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Animal Care and Use Committee (ACUC)

KETERANGAN KELAIKAN ETIK

" ETHICAL CLEARENCE "

No : 1.KE.199.12.2019

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PENELITIAN BERJUDUL	: Analisis Potensi Senyawa Aktif Capsaicin Terhadap Daya Hambat Aflatoksin B1 (AFB1) pada *Hepatoceluller Carcinoma* (HCC) Sebagai Kandidat Obat Spesifik AFB1-HCC
PENELITI UTAMA	: Moh. Sukmanadi
UNIT/LEMBAGA/TEMPAT PENELITIAN	: Program Studi S3 Sains Veteriner Jenjang Doktor Fakultas Kedokteran Hewan Universitas Airlangga
DINYATAKAN	: LAIK ETIK

Surabaya, 10 Desember 2019

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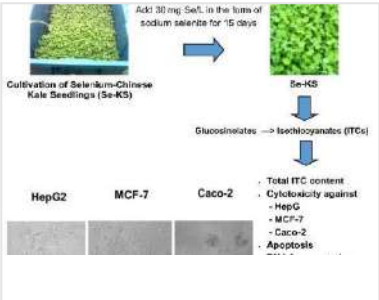

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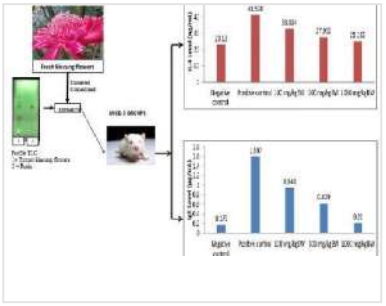

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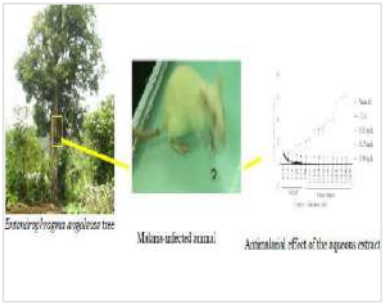

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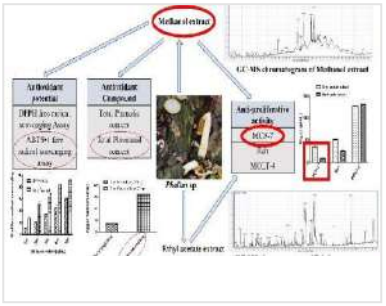

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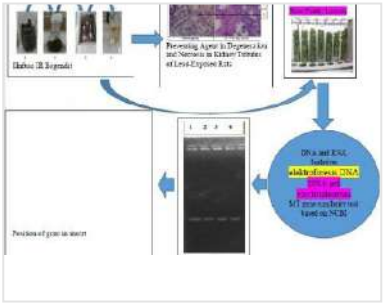

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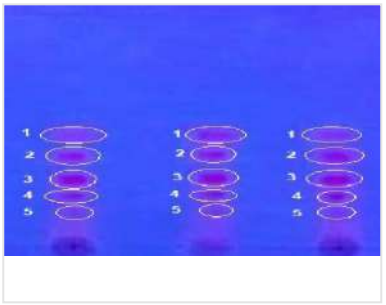

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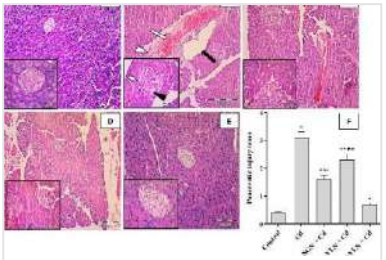

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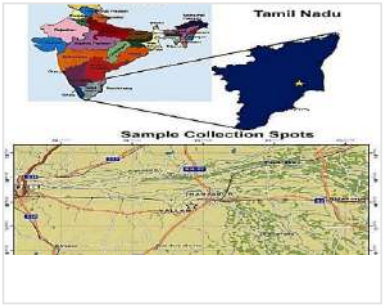

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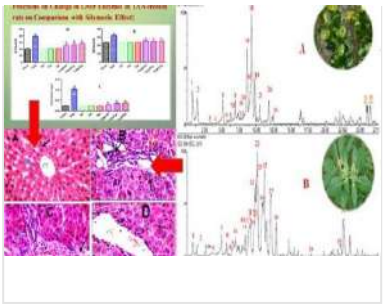

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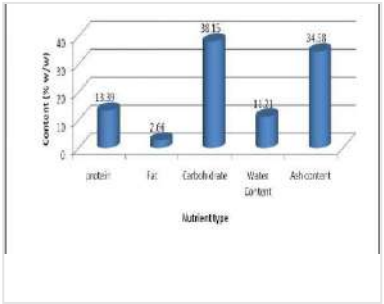

