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The Effect of *Ageratum conyzoides L* on Hepatocellular Carcinoma Rats Induced by DMBA based on *In Vivo-In Silico* Study

Heni Ratnasari
Department of Pharmacy,
Medical Faculty and Health Science
Universitas Muhammadiyah Yogyakarta
Yogyakarta, Indonesia
heniratnasari@gmail.com

Aulia Rahma
Department of Pharmacy,
Medical Faculty and Health Science
Universitas Muhammadiyah Yogyakarta
Yogyakarta, Indonesia
auliarahma217@gmail.com

Rifki Febriansah
Department of Pharmacy,
Medical Faculty and Health Science
Universitas Muhammadiyah Yogyakarta
Yogyakarta, Indonesia
briansyah_rifki@yahoo.com

***Abstract*— Liver cancer is the second most common cause of cancer deaths worldwide. Treatment that has been carried out was chemotherapy which has many disadvantages. Bandotan Herb *(Ageratum conyzoides L.)* containing flavonoid compounds was used as chemopreventive agents. This research was experimental study using molecular docking test of nobiletin compounds against VEGF, COX-2 and C-Myc compared to 5Fluorouracil. Carcinogenic test required 20 mice tails to be divided into 5 feeding groups. Induction was conducted on a per-oral using CMC-Na 0.5% 1 ml/200 gram, DMBA 20 mg/kg BW and BHCF at a dose of 750 mg/kg BW and 1500 mg/kg BW. DMBA was injected 2 times a week for 5 weeks. Histological observation was carried out using Immunohistochemistry and Haematoxylin-Eosin. TLC stated that BHCF contained flavonoid as a secondary metabolites. Molecular docking proved that nobiletin was better in inhibiting the expression of VEGF with an affinity value -7.6 kcal/mol. Induction of DMBA caused moderate over-expression of VEGF against liver tissue. Histologically, the introduction of BHCF at a dose of 1500 mg/kg BW provides better improvement degrees of histology than 750 mg/kg BW. The presence of flavonoid can be used as chemopreventive agents for liver cancer based on in silico and in vivo assay.**

Keywords—Ageratum comyzoides L., DMBA, hepatocellular carcinoma, in silico, in vivo

I. INTRODUCTION

Cancer is described by uncontrolled cell division. This is caused by DNA damage, mutations of vital genes that control cell division. Cell differentiation and cell growth regulated by protooncogen and tumor suppressor genes were found in all chromosomes. Protooncogens that have undergone changes give rise to cancer called oncogenes.[1] Hepatocellular carcinoma is a cancer with the third largest death in the world. The new cases have been recorded as 42,030 cases and 12,550 of them occurred in women. The death cases reached 31,780 in 2018. The case of liver cancer deaths has continued to increase almost 3% annually since 2000. [2]

Hepatocellular carcinoma is associated with viral infections of hepatitis B and C, cirrhosis, alcohol and exposure to carcinogenic compounds.[3] Complaints are generally occurred in the forms of a sense of discomfort in the abdomen and abdominal distention, weight loss, gastrointestinal bleeding, loss of appetite and jaundice.[4] Some of cancer treatment efforts such as surgery, chemotherapy, hormonal therapy and radiotherapy have been conducted. However, at present there has been no recognized method can prevent liver cancer and natural ingredient exploration is needed to find other chemopreventive.[5]

Bandotan Herb *(Ageratum conyzoides L.)* is known to have good chemopreventive activity. The plant has phytochemical content of flavonoids that is a secondary metabolite of polyphenol compounds that have biological effects in the form of anti-inflammatory and antitumor.[6] The previous research evaluated the chloroform fraction of bandotan herbs cytotoxic activity against the female cervical cancer cell IC50 was 27.48 µg/ml (greater than 5-fluorouracil synthetic drug (5-FU) that has a value of IC50 was 41.46 µg/ml. In addition nobiletin in flavonoid also has been tested in silico by comparing the potential of nobiletin and 5-FU to protein BCL-XL. The score of docking against 5-FU was -4.7 kcal/mol and it is against Nobiletin showing better results ie -8.0 kcal/mol. In vivo test, 1500 mg/kg BW extract has a good chemopreventive activity.[7]

