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Original Article

Effects of *Acalypha indica* L. Extract on Inflammatory Response in The Pathogenesis of Nonalcoholic Fatty Liver Disease: An Overview of TLR9, NFκB and TNFα Expression in Hepatocytes and Macrophages of Sprague-Dawley Rats

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ABSTRACT

Background: Complications of non-alcoholic fatty liver disease (NAFLD) include 67% of the criteria for metabolic syndrome. *Acalypha indica* L., (AI) which is one of a herbal plant had been known as anti-oxidant and anti-inflammatory effects. The effect of AI for therapy investigated by looking of the immune defense mechanisms. This researched was assessed by molecular docking approached on TLR9, NFκB, TNFα expression and liver morphological changes. **Methods:** Animal models of steatohepatitis were collected from high-fructose and cholesterol diet (HFCD) of Sprague-Dawley rats for 12 weeks and followed by therapy for 8 weeks. There were 5 groups from twenty five researched rats, include normal group (K1), HFCD group (K2), HFCD group supplemented with 400 mg *Acalypha indica* L. (K3), combination between 400 mg AI+Gemfibrozil (Gem) 31 mg (K4) and Gem 31 mg/kg (K5) in kgBW, respectively. **Results:** The results of molecular docking were carried out by assessing the interaction between hydrogen molecules of AI compounds and amino acid residues in TLR9, NFκB, TNFα. Morphological changes were assessed by scoring system. Statistical analyzed used Kruskall Wallis with post hoc Mann Whitney test continued by Spearman correlation test. **Conclusion:** The molecular docking analysis showed that, an alkaloid compounds were found besides the flavonoid compounds that can bind to the binding pocket of inflammatory markers with the best binding energies. Other compounds, there are dasycarpidan-1-methanol, acetate (ester), fenofibrate and quinine. Supplementation of AI would reduced hypertrophy (p=0.031), macrovesicular steatosis (p=0.018), inflammation foci (p=0.005) and also decreased of TLR9 (p=0.009), NFκB (p=0.009), TNFα (p=0.009) expression, but not as good as the combination of AI+Gem.
Key words: *Acalypha indica* L., NAFLD, TLR9, NFκB, TNFα.

INTRODUCTION

Nonalcoholic Fatty liver Disease (NAFLD) is one of a health problem for approximately 25% of the world's adult population. The largest number of cases is caused by a lack of monitoring of complications that cause disease progression and lack of early detection.[1,2] The most significant risk factor for NAFLD is metabolic syndrome. The metabolic system is closely related to the pathways of the TLR and NLR receptors. PRRs, such as TLRs is responsible for the recognition of immunogenic signals that induce changes in the liver. TLR9 regulate homeostasis under acute stress, acting on unmethylated CpG DNA. A study revealed that steatotic hepatocytes release mitochondrial DNA, located in microparticles into the plasma, activate TLR9 in the endosome to hyperactivation and produce inflammatory cytokines via regulation of NFκB by releasing cytokines, such as IL1β, TNFα and IL6, thereby promoting the development of steatohepatitis. Kupffer cells (liver macrophages) can trigger an inflammatory cascade then perform liver inflammation. Proinflammatory products and recruitment of chemokines such as CCL2, CXCL10 and fibrogenic monocytes released more injured tissue at the liver.[3]

Based on Rahman et al, in 2010,[4] *Acalypha indica* at the right dose can effect as an anti-inflammatory drug in the human body. The activity of *Acalypha indica* has an anti-inflammatory effect on rats using polar extract. Research studies have shown that *in vitro* and *in vivo* on all parts of the *Acalypha indica* plant are able to reduce inflammation. In addition to *in vitro* and *in vivo* studies, from *in silico* study based on Mojumder et al, in 2016,[5] the phytochemical compound 2-methylantraquinone contained in the *Acalypha indica* plant which had been predicted by molecular docking could inhibit COX1 in the inflammatory pathway. Based on its predictions of ADME and toxicity, it has been described a safety for using in humans. Sahukari, et al, in 2021,[6] also revealed that more than one hundred compounds predicted from the extract of the *Acalypha indica* plant with polar solutions in the form of methanolic extract of *Acalypha indica* roots were considered capable of having anti-inflammatory effects related to the NFκB pathway, as well as TNFα and IL1β cytokines. It was also had been proven by *in vivo* studied, where *Acalypha indica* roots extract can reduce the volume of leg edema in rats, using the paw test. Carrageenan-induced rats with paw test to see edema has been evaluated for its effectiveness as an anti-inflammatory from phytochemicals compounds.

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Zahidin et al, in 2017,[7] Phytochemicals contained in *Acalypha indica* are able to be anti-diabetic, anti-obesity, anti-hyperlipidemic, anti-oxidant, anti-inflammatory and even as anti-cancer. In addition, Rajasekaran, et al in 2013,[8] revealed that a dose of 400 mg / kgBB was the optimal dose of *Acalypha indica* L., as an anti-hyperlipidemic and a dose of 200-400 mg / kgBB of *Acalypha indica* L. extract was effective as an anti-oxidant[8] and anti-inflammatory.[7] In this study, histopathology and immunohistochemistry examinations were used to measured the parameters of TLR9, NFκB and TNFα which are known as the initial mechanism of inflammation in the liver, accompanied by in silico approached. This study used a stored biologic material, which had been induced by a high-fructose and cholesterol diet (HFCD) for 12 weeks, followed by therapy for 8 weeks. HFCD induced rodent models to be NAFLD with steatohepatitis spectrum. Rodent models were divided into 5 groups, including normal group, negative control, *Acalypha indica* L. (AI), *Acalypha indica* L., and Gemfibrozil (AI+Gem), and Gemfibrozil (Gem) groups. The liver was pre-researched by observing morphological changes in the liver including hypertrophy, microvesicular steatosis, macrovesicular steatosis and inflammatory foci, previously.[9] The aim of this study is to investigate anti-inflammatory effect of ethanolic extract of Acalypha indica roots on Liang's criteria of steatohepatitis spectrum of NAFLD rodent model induced by HFCD compared to Gemfibrozil.

METHOD

This study used in vivo experimental design, where the liver sample was obtained from 10 weeks male Sprague-Dawley rats aged (a stored biologic material in 10% neutral buffer formalin) from Department of Medical Pharmacy, Faculty of Medicine, Universitas Indonesia) with non alcoholic steatohepatitis rodent model induced by a high-fructose and cholesterol diet for 12 weeks with 55% liquid fructose (1 mL contained 550 mg of fructose and 0,5 mL of aqua added until 2 mL). The total fructose given once was 825 mg fructose, so that the total given twice a day was 1650 mg. The 10% of cholesterol diet derived from quail eggs, which is equivalent to 314.94 mg/100 gram of cholesterol mixed in feed, followed 8 weeks therapy.[10,11] This study used Liang's criteria to determine the morphological changes, followed by histopathology and immunohistochemistery assessment.

