CHAPTER III

MATERIALS AND METHODS

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3.1 Experimental Animals

Four hundred forty-four (444) albino mice were collected from the Animal house of the Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi. Mice were about 6-7 weeks old. An average weight was 20-25 grams. Experiments were carried out following the Ethical Committee Guidelines set down by the local committee regarding the care and use of animals for experimental procedures.

Figure 2: Albino mice were housed in this plastic case.

3.2 Experimental Area

The research work was done in the laboratory of clinical pathology, Department of Pathology and Parasitology, Hajee Mohammad Danesh Science and Technology University, Dinajpur. Adequate light and ventilation were provided in the room. Mice were housed in plastic containers with beds made of sawdust. The room temperature was 20 ± 5^{0}C. Mice were kept on 12-hour light and dark cycle (Dasari *et al.*, 2014).

Figure 3: Mice container setup

3.3 Ehrlich Ascites Carcinoma Cell (EAC)

Ehrlich ascites carcinoma (EAC) cells were used throughout the study. The cells were collected from the Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi. EAC cells were maintained by weekly intraperitoneal inoculation of 1×10^6 cells/mouse (Osman *et al.,* 2011). Mice gained weight within 4-5 days after EAC cell inoculation. Gradually the lower part of the stomach starts to swell. Mice died within 21-30 days of ever blooming.

Figure 4: Intraperitoneal inoculation of EAC cells on mice

3.4 Tumor Volume

The mice were sacrificed and the ascitic fluids were collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume determined by centrifuging at 1000 rpm for 10 minutes (Deepak *et al.,* 2007).

3.5 Tumor Cell Count

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the numbers of cells in the 64 small squares were counted (Siva *et al.,* 2007).

3.6 Viable/non-viable Tumor Cell Count

The cells were then stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the stain were nonviable. These viable and non-viable cells were counted (Deepak *et al.,* 2007).

$$\text{Cell count} = \left(\frac{\text{No. of cells} \times \text{dilution}}{\text{Area} \times \text{thickness of the liquid film}}\right)$$

3.7 Blood Sampling

Blood samples were collected from the heart following by heart puncture process (Pagano *et al.,* 2004). The blood was divided into two parts. The first part was collected into an EDTA tube for hematological examination. The second part was collected in a clot activator tube and then centrifuged to separate the serum.

3.8 Cannabis Leaves Collection

100gm dried leaves of *Cannabis sativa* were collected from the office of the Narcotics Control Authority, Dinajpur.

3.9 Preparation of Cannabis Extract

After collecting the cannabis leaves the following procedure was used for the extraction process (Harbone 1984; Golwala *et al.,* 2012).

The extraction process of Cannabis is given below-

1. **100g Cannabis Oven dry:** Gradually increase the temperature up to 30-50^{0}C. In this way, the moisture was removed from the cannabis.

Figure 5: Oven used for cannabis extract dry

2. **Blending:** Dry cannabis leaves were inserted into a blender machine to turn them into powder.

Figure 6: Blender machine used for powder extract formation

3. **Ethanol dissolves:** Cannabis power was dissolved in ethanol overnight at fridge temperature (Das *et al.*, 2010).

Figure 7: Cannabis powder was dissolved in the ethanol

4. **Filtration:** Whatman double-layer filter paper was used for this purpose. 3 times filtration was done to ensure that no solid material remains in the liquid.
5. **Rotatory evaporation method:** The stock solution was made from cannabis extract through vacuum evaporation (Peter *et al.*, 2011). This process was carried out in the Analytical Laboratory, Department of Chemistry, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

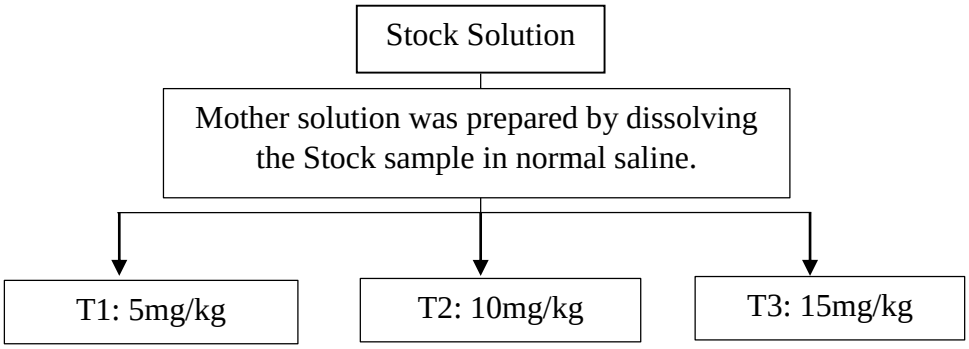
Figure 8: Rotatory evaporator machine

6. **Stock solution preparation:** After rotatory evaporation of cannabis extracts semisolid compound was found. This semisolid compound was called a stock sample. This stock sample was used for the 3 different doses preparation.

Figure 9: Stock solution

3.10 Doses Preparation

Three different doses of the cannabis extract were prepared by using this stock solution. Doses were 5mg/kg, 10mg/kg, and 15mg/kg.



3.10.1 Mother Solution Preparation

A 50mg stock sample was dissolved in 2 mL of normal saline. In this solution, 0.025mg/µL extract contain (Tourneau *et al.,* 2018).

2000µL saline contains 50mg extract

∴ 1µL saline contain = $\frac{50}{2000}$ = 0.025mg extract

= 0.025mg/µL solution

3.10.2 5mg/kg dose preparation

This dose was prepared by the body weight of mice. The average body weight of the mice was 20g. In one dose the mice contain 0.1mg of cannabis extract (Beyene 2008).

1000g mice inject extract per dose 5 mg

∴20g mice inject extract per dose = $\frac{5\times20}{1000}$ = 0.1mg/does [average body weight of mice 20g]

3.10.3 25µl Solution given per dose of injection

In a single dose, mice were injected with 25µl solution. So 25µl normal saline contains 0.1mg cannabis mother solution. Prepared a 2000µl solution. So in the 2000µl solution, the total extract was weighted 8mg.

25µl solution containing 0.1mg

∴ 2000 µl solution contain = $\frac{0.1\times2000}{25}$ = 8mg/2000µl solution

3.10.4 2000µl solution Preparation

In the 2000µl solution for the treatment of mice, the total amount of extract was 8mg. That extract dissolves in normal saline.

50mg extract dissolves in 2000µl solvent

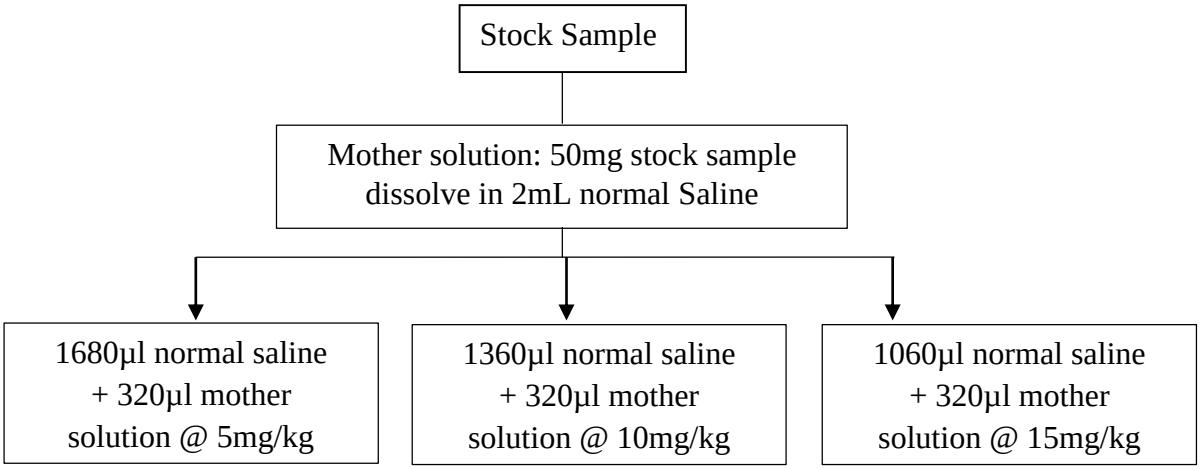
∴ 8mg extract dissolves in = $\frac{2000\times8}{50}$ = 320µl solvent

3.10.5 Three Different Doses of cannabis extract Preparation

The flowing steps were applied for the three different doses preparation-

1. By rotatory evaporator, stock samples were prepared.
2. A 50mg Stock sample was dissolved in 2mL normal saline.
3. In 5mg/kg dose- 0.1mg stock sample was injected into the mice.
4. In a 10mg/kg dose- 0.2mg stock sample was injected into the mice.

5. In a 15mg/kg dose- 0.3mg stock sample was injected into the mice.
6. Injected 25µL solutions in a single dose, and at a time prepared 2000 µL solution.
7. In 2000 µL solution total of 8mg stock sample was dissolved.
8. 5mg/kg=1680µl normal saline + 320µl mother solution was dissolved.
9. 10mg/kg=1360µl normal saline + 640µl mother solution was dissolved.
10. 15mg/kg=1060µl normal saline + 960µl mother solution was dissolved.



3.11 Cannabis Extract Injection

Mice were divided into different groups for the investigation of different objectives. The doses were injected into the intraperitoneal space of mice. The dose was injected every day into the mice and observed.

Figure 10: Cannabis extract was injected into the intraperitoneal space of mice.

3.12 Chemical identification by Gas chromatography-mass spectroscopy (GC-MS)

GC-MS test was done for the compound identification of cannabis extract. This test was carried out on BCSIR, Dhaka. Two ml of 10mg/kg extract was used for this purpose. GC-MS is a materials analysis method that employs gas chromatographs (GC) fitted with mass-selective detectors called mass spectrometers (MS).

3.12.1 GC-MS test procedure

Step-by-Step GC/MS Analysis Procedure (Sahil *et al.*, 2011)-

1. **Inject Sample into a Gas Chromatograph:** The sample was injected into a port that was heated to up to 300 °C causing the substance to volatilize.
2. **Separation of gaseous components as they pass through the column:** A customized oven that was heated to temperatures between 20° and 320°c was looped around the column. A substance was applied to the surface of the column to separate the different chemical components in the sample according to size and/or polarity. More volatile and smaller-sized sample components were passed through the column more quickly than larger ones.
3. **Mass Spectrometer (MS) Analysis:** Upon exiting the column, the separated components enter the MS, which comprises three internal steps:
    a. **The ionization source:** Components were bombarded with electrons, which causes them to disintegrate and produce positively charged ions.
    b. **Filter:** Based on their mass, the ions were filtered by an electromagnetic field as they travel through. Once particles leave the ionization source, analysts set a specified range of masses to be permitted through.
    c. **Detector:** A mass spectrum or distribution of ions of various sizes was produced by a computer after counting the number of filtered ions.

By comparing each mass spectrum to reference libraries of more than 275,000 different spectra, the components were identified from the mass spectrum. Analysts create a standard curve with known amounts of each substance to quantify chemicals within the sample under analysis.

