The Effects of Sponge *Haliclona sp* and Gorgonian *Isis hippuris* Combination Extract on Histopathological Feature of Swiss Mice Liver

RESEARCH ARTICLE

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Written by

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APPROVAL SHEET

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The Effects of Sponge *Haliclona sp* and Gorgonian *Isis hippuris* Combination Extract on Histopathological Feature of Swiss Mice Liver

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ABSTRACT

Backgrounds: Sponge *Haliclona* sp and Gorgonian *Isis hippuris* are marine organisms whose extracts are potential for anticancer. Nevertheless, there is only little information on both extracts toxicities which signifies subchronic toxicity study in this research. Histopathological feature of liver was chosen as an outcome concerning its role in body protection against toxicants. The purpose of this study was to investigate the effects of both extract's combination in graded doses on histopathological feature of Swiss mice liver after 3 months treatment.
Methods: This experimental study utilized post test only control group design. Fifty Swiss mice (male and female) were divided into 5 groups, treatment groups (T1, T2 and T3) were receiving 500 mg of food pellets that contained 0,15 mg; 1,5 mg; and 15 mg combination extract respectively. And as the control groups, C0 received food pellets contained merely ethanol while C negative received no treatment. Liver histopathology were observed and scored by modification of Knodell scoring system after 3 months treatment. Kruskal Wallis test was applied for all data.
Results: There were insignificant differences between each group with p value 0,914.
Conclusion: Sponge *Haliclona* sp and Gorgonian *Isis hippuris* combination extract caused no note-worthy liver damage after 3 months treatment in mice.

Key words: Sponge *Haliclona* sp, Gorgonian *Isis hippuris*, liver histopathological feature, toxicity, anticancer, subchronic

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Pengaruh Pemberian Campuran Ekstrak Sponge *Haliclona sp* dan Gorgonian *Isis hippuris* terhadap Gambaran Histopatologis Hepar Mencit Swiss

Widagdo[1], Neni Susilaningsih[2]

ABSTRAK

Latar Belakang: Sponge *Haliclona* sp dan Gorgonian *Isis hippuris* adalah dua dari banyak organisme laut yang terkenal akan potensinya sebagai agen antikanker. Namun, keduanya hanya memiliki sedikit informasi sehubungan dengan toksisitas, termasuk juga toksisitas subkronik. Gambaran histopatologi hepar terpilih mengingat peran hepar yang begitu besar dalam melindungi tubuh dari bahan-bahan toksik. Penelitian ini bertujuan mengetahui pengaruh pemberian campuran ekstrak Sponge *Haliclona* sp dan Gorgonian *Isis hippuris* dalam dosis bertingkat terhadap gambaran histopatologis hepar mencit Swiss setelah masa perlakuan selama 3 bulan.
Metode: Penelitian ini menerapkan desain *post test only control group* pada 50 mencit Swiss yang dibagi dalam 5 kelompok. Tiga kelompok perlakuan (T1, T2, dan T3) diberi pakan perlakuan sebanyak 500 mg yang masing-masing mengandung ekstrak etanol dari campuran Sponge *Haliclona* sp dan Gorgonian *Isis hippuris* sebanyak 0,15 mg; 1,5 mg; dan 15 mg. Kelompok kontrol yang pertama, C0 menerima pakan perlakuan yang mengandung etanol. Sedang kelompok control yang kedua, C negatif hanya menerima pakan standar. Setelah masa perlakuan selama 3 bulan, dilakukan pemeriksaan preparat histopatologis hepar dari seluruh mencit dan scoring dengan modifikasi *Knodell score*. Analisis data selanjutnya dilakukan dengan uji Kruskal Wallis, mengingat sebaran datanya yang tidak normal.
Hasil: Uji Kruskal Wallis menghasilkan nilai p=0,914, yang menandakan tidak adanya perbedaan yang bermakna antar tiap kelompok perlakuan.
Kesimpulan: Campuran ekstrak Sponge *Haliclona* sp dan Gorgonian *Isis hippuris* tidak menimbulkan kerusakan hepar yang bermakna setelah 3 bulan masa perlakuan pada mencit Swiss.