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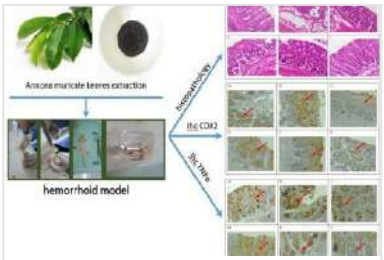

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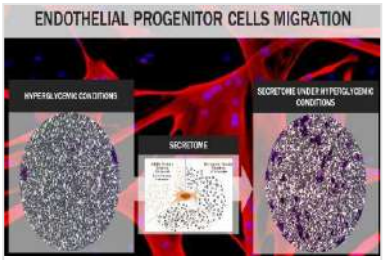

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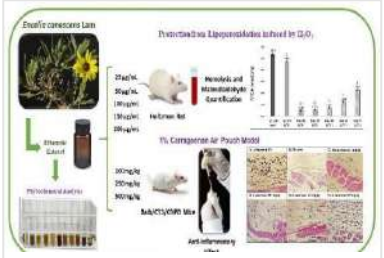

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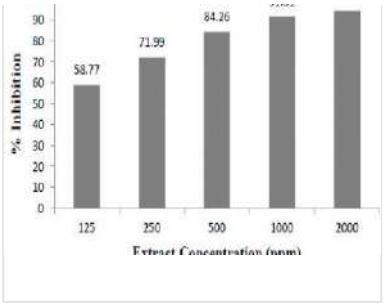

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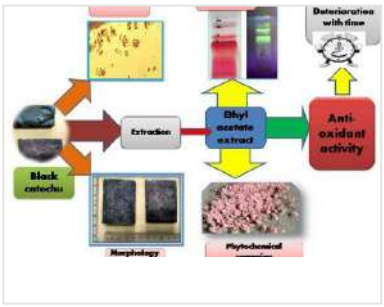

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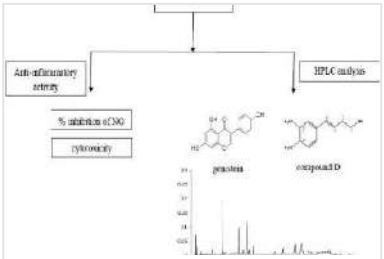

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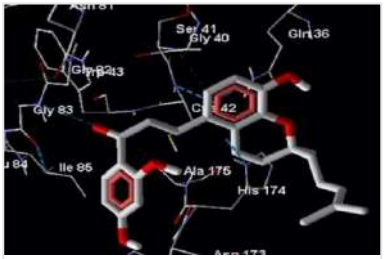

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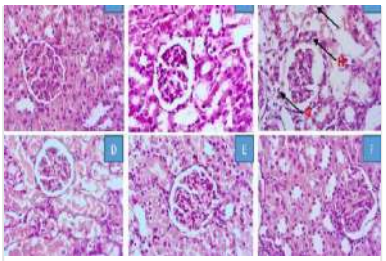

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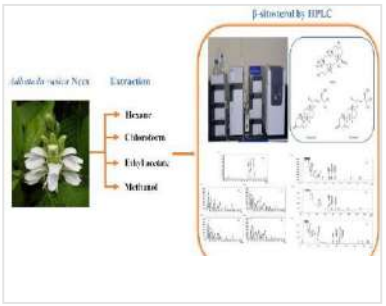

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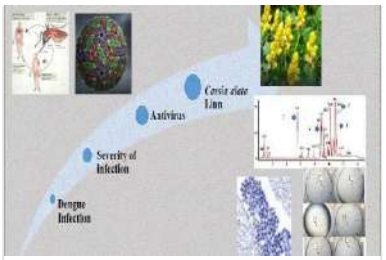

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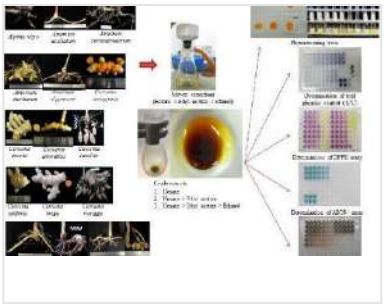

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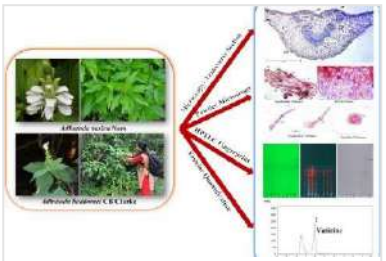

