CORRESPONDING AUTHOR

Judul :

Clove Flower Extract (Syzygium aromaticum) Has Anti-Cancer Potential Effect Analized by Molecular Docking and Brine Shrimp Lethality Test (BSLT

Penulis Utama dan Corresponding Author : Eduardus Bimo Aksono

Nama Jurnal : VETERINARY MEDICINE INTERNATIONAL. Volume 2022, Article ID 5113742. Pages: 1-7

COVER LETTER FOR SUBMISSION OF NEW MANUSCRIPTS

Dear Reviewers and Editor,

I am enclosing herewith a manuscript entitled "**Clove Flower Extract (Syzygium aromaticum) Has Anti-Cancer Potential Effect Analized by Molecular Docking and Brine Shrimp Lethality Test (BSLT)**" for possible evaluation and publication in your journal, after some revisions based on Reviewers comments and corrections

With the submission of this manuscript I would like to undertake that the above mentioned manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere; and that my Institute's **Airlangga University** representative is fully aware of this submission.

Best Regards
Eduardus Bimo
Airlangga University - Indonesia

eduardus bimo aksono h <eduardus-b-a-h@fkh.unair.ac.id>

Manuscript submitted to Veterinary Medicine International

2 messages

Veterinary Medicine International <sarah.viscarra@hindawi.com> Thu, Jun 16, 2022 at 4:05 PM
To: eduardus-b-a-h@fkh.unair.ac.id

Hindawi

Dear Dr. Aksono,

The manuscript titled "Clove Flower Extract (Syzygium aromaticum) Has Anti-Cancer Potential Effect Analized by Molecular Docking and Brine Shrimp Lethality Test (BSLT)" has been submitted to Veterinary Medicine International by herinda pertiwi.

To confirm the submission and view the status of the manuscript, please verify your details by clicking the link below.

Thank you for submitting your work to Veterinary Medicine International.

LOGIN

Kind regards,
Sarah Viscarra
Veterinary Medicine International

— Editorial Comments

Recommendation

hassan Al-Karagoly AE 03.08.2022

Major Revision Requested

Message for Author

Dear author,
I would like to thank you for submitting your manuscript entitled " Clove Flower Extract (Syzygium aromaticum) Has Anti-Cancer Potential Effect Analized by Molecular Docking and Brine Shrimp Lethality Test (BSLT)" to Veterinary Medicine International. Your manuscript has been revised, and a major revisions are required.

Please make the corrections listed below and resubmit the manuscript so that we can make a final decision.

Best regards,

The academic Editor

Editor comments

1. The English in the current manuscript is not of publication quality and requires some improvement. Please carefully proof-read spell check to eliminate grammatical errors
2. To maintain context unity, move this line of text " To measure the efficacy of traditional treatments, it is necessary to conduct scientific research, such as in the fields of

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Best regards,

The academic Editor

+ Response to Revision Request

+ Reviewer Reports 2 submitted

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Veterinary Medicine International <production.b@hindawi.com> Wed, Aug 31, 2022 at 7:13 PM
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Cc: eduardus-b-a-h@fkh.unair.ac.id, kautsatuh@gmail.com, aprliacl@gmail.com, luciats@gmail.com, aswathi.selvaraj@hindawi.com, production.b@hindawi.com

Dear Dr. herinda,

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From: **Veterinary Medicine International** <sarah.viscarra@hindawi.com>
Date: Wed, Aug 24, 2022, 11:24 AM
Subject: Your manuscript has been accepted for publication
To: <eduardus-b-a-h@fkh.unair.ac.id>

Hindawi

Dear Dr. aksono,

I am delighted to inform you that the review of your Research Article 5113742 titled Clove Flower Extract (Syzygium aromaticum) Has Anti-Cancer Potential Effect Analized by Molecular Docking and Brine Shrimp Lethality Test (BSLT) has been completed and your article has been accepted for publication in Veterinary Medicine International.

Please visit the manuscript details page to review the editorial notes and any comments from external reviewers. If you have deposited your manuscript on a preprint server, now would be a good time to update it with the accepted version. If you have not deposited your manuscript on a preprint server, you are free to do so.

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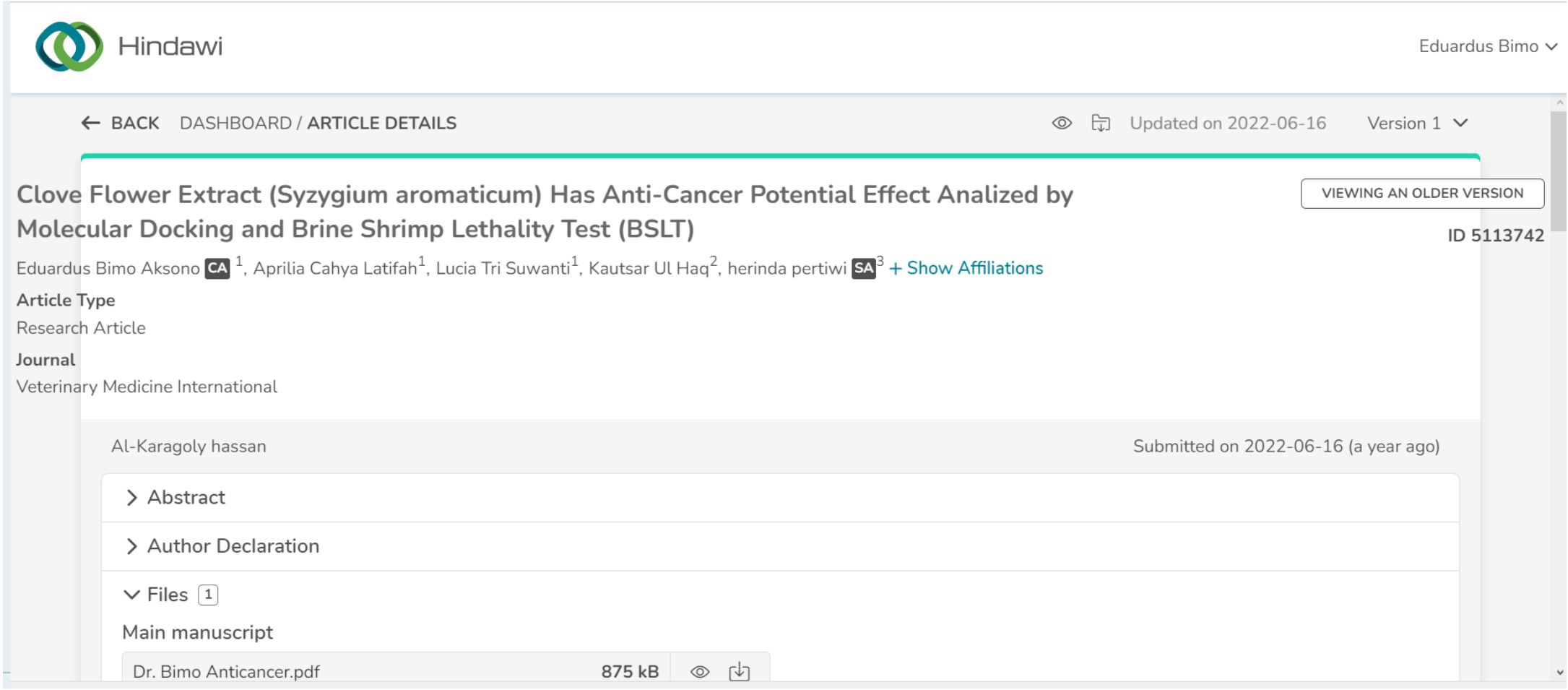
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Research Article
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— Editorial Comments

Decision

hassan Al-Karagoly 03.08.2022

Major Revision Requested

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Eduardus Bimo Aksono CA [1], Aprilia Cahya Latifah[1], Lucia Tri Suwanti[1], herinda pertiwi SA [2], Kautsar Ul Haq[3] + Show Affiliations

Article Type

Research Article

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Veterinary Medicine International

Al-Karagoly hassan

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> Abstract

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Decision

hassan Al-Karagoly 16.08.2022

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Best regards,

The academic Editor

— Response to Revision Request

Your Reply

14.08.2022

Dear Editor We have revised the manuscript based on your comments and correction. thank you very much Best Regards Herinda Pertiwi

Report Reviewer 2 16.08.2022

the manuscript is now available for publication

Report Reviewer 1 16.08.2022

The manuscript entitled “Clove Flower Extract (Syzygium aromaticum) Has Anti-Cancer Potential Effect Analized by Molecular Docking and Brine Shrimp Lethality Test (BSLT)”. This study investigated the effect of clove flower extract (Syzygium aromaticum) as a candidate for anti-cancer by indicating grid-score values using molecular docking and LC50 values using the Brine shrimp lethality test (BSLT) method. Overall, this is an interesting study topic with a good dataset; however, certain suggestions and comments need to be improved before it can be published.

Comments

1. The manuscript's English needs to be checked and corrected; it has a lot of punctuation and grammar mistakes.
2. Introduction and discussion should be focused more around the observations and novelty of this study, compared with other methods of preparation and can be supported with related references. For example:

doi: 10.1007/s13205-022-03117-2.
https://doi.org/10.3390/nano12122013
doi: 10.18502/ijm.v14i1.8810.
https://doi.org/10.3390/molecules26206140

3. Materials: Use a unique description for the appliances used in experiments: name (company, city, country)
4. Possible modes of action have not been discussed and as mentioned, the discussion is not appropriately carried out.
5. More organization is required in the manuscript Structure, particularly in the incorporation of tables and figures in the manuscript text.

Clove Flower Extract (Syzygium aromaticum) Has Anti-Cancer Potential Effect Analized by Molecular Docking and Brine Shrimp Lethality Test (BSLT)

ID 5113742

Eduardus Bimo Aksono CA [1], Aprilia Cahya Latifah[1], Lucia Tri Suwanti[1], Kautsar Ul Haq[2], herinda pertiwi SA[3] + Show Affiliations

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— Editorial Comments

Decision hassan Al-Karagoly 24.08.2022

Publish

— Response to Revision Request

Your Reply 22.08.2022

Dear Reviewer, I would like to send my manuscript after did some revision based on your comment and correction. thank you very much Best Regards Rinda

— Reviewer Reports 2 submitted

Report Reviewer 2 23.08.2022

the manuscript is suitable for publication.

Report Reviewer 1 22.08.2022

Report

Reviewer 1 22.08.2022

I think the manuscript now could be accepted for publication in Veterinary Medicine International

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Figure files

table and figures.docx	780 kB

Clove Flower Extract (*Syzygium aromaticum*) Has Anti-Cancer Potential Effect Analized by Molecular Docking and *Brine Shrimp Lethality Test* (BSLT)

Eduardus Bimo Aksono [1,2], Aprilia Cahya Latifah[1], Lucia Tri Suwanti [1], Kautsar Ul Haq[2,3], Herinda Pertiwi [4]

[1]Faculty of Veterinary Medicine, Airlangga University
[2]Engineering and Life Sciences Institute, Airlangga University
[3]Faculty of Science and Technology, Airlangga University
[4]Faculty of Vocational Studies, Airlangga University
Jl Mulyorejo, Surabaya, Jawa Timur, Indonesia, 60115

Corresponding Author: eduardus-b-a-h@fkh.unair.ac.id

ABSTRACT

This study is aimed to evaluate the effect of clove flower extract (*Syzygium aromaticum*) as a candidate of anti-cancer by indicate grid-score value using molecular docking and LC_{50} values using Brine shrimp lethality test (BSLT) method. A total of 300 larvae shrimp *Artemia salina* leach as animal model divided into 6 groups. Each group contains 10 larvae with 5 replication. The concentration of clove flower extract in the treatments media were 50 ppm (T1), 250 ppm (T2), 500 ppm (T3), 750 ppm (T4), 1000 ppm (T5) and 0 ppm as negative control (seawater). Mortality data percentage of *Artemia salina* leach analyzed by probit analysis. The results showed that the extract of clove flower (*Syzygium aromaticum*) has a toxic effect on larvae with LC_{50} values of 227,1 µg/ml or into the equation y = 2.8636 x – 1.7466 with R^2 value is 0,9062. Based on molecular docking, the proximity to the grid score value, eugenol acetate (grid-score -42.120934) has a close relationship with the cognate enzyme nitric oxide synthase (3E7G) (grid-score -61.271812). It concluded that clove flower extract is toxic to larval shrimp (*Artemia salina* Leach), thus clove flower extract has the potential effect as an anti-cancer drug. Based on the proximity to the grid-score value, eugenol acetate has close proximity to the cognate enzyme nitric oxide synthase (3E7G). Decrease of cancer cell growth suggested by inhibition of nitric oxide synthase.

Keywords : Brine shrimp lethality test, Clove flower, Molecular docking, *Syzygium aromaticum*

1. Introduction

Medicinal plants contain bioactive ingredients in one part or all parts of the plant that can be used to treat certain diseases. Parts of plants that can be used include leaves, fruit, flowers, seeds, roots, rhizomes, stems, bark, sap (Sada and Tanjung, 2010). To measure the efficacy of traditional treatments, it is necessary to conduct scientific research, such as in the fields of pharmacology, toxicology, identification, and isolation of active chemical substances contained in plants (Endarini, 2016).

Cloves (*Syzygium aromaticum*) are ancient spice plants that have been known and used for thousands of years before Christ. The tree itself is a native plant of the Maluku islands (Ternate and Tidore), formerly known to explorers as spice island (Sutriyono and Mahrus, 2018; Renggana *et al*., 2018).

Cloves contain substantial amounts of essential oil, in flowers (10–20%), stalks (5–10%), and leaves (1–4%) (Nurdjannah, 2004). Clove essential oil has the best quality because the yield is high and it contains eugenol, reaching 80–90%. Cloves are multi-beneficial and efficacious, both as food and beverage ingredients with high nutritional value as well as anticancer, antibacterial, antifungal, anti-inflammatory, antiproliferative, antifibrogenic, anti-insect, analgesic (Tulungen, 2019). Cloves have high antioxidant activity due to the high content of eugenol (Mu'nisa *et al*., 2012).

The phytochemicals contained in cloves are sesquiterpenes, monoterpenes, hydrocarbons, and phenolic compounds. Eugenol and β-caryophyllene are the most significant phytochemicals in clove oil. Eugenol has shown anticancer activity against colon, stomach, breast, prostate, melanoma, and leukemia cancers. β-caryophyllene exhibits anticancer properties against pancreatic, skin, lymphatic, and cervical cancers (Jaganathan and Supriyanto, 2012).