Kata kunci: Sponge *Haliclona* sp, Gorgonian *Isis hippuris*, gambaran histopatologis, toksisitas, antikanker, subkronik

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1. Introduction

Gorgosterol, Haliclonacyclamines A and B as examples of potential anticancer agents that so far have not been clinically certified, only have modest information on their toxicity contrasting their cytotoxicity.[1-3] Gorgosterol is isolated from Gorgonian *Isis hippuris*, while Haliclonacylamines A and B are from Sponge *Haliclona sp*.[2,3] All cytotoxicity experiments of both species were done in-vitro.[4-6] Methanol extracts of *Haliclona sp* and *Isis hippuris* had been proved to be cytotoxic towards L1210 cell line in 8 µg/ml dose for both extracts.[5,6] The only toxicity test ever performed on both extracts was preclinical trial on Nauplius *Artemia Salina* that yielded $LD_{50} \pm 17$ ppm.[6,7]

It is a logical deliberation to mix both extracts regarding their potentialities. Thus may increase the cytotoxicity effectiveness pharmacologically and yields practicality to enhance compliance. Compacting different compounds in single drug is necessitated in chronic treatment such as cancer.[5-10] Both *Haliclona sp* and *Isis hippuris* were separately extracted before blended. Since there was no chemical interaction, the combination yielded one single extract. Condensing the extract in food pellets is another practicality which makes oral administration applicable.[1,2,4,6]

Grading and amplifying the extract's effective dose was imperative in determining its safety spectrum.[5-7] Besides that, subchronic and chronic toxicity tests are also necessary to represent the toxicity of anticancer agents, since cancer often obliges a long time treatment. Subchronic test requires three months administration, while chronic needs six.[10]

Liver plays an important role in toxicology evaluation, regarding its function as a body defend against toxic substances.[2,10-12] Its damages can be observed by histopathological examination and biochemical test. Histopathological examination usually presents damages in later stage compared to biochemical test.[2,10] This experiment tried to estimate the effects of Sponge *Haliclona sp* and Gorgonian *Isis hippuris* combination extract on histopathological feature of Swiss mice liver.

2. Methods

This true experimental research applied post test only control group design. This research encompassed Toxicology, Histology and Pathology Anatomy. The sample size was determined based on World Health Organization Research Guidelines 1993. It explained that number of mice used in sub-chronic toxicity test should be at least 5 for each group.[11,13,14,15] Samples were fifty Swiss mice, both male and female from Pusat Antar Universitas (PAU) laboratory, Gajah Mada University, Yogyakarta that were healthy, mature (aged 5-6 weeks), behaviorally normal, weighted 20-25 gram and survived 3 months treatment period. This research used both gender (male and female) equally to avoid gender related confounding factors, for instance hormonal.[10] Mice that died during treatment were excluded from this experiment.

Samples grouping were done in random order (simple randomization) to avoid age and weight factor biases. Direct randomization was done instantly since the samples had already fulfilled inclusion criteria. Fifty Swiss mice were divided

into 5 groups. Three treatment groups (T1,T2,T3) and two control groups (C0 and C negative) were consisted of 5 males and 5 females each group. That distribution was done randomly after 7 days of acclimatization. This period of time was needed to adapt all mice in Histology laboratory's cages, where the 3 months treatment was implemented.

Treatment groups were receiving graded doses of treatment food. It was made from food pellets that condensed by combination extracts. Previously, *Haliclona* sp and *Isis hippuris* had been extracted separately by ethanol and mixtured afterwards. Each group had full access to normal daily food and water ad libitum. In additional, T1, T2 and T3 were receiving treatment foods contained 0,15 mg; 1,5 mg and 15 mg combination extract per 500 mg food pellets accordingly. C0 received 500 mg food pellets contain merely ethanol, while C negative received daily food only. Treatment was routinely done for 3 months.