This study uses the Federer formula to determine the number of sample to be used.

Formula : $(n-1)(t-1)\geq 15$

Explanation: n = Number sample of each group

t = Number of groups

number of samples in each group: $(n-1)(5-1) \geq 15$

$(n-1)(4) \geq 15$

$4n-4\geq 15$

$n \geq 4,75$

From calculations based on Federer's formula, the number of rats was 4.75, then the numbers were rounded up to 5 rats for each group with the total number of rats from this study was 25 rats. The research was conducted in the period 2021 – 2022, at the Departement of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia and *in silico* study in Department of Bioinformatics, Faculty of Medicine, Universitas Indonesia.

In silico

Preparations of target proteins for TLR9, NFκB and TNFα were taken from the database (RSCB PDB after screening database compounds with Herbaldb used Ligand Scout 4.4.8 software and from publication.[5,6,7] Meanwhile, the ligand preparation will be downloaded from the Pubchem database. The phytochemical compounds from *Acalypha indica* L., were used as ligands, based on molecular docking predictions. After obtaining of the 2D/3D ligands, it was followed by optimization with targeted protein (TLR9, NFκB and TNFα). The required software preparations was needed include Marvin Sketch to convert SDF files into pdb and the docking software with Autodock tools 1.5.7. If the docking validation was done using the re-docking method and good validation had been obtained, the docking compounds test would be carried out to visualization. Active compounds contained in *Acalypha indica* were predicted by the Computer Aided-Drug Design (CADD) method to determine they activity and probability of the compounds against the inflammatory pathways. In the molecular simulation, the binding affinity for the target protein was assessed by the most negative result in binding energy. Furthermore, visualization was carried out to determine the position when it was bind to the binding pocket of the inflammatory proteins (Ligplot software). In molecular simulation, the next step was determination of the adsorption, distribution, metabolism and excretion (ADME) to see the pharmacokinetics of the active compounds contained in *Acalypha indica*, as well as to see the effect of toxicity and its probabilities with SwissADME and ADMESAR (http://www.swissadme.ch/ and http://lmmd.ecust.edu.cn/admetsar1/predict/).

In vivo

Haematoxilin and eosin examination: Liver tissue was cut using a cutter/blade at a thickness of 2 mm, on the caudatus lobe, right lobe and left lobe liver tissue. Furthermore, the liver tissue was fixed using a 10% neutral buffered formalin solution. Then, tissue processing was carried out including dehydration, clearing, impregnation, embedding and rehydration through a graded alcohol series. Sequentially stained with hematoxylin eosin (HE) for six minutes, graded ethanol dehydration, purification process with xylene solution. The slides were observed with a light microscope, the cytoplasm was colored red, while the nucleus was blue. Five large visual fields were selected under 400x magnification an objective lens to view the histopathological features of hypertrophy, microvesicular steatosis, macrovesicular steatosis and inflammatory infiltration. The assessment of lesion degree's was started by using 100x and 200x magnification looking for the portal area and the lobular area, then with a 400x magnification analyzed liver morphological changes.

Histopathological scoring assessment of NAFLD: Inflammation is seen with a small number of inflammatory foci. It should consist of more than five inflammatory cells, then the five areas of inflammatory foci are counted and averaged. Assessment of microvesicular steatosis and macrovesicular steatosis seen from its location against the nucleus. If the steatosis supress against the nucleus, it means that macrovesicular steatosis and if its not supress the nucleus, this steatosis means microvesicular steatosis. Meanwhile, hypertrophy is an enlargement of cells with a size of more than one point half of normal cells compare to normal groups.

Immunohistochemistry examination: Immunohistochemical experimental procedures were followed according to the instructions from the kit. Liver tissue is put in paraffin, after FFPE block was performed, then it was cut using a cutter/blade at a thickness of 3µm., dried with xylene, dehydrated with graded ethanol. Endogenous peroxidase was eliminated with 3% H_20_2, Antigen Retrieval (AR) by Heat Induced Epitope Retrievel (HIER) with Tris EDTA PH 9 and temperature 96°C degrees in decloacking chamber, thirty minutes AR of TLR9 and NFκB. Meanwhile, TNFα was twenty five minutes AR. Incubation of mouse monoclonal antibody anti-TLR9 (Abcam) and rabbit polyclonal antibody anti-TNFα (Bioss antibody) need 1 hour for incubation at room temperature. Meanwhile rabbit polyclonal

antibody anti-NFκB needs 18 hours for incubation at four degrees. Each of antibodies was dilution at 1:100 followed by incubation of One Step Neopoly Detection Kit (Biogear) and visualized with DAB Chromogen (3,3-diaminobenzadine). DAB solution was added dropwise for one minute. Every step used PBS PH 7,4 for three minute to washed. The sections were slightly counterstained with hematoxylin, dehydrated with a series of ethanol, cleaned in xylene, were mounted and examined with a light microscope. Under microscope observation, it was found that the core of hematoxylin staining was blue and the positive result of DAB staining was brownish yellow. TLR9, NFκB and TNFα expressions were measured by Qupath software 0.3.2 at 400x magnification with ordinal data indicating the strength of the expression. TLR9 shows positive expression when brown color is seen on the cytoplasm of Kupffer cells or macrophage and TNFα shows positive expression when brown color is seen on the cytoplasm of hepatocyte, Kupffer Cells or macrophage.[12,13] Meanwhile, NFκB shows a positive expression when brown color is seen on the nuclei of Kupffer cells or macrophage. If the cell is not colored, it means negative value.[13] The scores were determined based on the intensity of the brown color with a score range of 0 to 3. Negative score 0, weak positive score 1, medium positive score 2 and positive strong score 3. Ordinal data in the form of scores from 10 fields for one sample of view with 400x magnification on each group, then analyzed by SPSS 24.0 software.

Data analysis

Data analysis used the SPSS 24.0 software. The researched data used ordinal data and more than two groups. Hence, the statistical analysis used the Kruskall-Wallis test. If the results of the Kruskall-Wallis test were significant at a p-value less than 0.05, then it was continued by the Mann-Whitney post hoc test. Meanwhile, to determine the positive or negative correlation of a relationship between variables, this researched used the Spearman correlation test.

