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IDENTIFYING PIRDOT LEAVES (*SAURAUIA VULCANI* KORTH) EFFECT ON LIVER BIOMARKER AND SPERMATOZOA QUALITY IN WHITE RATS (*RATTUS NORVEGICUS*) INDUCED CIGARETTE SMOKING THROUGH IN VIVO AND IN SILICO APPROACH

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Abstract

Cigarette smoking significantly leads to hepatocellular injury, inflammation, and infertility regarding the toxic agent and free radicals. The previous study revealed that the bioactive compound of Pirdot (*Saurauia vulcani Korth*.) has a high phenolic compound to be used as therapeutic agents such as immunostimulant and hepatoprotective. Our research explored more the potential of the ethanol extract of pirdot (Saurauia vulcani Korth.) (EEP) leaves on the quality of spermatozoa, hematocrit, SGPT, and SGOT of white rats (Rattus norvegicus) exposed to cigarette smoke through in vivo and silico approach. Firstly, the experiment used a completely randomized design (CRD) consisting of negative control (K-), positive control (K+), P1 treated group administered 100 mg/kg EEP; P2: 200 mg/kg EEP; and P3: 300 mg/kg EEP. Exposure to cigarette smoke was given to groups K+, P1, P2, and P3 as much as 2 stick cigarettes per day in 15 minutes. The observed data were achieved by One Way ANOVA test. Secondly, protein-protein interaction and molecular docking were utilized to investigate pathways of inhibition of bioactive compound Pirdot with CYP2A6 as protein target in nicotine metabolism. The results showed that EEP 200mg/kg BW had a significant effect on decreasing the hematocrit value and the SGPT and SGOT values. Furthermore, the white rats exposed to cigarette smoke and EEP 300 mg/kg BW had a significant effect on improving the quality of spermatozoa by increasing the morphology and motility of normal spermatozoa and elevating the concentration of spermatozoa ($p<0.05$). Moreover, the bioactive compound Pirdot, ursolic acid, showed the promising result to bind to active site protein CYP2A6 on -14.7 kcal/mol, while methoxsalen as a comparative compound has -7.1 kcal/mol. The compound- target pathway revealed six compounds from Pirdot linked with nicotine metabolism protein which TP53 protein plays an important role in inhibiting CYP2A6 as target of smoking therapy.

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Keywords: *Saurauia vulcani Korth., spermatozoa quality, liver biomarker and cigarette smoking.*

Introduction. One of the problems in public health is caused by cigarette smoking (CS) which potentially contributes to hepatocellular injury, inflammation, fibrosis and infertility[1]. It has lots of toxic agents such as nicotine, tar, carbon monoxide and polycyclic aromatic hydrocarbon (PAH) in those that give an impact to oxidize proteins, lipids and DNA [2]. Because of carrying lots of oxidants, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system is stimulated by cigarette smoking. Then, it also promotes free radical lipid peroxidation causing hepatocellular inflammation, activating hepatic stellate cells and inducing the proliferation of fibrotic mediators, matrix metalloproteinases, extracellular matrix proteins [3]. These evidences elevate Serum Glutamic Pyruvic Transaminase (SGPT) and Serum Glutamic Oxaloacetic Transaminase (SGOT) value. In addition, CS also produces carbon monoxide (CO) which becomes the primary factor in haemoglobin dissociation and toxicity. Hematocrit measurement was used to assess erythrocyte level, while it binds to CO causing increasing hematocrit value on blood.

Furthermore, the detoxification of endogenous compounds and xenobiotics is tackled by CYP450 enzymes in the liver. The toxic agents of CS induce expression of some CYP450 enzymes such as CYP1A2, CYP3A2, CYP2E1, CYP19A1 and CYP2A6[4]. Furthermore, CYP3A2 is related to testosterone production in that nicotine tends to play a role in the destructive impact on fertility in men [5]. In addition, CYP2A6 would be a protein target in nicotine metabolism which the loss of function of its enzyme tends to give impaired capacity for smoking less [6].

Saurauia vulcani (Pirdot) has been used in Batak tribes, North Sumatra-Indonesia[7] because of its antioxidant properties, wound healing, anti-inflammatory and immunomodulatory properties [8–10]. The previous study explored some bioactive compounds of Pirdot such as genistein, ursolic acid, stigmasterol and oleanolic acid which have contributed to those mechanisms [11]. However, a computational approach, used to identify the probable potential of the compound and define the underlying mechanism of target disease [12], has been unclear to predict the interaction of bioactive Pirdot in nicotine metabolism. This method characterized causal biological pathway interpretation and genes associated with smoking behaviors [13] in order to discover the potential therapeutic development.