Those three doses of T1, T2 and T3 were determined based on IC_{50} of both extracts towards L1210 cell line based on Trianto et al research, which was 8 μg/ml.[6] In this research, the density of mice cell was assumed to be equal with water density, 1 g/ml. By presuming weight of each mouse as ± 20 gram, Expected Effective Dose (EED) for each was 20 x 8 x 1 = 0,16 mg/mouse. In order to ease equations and extract's production, the dose was abridged into 0,15 mg/mouse. Graded doses 10 and 100 times of the EED was crucial to determine the safety standard of this extract.

After 3 months, all mice were terminated and opened to remove their liver. Those livers' histopathology preparations were observed microscopically with

400X magnification to collect data and subsequently scored by experienced histopathologist's recommended scoring system (modification of Knodell score[16]). Observation was done by light microscope in Histology and Biotechnology Laboratory. Each preparation will be observed for five fields, four at corner and one at the middle. Data was checked for its distribution by Saphiro-Wilk test and subsequently analyzed by Kruskal Wallis test. All analysis was performed by Windows SPSS 15.0.[17,18]

3. Results

Two mice from C0 and C negative that were died during 3 months treatment were excluded from this research. Consequently there were only forty eight liver preparations that were being observed and scored. The data was abnormally distributed based on Saphiro-Wilk test, especially for C negative, T2 and T3 group which significance values were below 0,05. They were 0,008; 0,044; and 0,011 respectively. This abnormal distribution could also be seen in box plot figure. Extreme data value was even noted in C negative group.

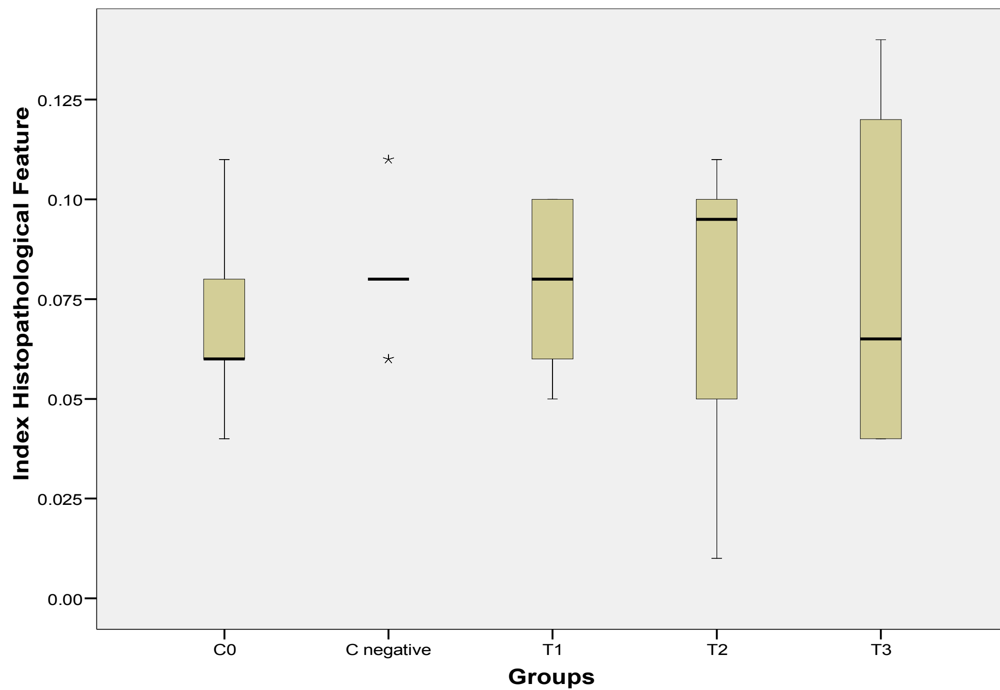

Figure 1. Box Plot of Each Group Index Histopathological Features Distribution

Regarding data's abnormal distribution, median, percentile 25 and 75 were chosen to describe liver index histopathological features descriptively. Those indicators are also mapped in box plot diagram above. The highest median was held by T2 group with 0,095; while the lowest was C0 group with 0,060.

