Group

Case Processing Summary

	Treatment groups	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
TNF-ALFA expresion	Grouup A	6	100.0%	0	.0%	6	100.0%
	Group B	6	100.0%	0	.0%	6	100.0%
	Group C	6	100.0%	0	.0%	6	100.0%
	Group D	6	100.0%	0	.0%	6	100.0%

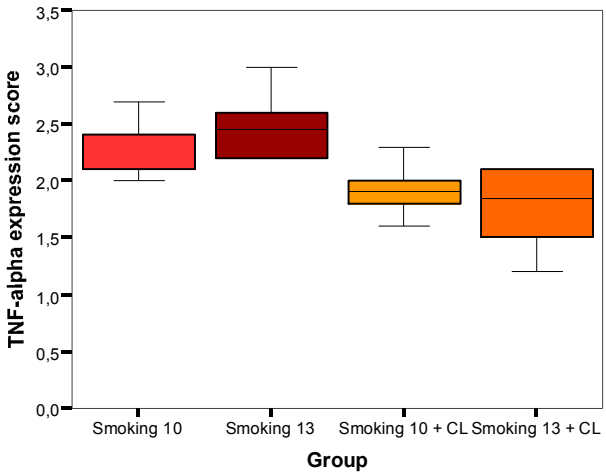
Means and median

Report

TNF-alpha expression score

Group	Mean	Std. Deviation	Median	Minimum	Maximum
Smoking 10 weeks	2,000	,63033	2,000	1,00	3,00
Smoking 13 weeks	2,250	,75944	2,2500	1.00	3,00
Smoking 10 weeks + curcuma L	1,8367	,40014	1,8300	1,00	2,00
Smoking 13 weeks + curcuma L	1,8367	,75697	2,000	1,00	3,00

Interactive Graph



Explore

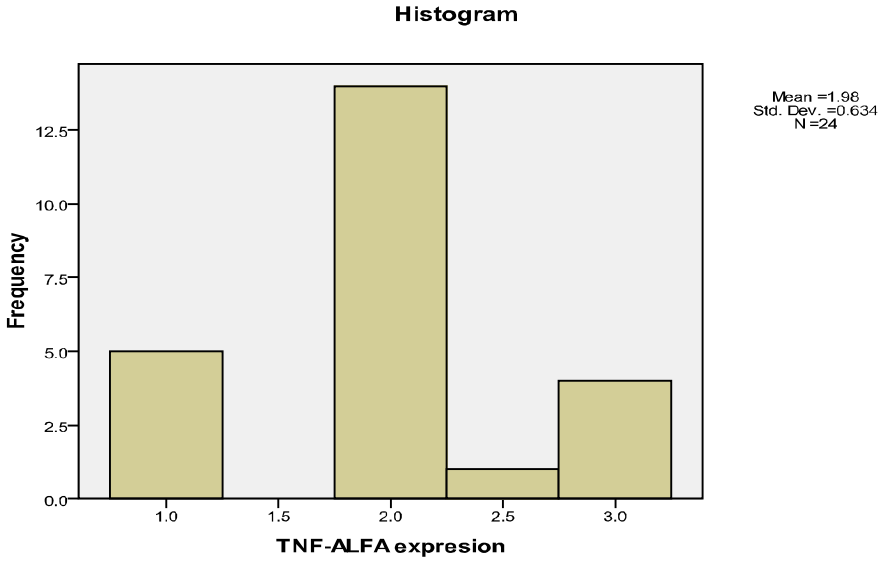
Descriptives

		Statistic	Std. Error
TNF-alpha expression score	Mean	1.979	.1294
	95% Confidence Interval for Mean	1.712 – 2.247	
	5% Trimmed Mean	1.977	
	Median	2.000	
	Variance	.402	
	Std. Deviation	.6338	
	Minimum	1.0	
	Maximum	3.0	
	Range	2.0	
	Interquartile Range	.0	
	Skewness	-.055-	.472
	Kurtosis	-.281-	.918

Tests of Normality

	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
TNF-alpha expression score	,305	24	0,000	,807	24	0,000

* This is a lower bound of the true significance.
a Lilliefors Significance Correction



Mann-Whitney Test

Ranks

	Treatment groups	N	Mean Rank	Sum of Ranks
TNF-ALFA expresion	Grouup A	6	6.92	41.50
	Group C	6	6.08	36.50
	Total	12		

Test Statistics[b]

	TNF-ALFA expresion
Mann-Whitney U	15.500
Wilcoxon W	36.500
Z	-.527-
Asymp. Sig. (2-tailed)	.598
Exact Sig. [2*(1-tailed Sig.)]	.699[a]

a. Not corrected for ties.

b. Grouping Variable: Treatment groups

Mann-Whitney Test

Ranks

	Treatment groups	N	Mean Rank	Sum of Ranks
TNF-ALFA expresion	Group B	6	8.50	51.00
	Group D	6	4.50	27.00
	Total	12		

Test Statistics[b]

	TNF-ALFA expresion
Mann-Whitney U	6.000
Wilcoxon W	27.000
Z	-2.035-
Asymp. Sig. (2-tailed)	.042
Exact Sig. [2*(1-tailed Sig.)]	.065[a]

a. Not corrected for ties.