Eugenol compounds, as the main component contained in clove oil, are potential candidates for further development in assisting modern chemotherapy treatments for cancer treatment. Eugenol works by inhibiting the proliferation and formation of tumors, increasing *Reactive Oxygen Species* (ROS), induced apoptosis, and having genotoxic effects on different cancer cells (Jaganathan and Supriyanto, 2012). Even Zari *et al*. (2021) also suggested that the anticancer effect of eugenol was achieved through various mechanisms such as induced apoptosis, cell cycle arrest, inhibition of proliferation, migration, angiogenesis, and metastasis

of several cancer cell lines. In addition, eugenol can be used as an adjunct therapy for patients treated with conventional chemotherapy. This combination leads to increased effectiveness with reduced toxicity.

Eugenol has the molecular formula $C_{10}H_{12}O_2$ with the IUPAC name 4-allyl-2-methoxyphenol. Eugenol also has other names, such as 4-alylguaikol, 1-allyl-4-hydroxy-3-methoxybenzene, caryophilic acid, 4-hydroxy-3-methoxyalylbenzene, 2-methoxy-4-alylphenol (Zari *et al*., 2021). The concentration of eugenol in clove oil varies from 70% to 96% (Bezerra *et al*., 2017), consisting of various functional groups, such as allyl (-CH2-CH=CH2), methoxy (-OCH3), and phenol (OH) (Towaha, 2012).

The third highest prevalence rate of all types of cancer worldwide is colorectal cancer (Mydhili *et al*, 2021). Medical treatment that are often used to treat colorectal cancer are chemotherapy or radiotherapy, surgery, immunoteraphy, hormonal therapy, and pharmacotherapy with a focus on tumor location and disease stage (Hagan and Donovan, 2013)

Developments in the health sector have found various kinds of anticancer drugs. The main purpose of using anticancer drugs is to selectively damage cancer cells without disturbing normal cells. This goal is frequently achieved, and there are currently very few anticancer drugs that work selectively for the treatment of specific types of cancer (Hardjono *et al*., 2016). Research in recent years has revealed that plants containing phytochemical compounds with anticancer properties are strongly associated with a reduced risk of cancer. In addition, natural products generally have minimal side effects, making them ideal candidates for cancer therapy (Muaja *et al*., 2013). However the studies concerned on the cloves as anticancer is remain limited, therefore this study is arrange to evaluate the potent effect of cloves as anticancer using *Brine Shrimp Lethality Test* (BSLT). This test is one of the initial methods that are often used to observe the level of toxicity of a compound and can be used as a screening test for the activity

of anticancer compounds in plant extracts (Zuraida, 2018). Utami and Yusi (2019) explained that the results of the toxicity test were expressed as Lethal Concentration 50 (LC_{50}), which is the optimum concentration of extract that is able to kill 50% of the population of *Artemia salina*. The lower of LC_{50} value indicate higher the toxicity effect. The advantages of this BSLT test are that it is simple, fast, easy, the results can be repeated, and it is not expensive (Hamidi *et al*., 2014). Tests using *Artemia salina* have a very high sensitivity to cytotoxic compounds (Anderson *et al*., 1991).

2. Materials and Methods

2.1. Study Design of BSLT Method

This study was an experimental study with a *post-test-only control group design* to test the toxicity of clove flower extract against *Artemia salina* using the *Brine Shrimp Lethality Test* (BSLT) method and molecular docking. The research design pattern used was a completely randomized design. This research has also followed the ethical procedure of the ethics commission at the Faculty of Veterinary Medicine, Airlangga University.

The larvae of *Artemia salina*, with a total of 10 samples for each concentration. In this study, five concentrations of clove flower extract were made in six experimental groups. Each concentration and control was repeated five times. So the total sample required is 300 tails.

This research was conducted in February 2022, approved by ethical committee of Faculty of Veterinary Medicine Airlangga University for animal experiment. The study was conducted at the Laboratory of the Veterinary Basic Medicine Division, Faculty of Veterinary Medicine, Airlangga University. Clove extract was made at the Unit Layanan Pengujian (ULP) of the Faculty of Pharmacy, Universitas Airlangga.

2.1.1. Clove Flower Extraction

The clove flower used was obtained in the Naringgul District, South Cianjur Regency, West Java Province. Whole and dried clove flowers weighed as much as 900 grams. Then it was processed with a set of distillation tools. The distilled extraction liquid was collected and to separate the essential oil from water, dichloromethane was added to the separating funnel in a ratio of 1:3. The volatile oil formed is accommodated and to remove the remaining water, Na2SO4 anhydrous is added (Dewi *et al*., 2018). Treatment solution was prepared from 20 mg of essential oil dissolved in 2 ml of ethanol then pipetted into vials as much as 25 µl, 125 µl, 250 µl, 375 µl, and 500 µl then left for 24 hours to evaporate solvent (Aini, 2018).

2.1.2. Hatching *Artemia salina*

Larval hatching is carried out in an aquarium. the aquarium was given a bulkhead whose bottom edge had been perforated so that the hatched eggs could come out of the hole and then be filled with seawater. Eggs are *Artemia salina* inserted in one of the chambers and then closed. In another room, it is lit using a 10 watt lamp to attract hatched shrimp. Larvae that have aged 48 hours will be used as test animals (Meyer *et al*., 1982).

2.1.3. Toxicity Test Using BSLT Method

One ml of seawater was added to treatment solution and homogenized with a vortex. Put 10 larvae of *Artemia salina* into vial and add seawater to a final volume of 5 ml so that the final results of the test solution are obtained with concentrations of 50 ppm, 250 ppm, 500 ppm, 750 ppm, and 1000 ppm. The control group was only given 5 ml of sea water without any clove extract. The toxicity test was repeated five times. Then observations were made for 1 day on the death of *Artemia salina*. The mortality results of *Artemia salina* at each concentration were compared with controls. Observations can be made after 24 hours of treatment. The standard criteria for assessing the mortality of *Artemia salina* larvae is that the larvae do not show movement for 10 seconds of observation.

The sample's toxicity test was determined by evaluate at the value of LC_{50}, which can kill *Artemia salina* up to 50% and statistical calculations were carried out using probit analysis with *Statistical Program and Service Solutions* (SPSS) for *windows* version 25 (Sumihe *et al*., 2014). From the percent mortality, monitor for the probit number or value of each group of test animals through the table, determine the dose log for each group, and then make a graph with a straight line equation of the relationship between the probit value and the concentration log, y = ax + b. Where y = probit number and x = concentration log, then a line is drawn from probit 5 (= 50% mortality) to the X axis, and the concentration log is obtained. Concentration logs are antiloged to get LC_{50} or LC_{50} values can also be calculated from the straight-line equation by entering the value 5 (probit of 50% of experimental animal deaths) as y so that x is produced as the log concentration value. LC_{50} is calculated and obtained from the antilog of the x value (Aini, 2018).

2.2. Study Design of Molecular Docking

Molecular docking is performed with the Dock 6.8. program package. The structures used are listed in Table 1. The structure and molecular surface preparations were carried out using the Dock Prep and Write DMS features in the Chimera 1.16 program (1). Spheres on the molecular surface were created using the SPHGEN program. Since the position of the active site of the enzyme is known with certainty, spheres were selected within a radius of 8.0 Å from the ligand using the SPHERE_SELECTOR program. After that, a simulation box is created with the appropriate size for the selected spheres, plus a margin of 8.0 Å in all directions using the SHOWBOX program. The grid was created at 0.25 Å resolution using the GRID program. The van der Waals interaction was modeled using the Lennard-Jones 12-6 potential and to model the electrostatic interaction, the Coulomb potential with a value ε=4r was used (2). The docking parameter validation is carried out by the redocking method, where the docking method is valid if the value of the symmetry-corrected root mean square deviation of

the heavy atom (HA_RMSDh) is less than 2.0 Å. The structure of eugenol and caryophyllene was optimized using the MMFF method in the Avogadro program (3), then the AM1-BCC electrostatic charge was added using the ANTECHAMBER program (4). Visualization of the docked pose using the Maestro 12.4 Release 2020-2 program (Schrodinger, Inc).

Table 1. Crystal structures used

No	Access code PDB	Description	Resolution	Reference (DOI)
1	2JF9	Estrogen receptor alpha LBD in complex with a tamoxifen-spesific peptide antagonist	2.10 Å	10.1074/jbc.M611424200
2	1HOV	Solution structure of a catalytic domain of MMP-2 complexed with SC-74020	NMR	10.1016/s0167-4838(02)00307-2
3	1UK0	Crystal structure of catalytic domain of human poly(ADP-ribose) polymerase with a novel inhibitor	3.00 Å	10.1016/s0014-5793(03)01362-0
4	2A4L	Human cyclin-dependent kinase 2 in complex with roscovitine	2.40 Å	10.1111/j.1432-1033.1997.0518a.x
5	2R0U	Crystal Structure of Chek1 in Complex with Inhibitor 54	1.90 Å	10.1016/j.bmcl.2007.09.007
6	2X7F	Crystal structure of the kinase domain of human Traf2- and Nck-interacting Kinase with Wee1Chk1 inhibitor	2.80 Å	-
7	3E7G	Structure of human INOSOX with inhibitor AR-C95791	2.20 Å	10.1038/nchembio.115
8	3RUK	Human Cytochrome P450 CYP17A1 in complex with Abiraterone	2.60 Å	10.1038/nature10743
9	4LXD	Bcl_2-Navitoclax Analog (without Thiophenyl) Complex	1.90 Å	10.1038/nm.3048
10	6GUE	CDK2/CyclinA in complex with AZD5438	1.99 Å	10.1016/j.chembiol.2018.10.015

3. Results and Discussion

3.1.Toxicity Test Using BSLT Method

The result show that the distillation of clove flowers as much as 900 grams have the average weight of essential oil as 9.75 (w/w) grams, with a yield of 1.083%.

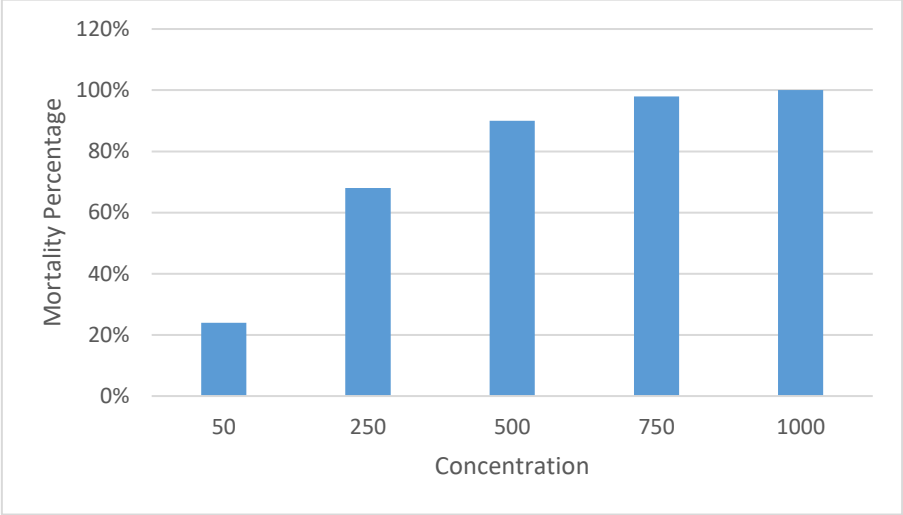


Figure 1. The results of the toxicity test of the concentration of clove flower extract (*Syzygium aromaticum*) on *Artemia salina* larvae

In the study of the toxicity test of clove flower extract using the BSLT method as an anticancer drug candidate, it was proven that clove flower extract was toxic to *Artemia salina* in the BSLT test (see Figure 1). Based on the results of the study, clove flower extract was toxic to *Artemia salina* aged 48 hours. In T1, with an extract concentration of 50 ppm, the mortality percentage was 24%. This is because the level of toxicity of clove flower extract is still low. Toxicity of insecticides in a species is strongly influenced by the concentration of chemical compounds of the insecticide in the body of the target species (Mardiana *et al*., 2005). At T2 with an extract concentration of 250 ppm, the mortality percentage was 68%. At this concentration, clove extract was toxic because it could kill *Artemia salina* by more than 50%. At a concentration of 250 ppm, the LC_{50} value can be calculated. In T3, with an extract concentration of 500 ppm, the mortality percentage was 90%, and in T4, with an extract concentration of 750 ppm, the mortality percentage was 98%. At this concentration, almost all the larvae died. This indicates that the clove flower extract is very toxic to *Artemia salina*. However, the highest lethal power was at T5, with an extract concentration of 1000 ppm. The

percentage of deaths was 100%. The increase in the concentration of the extract causes an increase in the content of the active ingredients in these substances, which function as insecticides that can kill organisms in large numbers (Kardinan, 2005).

In the control group, no dead larvae were found because at this treatment there was no addition of clove flower extract containing toxic substances. It showed that, the death of *Artemia salina* was occured by the administration of clove flower extract and not due to the influence of the external environment. Thus it can be seen that the higher the concentration of clove flower extract, the higher the number of deaths in *Artemia salina*. This result linear with a research conducted by Lisdawati *et al*. (2006), Sanjaya *et al*. (2013), and Sapulette *et al*. (2019), which showed that the higher concentration of plant extracts followed with the higher the number of death larvae. Based on this research, it means that the number of deaths caused by *Artemia salina* tends to be directly proportional to the increasing concentration of clove flower extract. Probit analysis revealed that the LC_{50} value of clove flower extract was 227.1 g/ml. Toxicity criteria for assessing the level of toxicity of plant extracts are classified in the following order: extracts with LC_{50} > 1000 g/ml are non-toxic, LC_{50} 500–1000 g/ml are low toxic, extracts with LC_{50} 100–500 g/ml are moderately toxic, while the extract with LC_{50} 0–100 g/ml was very toxic (Hamidi *et al*., 2014). From these criteria, clove extract is included in the moderate level of toxicity. The level of toxicity gives meaning to the potential activity of clove flower extract as an candidate anticancer. The smaller the LC_{50}, the more potential the plant has in cancer treatment.