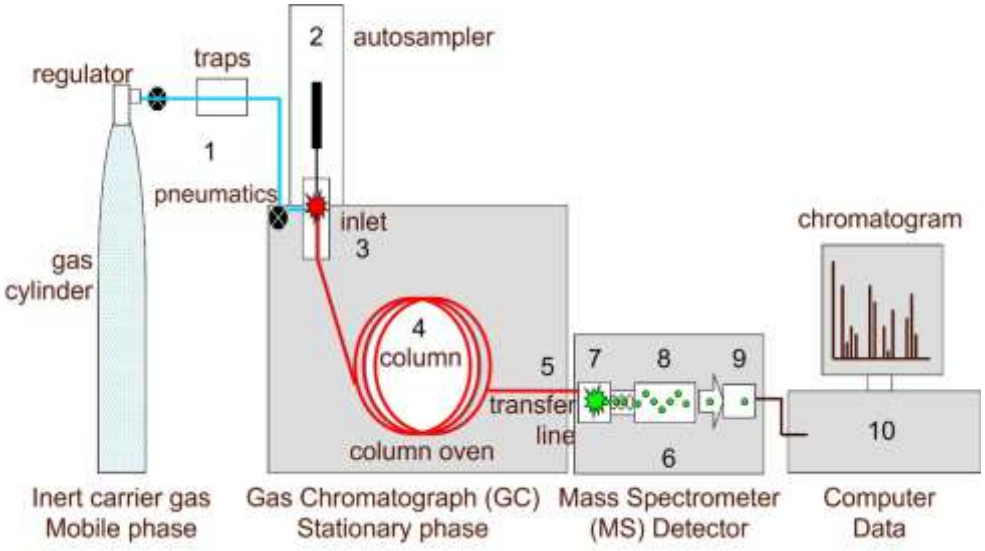


Figure 11: GC-MS procedure

3.12.2 GC-MS Analysis Spectrum

GC-MS analysis of cannabis extract found the major cannabinoid component in the ethanol extract. Major cannabinoids were THC, CBN, CBD, tetradecane, CBC, 9-Octadecenamide and D-Homo-24-nor-17-oxachola-20,22-diene-3,16-dione. The spectrum shows the presence of cannabinoids.

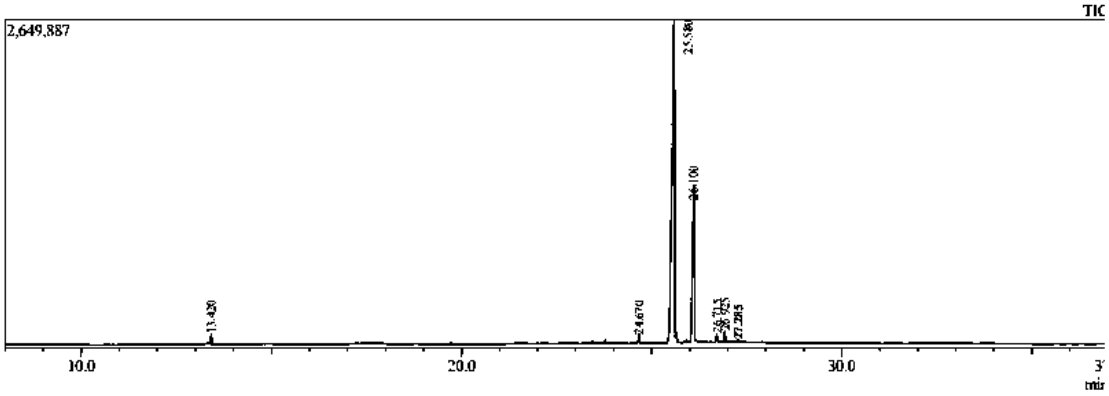


Figure 12: GC-MS chromatogram of *C. sativa* ethanolic extracts.

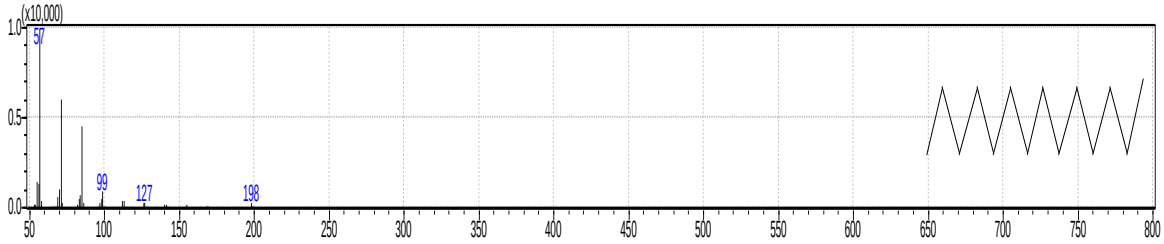


Figure 13: The GC-MS spectrum of tetradecane (Formula: $C_{14}H_{30}$, MW: 198) with x-axis m/z ratio and y-axis as intensity

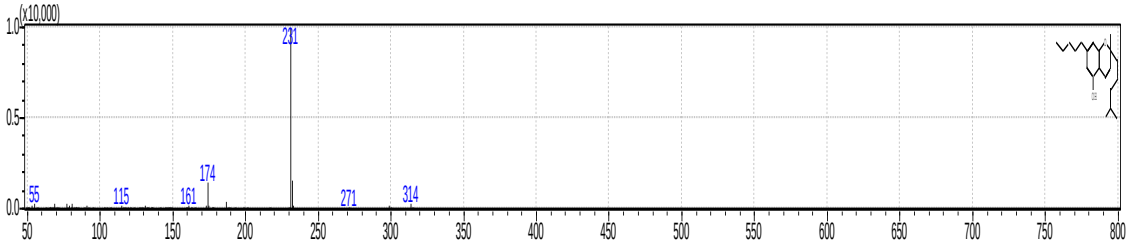


Figure 14: The GC-MS spectrum of cannabichrome (Formula: $C_{21}H_{30}O_2$, MW:314) with x-axis m/z ratio and y-axis as intensity

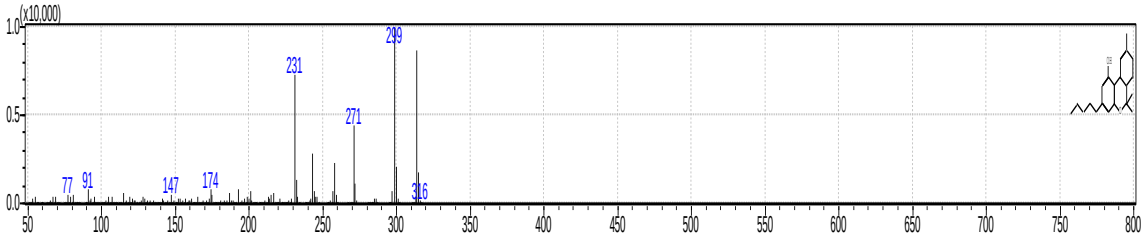


Figure 15: The GC-MS spectrum of delta-9-tetrahydrocannabinol (Formula: $C_{21}H_{30}O_2$, MW:314) with x-axis m/z ratio and y-axis as intensity

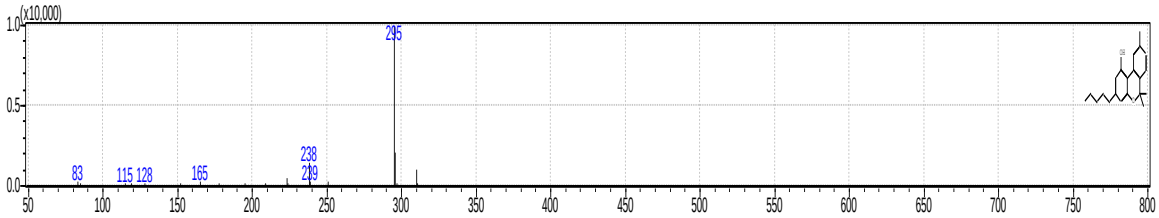


Figure 16: The GC-MS spectrum of cannabinol (Formula: $C_{21}H_{26}O_2$, MW:310) with x-axis m/z ratio and y-axis as intensity

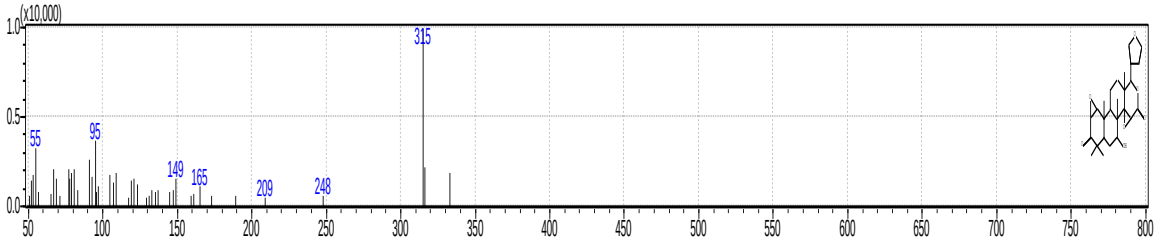


Figure 17: The GC-MS spectrum of D-Homo-24-nor-17-oxachola-20,22-diene-3,16-dione, (Formula: $C_{26}H_{32}O_7$, MW: 456) with x-axis m/z ratio and y-axis as intensity

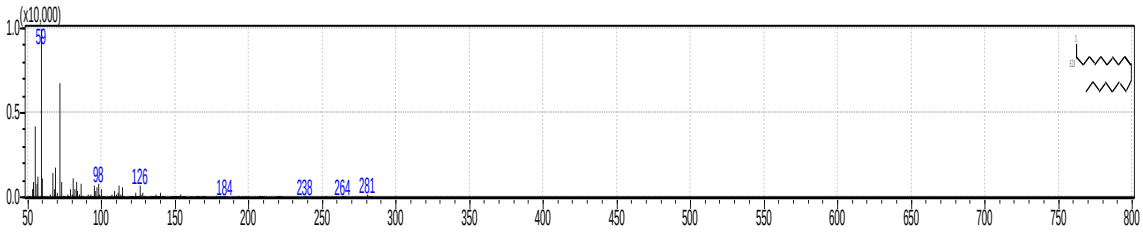


Figure 18: The GC-MS spectrum of 9-Octadecenamide (Formula: $C_{18}H_{35}NO$, MW: 281) with x-axis m/z ratio and y-axis as intensity

3.12.3 Structure of Cannabinoids

Figure 19 (a): Tetradecane

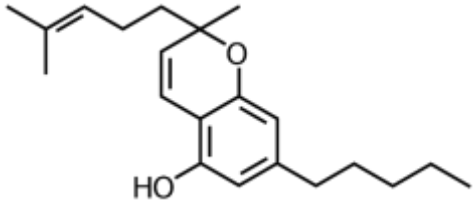


Figure-19 (b): Cannabichromene

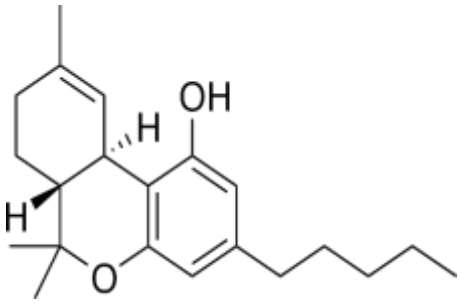


Figure 19 (c): Delta-9-Tetrahydrocannabinol

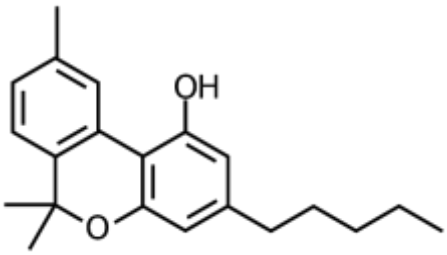


Figure 19 (e): D-Homo-24-nor-17-oxachola-20, 22-diene-3, 16-dione

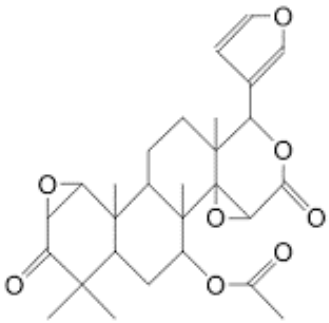


Figure 19 (d): cannabinol

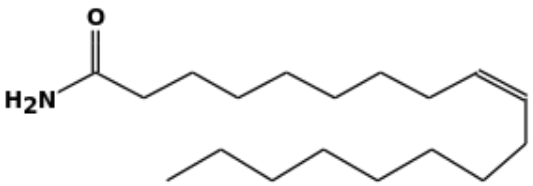
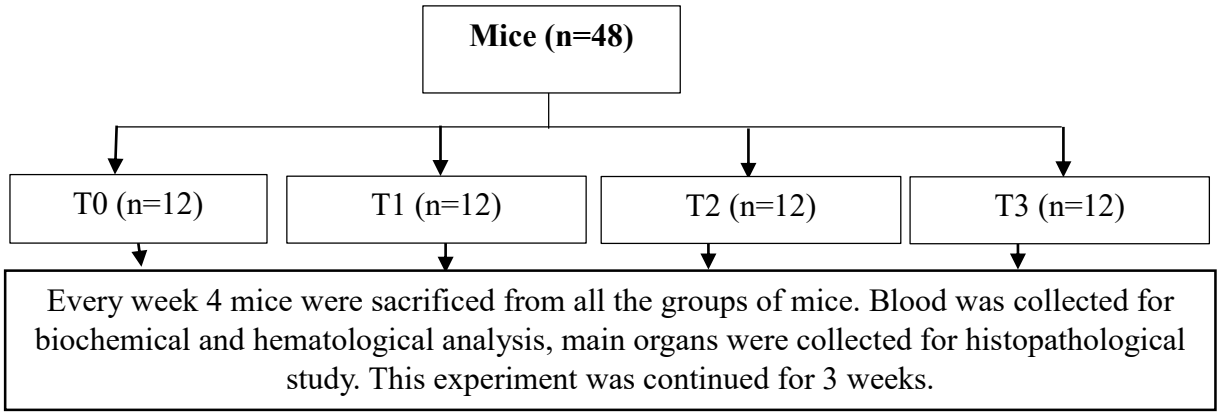