b. Grouping Variable: Treatment groups

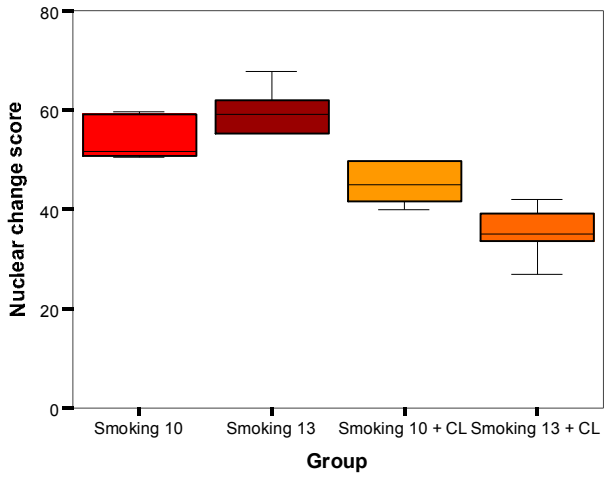
Means

Report

Liver cell change score

Group	Mean	Std. Deviation	Median	Minimum	Maximum
Smoking 10	50.9	6.40	48.8	50,40	59,60
Smoking 13	73.9	10.07	75.0	55,20	67,60
Smoking 10 + CL	43.3	1.92	42.9	40,00	49,60
Smoking 13 + CL	53.7	5.57	52.7	26,80	42,00
Total	48,4	10,38	50,0	26,80	67,60

Interactive Graph



Explore

Descriptives

			Statistic	Std. Error
Liver cell change score	Mean		50.9	2,12039
	95% Confidence Interval for Mean	Lower Bound	44,0803	
		Upper Bound	52,8530	
	5% Trimmed Mean		48,5963	
	Median		48.8	
	Variance		107,905	
	Std. Deviation		10,38773	
	Minimum		26,80	
	Maximum		67,60	
	Range		40,80	
	Interquartile Range		17,80	
	Skewness		-,230	,472
	Kurtosis		-,605	,918

Tests of Normality

	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Nuclear change score	,127	24	,200(*)	,976	24	,807

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Descriptives

Nuclear change score

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum
	Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower
Smoking 10	6	50.9	4,32049	1,76383	49,3326	58,4007	50,4
Smoking 13	6	73.9	4,66247	1,90345	54,8404	64,6263	55,2
Smoking 10 + CL	6	43.3	4,04508	1,65140	40,8216	49,3117	40,0
Smoking 13 + CL	6	53.7	5,21536	2,12916	29,7268	40,6732	26,8
Total	24	48,4	10,38773	2,12039	44,0803	52,8530	26,8

ANOVA

Liver cell change change score

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2061,973	3	687,324	32,742	,000
Within Groups	419,840	20	20,992		
Total	2481,813	23			

Post Hoc Tests

Multiple Comparisons

Dependent Variable:liver cell change score
Bonferroni

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
		Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower Bound
Smoking 10	Smoking 13	-5,86667	2,64525	0.01	-13,6096	1,8763
	Smoking 10 + CL	8,80000(*)	2,64525	,003	1,0570	16,5430
	Smoking 13 + CL	18,66667(*)	2,64525	,000	10,9237	26,4096
Smoking 13	Smoking 10	5,86667	2,64525	0.01	-1,8763	13,6096
	Smoking 10 + CL	14,66667(*)	2,64525	,000	6,9237	22,4096
	Smoking 13 + CL	24,53333(*)	2,64525	,001	16,7904	32,2763
Smoking 10 + CL	Smoking 10	-8,80000(*)	2,64525	,003	-16,5430	-1,0570
	Smoking 13	-14,66667(*)	2,64525	0.01	-22,4096	-6,9237
	Smoking 13 + CL	9,86667(*)	2,64525	,005	2,1237	17,6096
Smoking 13 + CL	Smoking 10	-18,66667(*)	2,64525	,000	-26,4096	-10,9237
	Smoking 13	-24,53333(*)	2,64525	,001	-32,2763	-16,7904
	Smoking 10 + CL	-9,86667(*)	2,64525	,005	-17,6096	-2,1237

* The mean difference is significant at the .05 level.

Appendix (2)

Groups	First pathologist result	Second pathologist result

groups of rats	Liver cell change (%)	TNF alpha expression	Liver cell change (%)	TNF alpha expression
control group(A 1)	57.8%	2 allred (5,6)	60%	2 allred (5,6)
control group (A 2)	46.8%	3 allred (7,8)	65%	3 allred (7,8)
control group (A 3)	59.8%	3 allred (7,8)	57.9%	2 allred (5,6)
control group(A 4)	45.4%	2 allred (5,6)	45%	2 allred (5,6)
control group(A 5)	45.3%	2 allred (5,6)	40.6%	3 allred (7,8)
control group(A 6)	50.8%	3 allred (7,8)	60%	2 allred (5,6)
control group (B 1)	71%	2 allred (5,6)	66%	3 allred (7,8)
control group (B 2)	80.6%	3 allred (7,8)	82%	3 allred (7,8)
control group (B 3)	72%	3 allred (7,8)	70%	3 allred (7,8)
control group (B 4)	77%	3 allred (7,8)	78%	3 allred (7,8)
control group (B 5)	85%	3 allred (7,8)	70%	2 allred (5,6)
Control group (B 6)	56%	3 allred (7,8)	40%	3 allred (7,8)