RESULTS

In silico

Molecular docking studies were used to analyze chemical molecules or small molecules with amino acids present at the active binding site of the protein. The docking simulation was able to identify the activity of a small molecule of *Acalypha indica* L. against TLR9 protein (PDB ID: 4RO9), NFκB (PDB ID: 4IDV) and TNFα (PDB ID: 6X82) related to their activity as anti-inflammatory on the NFκB pathway. In the molecular docking prediction results, the Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to identified several targeted proteins in the inflammatory pathways for molecular docking predictions. TLR9 which can be activated due to stress of endoplasmic reticulum and activate the NFκB pathway to release TNFα in steatohepatitis. Based on the prediction of molecular docking, the best validation of TLR9 is grid box 40x40x40 with the coordinates of the center on ligand are x: 46,430; y:-35,846; z:76.536 (Figure 1) binding energy -5.68 kcal/mol, RMSD 2.88 Å (Table 2). The RMSD value is still above 2, but its the best value that can be used with GA 100, besides the PDB ID had been published previously. Beta-sitosterol, stigmasterol and dasycarpidan-1-methanol, acetate (ester) are the lowest minus binding energy values with binding energy -8.67, -8.11, -7.04 kcal/mol inhibition constants (Ki) 615.02 uM, 486.06 uM and 10.55 uM), respectively (Figure 2). Based on the prediction of molecular docking, docking validation settings were also carried out into the active site of the NFκB protein. The best validation is grid box 40x40x40 with the coordinates of the center on ligand are x:16,220; y:13,917;z:87,361 (Figure 1) binding energy value -9.49 kcal/mol, RMSD 1.88 Å (Table 3). Based on the results of the docking simulation, beta-sitosterol, stigmasterol and fenofibrate are the lowest minus binding energy values (binding energy -11.00, -10.22, 9.61, kcal/mol and inhibition constant (Ki) 731.99 nM, 590.07 nM and 123.05 nM), respectively (Figure 2). Meanwhile, the best validation for TNFα is grid box 40x40x40 and the coordinates of the center on ligand are x: 7.213; y: -22,303; z: 34,736 (Figure 1) binding energy -6.65 kcal/mol and RMSD 0.68 Å) (Table 4). Beta-sitosterol, stigmasterol and quinine are the lowest minus binding energy values (binding energy -6.87, -6.87, -5.82 kcal/mol and the inhibition constants (Ki) values were 55.04 uM, 89.40 uM and 195.88 uM), respectively (Figure 2). Gemfibrozil as a standard drug also targeted to all three inflammatory markers, but the best hydrogen bond interactions between Gemfibrozil and inflammatory markers showed by NFκB amino acid residues (Figure 3) with the best binding energy -7.10 Kca/mol and the inhibition constants (Ki) values was 11.90 uM (Table 5).

Table 6 showed there are three selected therapeutic candidate compounds are not harmful according to AMES criteria and there are not carcinogenic compounds. Based on the Acute Oral Toxicity (AOT) study, beta-sitosterol and stigmasterol had an AOT value of category I, while fenofibrate and dasycarpidan-1-methanol, acetate (ester) had an AOT value of category III and quinine had an AOT value of category II. The five compounds are not carcinogenic or mutagenic.

In vivo

Based on the results of the study, there was found that the negative control group (HFCD without therapy) showed more liver morphological changes with hypertrophy, microvesicular steatosis, macrovesicular steatosis and inflammatory foci than *Acalypha indica* L. group/AI (supplemented with 400 mg/kgBW *Acalypha indica* L.), AI+Gem

Table 1: Grading system of NAFLD according to the mouse NAFLD/NASH Liang et al.[9]

Score	0	1	2	3
Steatosis				
Macrovesicular steatosis	<5%	5-33%	33-66%	>66%
Microvesicular steatosis	<5%	5-33%	33-66%	>66%
Hypertrophy	<5%	5-33%	33-66%	>66%
Inflammation				
Number of inflammatory foci/field	<0,5	0,5-1,0	1,0-2,0	>2,0

Table 2: Small molecules interaction with TLR9.

Compounds	Binding energy (Kcal/ mol)	Constant inhibition/ Ki (nM)/ (uM)/ (mM)
Native ligand	-5.68	47.52 uM
Beta-Sitosterol	-8.67	615.02 nM
Stigmasterol	-8.11	486.06 nM
Fenofibrate	-7.04	10.55 uM

Table 3: Small molecules interaction of NFκB.

Compounds	Binding energy (Kcal/ mol)	Constant inhibition/Ki (nM)/ (uM)/ (mM)
Native ligand	-8.15	1.05 uM
Beta-sitosterol	-11.00	731.99 nM
Stigmasterol	-10.22	590.07 nM
Dasycarpidan-1-methanol,acetate (ester)	-9.61	123.05 nM

Table 4: Small molecules interaction of TNFα.

Compounds	Binding energy (Kcal/ mol)	Constant inhibition/ Ki (nM)/ (uM)/ (mM)
Native ligand	-6.65	128.88 uM
Beta-sitosterol	-6.87	55.04 uM
Stigmasterol	-6.87	89.40 uM
Quinine	-5.82	195.88 uM

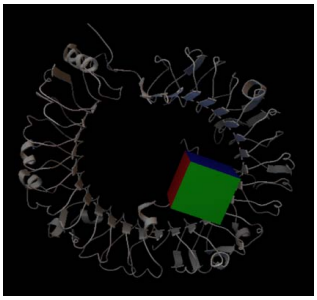
Table 5: The interaction between Gemfibrozil and The Inflammatory markers

Inflammatory Marker	Binding Energy Native ligan (Kcal/mol)	Binding Energy Gemfibrozil (Kcal/mol)	Constant Inhibition Native ligan (uM)	Constant Inhibition Gemfibrozil (mM/ uM)
TLR9	-5.68	-4.64	47.52 uM	1.79 mM
NFκB	-8.15	-7.10*	1.05 uM	11.90 uM
TNFα	-6.65	-3.27	128.88 uM	4.79 mM

Table 6: ADME and toxicity prediction.

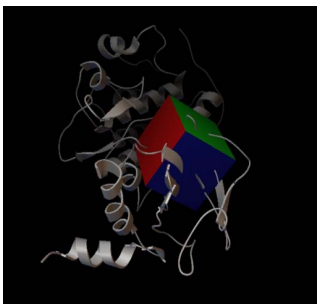
Ligands	Drug likeness				Pharmacokinetics						Toxicities		
	MW (g/mol)	HBA	HBD	LogP	GI Abs	Inhibitor CYP					AMES	Carcino-genesis	AOT
						CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4			
Beta- Sitosterol	414.71	1	1	7.19	low	no	no	no	no	no	-	-	I
Stigmasterol	412.69	1	1	6.97	low	no	no	yes	no	no	-	-	I
Fenofibrate	360.83	4	0	4.40	high	yes	yes	yes	yes	no	-	-	III
Dasycarpidan-1-methanol, acetate (ester)	326.43 g/mol	3	1	3.16	high	no	no	no	yes	no	-	-	III
Quinine	324.42 g/mol	4	1	2.81	high	no	no	no	yes	no	-	-	II
Gemfibrozil	250.33	3	1	3.35	high	no	no	no	yes	no	-	-	III

TLR9



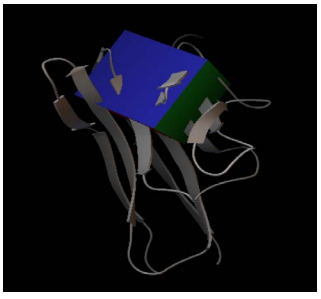
	40x40x40	50x50x50	60x60x60
Grid Center	-5.68 kcal/mol	-4.89 kcal/mol	-5.10 kcal/mol
x= 46.430			
y= -35.846			
z=76.536			
RMSD	2.88 Å	4.18 Å	3.21 Å
Inhibition constant	47.52 uM	7.31 mM	11.33 mM