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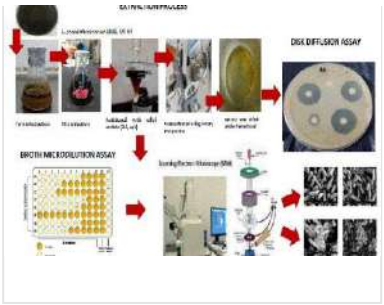

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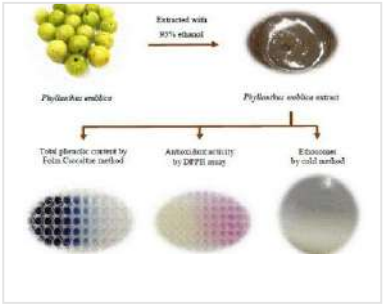

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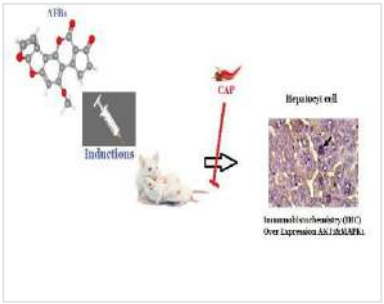

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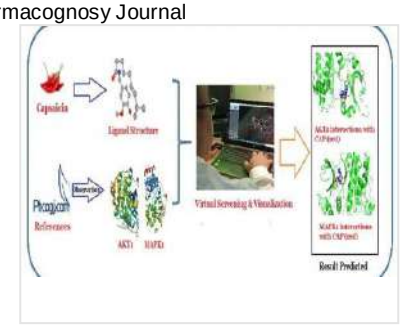

Mohammad Sukmanadi,Sri Agus Sudjarwo,Mustofa Helmi Effendi

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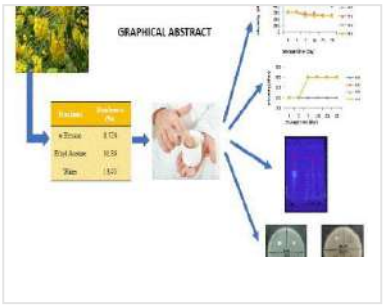

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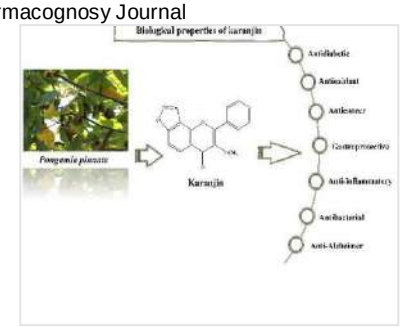

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Research Article

Capsaicin Bioactive in Cabai (*Capsicum Annum* L.) as Anticancer Through Inhibition of over Ekspresi Protein Target *RAC-alpha serine/threonine-protein kinase* (AKT1) and *Mitogen-activated protein kinase 1* (MAPK1) on Hepatocyt Cell Mice (*mus musculus*)

Mohammad Sukmanadi[1,*], Sri Agus Sudjarwo[2], Mustofa Helmi Effendi[3], Pudji Srianto[4], Aulanni'am[5], Rr. Sri Pantja Madyawati[4], Mirni Lamid[6], Hani Plumeriastuti[7]

ABSTRACT

Capsaicin is a secondary metabolite of the Chilean plant. In the pharmaceutical field in addition to relieving pain or pain, capsaicin is also known to have anticancer activity because it inhibits certain oncogenic proteins. Screening of components in *Capsicum Annum* L. against the target proteins AKT1 and MAPK1 is needed as an initial stage of drug discovery. Further screening of Capsaicin compounds for oncogenic proteins produced in Hepatocellular carcinoma (HCC) pathogenesis signaling. In silico data that have been obtained, Capsaicin in chili (*Capsicum Annum* L.) has a high affinity for MAPK1 and AKT1 receptor/protein targets with energy and potential activity score (Pa) 0.690 for preneoplastic treatment, 0.590 for apoptotic agonists, and 0.366 for antineoplastic activity. Statistical data using Kruskal Wallis obtained information that Capsaicin can inhibit the expression of AKT 1 and MAPK 1 on mice hepatocyte cells induced by AFB1 *in vivo* administration, therefore it can be a candidate for anticancer drugs.
Key words: Capsaicin, Hepatocellular carcinoma (HCC), MAPK1, AKT1, Anticancer.