Appendix (2)

Groups	First pathologist result	Second pathologist result

groups of rats	Liver cell change (%)	TNF alpha expression	Liver cell change (%)	TNF alpha expression
Treatment group(C 1)	45%	2 allred (5,6)	45%	2 allred (5,6)
Treatment group(C 2)	43%	1 allred (2,3,4)	40%	1 allred (2,3,4)
Treatment group(C 3)	45%	0 allred (0 ascore)	48%	1 allred (2,3,4)
Treatment group(C 4)	42%	2 allred (5,6)	41.5%	2 allred (5,6)
Treatment group(C 5)	41.4%	1 allred (2,3,4)	40%	1 allred (2,3,4)
Treatment group(C 6)	22%	2 allred (5,6)	23%	0 allred (0 ascore)
Treatment group(D 1)	51%	2 allred (5,6)	50%	2 allred (5,6)
Treatment group(D 2)	51.6%	1 allred (2,3,4)	54%	1 allred (2,3,4)
Treatment group(D 3)	62%	2 allred (5,6)	60%	2 allred (5,6)
Treatment group(D 4)	57%	1 allred (2,3,4)	56%	1 allred (2,3,4)
Treatment group(D 5)	53%	0 allred (0 ascore)	55%	0 allred (0 ascore)
Treatment group(D 6)	54%	0 allred (0 ascore)	55%	0 allred (0 ascore)

Appendix (3)

Reliability of measurement:

The current study calculated reliability coefficient of scores by measuring agreement between two observers, using Kappa coefficient test, which examines the agreement between two raters for a sureness whether there is a concordance in reading the data, the table below shows the result of Kappa test for tissue change and TNFα scores.

Number of Items	Variables	Chi square (χ^2) value	df	Sig (1) reading	Sig (2) reading
24	Tissue Change	16.7	19	0.70	0.76
24	TNFα	0.22	2	0.88	0.90

significant at level ($\alpha = 0.05$).

The result of Kappa test for tissue change and TNFα scores in measurement agreement

values of chi square (16.7 & 0.22) were not significant in two variables (tissue change and TNFα) the p values were more than (0.05), and that means the measurement was reliable.

Appendix (5)
Process of H&E STAINING

Tissue Sample .

Sample of the fabric . Tissue sample was tooked as a small section of tissue after termination of the rats . When taking the sample was tooked directly from the bodies after termination in order to avoid damage. The tissue was cuted carefully with a blunt object so as not to deform showing microscopy. Even the installation is good, should not increase the tissue mass about one centimeter, and the sample was immediately dipped in the installer.

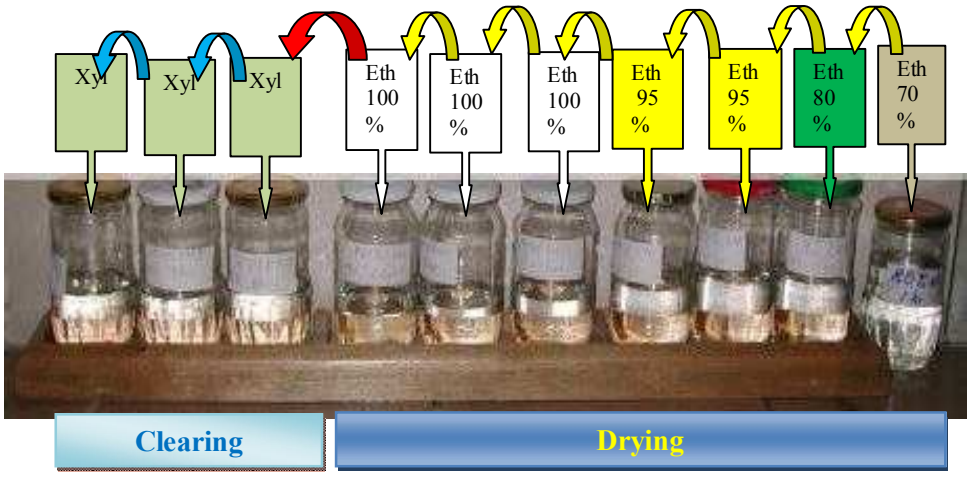
Fixation

The installation process was maked to prevent tissue damage, prevent erosion and chemical changes that occur as a result of activity of proteins in the tissue. The installation process was make thrombis the protein in the tissue. The chemical stabilizers, too for prevention of cellular enzymes (yeast) from the digestion of the cell, it also keeps carbohydrates and fat in the cells of the tissue.in this expiremental was used 4% neutral Formal dihiad as suitable for most routine work.