Table 1. Descriptive value of each group

Groups	N	Percentile 25	Median	Percentile 75
C0	9	0.055	0.060	0.085
C negative	9	0.055	0.080	0.080
T1	10	0.060	0.080	0.100
T2	10	0.050	0.095	0.100
T3	10	0.040	0.065	0.123
Total	48	0.060	0.080	0.100

Regarding its abnormal distribution, Kruskal Wallis test was performed afterwards. It capitulated p value = 0,914 which indicated insignificant difference between each group.

4. Discussion

Two mice from C0 and C negative groups died during 3 months treatment. Although there were no certain causes being diagnosed, cages' hygiene might stand as a strong reason. There were predicaments during treatment to keep all cages clean. Even though paddy husk in all cages was often layered, the complete cleaning process itself could only be done once a week because of human resources lacking. Low hygiene at last made mice vulnerable of infection and might end with death.[19] It also might induce non-specific reactive hepatitis, which is a reaction against endotoxin as a result of sepsis or an increased certain xenobiotics absorption through gastrointestinal tract.[20,21] This provision might explain why this research also discovered degeneration and hepatocytes necrosis in control groups. Although not scored in this research, portal infiltration by mononuclear cells (mainly lymphocytes), focal hepatocytes necrosis, lobular inflammation, and enlarged Kupffer's cells also noted in several liver histopathological preparations. Those features could be referred to microscopical findings of non-specific reactive hepatitis.[20]

The result of this experiment itself bears a resemblance to preliminary research held by Trianto et al. His acute toxicity research of this extract on mice with doses up to 100 mg/mice showed no behavior alteration or death. In other

words, no maximum dose of *Haliclona* sp and *Isis hippuris* combination extract that could be acquiesced.[22] The result of that preliminary research sounded as a primary premonition of the extract's safety. It is lastly verified by this experiment with no significant difference found between control and treatment groups.

The scoring system used in this research was only determining four types of liver histopatological damage: degeneration, picnotic nucleus, karyorexis and karyolysis. Those damages are quite contrast with the 3 months period of this research. Fibrosis and cirrhosis that usually found in long term toxicity research was not found in all liver histopathological preparations.[6]

The safety of this extract in this research is in fact also a shocking result concerning toxicology review of both Sponge *Haliclona* sp and Gorgonian *Isis hippuris*. Both have been reported producing toxin in environment. Genus Haliclona is rich of complex alkaloids, while *Isis hippuris* is a rich source of steroids.[5] Regardless on those details, *Haliclona* sp and *Isis hippuris* were not causing notable liver damages after 3 months treatment.

The result of this research is valuable information on *Haliclona* sp and *Isis hippuris* combination extract toxicity, regarding there is no toxicity study on mice had been performed before with this extract. Although it has been toxically proven on Nauplius *Artemia Salina* which succumbed LD_{50} ± 17 ppm,[7] it was corroborated safe for mice in this research. Therefore it is encouraging its usage in human as anticancer agent.

5. Conclusion

There was no significant difference on liver index histopathological feature between control and treatment groups after 3 months treatment with *Haliclona* sp and *Isis hippuris* combination extract. It means this extract is safe since no noteworthy liver damages were observed after 3 months treatment in mice.

6. Suggestion

Further researches on this theme are imperative in the future. Usage of specific substance derived from Sponge *Haliclona* sp and Gorgonian *Isis hippuris* instead of crude extracts is necessary, for instance using Haliclonacyclamine A, B and Gorgosterol instead of crude extracts from *Haliclona* sp and Gorgonian *Isis hippuris*. It is essential to develop those two marine organisms into anticancer drugs in the future.