Figure 19 (f): 9-Octadecenamide

3.13 Objective 1: To evaluate the effect of cannabis extract on albino mice

- Forty-eight (48) albino mice used for this study were divided into four groups of 12 mice each. Normal diet was maintained to all the groups.
- **Group 1: No treatment (T0).**
- **Group 2: Injected 5mg/kg cannabis extract (T1).**
- **Group 3: Injected 10mg/kg cannabis extract (T2).**
- **Group 4: Injected 15mg/kg cannabis extract (T3).**



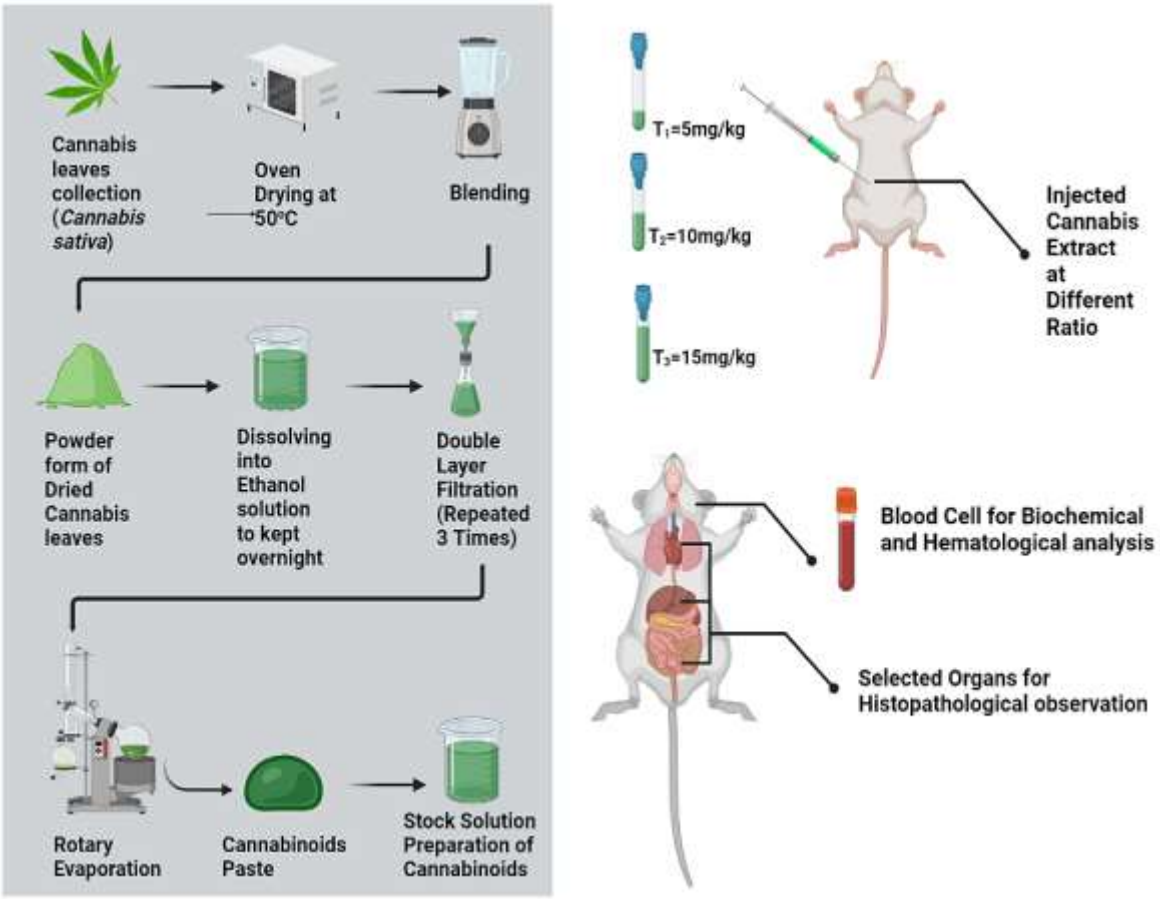
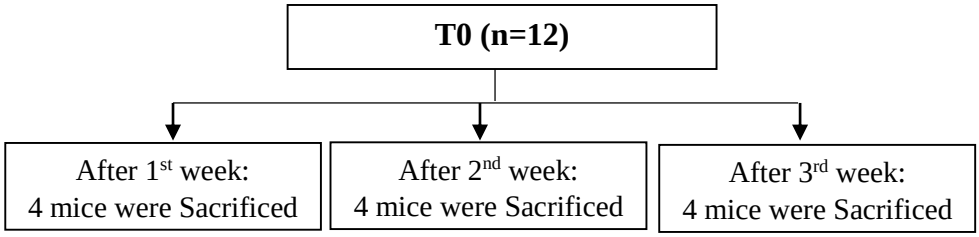


Figure 20: Schematic diagram of the process flow of objective 1.

3.13.1 Group-1: Control Group of Mice

- 12 mice were kept in a plastic case.
- Normal food and water were given to the mice.
- Every week four (4) mice were sacrificed for pathological observations.
- Blood was collected by the heart puncture method.
- For biochemical tests, blood was collected in the red tube (clot activator tube), and for the hematological test, blood was collected in a purple tube (anticoagulant tube).



3.13.2 Group-2: T1 Observation Group

- 12 mice were kept in a plastic case.
- Normal food and water were given to the mice.
- 5mg/kg cannabis extract was injected into the mice every day.
- Every week four (4) mice were sacrificed for pathological observation.
- Blood was collected by the heart puncture method.
- For biochemical tests, blood was collected in the red tube (clot activator tube), and for the hematological test, blood was collected in a purple tube (anticoagulant tube).

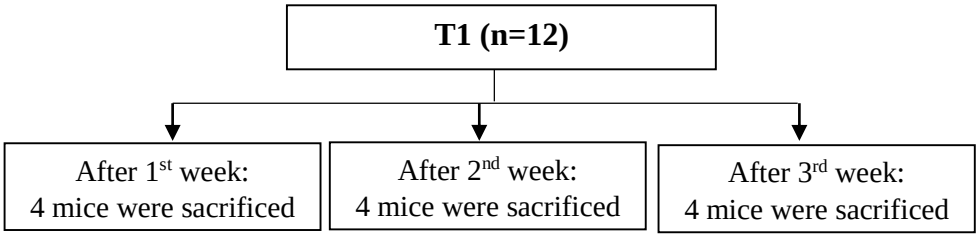
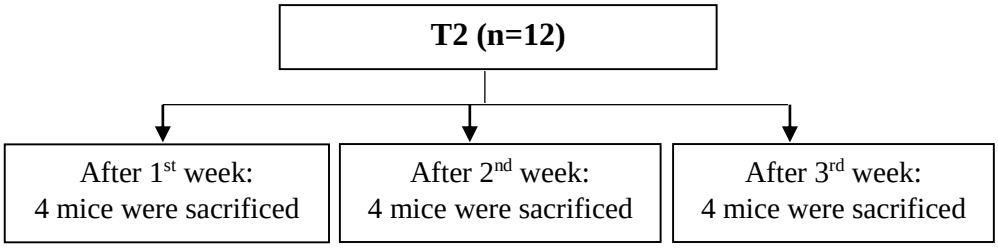


Figure 21: The main organs of mice were surgically removed and stored in formalin solution for histopathological study.

3.13.3 Group-3: T2 Observation Group

- 12 mice were kept in a plastic case.
- Normal food and water were given to the mice.
- 10mg/kg cannabis dose was injected into the mice every day.
- Every week four (4) mice were sacrificed for pathological observation.
- Blood was collected by the heart puncture method.
- For biochemical tests, blood was collected in the red tube (clot activator tube), and for the hematological test, blood was collected in a purple tube (anticoagulant tube).



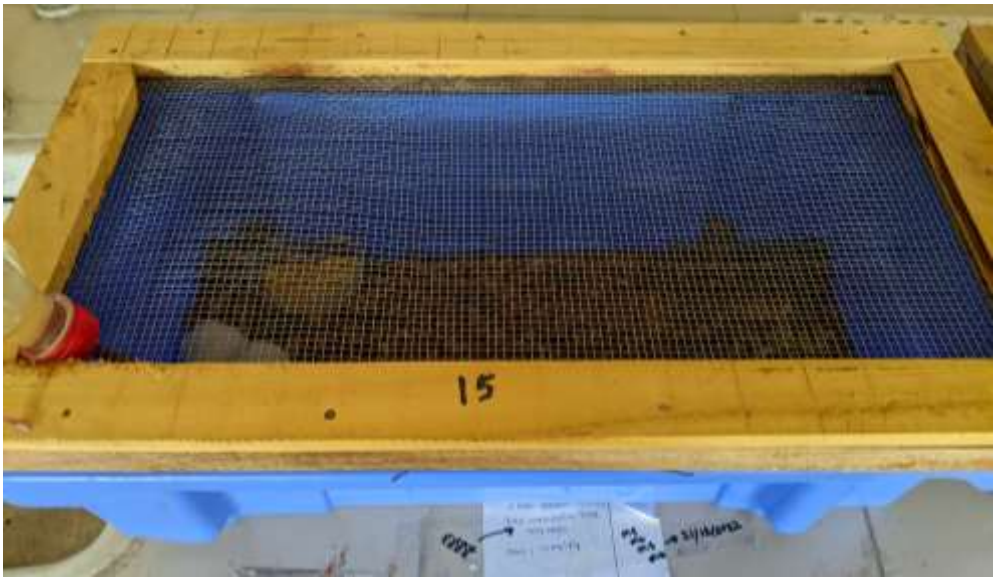
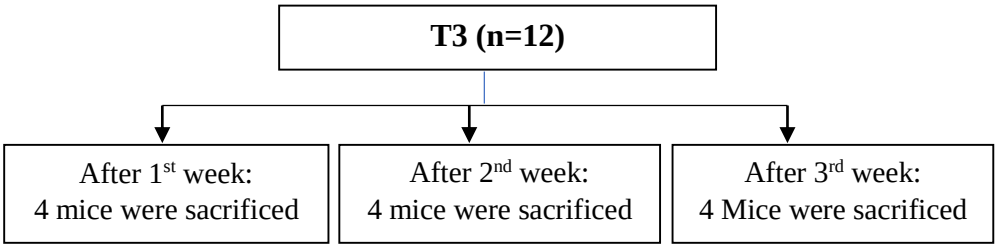


Figure 22: Mice were kept in a plastic case.