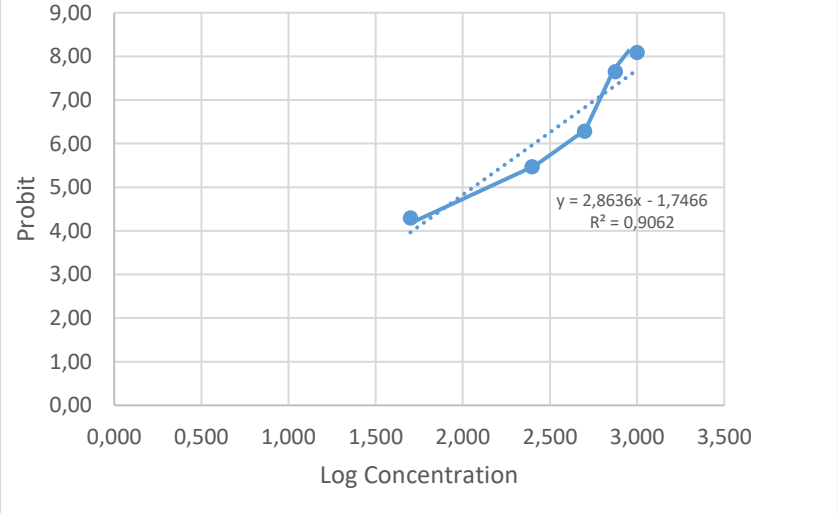


Figure 2. Graph of correlation of log concentration of clove flower extract (*Syzygium aromaticum*) with probit mortality rate of *Artemia salina* larvae

The value of LC_{50} can be confirme through the equation of the line y = ax + b, which is obtained from Figure 2. If the value of probit 5 (y = 5) is entered into the equation y = 2.8636 x – 1.7466, it will get the value of x = log 2.356, and then the value of x is antiloged to obtain a value of 227.1 g/ml. The results of the probit analysis are estimated values qualitatively so that it did not show the actual value. Therefore, in the results of the probit analysis, interval estimates also appear. From the analysis, the interval estimation is between the values with the lower limit of 135 and the upper limit of 293. Based on Figure 2. It is known that the R^2 value is 0.9062, which means that the effect of clove flower extract in killing *Artemia salina* is 90%. The coefficient of determination (R^2) is part of the total diversity of the dependent variable Y, which can be explained or taken into account by the diversity of the independent variable X. The value of R^2 is between 0 and 1, and the correlation is said to be better as R^2 approaches 1 (Sugiono, 2009). In the toxicity test, the R^2 value is 1, this indicates that the factors that affect the death of the test larvae are the result of the clove flower extract given.

Artemia salina larvae has a high sensitivity level to carry out a test, and based on their morphology, *Artemia salina* aged 48 hours has started to have a mouth and digestive tract to consume certain particles. Meanwhile, *Artemia salina* larvae that are 24 hours or in the second instar phase, even though they already have a digestive tract, are still unable to contact their environment, so that extracts or external compounds cannot be absorbed by the larvae (Hamidi *et al*., 2014).

Factors that need to be considered in achieving egg hatching and the growth of *Artemia salina* are salinity, pH, and seawater temperature. The salinity of the seawater used in this study

was 37 ppt. The pH of the seawater used is 8,1. A decrease in pH below 7 can cause death. Hatching cysts requires a slightly alkaline pH of 8–9 (Hiola *et al*., 2014). The seawater temperature in this study was 28.3^{o}C. *Artemia salina* cannot survive in hatcheries at temperatures below 6°C or above 35°C. Artemia growth, the desired temperature ranges from 26–31^{o}C (Bachtiar, 2003). During the hatching process, a lamp is added to the artemia larvae as a light source to maintain the optimum water temperature and can trigger the larvae to detach from their egg shells. The larvae that are actively moving will swim towards the light source because they have positive phototaxis properties, which are attracted to light (Martin and Davis, 2001).

Brine shrimp lethality test (BSLT) includes a top-ranking test in natural product screening for the presence of bioactive compounds. Although the use of the BSLT method is not adequate in determining the mechanism of action of bioactive substances in plants and is also not specific for anticancer activity, the BSLT method produces data that can be supported by more specific bioassays after the active compounds tested are proven to be toxic to *Artemia salina*, tending to be good candidates. good for anticancer research (Ogbole *et al*., 2017).

The phytochemicals contained in cloves are eugenol, β-caryophyllene, eugenol acetate, sesquiterpenes, monoterpenes, hydrocarbons, and phenolic compounds. Eugenol compounds are the main components contained in clove oil, with a content that can reach 78–95% (Shouny *et al*., 2019). The active ingredient eugenol functions as a larvae killer (anti-insect/anti-nematode) (Kardinan, 2005). Research conducted by Sanjaya *et al*. (2013) and Sapulette *et al*. (2019) showed that eugenol activity can cause death in disease-carrying vector larvae. This is also evidenced by the research of Cansian *et al*. (2017) and Gonzales *et al*. (2021), who stated that clove oil was toxic to *Artemia salina*.

The digestive tract of *Artemia salina* is a non-selective filter, making it easier for toxic substances to enter through the mouth. Toxic compounds from clove flower extract that enter can interact with cell membrane targets and enzymes so that they affect the larval body mechanism, which in turn can cause death (Isnansetyo and Kurniastuty, 1995). Based on the above research, it shows that the eugenol content in clove extract acts as a larvicide against *Artemia salina* through the mechanism to destruct cell membranes or interfering with larval metabolism.

3.2. Molecular Docking

Clove essential oil contains several compounds, the highest content in cloves is eugenol, eugenol acetate, and caryophyllene. In silico analysis was carried out by molecular docking of the three compounds to 9 proteins that are important in the proliferation of cancer cells (Jaganathan and Supriyanto, 2012). These three compounds can be evaluated by comparing the grid-score of their compounds with the grid-score of the original ligand (cognate). The results can be seen in Table 2. Based on these results, there is not compound that can produce a grid-score is better than cognate. It is because the structure of the clove essential oil bioactive have a few functional groups, especially caryophyllene, which is filled with hydrophobic interactions. There is not much interaction that can be carried out other than the van der Waals interaction. Therefore, caryophyllene mostly produced the worst grid-score compared to the other two compounds on 9 target proteins.

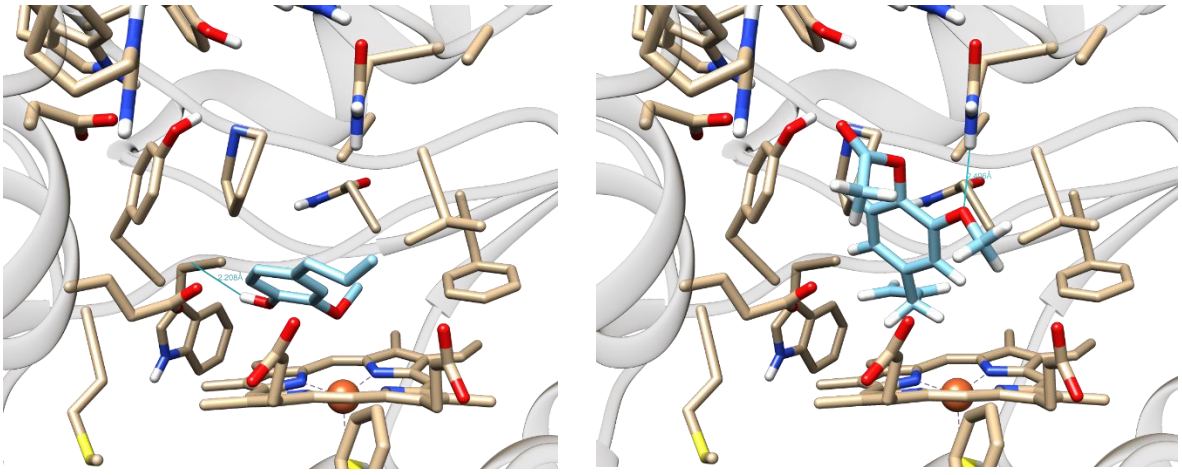
Table 2. Grid-score results and hydrogen bond interactions.

Protein	**Ligand**	**Grid-score**	**H-Bond number**	**Residue (H-Bond length, Å)**
Estrogen receptor-α	Eugenol	-29.758898	2	Lys145 (2.455); Ile82 (2.637)
	Eugenol acetate	-34.403477	0	
	Caryophyllene	-18.799114	0	
	Tamoxifen	**-66.970085**	1	Glu49 (1.954); Arg90 (2.046)

MMP-2	Eugenol	-41.468246	0	
	Eugenol acetate	-43.793243	1	Ser151 (2.388)
	Caryophyllene	-34.205738	0	
	SC-74020	**-109.474319**	4	Leu83 (2.017); Ala84 (2.441); His120 (3.054); Glu121 (2.421)
PARP	Eugenol	-31.103073	1	Ser243 (2.205)
	Eugenol acetate	-36.013237	1	Ser203 (2.585)
	Caryophyllene	-32.597618	0	
	FR257517	**-63.837357**	0	
CDK2	Eugenol	-36.910698	2	Asp74 (1.845); Lys77 (1.815)
	Eugenol acetate	-41.676758	1	Lys33 (2.136)
	Caryophyllene	-40.535130	0	
	Roscovitine	**-71.813713**	2	Leu71 (2.572; 1.992)
Chk1 kinase	Eugenol	-38.836273	3	Glu44 (1.504); Asn48 (2.658); Asp137 (1.801)
	Eugenol acetate	-39.099670	1	Asp137 (2.447)
	Caryophyllene	-35.456528	0	
	Cpd. 54	**-84.449051**	5	Glu74 (1.844); Cys76 (1.793); Glu80 (1.634); Glu123 (2.297); Asp137 (1.966)
NO synthase	Eugenol	-41.503204	1	Tyr291 (2.208)
	Eugenol acetate	-42.120934	1	Gln181 (2.406)
	Caryophyllene	-35.420425	0	
	AR-C95791	**-61.271812**	2	Tyr265 (2.129); Glu295 (1.937)
Human	Eugenol	-29.011292	0	
Cytochrome	Eugenol acetate	-32.352654	0	
P450	Caryophyllene	-31.890057	0	
CYP17A1	**Abiraterone**	**-61.819771**	1	Asn172 (1.864)
BCL-2	Eugenol	-35.300983	0	
	Eugenol acetate	-36.267906	0	
	Caryophyllene	-28.939451	0	
	ABT-199	**-79.199051**	1	Asn82 (2.186)
Cyclin A	Eugenol	-29.981400	2	Lys34 (2.082); Asp141 (2.623)
	Eugenol acetate	-31.090176	0	
	Caryophyllene	-25.151398	0	

	AZD5438	**-60.700954**	4	Lys34 (2.228); Leu79 (2.197); Leu79 (2.333); Asp82 (1.946)

The grid-score values of eugenol acetate has the closest grid-score to AR-C95791 (-42.12 vs.-61.27) in the NO synthase enzyme compared to other ligands and targets. When viewed from the docking pose, this compound can hold hydrogen bond interactions between oxygen in methyl ether and Gln181 residue. The allyl fragment points to Heme (Figure 3a). Eugenol has a similar grid-score (-41.50), but his docking pose is reversed. In eugenol, the phenolic group points to heme, and the phenolic OH group interacts with the backbone of the Tyr291 residue (Figure 3b). Although not better than cognate, eugenol has the ability to reduce NO produced by this enzyme (Prasad and Muralidhara, 2013). Eugenol also has the opportunity to be developed as a NO synthase inhibitor with several steps of chemical modification to add several functional groups to increase the number of interactions.



(a) (b)

Figure 3: *Docking pose* eugenol (a) and (b) eugenol acetat on the active site of the NO synthase enzyme

4. Conclusion

Clove flower extract was proven to be toxic to *Artemia salina* in the BSLT test with an LC_{50} value of 227.1 μg/ml based on probit analysis and classified as moderately toxic, so it has the potential to be developed as an anticancer drug. Based on the proximity to the grid-score value, eugenol acetate has close proximity to the cognate enzyme nitric oxide synthase (3E7G). If the in vitro test data shows inhibition of cancer cell growth, the most likely mechanism is through inhibition of nitric oxide synthase.

Data Avaibility

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Clove Flower Extract (*Syzygium aromaticum*) Has Anti-Cancer Potential Effect Analized by Molecular Docking and *Brine Shrimp Lethality Test* (BSLT)

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ABSTRACT

Developments in the health sector have found various kinds of anti-cancer drugs, but there are adverse side effects. On the other hand, anti-cancer medicines from natural products generally have minimal side effects, making them ideal candidates for cancer therapy. This study is aimed to evaluate the effect of clove flower extract (*Syzygium aromaticum*) as a candidate for anti-cancer by indicating grid-score values using molecular docking and LC_{50} values using the Brine shrimp lethality test (BSLT) method. Three hundred larvae shrimp *Artemia salina* leach as animal models were divided into 6 groups. Each group contains 10 larvae with 5 replication. The concentration of clove flower extract in the treatment media was 50 ppm (T1), 250 ppm (T2), 500 ppm (T3), 750 ppm (T4), 1000 ppm (T5), and 0 ppm as negativecontrol (seawater). Mortality data percentage of *Artemia salina* leach analyzed by probit analysis. The results showed that the extract of clove flower (*Syzygium aromaticum*) has a toxiceffect on larvae with LC_{50} values of 227,1 µg/ml or into the equation y = 2.8636 x – 1.7466 with R^2 value is 0,9062. Based on molecular docking, the proximity to the grid score value, eugenol acetate (grid-score -42.120934) has a close relationship with the cognate enzyme nitricoxide synthase (3E7G) (grid-score -61.271812). It concluded that clove flower extract is toxicto larval shrimp (*Artemia salina* Leach). Thus clove flower extract has the potential effect as ananti-cancer drug. Based on the proximity to the grid-score value, eugenol acetate has proximity to the cognate enzyme nitric oxide synthase (3E7G). In addition, the inhibition of nitric oxide synthase suggests a decrease in cancer cell growth.

Keywords: Brine shrimp lethality test, Clove flower, Molecular docking, *Syzygium aromaticum*

1. Introduction

Medicinal plants contain bioactive ingredients in one part or all parts of the plant that can be used to treat certain diseases. Features of plants that can be used include leaves, fruit, flowers, seeds, roots, rhizomes, stems, bark, and sap [1].

Cloves (*Syzygium aromaticum*) are ancient spice plants that have been known and used for thousands of years before Christ. The tree is a native plant of the Maluku islands (Ternate and Tidore), formerly known to explorers as spice islands [2,3].

Cloves contain substantial amounts of essential oil in flowers (10–20%), stalks (5–10%), and leaves (1–4%) [4]. Furthermore, clove essential oil has the best quality because the yield is high and contains eugenol, reaching 80–90%. Therefore, cloves are multi-beneficial and efficacious as food and beverage ingredients with high nutritional value and anti-cancer, antibacterial, antifungal, anti-inflammatory, antiproliferative, antifibrogenic, anti-insect, and analgesic [5]. Moreover, cloves have high antioxidant activity due to the high content of eugenol [6].

The phytochemicals contained in cloves are sesquiterpenes, monoterpenes, hydrocarbons, and phenolic compounds. Eugenol and β-caryophyllene are the most significant phytochemicals in clove oil. Eugenol has shown anti-cancer activity against colon, stomach, breast, prostate, melanoma, and leukemia. β-caryophyllene exhibits anti-cancer properties against pancreatic, skin, lymphatic, and cervical cancers [7].

