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Effect of Kelakai (*Stenochlaena palustris*) Extract on Organophosphate Pesticide Exposure: Cytotoxic Studies in Silico and in Ovo

Pengaruh Ekstrak Kelakai (*Stenochlaena palustris*) pada Paparan Pestisida Organofosfat: Kajian Sitotoksik in Silico dan in Ovo

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ABSTRACT

Pesticides are used by vegetable farmers in South Kalimantan to protect their crops from pests. The pesticide's active ingredient has a broad toxic effect on target and non-target organisms. Continuous exposure to pesticides causes cancer. Kelakai (Stenochlaena palustris) is believed to have cytotoxic activity against the proliferation of cancer cells. This study aimed to examine the potential activity of chemical compounds in anaplastic lymphoma kinase (ALK) exposed to organophosphate pesticides in silico and in ovo. The in silico study used molecular docking and virtual screening methods. The malachite methanol extract was obtained through maceration. The in ovo study was conducted by injecting free-range chicken eggs with pesticide compounds and methanol extracts at concentrations of 0.5 and 1 ppm. The in silico analysis revealed that ethion and neophytadiene had the most negative G values, at -8.62 and -8.39 kcal/mol, respectively, while the natural ligand 8 LY A 500 was -9.19 kcal/mol. The similarity in type and number of residues between the binding complexes of ethion and neophytadiene ligands and ALK protein suggests the possibility of competition between ethion and neophytadiene when bound to ALK protein. Neofitadiene is suspected of possessing anti-cancer properties by inhibiting the growth of ALK. Kelakai extract is considered to be able to slow down the rate of cell damage in chicken embryos caused by ethion with its inhibitory ability so that the cell surface is not damaged quickly.

Keywords: Anaplastic Lymphoma Kinase, molecular docking, virtual screening, ethion, neophyadiene.

ABSTRAK

Petani sayur di Kalimantan Selatan menggunakan pestisida untuk menjaga tanaman dari serangan hama. Bahan aktif pestisida memiliki efek toksisitas meluas pada organisme target dan non-target. Paparan pestisida yang terus menerus menyebabkan penyakit kanker. Tumbuhan kelakai (Stenochlaena palustris) diduga memiliki potensi aktivitas sitotoksik terhadap pertumbuhan sel kanker. Penelitian ini bertujuan mengkaji potensi aktivitas senyawa kimia kelakai pada protein Anaplastic Lymphoma Kinase (ALK) yang terpapar pestisida organofosfat secara in silico dan in ovo. Kajian in silico menggunakan metoda molecular docking dan virtual screening. Ekstrak metanol kelakai didapatkan dengan metode maserasi. Kajian in ovo dilakukan dengan menginjeksikan senyawa pestisida dan ekstrak metanol kelakai dengan dosis 0,5 dan 1 ppm terhadap telur ayam kampung. Hasil analisis in silico menunjukkan bahwa ethion dan neofitadiena mempunyai nilai ΔG paling negatif yaitu masing-masing sebesar -8.62 kkal/mol dan -8.39 kkal/mol, sedangkan ligan alami 8 LY A 500 sebesar -9.19 kkal/mol. Kemiripan jenis dan jumlah residu pada komplek ikatan antara ligan ethion dan neofitadiena dengan protein ALK menunjukkan potensi adanya kompetisi antara ethion dan neofitadiena ketika berikatan dengan protein ALK. Neofitadiena diduga sebagai senyawa yang berpotensi sebagai anti-kanker dengan menghambat pertumbuhan ALK. Ekstrak kelakai dianggap mampu memperlambat laju kerusakan sel pada embrio ayam yang disebabkan oleh ethion dengan kemampuan inhibitornya sehingga permukaan sel tidak cepat rusak.

Keywords: Anaplastic Lymphoma Kinase, docking molekular, skrining virtual, ethion, neofitadiena.

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1. INTRODUCTION

Vegetable farmers in South Kalimantan use pesticides to protect their crops from pest attacks. Pesticides are used to eradicate pests and weeds or nuisance plants. Apart from its benefits, pesticides have the potential to poison and eradicate other living creatures, including beneficial plants and insects, animals, and humans. The active ingredients in pesticides have widespread toxicity effects, affecting target and non-target organisms, humans, the environment, and the ecosystem (Souisa *et al.*, 2020). Individual organisms exposed to pesticides have a greater chance of developing cancer. The most common cancers caused by pesticides are blood cancer (leukemia), non-Hodgkins lymphoma, and brain cancer (Pamungkas, 2017). Cancer can be prevented by consuming vegetable plants (Marisa *et al.*, 2021).

