant extracts by gavages as described in the acute toxicity study. The dosage levels were logarithmically spaced as follows; 100 mg/kg, 300 mg/kg and 1000 mg/kg body weight daily. Controls were administered with untreated vehicle comprising 2.5 % Tween 80 in normal saline. Animals were dosed daily for 28 days with the test material on the basis of weekly mean group weight in accordance with OECD guideline (2008). Body weight of all animals was taken on weekly basis [13].

Clinical observations

Animals were pragmatic individually for clinical signs twice daily after dosing. Clinical explanations were recorded daily. The parameters of interest were changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, and autonomic and central nervous systems for signs of toxicity. Signs of toxicity included tremors, convulsions, salivation, diarrhea, lethargy, somnolence, or coma or death. The mortality rates for each dose group were recorded for the first 24 hours. Animals found declining or showing clinical signs of pain or distress was euthanized using diethyl ether [14].

Haematological tests

For haematological studies, 2-3 ml of blood was collected using a capillary tube from the orbital sinus of the lightly ether-anaesthetized Wister rat into a test tube containing ethylenediamine tetra acetic acid (EDTA). Haematological parameters of interest included; haemoglobin concentration (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), total erythrocyte count (RBC), haematocrit (PCV). Also collected was total white blood cell (WBC) and differential leukocyte count. Blood was collected before treatment and then thereafter fortnightly for 28^{th} days [15].

Clinical chemistry tests

About 2-3 ml of blood samples per animal were collected in heparinized tubes. Plasma was obtained by centrifuging heparinized blood at 12,000 rpm for 5 min. Plasma was separated and stored at -20 degree centigrade until use. Clinical chemistry parameters built-in concentration of total proteins, albumin and the activities of Aspartate aminotransferase (AST). These parameters were calculated using the liquid-chemistry photometric methods.

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The plasma protein concentration was strong-minded calorimetrically at 540 nm using the Biuret method while total plasma albumin was determined by the bromocresol green method at 630 nm. The enzyme activity was determined as per the guidelines from the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [16]. The plasma AST activity was determined by a photometric method with absorbance read at 340 nm.

STATISTICAL ANALYSIS

Data were expressed as the mean standard error of mean (S.E.M.) of the means and statistical analysis was carried out employing one-way ANOVA. Differences between the data were considered significant at $P < 0.05$.

RESULTS & DISCUSSION

TOXICITY STUDIES

Acute oral toxicity of *Cinnamomum Camphora* leaf extract

Medical Possessions

There was no death throughout acute oral toxicity test of *Cinnamomum Camphora* extract at 2000 mg/kg. All the animals showed clinical signs such as piroerectile, rubbing of nose and mouth and avoided feeds for the first 10 min post dosing. All the animals rubbed their mouth and nose with their front pawns and alongside the walls of the cage soon after dosing. All these symptoms moved out completely after 30 min of post dosing. The extract did not cause diarrhoea but the droppings in all test animals were wet and not well formed like pellets.

Unpleasant Pathology

Body weight changes during the 5 treatment days for the three animals dosed at 2000 mg/kg were not significantly different from the control group. All extracts treated animals showed a stable augment in body weights after 5th days. There was no confirmation of exudates in the peritoneal cavity during autopsy. The results are summarized in Table No.1.

Table No 1. Effect of ethyl acetate extract on weight of rats

S No.	Rat Groups	Weight at Zero day (gm)	Weight at 5th day after treatment (gm)	Weight gain (gm)
1	1	152	158	6
2	2	153	157	4
3	3	162	165	3

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4	Control	156	160	4

Acute toxicity study after intraperitoneal administration of leaf extract

Clinical Special Effects

Intraperitoneal administration of extract showed various serious complications and they lead to death of rats used in study. Initially, all the rats were treated by 2000 mg/kg, did not showed any toxic symptoms but all the rats died within two to three minutes. Immediately after dosing, the animals became restless, urbanized uncoordinated, jerky movements, then convulsed with their tails stretched and raised upward. In the 500 mg/kg body weight category, one rat died after 45 minutes, the second rat died after 4th hours and the third rat after 48th hours. However, symptoms were similar to 2000 mg/kg but milder. In third steps, 3 rats were dosed at 100 mg/kg survived without any observable symptoms for the next 24 hours and there was no mortality of rats. Dosing was continued for the next 5 days till the cumulative dose was equal to the next toxic dose of 500 mg/kg. The only observable transient signs that occurred within 30 minutes post extract administration included raised fur and mouth rubbing. From the above findings, it can be concluded that 100 mg/kg is safe dose for the pharmacological evaluation.