Dehydrated :

liver Tissue samples processing was done to remove water from the liver tissues, replacing such water with a medium that solidifies, setting very hard and so allowing extremely thin sections to be sliced. This process was done by using graded ethanol solutions as follows (70% , 80% , 95% , 95% , 100% ,100% , 100%) respectively , leaving the liver tissue samples in each solution for a sufficient period for replaced the water with alcohol. And despite the fact that paraffin is not soluble in

alcohol, therefor the alcohol replaces with the paraffin solvent has capable to soluble with paraffin .



Clearing :

Xylene solution used, usually, to clean the tissue mass by passing through graded xylene solutions ,, that ultimately lead to replacement of alcohol with xylene and then the liver tissue mass were became ready-to-Embed

Embedding

Before sectioning, tissue samples was embedded in a material with similar mechanical properties. This step allows the tissue to be cut easily. In order to sectioned the tissue with a microtome, it was embedded in paraffin. After fixation, tissues was got paraffin-embedded are dehydrated by first using graded ethanol solutions, then graded xylene solutions, then finally liquid paraffin. The graded

solutions gradually expose the sample to changes in hydrophobicity, minimizing damage to cells. After a short time in the liquid paraffin, the tissue is placed into a mold with more paraffin. The wax is allowed to solidify, forming a block that can be held in a microtome.

Sectioning

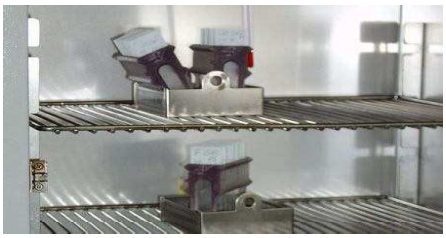
Samples embedded in paraffin are first mounted in a microtome. The microtome holds a sharp blade and is controlled by a crank that is turned to bring the paraffin block closer to the blade. As the crank is turned further, the blade cuts slices of paraffin, which containing tissue. After sectioning, the slices was placed on a slide

4.Mounting

After several slices of the paraffin-embedded tissue have been sectioned, the slices are removed from the blade and floated atop a warm water bath to smooth out the sample. The slices are teased apart and floated onto a slide . After the slides have dried, were placed in an oven to "bake" the paraffin.

5. drying oven

The unstained slides are then placed into a drying oven. The oven is warm and helps the section of tissue adhere to the slide.



TissueStaining

When the slides are removed from the oven, they are placed into an automated staining machine. The slides with the tissue were immersed in chemicals and dyes

that stain the cells(**H & E Staining)**. Hematoxylin stains the nucleus and the Eosin stains the cytoplasm of tissue cells.

Coverslipping A protective glass coverslip is attached to the slide with mounting medium was applied . This protects the tissue from being scratched. Better microscopic examination at various magnifications is also obtained by the use of coverslips.

B. Histology Specimen Preparation and staining (Liver cells change)

After the total duration of the experiment 10 weeks for (A&C),and 13 weeks for (B&D) at the end of it, the animals subjected to whole-body perfusion using normal saline and buffered formalin under light ether anesthesia. way to termination pay attention to the principles stated in Helsinki Declaration of 1975

and the National Guidelines for Health Research Ethics(PNEPK),The liver removed and stored immediately in buffered formalin for histopathologyy examination.

Histopathology feature of liver tissue on study groups at the end of experiment were shown on figure 11. Tissue damage were observed from H&E staining examination by Olympus PX51 light microscope with 1000x magnification in 10 fields from randomized choosing. The examiner counted the number of cells with nucleus changes (i.e, enlarge, karyorhexis, and karyolysis). The results were expressed in percentage (%) of abnormal cells per all cells counted on those fields. From total 24 rats, 24 livers of the rats were made into tissue slid

Appendix(6)

Immunohistochemistry staining

- Animals were sacrifice by overdose of ketamine injection.
- Liver was remove by clean surgery procedure.
- Liver tissue were fixed in formalin and embed in paraffin blocks according to standard procedures.
- Object glass slides were cleaned with 95% ethanol and treated with subbing solution and air dry, or by using pre-treated slides.
- Tissue sections were cut 4–6 micron thick and applied to slides. Tisue were deparaffinize in xylenes using three changes for 5 minutes each. Hydrate sections gradually through graded alcohols: wash in 100% ethanol twice for 10 minutes each, then 95% ethanol twice for 10 minutes each. Wash in deionized H2O for 1 minute with stirring. Aspirate excess liquid from slides.
- Antigen unmasking was performed at this point. Certain antigenic determinants are masked by formalin fixation and paraffin embedding and may be exposed by Pepsin: Incubate sections for 10–20 minutes in 0.1% pepsin in 0.01 N HCl at room temperature. Slides were washed several times in deionized H2O. Aspirate excess liquid from slides.
- For immunoperoxidase staining of tissue sections, will use, ABC Staining Systems ,The ABC Staining Systems utilize preformed avidin-biotinylated horseradish peroxidase complex as a detection reagent.[52]

- TNF-α immunohistochemical expression will quantified in accordance to Allred score by two independent pathologists and compared across histological categories using Kappa test.