Kelakai, scientifically known as *Stenochlaena palustris*, is a species of fern that exhibits robust growth in regions characterized by high levels of humidity, such as the peatlands in South Kalimantan (Nurinayah *et al.*, 2016). The Kelakai species exhibits a broad geographical range that remains mostly untapped and has not been cultivated. Kelakai is mainly employed in culinary applications and serves as a dietary component for the proboscis monkey *Nasalis larvatus* (Iskandar *et al.*, 2017). According to Maharani *et al.* (2000), the compound composition of kelakai has been associated with numerous health advantages. According to Erwin *et al.* (2016), the ethanol, n-hexane, and ethyl acetate fractions derived from the kelakai leaf extract contained alkaloid, steroid, and flavonoid components. Extracts from the n-hexane fraction and ethyl acetate fraction of kelakai herb had cytotoxic activity against cultured HepG2 liver cancer cells with an IC_{50} value of 865.84; 568.38 and 224.12 μg mL^{-1} (Yanti *et al.*, 2021). One method for extracting kelakai involves using the maceration technique, wherein the substance is soaked in a solvent. According to Damayanti and Fitriana (2013), the maceration method is widely regarded as an efficient approach for extracting substantial quantities. This process utilizes a relatively small amount of solvent, minimizing the potential for compound destruction. Additionally, the equipment required for maceration is simple.

Several studies have examined the relationship between pesticides and cancer risk (Agustin & Muhartono, 2018; Soeroso & Ananda, 2019). However, the precise process remains unclear. The potential impact of kelakai plants on the risk of cancer has been investigated in previous studies (Mashar & Annah, 2020; Margono *et al.*, 2016). However, further research is required to obtain additional insights in this area. Meanwhile, there is a lack of research investigating the cytotoxic effects of kelakai extract as a potential anticancer agent in both in silico and in ovo models. The present in silico investigation examined the possible carcinogenicity of organophosphate insecticides and the potential inhibitory effects of kelakai extract on the proliferation of cancer cells. An in-ovo investigation was undertaken on the proliferation of viable free-range chicken egg cells due to exposure to organophosphate insecticides and kelakai extract.

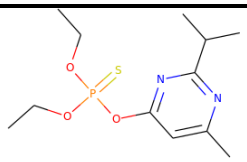
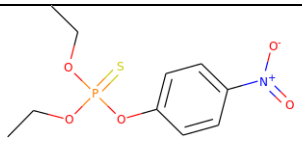
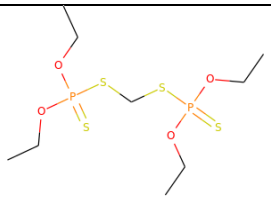
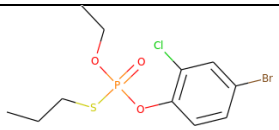
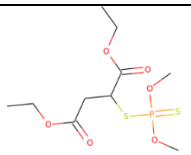
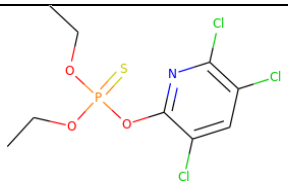
2. MATERIALS AND METHODS

2.1. Materials

The tools employed in the study comprised of a rotary evaporator, water bath, syringe, surgical scissors, tweezers, beaker apparatus, cellphone camera, and an egg incubator. The hardware used was a laptop connected to the internet. The software used were Windows 11 64-bit, USCF Chimera 1.16 program (https://www.cgl.ucsf.edu/chimera/download.html), Protein Data Bank database (https://www.rcsb.org/), PubChem database (https://pubchem.ncbi.nlm.nih.gov/), SwissDock web server (http://www.swissdock.ch/), pkCSM web server (http://biosig.unimelb.edu .au/pkcsm/prediction), and the web server Protox online tool (https://tox-new.charite.de/protox_II/).

The research material was kelakai plant taken from Banjarbaru, South Kalimantan. Other ingredients were n-hexane, methanol, 70% alcohol, Mition brand ethion pesticide, distilled water, and fertile free-range chicken eggs. The 3D structure of the docking target protein, Anaplastic Lymphoma Kinase (ALK) (Cao *et al.*, 2019), PDB code 5USQ, and native ligand 8LY A 500 were obtained from RCSB PDB. The organophosphate pesticide and kelakai compounds' structures were obtained from the PubChem database. Ten organophosphate compounds are presented in Table 1, and ten ligand compounds from kelakai extract are presented in Table 2 (Nurmilatina, 2017).

Table 1. The active ingredient compound of organophosphate pesticides

No	Compound Name	PubChem CID	Smiles Formula	Structure
1	Diazonin	3017	CCOP(=S)(OCC)OC1=NC(=NC(=C1)C)C(C)C	
2	Parathion	991	CCOP(=S)(OCC)OC1=CC=C(C=C1)[N+](=O)[O-]	
3	Ethion	3286	CCOP(=S)(OCC)SCSP(=S)(OCC)OCC	
4	Profenofos	38779	CCCSP(=O)(OCC)OC1=C(C=C(C=C1)Br)Cl	
5	Malation	4004	CCOC(=O)CC(C(=O)OCC)SP(=S)(OC)OC	
6	Kloropirifos (Dursban)	2730	CCOP(=S)(OCC)OC1=NC(=C(C=C1Cl)Cl)Cl	