Based on the results, it is very important to do in vivo test of chloroform fractions of the herbalists due to *Sprague dawley* strain of the DMBA-induced. DMBA is a carcinogen and imunosuppressor agent that is often used to induce cancer in rats. Some researchers reported that DMBA was able to induce hepatic cancer due to primary metabolism effects and exposure to xenobiotic substances. The benefit of this study is to know the state of histopathology as well as the severity of cancer occurring. This research is expected to be used as a reference and scientific foundation for the advanced Test in vivo and clinical trials as a step in the development of chemopreventive agents.

II. METHODOLOGY

A. Preparation of Bandotan Herb Chloroform Fraction

The material used in this study was Bandotan Herbs *(Ageratum conyzoides L.)* taken in Banguntapan, Bantul, Yogyakarta. A total of 1000 grams of powdered bandotan herb was macerated with a solution of ethanol 70% with a ratio of 1:10 for 5 days inside a sealed container, then it was filtered. The screening process would produce liquid/

macerate extracts and pulp. The pulp was then macerated for 2 days to maximize the compound content therein. The results of the first and second screening measured the volume, then the macerate were stored in a sealed container to avoid contaminants. Macerate was then fractionated using chloroform with a ratio of 1:1. The obtained chloroform fraction then was condensed by using rotary evaporator at 60˚C, then was steamed using a water bath at a temperature of 60˚C until a condensed extract was obtained for weighing.

B. Pytochemistry Compound Analysis

Analysis of phytochemical content was performed using thin layer chromatography method. The silent phase used is was silica gel F with the motion phase methanol:chloroform with a ratio of 7:2: done in the TLC plate with an elusion of 8 cm. The plate was inserted into the chambers that have been saturated. Plates were removed from the chambers after the eluent reached the plate boundary, then the plate was drained solvent/ motor phase. Plates were observed under UV rays of 254 nm and 366 nm and measured each Rf-spotting value. To know the content of flavonoid, the researchers observed color of the patches before and after that steamed with Ammoniac.

C. Molecular Docking

Molecular Docking is a computing method with a competitive model of protein, enzyme, or receptor tethering using cavity on the active side of proteins. The Pose has a function to determine the interaction between the ligands with the target protein and the docking score determines the affinity of the ligands interaction to the proteins.[8] The needed structure can be downloaded by visiting www.rcsb.org to look for PDB ID 5XV7 for VEGF, 5IKQ for COX-2 and 1MV0 for c-Myc protein.

- Preparations— *Autodock Vina, MGL Tools, Phyton, Open Babel and DS Visualizer* were used to prepare proteins and ligands. The proteins were ensured to be free of water and oxygen molecules by removing existing ligand in pdb format then converted to pdbqt file format. All files were placed in the same folder. The researchers created a new document conf.txt then opened command prompt dialog to determine the RMSD value, chose the conformation with the RMSD value less than 2 Å. The output produced several data to face out ligands and protein binding.
- The visualization was done using DS Visualizer, to know the overview of protein bands in each tested ligands. The previous document with the. pdbqt format file was changed to. pdb by Open Babel. Visualizations were displayed in three dimensions so that the ligands and protein bonds were clearly visible.

D. In Vivo Study

As much as 20 mice (*Rattus norvegicus*) of *Sprague Dawley* aged 40 days with a weight of 70-100 grams were separated. The animals were obtained from the experimental animal development unit (UPHP) Gadjah Mada University, placed in a cage with a temperature of 28-32 °C, 98% humidity and given pellet food and given a drink. The rats were adapted for 3 days. Female rats were divided into 5 groups according to the treatment. The injection was done by searching in the direction of the celestial edges to the esophagus.