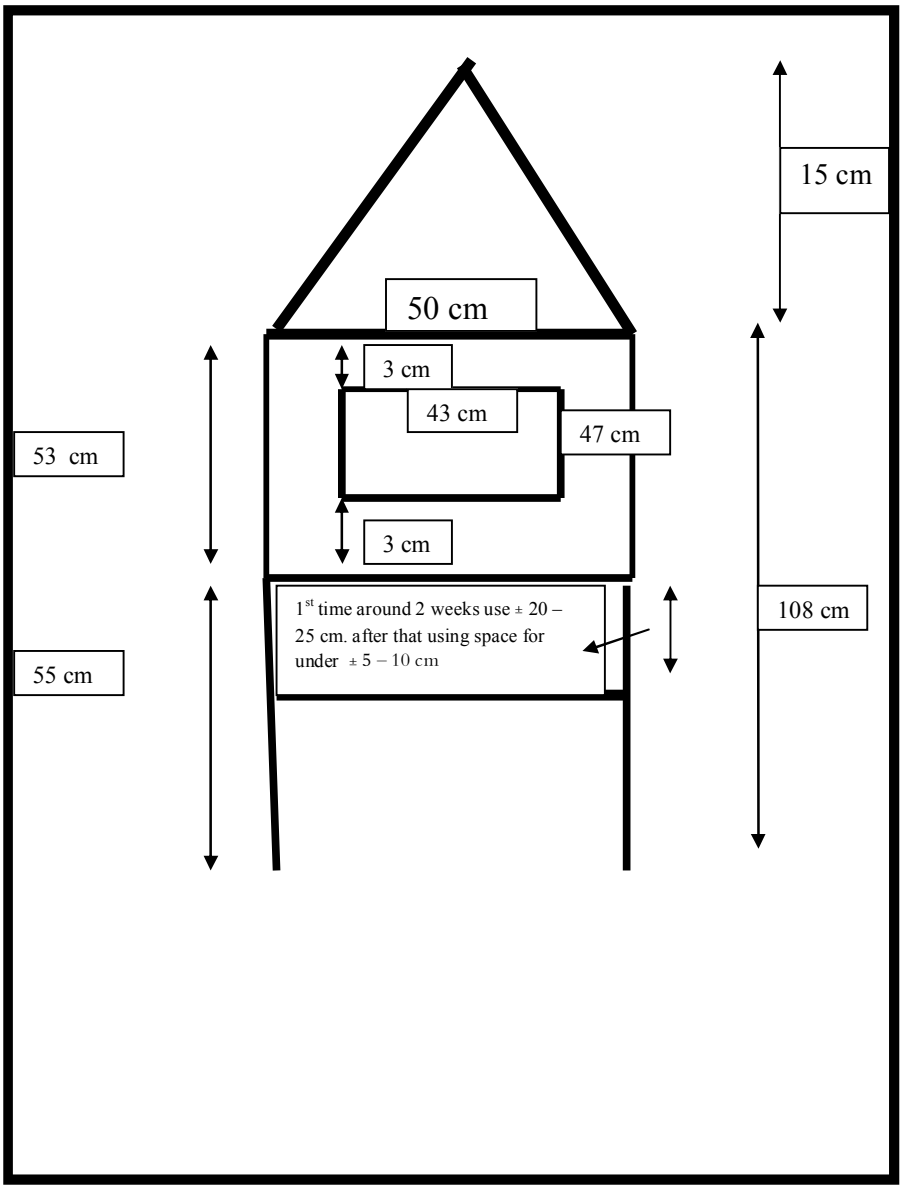
Liver cells TNF-α expression

Expression of TNFα observed by immunohistochemistry examination using TNFα antibody ,examination of slide done by Olymps PX51 light microscope using 10X ocular lens and 40X objective lens, examination and reading of the slides were done by counting the percentage of TNFα cells stained brown color in the cytoplasm as proportion score adding to it the intensity of the staining rated as none, mild, intermediate and strong, the result of the these two score is a number which is called Allred score which then categorized .quantified in accordance to Allred score. Allred score was established using a 0–8 scale based upon the sum of a proportion score (percent of stained cells) and intensity score (weak, intermediate, and strong). The possible values of Allred score are: 0 – Allred 0*; 1 – Allred 2, 3, 4; 2 – Allred 5, 6; 3 – Allred 7, 8 (*Allred score 1 is not possible). each slide rated 10 field of view with magnification 400X.