NFκB



	40x40x40	50x50x50	60x60x60
Grid Center	-9.49 kcal/mol	-8.93 kcal/mol	-9.49 kcal/mol
x= 16.220			
y= 13.917			
z=87.361			
RMSD	1.88 Å	1.06 Å	1.69 Å
Inhibition constant	1.05 µM	407.05 nM	297.97 nM

TNFα



	40x40x40	50x50x50	60x60x60
Grid Center	-6.65 kcal/mol	-7.05 kcal/mol	-8.01 kcal/mol
x= 7.213			
y= -22.303			
z= 34.736			
RMSD	0.68 Å	5.38 Å	5.50 Å
Inhibition constant	128.88 uM	42.94 uM	12.48 uM

Figure 1. Grid Docking Validation. Gridbox 40x40x40 shows the best validation with RMSD value 2.88 Å for TLR9, 1.88 Å for NFκB and 0.68 Å for TNFα.

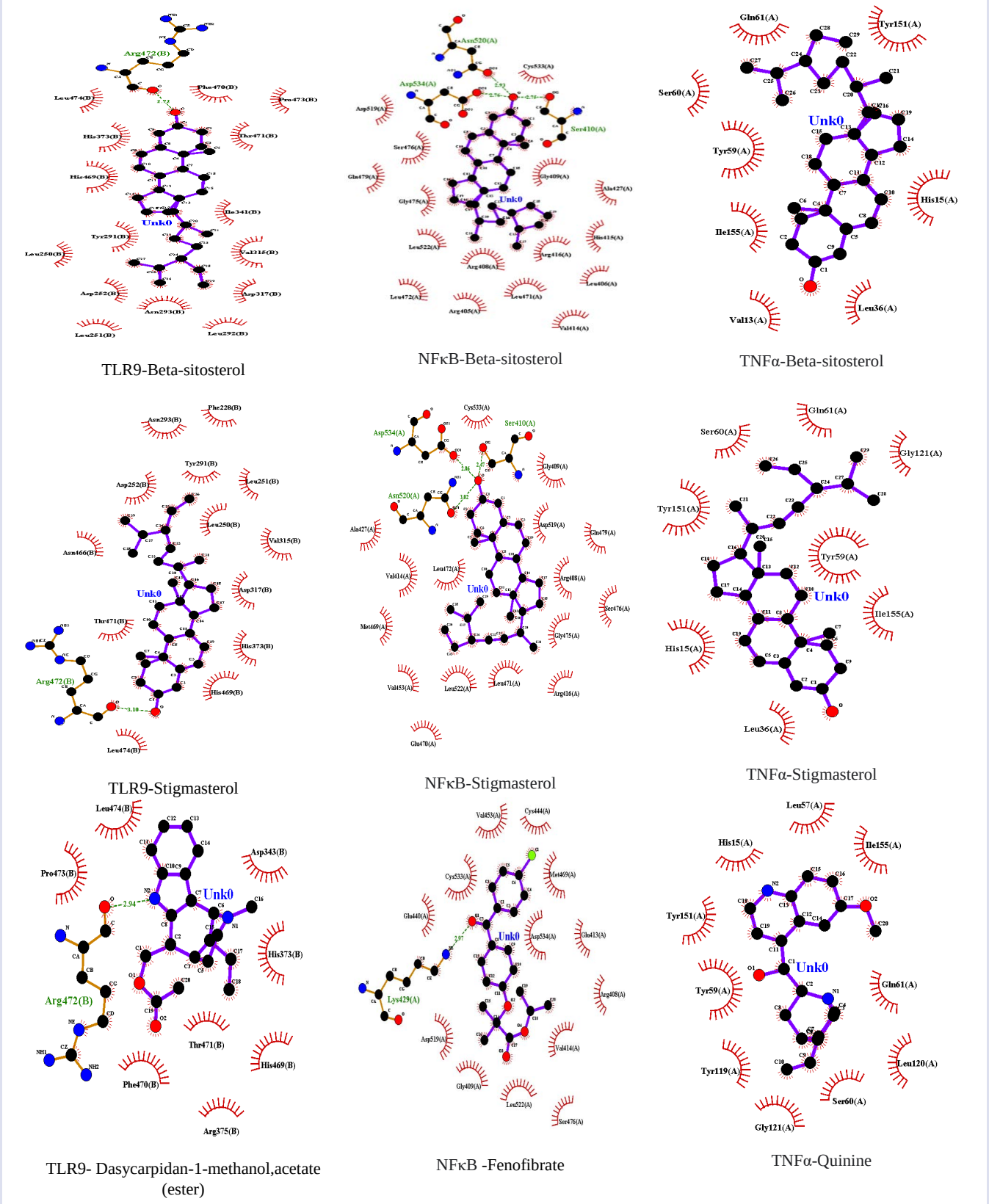


Figure 2. Ligplot analysis results are showing two-dimensional (2D) structures of TLR9, NFκB and TNFα interacting with *Acalypha indica* L., ethanolic roots extract. Those are hydrogen bond interactions between beta-sitosterol, stigmasterol, dasycarpidan-1-methanol, acetate (ester), quinine and fenofibrate with inflammatory markers.

