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RESEARCH REPORT

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DEVELOPMENT OF HALAL PHARMACEUTICAL PRODUCTS FROM *Chrysophyllum cainito* L. FOR THE TREATMENT OF OSTEOARTHRITIS

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CHAPTER I
INTRODUCTION

1.1 BACKGROUND

Osteoarthritis (OA) affects 240 out of every 100,000 people in Indonesia every year. This number goes up with age: 5% at age 40, 30% at age 40–60, and 65% at age 61 or older (Abdurrachman, Nurseptiani, and Adani, 2019). OA affects 15.5% of men and 12.7% of women in Indonesia (Siddik and Haryadi, 2020). The global prevalence of OA is 16% for those over the age of 16 and 22.9% for those over the age of 40. The incidence rate is 203:10,000 for adults over the age of 20, and 1:69 and 1:39, respectively, for men and women aged 70–79 years (Cui et al., 2020). This disease is the 12th most common disease in the world, and it affects the muscles and bones. It is anticipated that the prevalence of OA will double by 2020 as the population ages (Abdurrachman, Nurseptiani, and Adani, 2019).

Currently, the majority of OA therapies consist of glucosamine and chondroitin supplements. Utilized commonly are glucosamine and chondroitin derived from numerous sources, including porcine, bovine, shark, whale, bird, crustacean, and arthropod shells (Cantley, Rainsford, and Haynes, 2013; Rahma, Wulandari, and Nisa, 2019). Glucosamine is an amino-monosaccharide that is made in the body from glucose. It is used to make the glycoproteins and glycosaminoglycans that make up joint cartilage. When administered orally, glucosamine is considered a medicinal product of the class of symptomatic slow-acting drugs for OA, by inhibiting matrix metalloproteinase production and chondrocyte apoptosis and stimulating haeme oxygenase-1 (HO-1), a key enzyme regulating oxidative stress (Bannuru et al., 2019; Conrozier et al., 2019; Conrozier and Lohse, 2022). However, chondroitin increases joint glycosaminoglycan (GAG) concentrations, increases joint fluid viscosity, and heals joint structures (Kalim et al., 2018). Chondroitin works as an anti-inflammatory drug for OA by blocking the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and lowering the biomarkers of inflammation (inflammatory mediators) IL-1, IL-6, and tumor necrosis factor-alpha (TNF-alpha) (Singh et al., 2012).

The sources of glucosamine and chondroitin that are currently the most popular cause issues. In the decree of the Indonesian Religious Leader (MUI) number SKO8/Dir/LPPOM MUI/II/13, it is explained that glucosamine and chondroitin are required to be halal certified because they belong to the protein and XXIV groups. The types of products include: protein, amino acids, collagen, chondroitin, glucosamine, and so on (Kemenag, 2014). This reflects the teachings of Islam. Consuming what is lawful, holy, and good is a religious commandment,

and the law is obligatory. As stated in the Qur'an: "Hey, humans! Eat what is lawful and good from what is on earth, and do not follow the steps of the devil; for verily, the devil is a real enemy to you." (Surah al-Baqarah: 168) and the other verse: "O faithful! Eat of the good sustenance that we have given you and be grateful to Allah, if it is only Him you worship." (Surah al-Baqarah: 172).

Based on the decree of the MUI, the use of glucosamine and chondroitin derived from pigs is haram for Muslims. And if sharks and whales were used to make glucosamine and chondroitin, it would hurt the environment, and crab crustaceans and arthropods could cause patients to have life-threatening allergic reactions (Rahma, Wulandari, and Nisa, 2019). One alternative that can be used is to formulate herbal medicine from plants in the form of OA drugs that have anti-inflammatory and antinociceptive effects. In Indonesia, the use of plants as a treatment is widely carried out. This is due to the advantages of treatment by utilizing plants, including low side effects on plants that are used as drugs, which are relatively smaller when used correctly and appropriately, and complementary and synergistic effects in plant compounds (Harefa, 2020). One of the plants that can be used as traditional medicine to treat OA is kenitu (*Chrysophyllum cainito* L.) because of its high content of phytoestrogens (Ma'arif et al., 2019).

The bioinformatics test for anti-inflammatory and antinociceptive activity of *C. cainito* leaf extract was conducted in addition to in vivo assays. The in silico work commenced with metabolite profiling of *C. cainito* leaf extract using liquid chromatography–double mass spectrometry (LC-MS/MS) to forecast the chemical composition of *C. cainito* leaves. Effective and efficient usage of the LC-MS/MS instrument is demonstrated by its ability to provide good chromatographic resolution, boost analysis speed and sensitivity, save time, and decrease solvent consumption (Patil et al., 2011). On metabolite profiling compounds from *C. cainito* leaf extract, molecular docking and analysis of docking results were performed against the cyclooxygenase-2 (COX-2) receptor, using protein X-rays from the protein data bank (PDB) with the codes 3LN1 and 1PXX. Using PyRx 0.8 and Biovia Discovery Studio Visualizer 2016 to collect data on binding affinity, pharmacophore distance, bound amino acid type, and bond type (Muchtaridi et al., 2018). These criteria are utilized to forecast substances that are COX-2 inhibitors (Daina et al., 2017). Using the SwissADME webtool, the physicochemical, pharmacokinetic, and iteraturemics properties of the COX-2 inhibitor molecules were then determined (Pires et al., 2015; Daina et al., 2017). On the other hand, Sprague-Dawley (SD) rats were used to test the anti-inflammatory and antinociceptive effects of *C. cainito* leaf extract by using the writing test and the COX-2 inhibition test with western blot (Chen *et al.,* 2014).

C. cainito leaf extract will be formulated into a herbal tablet, and then the tablet will be evaluated to determine the appropriate formulation according to the guidelines in the Indonesian Pharmacopoeia VI (Kemenkes, 2020). At all stages of research, halal-critical points are constantly monitored. The perfect formula is then registered with the Badan Pengawas Obat dan Makanan (BPOM) as a pharmaceutical product and with the Badan Penyelenggara Jaminan Produk Halal (BPJPH) as a halal pharmaceutical product.

1.2 PROBLEMS OF RESEARCH

The absence of a theory about the halalness of pharmaceutical products from *C. cainito* as an OA treatment raises the question of whether 70% ethanol extract of *C. cainito* can be developed into halal products as an OA treatment that is guaranteed to be halal. This can be answered by observing the solvent residue test variable in the extract; metabolite profiling; in silico study; in vivo study by COX-2 serum observations; formulation in tablet, which includes physical characteristic and stability tests, which still pay attention to the halal critical point at each stage of the research.

1.3 AIMS OF RESEARCH

General

Create and develop a prototype of a halal pharmaceutical product from *C. cainito* as a treatment for OA that is registered with the BPOM and BPJPH.

Specific

1. Screening the chemical content of *C. cainito* leaf extract using the LC-MS/MS metabolite profiling technique.
2. Predicting in silico the anti-inflammatory and antinociceptive effects of *C. cainito* leaf extract on COX-2 receptors (PDB ID's 3LN1 and 1PXX).
3. Proving that the *C. cainito* leaf extract has anti-inflammatory and antinociceptive effects in living Wistar rats by evaluating the extent to which the animals stretch and by assessing the expression of COX-2 receptors using the western blot method.
4. Formulate and evaluate herbal tablets containing *C. cainito* leaf extract.
5. At each level of the research process, assess halal-critical points.
6. Register the prototype with BPOM and BPJPH to obtain a supplement product registration number and halal certificate.

CHAPTER II
LITERATURE REVIEW AND ROADMAP

2.1 LITERATURE REVIEW

2.1.1 *Chrysophyllum cainito* L.

C. cainito is a tropical tree belonging to the family Sapotaceae, and is cultivated as an orchard tree that bears fruit. Depending on where it is found, *C. cainito* is known by several names, including star apple, cainito, caimito, and "kenitu" in Indonesia. (Doan and Thao, 2020; Ma'arif et al., 2021; Sultana et al., 2021). Most *C. cainito* trees grow in areas with low to moderate elevations.

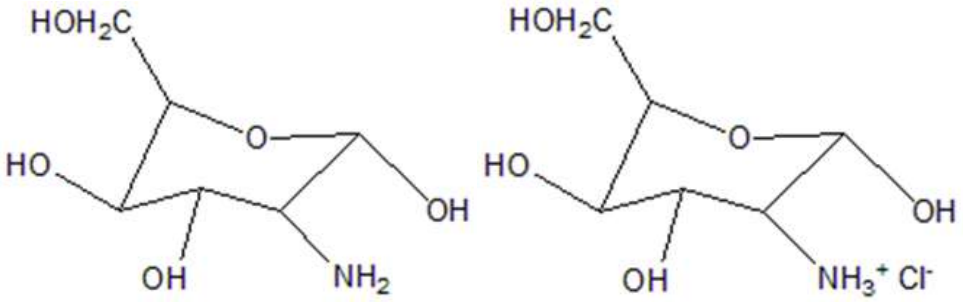
C. cainito leaf have been chemically screened and reported to possess alkaloids, flavonoids, phenols, sterols, and triterpenes (Shailajan and Gurjar, 2014). Phytoestrogen compounds are also found in *C. cainito* leaf extract at a high concentration (Ma'arif et al., 2019). This backs up a study that showed a 96% ethanol extract of *C. cainito* could increase the number of osteoblast cells in the trabecular vertebrae bone of male mice given dexamethasone. This activity occurs due to the presence of phytoestrogen compounds in the 96% ethanol extract of *C. cainito* (Ma'arif and Aditama, 2019; Ma'arif et al. 2021).

Figure 1. *Chrysophyllum cainito* L. (Ma'arif et al., 2019)

2.1.2 Glucosamine

Glucosamine is an endogenous amino-monosaccharide synthesized from glucose that is used in the biosynthesis of glycoproteins and glycosaminoglycans in joint cartilage (Conrozier and Lohse, 2022). It is made from glucose in almost every human tissue. Connective

tissue and cartilage have the most of it. Glucosamine can be extracted from chitin, which is found primarily in the exoskeletons of crustaceans (crabs, prawns, and lobsters), as well as in the cell membranes of mushrooms. Many of these proteoglycans, like hyaluronic acid, iterat sulfate, and iterat sulfate, can't be made without the amino sugar glucosamine. The production of glucosamine is one of the rate-limiting steps in proteoglycan production (Jerosch, 2011). There are two forms of glucosamine: glucosamine sulfate (GS) and glucosamine hydrochloride (GH) (Vasiliadis and Tsikopoulos, 2017).