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ATTACHMENT 1

TREATMENT FOOD DOSE CALCULATION

Based on previous research by Ir. Agus Trianto et al, IC_{50} of Sponge Haliclona sp and Gorgonian Isis hippuris towards Leukemia cancer cell-line (L1210) were 8μg/ml respectively.[12] It was assumed in this research that IC_{50} of both extracts' combination was also 8 μg/ml.

Density of each mouse cell was assumed to be equal with water density, which was 1 gr/ml.

Weight of each mouse in this research was approximately 20 gram.

By those data, dose for each mouse could be calculated = 8μg/ml x 1gr/ml x 20 gr
= 160 μg/mouse
= 0,16 mg/mouse

In order to ease calculation and extract production, Expected Effective Dose (EED was adjusted from 0,16 mg/mouse into 0,15 mg/mouse.

To investigate safety standard of this combination extract, EED was graded 10 and 100 times, which eventually yielded:

Dose I = 0,15 mg extract (EED)

Dose II = 10 x 0,15 mg = 1,5 mg extract/ mouse

Dose III = 100 x 0,15 mg/gr = 15 mg extract/ mouse

ATTACHMENT 2

HISTOPATHOLOGICAL PREPARATION

There are several methods to make histopathology preparation, such as Paraffin method, Frozen section, Celloidin, and Carbowax. This research will use Paraffin method, which used in Histology Laboratory of Diponegoro University. This method consists of seven steps procedures: tissue exclusion, fixation, dehydration, clearing, embedding, sectioning, staining and mounting.

I. Tissue exclusion

1. Mice are killed by neck dislocation after 3 months treatment.
2. Take the mice liver using sharp knife within less than 2 hours after killed to avoid autolysis or degeneration of tissue.
3. The maximum size of tissue exclusion is 1x1x1 cm^3.

II. Fixation

Soak tissue in formalin 10% (formulated by 10cc of formalin 38% and 90cc of aquadest) for 6 hours. Notes:

- ❖ Formalin is a non precipitant, non coagulant and does not form a new substance while fixation process is done. Other profits of using

Formalin as a fixation agent are long lasting and do not cause any damage to tissue.

❖ The functions of fixation are to stiffen tissue, to stop post mortem degeneration, to stop disintegration by bacteria and to increase tissue affinity toward dye substance.

III. Dehydration

a. Soak tissue in three different bottle of alcohol 30% respectively for 20 minutes in each bottle.

b. Then the soaking process is continued based on this list below:

  a. Alcohol 40% for 1 hour

  b. Alcohol 50 % for 1 hour

  c. Alcohol 70% for 1 hour

  d. Alcohol 80% for 1 hour

  e. Alcohol 90% for 1 hour

  f. Alcohol 96% for 1 hour

c. Dioxan and acetone are another solution that can be used to replace alcohol. But alcohol is good in expelling water from tissue and to stiffen it more.

Notes: The aim of dehydration is to expel water from tissue, so it becomes easier to penetrate by paraffin, remembrance that paraffin is not water soluble.

IV. Clearing

a. An intermediate solute from xylol and alcohol 96% (1:1) is usually used before clearing process for 2x20 minutes.

b. Soak tissue respectively in xylol based on the list below:

  a. Xylol I for 20 minutes

  b. Xylol II for 20 minutes

  c. Xylol III for 20 minutes

Notes: Xylol in clearing process is performed in order to purify the tissue. The purpose of clearing process itself is to make paraffin easier to penetrate and to increase transparency of tissue, so dye substance is easier to attach.

V. Embedding

In embedding, tissue is being infiltrated by soluble paraffin. There are two kinds of paraffin: hard paraffin and soft paraffin Embedding consists of 2 different processes that have to be done respectively. They are blocking and trimming.