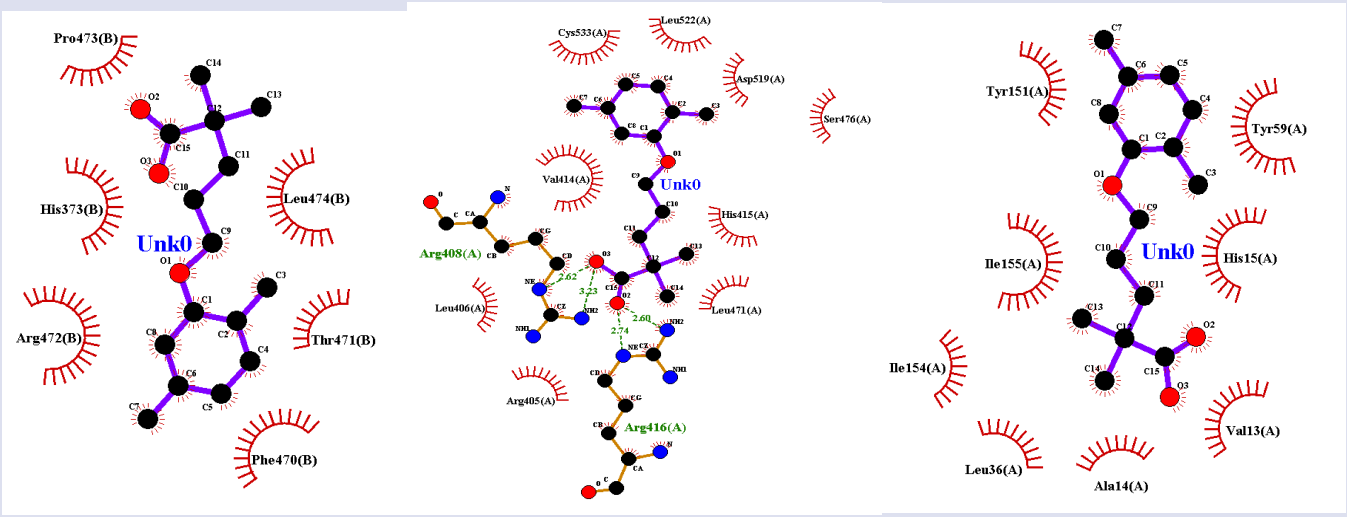


Figure-3 Ligplot analysis results are showing two-dimensional (2D) structures of TLR9, NFκB and TNFα interacting with *Acalypha indica* L., ethanolic roots extract. There are more hydrogen bond interactions between NFκB and Gemfibrozil.

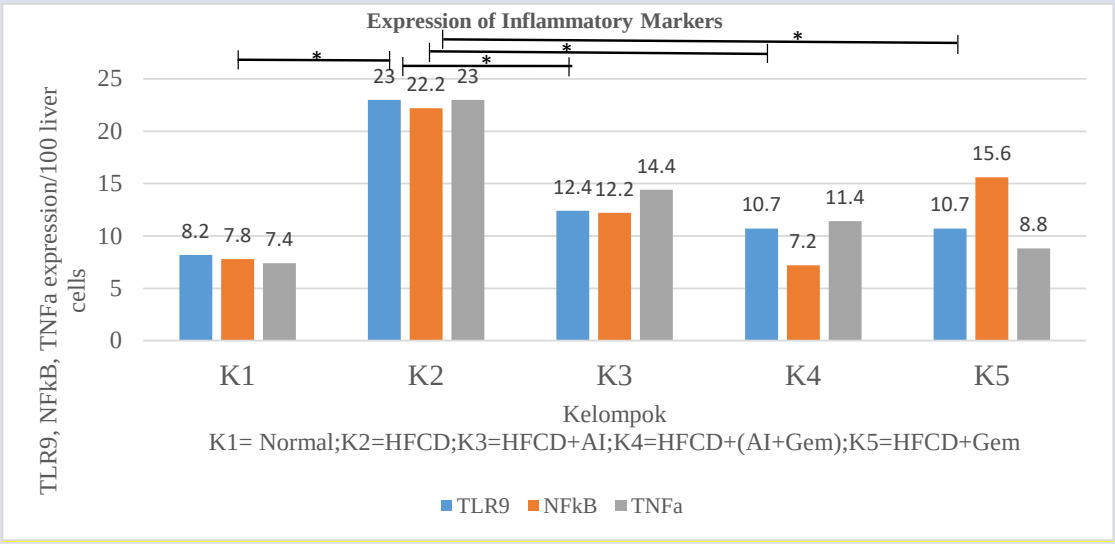


Figure 4. Statistical analysis of TLR9, NFκB and TNFα expression; Expression of the liver inflammation markers was carried out by Kruskall Wallis test followed by post hoc Mann Whitney test. Significant differences have a p value less than 0.05 (*). HFCD group without therapy (K2) showed the highest expression of TLR9, NFκB, TNFα compared to the HFCD group post therapy (K3, K4, K5).

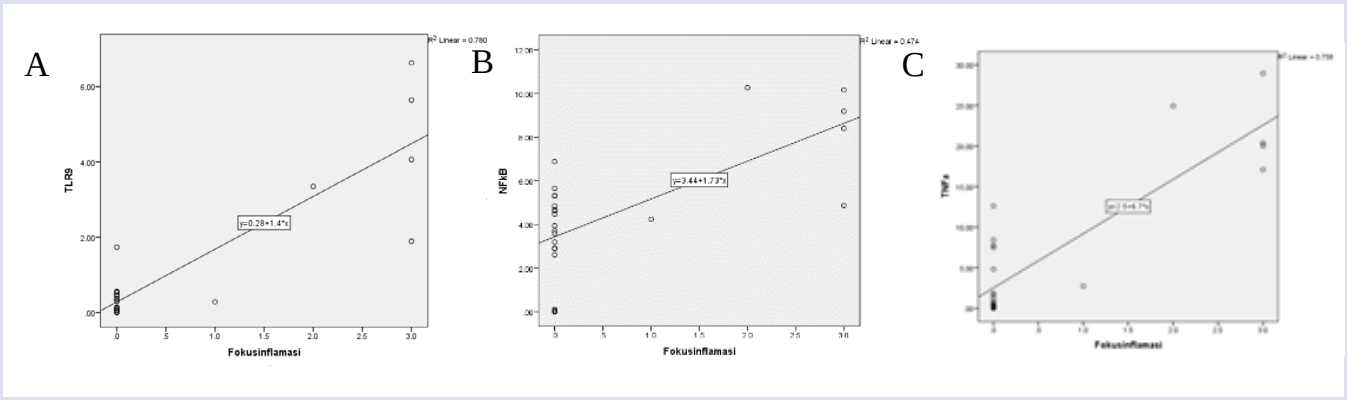


Figure 5. Spearman correlation test showed TLR9 (A), NFκB (B) and TNFα (C) \ expression positive correlation to liver inflammatory degrees. Significant differences have p value less than 0.05.

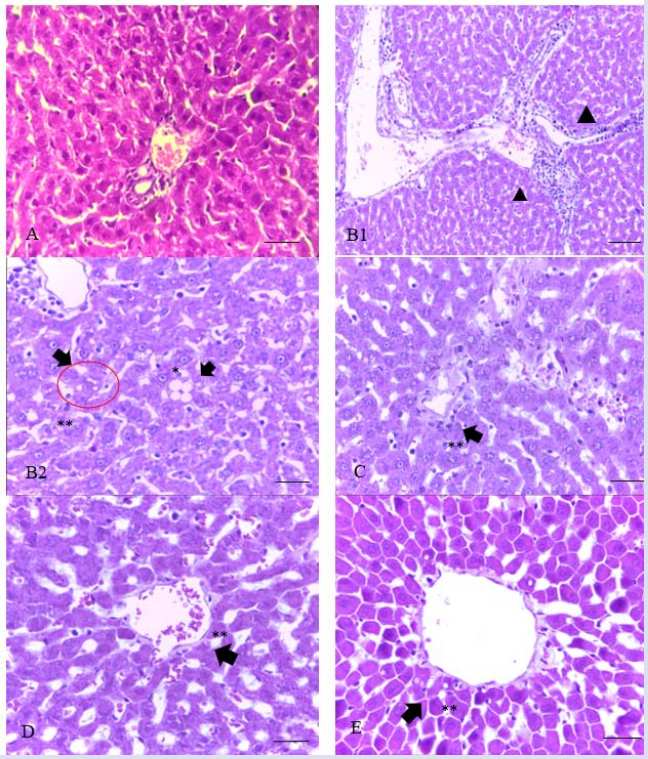


Figure-6. Liver cross-sections are from the NASH rodent models with Haematoxilin and Eosin staining; There is no morphological changes in normal group(A), but at 100x magnification (B1) HFCD group without therapy showed severe portal inflammation (▲) with (B2) macrovesicular (*) and microvesicular (**) steatosis. HFCD groups with AI (C), Gem+AI (D) and Gem (E) at 400x original magnification showed the decrease of hypertrophy, macrovesicular steatosis and inflammatory foci, but microvesicular steatosis (**) settled on morphological appearances post therapy.