(a) (b)

Figure 2. Chemical structures of (a) glucosamine sulfate; (b) glucosamine hydrochloride

2.1.3 Chondroitin

Chondroitin sulfate (CS) is one of the natural glycosaminoglycans (GAG) composed of the alternating sugars D-glucuronic acid (GlcA) and N-acetyl D-galactosamine (GalNAc). It is an important component of the extracellular matrix (ECM). CS is the most frequent GAG in the aggrecan molecule of the cartilage. Due to the negative charge of CS, it is responsible for the water retention of the cartilage, which is important for pressure resistance. It can be extracted from the cartilaginous tissue of cows, pigs, birds, and fish (sharks) and is ingested in the diet (Jerosch, 2011).

To treat the symptoms of osteoarthritis (OA), chondroitin sulfate (CS) compounds from a variety of sources are frequently employed, including porcine, bovine, fish, whales, and birds. This is because of a multitude of biological consequences, including inhibition of subchondral bone erosions mediated by the RANKL/OPG pathway, decreased chondrocyte apoptosis; enhanced production of articular cartilage proteoglycans, and decreased inflammatory mediators (Cantley, Rainsford, and Haynes, 2013). Commercially produced CS is made from raw materials derived from terrestrial sources like beef, pork, and chicken as well as marine sources including sharks, skates, and other bony fish. Additionally, a combination of all these sources is feasible, resulting in a CS end product with a variety of traits and qualities. The use

of potentially uncontrolled raw animal materials (tissues, bones, cartilages, but also soft organs) presents the issue of a final product with an uncontrolled structure, poor reproducibility, and an unknown origin, with the resultant biological effects, contaminants, clinical efficacy, and safety being variable grades of purity (Henrotin *et al.*, 2010).

2.1.4 The Principles of Halal in Islam

"Halal" is something that if used does not result in punishment (sin). Meanwhile, haram is something that is forbidden by Allah with a strict prohibition, where people who violate it are threatened with punishment by Allah in the hereafter. According to Islamic teachings, consuming what is lawful, holy, and good is a religious commandment, and the law is obligatory (Putri, 2021).

According to the Majelis Ulama Indonesia (MUI), the causes of prohibition are divided into six categories, namely:

1. Najis (An-Najasah)

   Everything that is unclean is forbidden to be consumed, for example, everything that comes from pigs and dogs, carrion, blood, animals that are not slaughtered according to syar'i, and food that is exposed to the unclean (mutanajjis).
2. Dirty and place (Istiqdzar)

   Some examples of items that are haram because they are dirty and unclean are saliva, semen, maggots, and cockroaches.
3. Harmful to health (Dharar)

   Some examples of materials that are prohibited because they are harmful to health are harmful animals and plants, as well as chemicals that are harmful to health and the body.
4. Unlock (Iskar)

   In this case, intoxicating substances include the use of khamr and alcohol. For the use of khamr and alcohol, there are several policies, namely:

   - Every khamr must contain alcohol, but not all alcohol is categorized as khamr.
   - Khamr is any intoxicating drink, whether from grape juice or anything else.
   - In addition to intoxicating drinks, it is not automatically categorized as khamr, even though it is a liquid, such as syrup.
   - Every khamr is unclean and haram, even if it's a little
   - Some alcohol comes from the khamr industry, and some is pure alcohol.

5. Beasts

   The reason why goods are forbidden is because they come from wild animals, which means animals with sharp fangs and nails.

6. There are arguments against it (‘Adam al-idzn syar’an)

   For example, the item can be forbidden if there is evidence that forbids it. For example, the item is in accordance with the hadith of Aisyah RA: Rasulullah SAW said, “Five animals that can be killed outside or inside the ground are forbidden, namely; snakes, crows, rats, fierce dogs, and eagles.’’ (HR. Muslim)

2.1.5 The Basis for Determining Halal Products

In determining the legal fatwa of halal and haram for an item, MUI uses the following provisions:

1. Determination of fatwa for halal products using the MUI Fatwa Determination System and Procedure, which refers to sources of Islamic law, namely: Al-Quran, Sunnah, Ijma’, and Qiyas.
2. Special Principles for Determining Fatwa for Halal Products
   - Using the opinion of moderate scholars (tawassuth) not harsh and rigid (tasyaddud) or too easy (tasahhul).
   - In terms of determining product halalness, applying the most careful principle, al-akhdu bi al-ahwath, and the majority agreed (jumhur) aka avoiding differences, al-Khuruj min al-khilaf.

   In determining the fatwa for halal products, it is based on the following points:

a. The materials used include raw materials, additives, processing aids, packaging, materials that are in direct contact with materials or products, and media for validation that are in direct contact with materials or products.
b. Proses Produk Halal (PPH)

   PPH is an activity to guarantee the halalness of a product, which includes the supply of materials, processing, storage, packaging, distribution, sales, and presentation of the product. PPH criteria are met by taking into account several things: location, place, and equipment; PPH equipment and tools; and PPH procedures.
c. Sistem Jaminan Produk Halal (SJPH)

   SJPH is a measure that becomes the basis for assessing or determining halal product guarantees, covering five aspects, namely commitment and responsibility, materials, halal product processes, products, and monitoring and evaluation.

2.1.6 The Pathophysiology of Osteoarthritis (OA)

Osteoarthritis causes joint stiffness, inflammation, and cartilage degeneration, causing discomfort in the hands, neck, back, waist, and knees. Osteoarthritis causes cartilage thinning, discomfort, and stiffness. Loss of cartilage, bone hypertrophy, and bone capsule thickening cause osteoarthritis (Kalim and Wahono, 2019).

Changes in cartilage metabolism cause osteoarthritis, especially in the knee. Increased enzyme activity damages cartilage matrix macromolecules and reduces proteoglycans and collagen production. Degeneration of articular cartilage produces chemicals that drive macrophages to generate IL-1, boosting proteolytic enzymes for extracellular matrix destruction. Changes in proteoglycans make cartilage resistant to joint pressure and other overloading factors. Damaged chondrocytes and non-collagen alterations accompany decreased cartilage strength. The molecular composition and matrix of articular cartilage will alter, followed by matrix dysfunction. Cartilage loss reduces joint space. Osteophytes form on injured joint margins. Osteophyte development repairs and reshapes joints. Osteophytes may help cartilage alterations in osteoarthritis by increasing joint surface area. Progressive erosion eventually erodes the underlying bone. At a pressure that surpasses the bone's biomechanical strength, the subchondral bone thickens and becomes dense. Eburnation causes subchondral bone sclerosis. Joint cartilage deteriorates, causing osteoarthritis symptoms like pain, stiffness, and deformity (Sembiring, 2018).

2.1.7 Pharmacological Therapy for Osteoarthritis

OA pharmacological therapy uses painkillers. Besides aiming to be a symptomatic aid (reduction of pain and discomfort), pharmacological therapy is also an exacerbation of inflammation, relieving or reducing depression and anxiety in patients. Patients with OA and inflammation using NSAIDs (Non-Steroid Anti-Inflammatory Drugs) (Sembiring, 2018).

Analgesic NSAIDs have a relatively varied chemical structure, but their mechanism of action on the cyclooxygenase (COX) enzyme is identical. Aspirin and indomethasone block the enzymatic generation of prostaglandins (PG) in in vitro tests; if cells are injured, PG is released (Yubo et al., 2017).

NSAIDs inhibit COX, so arachidonic acid isn't converted to PGG2. Chemical structure, acidity, and first availability classify NSAIDs. This group is categorized by the selective suppression of constitutive cyclooxygenase-1 (COX-1) and inducible cyclooxygenase-2 (COX-2). COX-1 is always present in numerous human tissues and acts to maintain body physiology, such as stomach mucus production. COX-2 is an inducible enzyme that is not

monitored in most tissues but increases in inflammatory or pathological circumstances (Faki et al., 2021).

NSAIDs that block COX-1 and/or COX-2 bind to the enzyme's active site, rendering it unable to convert arachidonic acid into inflammatory prostaglandins. Ibuprofen, indomethacin, and naproxen inhibit COX-1 and COX-2. Acetosal and ketorocal inhibit COX-1 selectively. Piroxicam blocks COX-1, while diclofenac, meloxicam, and nimesulide block COX-2. Celecoxib and rofecoxib suppress COX-2 (Faki et al., 2021)

OA treatments include NSAID analgesics and phytoestrogens. Phytoestrogens have the same structure, action, and affinity as estrogens in mammals. Phytoestrogens provide their activity by binding to ERs (ER-independent) or other mechanisms (ER-independent). The ER-dependent pathway can offer direct action by binding to ER-β (Mirza et al., 2021). Estrogen aids in bone growth and inhibits OA progression. Estrogen impacts bone and blood cell development. If estrogen levels drop, osteoblast TGF-α and endothelial nitric oxide levels drop, leading to enhanced osteoclast differentiation and maturation (Prices & Wilson, 2013), which can lead to OA severity. In previous studies, *C. cainito* was proven to contain high levels of phytoestrogens. Therefore, it has great potential to be developed as a treatment for OA through its anti-inflammatory and antinociceptive effects.