Hence, this study investigates the potential of Pirdot to prevent liver injury and increase concentration of spermatozoa through in vivo and in silico approaches. The assessment of quality of spermatozoa, hematocrit, SGPT, and SGOT of white rats (*Rattus norvegicus*) exposed to cigarette smoke (CS) is analyzed by given the ethanol extract of pirdot (*Saurauia vulcani* Korth.) leaves. Moreover, the correlation pathway between liver enzyme, testosterone, nicotine and compounds of Pirdot are explored by in silico approach such as protein-protein interaction and molecular docking study.

Method. Experimental Animal and Cigarettes

The study was held for 30 days and twenty- five of 10±2-week-old male Wistar rats with initial weights of 200 ± 20 gr were used. The animals were taken from the Biology Laboratory State

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University of Medan, Indonesia. The animals were maintained on a standard diet and water was given ad libitum. The rats were completely randomized into five groups containing five rats in each group as follows previous study [14] : K^- : negative control group (only given water and food); K^+: positive control group (given cigarette smoking); P1 : smoke-exposed groups were given 100 mg/kg ethanol extract Pirdot leaves (EEP); P2 : smoke-exposed groups were given 200 mg/kg EEP; P3 : smoke-exposed groups were given 300 mg/kg EEP. The rats were given 2 stick cigarettes a day in group treatment for 15 minutes. In addition, non-filter cigarettes were applied from a commercial brand (Kretek Gudang Garam, Indonesia). According to the previous study, one stick cigarette has 1.83 mg nicotine, 39 mg tar and 17.8 mg carbon monoxide [15].

Preparation of Ethanol extract Pirdot leaves

Pirdot Fresh leaves were obtained from Tarutung, North Sumatra- Indonesia. The dried leaves of Pirdot (1050 g) were macerated with 96 % ethanol for 60 h at room temperature and then filtered by using Whatman paper. The filtrate was concentrated by a rotary evaporator separating the solvent with the final result [16]. The yield of ethanolic of Pirdot was distilled to obtain three concentration extract (100, 200, and 300 mg/kg BW).

Hematocrit, SGPT and SGOT Measurement

At the end of treatment, the animals were euthanized and fresh blood from all groups of treatment was collected. Hematocrit value on blood was measured by microhematocrit method [17] following the process, that is pouring fresh blood samples into 2/3 tubes containing EDTA as anticoagulant. It is used for avoiding the clotting process. Then, the sample was centrifuged at 3500 rpm for 10 mins and the supernatant was collected for further analysis.

Furthermore, to collect blood serum, the sample was placed into tube, then serum solution (25µL) was mixed with 250 µL mono-reagent SGPT and SGOT, later shaken well followed by stored for 50 sec. Thereafter, serum was separated into sterilized Eppendorf vials, then these biochemical parameters were estimated by photometer Microlab 300 LX during 150 sec, 340 nm absorbance of wavelength at 37^0 C using photometric method.

Motility, concentration and morphology Spermatozoa

After 30 days treatment, Epididymal spermatozoa were collected and the sample was diluted with 1 ml NaCl 0.9% and mixed well to form spermatozoa suspension. After that, sperm motility was assessed by transferring 5µl samples onto a clean glass slide and covered with a coverslip. The slide was examined under a microscope at a magnification of x100 [18]. For assessment of sperm concentration, sample was mixed and 5µl diluted specimen was placed onto an improved Neubauer ruled hemocytometer. The average of five chambers of the hemocytometer were counted to analyze sperm concentration[19]. Furthermore, sperm morphology was determined by the Eosin-Nigrosin method. Sperm suspension with eosin 1 % transferred onto a clean slide and at least 100 spermatozoa observed under microscope (100x) for the analysis of morphological features.

Statistical analysis

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Biochemical data (hematocrit, SGPT and SGOT value) and the quality of spermatozoa parameters were evaluated by one-way (ANOVA) using SPSS software version 22. The data were presented as mean ± SD and p-values < 0.05 was considered statistically significant.