3.13.4 Group-4: T3 observation group

- 12 mice were kept in a plastic case.
- Normal food and water were given to the mice.
- 15mg/kg cannabis extract dose was injected into the mice daily.
- Every week four (4) mice were sacrificed for pathological observation.
- Blood was collected by the heart puncture method.
- For biochemical tests, blood was collected in the red tube (clot activator tube), and for the hematological test, blood was collected in the purple tube (anticoagulant tube).



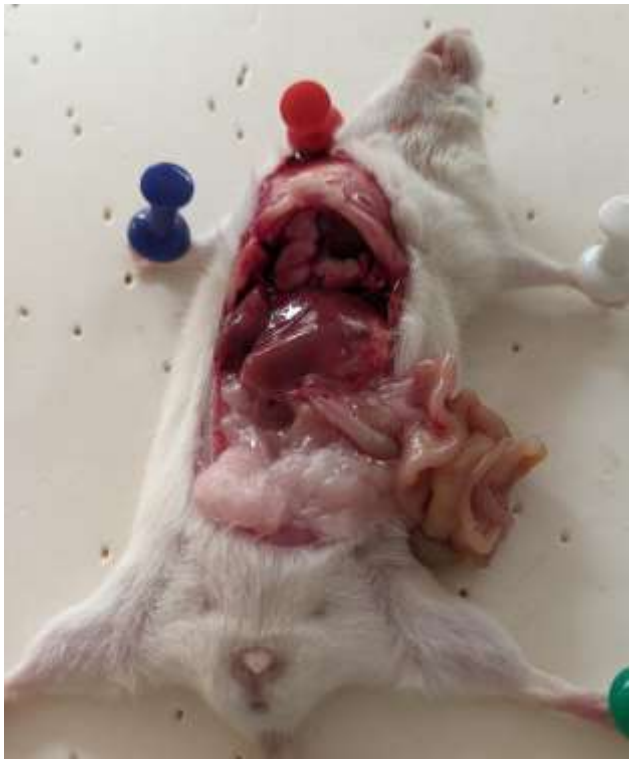
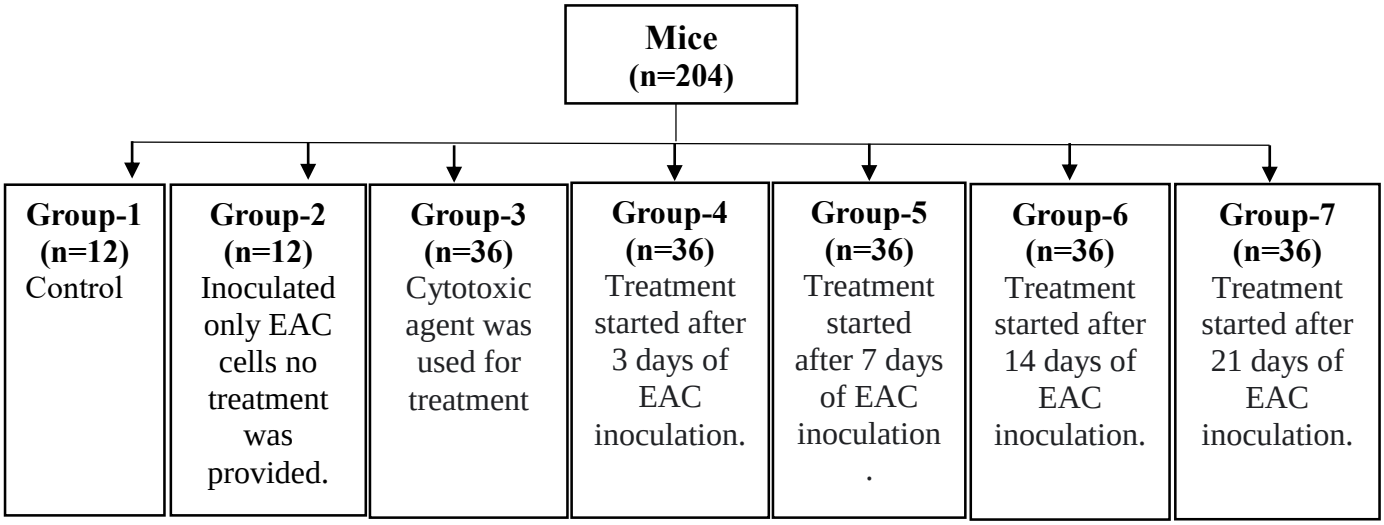


Figure 23: Mice were sacrificed and collection of blood and main organs for pathological investigations.

3.14 Objective 2: To evaluate the responses of cannabis extract for the treatment of EAC cells induced cancer

- Two hundred four (204) albino mice were used for this study. Mice were divided into seven groups.
- **Group- 1: Control group of mice**
- **Group- 2: Inoculated only EAC cells no treatment was provided**
- **Group- 3: Cytotoxic agent was used for treatment purposes**
- **Group- 4: Treatment started after 3 days of EAC inoculation**
- **Group- 5: Treatment started after 7 days of EAC inoculation**
- **Group- 6: Treatment started after 14 days of EAC inoculation**
- **Group- 7: Treatment started after 21 days of EAC inoculation**



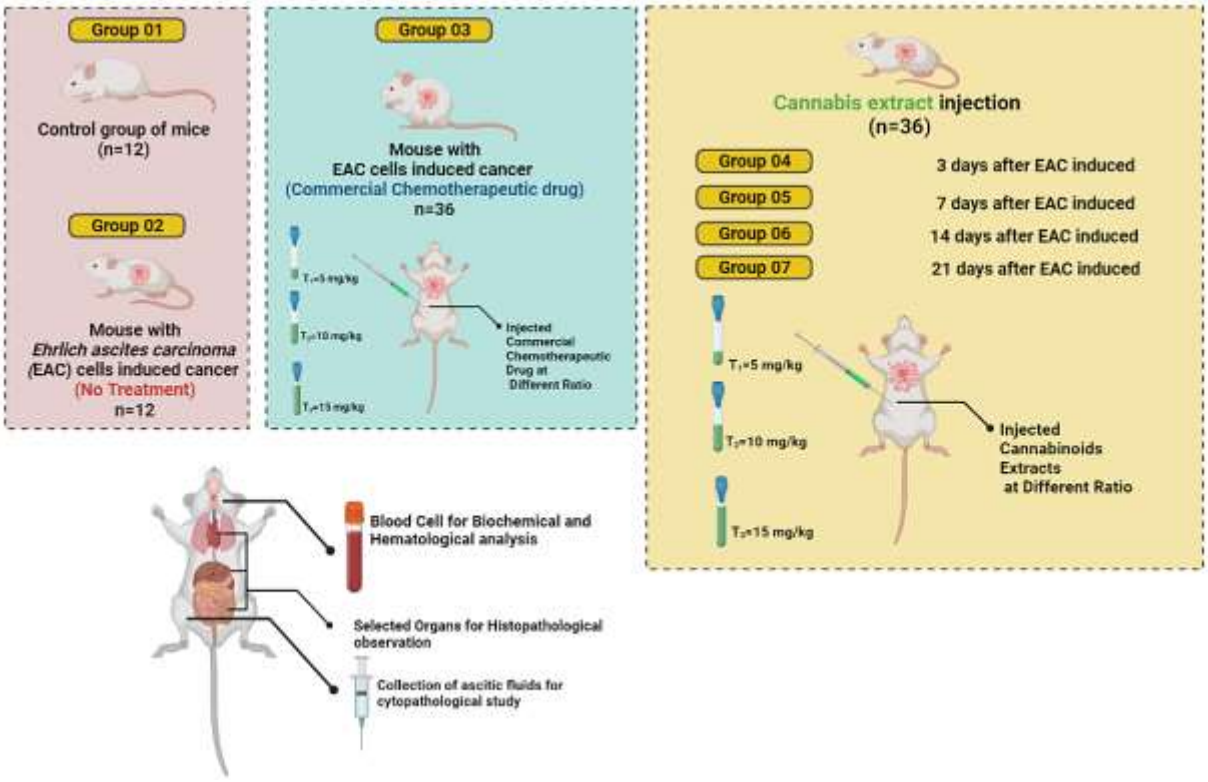
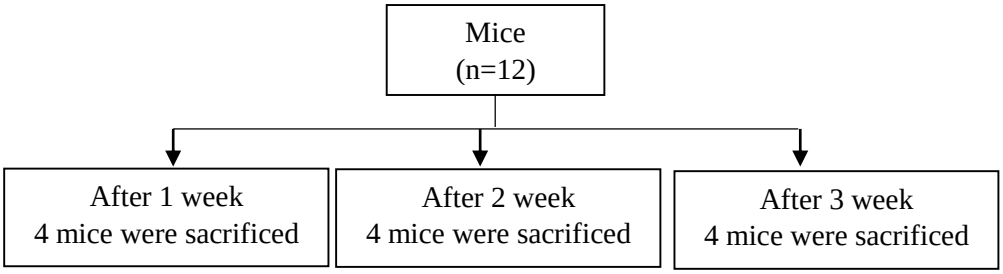


Figure 24: Schematic diagram of the process flow of the response of cannabis extract for the EAC cells induced cancer.

3.14.1 Group-1: Control Group of Mice

- 12 mice were used for this purpose.
- Normal diet was maintained.
- 4 mice sacrifice every week. Observation period 3 weeks long.
- Blood was collected for biochemical and hematological observations.



3.14.2 Group-2: Inoculated only EAC cells no treatment was provided.

- 12 mice were used for this purpose.
- 1×10^6 EAC cells/mouse were inoculated and a normal diet was maintained.

- Mice weight was measured daily.
- 4 mice per week were sacrificed in this study. Observation period 3 weeks long.
- Blood was collected for biochemical and hematological study. Ascitic fluids were collected for cytopathological study and mice main organs were collected for histopathological study.

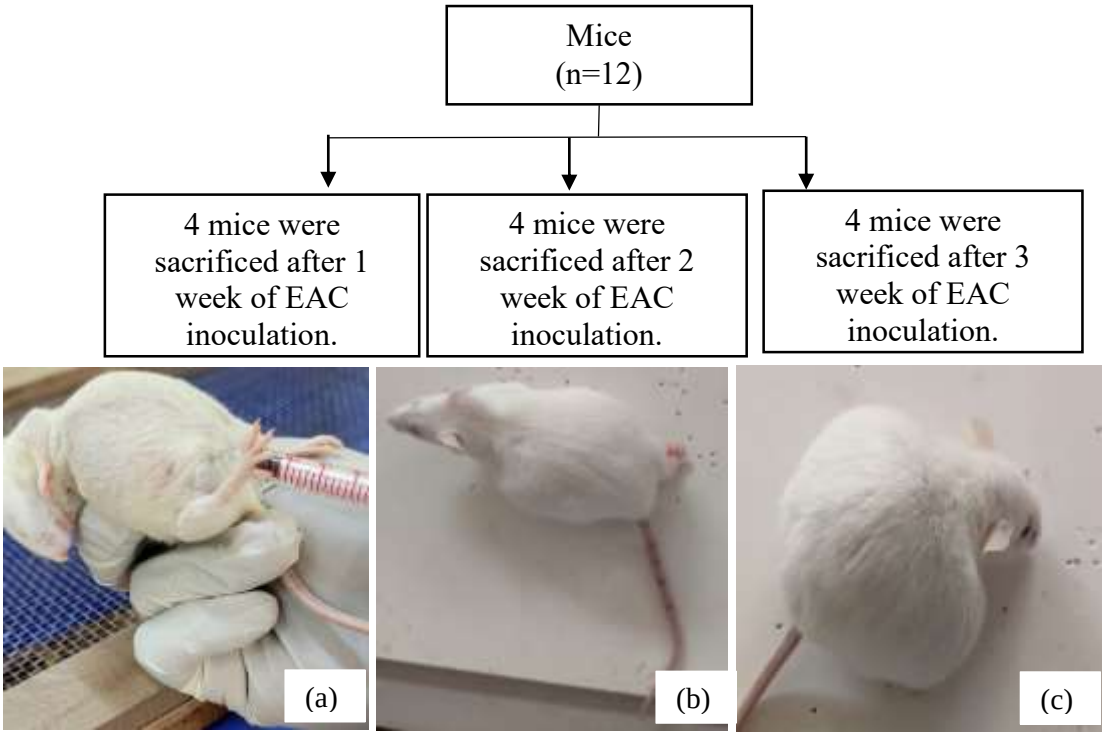
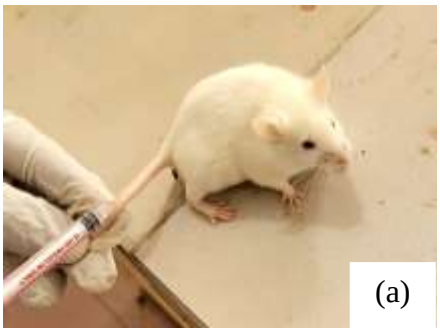


Figure 25: Mice were injected (a) EAC cells (1×10^6/mouse) intraperitoneal space (b) after one week of EAC injection, (c) after two weeks of EAC injection.