2.2 ROADMAP OF RESEARCH

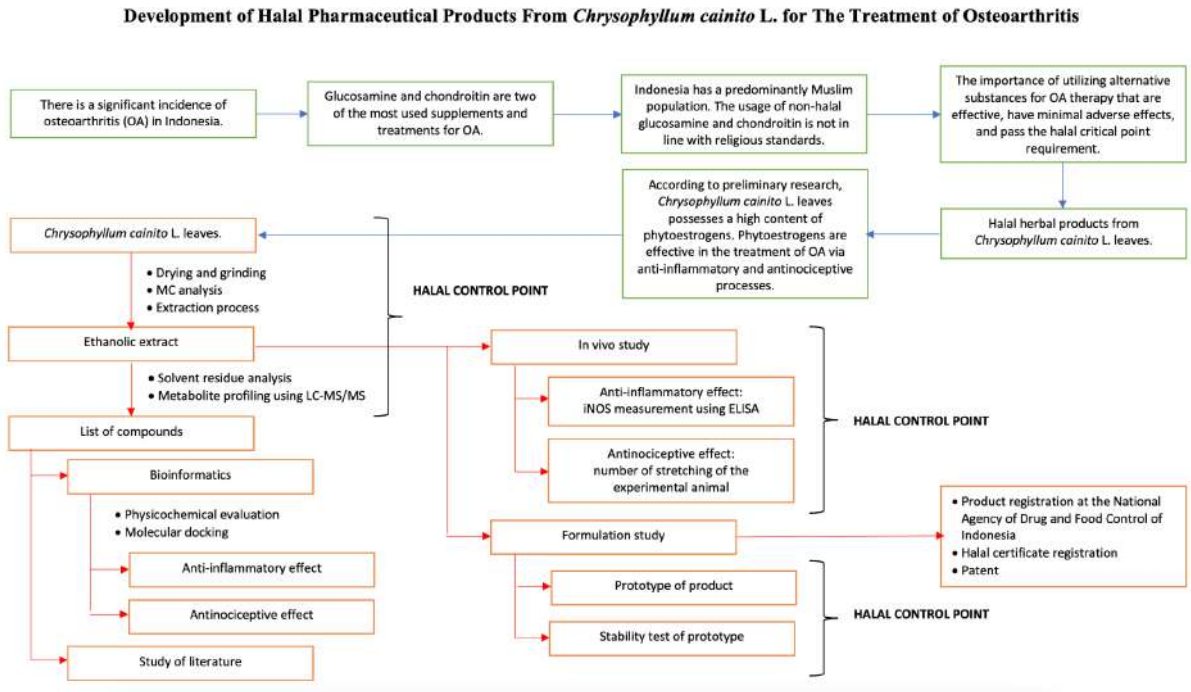


Figure 3. Roadmap of Research: *C. cainito* for the Treatment of Osteoarthritis

CHAPTER III

RESEARCH METHODS, RESULTS, AND DISCUSSIONS

Table 1. Progress report summary

No.	Activity	Progress	Information
1	Preparation and extraction	100%	Yield value of 27.3%, The amount of dry extract is 65.62 g.
2	Metabolite Profiling (GC-MS, LC-MS)	100%	GC-MS + LC-MS: 18 compounds were matched, but only 11 compounds have matching quality above 80%.
3	In silico studies	100%	3LN1 and 1PXX: Both have 3 compounds with best activity, and 7 compounds with normal activity compared to native ligand
4	in vivo studies		
	Acute Toxicity Test	100%	LD_{50} of 70.028 g/kgBB (practically non-toxic)
	Extract Antinociceptive Test	100%	The optimum dose at dose 2 (using 3x1 tablet/day), exceeds Ibuprofen as K(+)
	Tablet Antinociceptive Test	100%	The optimum dose at dose 2 (using 3x1 tablet/day), exceeds Ibuprofen as K(+)
5	Tablet Formulation	100%	Tablet Prototype Completed
6	Formula Stability Test	100%	Pass the standart
7	BPOM and BPJPH Registration	50%	Process
8	Publication	100%	Published in the Biomedical and Pharmacology Journal (Scopus Q3)

3.1 Preparation and Extraction

C. cainito was obtained in powder form from UPT Materia Medika Batu, East Java, Indonesia, and met the requirements for water content (<10%). *C. cainito* leaf powder was mixed with 70% ethanol at a ratio of 1:20 and extracted ultrasonically for 3x2 minutes. The filtrate is separated and evaporated using a rotary evaporator. These steps can be repeated until a certain amount of 70% ethanol extract is obtained.

The results obtained in the extraction of *C. cainito* are:

- Extract yield of 27.3%.
- The total amount of simplicia powder used was 240 g.
- The amount of dry extract produced is 65.62 g.

Figure 4. Preparation and Extraction of *C. cainito*

3.2 Metabolite Profiling

The implementation was carried out at the University of Kuala Lumpur, Royal College of Medicine Perak, Malaysia.

Figure 5. Research Collaboration with UniKL, RCMP

3.2.1 GC-MS

C. cainito leaves contain high phytoestrogen compounds, which have been proven through preliminary research in the form of me/tabolite profiling with UPLC-QToF-MS/MS. To ascertain the type and amount of compounds contained in *C. cainito*, further metabolite profiling was carried out using GC-MS and LC-MS. Later metabolite profiles the results of metabolite profiling can be seen in the following figure and table:

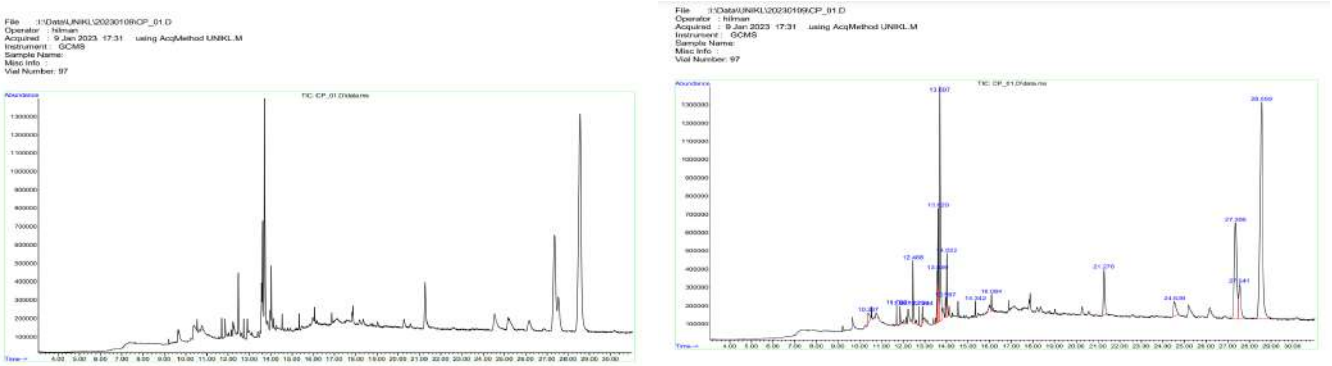


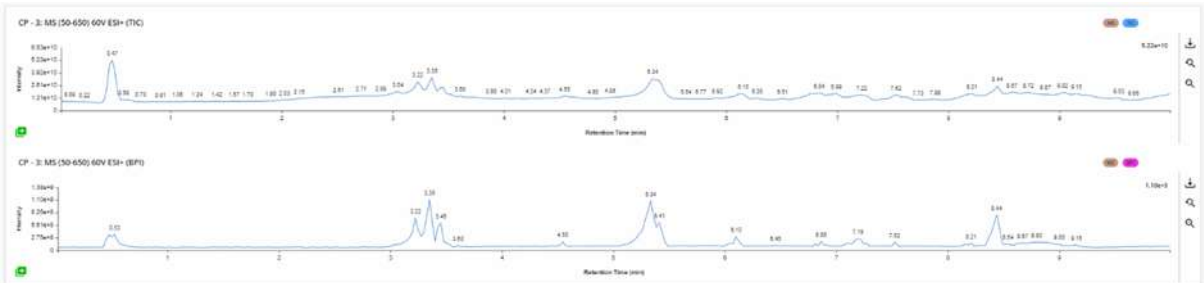
Figure 6. GC-MS chromatogram of a 70% ethanol extract of *C. cainito*

Table 2. Compounds in the 70% ethanol extract of *C. cainito* that have SI > 85% in GC-MS analysis

No	RT	Nama Senyawa	Quality	BM	CAS
1	8,148	2,4-Di-tert-butylphenol	94	206,167	000096-76-4
2	10,273	Hexadecanoic acid, methyl ester	95	270,256	000112-39-0
3	11,113	9-Octadecenoic acid (Z)-, methyl ester	96	296,272	000112-62-9
4	11,141	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	81	292,24	000301-00-8
5	11,189	Phytol	81	296,308	000150-86-7
6	11,397	Linoleic acid ethyl ester	95	308,272	000544-35-4
7	11,432	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	99	306,256	001191-41-9
8	11,508	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	87	264,245	000506-44-5
9	17,75	Vitamin E (dl-α-Tocopherol)	96	430,381	010191-41-0
10	20,797	Stigmasterol	74	412,371	000083-48-7
11	23,394	12-Oleanen-3-yl acetate, (3.alpha.)-	97	468,397	033055-28-6
12	23,574	Germanicol	53	426,386	000465-02-1
13	24,47	Lup-20(29)-en-3-ol, acetate, (3.beta.)-	99	468,397	001617-68-1

The types of compounds detected in the 70% ethanol extract of *C. cainito* leaves can be seen in the table above. There are 18 types of volatile compounds with 11 compounds having a similarity index (SI) above 85% and 7 compounds below 85%. The 11 compounds that had SI > 85% were chosen because SI > 85% indicated that the compounds were "valid" or true. Two compounds with SI < 85% (stigmasterol and germanicol) were also selected to be combined with 11 compounds with SI > 85% for in silico studies.