Protein – protein interaction and molecular docking study

Construction protein-protein interaction (PPI) network [20] is used to characterize interaction between Pirdot's bioactive compound and protein target**.** The STITCH database (http://stitch.embl.de/) was used to construct the PPI with "*Homo sapiens"* chosen as the species target. The confidence score was limited to 0.7 in the minimum (low confidence < 0.4; medium ≤ 0.7; and high > 0.7). For further analysis, the data was formed as a .tsv file to evaluate the PPI network at Cytoscape 3.9.0 (https://cytoscape.org/). Betweenness centrality was a parameter to calculate the value of the bridge centrality of the node [21]. Furthermore, AutoDock Vina version 4.2 was a docking program used to identify the active compound with high affinity for a protein CYP2A6. This program has high speed performance, accuracy, and reliability to screen structure-based virtual [22]. Firstly, both protein and bioactive compounds would be formatted in .PDBQT format. Then the grid box center would be set at X = 51.445; Y = 79.05; and Z = 55.35 Å. In addition, Methoxsalen was used as a control to compare the binding patterns analysis.

Result and Discussion

Hematocrit, SGPT and SGOT Measurement

The Data analysed by ANOVA showed the ethanol extract Pirdot administration had a significant differences (P ≤ 0.05) on hematocrit level in various doses. As displayed in Table 1, EEP had lower of hematocrit level (41.29 ± 0.70 %) in comparison with control group induced smoke (46.20 ± 0.91 %). According to a previous study[23], hematocrit level was increasing dramatically in active smokers. Increasing oxidative and nitrosative stress tend to relieve antioxidant status in biochemical markers of blood profile[24]. Pirdot could play a role in cigarette therapy in which its bioactive compound enters into blood through enterohepatic circulation and scavenges free radicals.

Similarly, the statistical analysis shows EEP significantly improved the oxidative biomarker. Pirdot has a potential prevention of hepatocyte damage[25] which its bioactive compound tackles free radicals. As shown in Table 1, CS (K+ group) significantly (p < 0.05) elevated SGPT and SGOT level, meanwhile the extract Pirdot resulted in a significant decrease in SGPT at dose 100 mg/kg and SGOT value at dose 200 mg/kg. Likewise, the administration of extract of tobacco leaves orally at dose 200 and 400 mg/kg could increase ALT (Alanine Aminotransferase) and AST (Aspartate Aminotransferase) serum in order to indicate the consumption of tobacco in a smokeless form contributed in liver damage[26]. However, the potential of flavonoid could give a protection of hepatocyte injury by decreasing the necrosis value and inhibiting the formation of malondialdehyde (MDA)[14].

Table 1. The Changes of SGPT, SGOT, Hematocrit level in the serum of white rats.

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Group	SGPT (IUl^{-1})	SGOT (IUl^{-1})	Hematocrit (%)
K- (negative control)	44.58 ± 1.73	73.50 ± 2.43	40.38 ± 1.54
K+ (positive control)	158.76 ± 1.24	160.88 ± 2.46	46.20 ± 0.91
P1 (100 mg/kg EEP)	58.24 ± 1.53	115.08 ± 2.66	44.72 ± 0.63
P2 (200 mg/kg EEP)	69.92 ± 1.17	76.50 ± 0.85	41.29 ± 0.70
P3 (300 mg/kg EEP)	63.40 ± 1.81	92.80 ± 1.81	43.86 ± 1.21

Motility, concentration and morphology Spermatozoa

The statistical results show the significant effect of administration EEP in sperm parameters buy comparing spermatozoa morphology, motility, and concentration are shown in Table 2. The results indicate a statistically significant difference in all groups ($p \leq 0.05$). While Group K+ induced CS had significantly decreased value of sperm parameter, the administration EEP at dose 300 mg/kg significantly increased sperm quality ($p \leq 0.05$). Likewise previous study, the polyphenol compounds enhanced sperm concentration and sperm vitality control[18].

Table 2. The effect of consumption EEP for 30 days in measurement of spermatozoa quality in rats treated CS ($p \leq 0.05$)

Treatment	Spermatozoa Morphology (%) ± STDEV	Spermatozoa motility (%)	Spermatozoa concentration ($x10^5$)/ ml
K-	95,00 ± 3,39	85,20 ± 2,59	250,60 ± 15,85
K+	73,80 ± 4,15	50,20 ± 2,59	180,20 ± 13,66
P1	87,40 ± 2,07	58,80 ± 3,49	184,00 ± 7,97
P2	86,20 ± 3,27	61,40 ± 2,07	208,80 ± 16,02
P3	87,60 ± 5,73	65,40 ± 3,65	223,60 ± 23,42