Group A (control without treatment) was given a solution of CMC-Na 0.5% at a dose of 1 ml/200 grams. Group B (control DMBA) was inducted with DMBA (7.12-Dimetilbenzen [a] antrasena) at a dose of 20mg/kg BW twice a week for 5 weeks. DMBA solution was obtained by discontinuing it with corn oil in conical tube then vortex for 15 minutes. Group C (extract control) was administered at a dose of 1500 mg/kg BW every day for 5 weeks. Group D and E (DMBA + extract) were subsequently given treatment with a dose of 750mg/kgBW and 1500 mg/kg BW on the fourth and fifth weeks.

E. Histology Dye Test

The test began with the paraffin removal process of the cutting organ histology and it was done by doing immersion in Xylol III for 3 minutes. This process was continued by soaking with Xylol II for 3 minutes. Slides were dehydrated (water phase removal) with alcohol levels sequential to 100%, 95% and 70%. Then the researchers dipped the slide inside the Deionized water (D. I), each levels for 3 minutes. The slide was oxidized using H_2O_2 (hydrogen peroxide), incubated for 10 minutes in D. I water. After that, slides were rinsed with tap water. The slide was washed with PBS 2 times, each for 2 minutes. To open the pores of the organ histology and the elimination of residual paraffin, it was done by warming in the cooker (temperature 100 ° C) for 10 minutes. After boiling, the slide was cooled in room temperature for 20 minutes. The Slide was then washed with tap water and rinsed with the aquadest. Rinse with water in D. I water. The slide was given a Haematoxylin-Eosin dye set, let stand for 2 minutes and rinsed with tap water. Then it was dehydrated (water phase removal) with alcohol 100% and cleaned it in xylene stage. And the last one at this stage was the Mountling and coverslip process.

While, in the Immunohistochemistry process, the procedure began with the deparafination using xylene 3 times for 3 minutes. Furthermore, it was rehydrated using ethanol 100% for 2 minutes, ethanol 95% for 2 minutes and 70% ethanol for 1 minute, then was continued with water for 1 minute. The preparation soaked for 10 minutes at room temperature in the peroxidase blocking solution, incubated in a serum prediluted blocking of 25º C for 10 minutes. The preparation soaked in monoclonal antibodies anti-VEGF 25º C for 10 minutes. Subsequent preparations were washed with Phospate Buffer Saline (PBS) for 5 minutes, then incubated with a chromogenous Diaminobenzinidine (DAB) 25º C for 10 min, continued incubation with Haematoxylin-Eosin for 3 minutes. The preparation was washed with flowing water, then cleaned and added with mounting media.

III. RESULTS AND ANALYSIS

A. Preparation of Bandotan Herb Chloroform Fraction

Extraction was carried out using maceration method for 5 days with a ratio of 1:10 then was remacerated for 2 days. It produced 18 L of liquid extract then continued to be

separated using chloroform. As much as 5 L of liquid extract was separated with liquid-liquid partition method with a ratio of 1:1 and produced 8.1 L of BHCF. Furthermore, the BHCF was evaporated using a rotary evaporator and obtained by a concentrated FKB of 2 L and the remaining separation of ethanol. Condensed FKB was obtained through the heating process using water bath at a temperature of 60° C with a rotation speed of 90 rpm and produced a condensed extract 17.4 grams with a yield value of 4.22%.

The extraction was described by the broken cell wall due to the pressure differences both inside and outside the cell so that the secondary metabolite in the cytoplasm will be dissolved.[9] A liquid-liquid fraction process was performed using chloroform used to obtain the better and optimum flavonoid compound based on its polarity index. The separation funnel showed 2 phases whereas the upper phase (ethanol fraction 70%) and the lower phase (chloroform fraction) due to the difference in the density of both solvents.

B. Pytochemistry Compound Analysis

Identification of flavonoid was performed using the TLC by spotting BHCF above silica gel plate. The result was identified using the Rf value, i.e. distance driven by the compound from the origin point was divided by distance driven by the solvent from the origin point. Color reagent was done using ammonia vapor. The resulting colors performed color interpretation of spotting and qualitative analysis of flavonoid content and it can be seen in Figure 1.