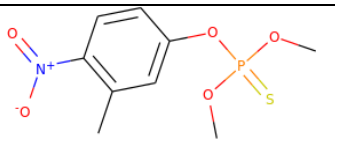
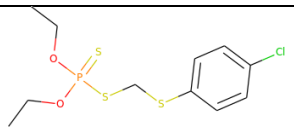
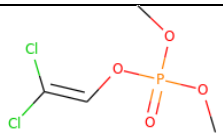
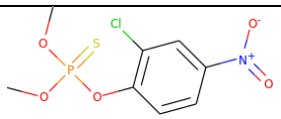
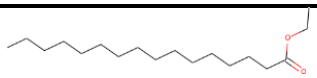
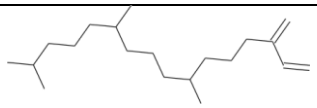
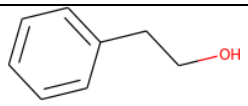
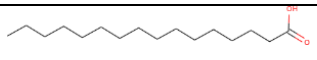
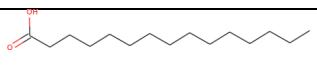
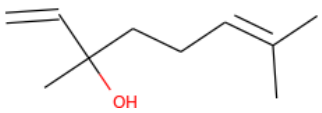
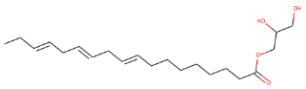
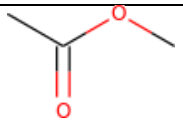
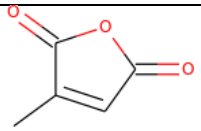
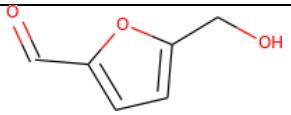
7	Fenitrothion	31200	CC1=C(C=CC(=C1)OP(=S)(OC)OC)[N+](=O)[O-]	
8	Carbopheno-thion	13081	CCOP(=S)(OCC)SCSC1=CC=C(C=C1)Cl	
9	Dichlorvos	3039	COP(=O)(OC)OC=C(Cl)Cl	
10	Dicapthon (Dicaptan)	17168	COP(=S)(OC)OC1=C(C=C(C=C1)[N+](=O)[O-])Cl	

Table 2. Ligand compounds from kelakai extract

No	Compound Name	PubChem CID	Smiles Formula	Structure
1	Hexadecanoic acid ethyl ester	12366	CCCCCCCCCCCCCCC(=O)OCC	
2	Neophytadiene	10446	CC(C)CCCC(C)CCCC(C)CCCC(=C)C=C	
3	Phenethyl alcohol	6054	C1=CC=C(C=C1)CCO	
4	Hexadecanoic acid	985	CCCCCCCCCCCCCCC(=O)O	
5	Pentadecanoic acid	13849	CCCCCCCCCCCCCCC(=O)O	

6	Linalool	6549	CC(=CCCC(C)(C=C)O)C	
7	Linoleic acid	5280450	CCC=CCC=CCC=CCCCCCCC(=O)OCC(CO)O	
8	3-Methyl acetate (3-methyl acetate)	6584	CC(=O)OC	
9	3-metil-2,5-furandion (Citraconic anhydride)	12012	CC1=CC(=O)OC1=O	
10	5-hidriksimetil-2furankarboksaldehid	237332	C1=C(OC(=C1)C=O)CO	

2.2. In silico analysis

The ALK protein structure (PDB ID: 5USQ) was downloaded from the RCSB PDB site in *.pdb format. Proteins were prepared using the UCSF Chimera 1.16 program by eliminating all residues. The natural ligand, 8LY, was isolated from the protein and stored in *.mol2 format. Organophosphate pesticide ligands and kelakai compound ligands were also prepared using UCSF Chimera 1.16. Molecular docking using the SwissDock web server. The docking parameter taken was the Gibbs free energy value (ΔG). The visualization of docking results was conducted using the USCF Chimera 1.16 software to illustrate the specific interactions between the ligand and residues of the target protein. The virtual screening process used the pkCSM and ProTox web servers.

2.3. Kelakai extraction

A total of 250.0 grams of kelakai powder was carefully measured and subsequently combined with 1.0 mL of 95% methanol. The resulting mixture was subjected to a maceration process, which involved three cycles of 24 hours each. The mixture was subjected to filtration to isolate a filtrate, which was subsequently concentrated to one-fourth of the original solvent volume using a rotary evaporator. The evaporation process was then continued using a water bath. The methanol filtrate, which was concentrated, was next subjected to partitioning using a solvent of n-hexane in order to extract the n-hexane fraction of kelakai (Damayanti & Fitriana, 2013).

2.4. In ovo activity assay

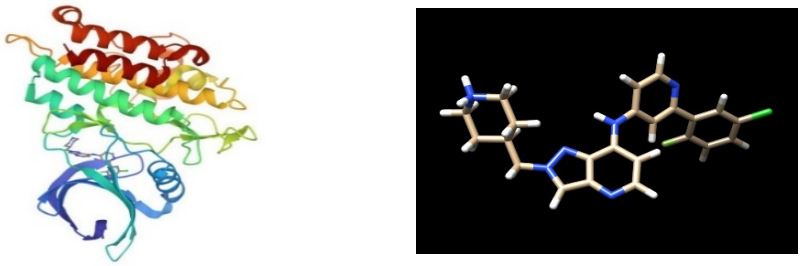
A total of twenty-four chicken eggs, which were viable and obtained from free-range sources, were allocated into three distinct groups. Group 1 served as the untreated control group, Group 2 received injections of organophosphate pesticides, and Group 3 received injections of organophosphate pesticides along with methanol extract derived from kelakai plants. The eggs underwent a process of initial cleansing with running water, followed by sterilization with a 70% alcohol solution. Subsequently, the eggs were carefully transferred to an incubator for further development. Embryonic day 0 (E0) refers to the specific day the egg is introduced into the incubator. The eggs were subjected to incubation at a temperature of 37.8°C, with 55% humidity.