SUB-ACUTE TOXICITY

Clinical Signs

All the animals in 100 mg/kg, 300 mg/kg and 1000 mg/kg dose categories did not exhibit any abnormality throughout the 28th day's oral management of *Cinnamomum Camphora* extract. The only fleeting clinical signs that were most marked at 1000 mg/kg and lasted for about 30 minutes included raised fur, fast rates of respiration and rubbing at the oral cavity representative irritation. The animals looked dull and motionless immediately after dosing but this signs moved out after a few minutes. The motor functions were normal with no signs of gait irregularity. The mucous membranes were ordinary in all animals and there were no noticeable changes to the color of the eyes. All the animals except the control groups defecated semi-formed wet droppings that could not fit the description of outright diarrhoea. All the treatment groups gained weight increasingly as compared to the control. There was thus a significant weight difference from week 0 to week 1, week 1 to week 2, week 2 to week 3 and week 3 to week 4. Although there was a moderate increase in weight in all

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groups, the weight changes in the treatment groups 100 to 1000 mg/kg did not differ significantly. Weight changes between the test groups and control were significantly different, the control group gaining significantly more weight than any of the test groups at each time point. The animals in 100 mg/kg category showed a mean drop in weight during the first week of treatment and this is corroborated by a negative percentage body weight change. However, this occurrence could not be explained to be dose-related since the weight at high dosage levels was not affected at this time point. The control showed a higher increase in weight compared to all the test groups indicating that the extract had some effects either on the feeding patterns or on absorption of nutrients from the gut. The organ weight indices for various organs remained almost at a constant across the four treatment categories, that is, 1000, 300, 100 mg/kg body weight and in the control group implying that the oral doses tested had little or no impact on the weighed organs.

Table No 2. Effects of the *Cinnamomum Camphora* extract on mean body weight of rats

S No	Dosage mg/kg	Mean Body Weight				
		Week 0	Week 1	Week 2	Week 3	Week 4
1	Control	175.4 ± 2.45	188.4 ± 3.11	195.4 ± 2.93	210.5 ± 2.23	220.3 ± 3.22
2	100	180.7 ± 2.41	185.7 ± 3.56	198.6 ± 3.96	210.7 ±3.45	222.6 ± 3.32
3	300	177.3 ± 3.67	180.3 ± 3.33	187.5 ± 4.48	195.6 ±6.66	212.8 ± 3.67
4	1000	185.7 ±4.77	190.9 ± 2.99	198.8 ± 3.51	210.4 ± 4.26	205.7 ± 4.83

Table No 3. Effects of extract on actual weights & organ Weight indices of rats

S No.	Dosage	Organ Weight				
		Liver AMW (mg)	Kidney AMW (mg)	Adrenal AMW (mg)	Heart AMW (mg)	Spleen AMW (mg)
1	Control	713.33±1.77	1.42 ±0.23	58.3 ±2.11	494.1±8.22	774.7±4.41
2	100	713.22±2.88	1.44 ±0.12	61.5±3.23	498.4±7.34	780.3±8.66
3	300	721.14±2.33	1.41 ±0.14	62.7 ±4.45	510.6±7.33	784.8±9.78
4	1000	721.45±2.13	1.23 ±0.12	57.2± 2.11	522.7±4.55	785.2±6.62

Where- AMW-Average Mean Weight

Haematological effects

The haematological variations throughout the 4 weeks of sub-acute testing in rats. The extract

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caused a modest increase in RBC count at dosages 100, 300 and 1000 mg/kg compared to the control. However, this augment was neither dose nor time related. There was a slight significant dissimilarity in RBC values ($p<0.0001$.) at dose 1000 mg/kg and the control in the fourth week at which summit RBC dropped significantly beneath the control values dropped. All the treatment groups and the control had a measured rise in haemoglobin, then a slight fall during week 2 to 4 but the levels remained higher than those taken before treatment. The variations between treatment groups were not significant in all time points. Red blood cell levels and haemoglobin levels showed an almost common trend. PCV levels did not show any meaning dissimilarity between all the treatment groups and the controls. The values for immature RBC in doses 100, 300 and 1000 mg/kg were below a digit while that for control was zero. There was dose unconnected fluctuations in the levels of MCH in all treatment groups with that of control experiencing a restrained fall from week 1 to week 4. In 1000 mg/kg category, the MCH level fell during week 1 to 2 then rose progressively during week 3 to 4.