The chosen motion phase chloroform:methanol (7:2 V/V) have been widely used in some cases using chloroform:methanol:water (65:25:4 V/V).[10] The silent phase selection also relates to the polarity, solubility, molecular weight, ionizing ability and analyzed size. A total of 64% of researchers used silica sorbent and 9% followed by cellulose as well as alumina 3%.

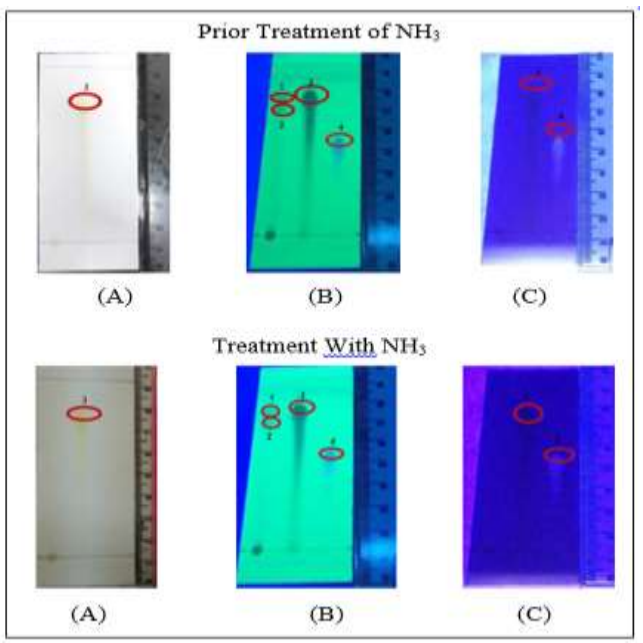


Fig. 1.Identification of Compounds with Thin Layer Chromatographic (A) Visible Light (B) UV 254 nm (C) UV 366 nm.

TABLE 1. TLC RESULTS DUE TO NH_3 VAPOR

Spot	Rf	Color Spots with NH_3		
		Visible Light	*UV 254 nm*	*UV 366 nm*
1	0,75	-	Bright Purple	-
2	0.81	-	Bright Purple	-
3	0.83	Yellow	Dark Purple	White
4	0.53	-	Bright Purple	White

The sample observation of BHCF under UV rays 254 nm produced purple light while under UV 366 did not show the color of the spotting. Based on the Rf values, BHCF contained flavonoid compounds with the type of non polar aglycones. The purple spots seen in the visual observation of TLC were estimated as the flavones and flavonols. One of the identified compounds was methoxylated flavones that contain nobiletin compounds and their derivatives.[11]

Yellow and violet color in UV rays indicate quercetine and flavonoids glycosides. Flavonoids glycosides are flavonoids that are often found in plants and this compound binds to sugar. Routine (comparator compounds) is an example of flavonoid glycosides. So on the 3rd spots, yellow color indicating that the spotting is a flavonoids glycosides. While the 4th spots are alkaloid compounds. Further for quantifying process can be continued with TLC-Densitometry and HPLC study to make sure the presence and the level of nobiletin in flavones group.

C. Molecular Docking Test

Molecular Docking is performed to analyze the strength of the oncogene bond of VEGF against nobiletin. The data interpretation was shown in Table 2 using the docking score.

TABLE 2. MOLECULAR DOCKING SCORE

Compound	Protein	RMSD	Score	Conformation
Nobiletin	VEGF	1.122	-7.6	6
5-Fluorouracil	VEGF	1.534	-4.7	3
Nobiletin	COX-2	1.038	-7.5	3
5-Fluorouracil	COX-2	1.927	-5.2	6
Nobiletin	c-Myc	1.395	-4.9	5

Each compound produced 9 conformations. RMSD is the deviation value between a ligand and its comparison, that is, if the deviation is too large, the greater the prediction error of ligand and protein interactions.[12] The best results means that the needed energy for nobiletin to interact with VEGF (-7.6 kcal/mol) was lower than 5-FU to VEGF (-4.7 kcal/mol) resulting in less stable bonds. Based on previous research, nobiletin has been shown to have anti tumor activity through deregulation of cell cycle, anti proliferation, induction of apoptosis, anti angiogenesis, anti-inflammatory and anti carcinogenic.[13-15]