Group 1 was resolved at embryo ages E0, E3, E7, E8, E9, E11, E14, E17, and E21. The eggs were transferred to a petri dish using tweezers and scissors to observe the embryo's development. Groups 2 and 3 were tested on eggs on day 9 (E9) of the incubation period. The test solution was administered into the air cavity of the egg at concentrations of 0.5 ppm and 1.0 ppm, utilizing a sterile syringe for injection. Following the injection, the aperture was subsequently sealed using plaster. After 6, 12 and 24 hours, it was observed to monitor changes in blood cell growth and toxic symptoms in chicken embryos.

3. RESULTS AND DISCUSSION

3.1. Docking Molecular

The process of preparing target proteins and ligands for molecular docking was conducted with UCSF Chimera 1.16. The target protein was anaplastic lymphoma kinase (ALK), located on the short arm of chromosome 2 (2p23) and exchanges segments of the chromosome with other genes. This protein contributes to the pathogenesis of various hematological malignancies and solid tumors, including lymphoma, lung cancer, inflammatory myofibroblastic tumor (IMT), Spitz tumor, renal carcinoma, thyroid cancer, gastrointestinal cancer, breast cancer, leukemia and ovarian carcinoma (Cao *et al.* al., 2019). Figure 1a depicts the three-dimensional configuration of ALK. The 8LY A 500 ligand was extracted from the target protein and subjected to energy minimization for preparation. Figure 1b displays the 3D structural representation of the 8LY A 500 ligand.



(a) (b)

Figure 1. 3D structure of (a) protein ALK, (b) ligand 8LY A 500

The molecular docking analysis was conducted using the SwissDock website to examine the interaction between the ALK receptor protein (PDB ID: 5USQ) and a set of 10 pesticide compound ligands, as well as another set of 10 compound ligands found in kelakai. Additionally, the docking analysis included the natural ligand 8LY A 500 (Figure 1b). The parameters resulting from docking were Gibbs free energy (ΔG) values and interactions between ligand bonds and amino acid residues. The molecular docking data between the ligand and the target protein are presented in Table 3. The Gibbs free energy (ΔG) demonstrates the robust affinity between the protein and the ligand. A ΔG value that is increasingly negative (low) indicates that the compound requires less energy when binding occurs. This indicates that this compound has a greater potential and activity to interact with and establish strong bonds with its target proteins (Rena *et al.*, 202).

Table 3. Results of molecular docking of Anaplastic Lymphoma Kinase (ALK) ligand and protein

Ligand	ΔG (kkal/ mol)	Amino Acid Residues	
		Hydrogen Bond	Hydrophobic Interactions
Diazonin	-8.16	-	LEU260, GLY286, LEU340, ALA230, VAL219, SER280, ALA350, TYR249, PHE262, ASP351, LYS232, ILE211, ALA 230, GLU245.
Parathion	-7.68	-	TYR249, LEU260, ASP351, LEU340, GLY286, ASP281, TYR282, ALA230, SER280, VAL219, LYS232, LEU278