Protein-protein Interaction and Docking Simulation

In this study, we analysed six active compounds Pirdot with targets such as nicotine, testosterone, and CYP2A6 in **Fig.1**. Betweenness centrality value visualized that TP53, CDH1, TNFRSF10B, CTNNB1, CASP3, CDKN1A and CNTN2 could be the key component in the pathway. The stitch result showed that Ursolic acid activated CASP3 with confidence score 0.9, then inhibited the nicotine at 0.7 score. In a similar way, Oleanolic acid and Sitosterol activated CASP3 at 0.7 score. The corosolic acid resulted in increased degradation of CTNNB1 protein at 0.7. In addition, the maslinic acid compound has downregulated with E-cadherin at 0.8, then it has a catalysis interaction with CASP3 at 0.9 score. Nicotine is bound to CYP2A6 with 0.8 score, while genistein is probably bound to CYP19A1 at 0.7 [50][51].

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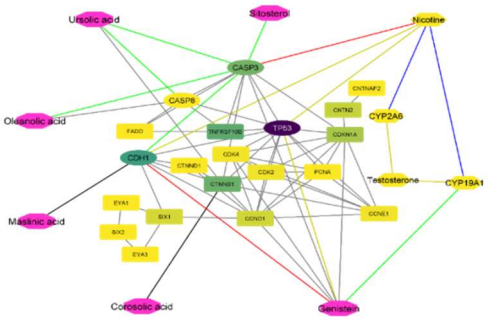

Figure 1. Protein-protein interaction Pirdot smoking; the octagon nodes represent bioactive compound Pirdot, hexagon nodes represent targets, and the circular nodes are the major protein involved in mechanism. (The red line is an inhibition, the green line is an activation, the blue line is a binding, and the brown line is a transcriptional regulation) [52][53].