3.14.3 Group-3: Cytotoxic agent was used for treatment purposes.

- 36 mice were used for this purpose, which were divided into 3 groups.
- 1×10^6 EAC cells/mouse were inoculated and a normal diet was maintained.
- Mice weight was measured daily.
- 5mg/kg, 10mg/kg and 15 mg/kg of cytotoxic agent (ifodex) was injected into the 3 groups of mice respectively. After 3,7,14 and 21 days of injection randomly selected 3 mice in every group and sacrificed, and blood was collected

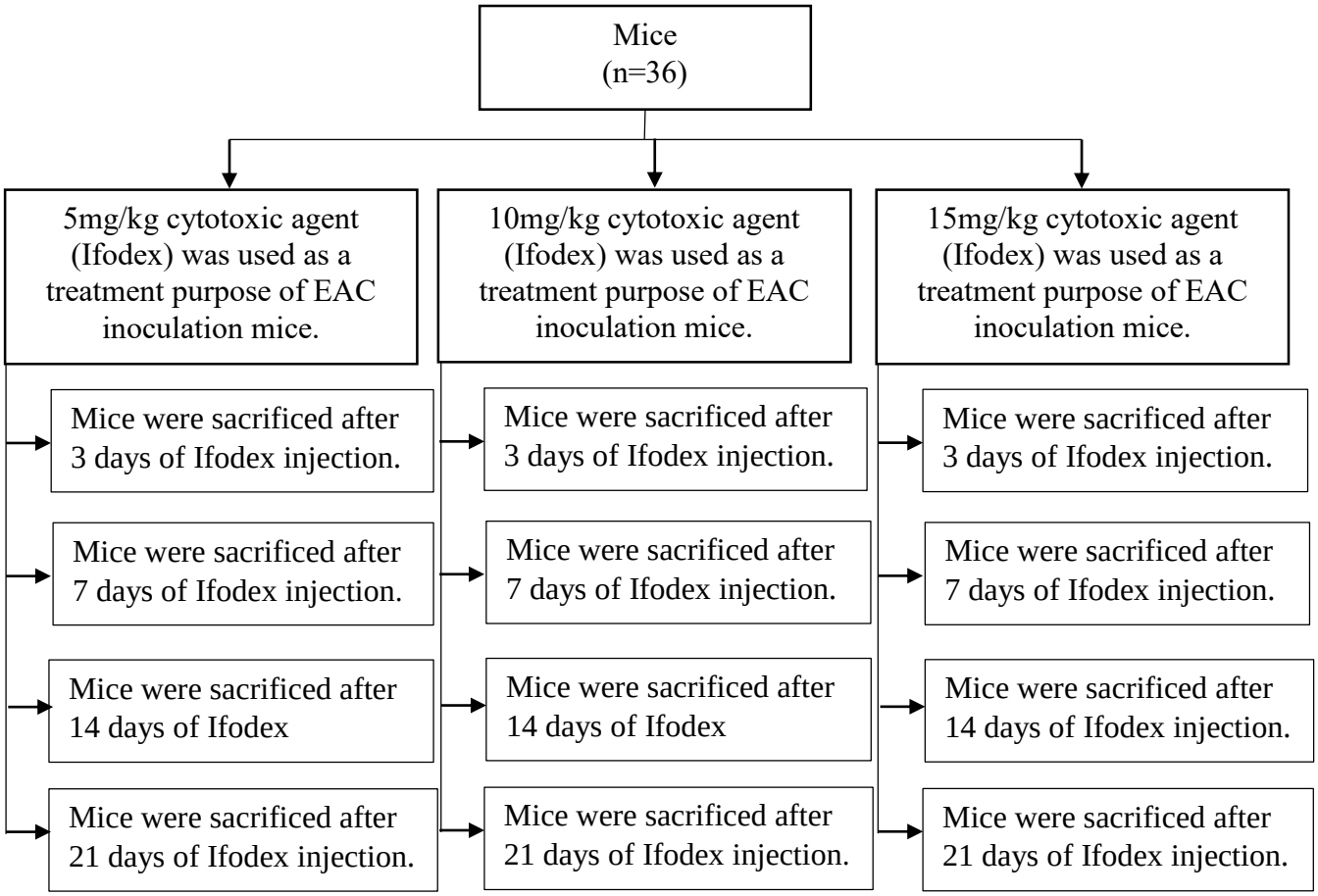
for biochemical and hematological study. Ascitic fluid was collected for cytological study and mice main organs were collected for histological study.



(a)

(b)

Figure 26: Figure shows the (a) Ifodex injected into the tail vein of albino mice. (b) Ifodex cytotoxic agent.



3.14.4 Group-4: Treatment started after three days of EAC inoculation.

- Firstly 1×10^6 EAC cells/mouse were inoculated and a normal diet was maintained.
- Mice weight was measured daily.
- After 3 days of EAC cells inoculation, mice were started treatment with cannabis extract daily in three different (T1, T2 and T3) concentrations.
- After 3 days of cannabis extract injection, mice were sacrificed from all groups and blood was collected for biochemical and hematological study, ascitic fluids were collected for cytopathological study and mice main organs were collected for histopathological study.
- In the same way, after 7, 14 and 21 days of treatment with cannabis extract mice were sacrificed for pathological observations.
- Blood was collected by heart puncture method and stored in a clot activator tube and EDTA tube.
- Main organs such as the heart, lung, liver, kidney, and pancreas were collected in 10% formalin solution for histopathological study.

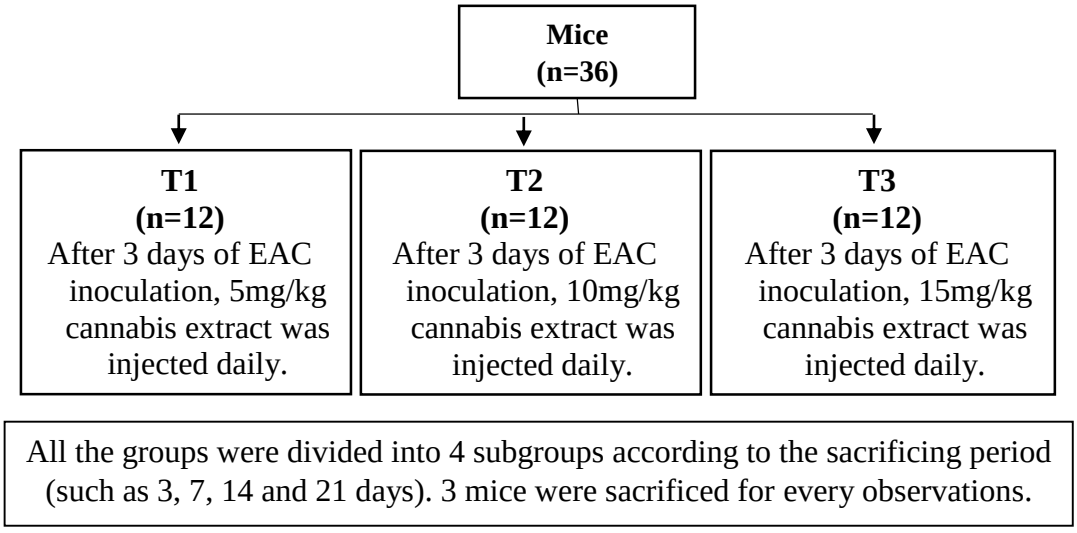
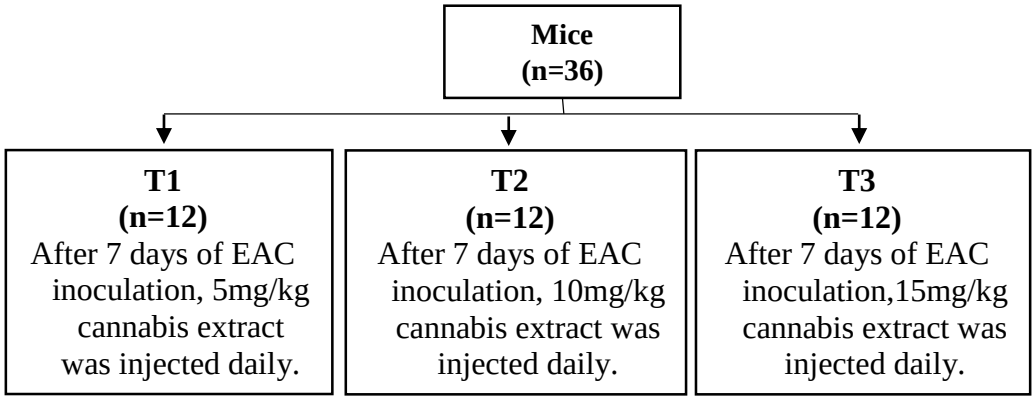


Figure 27: Blood was collected by the heart puncture process.

3.14.5 Group-5: Treatment started after seven days of EAC inoculation.

- Firstly 1×10^6 EAC cells/mouse were inoculated and a normal diet was maintained.
- Mice weight was measured daily.
- After 7 days of EAC cells inoculation, mice were started treatment with cannabis extract daily in three different (T1, T2 and T3) concentrations.
- After 3 days of cannabis extract injection, mice were sacrificed from all groups and blood was collected for biochemical and hematological study, ascitic fluids were collected for cytopathological study and mice main organs were collected for histopathological study.
- In the same way, after 7, 14 and 21 days of treatment with cannabis extract mice were sacrificed for pathological observations.
- Blood was collected by heart puncture method and stored in a clot activator tube and EDTA tube.
- Main organs such as the heart, lung, liver, kidney, and pancreas were collected in 10% formalin solution for histopathological study.
- Ascitic fluids were collected for cytopathological study.