Ligand	ΔG (kkal/ mol)	Amino Acid Residues	
		Hydrogen Bond	Hydrophobic Interactions
Ethion	-8.62	-	TYR282, GLY286, SER287, LEU340, ILE211, ALA230, VAL279, LYS232, LEU278, GLU245, ASP351, LEU260, SER280, VAL231, TYR282
Profenofos	-8.05	-	SER280, LEU340, LEU260, TYR282, ALA230, ALA350, VAL219, LYS232, ILE 211, ASP281, ASP351, GLY286, LYS337.
Malation	-7.95	-	SER280, LEU340, LEU260, TYR282, TYR249, PHE262, ALA230, VAL219, LYS232, VAL231, LEU278, GLU245, PHE262.
Kloropirifos (Dursban)	-7.63	-	GLY286, LEU340, TYR282, ALA350, ASP351, ILE211, VAL219, LYS232
Fenitrothion	-7.59	ASP351 (2.261 Å)	SER280, LEU340, TYR282, ALA230, ALA350, VAL219, LYS232, ILE 211, ASP281, ASP351, GLY286, LY232, TYR249.
Carbopheno-thion	-8.24	-	SER280, LEU278, TYR282, ALA230, VAL219, LYS232, ILE 211, PHE262, GLY286, TYR249, LEU260, LEU340.
Dichlorvos	-7.40	ALA380 (2.129 Å)	ALA403, ALA399, ASP400, LEU334, TYR378, THR375, ASP333, GLY374, VAL373, ARG332, VAL383, MET379
Dicapthon	-8.16	ASP351 (2.426 Å)	ALA350, LEU340, GLY286, LEU260, ASP281, TYR282, ILE211, VAL219, ALA230, LYS232, TYR249.
Hexadecanoic acid ethyl ester	-7.08	LYS337 (1.878 Å)	ILE211, ALA230, ASP351, LEU340, ASN338.
Neophytadiene	-8.39	-	ILE211, VAL219, LYS232, LEU278, ALA278, ALA230, TYR282, SER280, PHE262, TYR249, GLU284, LEU260, ASP351, GLY286, ALA350, LEU 340.
Phenethyl alcohol	-6.55	ARG332 (2.011 Å)	MET379, VAL383, THR375, ALA399, GLY374, VAL373, ASP400, LEU334, ALA403, TYR378.
Hexadecanoic acid	-8.09	-	ILE211, VAL219, LYS232, LEU278, ALA350, ALA230, TYR282, SER280, PHE262, TYR249, LEU360, ASP351, ASP290, GLY286, ALA350, LEU 340, ARG294.
Pentadecanoic acid	-8.00	-	ILE211, VAL219, LYS232, LEU278, ALA330, ALA230, TYR249, GLU245, LEU260, ASP351, SER280, GLY286, TYR282.
Linalool	-6.90	TYR378 (1.853 Å)	THR375, GLY374, ASP333, MET379, VAL383, ASP400, ALA403, ALA380, ALA399, ILE388, ARG372, VAL373.
Linoleic acid	-8.01	-	ILE211, VAL219, LYS232, LEU278, ALA230, TYR282, SER280, LEU260, PHE262, GLY286, LEU340, TYR249, SER287, ASP290, PHE289, LYS337.
3-Methyl acetate	-5.88	-	MET379, VAL383, THR375, ALA399, ARG332, ASP333, LEU334, ALA403, TYR378.
3-methyl-2,5-furandione	-6.54	THR375 (2.210 Å)	MET379, VAL383, THR375, ALA399, ARG332, ASP400, LEU334, ALA403, TYR378, GLY374, ASP333.
5-hydroxy methyl-2-furan carboxaldehyde	-6.58	VAL373 (2.196Å) dan ALA403 (2.196 Å)	MET379, VAL383, THR378, ALA399, ARG332, ASP400, LEU334, TYR378, GLY374.
Natural ligand 8LYA 500	-9.19	-	ILE211, VAL219, VAL231, VAL279, LYS232, LEU278, ALA230, TYR282, SER280, LEU340, ASP281, PHE262, GLY286, ASP351, TYR249, GLU284, GLY286, ARG294.

The molecular docking data of 10 pesticide ligands, 10 chemical compounds, and natural ligands against the ALK protein are presented in Table 3. The results of the study revealed that ethion and neophytadiene exhibited the most pronounced negative ΔG values, specifically -8.62 kcal/mol and -8.39 kcal/mol, respectively. According to Suhadi *et al.* (2019), a higher magnitude of the ΔG value indicates a greater strength in the link between the target protein and ligand. This can be attributed to the enhanced stability and strength of noncovalent contacts. It is hypothesized that ethion possesses a higher propensity to induce detrimental effects on the ALK protein receptor compared to alternative insecticides. Despite the fact that the ΔG value of neophytadiene remains higher than that of the natural ligand (8LY A 500) for the target protein, the observed ΔG value exhibits a rather small disparity. Neophytadiene exhibits the capability to displace endogenous ligands and function as a competitive inhibitor.

It is hypothesized that neophytadiene exhibits potential anticancer properties through its ability to inhibit the ALK protein. ALK is involved in the process of signal transduction. The initiation of this process involves the activation of kinases located on the cellular membrane and the subsequent formation of kinase complexes by dimerization. The subsequent step after dimerization is phosphorylation, wherein phosphate groups are transferred from one protein to another within the signal transduction pathway. The transfer of phosphate groups plays a crucial role in regulating the transmission of signals from the cell surface to the nucleus, thereby dictating cellular processes such as proliferation, division, differentiation, apoptosis, and metastasis. The occurrence of mutations in the ALK protein leads to the activation of autophosphorylation activity within the signal transduction system, hence promoting uncontrolled cellular proliferation and facilitating cancer progression. ALK mutations have been observed in various types of cancer, such as lung cancer, neuroblastoma cancer cells, anaplastic large-cell lymphoma, and NSCLC (Ridwanuloh, 2017).