3.2.2 LC-MS



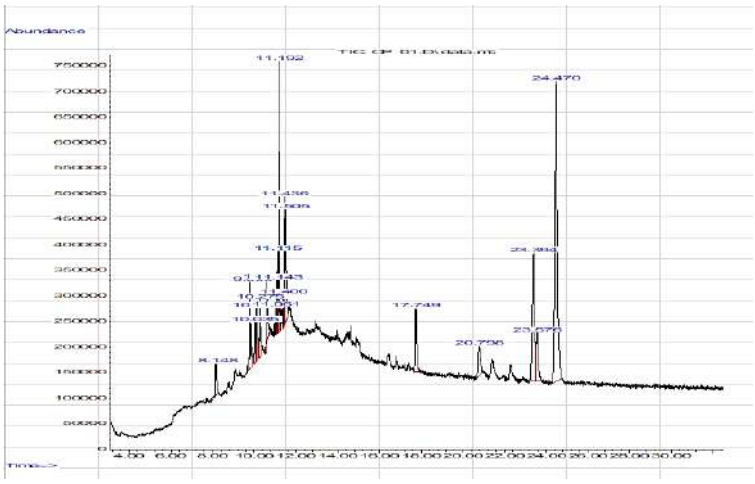


Figure 7. LC-MS chromatogram of a 70% ethanol extract of *C. cainito*

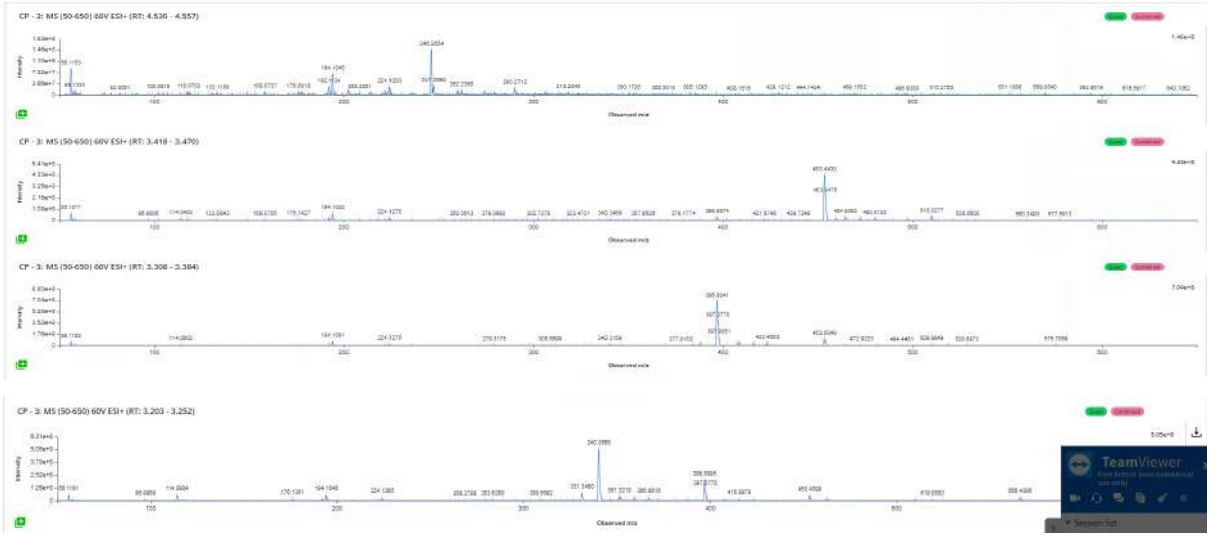
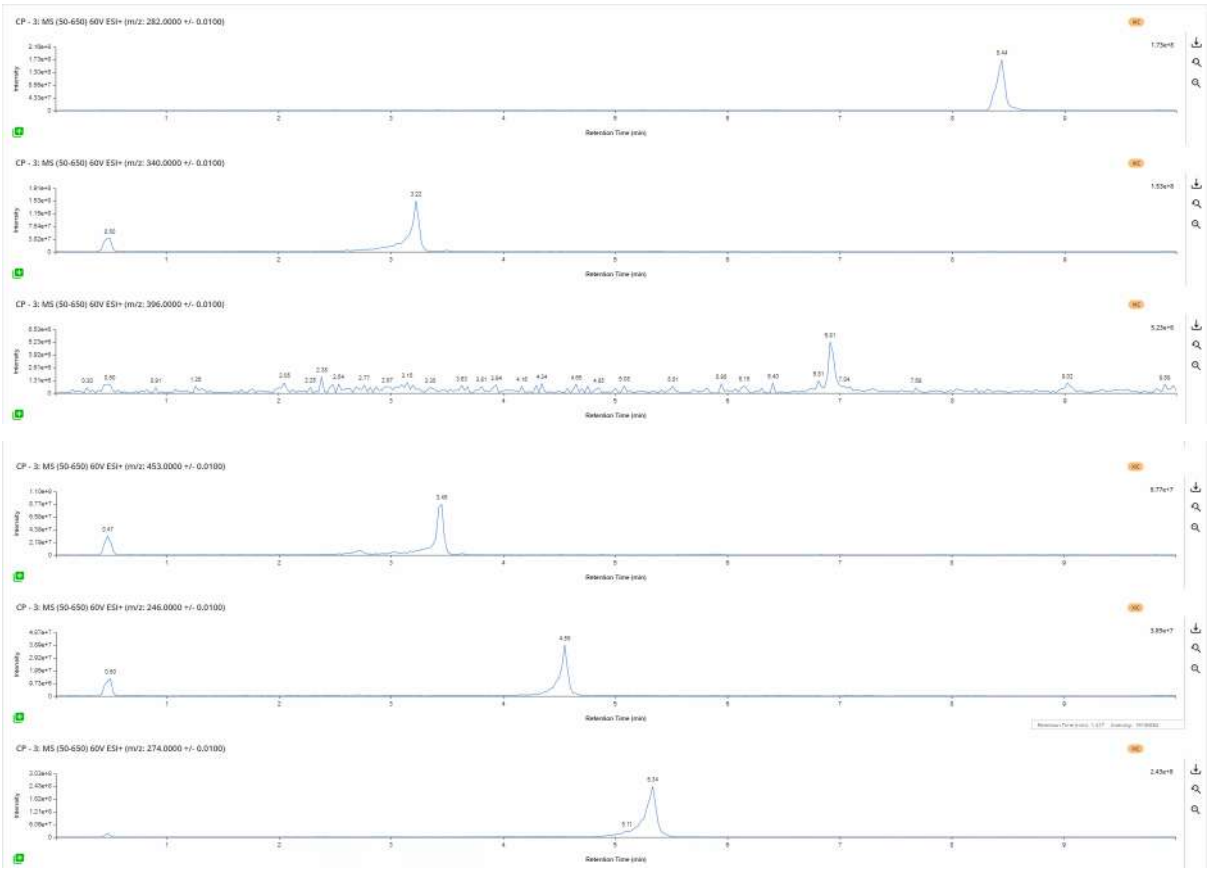


Figure 8. Mass spectrum of various peaks



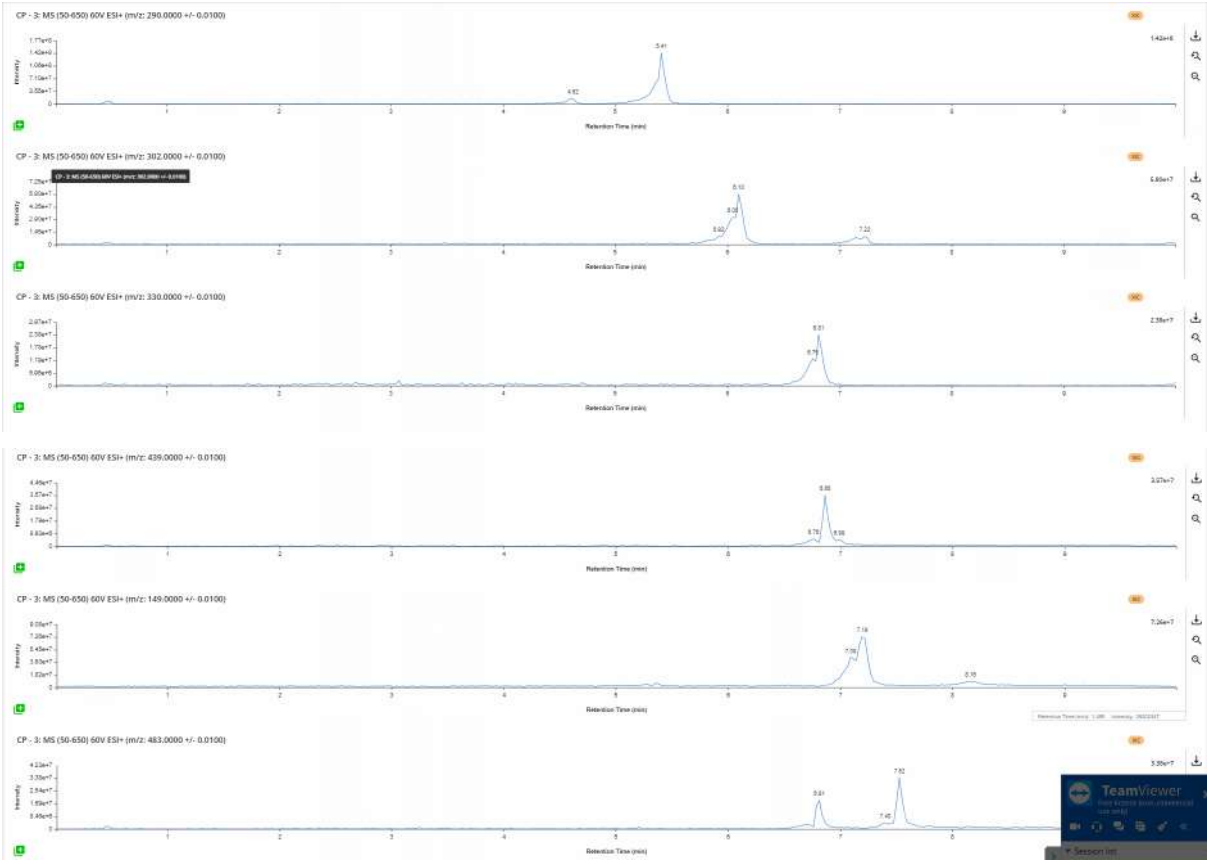


Figure 9. Chromatograms of extract ions from the major and minor ions of different peaks

Table 3. The major and minor ions from various peaks, as shown in Figure 7.

Using MS scan mode (50-650) 60V ESI+ BPI

file CP_20230310; 2ng\ul ; 10ul

RT	m/z major	minor			
8.405-8.465	282				
7.498-7.557	483		73	194	505
7.16-7.226	149		301	90	285
7.073-7.123	same like 7.16-7.22				
6.848-6.885	439		461	194	330
6.798-6.826	330		483	194	505
6.079-6.132	302		346	194	
5.407-5.449	290		318	194	334
5.274-5.361	274		318	194	230
4.530-4.557	246		194		
3.418-3.470	453				
3.308-3.384	396				
3.203-3.252	340		396		

belong to the same compounds

belong to different compounds

Based on the GCMS database search, 18 compounds were matched, but only 11 compounds (yellow box) have matching quality above 80%. However, only two compounds can be detected in the LCMS based on the ions of these compounds. They are linoleic acid ethyl ester (m/z 308; RT 11.397min) and 9,12,15-Octadecatrien-1-ol, (Z,Z,Z)- (m/z 264; RT 11.508min). Upon further inspection with LCMS spectra, linoleic acid ethyl ester possibly exists with [M+H]+ of 309 and appears at 7.73 min with its base peak ions of [M+H]+ of 194. While, 9,12,15-Octadecatrien-1-ol, (Z,Z,Z)- may possibly exist with [M+H]+ of 265 it appears at 8.44 min with its base peak ions of [M+H]+ of 282.