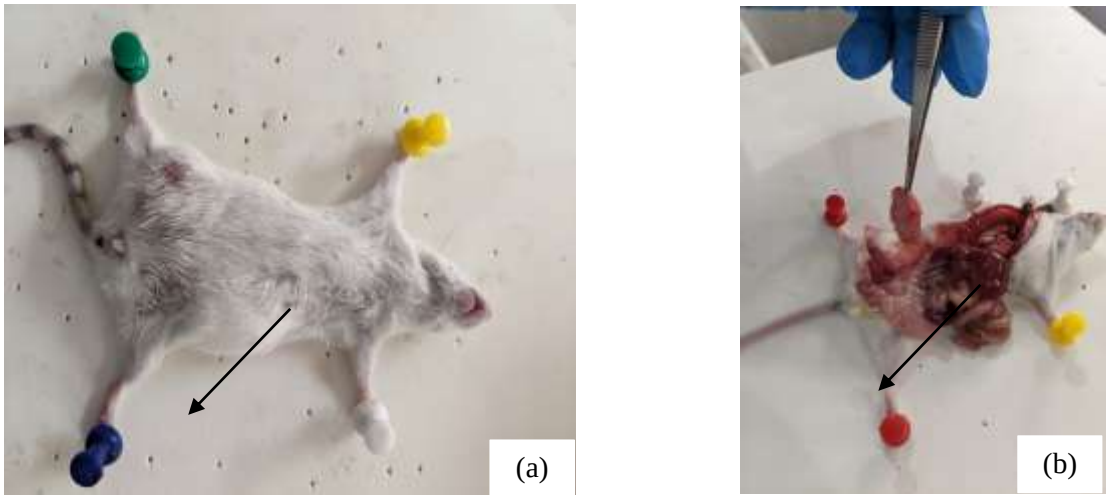
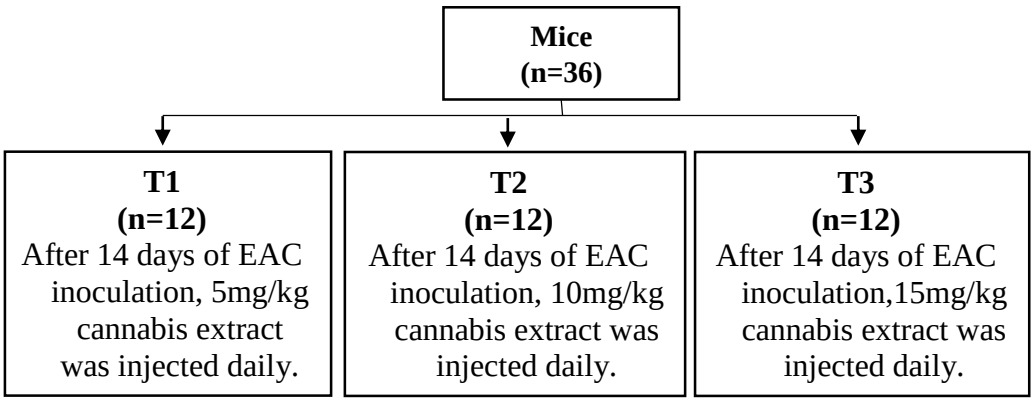


Figure 28: The indicating area on the picture shows (a) the tumor bringing mice and (b) the tumor was surgically removed from the mice.

3.14.6 Group-6: Treatment started after fourteen days of EAC inoculation

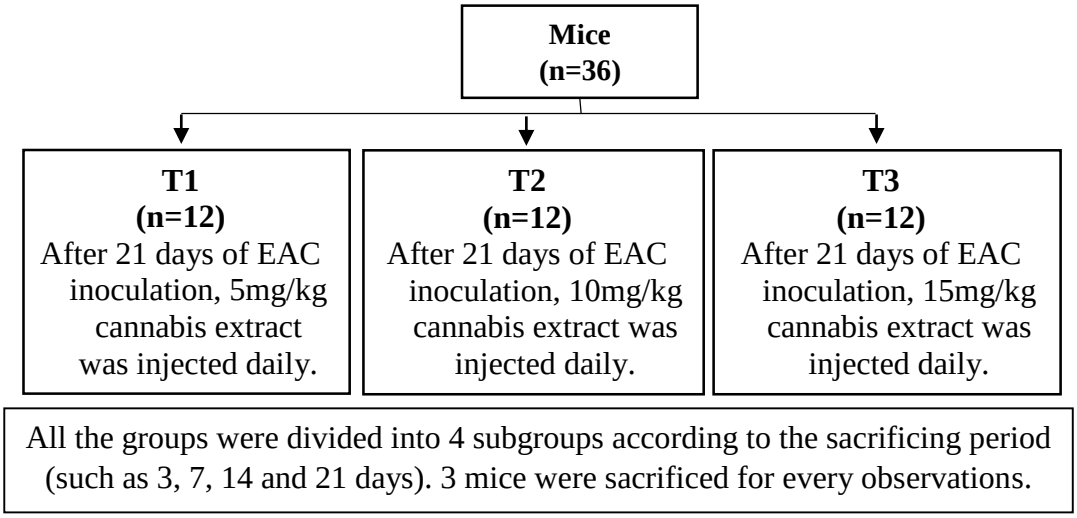
- Firstly 1×10^6 EAC cells/mouse were inoculated and a normal diet was maintained.
- Mice weight was measured daily.
- After 14 days of EAC cell inoculation, mice were started treatment with cannabis extract daily in three different (T1, T2 and T3) concentrations.
- After 3 days of cannabis extract injection, mice were sacrificed from all groups and blood was collected for biochemical and hematological study, ascitic fluids were collected for cytopathological study and mice main organs were collected for histopathological study.
- In the same way, after 7, 14 and 21 days of treatment with cannabis extract mice were sacrificed for pathological observations.
- Blood was collected by heart puncture method and stored in a clot activator tube and EDTA tube.
- Main organs such as the heart, lung, liver, kidney, and pancreas were collected in 10% formalin solution for histopathological study.
- Ascitic fluids were collected for cytopathological study.



All the groups were divided into 4 subgroups according to the sacrificing period (such as 3, 7, 14 and 21 days). 3 mice were sacrificed for every observations.

3.14.7 Group-7: Treatment started after twenty one days of EAC inoculation

- Firstly 1×10^6 EAC cells/mouse were inoculated and a normal diet was maintained.
- Mice weight was measured daily.
- After 21 days of EAC cell inoculation, mice were started treatment with cannabis extract daily in three different (T1, T2 and T3) concentrations.
- After 3 days of cannabis extract injection, mice were sacrificed from all groups and blood was collected for biochemical and hematological study, ascitic fluids were collected for cytopathological study and mice main organs were collected for histopathological study.
- In the same way, after 7, 14 and 21 days of treatment with cannabis extract mice were sacrificed for pathological observations.
- Blood was collected by heart puncture method and stored in a clot activator tube and EDTA tube.
- Main organs such as the heart, lung, liver, kidney, and pancreas were collected in 10% formalin solution for histopathological study.
- Ascitic fluids were collected for cytopathological study.



Objective 3: To determine the preventive efficacy of cannabis extract on EAC cells induced cancer.

One hundred ninety-two (192) albino mice used for this study were divided into four groups.

1. **Group 1: Effectiveness of three doses of cannabis extract in preventing cancer.**
2. **Group 2: Effectiveness of seven doses of cannabis extract in preventing cancer.**
3. **Group 3: Effectiveness of fourteen doses of cannabis extract in preventing cancer.**
4. **Group 4: Effectiveness of twenty one doses of cannabis extract in preventing cancer.**

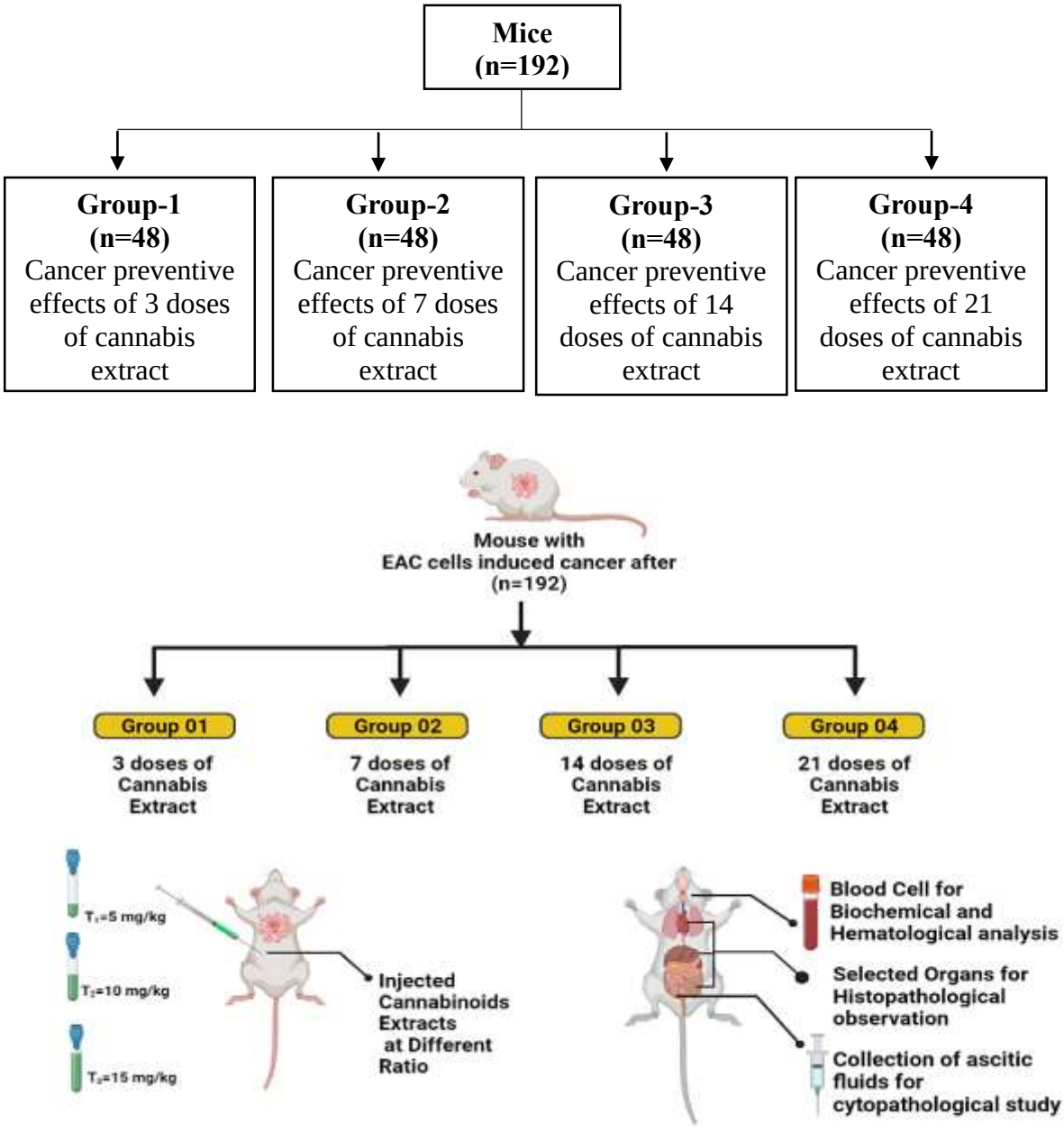
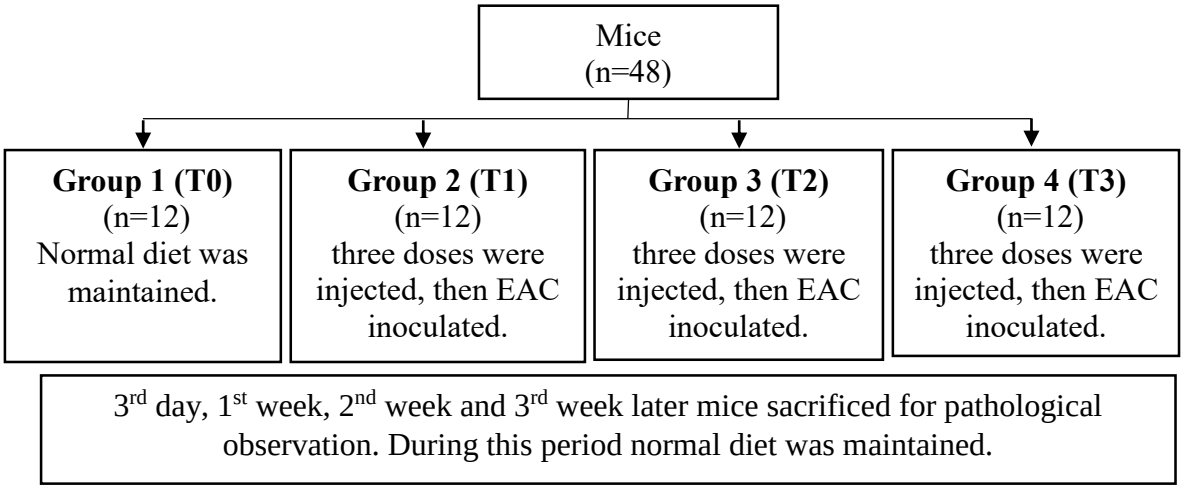


Figure 29: Schematic diagram of the process flow of the response of cannabis extract preventive purpose of EAC cells induced cancer.