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BACKGROUND

Hepatocellular carcinoma (HCC) is a malignant tumor that attacks the liver. The prevalence of malignant tumors is very high, ranked fourth globally as cancer with an incidence rate of 5.3% compared to other cancers.[1] Pathophysiological HCC arises because of chronic exposure to hepatocytes that can cause epigenetic and genetic changes that lead to the induction of oncogenic proteins and/or activation of tumor suppressor genes. This pathogenesis occurs in four important signaling pathways (WNT, TGFß, PI3K / Akt, and RAF / MEK / ERK) involved in the development of HCC.[2]

The RAS / RAF / MEK / ERK pathway plays an important role in the development of liver cancer. Similar to many signaling networks, this pathway is activated at HCC through a variety of mechanisms. RAS activation is initiated by the binding of extracellular signaling compounds such as hormones and growth factors with VEGFR and PDGFR. The bond between signaling compounds will cause a phosphorylation reaction in the RAS and initiate a series of cascades that lead to the activation of ERK for the proliferation and differentiation and angiogenesis of HCC. Certain mutated components or Ras-Raf-MEK-ERK / MAPK overexpression are increasingly being studied in HCC carcinogenesis. The abnormal target protein signaling pathway contributes to cell proliferation, differentiation, survival, and uncontrolled cell apoptosis are biomarkers of carcinogenic processes.[3]

The PI3K pathway is activated in HCC through various mechanisms such as IGF-1 binding to IGFR thus initiating the P13K signaling series and activating Akt which directly influences mTOR which will regulate the proliferation and angiogenesis of HCC.[2]

Interventions can inhibit the proliferation and angiogenesis of HCC and reduce the concentration of these compounds due to excessive expression due to genetic and epigenetic factors.[4] The use of drugs to block the signaling pathway has been carried out and has reached clinical trials such as Salisarib, Sorafenib, and Selumetinib, but the use of these drugs in the long term will cause side effects such as decreased peripheral blood supply, heart attacks and acute heart failure,[3] It is suspected that capsaicin acting at this signaling point inhibits the viability of hepatocellular carcinoma cells when exposed to herbal medicine agents, so alternative herbal medicines are needed to inhibit the HCC signaling.

Chili has natural compounds that can provide enormous benefits for humans and animals. One of the most important compounds in chili is capsaicin. Capsaicin (8-methyl-N-vanillyl-6-none amid) is an active component of chili which is irritant to mammals including humans and causes burning and heat in any tissue that is touched.

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In the pharmaceutical field in addition to relieving pain or pain, capsaicin is also known to have anticancer activity.[5] High potential in the pharmaceutical field as anticancer, anti-arthritis and analgesia in addition to having a commercial value in the food industry.[6,7]

Chili (*Capsicum Annum* L.) has a spicy taste and has a very sharp aroma, a spicy flavoring chili is a capsaicinoid compound. Capsaicinoids include nordihydrocapsaicin, capsaicin, dihydrocapsaicin, nor capsaicin, homodihydrocapsaicin, homocapsaicin, nonivamide.[8] One of the most important compounds in chili is capsaicin, capsaicin is a secondary metabolite of the chili plant. In the pharmaceutical field in addition to relieving pain or pain, capsaicin is also known to have anticancer activity because it inhibits certain oncogenic proteins.[5] Immunohistochemical examination between capsaicin compounds and oncogenic proteins produced in the signaling of HCC pathogenesis.

Screening components in Capsicum Annum L against MAPK1 and AKT1 target proteins is the initial stage of drug discovery. Screening usually uses a computer method or commonly known as in silico. In silico consists of various methods and the most commonly used for drug discovery and prediction of the drug, effects are *Molecular Docking*.[9]

From this background, this study aims to test the bioactive component of *Capsicum Annum* L on MAPK1 and AKT1 target proteins using the Immunohistochemistry (IHC) method to determine the reactions between receptor-ligand (antigen-antibody) complexes formed and become a reference *in vivo* tests.

METHOD

This study used an experimental laboratory research design with experimental animals (*In vivo*) with an examination of the immunohistochemical method.

Immunohistochemical sample preparation

The preparations are immersed in xylol 2 times, sequential alcohol (96%, 90%, 80%, 70%) for the hydration process. Washed in PBS pH 7.4 3 times each for 5 minutes. Soaked in 3% hydrogen peroxide (in distillate water) for 20 minutes. Washed in PBS pH 7.4 for 3x5 minutes. Soak in 1% BSA for 10-30 minutes at room temperature. Washed in PBS pH 7.4 for 3x5 minutes. Primary antibodies are added for 1 hour at room temperature. Then incubated overnight. Then washed in PBS pH 7.4 for 3x5 minutes. Then added a secondary antibody labeled Strep avidin horseradish peroxidase (SA-HRP) for 1 hour at room temperature. Washed in PBS pH 7.4 for 3x5 minutes. Preparations are added chromogen DAB (3,3-diaminobenzidine tetrahydrochloride) for 10-20 minutes at room temperature. Wash in distilled water for 3x5 minutes. Counterstained with hematoxylin for 5 minutes at room temperature. Wash in distilled water for 3x5 minutes. Mounting with insert. Observation using a microscope at magnification 100 and 400 times.