The chromatogram of CP from the LCMS data shows various patterns (Fig 1, Table 1). The mass spectrum of the various peaks is depicted in Fig 2. The major and minor ions of the peaks were used to construct the chromatogram of the extract ions as shown in Fig 3. In Table 1, LCMS data suggests there are other compounds in the extract that appear at different retention times with their unique [M+H]+ ions. However, these compounds cannot be determined by the current technique. Using High-Resolution LCMS with a database search may help identify these compounds. The crude extract suggests some peaks are overlapped and may need further separation.

3.3 In Silico Studies

Table 4. PDB ID: 3LN1

Compound	Binding Energy	Inhibition Constant	RMSD
Ligand Native	-10,83	11.60 nM (nanomolar)	0.891 A
Stigmasterol	-9,96	50.13 nM (nanomolar)	37.387 A
Vitamin E (dl-α-Tocopherol)	-9,93	52.44 nM (nanomolar)	39.278 A
Phytol	-8,21	958.36 nM (nanomolar)	39.230 A
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	-8,12	1.12 uM (micromolar)	36.251 A
Linoleic acid ethyl ester	-8,07	1.22 uM (micromolar)	37.772 A
9 octadeconoic	-7,68	2.33 uM (micromolar)	37.822 A
9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	-7,63	2.55 uM (micromolar)	31.820 A
9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	-7,53	3.05 uM (micromolar)	37.273 A
2,4-Di-tert-butylphenol	-7,41	3.70 uM (micromolar)	37.492 A
Hexadecanoic acid, methyl ester	-7,09	6.31 uM (micromolar)	39.705 A
Germanicol	+1,83	0	38.116 A
Lup-20(29)-en-3-ol, acetate, (3.beta.)-	+6,42	0	37.326 A
12-Oleanen-3-yl acetate, (3.alpha.)-	+10,5	0	37.824 A

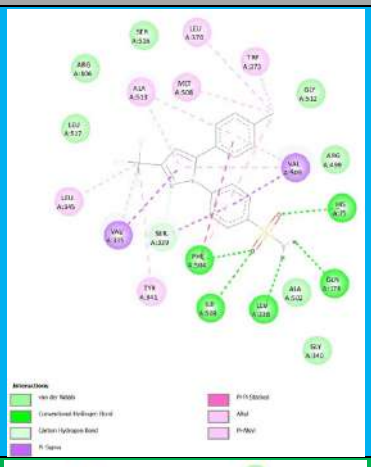
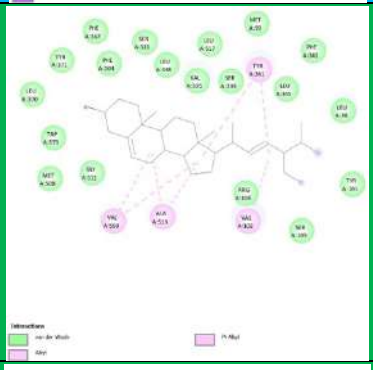
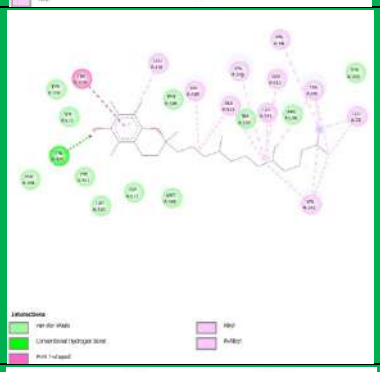
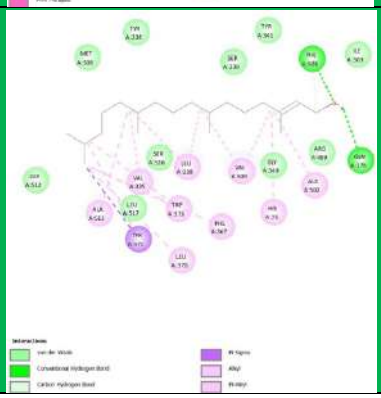
Blue : Native Ligand

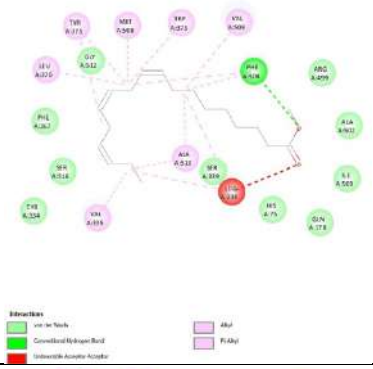
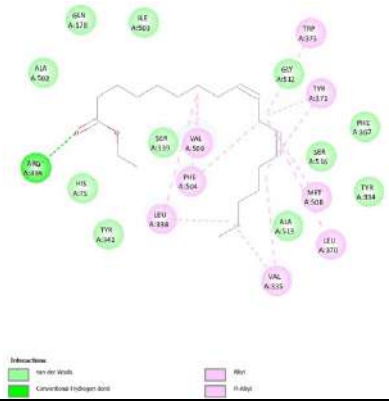
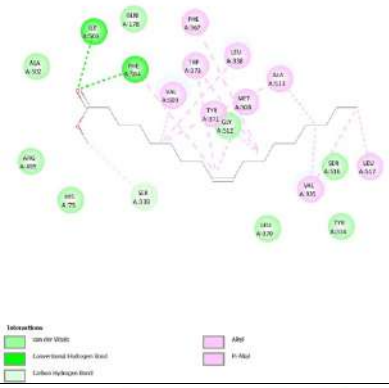
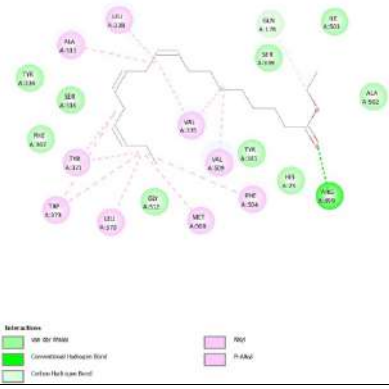
Green : Best activity

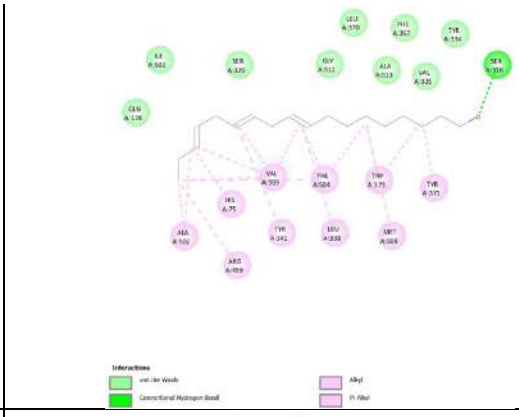
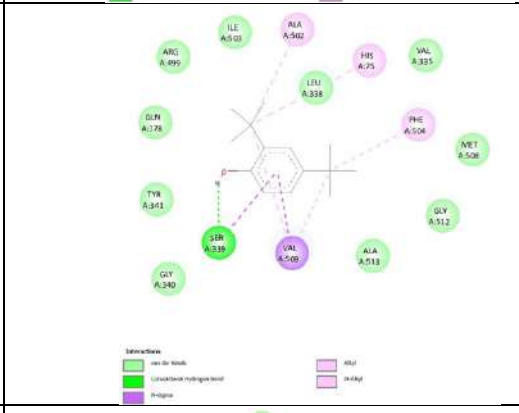
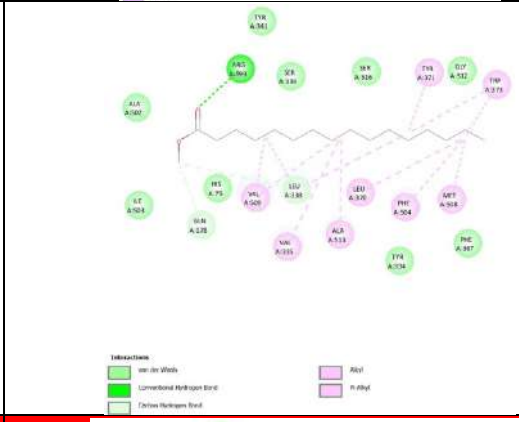
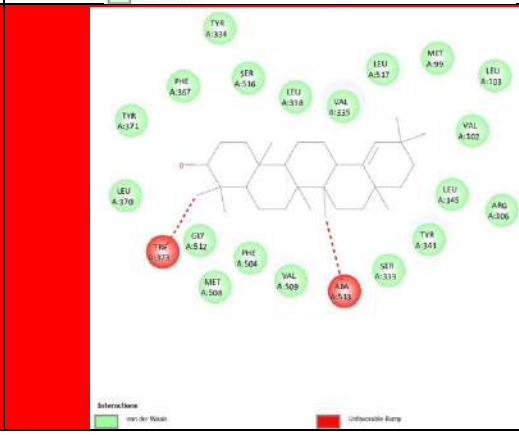
White : Normal activity

Red : Don’t have activity

Table 5. Interactions of Amino Acid Residues of Each Compound

Compound	Amino Acid Residue Interactions
Ligand Native	
Stigmasterol	
Vitamin E (dl-α-Tocopherol)	
Phytol	

9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	
Linoleic acid ethyl ester	
9 octadeconoic	
9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	

9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	
2,4-Di-tert-butylphenol	
Hexadecanoic acid, methyl ester	
Germanicol	