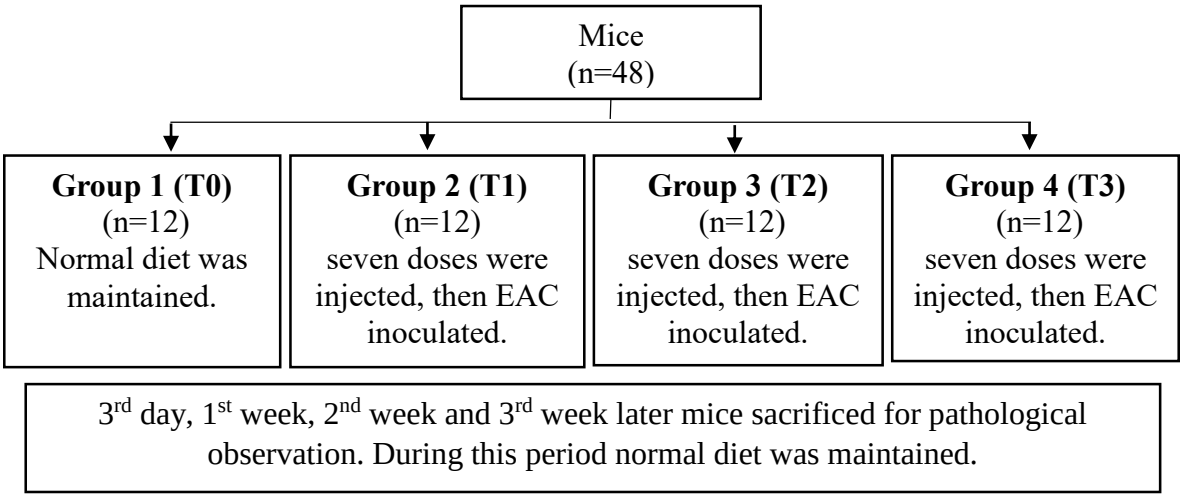
3.15.1 Group-1: Effectiveness of three doses of cannabis extract in preventing cancer

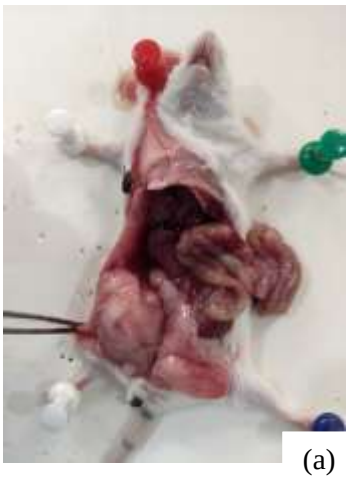
- 48 albino mice were divided into four groups.
- In the group 1 (control) normal diet was maintained. Group 2 mice were injected T1 cannabis extract into the intraperitoneal space with a maintained normal diet, group 3 mice were injected T2 cannabis extract into the intraperitoneal space with a maintained normal diet, group 4 mice were injected T3 cannabis extract into the intraperitoneal space with a maintained normal diet.
- After 3 days of cannabis extract injection (daily one dose), 1×10^6 EAC cells/mouse were inoculated into group 2, group 3 and group 4.
- After inoculation of 3 days, 7 days, 14 days, and 21 days of observation, mice were sacrificed. Blood samples were collected for biochemical and hematological study. Ascitic fluids were collected for cytopathological study and the main organs of mice were collected for histopathological study.



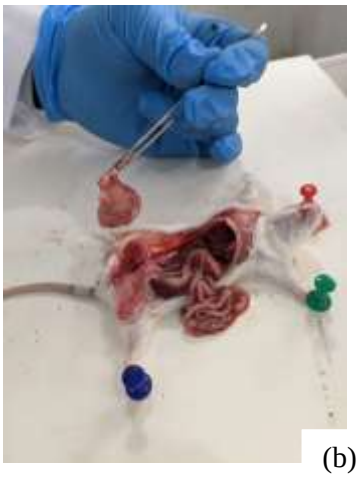
3.15.2 Group-2: Effectiveness of Seven doses of cannabis extract in preventing cancer

- 48 albino mice were divided into four groups.
- In the group 1 (control) normal diet was maintained. Group 2 mice were injected T1 cannabis extract into the intraperitoneal space with a maintained normal diet, group 3 mice were injected T2 cannabis extract into the intraperitoneal space with a maintained normal diet, group 4 mice were injected T3 cannabis extract into the intraperitoneal space with a maintained normal diet.
- After 7 days of cannabis extract injection (one dose/day), 1×10^6 EAC cells/mouse were inoculated into group 2, group 3 and group 4.
- After inoculation of 3 days, 7 days, 14 days, and 21 days of observation, mice were sacrificed. Blood samples were collected for biochemical and hematological study. Ascitic fluids were collected for cytopathological study. Tumors and main organs of mice were collected for histopathological study.





(a)

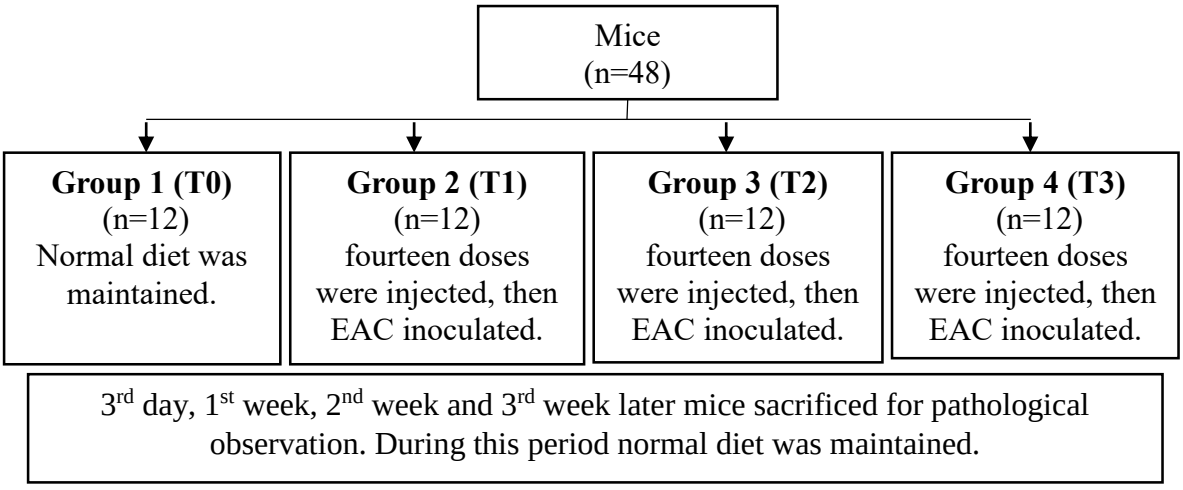


(b)

Figure 30: Mice were sacrificed and the tumor was surgically removed for histopathological study. (a) the picture shows the tumor, (b) the picture shows surgically removed the tumor.

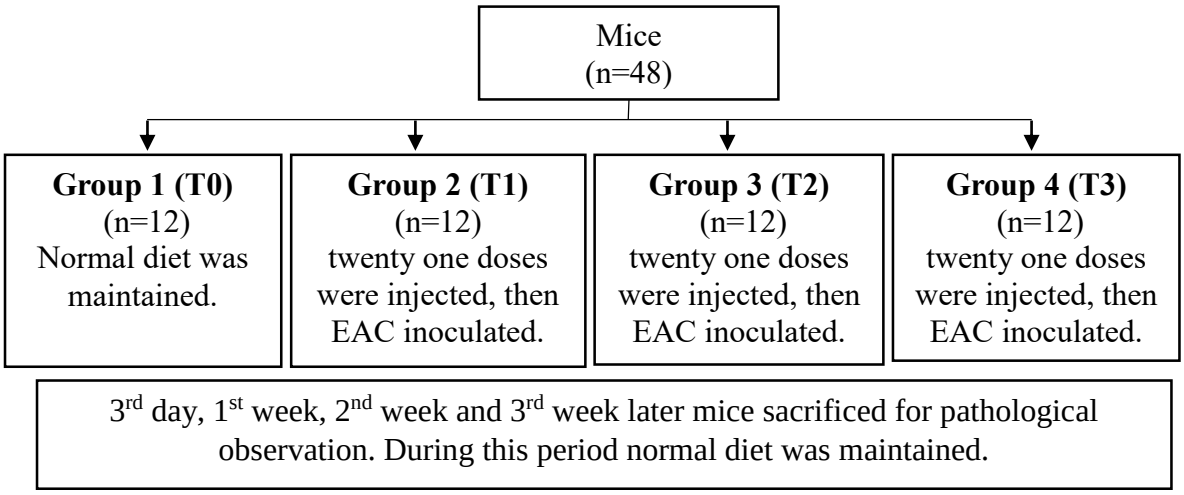
3.15.3 Group-3: Effectiveness of fourteen doses of cannabis extract in preventing cancer

- 48 albino mice were divided into four groups.
- In the group, 1 (control) normal diet was maintained. Group 2 mice were injected T1 cannabis extract into the intraperitoneal space with a maintained normal diet, group 3 mice were injected T2 cannabis extract into the intraperitoneal space with a maintained normal diet, group 4 mice were injected T3 cannabis extract into the intraperitoneal space with a maintained normal diet.
- After 14 days of cannabis extract injection (one dose/day), 1×10^6 EAC cells/mouse were inoculated into group 2, group 3 and group 4.
- After inoculation of 3 days, 7 days, 14 days, and 21 days of observation, mice were sacrificed. Blood samples were collected for biochemical and hematological study. Ascitic fluids were collected for cytopathological study. Tumors and main organs of mice were collected for histopathological study.



3.1.5.4 Group-4: Effectiveness of twenty one doses of cannabis extract in preventing cancer

- 48 albino mice were divided into four groups.
- In the group 1 (control) normal diet was maintained. Group 2 mice were injected T1 cannabis extract into the intraperitoneal space with a maintained normal diet, group 3 mice were injected T2 cannabis extract into the intraperitoneal space with a maintained normal diet, group 4 mice were injected T3 cannabis extract into the intraperitoneal space with a maintained normal diet.
- After 21 days of cannabis extract injection (one dose/day), 1×10^6 EAC cells/mouse were inoculated into group 2, group 3 and group 4.
- After inoculation of 3 days, 7 days, 14 days, and 21 days of observation, mice were sacrificed. Blood samples were collected for biochemical and hematological study. Ascitic fluids were collected for cytopathological study. Tumors and main organs of mice were collected for histopathological study.



3.16 Pathological and hematological tests

For this research purpose, four types of pathological tests were done. Those were-

- Biochemical tests
- Hematological tests
- Histopathological study
- Cytopathological study

3.16.1 Biochemical test

- Blood was collected by heart puncture method in a 1ml syringe.
- Blood was stored in a clot activator tube (red color tube).
- After the collection of blood, was centrifuged at 1000rpm for 10 minutes in a centrifugation machine and separated the serum.
- RBS, S. creatinine, S. cholesterol, S. triglyceride, S. uric acid, SGPT, SGOT and alkaline phosphatase test were done in the indigo plus fully automated clinical chemistry analyzer.