Eugenol compounds, the main component of clove oil, are potential candidates for further development in assisting modern chemotherapy treatments for cancer treatment. Eugenol works by inhibiting the proliferation and formation of tumors, increasing Reactive Oxygen Species (ROS), inducing apoptosis, and having genotoxic effects on different cancer cells [7]. Even Zari et al. [8] also suggested that the anti-cancer effect of eugenol was achieved through various mechanisms such as induced apoptosis, cell cycle arrest, inhibition of proliferation, migration, angiogenesis, and metastasis of several cancer cell lines. Moreover, eugenol can be used as an adjunct therapy for patients treated with conventional chemotherapy. This combination leads to increased effectiveness with reduced toxicity. Several research results show that eugenol plant extracts have various biological activities, such as antifungal, anti-cancer, and anti-inflammatory. For example, as an anti-cancer activity,

eugenol is proapoptotic in breast cancer by downregulating E2F1/survivin [9].

Eugenol has the molecular formula $C_{10}H_{12}O_2$ with the IUPAC name 4-allyl-2-methoxyphenol. Moreover, eugenol has other names, such as 4-alylguaikol, 1-allyl-4-hydroxy-3- methoxybenzene, caryophilic acid, 4-hydroxy-3-methoxyalylbenzene, 2-methoxy-4- alylphenol [8]. The concentration of eugenol in clove oil varies from 70% to 96% [10], consisting of various functional groups, such as allyl (-CH2- CH=CH2), methoxy (-OCH3), and phenol (OH) [11].

Colorectal cancer is the third highest prevalence rate of all types of cancer worldwide [12]. Medical treatments often used to treat colorectal cancer are chemotherapy or radiotherapy, surgery, immunotherapy, hormonal therapy, and pharmacotherapy with a focus on tumor location and disease stage [13].

Developments in the health sector have found various kinds of anti-cancer drugs. The primary purpose of using anti-cancer drugs is to damage cancer cells without disturbing normal cells selectively. This goal is frequently achieved, and very few anti-cancer drugs currently work selectively to treat specific types of cancer [14]. Research in recent years has revealed that plants containing phytochemical compounds with anti-cancer properties are strongly associated with a reduced risk of cancer. In addition, naturalproducts generally have minimal side effects, making them ideal candidates for cancer therapy [15]. However, the studies on cloves as anti-cancer remain limited. Therefore, this study is arranged to evaluate the potent effect of cloves as anti-cancer using Brine Shrimp Lethality Test (BSLT). A straightforward benchtop bioassay that has produced positive results for screening plant extracts for biological activity is the Brine Shrimp lethality test [16]. The brine shrimp is toxic to a wide range of chemicals and natural products; the toxicity test is based on the brine shrimp dying after exposure to different amounts of plant extracts. This assumption has been applied to screening medicinal plant extracts in the BSLT since toxicology may be characterized as pharmacology at greater dosages because bioactive chemicals are almost always poisonous in high quantities [17].

The approach was initially explained by Meyer et al. [18], brine shrimp eggs begin to hatch 48 hours after being submerged in the brine. Each plant extract is diluted in a mixture of 20 ml of methylene chloride and methanol to produce a stock solution with a concentration of 10 mg/ml (1:1). Then, triplicate 500, 50, and 5 g/ml aliquots from the stock solutions are transferred to the vials, and the solvent is allowed to evaporate. After evaporation, 5 ml of brine are added to each vial to produce concentrations of 1000, 100, and 10 ppm. Ten nauplii are collected in each vial (30 shrimps per concentration). Lethal Concentrations at 50% Mortality (LC50) values are calculated using the Finney computer program with the number of survivors at each concentration.

Utami and Yusi [19] explained that the toxicity test results were expressed as Lethal Concentration 50 (LC50), the optimum concentration of extract that can kill 50% of the population of *Artemia salina*. Therefore, the lower LC_{50} value indicates a higher toxicity effect. The advantages of this BSLT test are that it is simple, fast, easy, the results can be repeated, and it is not expensive [20]. In addition, tests using *Artemia salina* have a very high sensitivity to cytotoxic compounds [21].

It is required to do a scientific study, such as in the areas of pharmacology, toxicology, identification, and isolation of active chemical compounds present in plants, to evaluate the effectiveness of conventional treatments [22]. Therefore, this study aims to determine the effect of clove flower extract (*Syzygium aromaticum*) as an anti-cancer candidate by indicating grid-score values using molecular docking and LC_{50} values using the Brine shrimp lethality test (BSLT) method.

2. Materials and Methods

2.1. Study Design of BSLT Method

This study was an experimental study with a *post-test-only control group design* to test the toxicity of clove flower extract against *Artemia salina* using the *Brine Shrimp Lethality*

Test (BSLT) method and molecular docking. The research design pattern used was a completely randomized design. This research has also followed the ethical procedure of the ethics commission at the Faculty of Veterinary Medicine, Airlangga University.

Ten samples total were collected for each concentration of Artemia salina larvae. In this study, five concentrations of clove flower extract were made in six experimental groups. Each concentration and control was repeated five times. So the total sample required is 300 tails.

This research was conducted from February to March 2022 and approved by the ethical committee of the Faculty of Veterinary Medicine at Airlangga University for animal experiments. The study was conducted at the Laboratory of the Veterinary Basic Medicine Division, Faculty of Veterinary Medicine, Airlangga University. Clove extract was made at the Unit Layanan Pengujian (ULP) Faculty of Pharmacy, Universitas Airlangga.

2.1.1. Clove Flower Extraction

The clove flower was obtained in the Naringgul District, South Cianjur Regency, West Java Province. Whole and dried clove flowers weighed as much as 900 grams. Then it was processed with a set of distillation tools. First, the distilled extraction liquid was collected, and to separate the essential oil from water, dichloromethane was added to the separating funnel in a ratio of 1:3. After accommodating the generated volatile oil, Na2SO4 anhydrous was applied to eliminate the remaining water [23]. Treatment solution was prepared from 20 mg of essential oil dissolved in 2 ml of ethanol, then pipetted into vials as much as 25 µl, 125 µl, 250 µl, 375 µl, and 500 µl, then left for 24 hours to evaporate solvent [24].

2.1.2. Hatching *Artemia salina*

Artemia salina larvae (Supreme plus®) were obtained from the Gunung Sari Ornamental Fish Market, Surabaya, East Java. Larval hatching is carried out in an aquarium. The environmental conditions for hatching *Artemia salina* larvae are pH 8-9; Water salinity ranges from 5-70 ppt; Temperature 26-31C^0. While aeration is controlled using an aerator.

The aquarium was given a bulkhead whosebottom edge had been perforated so that the hatched eggs could come out of the hole and thenbe filled with seawater. Eggs are *Artemia salina* inserted in one of the chambers and then closed. In another room, it is lit using a 10-watt lamp to attract hatched shrimp. Larvae aged 48 hours will be used as test animals [18].

2.1.3. Toxicity Test Using BSLT Method

One ml of seawater was added to the treatment solution and homogenized with a vortex. Put 10 larvae of *Artemia salina* into the vial and add seawater to a final volume of 5 ml so that thefinal results of the test solution are obtained with concentrations of 50 ppm, 250 ppm, 500 ppm,750 ppm, and 1000 ppm. The control group was only given 5 ml of seawater without cloveextract. The toxicity test was repeated five times. Then observations were made for 1 day on the death of *Artemia salina*. The mortality results of *Artemia salina* at each concentration werecompared with controls. Observations can be made after 24 hours of treatment. The standard criteria for assessing the mortality of Artemia salina larvae is that the larvae do not show movement for 10 seconds of observation.

The sample's toxicity test was determined by evaluating the value of LC50, which can kill Artemia salina up to 50%. Statistical calculations were performed using probit analysis with Statistical Program and Service Solutions (SPSS) for windows version 25 [25]. From the percent mortality, monitor for the probit number or value of each group of test animals through the table, determine the dose log for each group, and then make a graph with a straight line equation of the relationship between the probit value and the concentration log, $y = ax + b$. Where y = probit number and x = concentration log, a line is drawn from probit 5 (= 50% mortality) to the X axis, and the concentration log is obtained. Concentration logs are antiloged to get LC50, or LC50 values can also be calculated from the straight-line equation by entering the value 5 (probit of 50% of experimental animal deaths) as y so that x is produced as the log concentration value. LC50 is calculated and obtained from the antilog of the x value [26].

2.2 Study Design of Molecular Docking

Molecular docking is performed with Dock 6.8. program package. The structures used are listed in Table 1. The structure and molecular surface preparations were carried out using the Dock Prep and Write DMS features in the Chimera 1.16 program (1). Spheres on the molecular surface were created using the SPHGEN program. Since the position of the active site of the enzyme is known with certainty, spheres were selected within a radius of 8.0 Å from the ligand using the SPHERE_SELECTOR program. After that, the SHOWBOX program creates a simulation box with the appropriate size for the selected spheres, plus a margin of 8.0 Å in all directions. The grid was made at 0.25 Å resolution using the GRID program. The van der Waals interaction was modeled using the Lennard-Jones 12-6 potential, and to model the electrostatic interaction, the Coulomb potential with a value ε=4r was used (2). The docking parameter validation is carried out by the redocking method, where the docking method is valid if the value of the symmetry-corrected root mean square deviation of the heavy atom (HA_RMSDh) is less than 2.0 Å. The structure of eugenol and caryophyllene was optimized using the MMFF method in the Avogadro program (3), and then the AM1-BCC electrostatic charge was added using the ANTECHAMBER program (4). Visualization of the docked poses using the Maestro 12.4 Release 2020-2 program (Schrodinger, Inc).

Table 1. Crystal structures used

No	Access code PDB	Description	Resolution	Reference (DOI)
1	2JF9	Estrogen receptor alpha LBD in complex with a tamoxifen-specific peptide antagonist	2.10 Å	10.1074/jbc.M611424200
2	1HOV	Solution structure of a catalytic domain of MMP-2 complexed with SC-74020	NMR	10.1016/s0167-4838(02)00307-2
3	1UK0	Crystal structure of the catalytic domainof human poly(ADP-ribose) polymerase with a novel inhibitor	3.00 Å	10.1016/s0014-5793(03)01362-0
4	2A4L	Human cyclin-dependent kinase 2 in complex with roscovitine	2.40 Å	10.1111/j.1432-1033.1997.0518a.x

5	2R0U	Crystal Structure of Chek1 in Complex with Inhibitor 54	1.90 Å	10.1016/j.bmcl.2007.09.007
6	2X7F	Crystal structure of the kinase domain of human Traf2- and Nck-interacting Kinase with Wee1Chk1 Inhibitor	2.80 Å	-
7	3E7G	Structure of human INOSOX with inhibitor AR-C95791	2.20 Å	10.1038/nchembio.115
8	3RUK	Human Cytochrome P450 CYP17A1 in complex with Abiraterone	2.60 Å	10.1038/nature10743
9	4LXD	Bcl_2-Navitoclax Analog (without Thiophenyl) Complex	1.90 Å	10.1038/nm.3048
10	6GUE	CDK2/CyclinA in complex with AZD5438	1.99 Å	10.1016/j.chembiol.2018.10.015

3. Results and Discussion

3.1 Toxicity Test Using BSLT Method

The result shows that the distillation of clove flowers of as much as 900 grams has the average weight of essential oil as 9.75 (w/w) grams, with a yield of 1.083%.

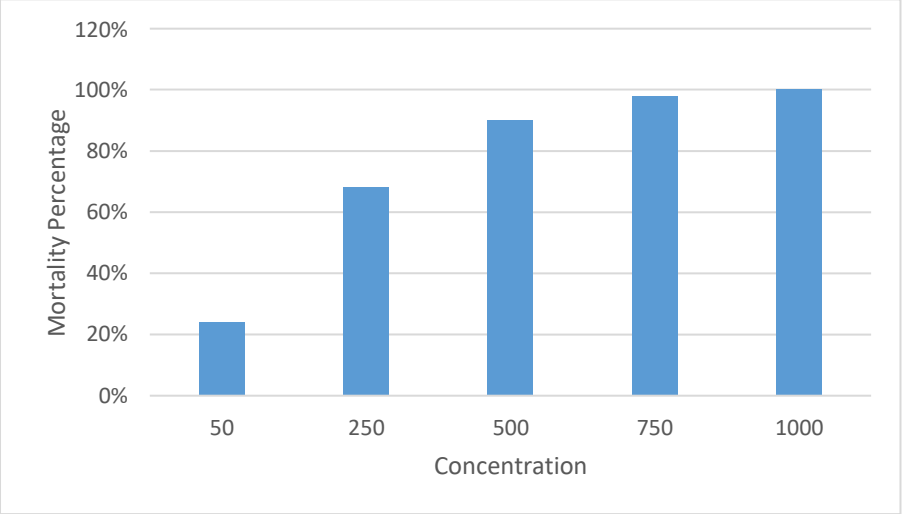


Figure 1. The results of the toxicity test of the concentration of clove flower extract (*Syzygium aromaticum*) on *Artemia salina* larvae

In the study of the toxicity test of clove flower extract using the BSLT method as an anti-cancer drug candidate, it was proven that clove flower extract was toxic to *Artemia salina* in the BSLT test (see Figure 1). Based on the study's results, clove flower extract was toxic to *Artemia salina* aged 48 hours. In T1, with an extract concentration of 50 ppm, the mortality

percentage was 24%; this is because clove flower extract's toxicity level is still low. The toxicity of insecticides in a species is strongly influenced by the concentration of the insecticide's chemical compounds in the target species' body [26]. At T2, with an extract concentration of 250 ppm, the mortality percentage was 68%. At this concentration, clove extract was toxic because it could kill *Artemia salina* by more than 50%. Therefore, at a concentration of 250 ppm, the LC_{50} value can be calculated. In T3, with an extract concentration of 500 ppm, the mortality percentage was 90%; in T4, with an extract concentration of 750 ppm, the mortality percentage was 98%. At this concentration, almost allthe larvae died; this indicates that the clove flower extract is very toxic to *Artemia salina*. However, the highest destructive power was at T5, with an extract concentration of 1000 ppm. The percentage of deaths was 100%. The increase in the concentration of the extract causes an increase in the content of the active ingredients in these substances, which function as insecticides that can kill organisms in large numbers [27].

In the control group, no dead larvae were found because, at this treatment, there was no addition of clove flower extract containing toxic substances. It showed that the death of *Artemia salina* occurred by the administration of clove flower extract and not due to the influence of the external environment. Thus it can be seen that the higher the concentration of clove flower extract, the higher the number of deaths in Artemia salina. This result is linear with research conducted by Lisdawati et al. [28], Sanjaya et al. [29], and Sapulette et al. [30], which showed that a higher concentration of plant extracts followed by a higher number of dead larvae. This research means that the number of deaths caused by *Artemia salina* tends to be directly proportional to the increasing concentration of clove flower extract. Probit analysis revealed that the LC50 value of clove flower extract was 227.1 g/ml. Toxicity criteria for assessing the level of toxicity of plant extracts are classified in the following order: extracts with LC50 > 1000 g/ml are non-toxic, LC50 500–1000 g/ml is low toxic, extracts with LC50 100–500 g/ml are moderately toxic, while the extract with LC50 0– 100 g/ml was very toxic [20]. From these criteria, clove extract is included in the moderate level of toxicity. The level of toxicity gives

meaning to the potential activity of clove flower extract as a candidate anti-cancer. The smaller the LC50, the more potential the plant has in cancer treatment.