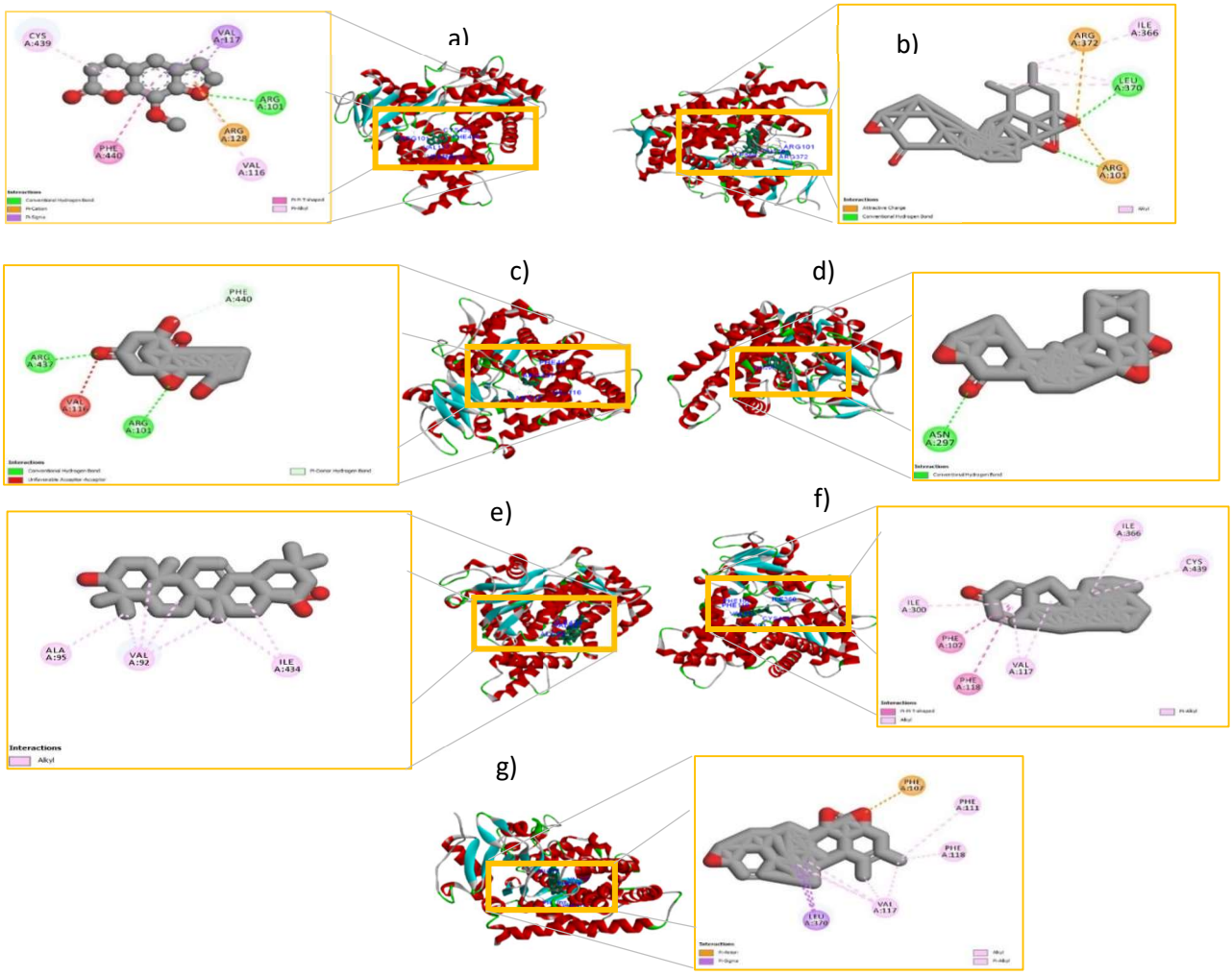

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Figure 2. Docking poses of CYP2A6 protein with active compound (the green color) a) Methoxsalen; b) Corosolic acid; c) Genistein; d) Maslinic acid; e) Oleanolic acid; f) Sitosterol; g) Ursolic acid

We identify the accuracy of the prediction of the protein interaction network through docking simulation. The promising binding affinity has lower energy calculations that the result showed that they were bound to the active site of CYP2A6 in **Fig.2**. The best inhibitor pose energy was found in Ursolic acid -14.7 kcal/mol which has hydrophobic interaction at Val117, Phe118, Asn297, Ile366, Leu379 and Phe480, the hydrogen bond was at Phe432. The all of compound pirdot bound to active site CYP2A6 were at Asn297, Ile300, Leu296, Leu370, Phe107, Phe111, Phe118, Phe480, and Val117 are listed in **Table 3**. [54][55].

In addition, the previous study has reported that coumarin affected the CYP2A6 [27] which has binding pockets in Glu 442, Gly 443, Arg 136-446 and Phe 440 [28]. However, Methoxsalen as a control compound bind to active site CYP2A6 -7.1 kcal/mol which hydrogen bond was found in Thr305 and hydrophobic interaction was in Val116, Val117 and Phe440 [56][57].

Table 3. Binding affinity of top candidate compound and CYP2A6 Protein

Bioactive compound	Compound ID	Molecular interaction with CYP2A6 Protein		Binding Affinity (kcal/mol)
		Hydrogen Bond	Hydrophobic Interaction	
Methoxsalen	4114	Thr305	Val116, Val117, Phe440	-7.1
Corosolic acid	6918774	Arg101, Ser369, Leu 370	Val117, Ile366, Leu370, Phe440	-14.5
Genistein	5280961	Arg101, Arg 437	Val117, Leu 370	-8.4
Maslinic acid	73659	Asn297	Phe107, Val117, Phe209, Ile300, Leu370, Phe480	-14.4
Oleanolic acid	10494	Asn438	Val92, Ala95, Ile434	-6.8
Sitosterol	222284	-	Val117, Ile366, Leu370, Leu395, Phe480	-12.4
Ursolic acid	64945	Phe432	Val117, Phe118, Asn297, Ile366, Leu370, Phe480	-14.7

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Discussion. Oxidative stress plays to be crucial role of liver dysfunction and hepatic injury [29]. Our findings revealed that CS has contributed to alleviating oxidative stress and hematocrit levels. Toxic agents of cigarette smoking, for instance CO are associated with hematocrit level in blood [30]. In this study, the administration of Pirdot decreased hematocrit level induced CO at 200mg/kg EEP oral doses. The potential of EEP significantly improves red blood cells which are damaged by cigarette smoking. Bioactive compounds of Pirdot, for instance genistein, neutralize free radicals and elevate erythrocytes value. Due to flavonoid compound was poor radical scavengers, it was involved in mitochondrial respiration and erythrocyte hemolysis [31].