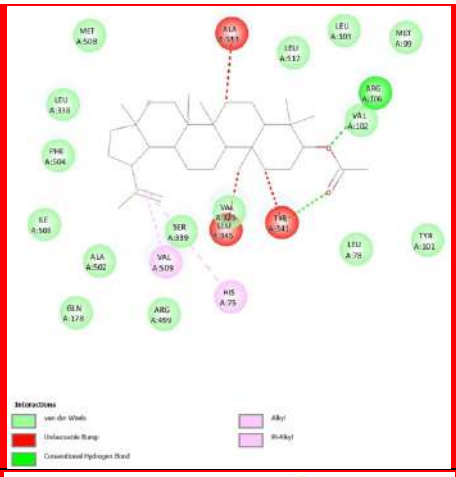
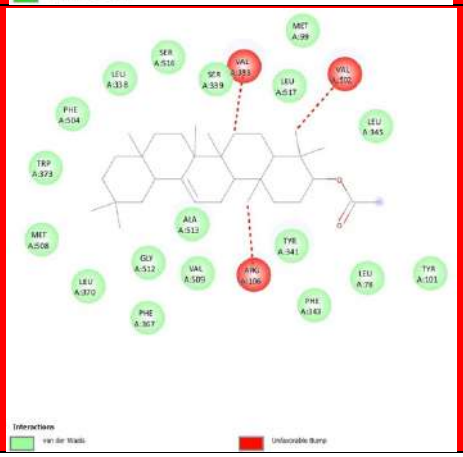
Lup-20(29)-en-3-ol, acetate, (3.beta.)-	
12-Oleanen-3-yl acetate, (3.alpha.)-	

Table 6. PDB ID: 1PXX

Compound	Binding Energy	Inhibition Constant	RMSD
Vitamin E (dl-α-Tocopherol)	-10,46	21.58 nM (nanomolar)	37.149 A
Stigmasterol	-10,17	35.22 nM (nanomolar)	34.686 A
Ligand Native	-7,91	1.60 uM (micromolar)	1.566 A
Phytol	-7,49	3.24 uM (micromolar)	37.458 A
Linoleic acid ethyl ester	-7,2	5.28 uM (micromolar)	36.554 A
9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	-7,15	5.73 uM (micromolar)	37.721 A
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	-7,06	6.70 uM (micromolar)	35.602 A
9 octadeconoic	-6,84	9.66 uM (micromolar)	33.180 A
9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	-6,78	10.78 uM (micromolar)	33.754 A
2,4-Di-tert-butylphenol	-6,42	19.73 uM (micromolar)	35.462 A
Hexadecanoic acid, methyl ester	-6,38	21.05 uM (micromolar)	33.194 A
Germanicol	-0,36	0	35.690 A
Lup-20(29)-en-3-ol, acetate, (3.beta.)-	+0,87	0	36.461 A
12-Oleanen-3-yl acetate, (3.alpha.)-	+8,88	0	33.507 A

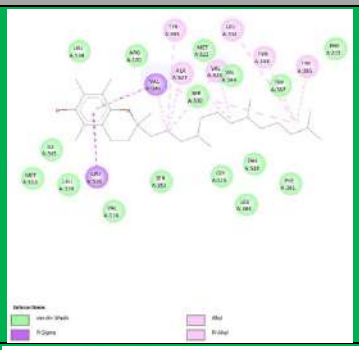
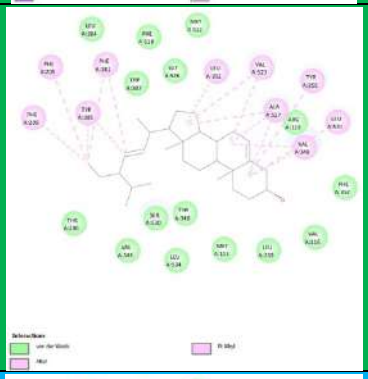
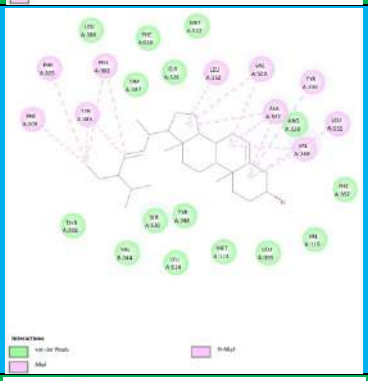
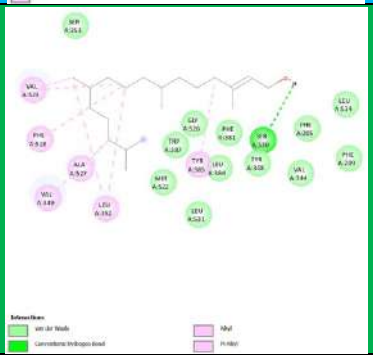
Blue : Native Ligand

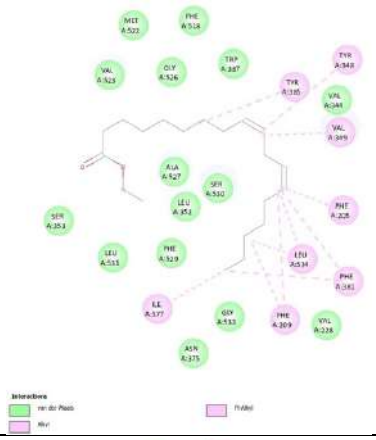
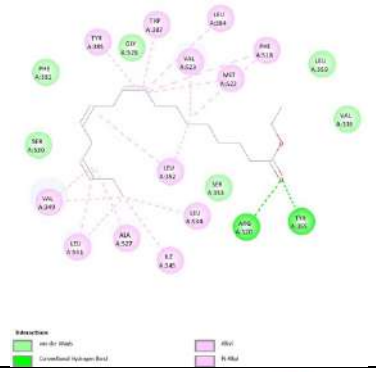
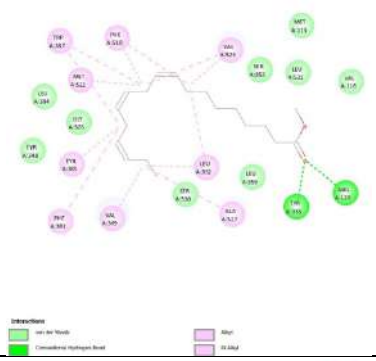
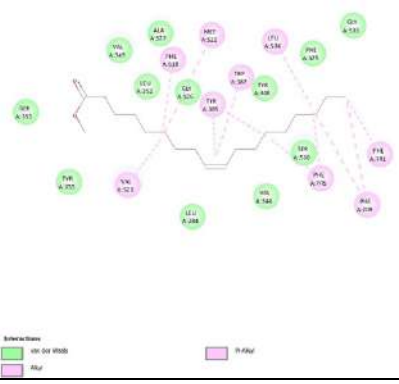
Green : Best activity

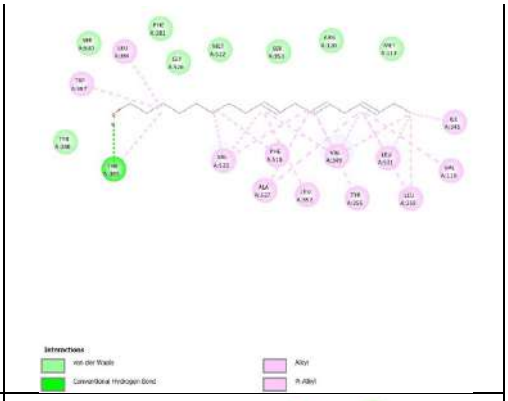
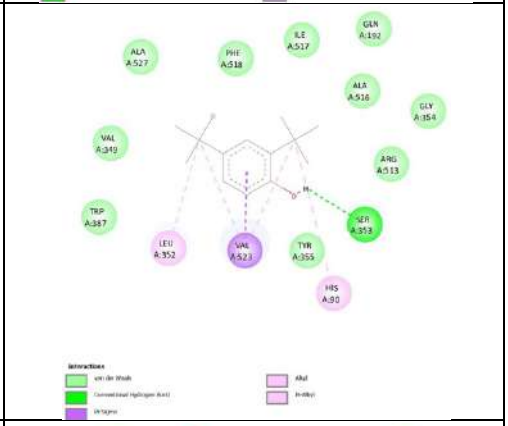
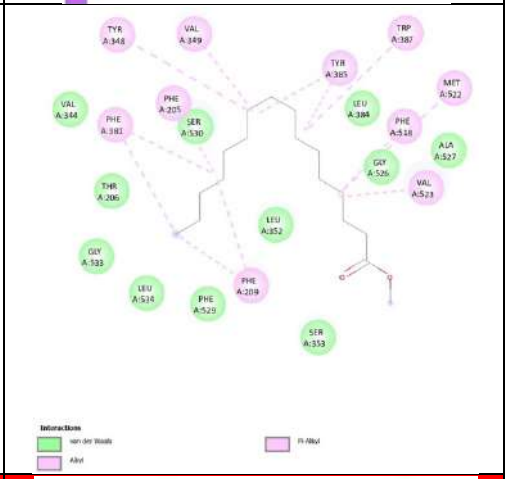
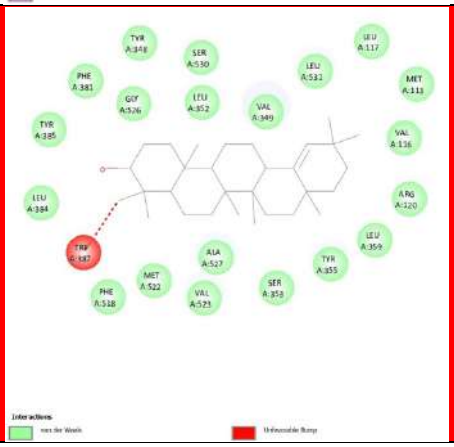
White : Normal activity