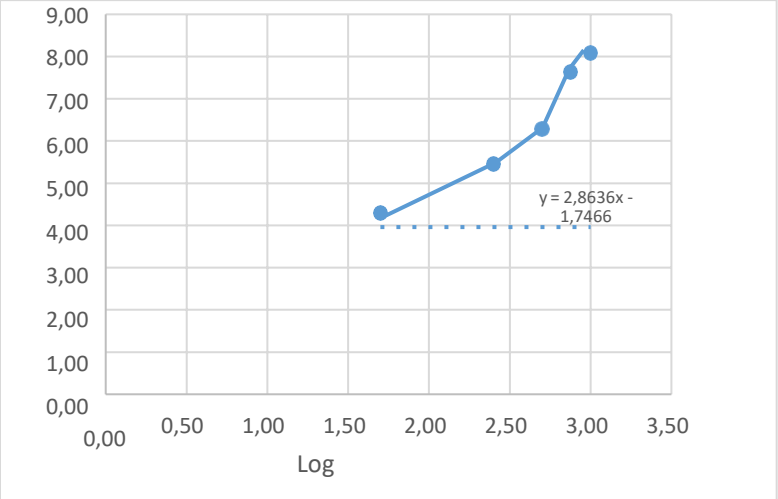


Figure 2. Graph of correlation of log concentration of clove flower extract (*Syzygium aromaticum*) with a probit mortality rate of *Artemia salina* larvae

The value of LC_{50} can be confirmed through the equation of the line y = ax + b, which is obtained from Figure 2. If the value of probit 5 (y = 5) is entered into the equation y = 2.8636 x – 1.7466, it will get the value of x = log 2.356, and then the value of x is antiloged to obtain a value of 227.1 g/ml. The probit analysis results are qualitatively estimated values, so they did not show the actual value. Therefore, interval estimates also appear in the probit analysis results. From the analysis, the interval estimation is between the values with thelower limit of 135 and the upper limit of 293. Based on Figure 2. It is known that the R^2 valueis 0.9062, meaning that clove flower extract's effect in killing *Artemia salina* is 90%.The coefficient of determination (R^2) is part of the total diversity of the dependent variable Y,which can be explained or taken into account by the diversity of the independent variable X. The value of R^2 is between 0 and 1, and the correlation is said to be better as R^2 approaches 1 [31]. In the toxicity test, the R^2 value is 1, which indicates that the factors that affectthe death of the test larvae are the result of the clove flower extract given.

Artemia salina larvae have a high sensitivity level to carrying out a test. Based on their morphology, *Artemia salina* aged 48 hours has started to have a mouth and digestive tract to

consume certain particles. Meanwhile, *Artemia salina* larvae that are 24 hours or in the second instar phase, even though they already have a digestive tract, are still unable to contact their environment, so extracts or external compounds cannot be absorbed by the larvae [20].

Factors that need to be considered in achieving egg hatching and the growth of *Artemia salina* are salinity, pH, and seawater temperature. The saltiness of the seawater used in this study was 37 ppt. The pH of the seawater used is 8,1. A decrease in pH below 7 can cause death. Hatching cysts require a slightly alkaline pH of 8–9 [32]. The seawater temperature in this study was 28.3oC. Artemia salina cannot survive in hatcheries at temperatures below 6℃ or above 35℃. In Artemia growth, the desired temperature ranges from 26–31oC [33]. During the hatching process, a lamp is added to the artemia larvae as a light source to maintain the optimum water temperature and can trigger the larvae to detach from their egg shells. The larvae that are actively moving will swim toward the light source because they have positive phototaxis properties that attract light [34].

Brine shrimp lethality test (BSLT) includes a top-ranking test in natural product screening for the presence of bioactive compounds. Although the use of the BSLT method is not adequate in determining the mechanism of action of bioactive substances in plants and is also not specific for anti-cancer activity, the BSLT method produces data that can be supported by more specific bioassays after the active compounds tested are proven to be toxic to *Artemia salina*, tending to be good candidates for anti-cancer research [35].

The phytochemicals contained in cloves are eugenol, β-caryophyllene, eugenol acetate, sesquiterpenes, monoterpenes, hydrocarbons, and phenolic compounds. Eugenol compounds are the main components in clove oil, with content reaching 78–95% [36]. The active ingredient eugenol functions as a larvae killer (anti-insect/anti- nematode) [27]. Sanjaya *et al*. [29] and Sapulette *et al*.[30] showed that eugenol activity could cause death in disease-carrying vector larvae; this is also evidenced by the research of Cansian *et al*. [37] and Gonzales *et al*. [38], who statedthat clove oil was toxic to *Artemia salina*.

The digestive tract of Artemia salina is a non-selective filter, making it easier for toxic substances to enter the mouth. Toxic compounds from clove flower extract that enter can interact with cell membrane targets and enzymes to affect the larval body mechanism, which can cause death [39]. The above research shows that the eugenol content in clove extract acts as a larvicide against Artemia salina by destroying cell membranes or interfering with larval metabolism.

3.1. Molecular Docking

Clove essential oil contains several compounds; the highest content in cloves is eugenol, eugenol acetate, and caryophyllene. In silico analysis was carried out by molecular docking of the three compounds to 9 proteins that are important in the proliferation of cancer cells [7]. These three compounds can be evaluated by comparing thegrid score of their compounds with the grid score of the original ligand (cognate). The resultscan be seen in Table 2. Based on these results, no compound can produce a grid-score better than cognate. It is because the clove essential oil bioactive structure hasa few functional groups, especially caryophyllene, which is filled with hydrophobic interactions. There is not much interaction that can be carried out other than the van der Waalsinteraction. Therefore, caryophyllene primarily produced the worst grid score compared to the other two compounds on 9 target proteins.

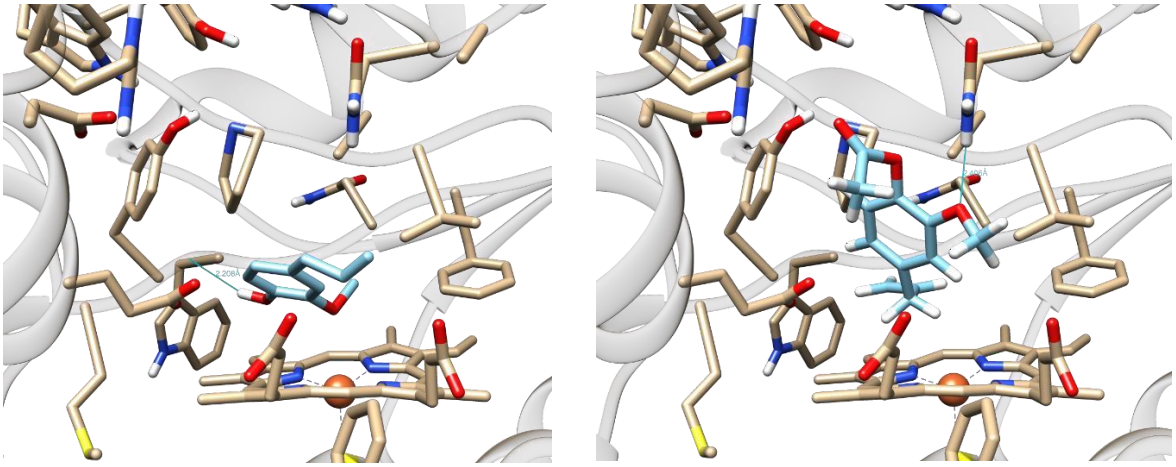
Table 2. Grid-score results and hydrogen bond interactions.

Protein	Ligand	Grid-score	H-Bond number	Residue (H-Bond length, Å)
Estrogen receptor-α	Eugenol	-29.758898	2	Lys145 (2.455); Ile82 (2.637)
	Eugenol acetate	-34.403477	0	
	Caryophyllene	-18.799114	0	
	Tamoxifen	**-66.970085**	1	Glu49 (1.954); Arg90 (2.046)
MMP-2	Eugenol	-41.468246	0	
	Eugenol acetate	-43.793243	1	Ser151 (2.388)
	Caryophyllene	-34.205738	0	
	SC-74020	**-109.474319**	4	Leu83 (2.017); Ala84

				(2.441); His120 (3.054); Glu121 (2.421)
PARP	Eugenol	-31.103073	1	Ser243 (2.205)
	Eugenol acetate	-36.013237	1	Ser203 (2.585)
	Caryophyllene	-32.597618	0	
	FR257517	**-63.837357**	0	
CDK2	Eugenol	-36.910698	2	Asp74 (1.845); Lys77 (1.815)
	Eugenol acetate	-41.676758	1	Lys33 (2.136)
	Caryophyllene	-40.535130	0	
	Roscovitine	**-71.813713**	2	Leu71 (2.572; 1.992)
Chk1 kinase	Eugenol	-38.836273	3	Glu44 (1.504); Asn48 (2.658); Asp137 (1.801)
	Eugenol acetate	-39.099670	1	Asp137 (2.447)
	Caryophyllene	-35.456528	0	
	Cpd. 54	**-84.449051**	5	Glu74 (1.844); Cys76 (1.793); Glu80 (1.634); Glu123 (2.297); Asp137 (1.966)
NO synthase	Eugenol	-41.503204	1	Tyr291 (2.208)
	Eugenol acetate	-42.120934	1	Gln181 (2.406)
	Caryophyllene	-35.420425	0	
	AR-C95791	**-61.271812**	2	Tyr265 (2.129); Glu295 (1.937)
Human Cytochrome P450	Eugenol	-29.011292	0	
	Eugenol acetate	-32.352654	0	
	Caryophyllene	-31.890057	0	
CYP17A1	**Abiraterone**	**-61.819771**	1	Asn172 (1.864)
BCL-2	Eugenol	-35.300983	0	
	Eugenol acetate	-36.267906	0	
	Caryophyllene	-28.939451	0	
	ABT-199	**-79.199051**	1	Asn82 (2.186)

Cyclin A	Eugenol	-29.981400	2	Lys34 (2.082); Asp141 (2.623)
	Eugenol acetate	-31.090176	0	
	Caryophyllene	-25.151398	0	
	AZD5438	**-60.700954**	4	Lys34 (2.228); Leu79 (2.197); Leu79 (2.333); Asp82 (1.946)

The grid-score values of eugenol acetate have the closest grid score to AR-C95791 (-42.12 vs.-61.27) in the NO synthase enzyme compared to other ligands and targets. This compound can hold hydrogen bond interactions between oxygen in methyl ether and Gln181 residue when viewed from the docking pose. The allyl fragment points to Heme (Figure 3a). Eugenol has a similar grid score (-41.50), but his docking pose is reversed. In eugenol, the phenolic group points to heme, and the phenolic OH group interacts with the backbone of the Tyr291 residue (Figure 3b). Although not better than cognate, eugenol can reduceNO produced by this enzyme [40]. Eugenol also has the opportunityto be developed as a NO synthase inhibitor with several steps of chemical modification to addseveral functional groups to increase the number of interactions.



(a) (b)

Figure 3: *Docking pose* eugenol (a) and (b) eugenol acetate on the active site of the NO synthase enzyme

4. Conclusion

Clove flower extract was proven toxic to *Artemia salina* in the BSLT test with an LC_{50} value of 227.1 μg/ml based on probit analysis and classified as moderately toxic, so it has the potential to be developed as an anti-cancer drug. Furthermore, based on the proximity to the grid-score value, eugenol acetate has proximity to the cognate enzyme nitric oxide synthase (3E7G).Therefore, if the in vitro test data shows inhibition of cancer cell growth, the most likely mechanism is through inhibition of nitric oxide synthase.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Clove Flower Extract (*Syzygium aromaticum*) Has Anti-Cancer Potential Effect Analized by Molecular Docking and *Brine Shrimp Lethality Test* (BSLT)

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Abstract

Various anticancer medications have been discovered due to advances in the health care industry, but they have undesirable side effects. On the other hand, anticancer drugs derived from natural sources have low side effects, making them excellent for cancer therapy. This study aims to evaluate the effect of clove flower extract (*Syzygium aromaticum*) as a potential anticancer agent by determining grid-score values using molecular docking and LC50 values using the Brine shrimp lethality test (BSLT) technique. As animal models, three hundred larvae of *Artemia salina* leach were divided into six groups. Each group has ten larvae that have undergone five replications. The clove flower extract concentration in the treatment media was 50 ppm (T1), 250 ppm (T2), 500 ppm (T3), 750 ppm (T4), 1000 ppm (T5), and 0 ppm (seawater) as the control. The probit analysis of *Artemia salina* leach mortality percentage data. The results indicated that the clove flower extract (*Syzygium aromaticum*) is harmful to larvae with LC50 values of 227,1 g/ml or into the equation y = 2,8636 x – 1,7466 with an R2 value of 0.9062. According to molecular docking, eugenol acetate (grid-score -42.120834) has a close relationship with the cognate enzyme nitric oxide synthase (3E7G) based on its proximity to the grid score value (grid-score -61.271812). Therefore, clove flower extract has the potential to act as an anticancer medication. Based on the grid-score proximity, eugenol acetate is close to the homologous enzyme nitric oxide synthase (3E7G). Inhibition of nitric oxide synthase also shows a reduction in cancer cell proliferation.

Keywords: Brine shrimp lethality test, Clove flower, Molecular docking, *Syzygium aromaticum*

1. Introduction

Some or all components of medicinal plants contain bioactive compounds that can be used to treat specific diseases. Leaves, fruit, flowers, seeds, rhizomes, stems, bark, and sap are all functional plant parts [1]. Cloves (*Syzygium aromaticum*) are ancient spice plants known and utilized for centuries. The tree is native to the Maluku islands (Ternate and Tidore) Indonesia, which explorers formerly referred to as the spice islands [2,3].

The essential oil content of clove flowers (10–20%), stems (5–10%), and leaves (1–4%) is substantial [4]. Additionally, clove essential oil has the highest quality due to its high yield and 80–90% eugenol content. Cloves are, therefore multi-beneficial and effective as food and beverage additives with high nutritional value, anticancer, antibacterial, antifungal, anti-inflammatory, antiproliferative, antifibrogenic, anti-insect, and analgesic properties [5]. In addition, cloves have a significant antioxidant activity due to their high eugenol concentration [6].

Cloves contain sesquiterpenes, monoterpenes, hydrocarbons, and phenolic substances as phytochemicals. The two most essential phytochemicals in clove oil are eugenol and -caryophyllene. Eugenol has demonstrated anticancer properties against colon, stomach, breast, prostate, melanoma, and leukemia cancers, while -caryophyllene has anticancer effects on pancreatic, cutaneous, lymphatic, and cervical malignancies [7].