(A) (B)

Fig. 2. Interaction of 2 Visualization of Active Compounds Test on VEGF Protein: (A) Nobiletin (B) 5-Fluorouracil

As for the results of this study, Nobiletin has potential as anticancer activity through an apoptotic mechanism involving VEGF. The role of polymethoxyflavone (nobiletin) compounds is against inhibitory expression of the VEGF pursued anti angiogenesis and cell cycle regulation by inhibit VEGF and FGF through the down regulation of ERK 1/2 receptors, C-JNK and caspase line activation.[16] There are two types of amino acid bonds formed in the binding of nobiletin against VEGF proteins, namely phi sigma bonds and phi alkyl bonds (figure 2). In chemistry, sigma belongs to the strongest covalent bond.

The docking score is contrary to the affinity energy, the more negative docking score, the higher affinity. Based on the information, the Nobiletin compound has better potential for VEGF proteins due to a very strong form of bonding, so it only takes a bit of energy to maintain a stable state. Nobiletin is a polymethoxy flavonoids isolated from nature and has inhibitory activity on cancer growth through anti angiogenesis and anti apoptosis. Anti angiogenesis is done by reducing of Akt levels, HIF-1 α, NF-κB and VEGF in the case of ovarian cancer.[17]

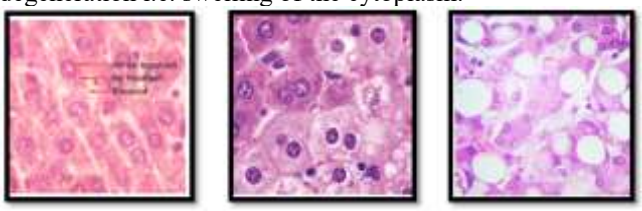
D. In Vivo Test

DMBA (7,12-Dimethylbenz(a)anthracene) is a member of polycyclic aromatic hydrocarbons (PAHs) used for medical purposes. It is one of the most powerful synthetic carcinogens. DMBA leads necrosis by damaging cell structures through oxidative stress leading to lobular hyperplasia which then triggers tubular adenomas which are non-invasive mammalian pathology.[18] This condition occurs in situ carcinoma and develops into an invasive lobular carcinoma.[19] Vasularity is related to in situ carcinoma and is initiated in vitro angiogenesis by producing VEGF as a potent angiogenic growth factor. DMBA affects sinusoid tissue and proliferation cycle. Angiogenesis depicted oxygen supply as well as other nutrient to the tumor tissues and it is a crucial point in hepatocarsinogenesis, progression and metastatic cancer on liver.[20]

Liver tissue has undergone a microscopic change (can be observed through histology observation using HE and IHC) on microscopic observation by scarifying all of feeding groups after the sixth weeks through thoracic surgery. Macroscopic observation used to know the number and size of nodules formed in rats. But in macroscopic observation it takes a long time until the nodules arise.

E. Histology Dye Test

Histopathology observation is performed by microscopic viewing with *Toupview*® software to observe the influence of the administration of BHCF due to the hepatic cell tissue of *Sprague dawley* rodent induced by DMBA. The properties of carcinogenesis are examining the normal and broken cell structure using Haematoxylin and Eosin dye test by observing cytoplasmic changes, especially around the centralist vein and the dilation of sinusoid. In addition, the damaging of liver cancer can also be described through the process of hydropic degeneration i.e. swelling of the cytoplasm.



(A) (B) (C)

Fig. 3.(A) The depiction of normal hepatocytes (B) liver tissue suffered from hyperphonic degeneration 40x10 magnification (C) liver tissue experiencing fatty degeneration[21]

Based on IHC staining, the preparation produced brown color when there was high protein expression in the preparation and the normal preparation will show the blue color. The degree of damaging assessment was done qualitatively and continued with scoring semi-quantitative analysis (table 3).