group (supplemented with combination between 400 mg/kgBW *Acalypha indica* L. and 31 mg/kgBW Gemfibrozil) and Gem group (supplemented with 31 mg/kgBW Gemfibrozil) with a significancies value of $p<0.05$. The statistical analysis results showed hypertrophy is improved after being given therapy (p=0.31), as well as the same improvement in macrovesicular steatosis in the AI (p=0.018), AI+Gem (p=0.018) and Gem (p=0.018) groups. The statistical analysis results also showed that AI (p=0,05), AI+Gem (p=0,004) and Gem (p=0,004) showed improvement in inflammatory foci compared to the HFCD group without therapy. Meanwhile, steatosis microvesicular settled on post therapy groups AI (p=0.058), AI+Gem (0.478) and Gem (0.513), although those are a decrease of histopathological appearances. Based on statistical analysis also showed that the HFCD group without therapy is show more morphological changes of the liver either hypertrophy (p=0.001), microvesicular steatosis (p=0.009), macrovesicular steatosis (p=0.001) and inflammation foci (p=0.001) (Figure 6).

Based on the results of the study, there was a decrease in TLR9 expression in the group induced by HFCD supplemented with therapy (AI p=0.09, AI+Gem p=0.009, Gem=0.009) compared to positive control (HFCD group without therapy). The results also showed that the decrease in NFκB expression in the group induced by HFCD which is supplemented with therapy (AI p=0.009, AI+Gem p=0.009, Gem=0.047). Based on the results of the study also showed a decrease in TNFα expression in the HFCD supplemented with therapy groups (AI p=0.009, AI+Gem p=0.009, Gem p=0.009). Decreased expression of NFκB was more significant to measuring inflammatory protein expression in steatohepatitis rodent model than TLR9 and TNFα (Figure 4). In addition, the results showed a significant relationship liver inflammation and inflammatory markers expression of TLR9 (p=0.001 with r=0.642 positive, strong and unidirectional correlation), NFκB (p=0.001 with r=0.598 positive, strong and unidirectional correlation), TNFα (p=0,001 r=0,688 positive, moderate, unidirectional correlation) (Figure 5). This researched had proved that, atherogenic diet to NAFLD rodent models would induced steatohepatitis, where it can activate TLR9 and its inflammatory pathway NFκB, by releasing proinflammatory cytokines such as TNFα in accordance with the inflammatory mechanism of the NAFLD theory.[7,8,12]

DISCUSSION

In silico

Based on KEGG, many pathways can be used as protein target therapy for NAFLD. In addition, this pathway was also investigated to determine anti-inflammatory and anti- apoptotic effect. In this study, the target of the Toll receptor (TLR) and the NFκB pathway was in one way that indicated a cause and effect of protein activation until it release cytokine products.[3] This study is no longer looking for proteins that can be bound to the compounds contained in *Acalypha indica* L., but it aims to determine the binding mechanism of compounds contained in *Acalypha indica* L. with targeted proteins (TLR9, NFκB and TNFα) which had been mentioned in the literature about systemic inflammation in NAFLD.[14,15,16]

Zatsepin *et al*, in 2016,[17] through their study revealed that the discovery of TLR9 antagonists with computational techniques, which were followed by *in vitro* experimental investigations. TLR9 is a promising target for drug discovery, because it is involved in several disease pathologies both acute and chronic inflammatory processes. The study carried out a combined approached to discover the structure of new TLR9 antagonists with computational techniques and cell-based assays. It distinguishes between 'active' and 'inactive' TLR9 antagonist molecules, based on the physico-chemical properties of the molecule.[17] It was based on the structure of TLR9 with a ligand-based approached is not yet known. The study revealed that there are 5 antagonistic structures founded that have not been mentioned in the literature such as the PDB database, or patents. TLR9 has a complex structure, where from the PDB database, the structure of TLR9 has protein-protein or protein-nucleotide bonds, while the desired bond is between proteins and small molecules to study using computational techniques. However, several TLR structures have been described and used for prediction and *in silico* modeling, which is also included in the PDB database.[1]

Sribar *et al*, in 2021,[16] revealed that the protein code 4R09 for TLR8 can be used as a target protein for prediction of molecular simulations by identifying the bond between TLR proteins and small molecules or chemical compounds for drug discovery. These proteins have a similar crystal structure and are located on the endosome with TLR7 and TLR9. All of these proteins have binding pockets or binding sites with ligand surrounded by leucine-rich repeats (LRRs). The structure has a resolution less than 2.75 Å, it can bind to its native ligand (small molecule), mutation 0, with a better assessment quality.

In vivo

Observations started with 100x, 200x, 400x magnification looking for portal areas (biliary ducts, hepatic arteries and portal veins), central veins and zones 1,2,3 then at 400x magnification were analyzed for morphological changes in liver cells using the Liang score for NAFLD on rodent models. Calculating inflammatory foci was by counting the number of inflammatory foci of small visual field (10 fields of view), then five inflammatory foci in five different fields were counted and averaged. An inflammatory focus is called a cluster consisting of 5 inflammatory cells. Based on Liang score (0.5-1 is a light focus, 1.0-2.0

is a moderate focus and a more than 2.0 is heavy focus).[9] Although, in one field of view there is one focus of inflammation but the value is less than 0.5, it is still normal criteria. Meanwhile, the assessment of microvesicular and macrovesicular steatosis is determined by its location to the nucleus. If it is suppress the nucleus, it will determined as macrovesicular steatosis, whereas if it is not suppress the nucleus, it will determined as microvesicular steatosis. Besides that, hypertrophy was assessed based on cells with a size of more than one half times normal cells compare to normal groups.