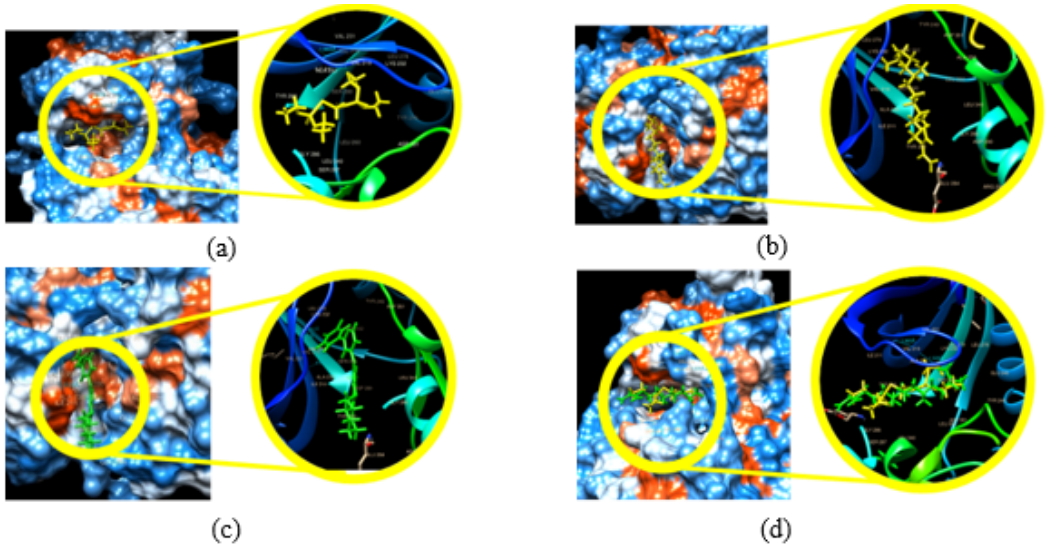


Figure 2. Molecular docking results with Anaplastic Lymphoma Kinase (ALK) protein (a) Ethion; (b) Neophytadiene (c) 8LY A 500; (d) Neophytadiene and ethion

The visualization of the molecular docking data is depicted in Figure 2. The ethion ligand exhibits binding affinity towards 15 specific residues of the ALK protein, which include TYR282, GLY286, SER287, LEU340, ILE211, ALA230, VAL279, LYS232, LEU278, GLU245, ASP351, LEU260, SER280, VAL231, and TYR282. Neophytadeien exhibits binding affinity towards 16 anaplastic lymphoma kinase (ALK) residues, namely ILE211, VAL219, LYS232, LEU278, ALA278, ALA230, TYR282, SER280, PHE262, TYR249, GLU284, LEU260, ASP351, GLY286, ALA350, LEU 340. The binding location of ethion and neophytadiene with ALK protein was right in the active site, which has similarities in the interaction of the amino acid residues of the natural ligand 8LY A 500 with the ALK protein. The binding site, also known as

the active site, refers to a specific region on the protein molecule that serves as the location of interaction between the protein and its ligand. The similarity in the type and number of residues in the binding complex between ethion and neophytadiene ligands and the ALK protein indicates the potential for competition between ethion and neophytadiene when binding to the ALK protein.

3.2. In silico pharmacotoxicity test

Virtual pharmacotoxicity test using pkCSM and Protox web servers with parameters Ames Toxicity, Hepatotoxicity, Skin Sensitization, LD50Acute (mg/kg) and Class. The analysis results are presented in Table 4.

Table 4. Results of pharmacokinetic and toxicity prediction analysis of ligands

Compound Name	Ames Toxicity*	Hepatotoxicity*	Skin Sensitization*	LD_{50}Acute** (mg/kg)	Class**
Diazonin	No	Yes	No	17	2
Parathion	No	No	No	19	1
Ethion	No	No	No	13	2
Profenofos	No	No	No	162	3
Malation	No	No	No	190	3
Kloropirifos	No	No	No	60	3
Fenitrothion	Yes	No	No	229	3
Carbopheno-thion	No	No	No	7	2
Dichlorvos	Yes	No	Yes	17	2
Dicapthon	Yes	No	No	284	3
Hexadecanoic acid ethyl ester	No	No	Yes	5000	5
Neophytadiene	No	No	Yes	5050	6
Phenethyl alcohol	No	No	Yes	800	4
Hexadecanoic acid	No	No	Yes	900	4
Pentadecanoic acid	No	No	Yes	900	4
Linalool	No	No	Yes	2200	5
Linoleic acid	No	No	Yes	10000	6
3-Methyl acetate	No	No	No	5000	5
3-methyl-2,5-furandione	No	No	Yes	2600	5
5-hydroxy methyl-2-furan carboxaldehyde	No	No	No	2500	5
Natural ligands	No	Yes	No	200	3

Note: pkCSM (*) and Protox (**) online tool

Toxicity prediction aims to predict the level of toxicity of compounds in the human body. The LD_{50} toxicity parameter is classified based on the Globally Harmonized System (GHS), divided into toxicity classes I to VI. The six toxicity classes use LD_{50} thresholds of 5, 50, 300, 2000, and 5000 mg/kg body weight with the following details. Class I (fatal if swallowed) has an LD_{50} value range of ≤ 5 mg/kg. Class II (fatal if swallowed) has a value range of $5 < LD_{50} \leq 50$ mg/kg. Class III (toxic if swallowed) has a value range of $50 < LD_{50} \leq 300$ mg/kg. Class IV (dangerous if swallowed) has a value range of $300 < LD_{50} \leq 2000$ mg/kg. Class V (can be dangerous if swallowed) has a value range of $2000 < LD_{50} \leq 5000$ mg/kg and class VI (non-toxic) has a value range of $LD_{50} > 5000$ mg/kg. The Ames toxicity test was employed to assess the mutagenesis capacity of various chemicals using bacterial organisms. According to Naufa *et al.* (2022), a positive substance exhibiting Ames toxicity demonstrates mutagenic properties and has the potential to function as a carcinogen.