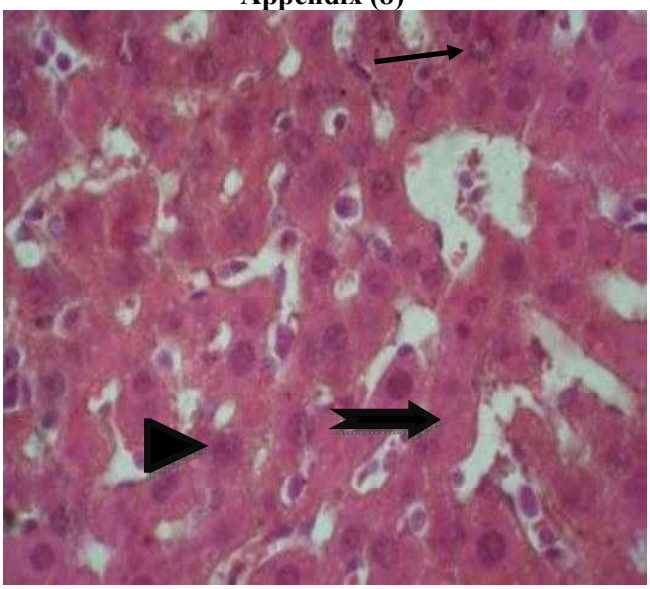
Proportion Score (PS)		**Intensity Score (IS)**	
Value	**Significance**	**Value**	**Significance**
0	none	0	none
1	<1%	1	weak
2	1- 10%	2	intermediate
3	10- 33%	3	strong
4	33- 66%		
5	> 66%		

Appendix (7)

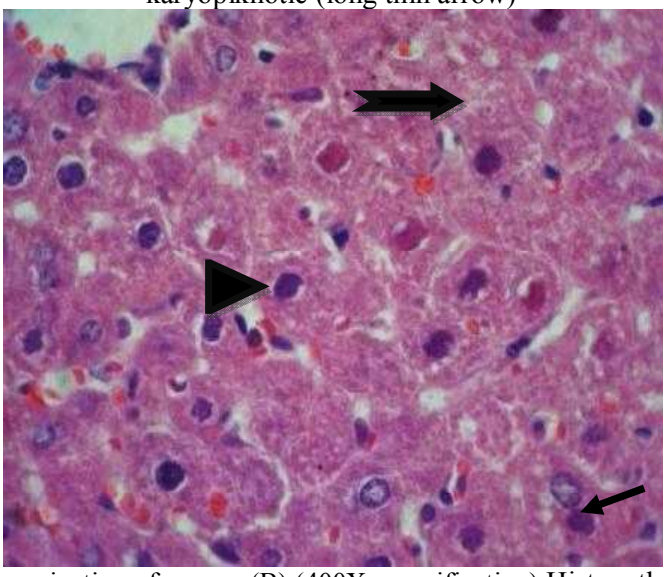
55fffo



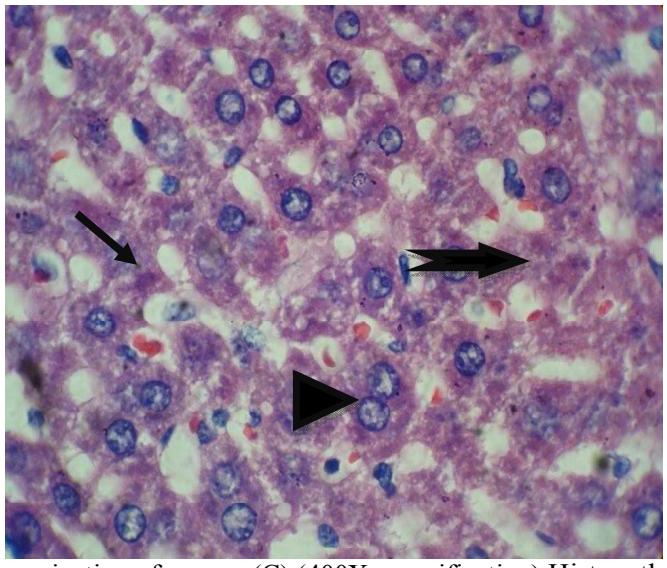
Appendix (8)



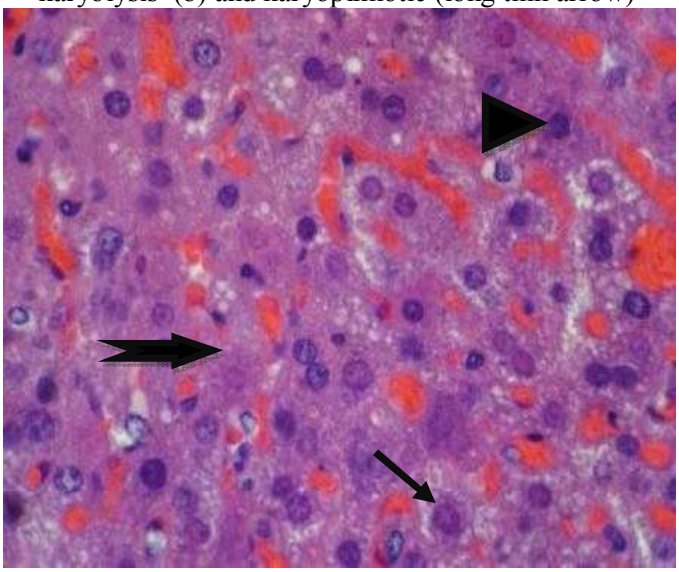
HE staining examination of group (A) (400X magnification) Histopathology feature of liver cells changes of SD rats after SD rats after 13 weeks cigarette smoke exposure . (Head Arrow) point cell with odeama ,(tailed arrow) karyolysis (b) and karyopiknotic (long thin arrow)