TABLE 3. SCORING OF IHC STAINING [22]

IHC Painting Pattern	Score	Classification
No membrane staining of IHC painting results	0	Negative
Membrane staining is incomplete (< 10%) and weak	1+	Negative
Membrane staining is complete (not uniform (10% - 30%))	2+	Equivocal
Staining of the uniform membrane intensity (> 30%)	3+	Positive

This dye based is a microscopic observation to analyze the histopatology of SD due to the chemopreventive effect of BHCF based on damaged and cell repairs. It consists of Capsula Glisson and hepatic lobules. Lobules are composed of hepatocyte and sinusoid cells. Lobulus is a hexagon-shaped prism and is formed by liver cells accompanied with sinusoids. Normally, the microscopic hepatocytes cells are cuboid and have a central rounded cell core.

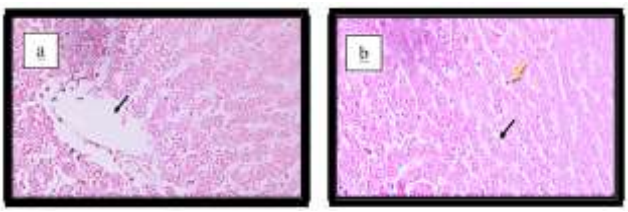


Fig. 4. Overview of hepatocyte-functional cell of liver tissue due to HE test magnification 40x, (a) centralist vein (→) (b) sinusoid (→) endothelium (→)

Cellular damage can be described through a degenerative process and necrosis. Hydro-pic degeneration is the process of swelling of the cytoplasm due to cell membrane damage due to hypoxia caused by chemical poisoning.[23]

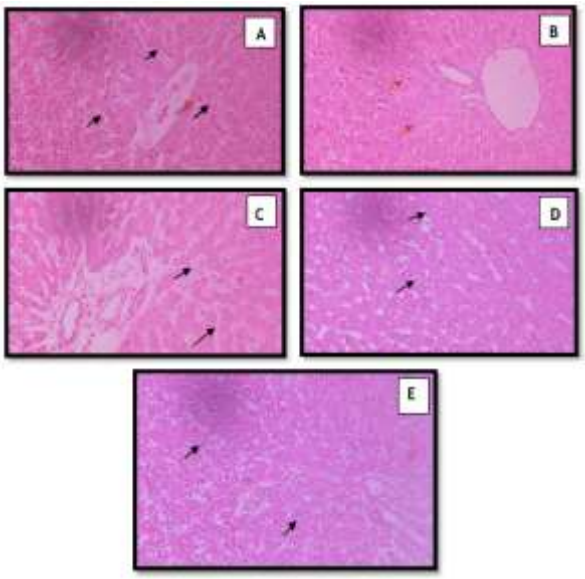


Fig. 5. Observation using HE staining, normal cell (→)broken cell (→) (A) CMC-Na 0,5% solvent (B) DMBA 20 g/kgBW (C) BHCF 1500 mg/kg BW (D) DMBA + BHCF 750 mg/kg BW (E) DMBA + BHCF 1500 mg/kgBW

In A and C feeding groups, there was no significant differences. Both received solvent control and BHCF. Sample B with DMBA induction of 20 mg/kg BW showed noticeable difference in hepatocytes. Hepatocyte cells undergoing changes (inflammation) can be described by the boundary between cells, examined cytoplasm and irregularities of the cell form. Sinusoid is was not seen so clear clearly as in other samples. Liver tissue has hyperproliferation leading to moderate carcinogen. Some histological repairs on D and E feeding groups observed had the better result on 1500 mg/kgBW treatment.