3.16.2 Biochemistry analyzer

Thermo Scientific™ Indiko plus Clinical Chemistry Analyzer is a fully-automated random access bench top system for clinical chemistry testing. Combined the system with reagents and consumables.

Figure 31: Indiko plus fully automated Clinical Chemistry Analyzer.

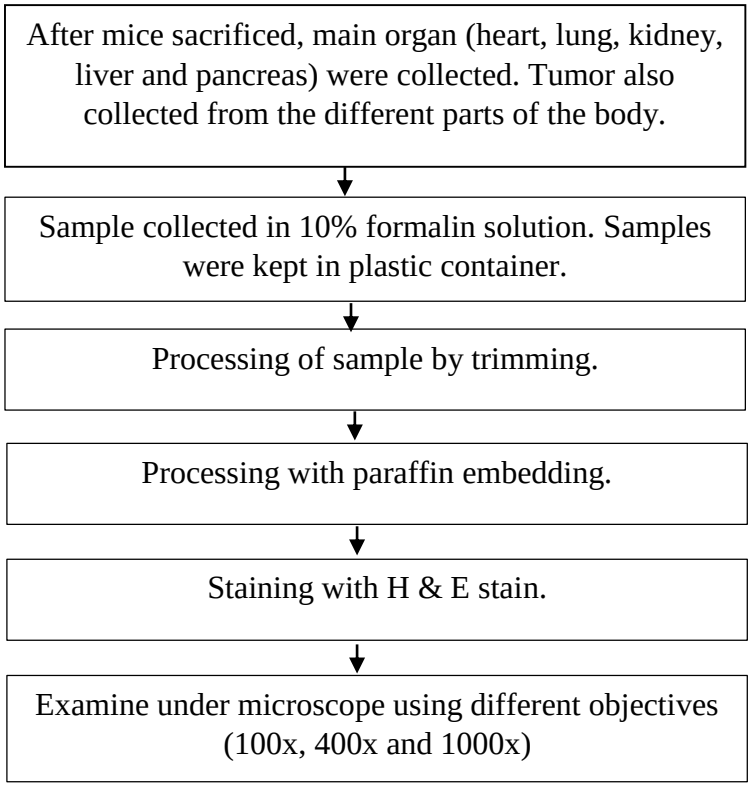
3.16.3 Hematological test

A complete blood count (CBC) test measures the number of blood cells circulating in the bloodstream. Specifically, this research blood test measured a blood sample for the level of red blood cells, which carry oxygen throughout the body; white blood cells, which fight infection; and platelets, which help with blood clotting. Also measure hemoglobin, a protein in red blood cells that carries oxygen, and hematocrit, the ratio of red blood cells to plasma. CBC test was used to detect a variety of conditions, including leukemia, anemia, and infection. CBC test was done in Mindray, BC-5150 fully automated hematology analyzer.

As the lightest and most compact 5-part hematology analyzer so far from Mindray, BC-5150 is a highly user-friendly and innovative analyzer that offers cost-efficient CBC and 5-part white cell differential results.

Figure 32: Mindray, BC-5150 fully automated hematology analyzer.

3.17 Histopathological procedure



3.17.1 Tissue processing and sectioning procedure

The tissues were processed and sectioned as followed:

Collection of tissue and Processing: During tissue collection, the following point were taken into consideration.

The tissues were collected in conditions as fresh as possible. Normal and abnormal tissues were collected side by side. The thickness of the tissues was as less as possible (5mm approximately).

Fixation: 10% formalin was taken in the plastic container. (10 folds of the tissue size and weight) and fixed tissue for 3-5 days.

Washing: The tissues were trimmed into thin sections and washed overnight in running tape water to remove formalin.

Dehydration: The tissues were dehydrated by ascending ethanol series to prevent shrinkage of cells as per the following schedule. The tissues were dehydrated in 50%, 70%, 80%, 95%, 100%, 100%, and 100% ethanol for one hour each.

Impregnation: Impregnation is done in melted paraffin at 56-60°C for 3 hours.

Sectioning: Then the tissues were sectioned with a microtome at 5-pm thickness. A small amount of gelatin was added to the water bath for better adhesion of the section to the slide. The sections were allowed to spread in a warm water bath at 40 to 42°C. Then the sections were taken on grease-free clear slides.

Drying: The slides containing the section were air-dried and kept in a cool place until staining.

Figure 33: Samples were sectioned for histopathological study.

3.17. 2 Routine hematoxylin and eosin staining procedure

The sectioned tissues were stained as described below-

- The sectioned tissues were deparaffinized in three changes of xylene (three minutes in each). Then the sectioned tissues were rehydrated through descending grades of alcohol (three changes in absolute alcohol, three minutes in each, 95% alcohol for two minutes, 80% alcohol for two minutes, 70% alcohol for two minutes) followed by distilled water for 5 minutes. The tissues were stained with hematoxylin for fifteen minutes and washed in running tap water for 10-15 minutes.
- Then the tissues were differentiated in acid alcohol by 2 to 3 quick dips (1 part HCl and 99 parts 70% alcohol) and washed in tap water for five minutes followed by 2-3 dips in ammonia water until sections were bright blue.
- Then the section on the slide was stained with eosin for one minute.
- The section was differentiated and dehydrated in alcohol (95% alcohol: three changes, 2-3 dips each, absolute alcohol: three changes 2-3 minutes for each cleaned in xylene three changes, five minutes in each).
- Tissues were mounted with coverslip by using DPX.
- The slide was dried at room temperature and examined a high (400X) power microscopic field.
- Then the images of the stained section were taken with a digital camera (Sony 14.2 Megapixel).

3.18 Procedure for Fine needle aspiration cytology (FNAC)

The abbreviation FNAC stands for Fine Needle Aspiration Cytology. The method is a low-cost, straightforward, and rapid means to sample extraneous lumps and masses that may be seen in the breasts, neck, etc. A small gauge needle is used to take a sample, which is then examined under a microscope after being inserted into the suspicious mass. As was already explained, it includes a straightforward surgical operation. The test primarily aids in the diagnosis of cancer and inflammatory diseases. Contrary to open surgery, a biopsy is significantly easier, faster, and safer. The chance of any difficulties following the test was quite low.

1. The sterile technique was used on all mice. Gloves were always worn for personal safety.

2. FNA specimens were submitted as smeared slides or fluid specimens made by rinsing the needle and syringe.
3. While maintaining aseptic conditions, the contents of the syringe and needle were partially expelled onto a glass slide, which was appropriately labeled with the mice number of the pass on its frosted side. Working quickly, the specimen was gently smeared between two slides by lightly sandwiching them together (do not compress) and then separating them as if opening a book.
4. Immediately fix one super frost plus slide in 95% ethanol with 2% acetic acid for each pass. All specimens were fixed for at least 15 minutes before staining to preserve cellular morphology.
5. All other slides (plain slides) were air-dried and immediately stained by the modified Giemsa (e.g.: Diff-Quik) method and were screened by the aspirator for immediate assessment.
6. The remaining material in the needle was rinsed in a preservative fluid.
7. If fixed smears were received, these were usually stained with Pap stain unless there was a request for special stains.
8. If a fluid specimen was received, one Pap (plus) and one Diff-Quik (plain) slide was prepared from each fluid specimen unless special stains were required. Discuss requests for special stains with a pathologist.
9. Specimens from different sites were combined.
10. Specimens were cleaned in containers to avoid contamination.
11. Collection errors or problems compromised the results by obscuring cellular morphology.

Figure 34: Ascitic fluids were collected at this location.

3.18.1 Slide Preparation

1. Following the FNA, gently removed the needle from the syringe while holding the needle above the slide in case of accidental spillage of the specimen.
2. Draw air into the syringe and replace the needle with the syringe.
3. Squirted only 1 - 2 drops to the center of one of the labeled slides.
4. Placed the labeled slide face down on the other slide and let the specimen slowly spread out between the two slides.
5. Quickly separated the two slides like opening a book. The result was a mirror image of the specimen on each slide.
6. Immediately fixed one slide in 95% ethanol. Allow the other slide to air-dry. Return to the syringe and needle and gently flush the needle in the solution 2 - 3 times. Then, place the needle in an appropriate sharps disposal container.
7. The other air-dried slide was stained by immediately using the Diff-Quick staining method, so it can be reviewed for adequacy. The Diff-Quick staining method was performed as follows:
    - **Greenish-Blue Quick Link III Fixative (Methanol):** Dipped the slide in the greenish-blue methanol fixation 20 times.
    - **Dark Orange Quick Link III Solution I (Sodium Azide):** Dipped the slide in the dark orange sodium azide solution 20 times.
    - **Dark Blue Quick Link III Solution II (Hematoxylin):** Dipped the slide in the dark blue hematoxylin solution 20 times.
    - **Water:** Dipped the slide in the water 20 times to rinse it.
8. After the slide was stained and air-dried, view the slide with a microscope.

3.19 Materials

3.19.1 Materials Used for keeping mice

No.	Items	Description
1.	Plastic Rag	Hight-18 inches, length- 36 inches
2.	Water Battle	250mL
3.	Food plate	Small round plastic pot.
4.	Wood Powder	Wood dust collects from the wood mill.

3.19.2 Food Items for Mice

No.	Items	Description
1.	Cracked corn	5kg
2.	Wheat flour	5kg
3.	Soyabil	1kg
4.	L-premix	0.5 kg
5.	Water	Normal water

3.19.3 Blood sample collection and processing equipment

No.	Equipment	Company	Origin
1.	Surgical scissors	Putex	China
2.	Surgical knife	Putex	China
3.	Surgical clips	Putex	China
4.	Surgical forceps	Putex	China
5.	Surgical needle	Putex	China
6.	1cc syringe	GMI	Bangladesh

7.	5cc syringe	GMI	Bangladesh
8.	Refrigarator	LG	Korea
9.	Aultoclave	Stanton	UK
10.	Slide	Slammed	Germany
11.	Coverslip	Cypress	China
12.	Fluorescent Microscope	Olympics	Japan
13.	Forceps	Cypress	China
14.	Graduated cylinder	Cypress	China
15.	Micro-pipette(2-20 µl, 10-100µl,100-1000µl)	Slammed	Germany
16.	Analytical balance	Stanton	UK
17.	Water bath	Memmert	Germany
18.	Light Microscope	Olympus	Japan
19.	Digital balance	Stanton	UK
20.	Centrifuge	Hetttich	Germany

3.19.4 Organ collection equipment for histopathological study

No.	Items	Company	Origin
1.	Plastic bottle	RFL	Bangladesh
2.	Formalin Solution	Sigma –Aldrich	USA

3.19.5 Laboratory glassware

Items	Description	Company	Origin
Beaker	100mL, 250mL, 500mL and 1L	Labtex	China
Conical flask	250mL, 500mL	Labtex	China

Measuring Cylinder	100mL, 250mL, 500mL	Bellco	USA
Volumetric flask	250mL, 500mL	Bellco	USA
Test tube	6cc	Labtex	China
Dropper bottle	100mL	Labtex	China
Pipette	10mL	Labtex	China
Reagent bottle	10mL	Labglass	USA

3.19.6 Chemicals Used for biochemical study

No.	Items	Company	Origin
1.	Glucose	Genetic	Germany
2.	Creatinine (Enzymatic)	Genetic	Germany
3.	SGPT	Chronolab	USA
4.	SGOT	Chronolab	USA
5.	Serum Cholesterol	Genetic	Germany
6.	Triglyceride	Genetic	Germany
7.	Uric acid	Genetic	Germany

3.19.7 Chemical used for hematological study

No.	Items	Company	Origin
1.	Diluent	Biotrade	China
2.	Diff lyse	Biotrade	China
3.	LH lyse	Biotrade	China
4.	Probe cleanser	Biotrade	China