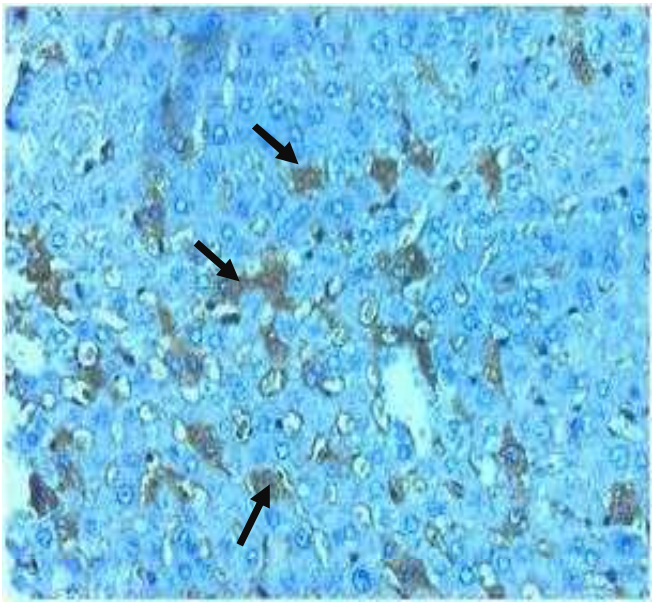
HE staining examination of group (B) (400X magnification) Histopathology feature of liver cells changes of SD rats after SD rats after 13 weeks cigarette smoke exposure . (Head Arrow) point cell with odeama ,(tailed arrow) karyolysis (b) and karyopiknotic (long thin arrow)



HE staining examination of group (C) (400X magnification) Histopathology feature of liver cells changes of SD rats after SD rats after 10 weeks cigarette smoke +curcuma Lexposure . (Head Arrow) point cell with odeama ,(tailed arrow) karyolysis (b) and karyopiknotic (long thin arrow)

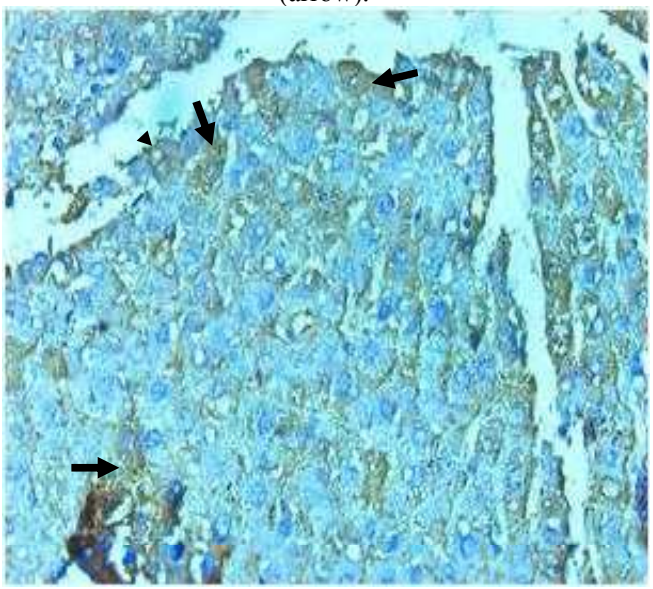


HE staining examination of group () (400X magnification) Histopathology feature of liver cells changes of SD rats after SD rats after 13 weeks cigarette smoke +curcuma Lexposure . (Head Arrow) point cell with odeama ,(tailed arrow) karyolysis (b) and karyopiknotic (long thin arrow)



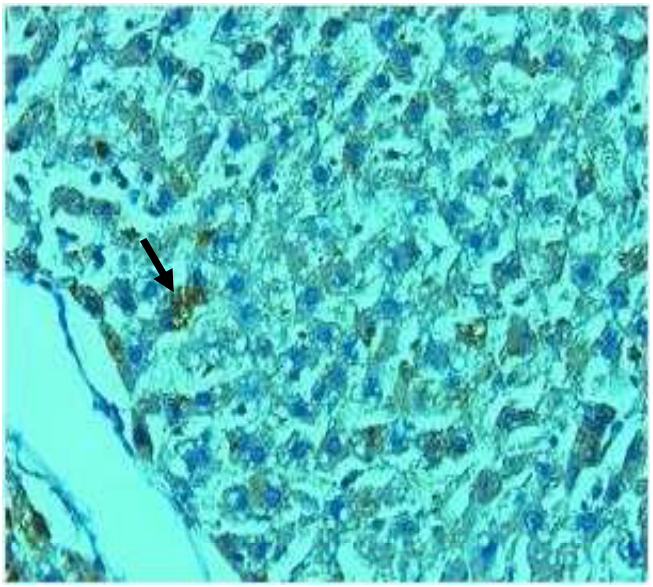
IHC staining examination of control group(A)

Morphology of TNF-α expression of the SD Rats liver cells in control group(A),using 400X magnification after 10 weeks cigarette smoke The cells were brown coloured (arrow).