The major component of clove oil, eugenol, is a possible contender for future development as an aid to current chemotherapeutic cancer therapies. Eugenol inhibits the growth and development of tumors, increases reactive oxygen species (ROS), induces apoptosis, and has genotoxic effects on many cancer cells [7]. Even Zari et al. [8] suggested that the anticancer action of eugenol was achieved via multiple mechanisms, including induced apoptosis, cell

cycle arrest, suppression of proliferation, migration, angiogenesis, and metastasis of multiple cancer cell lines. Additionally, eugenol can be used as adjuvant therapy for individuals undergoing standard chemotherapy. This combination increases efficacy while decreasing toxicity. Several research findings indicate that eugenol plant extracts possess various biological properties, including antifungal, anticancer, and anti-inflammatory properties. For instance, as an anticancer agent, eugenol promotes apoptosis by downregulating E2F1/survivin in breast cancer [9].

Eugenol's chemical formula is C10H12O2, and its IUPAC name is 4-allyl-2- methoxyphenol. In addition, eugenol is also known as 4-alylguaikol, 1-allyl-4-hydroxy-3-methoxybenzene, cryophilic acid, 4-hydroxy-3-methoxyalylbenzene, and 2-methoxy-4- alylphenol [8]. The percentage of eugenol in clove oil ranges from 70% to 96% [10], and its functional groups include allyl (-CH2-CH=CH2), methoxy (-OCH3), and phenol (OH) [11].

Various anticancer medications have been discovered due to advancements in the medical field. The primary objective of anticancer medications is to harm cancer cells without affecting normal cells selectively. This objective is typically attained, and very few anticancer medications now target specific cancer types [12, 13]. Recent studies have shown that plants containing phytochemical substances with anticancer capabilities are strongly connected with a decreased cancer risk. Moreover, natural products typically have few side effects, making them attractive candidates for cancer therapy [14]. However, there are few investigations on cloves as an anticancer agent.

This study is designed to examine the anticancer efficacy of cloves using the Brine Shrimp Lethality Test (BSLT). The Brine Shrimp lethality test is a straightforward benchtop bioassay that has provided favorable results for screening plant extracts for biological activity [15]. The brine shrimp is toxic to various chemicals and natural products; the toxicity test is based on the

brine shrimp's death after exposure to varying plant extracts. This premise has been used in screening therapeutic plant extracts in the BSLT, as toxicology can be described as pharmacology at higher doses because bioactive compounds are almost often harmful at high doses [16].

Utami and Yusi [18] indicated that the toxicity test results were expressed as Lethal Concentration 50 (LC50), which is the optimal concentration of extract that can kill 50% of the *Artemia salina* population. Consequently, a lower LC50 value suggests a more significant hazardous effect. The benefits of this BSLT test are that it is simple, quick, straightforward, repeatable, and inexpensive [19]. Moreover, *Artemia salina* tests are susceptible to harmful chemicals [20].

To evaluate the efficacy of conventional treatments [21], a scientific investigation, such as in the fields of pharmacology, toxicology, identification, and isolation of active chemical components present in plants, is required. Therefore, this study intends to establish the anticancer efficacy of clove flower extract (*Syzygium aromaticum*) by determining grid-score values using molecular docking and LC50 values using the Brine shrimp lethality test (BSLT) method.

2. Materials and Methods

Study Design of BSLT Method

This study was an experimental investigation with a post-test-only control group design to determine the toxicity of clove flower extract to *Artemia salina* utilizing the Brine Shrimp Lethality Test (BSLT) and molecular docking. The research design had completely randomized design. This research has also adhered to the guidelines of the Faculty of Veterinary Medicine's ethics commission of Airlangga University.

Ten samples were taken for every concentration of *Artemia salina* larvae. This study prepared five strengths of clove flower extract in six experimental groups. Five repetitions of each concentration and control were conducted. The required sample size is, therefore 300 larvae.

This research was conducted between February and March 2022 in the Laboratory of the Veterinary Basic Medicine Division, Faculty of Veterinary Medicine, Airlangga University, and Unit Layanan Pengujian (ULP) Faculty of Pharmacy Airlangga University to produce clove extract. The ethical Committee of the Faculty of Veterinary Medicine Airlangga University approved all the procedures for laboratory animals.

Clove Flower Extraction

The clove flower was obtained from local farm in the Naringgul District, South Cianjur Regency, West Java Province, Indonesia. Whole and dried clove flowers weighed as much as 900 grams. Then it was processed with a set of distillation tools. First, the distilled extraction liquid was collected, and to separate the essential oil from water, dichloromethane was added to the separating funnel in a ratio of 1:3. After accommodating the generated volatile oil, Na2SO4 anhydrous was applied to eliminate the remaining water [23]. Treatment solution was prepared from 20 mg of essential oil dissolved in 2 ml of ethanol, then pipetted into vials as much as 25 µl, 125 µl, 250 µl, 375 µl, and 500 µl, then left for 24 hours to evaporate solvent [24].

Hatching *Artemia salina*

The larvae of *Artemia salina* (Supreme plus® Goldenwest, origin from Great Salt Lake USA) were obtained from the Gunung Sari Ornamental Fish Market in Surabaya, East Java. The hatching of larvae occurs in an aquarium. The environmental conditions for hatching *Artemia salina* larvae are pH 8-9; water salinity between 5 and 70 ppt; and temperatures between 26 and 31 degrees Celsius. At the same time, aeration is regulated by utilizing an aerator.

The aquarium was equipped with a bulkhead whose bottom edge was perforated, allowing hatched eggs to escape through the hole before being refilled with seawater. *Artemia salina* eggs are put into one compartment, which is subsequently sealed. In another area, a 10-watt lamp attracts newly hatched shrimp. 48-hour-old larvae will serve as test subjects [18].

Toxicity Test Using BSLT Method

One ml of seawater was added to the treatment solution and homogenized with a vortex. Put 10 larvae of Artemia salina into the vial and add seawater to a final volume of 5 ml so that the final results of the test solution are obtained with concentrations of 50 ppm, 250 ppm, 500 ppm, 750 ppm, and 1000 ppm. The control group was only given 5 ml of seawater without clove extract. The toxicity test was repeated five times. Then observations were made for one day on the death of Artemia salina. The mortality results of Artemia salina at each concentration were compared with controls. Observations can be made after 24 hours of treatment. The standard criteria for assessing the mortality of Artemia salina larvae is that the larvae do not show movement for 10 seconds of observation.

The sample toxicity test was determined by evaluating the value of LC50, which can kill Artemia salina up to 50%. Statistical calculations were performed using probit analysis with Statistical Program and Service Solutions (SPSS) for windows version 25. From the percent mortality, monitor for the probit number or value of each group of test animals through the table, determine the dose log for each group and then make a graph with a straight line equation of the relationship between the probit value and the concentration log, $y = ax + b$. Where y = probit number and x = concentration log, a line is drawn from probit 5 (= 50% mortality) to the X axis, and the concentration log is obtained. Concentration logs are antiloged to get LC50, or LC50 values can also be calculated from the straight-line equation by entering the value 5 (probit of 50% of experimental animal deaths) as y so that x is

produced as the log concentration value. LC50 is calculated and obtained from the antilog of the x value [25].

Design of Molecular Docking

Molecular docking is conducted using the Dock 6.8 software package. The employed structures are listed in Table 1. The structural and molecular surface preparations were carried out using Chimera 1.16's Dock Prep and Write DMS functions (1). Using the SPHGEN tool, spherical structures were formed on the surface of molecules. Since the precise location of the enzyme's active site is known, the SPHERE SELECTOR tool was used to choose spheres within a radius of 8.0 from the ligand. The SHOWBOX application then generates a simulation box with the correct dimensions for the selected spheres plus a margin of 8.0 in all directions. Using the GRID software, a 0.25 resolution grid was created. Van der Waals interaction was modeled using the Lennard-Jones 12-6 potential, while electrostatic interaction was modeled using the Coulomb potential with a value of =4r (2). Redocking is used to validate the docking parameters, where the docking approach is considered legitimate if the symmetry-corrected root means square deviation of the heavy atom (HA RMSDh) is less than 2.0. Using the MMFF approach in the Avogadro software (3), the structures of eugenol and caryophyllene were optimized, and then the AM1-BCC electrostatic charge was applied using the ANTECHAMBER program (4). Visualization of the docked positions utilizing Maestro 12.4 Release 2020-2 (Schrodinger, Inc).

Table 1. Crystal structures used

No	Access code PDB	Description	Resolution	Reference (DOI)

1	2JF9	Estrogen receptor alpha LBD in complex with a tamoxifen-specific peptide antagonist	2.10 Å	10.1074/jbc.M611424200
2	1HOV	Solution structure of a catalytic domain of MMP-2 complexed with SC-74020	NMR	10.1016/s0167-4838(02)00307-2
3	1UK0	Crystal structure of the catalytic domainof human poly(ADP-ribose) polymerase with a novel inhibitor	3.00 Å	10.1016/s0014-5793(03)01362-0
4	2A4L	Human cyclin-dependent kinase 2 in complex with roscovitine	2.40 Å	10.1111/j.1432-1033.1997.0518a.x
5	2R0U	Crystal Structure of Chek1 in Complex with Inhibitor 54	1.90 Å	10.1016/j.bmcl.2007.09.007
6	2X7F	Crystal structure of the kinase domain of human Traf2- and Nck-interacting Kinase with Wee1Chk1 Inhibitor	2.80 Å	-
7	3E7G	Structure of human INOSOX with inhibitor AR-C95791	2.20 Å	10.1038/nchembio.115
8	3RUK	Human Cytochrome P450 CYP17A1 in complex with Abiraterone	2.60 Å	10.1038/nature10743
9	4LXD	Bcl_2-Navitoclax Analog (without Thiophenyl) Complex	1.90 Å	10.1038/nm.3048
10	6GUE	CDK2/CyclinA in complex with AZD5438	1.99 Å	10.1016/j.chembiol.2018.10.015

3. Results and Discussion

BSLT Result

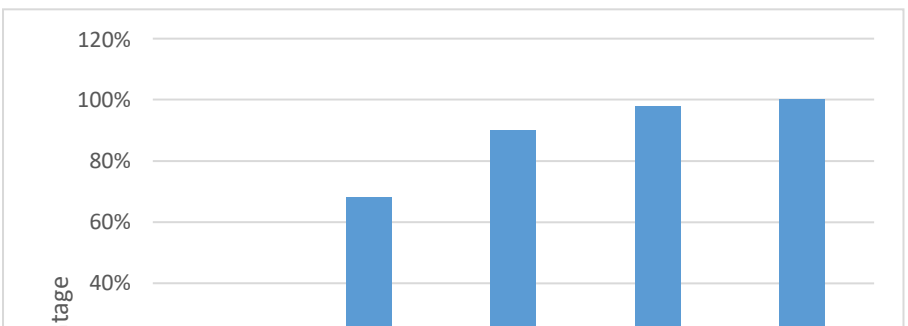


Figure 1. The results of the toxicity test of the concentration of clove flower extract(*Syzygium aromaticum*) on *Artemia salina* larvae

In the study of the toxicity test of clove flower extract as a potential anticancer medicine utilizing the BSLT method, it was determined that clove flower extract was poisonous to *Artemia salina* in the BSLT test (see Figure 1). Based on the investigation results, 900 grams of clove flowers distillation produced essential oil as 9.75 (w/w) grams, with a yield of 1.083%. This extract proved poisonous to 48-hour-old Artemia salina. In T1, where the extract concentration was 50 ppm, the mortality rate was 24% because clove flower extract's toxicity level is still low. The concentration of the pesticide's chemical components within the target species' body significantly impacts the insecticide's toxicity [25]. At T2, with a 250 ppm extract concentration, the mortality rate was 68%. At this dosage, clove extract was poisonous, as it could kill *Artemia salina* by more than 50 percent. At a concentration of 250 ppm, it is possible to determine the LC50 value. In T3, where the extract concentration was 500 ppm, the percentage of death was 90%; in T4, where the extract concentration was 750 ppm, the percentage of mortality was 98%. This result suggests that the clove flower extract is highly toxic to *Artemia salina* at this dose. T5 possessed the most destructive potential, with an extract concentration of 1000 ppm. The proportion of deaths was 100 percent. The rise in extract concentration induces an increase in the number of active components in these substances, which function as insecticides capable of killing numerous species [27].

In the control group, there were no dead larvae since there was no addition of clove flower

extract containing poisonous compounds. It demonstrated that the death of *Artemia salina* was caused by the administration of clove flower extract and not by environmental factors. Thus, it can be seen that the number of *Artemia salina* fatalities is proportional to the concentration of clove flower extract. This result is consistent with the findings of Lisdawati et al. [28], Sanjaya et al. [19], and Sapulette et al. [29], who discovered a correlation between the concentration of plant extracts and the number of dead larvae. This research indicates that the number of Artemia salina-related fatalities is often proportional to the concentration of clove flower extract. The LC50 of clove flower extract was calculated to be 227.1 g/ml using the Probit method. LC50 500–1000 g/ml is mildly toxic, LC50 100–500 g/ml is highly toxic, and LC50 0–100 g/ml is extremely poisonous [20]. Based on these factors, clove extract is classified as moderately hazardous. The level of toxicity provides context for the potential anticancer activities of clove flower extract. The lower the LC50, the greater the plant's potential as a cancer treatment.

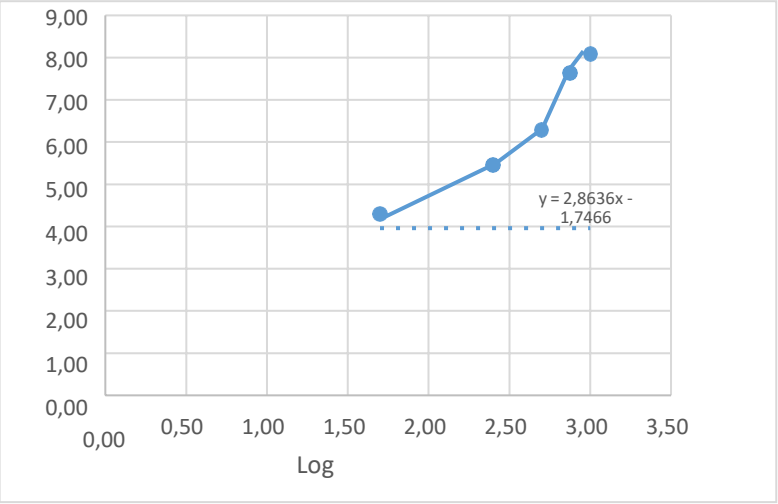


Figure 2. Graph of correlation of log concentration of clove flower extract (*Syzygium aromaticum*) with a probit mortality rate of *Artemia salina* larvae

Figure 2 provides the equation for the line y = ax + b, which can be used to confirm the LC50 value. Inputting the value of probit 5 (y = 5) into the equation y = 2.8636 x – 1.7466 yields the result of x = log 2.356, which is then antiloged to yield the value of 227.1 g/ml. The results of

the probit analysis are qualitatively estimated values, so they did not reflect the actual value. Consequently, interval estimates also emerge in the findings of the probit analysis. Based on the investigation, the estimated interval between the values range is 135 - 293. Figure 2 describes that The R2 value is known to be 0.9062, indicating that clove flower extract is 90% effective at killing Artemia salina. The coefficient of determination (R2) is a component of the total diversity of the dependent variable Y, which may be accounted for or explained by the variety of the independent variable X. The value of R2 is between 0 and 1, and it is argued that the correlation improves as R2 approaches 1 [30]. In the toxicity test, the R2 value is 1, indicating that the clove flower extract administered is the cause of the mortality of the test larvae.