1. Blocking

  a. Tissue is soaked in soluble paraffin and xylol (1:1) for 20 minutes inside 60ºC oven to avoid congealing of paraffin.

  b. Carry on the blocking process by soaking the tissue in Paraffin I for 20 minutes, in Paraffin II for 20 minutes and in Paraffin III for 20 minutes respectively.

c. Next the tissue is being placed inside a mold made by metal as the wall and porcelain as the base.

d. Then freeze the tissue by soaking it with cold water until paraffin block is well formed.

2. Trimming

Cut the paraffin block that is yielded by blocking process

VI. Sectioning

1. Cut the tissue by rotary microtome to yield 3-10µm thickness. The best form of cut is called paraffin ribbon.
2. Removed the tissue using needle into warm water (40-45ºC) to make it swell.
3. Object glass covered by egg albumin (1:1) is used to put out the tissue from the water.
4. Dried the object glass and the tissue.
5. Timol can be added to avoid disintegration of egg albumin and to retain the growth of fungus.

VII. Staining

1. Place the preparation into the staining jar.
2. Deparaffinisation is performed before staining to expel formalin from the tissue. In this process, the preparation is soaked in xylol I for 10 minutes, xylol II for 10 minutes and xylol III for 10 minutes respectively.

3. Then rehydrate the preparation by soaking it in these solutes below:
    a. Xylol alcohol (alcohol 96% + xylol) for 5 minutes
    b. Alcohol 80% - 70% - 50% - 40% - 30% respectively
    c. Water
4. Stain the preparation with Hematoxyllin
    a. Soak the preparation in Hematoxyllin solute for 10 minutes
    b. Flush it with aquadest
    c. Flush it again with acid alcohol (Alcohol + NaCl 90%)
    d. Soak the preparation in graded concentration of alcohol from 50% - 96% respectively.
5. Stain the tissue with Eosin
    a. Soak the preparation in Eosin for 6 minutes
    b. Flush it with alcohol 96%
    c. Soak the preparation again in xylol alcohol
    d. Dry the preparation with filtration paper
    e. Clean the object glass with cotton drown in alcohol
    f. Soak it in xylol I
    g. Soak it in xylol II
    h. Drip it with Canadian balsam

VIII. Mounting

Covering the preparation with deck glass as the last step of making histopathology preparation.

ATTACHMENT 3
SCORING SYSTEM

Liver histopathological preparations in this research were scored for their damages with modification of Knodell score by dr. Kasno, Sp PA.

Table 3.1. Scoring System of Liver Histopathological Feature

Degeneration	Score	Picnotic nucleus	Score	Karyorexis	Score	Karyolysis	Score
0%	0	0%	0	0%	0	0%	0
< 25%	1	< 25%	1	< 25%	1	< 25%	1
25 - < 50%	2	25 - < 50%	2	25 - < 50%	2	25 - < 50%	2
50 - < 75%	3	50 - < 75%	3	50 - < 75%	3	50 - < 75%	3
75 - 100%	4	75 - 100%	4	75 - 100%	4	75 - 100%	4
Index Histopathological = Score summarize / 16							

ATTACHMENT 4
STATISTIC ANALYSIS

Table 4.1. Descriptive Data

Groups	Mean	N	Median	Std. Deviation	Minimum	Maximum
C0	.07	9	.06	.022	.04	.11
C negative	.08	9	.08	.015	.06	.11
T1	.08	10	.08	.019	.05	.10
T2	.08	10	.10	.033	.01	.11
T3	.08	10	.07	.043	.04	.14
Total	.08	48	.08	.028	.01	.14