Similarly, continuous CS tends to induce oxidative stress and reduce antioxidants in those caused hepatocellular injury. This incidence caused by exceeding the production of reactive oxygen species (ROS) in order to contribute to liver damage [32]. During hepatic damage, the concentration of biochemical markers, such as SGPT and SGOT, is elevated in those to measure exposure of hepatotoxin [33]. However, the data show that EEP treatment potentially reduces lipid peroxidation in rat blood, which 100 and 200 mg/kg EEP are the best measurement to treat SGPT value. In addition, SGOT value as an indicator of liver damage was slightly increased in K+ control group by CS exposure. The SGOT value in P2 group indicates 200 mg/kg EEP administration significantly returned SGOT level to their relative control negative levels. Soliman [34] noted that *T.vulgaris* extract has significant levels of polyphenols and flavonoids which normalized ALT and AST level induced $NaNO_3$ to the control levels. Abu [35] has proven that hepatoprotective properties of plants has a potential active compound such as polyphenol and flavonoids which is involved in promoting the expression of glutamylcysteine synthetase. Our studies confirmed that increasing SGPT and SGOT, as the indicator of liver damage [8,25], levels has significantly correlated with serum cytokine levels for instance IL-1β and TNF-α [36], while bioactive compound Pirdot reduced the value to the negative control level [58][59].

Additionally, our finding was purposed to evaluate the effectiveness of Pirdot in order to improve the quality sperm in rats induced CS. Spermatozoa morphology and motility are the primary biomarkers used to assess and determine the fertilizing ability of spermatozoa [37]. The data presented the percentage of sperm parameter of group induced CS and administered orally Pirdot extract were significantly different compared to positive control group ($p \leq 0.05$). The results indicate that CS exposure has been a critical factor for the low levels of sperm parameters. Moreover, the previous study presumed that CS has a role to increase oxidative stress in passive smokers compared with healthy non-smoker by altering sperm parameters [38].

On the other hand, our study develops a network target between bioactive compound Pirdot with potential target protein related to nicotine metabolism. As shown in Fig.1, the system pharmacology provides a molecular mechanism in which six compound Pirdot with 23 target proteins have synergy probability indicating this regulation tends to be the treatment of smoking. According to Betweenness centrality analysis, TP53 protein is one of the key components in those pathways in which all active compounds Pirdot have a connection to them. These pathway also

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revealed that TP53 has a significant potency to relieve DNA damage of lung cancer in smoking induced [39]. A causal relationship among hepatocellular injury, spermatozoa, hematocrit and CS exposure is further related by genetic susceptibility to cigarette smoking. For example, genetic association with smoking were conducted to gene-lifestyle interaction, such as LOC105378783, PTPRZ1, CNTNAP2, PRKAG2, MIR4686, ZNF729, DHCR8, EYA3, ETV5, TMEM175, CREB3l2, EYA1, and B3GNT [40]. Hereafter, gene CYP2A6 tends to play a crucial role involved in hepatocellular injury and severe liver inflammation [41-48] which demonstrated that nicotine metabolism is related to cytochrome P450 2A6 [42]. This isoform has contributed to the metabolism of therapeutic drugs and xenobiotics such as nicotine and tobacco-specific nitrosamines [28,47,49]. Because of their capacities, it would be a promising inhibition to prevent and treat tobacco-dependent smoking by converting nicotine to cotinine and reducing the ratio of cotinine as a biomarker of removing nicotine [43].

The binding affinity score (Table 3) states that four bioactive compounds Pirdot have lower scores than Methoxsalen. The docking poses of methoxsalen and Pirdot compounds are illustrated in Figure 2. Ursolic acid compounds tend to be one of top Pirdot compounds that prevent damage from cigarette smoke-induced by oxidative stress. The previous study reported that ursolic acid attenuated the damage of cigarette smoke -induced in rat lungs [44]. Moreover, triterpene acid such as ursolic acid, corosolic acid, maslinic acid and oleanolic acid have a protective effect to treat CS-induced [45-46].

Conclusion

In summary, cigarette smoking has contributed to an increased oxidative biomarker (SGPT, SGOT, Hematocrite) and a lower sperm parameter. However, the effect of EEP was significantly involved in improved oxidative biomarker and sperm parameters, specifically at a dose of 200 and 300 mg/kg BW in this study. In addition, the molecular interaction mechanism and target protein showed that bioactive compounds of Pirdot could be effective candidate therapy for smoking to inhibit CYP2A6 protein in nicotine metabolism. Further analysis of consumption of EEP in rats induced electrical smoke is needed to reveal the capacity of antioxidants of Pirdot.

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