Table 4 presents the results of the toxicity test conducted using pkCSM Online Tools. The findings indicate that ethion and neophytadiene exhibited no toxicity in the Ames toxicity and hepatotoxicity assessments. Neophytadiene in skin sensitization analysis shows a positive value,

so it is considered to cause skin sensitization. The natural ligand (8LY A 500) has no toxicity in the Ames toxicity and skin sensitization analysis. However, it has hepatotoxicity, so it can be predicted that the natural ligand is toxic to the target protein (ALK). Lethal dose 50 (LD_{50}) toxicity analysis reveals that the Ethion compound has an LD_{50} value of 13 mg/kg; therefore, it is classified as class II (fatal if ingested): 5 LD_{50} 50 mg/kg, indicating that the compound has a high toxicity effect. Therefore, ethion has activator activity against the ALK protein. The natural ligand (8LY A 500) has an LD_{50} value of 200 mg/kg, placing it in class II (toxic if ingested): 50 LD_{50} 300 mg/kg, indicating the compound has a relatively high toxicity effect. The LD_{50} value of the neophytadiene compound is 5050 mg/kg, so it is classified in class 6 class VI (non-toxic): LD_{50} > 5000 mg/kg, indicating a very minimal toxicity effect. According to the LD_{50} value, the greater the toxic value, the less toxic a substance is, and vice versa (Faqiha *et al.*, 2022).

3.3. Kelakai extract

Kelakai (*Stenochlaena palustris*) was extracted using a maceration technique, repeated three times for 24 hours each. A total of 17.60 grams of methanol extract and 27.43 grams of n-hexane fraction derived from kelakai were acquired. Subsequently, the extract underwent analysis with GC-MS (Gas Chromatography Mass Spectrometry). The study revealed the presence of 10 peaks in the methanol extract and 20 peaks in the n-hexane kelakai fraction. The predominant constituent within the kelakai methanol extract was at peak 4, exhibiting a retention area value of 30.39% and a retention time of 32.026. Peak 4 potentially comprises of 9-octadecenoic acid (Z)- and its methyl ester (CAS). In the kelakai extract of n-hexane, the most prominent chemical constituent was observed at peak 13, with a retention area value of 19.21% and a retention time of 32.031. Peak 13 is believed to contain the following compounds: Cyclopentanemethylamine, 2-isopropylidene-N,N,5-trimethyl-, and (1R,5R)-(-)- (CAS). The outcomes of the extraction process are depicted in Figure 3. The methanol extract's results were afterward utilized for further in-ovo examinations concerning the exposure of fertile chicken eggs.

(a) (b)

Figure 3. Extraction results (a) kelakai methanol; (b) n-hexane kelakai

3.4. In ovo cytotoxic activity assay

The developmental progression of free-range chicken embryos from 0 to 21 days is depicted in Figure 4. Figure 4a depicts a day 0 embryo, whereby the zygote blastoderm cells are situated in the central region, characterized by its white appearance. Figure 4b depicts the presence of red lines on the blastoderm, signifying the initiation of the blood circulation system. The observation of embryonic development is facilitated by the extended morphology of the central blastoderm (Fitriani *et al.*, 2021). After an incubation period exceeding 7 days, it becomes evident that the limbs have undergone development, the eyes have become prominent, and the limbs have formed, as seen in Figure 4c. The maturation of the embryo's organs has reached its culmination, exhibiting a progressive manifestation of the structural formation of the avian embryo's organs. According to the findings of Kusumawati *et al.* (2016), it has been established that the bodily organs of chicken embryos exhibit a significant level of development during an incubation period beyond 7 days. A duration of incubation beyond 10 days signifies the existence of cerebral matter, a beak structure, feathers, and wings (Figures 4d, 4e, and 4f). The process of achieving optimal organ development commences on the fifteenth day and continues after that (Figures 4g, 4h, and 4i). Negative control observations (without treatment) showed good results from 0 to 21 days until the chicks were ready to hatch. Embryo development is normal, and there are no growth disorders.

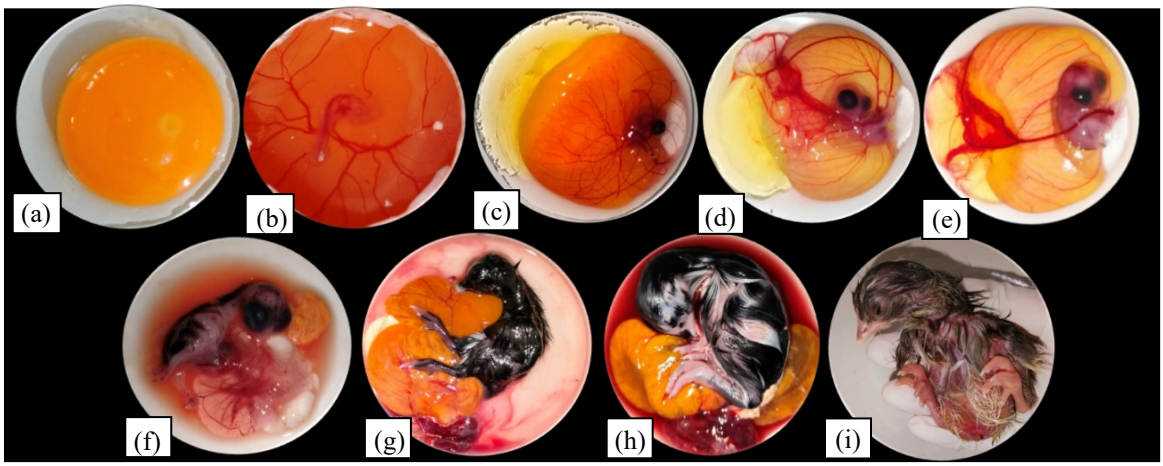


Figure 4. Negative control test results (a) 0-day embryo (b) 3-day embryo (c) 8-day embryo; (d) 9-day embryo; (e) 10-day embryo; (f) 12-day embryo; (g) 15-day embryo; (h) 18-day embryo; (i) 21-day