Artemia salina larvae exhibit a high level of sensitivity to testing. Based on their morphology, 48-hour-old *Artemia salina* has developed a mouth and digestive system to absorb specific particles. *Artemia salina* larvae that are 24 hours old or in the second instar phase, despite having a digestive system, cannot interact with their surroundings and cannot absorb extracts or external substances [20].

In order to achieve *Artemia salina* egg hatching and growth, salinity, pH, and seawater temperature must be considered. This investigation utilized seawater with a salinity of 37 ppt. The pH of the used seawater is 8.1. A pH fall below 7 can be fatal. [31] Hatching cysts require a pH of 8–9, which is slightly alkaline. In this investigation, the seawater temperature was 28.3oC. *Artemia salina* cannot thrive at temperatures below 6°C or above 35°C in hatcheries. The optimal Artemia growth temperature varies from 26 to 31 degrees Celsius [32]. During the hatching process, a lamp is added to the artemia larvae as a light source to maintain the optimal water temperature and to stimulate the larvae to shed their egg shells. The actively moving larvae swim toward the light source due to their positive phototaxis features that attract light [33].

Artemia salina's digestive tract is a nonselective filter, which makes it easier for poisonous chemicals to enter the mouth. Phyto-chemicals from clove flower extract can interact with cell membrane targets and enzymes, resulting in death [34]. It is in agreement with the initial researches that demonstrate the eugenol component of clove extract acts as a larvicide against *Artemia salina* by damaging cell membranes or interfering with the metabolism of the larvae [35,36].

The Brine Shrimp Lethality Technique (BSLT) is a leading method for evaluating the presence of bioactive compounds in natural products, with results typically related to cytotoxic and anticancer activity [37]. Meyer et al. [17] initially elucidated this method, 48 hours after being soaked in brine, brine shrimp eggs will begin to hatch. Each plant extract is diluted in 20 ml of a mixture of methylene chloride and methanol to form a stock solution with a 10 mg/ml concentration (1:1). Then, aliquots of 500, 50, and 5 g/ml are transferred in triplicate from the stock solutions to the vials, and the solvent is allowed to evaporate. After evaporation, 5 ml of brine are added to each vial to generate 1000, 100, and 10 ppm concentrations. Each vial is filled with ten nauplii (30 shrimps per concentration). Lethal Concentrations at 50% Mortality (LC50) values are determined using the Finney computer program's number of survivors at each concentration.

As a preliminary anticancer screening test, BSLT method is insufficient for determining the mechanism of action of bioactive substances in plants and is not specific for anticancer activity. However the BSLT method generates data that can be supported by more specific bioassays after the active compounds tested are toxic to Artemia salina, indicating that they are likely potential candidates for anticancer research. Further evaluation to indicate cytotoxicity of *Syzygium aromaticum* can be conducted in vitro by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay [38], to consider the safety of the product application in the human model cells.

Molecular Docking

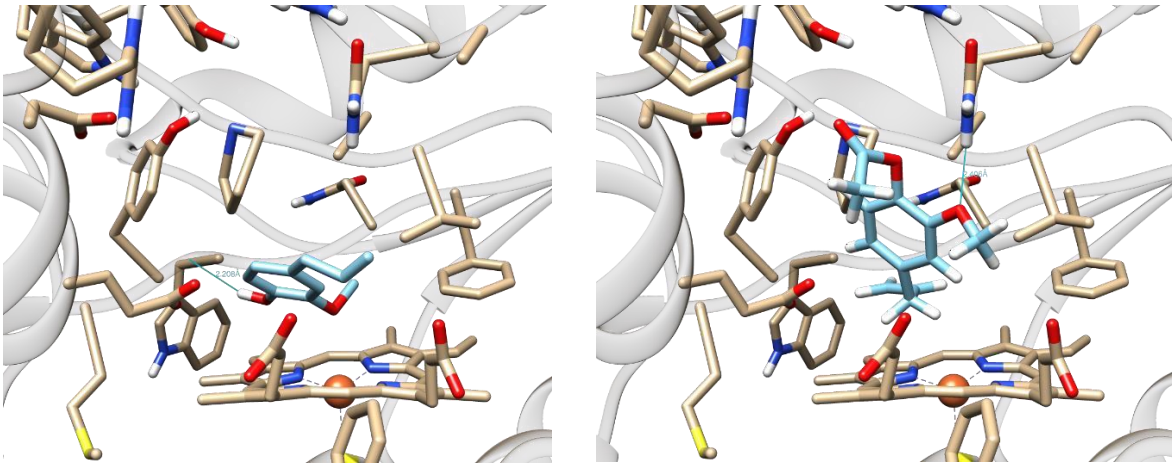
Clove essential oil includes many components, with eugenol, eugenol acetate, and caryophyllene constituting the majority. The three chemicals were analyzed in silico by molecular docking with nine proteins essential for cancer cell proliferation [7]. These three compounds can be evaluated by comparing their grid score to the original ligand's grid score (cognate). The outcomes are shown in Table 2. According to these findings, no substance can produce a higher grid score than cognate due to the clove essential oil's bioactive structure, including a small number of functional groups, particularly caryophyllene, which is rich in hydrophobic interactions. Other than the van der Waals interaction, there are few other possible interactions. In addition, caryophyllene produced the lowest grid score on nine target proteins compared to the other two chemicals.

Table 2. Grid-score results and hydrogen bond interactions.

Protein	Ligand	Grid-score	H-Bond number	Residue (H-Bond length, Å)
Estrogen receptor-α	Eugenol	-29.758898	2	Lys145 (2.455); Ile82 (2.637)
	Eugenol acetate	-34.403477	0	
	Caryophyllene	-18.799114	0	
	Tamoxifen	**-66.970085**	1	Glu49 (1.954); Arg90 (2.046)
MMP-2	Eugenol	-41.468246	0	
	Eugenol acetate	-43.793243	1	Ser151 (2.388)
	Caryophyllene	-34.205738	0	
	SC-74020	**-109.474319**	4	Leu83 (2.017); Ala84 (2.441); His120 (3.054); Glu121 (2.421)
PARP	Eugenol	-31.103073	1	Ser243 (2.205)
	Eugenol acetate	-36.013237	1	Ser203 (2.585)
	Caryophyllene	-32.597618	0	
	FR257517	**-63.837357**	0	
CDK2	Eugenol	-36.910698	2	Asp74 (1.845); Lys77 (1.815)
	Eugenol acetate	-41.676758	1	Lys33 (2.136)
	Caryophyllene	-40.535130	0	
	Roscovitine	**-71.813713**	2	Leu71 (2.572; 1.992)
Chk1 kinase	Eugenol	-38.836273	3	Glu44 (1.504); Asn48 (2.658); Asp137 (1.801)
	Eugenol acetate	-39.099670	1	Asp137 (2.447)

	Caryophyllene	-35.456528	0	
	Cpd. 54	**-84.449051**	5	Glu74 (1.844); Cys76 (1.793); Glu80 (1.634); Glu123 (2.297); Asp137 (1.966)
NO synthase	Eugenol	-41.503204	1	Tyr291 (2.208)
	Eugenol acetate	-42.120934	1	Gln181 (2.406)
	Caryophyllene	-35.420425	0	
	AR-C95791	**-61.271812**	2	Tyr265 (2.129); Glu295 (1.937)
Human Cytochrome P450	Eugenol	-29.011292	0	
	Eugenol acetate	-32.352654	0	
	Caryophyllene	-31.890057	0	
CYP17A1	**Abiraterone**	**-61.819771**	1	Asn172 (1.864)
BCL-2	Eugenol	-35.300983	0	
	Eugenol acetate	-36.267906	0	
	Caryophyllene	-28.939451	0	
	ABT-199	**-79.199051**	1	Asn82 (2.186)
Cyclin A	Eugenol	-29.981400	2	Lys34 (2.082); Asp141 (2.623)
	Eugenol acetate	-31.090176	0	
	Caryophyllene	-25.151398	0	
	AZD5438	**-60.700954**	4	Lys34 (2.228); Leu79 (2.197); Leu79 (2.333); Asp82 (1.946)

Eugenol acetate has the closest grid score to AR-C95791 (-42.12 vs. -61.27) in the NO synthase enzyme compared to other ligands and targets. This molecule can maintain hydrogen bond contacts between oxygen in methyl ether and Gln181 residue when examined from the docking stance. The fragment allyl points to Heme (Figure 3a). Eugenol has a comparable grid score (-41.5). However, his docking position is the opposite. The phenolic group in eugenol points to heme, and the phenolic OH group interacts with the Tyr291 residue's backbone (Figure 3b). Although not as effective as its homolog, eugenol can inhibit the production of NO by this enzyme [36]. Eugenol may also be created as a NO synthase inhibitor by including multiple functional groups to enhance the number of contacts through a series of chemical modifications.



(a) (b)

Figure 3: *Docking pose* eugenol (a) and (b) eugenol acetate on the active site of the NO synthase enzyme

4. Conclusion

Clove flower extract was determined to be moderately toxic to *Artemia salina* in the BSLT test with an LC50 value of 227.1 g/ml based on probit analysis. Hence it has the potential to be developed as an anticancer medicine. In addition, eugenol acetate is close to its cognate enzyme nitric oxide synthase based on its proximity to the grid-score value (3E7G). Therefore, if in vitro test results indicate that cancer cell proliferation is inhibited, the most likely mechanism is the suppression of nitric oxide synthase.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Clove Flower Extract (*Syzygium aromaticum*) Has Anti-Cancer Potential Effect Analized by Molecular Docking and *Brine Shrimp Lethality Test* (BSLT)

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Abstract

Various anticancer medications have been discovered due to advances in the health care industry, but they have undesirable side effects. On the other hand, anticancer drugs derived from natural sources have low side effects, making them excellent for cancer therapy. This study aims to evaluate the effect of clove flower extract (*Syzygium aromaticum*) as a potential anticancer agent by determining grid-score values using molecular docking and LC50 values using the Brine shrimp lethality test (BSLT) technique. As animal models, three hundred larvae of *Artemia salina* leach were divided into six groups. Each group has ten larvae that have undergone five replications. The clove flower extract concentration in the treatment media was 50 ppm (T1), 250 ppm (T2), 500 ppm (T3), 750 ppm (T4), 1000 ppm (T5), and 0 ppm (seawater) as the control. The probit analysis of *Artemia salina* leach mortality percentage data. The results indicated that the clove flower extract (*Syzygium aromaticum*) is harmful to larvae with LC50 values of 227,1 g/ml or into the equation y = 2,8636 x – 1,7466 with an R2 value of 0.9062. According to molecular docking, eugenol acetate (grid-score -42.120834) has a close relationship with the cognate enzyme nitric oxide synthase (3E7G) based on its proximity to the grid score value (grid-score -61.271812). Therefore, clove flower extract has the potential to act as an anticancer medication. Based on the grid-score proximity, eugenol acetate is close to the homologous enzyme nitric oxide synthase (3E7G). Inhibition of nitric oxide synthase also shows a reduction in cancer cell proliferation.

Keywords: Brine shrimp lethality test, Clove flower, Molecular docking, *Syzygium aromaticum*

1. Introduction

Some or all components of medicinal plants contain bioactive compounds that can be used to treat specific diseases. Leaves, fruit, flowers, seeds, rhizomes, stems, bark, and sap are all functional plant parts [1]. Cloves (*Syzygium aromaticum*) are ancient spice plants known and utilized for centuries. The tree is native to the Maluku islands (Ternate and Tidore) Indonesia, which explorers formerly referred to as the spice islands [2,3].

The essential oil content of clove flowers (10–20%), stems (5–10%), and leaves (1–4%) is substantial [4]. Additionally, clove essential oil has the highest quality due to its high yield and 80–90% eugenol content. Cloves are, therefore multi-beneficial and effective as food and beverage additives with high nutritional value, anticancer, antibacterial, antifungal, anti-inflammatory, antiproliferative, antifibrogenic, anti-insect, and analgesic properties [5]. In addition, cloves have a significant antioxidant activity due to their high eugenol concentration [6].

Cloves contain sesquiterpenes, monoterpenes, hydrocarbons, and phenolic substances as phytochemicals. The two most essential phytochemicals in clove oil are eugenol and -caryophyllene. Eugenol has demonstrated anticancer properties against colon, stomach, breast, prostate, melanoma, and leukemia cancers, while -caryophyllene has anticancer effects on pancreatic, cutaneous, lymphatic, and cervical malignancies [7].

The major component of clove oil, eugenol, is a possible contender for future development as an aid to current chemotherapeutic cancer therapies. Eugenol inhibits the growth and development of tumors, increases reactive oxygen species (ROS), induces apoptosis, and has genotoxic effects on many cancer cells [7]. Even Zari et al. [8] suggested that the anticancer action of eugenol was achieved via multiple mechanisms, including induced apoptosis, cell

cycle arrest, suppression of proliferation, migration, angiogenesis, and metastasis of multiple cancer cell lines. Additionally, eugenol can be used as adjuvant therapy for individuals undergoing standard chemotherapy. This combination increases efficacy while decreasing toxicity. Several research findings indicate that eugenol plant extracts possess various biological properties, including antifungal, anticancer, and anti-inflammatory properties. For instance, as an anticancer agent, eugenol promotes apoptosis by downregulating E2F1/survivin in breast cancer [9].

Eugenol's chemical formula is C10H12O2, and its IUPAC name is 4-allyl-2- methoxyphenol. In addition, eugenol is also known as 4-alylguaikol, 1-allyl-4-hydroxy-3-methoxybenzene, cryophilic acid, 4-hydroxy-3-methoxyalylbenzene, and 2-methoxy-4- alylphenol [8]. The percentage of eugenol in clove oil ranges from 70% to 96% [10], and its functional groups include allyl (-CH2-CH=CH2), methoxy (-OCH3), and phenol (OH) [11].