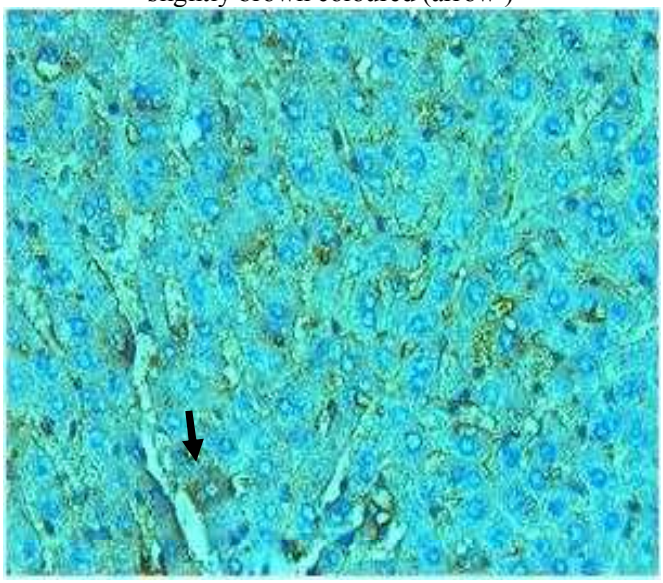
IHC staining examination of control group(B)

Morphology of TNF-α expression of the SD Rats liver cells in group(B),using 400X magnification after 13 weeks cigarette smoke . The cells were brown coloured (arrow).



IHC staining examination of Treatment group(C)

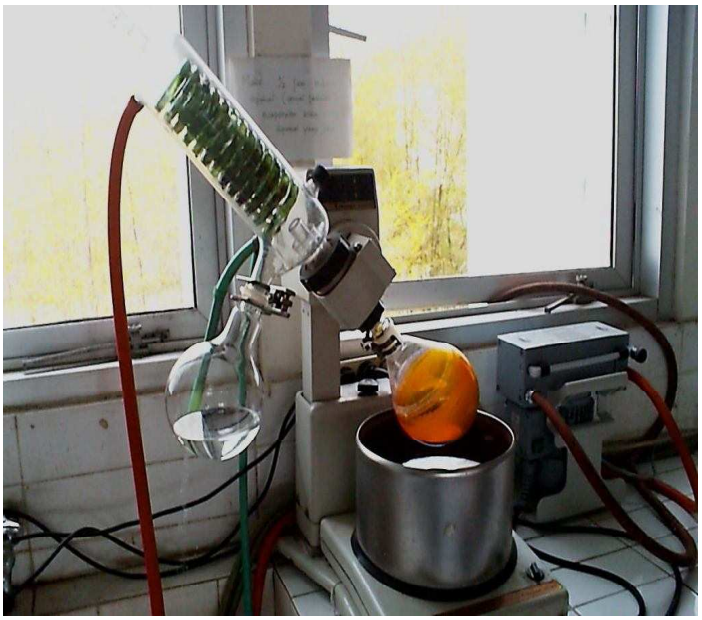
Morphology of TNF-α expression of the SD Rats liver cells in group(C),using 400X magnification after 10 weeks cigarette smoke +curcuma L exposure. The cells were slightly brown coloured (arrow)



IHC staining examination of Treatment group(D)

Morphology of TNF-α expression of the SD Rats liver cells in group (D),using 400X magnification after 13 weeks cigarette smoke +curcuma L exposure. The cells were more slightly brown coloured (arrow).

Curcuma longa rhizoma dry in the oven



Curcuma longa rhizoma extraxt in the soxhelst

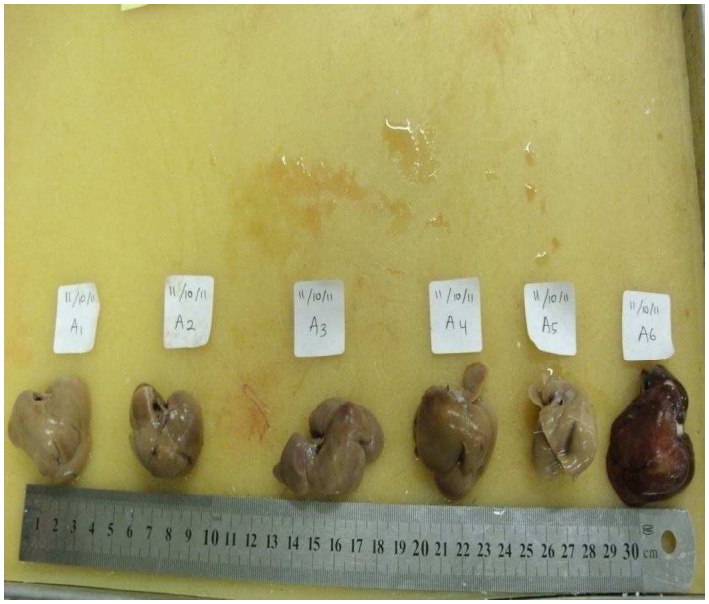
Key of the expirement
(Marlboro cigar and curcuma longa rhizoma extract)

Spraque Dawley Rats

Operation Tools of SD Rats termination

Perfusion of the rats and fixation

Operation of Rats were taken its liver tissue



Liver of the SD Rats

Appendix plce of the study

DIPONEGORO UNIVERSITY

SEMARANG INDONESIA