Based on Liang et al. in 2014,[9] the Liang scoring is uniform and well defined, especially in the acute condition of NAFLD, namely, non-alcoholic steatohepatitis (NASH). NASH is a prerequisite for diagnosis before determining the accuracy in giving therapy. Furthermore, precise and accurate diagnosis is needed for preclinical research and drug development. Rodent model induced by a high-fat diet will cause non-alcoholic fatty liver with a spectrum of steatohepatitis as indicated by the Liang scoring.[9] In Zhang et al. study in 2015,[19] rodent model induced by a high-fat diet for 8-24 weeks, showed changes in liver morphology to NASH with increased expression of inflammatory proteins. In another study by Mridha, et al in 2017,[20] also revealed that there were morphological changes in the liver of rats induced by a diet high in 23% fat, 45% carbohydrates, 20% protein, 0.2% cholesterol or an atherogenic diet into a metabolic syndrome with steatohepatitis liver tissue features, accompanied by increased expression of inflammatory proteins.[20] In addition, based on Taskinen, et al in 2019, a high-fructose diet in the long term can also increase the risk of type 2 diabetes and cardiovascular disease. Fructose is absorbed in the small intestine and metabolized in the liver, where it stimulates fructose, glycolysis, lipogenesis and hepatic glucose production. These mechanisms can lead to hypertriglyceridemia and non-alcoholic fatty liver disease, with the etiology of metabolic syndrome.[21] Sahukari et al, in 2021,[6] also showed that the compounds contained in the roots of Acalypha indica L., can be attached to inflammatory proteins such as NFκB, as well as cytokines TNFα and IL1β based on molecular docking predictions, as well as in vivo tests including the results of haematological examination level of leukocytes and platelets on rat serum with histopathological examination and analysis of carrageenan-induced edema (paw test).[6]

TLR9 is well expressed on Kupffer cells either in the foci of inflammation or between the sinusoidal spaces. These results are in agreement with Wang and Mehal in 2021,[16] where is TLR9 in Kupffer cells can be activated in early nonalcoholic fatty liver. Kupffer cell activation is caused by the expression signal of intestinal bacteria or intestinal bacterial products that are translocated to the liver through the gut-liver axis which flow into the portal, and between sinusoidal spaces carried by microparticles, or the inflammation can be caused by damage to hepatocytes due to excessive accumulation of lipids in the liver. in the form of a DAMP signal.[16] Handa, et al in 2016,[14] and Shepard et al,in 2021,[15] revealed that PAMP and DAMP signals can activate Kupffer cells, hepatocytes, and stellate cells by activating TLR9, and their inflammatory pathways such as NFκB or receptors in the cytoplasm, namely NLR. Feng et al, 2020,[3] revealed that the contribution of Kupffer cells (liver macrophages) can lead to the progression of non-alcoholic fatty liver disease by inducing inflammation, recruiting hepatocytes to secrete inflammatory mediators including proinflammatory cytokines, and chemokines such as CCL2, CXCL10 and recruiting fibrogenic monocytes to the liver injury.[3]

In this research showed that, a high fructose-cholesterol diet would increase lipid absorption from the intestine to the liver, accompanied by changes in the intestinal microbiota, and disruption of the integrity of the intestinal epithelial barrier, where PAMP became from bacterial product or LPS was expressed, and translocation entered gut-liver axis[12,13] (1), DAMP is also released to activate TLR on endosomes[12] (2), which in turn activates the MYD88 adapter protein, phosphorylates IRAK, TRAF, Ikkα, and promotes proteasome degradation (3); RelA (NFκB p65) will be released and bound to the p50 subunit to form a dimer in the canonical NFκB pathway (4). Furthermore, the NFκB dimer will be translocated to the nucleus to activate inflammatory genes and apoptosis (5).[22] AI+Gem will increase the activity of PPARα agonists, inhibit SREBP1c, ApoB, leptin as anti-hyperlipidemia, and obesity,[11] and malondialdehyde as an anti-oxidant (6). Increasing the PPARα agonist action by AI+Gem can dampen TLR9 activation by inhibiting endosomal signaling (7) and inhibiting NFκB translocation to the nucleus, thereby preventing inflammation and apoptosis. AI, AI+Gem and Gem can reduce steatosis and liver inflammation, accompanied by a decrease in the expression of inflammatory markers. Based on molecular docking analysis and liver histopathology, the AI+Gem combination was the best at reducing TLR9, NFκB, and TNFα expression (10). The induction of HFCD for 12 weeks also can cause a steatotic liver condition accompanied by inflammatory foci followed with positive expression of inflammatory markers. The pathomechanism of AI mainly reduces the expression of TLR9 markers, and NFκB on Kupffer cells or macrophages. Meanwhile, Gem decreased the expression of NFκB in Kupffer cells or macrophages, as well as decreased TNFα expression in hepatocytes, along with the disappearance of hypertrophy, macrovesicular steatosis and inflammatory foci. The combination of AI and Gem can reduce the expression of the three markers along with the disappearance of hypertrophy and macrovesicular steatosis, as well as inflammatory foci.

CONCLUSION

From this study, it could be concluded that besides the flavonoid compounds contained in Acalypha indica L., alkaloid compounds such as beta-sitosterol and stigmasterol have the best interactions with the three inflammatory markers. Based on the statistical analysis also showed that HFCD group without therapy was significant differences in liver morphological changes either hypertrophy, microvesicular steatosis, macrovesicular steatosis, and foci of inflammation between normal and HFCD group with therapy. The combination of AI and Gem can reduce the expression of the three markers along with the disappearance of hypertrophy, macrovesicular steatosis and inflammatory foci. Meanwhile, microvesicular steatosis settled in HFCD group with therapy.

CONFLICTS OF INTEREST

There is no conflicts of interest in this research.