TABLE 4. AN OVERVIEW OF HE STAINING

Group	Number of Broken Cells			Average (%)
	Replication 1	*Replication 2*	*Replication 3*	
Group A	3 of 60 cells	3 of 56 cells	2 of 50 cells	± 6%
Group B	25 of 68 cells	21 of 65 cells	27 of 76 cells	± 46.1%
Group C	3 of 43 cells	5 of 55 cells	3 of 36 cells	± 8.2
Group D	18 of 65 cells	13 of 46 cells	17 of 69 cells	± 26
Group E	10 of 68 cells	7 of 62 cells	9 of 65 cells	± 13.2%

Group A and C showed an average breakdown of 6% and 8.2% cells which showed low damage value. CMC Na in rats affected cell regeneration process. DMBA administration (group B) showed ± 46.1% damaging cells indicating the highest level. Furthermore, the feeding group D and E showed an average ± 26% and ± 13.2% damaging cell. Both treatment groups, administration of BHCF extract dose 1500 mg/kgBW showed better cells repairs.

VEGF expression can be known by staining immunohistochemistry through the principle of binding antibodies (Ab) and antigen (AG). This method is often used to detect distribution as well as localization of certain proteins on a body tissues (figure 6).

DMBA carcinogenesis (group B) in liver tissue rated less significantly. The immunological properties seen by brown spots indicated the presence of VEGF protein.

Fig. 6. The Presence of VEGF on Liver Tissue (→)

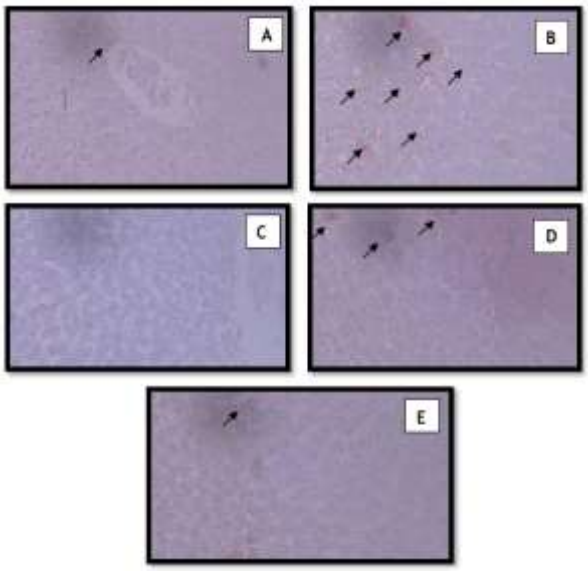


Fig. 7. IHC staining, VEGF expression (→); (A) CMC-Na 0,5% (B) DMBA 20 mg/kg BW (C) BHCF 1500 mg/kg BW (D) DMBA + BHCF 750 mg/kg BW (E) DMBA + BHCF 1500 mg/kg BW

TABLE 5. SCORING AND CLASSIFICATION OF IHC STAINING

Group	IHC Score		
	Replication 1	*Replication 2*	*Replication 3*
Group A	1+	0	1+
Group B	2+	2+	1+
Group C	0	1+	0
Group D	0	1+	1+
Group E	0	0	1+

Semi-quantitative data were performed with scoring degree. The positive results were shown with brown spots. Group B got results of 2 +, 2 + and 1 + showed the highest VEGF protein expression. This is related with the induction of DMBA due to DNA adducts and an over-expression of VEGF proteins. The VEGF expression of group B figure out < 30% (moderate). The results for group D and E are 2+ and 1+. Both compared and be concluded that the administration of BHCF 1500 mg/kg BW proved better repair to *Sprague dawley* rats induced by DMBA.

IV. CONCLUSION

Bandotan herb *(Ageratum conyzoides L)* contains a secondary metabolite of a flavonoid compound. One of the flavonoid compounds tested was in the form of nobiletin which has the inhibitory activity of VEGF protein expression and better than 5-FU as the comparator and got docking score -7.6 kcal/mol. The administration of DMBA for 5 weeks was able to affect cellular histology and the administration of 1500 mg/kg BW BHCF was able to reduce the severity and repair the cell structure of hepatocyte liver tissue.

It is suggested to test bandotan herb using TLC-Densitometry and HPLC method to determine the more specific active compound. Histological observation using

IHC staining should be done against some proteins (against COX-2 proteins, VEGF and C-Myc) to compare the level of proteins over expression in liver tissue due to *Sprague dawley* rats.

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