RESULTS AND DISCUSSION

Immunohistochemistry (IHC)

The immunohistochemical examination is intended to determine the expression of AKT 1 and MAPK1 (ERK) on cells of mice hepatocytes (Mus musculus). AKT 1 and MAPK 1 (ERK) expression score data were obtained using the modified Remmele method.[10] Remmele scale index (Immuno Reactive Score / IRS) is the result of multiplying the percentage score of immunoreactive cells with the color intensity score on immunoreactive cells. Data for each sample is the average value of the IRS observed in 5 (five) Field View (LP) at 400x magnification (Figures 1-3).

Comparison of AKT 1 expression (chromogen brown) in hepatocyte (arrow) cells between treatment groups. AKT1 is expressed in both

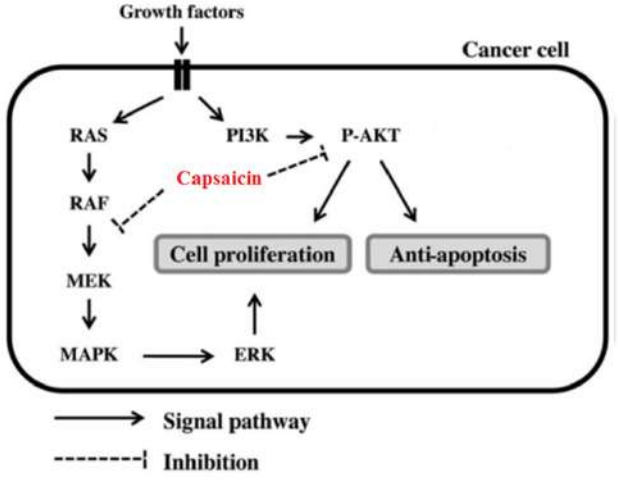

Figure 1: Schematic diagram of the probable mechanism of Capsaicin. The RAS / RAF / MAPK pathway and the PI3K-AKT pathway control cellular proliferation and apoptosis (modification. Morisaki *et al*, 2013). Barriers from HCC signaling compounds such as MAPK1 (ERK) and AKT using special.

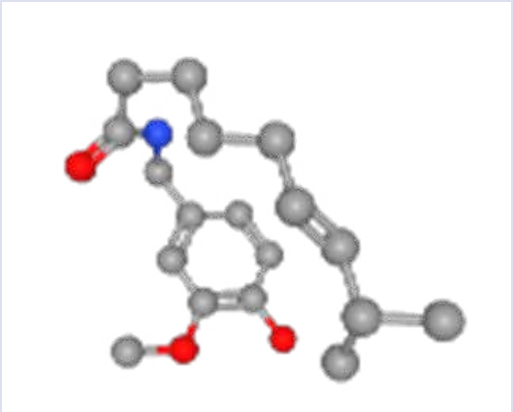

Figure 2: Test Ligand Structure (Capsaicin) [*https://pubchem.ncbi.nlm.nih.gov/*].

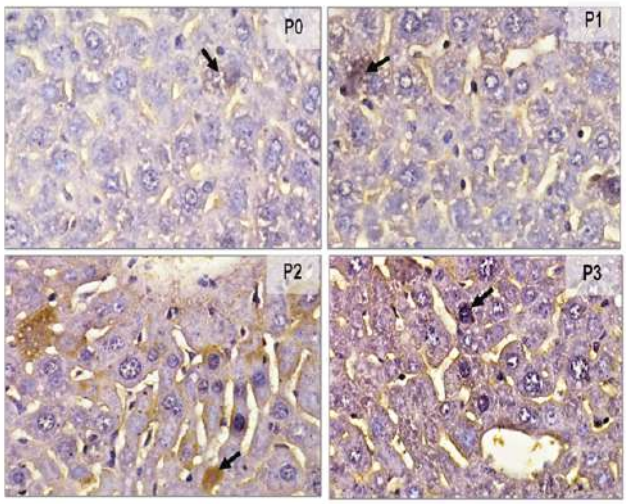

Figure 3: Immunohistochemical staining AKT 1, 1000x magnification; Nikon H600L microscope; DS Fi2 300 megapixel camera.

the cytoplasm and the nucleus of hepatocyte cells. The results of the examination showed that the expression of AKT1 in the treatment group Giving AFB1 (3 mg / Kg BW) (P2) and Giving AFB1 (3 mg / Kg BB) and Capsaicin (10 mg / Kg BB) (P3) looked the same but was stronger compared to the control treatment group (P0) and the treatment group giving Capsaicin (10 mg / Kg BW) (P1).