Table No 4. Overall cumulative effect of the extract on haematological values in rats

Dose	WBC	RBC	PCV %	Haemoglobin (g/dL)	MCV (fL)	MCHC (g/dl)	Thrombocytes (×103 /_l)	Neutrophils (%)	Total Mature Neutrophils (%)	MCH (pictogram)
1000 mg/kg	24022 ±3.33	5.33 ±0.5	42.16 ±2.4	15.44 ±2.34	62.9 ±3.13	40.95 ±3.6	424.3 ±7.12	25.7 ±4.72	24.4 ±4.72	22.3 ±4.24
300 mg/kg	2434 ±3.54	5.9 ±0.6	41.2 ±3	15.67 ±3.12	61.5 ±3.83	39.1 ±3.92	430.5 ±8.52	26.8 ±2.54	25.3 ±4.32	24.4 ±4.73
100 mg/kg	12135 ±5.44	5.6 ±0.2	40.2 ±2.1	15.98 ±4.33	61.3 ±4.33	39.4 ±3.22	432.6 ±5.33.4	27.2 ±4.34	24.4 ±4.22	25.8 ±3.84
Control	28098 ±4.02	6.2 ±0.8	43.3 ±1.22	16.22 ±3.12	62.6 ±3.99	40.4 ±3.91	433.8 ±4.69	26.7 ±4.23	26.4 ±4.24	26.7 ±3.39

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Thrombocytes levels knowledgeable a gradual augment in all the treatment groups together with their controls but these trends were not significantly different from the control and between treatment and at dissimilar times. There was a significant difference in the levels of thrombocytes at 1000 mg/kg treatment group and in the levels of WBC in the 300 and 100 mg/kg categories.

The WBC standards at dosage levels of 300 and 1000 mg/kg remained steady around 25,000 cell/µL between week 2 and 4. The increase in WBC values at 100 mg/kg compared personally with that of the control group between week 2 and 4 with the control group showing a slight increase on top of those values at dosage 100 mg/kg. WBC levels practiced a drop in week 0 to week 2 and then a small rise until week 4. The high WBC levels for dosage 100 and 300 mg/kg at the establishment of the conduct experiment could have been due to laboratory errors or personage animal factors since they could not be connected to extract administration.

In general, the lymphocyte levels remained almost a constant in all treatment groups, variable between 65 and 75%. The was no noticeable transform in lymphocyte level in control group, but there was a slight boost in 300 and 1000 mg/kg categories. The levels in 100 mg/kg group fell somewhat from week 1 to 2 then rose from week 3 to 4.

There was a non-dose correlated slight MCV increase in all treatment groups between week 2 to 4 with control almost outstanding as a constant. However, the level in 100 mg/kg category knowledgeable a drop from week 1 to 2 followed by a rise during week 3 and 4.

Total neutrophils and mature neutrophils levels did not show signs of any appreciable differences amongst all the groups. The values were a steady during week 1 to 2 in 100 mg/kg category but there was a slight reduction during week 3 to 4. In 300 mg/kg group the principles fell steadily throughout the treatment period. In 100 mg/kg category, there was a small increase followed by diminish during week 1 to 2 and week 3 to 4 respectively.

Clinical Parameters

Throughout the treatment of experimental rats with the extract, there was a small dose un-related alter in protein levels during week 0 to week 2. The protein levels for dosage 1000 mg/kg and the control greater than before by less than half a unit whereas those for dosage 100 and 300 mg/kg abridged the same margin. From week 2 to week 4, the levels of protein in all behavior groups increase by a minute value.

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All the dosage levels of the extract and the control showed similar trends in albumin concentration that was intimately similar to those in control group with values in all groups and the control changeable around 3.5 g/dL from weeks 2 to 4. There was therefore no important dissimilarity in albumin levels between all the treatment groups and the control.

Aspartate aminotransferase (AST) levels dropped progressively from week 1 to week 4 but levels in control group rose again reasonably between weeks 2 and 4. The decreasing trend in AST levels predicted that any continuous dosing of the animals ahead of week 4 could cause further decline in the levels of AST.

Table No 5. Effects of different flower extracts on the clinical chemistry in rats

Dosage	Total Protein level (g/dL)			Mean Albumin level (mg/dl)			Mean AST level IU/L		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
1000 mg/kg	7.8± 1.3	7.7 ± 1.1	7.5± 1.2	4.6±0.41	3.5± 0.21	4.9± 0.22	20.4± 2.11	14.5± 2.11	12.2± 2.13
300 mg/kg	7.7± 1.6	7.3 ± 1.2	7.8± 1.4	4.5± 0.53	3.6± 0.12	4.1± 0.25	19.4± 2.22	13.7± 2.23	13.4± 3.21
100 mg/kg	6.3 ± 1.6	7.9± 2.4	7.4± 1.1	4.8± 0.21	3.7± 0.23	4.7± 0.11	19.6± 3.12	14.4± 1.11	12.2± 1.31
Control	6.2 ± 2.2	7.6± 1.5	7.9± 1.3	4.7± 0.12	3.7± 0.11	4.8 ± 0.23	18.8± 2.16	14.7± 2.14	12.6± 3.24

CONCLUSION

The results showed that no death of experimental rats at an oral dose of 2000 mg/kg body weight symptomatic of that the ethyl acetate extract of *Cinnamomum Camphora* leaf is virtually non-toxic after oral dose exposure presuming that its components were engrossed. The extract can then be concluded to be safe for oral use as a traditional herbal remedy for the different ailments.

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