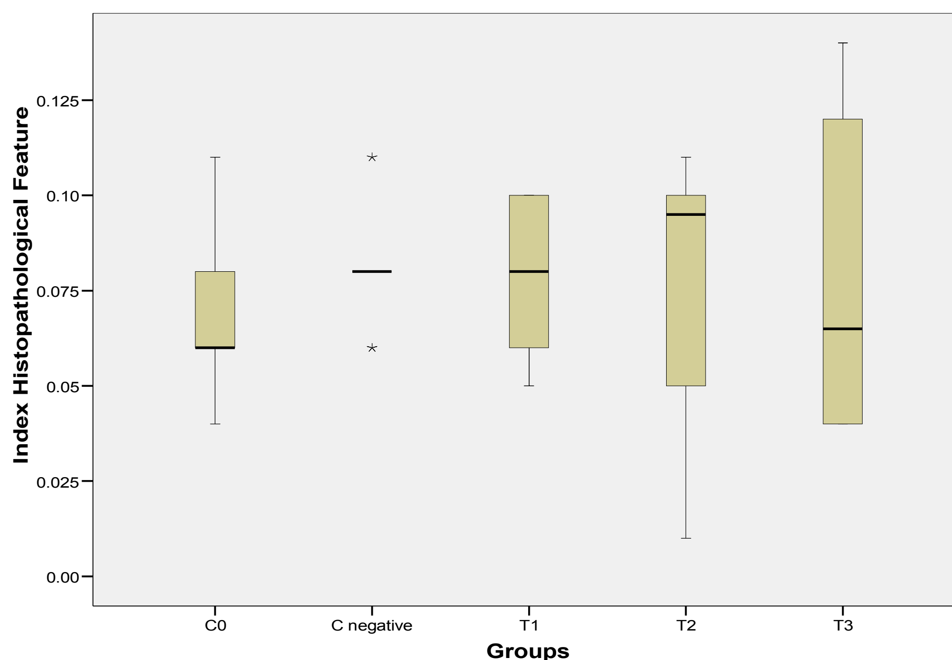

Figure 4.1. Box Plot of Index Histopatological Features Distribution

Table 4.2. Tests of Normality

		Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Groups	Statistic	df	Sig.	Statistic	df	Sig.
Index Histopathological Feature	C0	,232	9	,176	,949	9	,676
	C negative	,358	9	,001	,764	9	,008
	T1	,217	10	,198	,876	10	,116
	T2	,259	10	,055	,840	10	,044
	T3	,317	10	,005	,789	10	10

a Lilliefors Significance Correction

Table 4.3. Kruskal Wallis test

	Groups	N	Mean Rank

Index Histopathological Feature	C0	9	20,78
	C negative	9	25,28
	T1	10	25,95
	T2	10	26,30
	T3	10	23,90
	Total	48	

Test Statistics(a,b)