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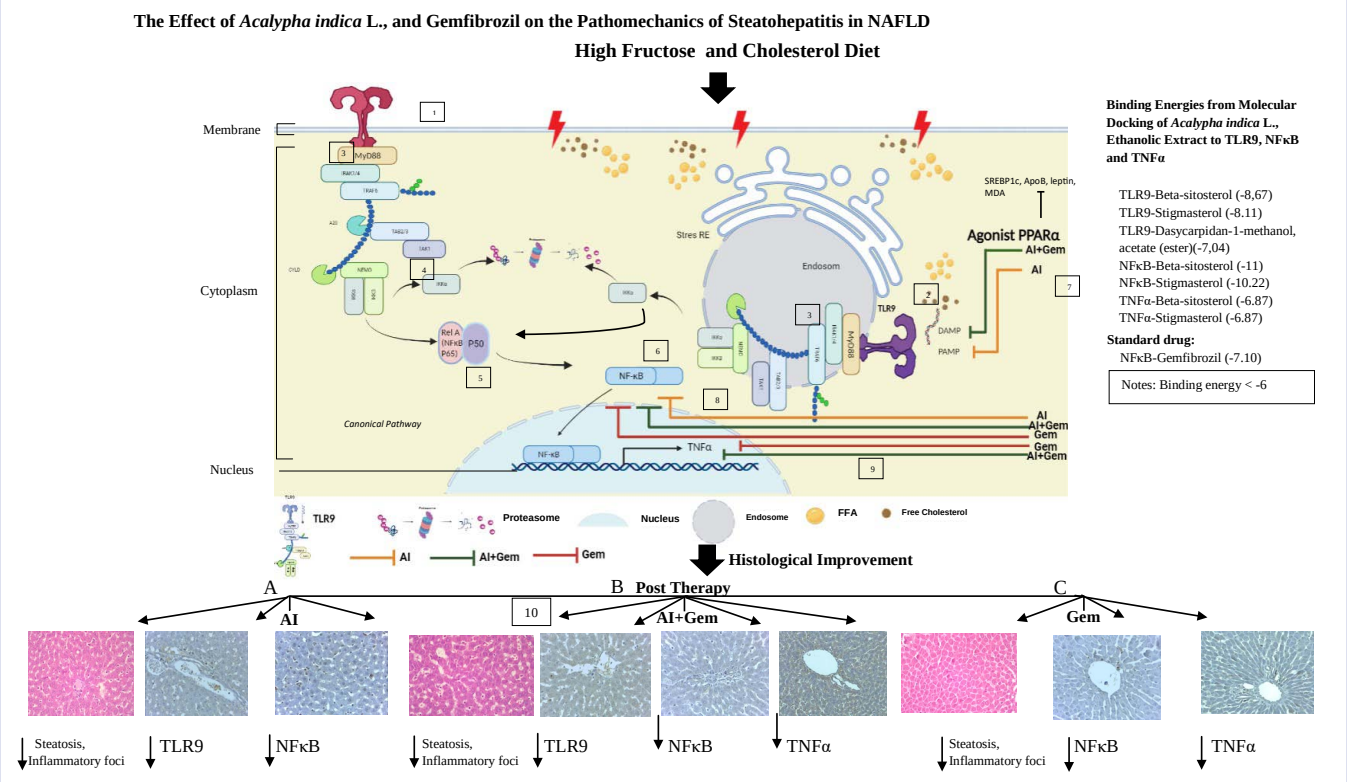
REFERENCES

1. De Vries M, Westerink J, Kaasjager KHAH, De Valk HW. Prevalence of nonalcoholic fatty liver disease (NAFLD) in patients with type 1 diabetes mellitus: A systematic review and meta-analysis. J Clin Endocrinol Metab. 2020;105(12):3842-53.
2. Poznyak V, Fleischmann A, Rekve D, Rylett M, Rehm J, Gmel G. The World Health Organization's global monitoring system on alcohol and health. Alcohol Res Curr Rev. 2013;35(2):244-9.
3. Feng D. The alteration of immune cells in the pathogenesis of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Liver Res. 2020;4(1):23-7.
4. Rahman MA, Bachar SC, Rahmatullah M. Analgesic and anti-inflammatory activity of methanolic extract of Acalypha indica Linn. Pak J Pharm Sci. 2010;23(3):256-8.

5. Mojumder M, Paul Arkajyoti, Kabir MSH, Rahman MG. Molecular docking and pass prediction for analgesic activity of some isolated compounds from *Acalypha indica* L and ADME/T property analysis of the compounds. Semantic Scholar. 2016;5(7):1761-70.
6. Sahukari R, Punabaka J, Bhasha S, Ganjikunta VS, Ramudu SK. Phytochemical profile, free radical scavenging and anti-inflammatory properties of *Acalypha indica* root evidence from in vitro and in vivo studies. Molecules. 2021;26(6251):1-21.
7. Zahidin NR, Saidin S, Zulkifli RM, Muhamad II, Ya'akob H. A review of *Acalypha indica* L., (Euphorbiaceae) as traditional medicinal plant and its therapeutic potential. J Ethnopharmacol. 2017;(207):146-73.
8. Rajasekaran S, Anandan R, Nishad K. Activity of Acalypha indica Linn. on atherogenic diet induced hyperlipidemia. Int. J. Pharm. Pharm. Sci.2013;5:699-701.
9. Liang W, Menke AL, Driessen A, Koek GH, Lindeman JH. Establishment of a general NAFLD *scoring* system for rodent models and comparison to human liver pathology. Plos One. 2014;9(2):1-17.
10. Dewi DAAP, Estuningtyas A. Purwaningsih EH. Effect of extract *Aclypha indica* L. on lipid profile and cardiac troponin-T of rats fed with high fructose and cholesterol diet. J southwest jiantong univ. 2022;57(1):1-8.
11. Hakim RW, Leviana M, Krisnamurti DGB, Budianto T, Gustada H.Antihypercholesterolemia effect of *Acalypha indica* L. to serum lipid profile and histopathology liver on *Sprague-Dawley* rats: Focused on gemfibrozil as a control. Advance Science Letters. 2017;23(7):6966-9.
12. Mohamed FE, Al-Jehani RM, Minogue SS, Andreola F, Winstanley A. Effect of toll-like receptor 7 and 9 targeted therapy to prevent the development of hepatocellular carcinoma. Liver Int. 2015;35(3):1061076.
13. Li H, Wang Y, Zhang M, Hu J, Li Z. The high expression of TNF-α and NF-κB in tumor microenvirontment predicts good prognosis of patients with BCLC-0-B hepatocellular carcinoma. Transl Cancer Res. 2018;8(2):532-41.
14. Handa P, Vemulakonda A, Kowdley KV, Uribe M, Méndez-Sánchez N. Mitochondrial DNA from hepatocytes as a ligand for TLR9: Drivers of nonalcoholic steatohepatitis? World J Gastroenterol. 2016;22(31):6965-71.
15. Shepard CR. TLR9 in MAFLD and NASH: At the intersection of inflammation and metabolism. Front Endocrinol (Lausanne). 2021;11(1):1-21.
16. Wang H, Mehal W, Nagy LE, Rotman Y. Immunological mechanisms and therapeutic targets of fatty liver diseases. Cell Mol Immunol. 2021;18(1):73-91.
17. Zatsepin M, Mattes A, Rupp S, Finkelmeier D, Arijit B. Computational discovery and experimental confirmation of TLR9 receptor antagonist leads. J Chem Inf Model. 2021;56(9):1835-46.
18. Sribar AD. Tailoring toll-like receptor 8 ligands for balancing immune response and inflammation. Department of Biology, Chemistry, Pharmacy of Freie Universität Berlin 2021;1-140.
19. Zhang RN, Pan Q, Zhang Z, Cao HX, Shen F. Saturated fatty acid inhibits viral replication in chronic hepatitis B virus infection with non-alcoholic fatty liver disease by toll-like receptor 4-mediated innate immune response. Hepat Mon. 2015;15(5):1-9.
20. Mridha AR, Haczeyni F, Yeh MM, Haigh WG, Ioannou GN. TLR9 is up-regulated in human and murine nash: pivotal role in inflammatory recruitement and cell survival. Clin Sci (lond). 2017;131(16):2145-59.
21. Taskinen MR, Packard CJ, Boren J. Dietary fructose and the metabolic syndrome. Nutrients. 2019;11(9):1-16.
22. Liu T, Zhang L, Joo D, Sun SC. NFκB signaling in inflammation. Nature. 2017;(2):1-9.

GRAPHICAL ABSTRACT



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