Comparison of MAPK1 (brown chromogen) expression in hepatocyte (arrow) cells between treatment groups. MAPK1 is expressed in both the cytoplasm and the hepatocyte cell nucleus. The results of the examination showed that the expression of MAPK1 in the treatment group Giving AFB1 (3 mg / Kg BB) (P2) and Giving AFB1 (3 mg / Kg BB) and Capsaicin (10 mg / Kg BB) (P3) looked the same but was stronger compared to the control treatment group (P0) and the treatment group giving Capsaicin (10 mg / Kg BW) (P1) (Figures 3 and 4).

The data obtained in the form of a Remmele Scale Index score (Immuno Reactive Score / IRS) results from the multiplication score of immunoreactive cells with a color intensity score, were analyzed by Kruskal Wallis followed by the Z Test, statistical analysis using the IBM SPSS Corp. computer statistical program. Real 21.

Statistical analysis, data are presented as mean (mean) ± standard error. Differences between groups were assessed for statistical significance using the Kruskal-Wallis test or the Multiple comparison test with the Z test (Multiple comparisons by Z test), depending on data distribution. P values <0.05 were considered statistically significant.

The data obtained indicate the number of assessment scores with Immuno Reactive Score (IRS) on the expression of AKT 1 under each capsaicin treatment condition (mean ± SEM of six replications). Statistically significant difference from controls (*p<0.05;**p<0.01). The expression of AKT1 on giving AFB 1 is the same as the expression of AKT 1 on giving AFB1 and Capsaicin; The expression of AKT1 in giving Capsaicin is the same as the expression of AKT 1 in giving AFB1 and Capsaicin while giving AFB1 is different from giving Capsaicin in AKT 1 expression.

The data obtained shows the number of assessment scores with the Immuno Reactive Score (IRS) on the expression of MAPK 1 under each capsaicin treatment condition (mean ± SEM from six replications). Statistically significant difference from controls (* p <0.05; ** p <0.01).

The highest MAPK1 expression in AFB1 (P2) treatment, with a mean rank of 18.41 ± 1.4108, showed a significant difference (p <0.05) with other treatments, namely CAP (P1); CAP and AFB1 (P3), and control (P0) each with a mean rank of 11.42 ± 2.1269; 11.42 ± 3.0067 and 8.75 ± 3.5137. It is also known that the last three (the) treatments did not differ significantly (p> 0.05).

Based on the results of testing using Kruskal Wallis can provide information that Capsaicin can inhibit the expression of AKT 1 and MAPK 1 on mouse hepatocytes induced by AFB1 *in vivo* administration (Figures 5-7).

Immunohistochemistry technique is a method that aims to identify specific cells based on antigenic components or cellular products with complex antigen-antibody reactions. Immunohistochemistry is used as a basis for diagnosis and identification of cell types based on cytomorphology. This examination is often done in cases of tumors or malignancies. In addition, immunohistochemistry is also often used for research to determine the distribution and location of biomarkers or expressed proteins in various body tissues.

Immunohistochemistry is a combination of histological or cytological examination with immunology. The method of coloring substances or active ingredients in tissues uses the basic principle of immunology, namely by binding the active ingredients or antigens on the specific active side by the active ingredients or antibodies. The results of this reaction can be identified in the specimen if the antibodies are bound by a marker that can be in the form of fluoresin, enzymes, particulate matter, or isotopes that can be visualized, so as to indicate the presence of active ingredients in the tissue. Active ingredients can be in the form of proteins, carbohydrates, nucleic acids, fats, other natural ingredients and synthetic materials. Immunohistochemical examination has a high ability to separate, select, and be specific. This examination is to detect the presence of antigens, this is due to the presence of specific bonds between antigens and antibodies.

The results of this study obtained a reaction to AKT1 and MAPK-1 based on immunohistochemistry. In addition, based on the results of Scoring with Immuno Reactive Score / IRS showed the expression of MAPK 1 in each treatment.

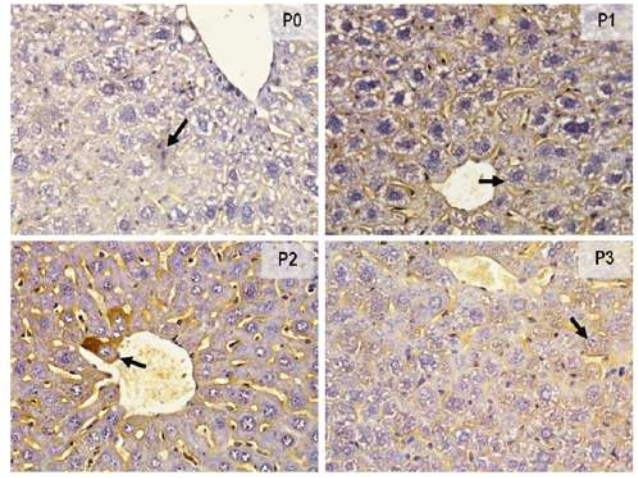

Figure 4: Immunohistochemical staining MAPK 1, 1000x magnification; Nikon H600L microscope; DS Fi2 300-megapixel camera.