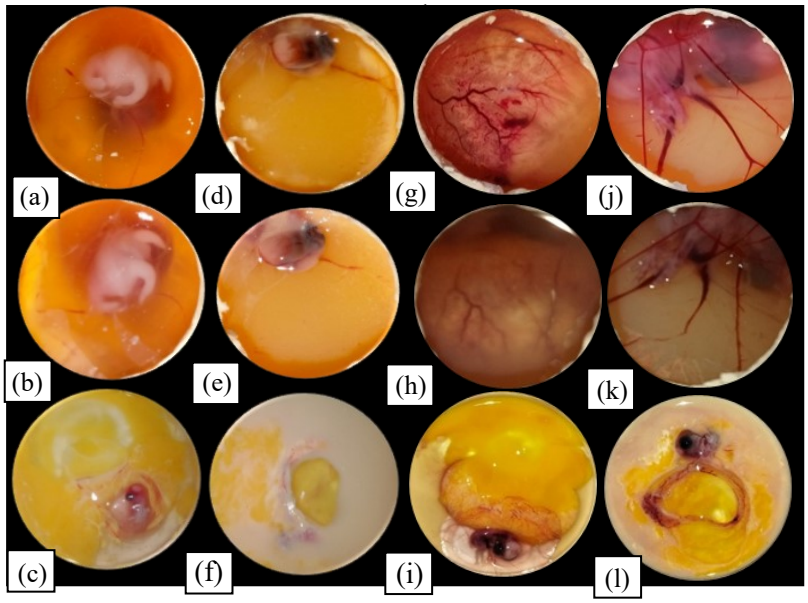


Figure 5. Activity test results for ethion (E) and methanol extract (M) (a) E 0.5 ppm, 6 hours; (b) E 0.5 ppm, 12 hours; (c) E 0.5 ppm, 24 hours; (d) E 1 ppm, 6 hours; (e) E 1 ppm, 12 hours; (f) E 1 ppm, 24 hours; (g) ME 0.5 ppm, 6 hours; (h) ME 0.5 ppm, 12 hours; (i) ME 0.5 ppm, 24 hours; (j) ME 1 ppm, 6 hours; (k) ME 1 ppm, 12 hours; (l) ME 1 ppm, 24 hours.

Observations pertaining to the development of chicken eggs injected with ethion concentrations of 0.5 and 1 ppm are presented in Figure 5. Figures 5a, 5b and 5c depict alterations in the cellular surface of the embryo characterized by the presence of numerous white spots. These aberrations lead to irregular cell aggregation, causing anomalous embryonic development and potentially resulting in embryonic mortality. Furthermore, it should be noted that the presence of ethion can lead to the formation of residues within egg yolk. This, in turn, could impede the proper growth and development of chicken embryos, ultimately manifesting developmental abnormalities. This is primarily due to the fact that egg yolk serves as a vital source of nutrition for these embryos, as depicted in Figures 5d, 5e, and 5f. Therefore, exposure to ethion induces alterations in embryonic development compared to the development of control embryos. The combined administration of kelakai methanol extract and ethion demonstrated superior outcomes compared to the administration of ethion alone, as seen in Figures 5g, 5h, and 5i. The inclusion of kelakai extract is hypothesized to possess compensatory effects on cellular damage induced by ethion through its inhibitory properties, hence mitigating excessive damage to the cell surface. This is evident from the observed presence of residual ethion on the cell surface, albeit in minimal

quantities, as depicted in Figures 5j, 5k, and 5l. The confirmation of this assertion is supported by the in silico analysis of neophytadiene and ethion compounds utilizing the ALK target protein. The results indicate that both compounds exhibit binding interactions with around 15-16 amino acid residues at a shared binding region. Consequently, a competitive interaction arises between the two entities as they vie for occupancy on the active site of the target protein. Hence, it is plausible that ethion and neophytadiene exhibit comparable affinities for cellular binding, as shown by Dewi (2020) and Firdaus *et al.* (2021).

4. CONCLUSION

Ethion, an organophosphate pesticide, is considered highly toxic and can potentially cause cytogenic effects with ΔG= -8.62 kcal/mol. The compound neophytadiene, derived from kelakai, exhibits promising potential as a competitive inhibitor of the Anaplastic Lymphoma Kinase (ALK) protein, with ΔG value of -8.39 kcal/mol. The observation of a shared pattern and quantity of binding residues between ethion and neophytadiene inside the ALK protein suggests a competitive relationship between these two compounds. It is hypothesised that the kelakai extract possesses the capability to decelerate the cellular deterioration in chicken embryos induced by ethion.

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