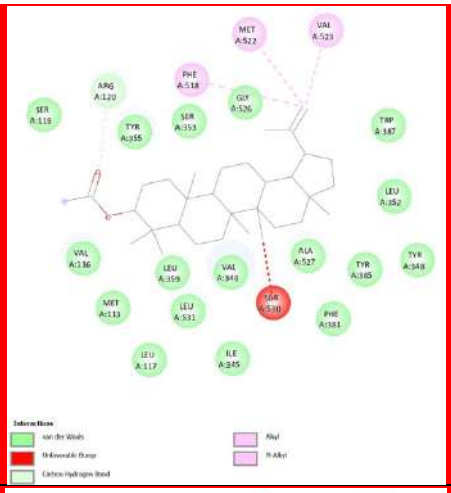
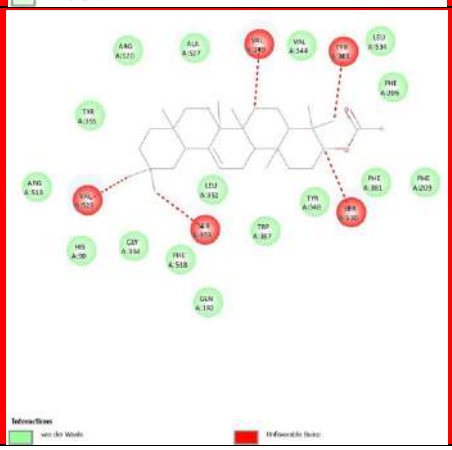
Red : Don’t have activity

Table 7. Interactions of Amino Acid Residues of Each Compound

Compound	Amino Acid Residue Interactions
Vitamin E (dl-α-Tocopherol)	
Stigmasterol	
Ligand Native	
Phytol	

Linoleic acid ethyl ester	
9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	
9 octadeconoic	

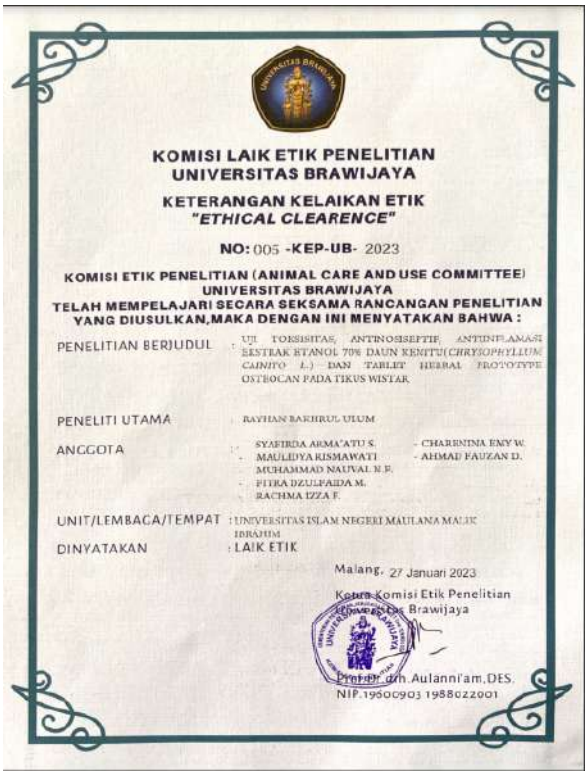
9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	
2,4-Di-tert-butylphenol	
Hexadecanoic acid, methyl ester	
Germanicol	

Lup-20(29)-en-3-ol, acetate, (3.beta.)-	
12-Oleanen-3-yl acetate, (3.alpha.)-	

3.4 In Vivo Study

3.4.1 Animal Preparation

Male Wistar rats (weight, 150-250 grams; age, 8-10 weeks) were used. All research procedures were approved for testing on research animals by the Experimental Animal Ethics Committee. The rats used in the study were maintained under standard laboratory environmental conditions and fed ad libitum with a controlled ambient temperature of 22 ± 2˚C and 50-60% humidity. A 12-hour light/dark cycle is maintained at all times. Mice were housed with seven animals in each cage, adapted to the conditions of the cage, and acclimatized for 1 week before the experiment (Chen *et al.,* 2014).



KOMISI LAIK ETIK PENELITIAN
UNIVERSITAS BRAWIJAYA

KETERANGAN KELAIKAN ETIK
"ETHICAL CLEARENCE"

NO: 005 -KEP-UB- 2023

KOMISI ETIK PENELITIAN (ANIMAL CARE AND USE COMMITTEE)
UNIVERSITAS BRAWIJAYA
TELAH MEMPELAJARI SECARA SEKSAMA RANCANGAN PENELITIAN YANG DIUSULKAN,MAKA DENGAN INI MENYATAKAN BAHWA :

PENELITIAN BERJUDUL : UJI TOKSISITAS, ANTINOSISEPTIF, ANTIINFLAMASI EKSTRAK ETANOL 70% DAUN KENITU (*CHRYSOPHYLLUM CAINITO* L.) DAN TABLET HERBAL PROTOTYPE OSTEOCAN PADA TIKUS WISTAR

PENELITI UTAMA : RAYHAN BAKHRUL ULUM

ANGGOTA : - SYAFIRDA ARMA'ATU S. - CHARENINA ENY W.
- MAULIDYA RISMAWATI - AHMAD FAUZAN D.
- MUHAMMAD NAUVAL N.F.
- PITRA DZULFAIDA M.
- RACHMA IZZA F.

UNIT/LEMBAGA/TEMPAT : UNIVERSITAS ISLAM NEGERI MAULANA MALIK IBRAHIM

DINYATAKAN : LAIK ETIK

Malang, 27 Januari 2023
Ketua Komisi Etik Penelitian
Fakultas Kedokteran
Universitas Brawijaya

Prof. dr. Aulanni'am, DES.
NIP. 19600903 198802 2001

Figure 10. Ethical clearance

3.4.2 Acute Extract Toxicity Test

The 70% ethanol extract of *C. cainito* leaves proved to be practically non-toxic to wistar rats (*Rattus norvegicus*) with an LD50 value of 70.028 g/kg. The extract is said to be practically non-toxic if the LD50 value is > 15.00 g/kg.

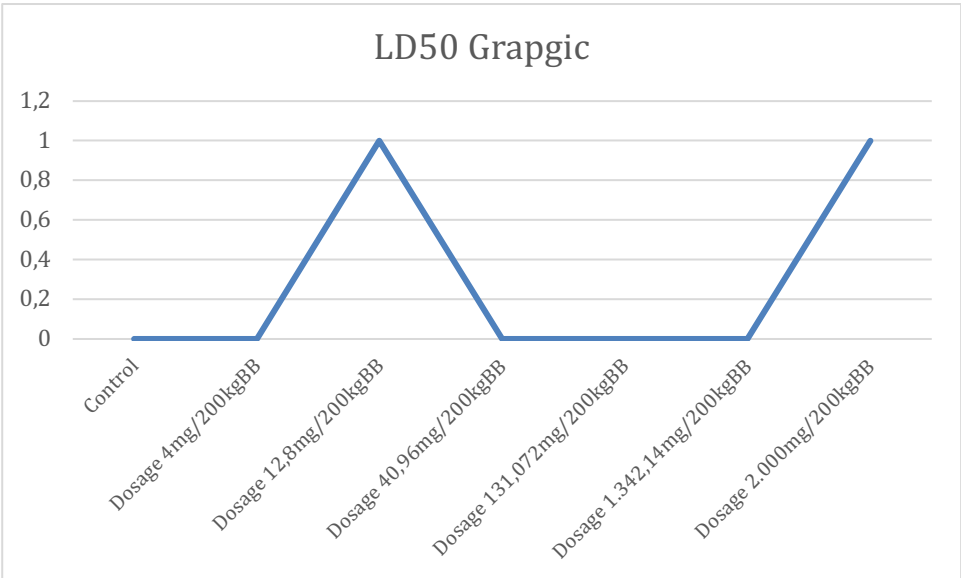
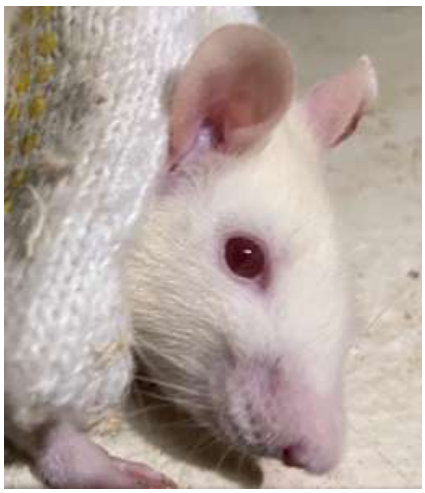


Figure 11. LD_{50} graphic

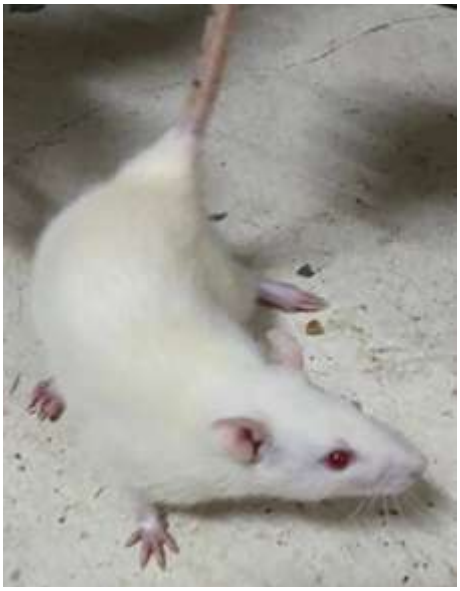
Apart from being quantitative, the safety of the 70% ethanol extract of kenitu leaves was also proven qualitatively by observing the morphology and physiology of experimental

animals. The results of the descriptive qualitative test showed no difference in the body morphology and physiology of the rats in the negative control group and the 2,000 mg/kg BW group after 28 days, so it can be said that the extract is safe.



The eye condition of the control rats was negative.

The eye condition of the rats in the extract group at a dose of 2,000 mg/kgBB



The body condition of the control rats was negative.

The body condition of the rats was affected by the dose of cainito extract at a dose of 2,000 mg/kg BB.

Figure 12. Qualitative assessment of rats

3.4.3 Antinociceptive Extract Test

Extract dosage is as follows:

- Based on dosage optimization and preliminary research results, the dose of 70% ethanol extract for humans is 450 mg/70kg weight/day.
- The dosage per tablet is 900 mg containing 150 mg of ethanol extract of 70% of the bladder.
- The dosage is 3 times a day, 1 tablet.