Uji Z untuk Skor AKT 1

Mean Rank		$R_{i.1}$	$R_{i.2}$	Differents of Mean Rank ($R_{i.1} - R_{i.2}$)	λ [1]	p [2]
Kontrol	CAP	4.5	11.33	-6.83	-1.67	s
	AFB1	4.5	19.33	-14.83	-3.63	s
	CAP+AFB1	4.5	14.83	-10.33	-2.53	s
CAP	AFB1	11.33	19.33	-8	-1.96	s
	CAP+AFB1	11.33	14.83	-3.5	-0.86	ns
AFB1	CAP+AFB1	19.33	14.83	4.5	1.10	ns

[1] $\lambda=(R_{i.1}-R_{i.2})/4{,}082$; [2] Z_{tabel} Distribusi Normal, s = signifikan (p<0,05), ns = non signifikan (p>0,05)

Figure 5: AKT-1 Z test results.

Uji Z untuk Skor MAPK 1

Mean Rank		$R_{i.1}$	$R_{i.2}$	Differents of Mean Rank ($R_{i.1} - R_{i.2}$)	λ [1]	p [2]
Kontrol	CAP	8.75	11.42	-2.66667	-0.65	ns
	AFB1	8.75	18.42	-9.66667	-2.37	s
	CAP+AFB1	8.75	11.42	-2.66667	-0.65	ns
CAP	AFB1	11.42	18.42	-7	-1.71	s
	CAP+AFB1	11.42	11.42	0	0.00	ns
AFB1	CAP+AFB1	11.42	11.42	0	0.00	ns

[1] $\lambda=(R_{i.1}-R_{i.2})/4{,}082$; [2] Z_{tabel} Distribusi Normal, s = signifikan (p<0,05), ns = non signifikan (p>0,05)

Figure 6: MAPK-1 Z test results.

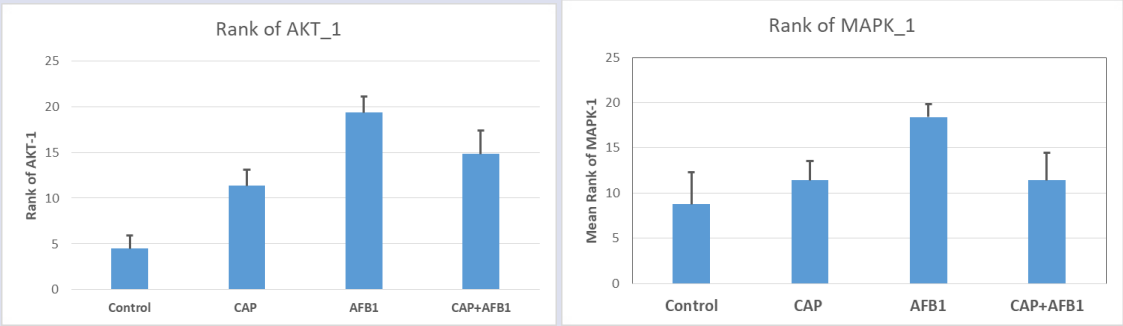

Figure 7: Average data diagram (mean) ± standard error AKT 1 (A) and average data (mean) ± *standard error* MAPK 1 (B) with differences between groups for significance statistics.

Capsaicin can inhibit the formation of all metabolites 4-(methylnitrosamino) -1- (3-pyridyl) -1-butanone (NNK) by all microsomal fractions and inhibit α-hydroxylation by microsomes. This shows that capsaicin as a natural dietary constituent, has antimutagenic and anticarcinogenic properties through inhibition of xenobiotic metabolic enzymes.[11] Capsaicin (8-methyl-N-vanillyl-6-nonenamide) in research shows that capsaicin can inhibit mutagenicity and DNA binding in several carcinogenic chemicals suspected by suppressing the activation of metabolism in cells, capsaicin inhibits AFB1 biotransformation by modifying the activity of liver enzymes in the phase of carcinogenic chemicals suspected by suppressing the activation of metabolism in cells, capsaicin inhibits AFB1 biotransformation by modifying the activity of liver enzymes in the phase of carcinogenic chemicals.[12]

This shows that when AFB1 enters the body it is predicted to potentially influence the activation of the AKT1 and MAPK1 oncogenic protein so that it triggers hepatocellular carcinoma (HCC) and AFB1 to have a more stable binding energy compared to other proteins to enable the formation of AFB1-AKT1 molecular complexes. It is predicted that AKT1 is an oncoprotein that has become a target of inhibition by Capsaicin. So that there is an AFB1 barrier indirectly interfering with the activation of AKT1 and MAPK1 proteins.