	Index Histopathological Feature
Chi-Square	,971
df	4
Asymp. Sig.	,914

a Kruskal Wallis Test
b Grouping Variable: Groups

ATTACHMENT 5

LIVER HISTOPATHOLOGICAL FEATURES OF EACH GROUP

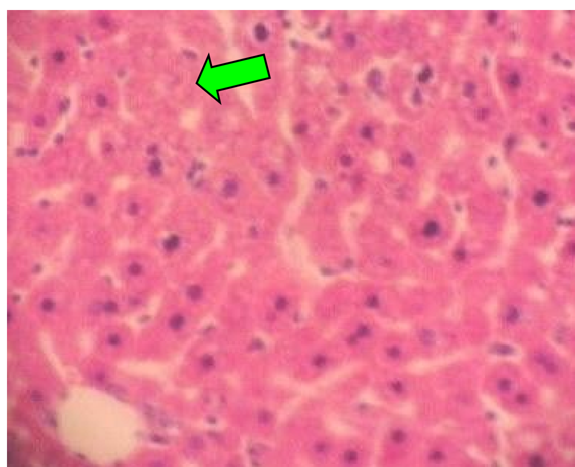

Figure 5.1. Liver histopathological feature of C0 group

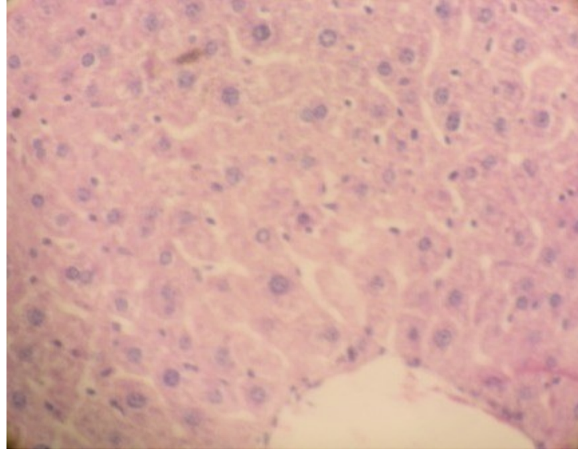

Figure 5.2. Liver histopathological feature of C negative group

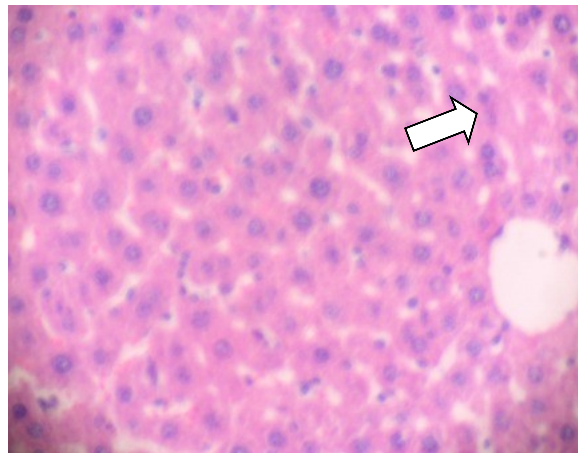

Figure 5.3. Liver histopathological feature of T1 group

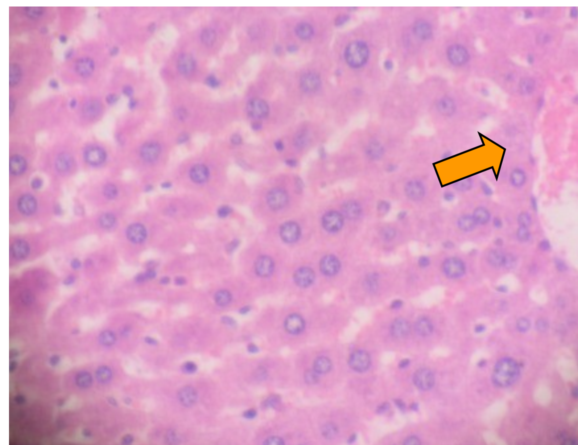

Figure 5.4. Liver histopathological feature of T2 group

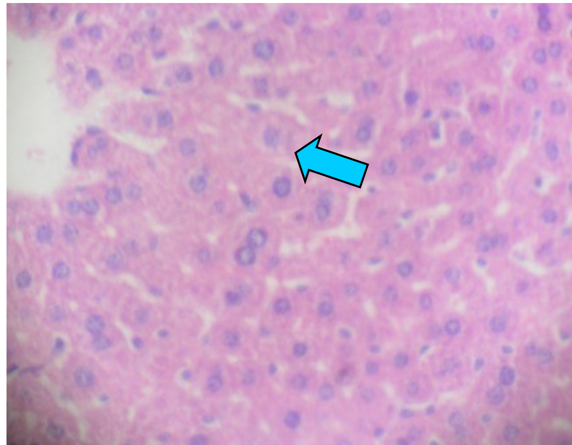

Figure 5.5. Liver histopathological feature of T3 group

Figure index:

- Green arrow = Karyolysis
- White arrow = Picnotic nucleus
- Orange arrow = Karyorexis
- Blue arrow= Degeneration