  Dose 1 = 4.05 mg/200g weight of rats

  Dose 2 = 8,1 mg/200g weight of rats

  Dose 3 = 16,2 mg/200g weight of rats

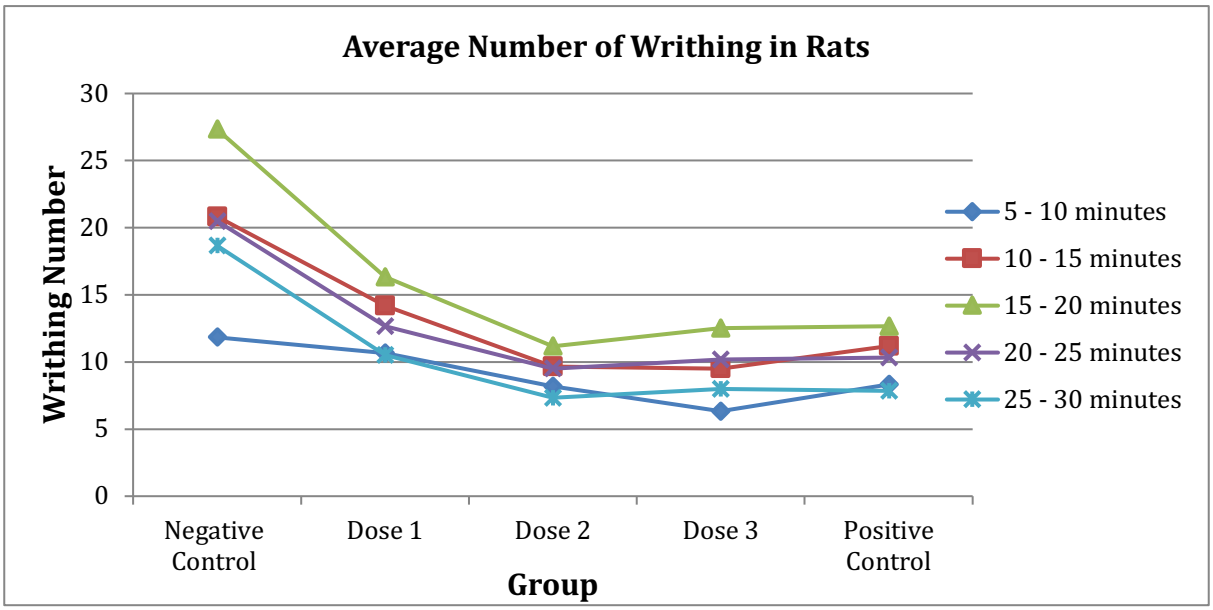


Figure 13. Average Number of Writhing in Rats Extract Test Antinociceptive Extract Test

3.4.4 Tablet Antinociceptive Test

The dosage of the tablets is as follows:

- Dose 1 = 24,3 mg/200g weight of rats
- Dose 2 = 48,6 mg/200g weight of rats
- Dose 3 = 97,2 mg/200g weight of rats

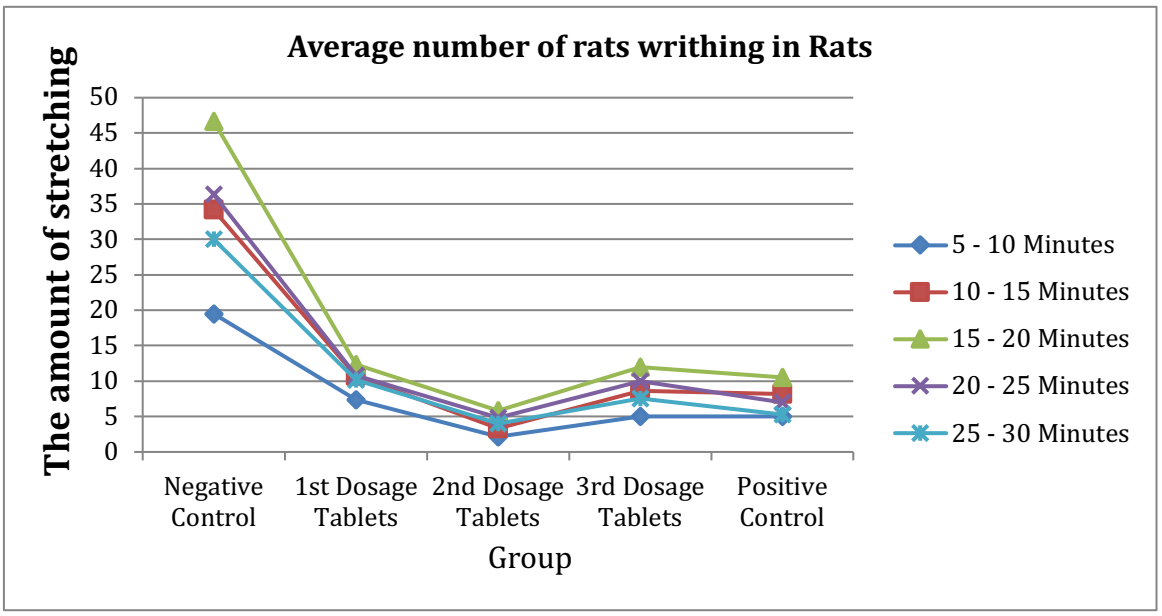


Figure 14. Average Number of Writhing in Rats Tablet Antinociceptive Test

The results of the study showed that tablets at all doses had a good analgesic effect compared to negative controls, with dose 2 (use 3 times a day of 1 tablet) having the best analgesic effect in comparison to other groups. The analgesic effect of 2-dose tablets is higher than that of **Ibuprofen** (positive control).

3.4.5 Extract Anti-inflammatory Tests

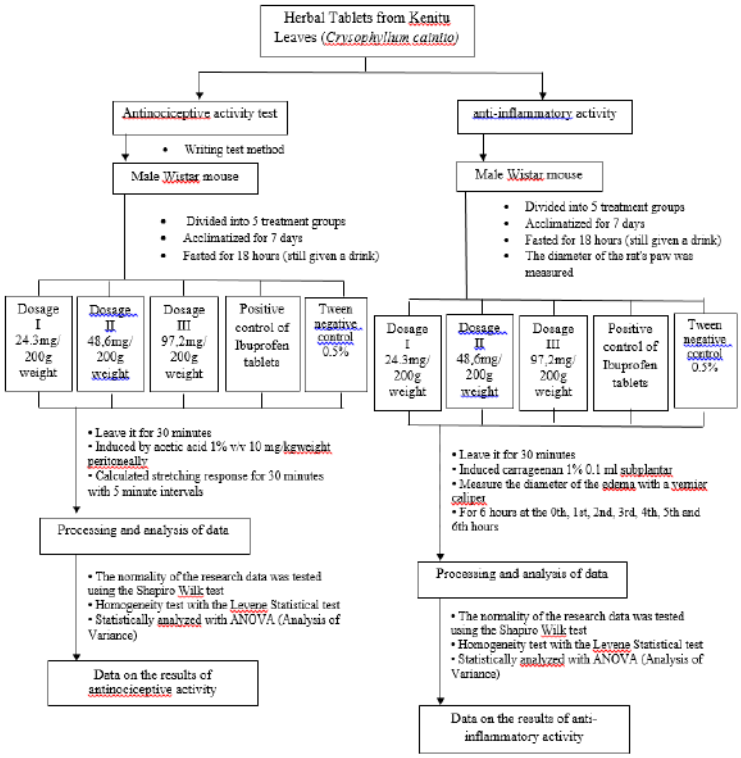


Figure 15. Extract Inflammatory Test Method

3.5 Osteocan Product Formulations

Preparation

- The average extract yield is 15%.
- Based on dose optimization and preliminary research results, the dose of kenitu leaf 70% ethanol extract for humans is 450 mg/70 kg of body weight/day.
- The weight per tablet is 900 mg containing 150 mg of a 70% ethanol extract of *cainito* leaves.

The Osteocan product formula is as follows:

1. Lactose
2. Microcristalline Cellulose
3. Sodium Starch Glycolate
4. Hydrated Silica
5. Magnesium Stearate
6. Copovidone
7. Methyl Paraben

The tablet prototype is then evaluated to determine the right formulation according to the guidelines in the Indonesian Pharmacopoeia VI (Ministry of Health, 2020). At all stages of

research, halal critical points will be continuously monitored. Tablet formula stability tests include organoleptic tests, weight uniformity tests, tablet hardness tests, tablet friability tests, tablet disintegration tests, and tablet physical stability tests.

Figure 16. Osteocan Products

3.8 Publication

Biomedical & Pharmacology Journal, December 2022. Vol. **15**(4), p. 1853-1865

A Systematic Review: Comparison of Immunocytochemistry, ELISA, and Western Blot Methods in Alkaline phosphatase Measurement at Genistein-induced Osteoblast Cell

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Osteoporosis is a bone disorder characterized by the decrease of bone mass along with bone micro-architecture damage and has a risk become a fracture. One of the causes of osteoporosis is estrogen deficiency. Genistein is a phytoestrogen compound in the isoflavone group containing a similar structure compared to 17ß-estradiol, thus it can bind to estrogen receptors and produce an estrogenic effect. Genistein induction can stimulate bone formation and promote the increase of alkaline phosphate (ALP) activities in osteoblast cells which can be observed by immunocytochemistry or Enzyme-linked Immunosorbent Assay (ELISA) or Western blot method. Using the PRISMA guideline technique, choose and strategize article searches by reading the title, abstract, and then the whole text of the article. Articles with the keywords "genistein or osteoblast cells or alkaline phosphate or immunocytochemistry or immunofluorescence or ELISA or western blot" were retrieved from databases including Google Scholar, PubMed, Researchgate, and Sciencedirect. 24 relevant research articles were uncovered as a result of this systematic review. Comparison of immunocytochemistry and ELISA methods in order to analyze the activities of ALP in osteoblast induced by genistein includes selectivity, sensitivity, processing time, and cost efficiency parameters. The immunocytochemistry method has a higher level of sensitivity and a faster processing time, whereas the ELISA method has a higher level of selectivity and less cost efficiency. The western blot method has selectivity for detecting complex-level protein expression.

Keywords: Cost Efficiency; ELISA; Immunocytochemistry; Selectivity; Sensitivity, Processing Time; Western Blot.

Figure 17. Publish a Journal 'A Systematic Review: Comparison of Immunocytochemistry, ELISA, and Western Blot Methods in Alkaline phosphatase Measurement at Genistein-induced osteoblast Cell'

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