Based on the results of in silico research, there is super-expression of AKT-1 and MAPK-1 as evidence of the molecular mechanism of capsaicin inhibition against AFB-1 in Hepatocellular Carcinoma (HCC).

Analysis of amino acid residues, Capsaicin to AKT1 and Capsaicin to MAPK1 have relatively similar residues so that it can be concluded that the binding sites of the two compounds are close to similarities and affect receptors at relatively similar sites, namely oncogenic receptors/proteins.[13-15]

Further analysis determined the effect of capsaicin on AKT1 and MAPK1 needed to analyze the potential of Capsicum sp. based on their chemical structure. After the activity test was obtained the Potential Activity Score (Pa) score indicator, the capsaicin compound had Pa 0.690 for preneoplastic treatment, 0.590 for apoptosis agonist and 0.366 antineoplastic activity.

The more the value (Pa) approaches one, the better the potential for activity. Through this test, capsaicin has good activity value and has the potential as an anti-cancer drug because it inhibits the signaling of AKT1 and MAPK1 / ERK which can inhibit the proliferation of cancer cells because it has apoptotic effects of agonists and antineoplastic agents.

CONCLUSIONS AND RECOMMENDATIONS

Conclusion

In conclusion, our data show that capsaicin in chilies (*Capsicum Annum* L.) can synergistically inhibit cell viability through reactions in the anti-apoptotic AKT signaling pathway and MAPK 1 potentiates the antiproliferative action that can promote HCC cell apoptosis, thereby allegedly having the potential to prevent HCC in patients and accordingly become candidates for anticancer drugs, a finding that demands further clinical testing.

Suggestion

Conducting further tests through pre-clinical and clinical test methods and standardization of capsaicin formulations as anticancer drugs.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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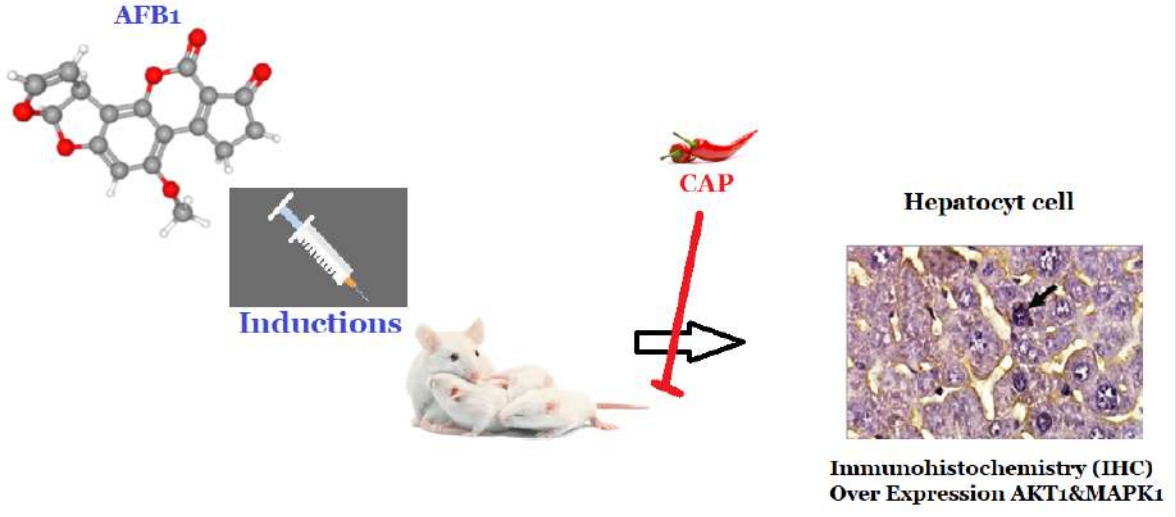
GRAPHICAL ABSTRACT



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Prof. Sri Agus Sudjarwo, Ph.D., DVM., one expert in the field of pharmacology from Universitas Airlangga. He had dreamed of becoming an expert in pharmacology since his childhood because of the many research fields that this science could investigate.

Dr. Mustofa Helmi Effendi, DVM., DTAPH is Associate Professor in Veterinary Epidemiology Head of Department of Veterinary Public Health, Faculty of Veterinary Medicine, Airlangga University;Head of Halal Research Center, Airlangga University.

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