Various anticancer medications have been discovered due to advancements in the medical field. The primary objective of anticancer medications is to harm cancer cells without affecting normal cells selectively. This objective is typically attained, and very few anticancer medications now target specific cancer types [12, 13]. Recent studies have shown that plants containing phytochemical substances with anticancer capabilities are strongly connected with a decreased cancer risk. Moreover, natural products typically have few side effects, making them attractive candidates for cancer therapy [14]. However, there are few investigations on cloves as an anticancer agent.

This study is designed to examine the anticancer efficacy of cloves using the Brine Shrimp Lethality Test (BSLT). The Brine Shrimp lethality test is a straightforward benchtop bioassay that has provided favorable results for screening plant extracts for biological activity [15]. The brine shrimp is toxic to various chemicals and natural products; the toxicity test is based on the

brine shrimp's death after exposure to varying plant extracts. This premise has been used in screening therapeutic plant extracts in the BSLT, as toxicology can be described as pharmacology at higher doses because bioactive compounds are almost often harmful at high doses [16].

Utami and Yusi [18] indicated that the toxicity test results were expressed as Lethal Concentration 50 (LC50), which is the optimal concentration of extract that can kill 50% of the *Artemia salina* population. Consequently, a lower LC50 value suggests a more significant hazardous effect. The benefits of this BSLT test are that it is simple, quick, straightforward, repeatable, and inexpensive [19]. Moreover, *Artemia salina* tests are susceptible to harmful chemicals [20].

To evaluate the efficacy of conventional treatments [21], a scientific investigation, such as in the fields of pharmacology, toxicology, identification, and isolation of active chemical components present in plants, is required. Therefore, this study intends to establish the anticancer efficacy of clove flower extract (*Syzygium aromaticum*) by determining grid-score values using molecular docking and LC50 values using the Brine shrimp lethality test (BSLT) method.

2. Materials and Methods

Study Design of BSLT Method

This study was an experimental investigation with a post-test-only control group design to determine the toxicity of clove flower extract to *Artemia salina* utilizing the Brine Shrimp Lethality Test (BSLT) and molecular docking. The research design had completely randomized design. This research has also adhered to the guidelines of the Faculty of Veterinary Medicine's ethics commission of Airlangga University.

Ten samples were taken for every concentration of *Artemia salina* larvae. This study prepared five strengths of clove flower extract in six experimental groups. Five repetitions of each concentration and control were conducted. The required sample size is, therefore 300 larvae.

This research was conducted between February and March 2022 in the Laboratory of the Veterinary Basic Medicine Division, Faculty of Veterinary Medicine, Airlangga University, and Unit Layanan Pengujian (ULP) Faculty of Pharmacy Airlangga University to produce clove extract. The ethical Committee of the Faculty of Veterinary Medicine Airlangga University approved all the procedures for laboratory animals.

Clove Flower Extraction

The clove flower was obtained from local farm in the Naringgul District, South Cianjur Regency, West Java Province, Indonesia. Whole and dried clove flowers weighed as much as 900 grams. Then it was processed with a set of distillation tools. First, the distilled extraction liquid was collected, and to separate the essential oil from water, dichloromethane was added to the separating funnel in a ratio of 1:3. After accommodating the generated volatile oil, Na2SO4 anhydrous was applied to eliminate the remaining water [23]. Treatment solution was prepared from 20 mg of essential oil dissolved in 2 ml of ethanol, then pipetted into vials as much as 25 µl, 125 µl, 250 µl, 375 µl, and 500 µl, then left for 24 hours to evaporate solvent [24].

Hatching *Artemia salina*

The larvae of *Artemia salina* (Supreme plus® Goldenwest, origin from Great Salt Lake USA) were obtained from the Gunung Sari Ornamental Fish Market in Surabaya, East Java. The hatching of larvae occurs in an aquarium. The environmental conditions for hatching *Artemia salina* larvae are pH 8-9; water salinity between 5 and 70 ppt; and temperatures between 26 and 31 degrees Celsius. At the same time, aeration is regulated by utilizing an aerator.

The aquarium was equipped with a bulkhead whose bottom edge was perforated, allowing hatched eggs to escape through the hole before being refilled with seawater. *Artemia salina* eggs are put into one compartment, which is subsequently sealed. In another area, a 10-watt lamp attracts newly hatched shrimp. 48-hour-old larvae will serve as test subjects [18].

Toxicity Test Using BSLT Method

One ml of seawater was added to the treatment solution and homogenized with a vortex. Put 10 larvae of Artemia salina into the vial and add seawater to a final volume of 5 ml so that the final results of the test solution are obtained with concentrations of 50 ppm, 250 ppm, 500 ppm, 750 ppm, and 1000 ppm. The control group was only given 5 ml of seawater without clove extract. The toxicity test was repeated five times. Then observations were made for one day on the death of Artemia salina. The mortality results of Artemia salina at each concentration were compared with controls. Observations can be made after 24 hours of treatment. The standard criteria for assessing the mortality of Artemia salina larvae is that the larvae do not show movement for 10 seconds of observation.

The sample toxicity test was determined by evaluating the value of LC50, which can kill Artemia salina up to 50%. Statistical calculations were performed using probit analysis with Statistical Program and Service Solutions (SPSS) for windows version 25. From the percent mortality, monitor for the probit number or value of each group of test animals through the table, determine the dose log for each group and then make a graph with a straight line equation of the relationship between the probit value and the concentration log, $y = ax + b$. Where y = probit number and x = concentration log, a line is drawn from probit 5 (= 50% mortality) to the X axis, and the concentration log is obtained. Concentration logs are antiloged to get LC50, or LC50 values can also be calculated from the straight-line equation by entering the value 5 (probit of 50% of experimental animal deaths) as y so that x is

produced as the log concentration value. LC50 is calculated and obtained from the antilog of the x value [25].

Design of Molecular Docking

Molecular docking is conducted using the Dock 6.8 software package. The employed structures are listed in Table 1. The structural and molecular surface preparations were carried out using Chimera 1.16's Dock Prep and Write DMS functions (1). Using the SPHGEN tool, spherical structures were formed on the surface of molecules. Since the precise location of the enzyme's active site is known, the SPHERE SELECTOR tool was used to choose spheres within a radius of 8.0 from the ligand. The SHOWBOX application then generates a simulation box with the correct dimensions for the selected spheres plus a margin of 8.0 in all directions. Using the GRID software, a 0.25 resolution grid was created. Van der Waals interaction was modeled using the Lennard-Jones 12-6 potential, while electrostatic interaction was modeled using the Coulomb potential with a value of =4r (2). Redocking is used to validate the docking parameters, where the docking approach is considered legitimate if the symmetry-corrected root means square deviation of the heavy atom (HA RMSDh) is less than 2.0. Using the MMFF approach in the Avogadro software (3), the structures of eugenol and caryophyllene were optimized, and then the AM1-BCC electrostatic charge was applied using the ANTECHAMBER program (4). Visualization of the docked positions utilizing Maestro 12.4 Release 2020-2 (Schrodinger, Inc).

Table 1. Crystal structures used

No	Access code PDB	Description	Resolution	Reference (DOI)

1	2JF9	Estrogen receptor alpha LBD in complex with a tamoxifen-specific peptide antagonist	2.10 Å	10.1074/jbc.M611424200
2	1HOV	Solution structure of a catalytic domain of MMP-2 complexed with SC-74020	NMR	10.1016/s0167-4838(02)00307-2
3	1UK0	Crystal structure of the catalytic domainof human poly(ADP-ribose) polymerase with a novel inhibitor	3.00 Å	10.1016/s0014-5793(03)01362-0
4	2A4L	Human cyclin-dependent kinase 2 in complex with roscovitine	2.40 Å	10.1111/j.1432-1033.1997.0518a.x
5	2R0U	Crystal Structure of Chek1 in Complex with Inhibitor 54	1.90 Å	10.1016/j.bmcl.2007.09.007
6	2X7F	Crystal structure of the kinase domain of human Traf2- and Nck-interacting Kinase with Wee1Chk1 Inhibitor	2.80 Å	-
7	3E7G	Structure of human INOSOX with inhibitor AR-C95791	2.20 Å	10.1038/nchembio.115
8	3RUK	Human Cytochrome P450 CYP17A1 in complex with Abiraterone	2.60 Å	10.1038/nature10743
9	4LXD	Bcl_2-Navitoclax Analog (without Thiophenyl) Complex	1.90 Å	10.1038/nm.3048
10	6GUE	CDK2/CyclinA in complex with AZD5438	1.99 Å	10.1016/j.chembiol.2018.10.015

3. Results and Discussion

BSLT Result

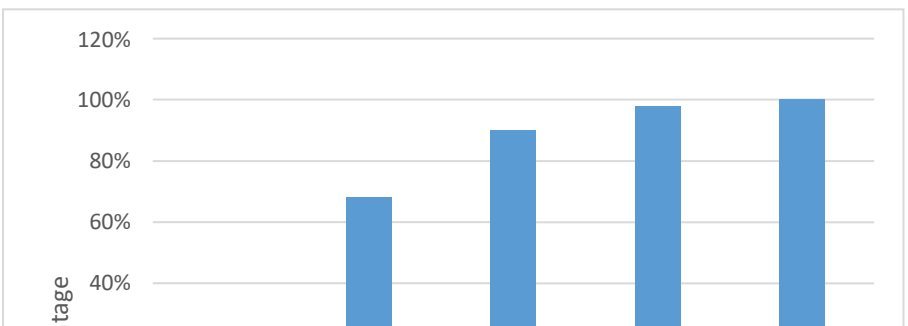


Figure 1. The results of the toxicity test of the concentration of clove flower extract(*Syzygium aromaticum*) on *Artemia salina* larvae

In the study of the toxicity test of clove flower extract as a potential anticancer medicine utilizing the BSLT method, it was determined that clove flower extract was poisonous to *Artemia salina* in the BSLT test (see Figure 1). Based on the investigation results, 900 grams of clove flowers distillation produced essential oil as 9.75 (w/w) grams, with a yield of 1.083%. This extract proved poisonous to 48-hour-old Artemia salina. In T1, where the extract concentration was 50 ppm, the mortality rate was 24% because clove flower extract's toxicity level is still low. The concentration of the pesticide's chemical components within the target species' body significantly impacts the insecticide's toxicity [25]. At T2, with a 250 ppm extract concentration, the mortality rate was 68%. At this dosage, clove extract was poisonous, as it could kill *Artemia salina* by more than 50 percent. At a concentration of 250 ppm, it is possible to determine the LC50 value. In T3, where the extract concentration was 500 ppm, the percentage of death was 90%; in T4, where the extract concentration was 750 ppm, the percentage of mortality was 98%. This result suggests that the clove flower extract is highly toxic to *Artemia salina* at this dose. T5 possessed the most destructive potential, with an extract concentration of 1000 ppm. The proportion of deaths was 100 percent. The rise in extract concentration induces an increase in the number of active components in these substances, which function as insecticides capable of killing numerous species [27].

In the control group, there were no dead larvae since there was no addition of clove flower

extract containing poisonous compounds. It demonstrated that the death of *Artemia salina* was caused by the administration of clove flower extract and not by environmental factors. Thus, it can be seen that the number of *Artemia salina* fatalities is proportional to the concentration of clove flower extract. This result is consistent with the findings of Lisdawati et al. [28], Sanjaya et al. [19], and Sapulette et al. [29], who discovered a correlation between the concentration of plant extracts and the number of dead larvae. This research indicates that the number of Artemia salina-related fatalities is often proportional to the concentration of clove flower extract. The LC50 of clove flower extract was calculated to be 227.1 g/ml using the Probit method. LC50 500–1000 g/ml is mildly toxic, LC50 100–500 g/ml is highly toxic, and LC50 0–100 g/ml is extremely poisonous [20]. Based on these factors, clove extract is classified as moderately hazardous. The level of toxicity provides context for the potential anticancer activities of clove flower extract. The lower the LC50, the greater the plant's potential as a cancer treatment.

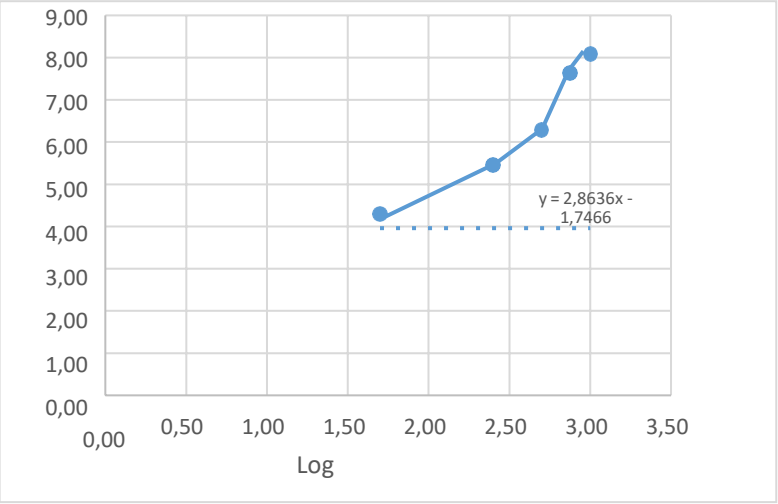


Figure 2. Graph of correlation of log concentration of clove flower extract (*Syzygium aromaticum*) with a probit mortality rate of *Artemia salina* larvae

Figure 2 provides the equation for the line y = ax + b, which can be used to confirm the LC50 value. Inputting the value of probit 5 (y = 5) into the equation y = 2.8636 x – 1.7466 yields the result of x = log 2.356, which is then antiloged to yield the value of 227.1 g/ml. The results of

the probit analysis are qualitatively estimated values, so they did not reflect the actual value. Consequently, interval estimates also emerge in the findings of the probit analysis. Based on the investigation, the estimated interval between the values range is 135 - 293. Figure 2 describes that The R2 value is known to be 0.9062, indicating that clove flower extract is 90% effective at killing Artemia salina. The coefficient of determination (R2) is a component of the total diversity of the dependent variable Y, which may be accounted for or explained by the variety of the independent variable X. The value of R2 is between 0 and 1, and it is argued that the correlation improves as R2 approaches 1 [30]. In the toxicity test, the R2 value is 1, indicating that the clove flower extract administered is the cause of the mortality of the test larvae.

Artemia salina larvae exhibit a high level of sensitivity to testing. Based on their morphology, 48-hour-old *Artemia salina* has developed a mouth and digestive system to absorb specific particles. *Artemia salina* larvae that are 24 hours old or in the second instar phase, despite having a digestive system, cannot interact with their surroundings and cannot absorb extracts or external substances [20].

In order to achieve *Artemia salina* egg hatching and growth, salinity, pH, and seawater temperature must be considered. This investigation utilized seawater with a salinity of 37 ppt. The pH of the used seawater is 8.1. A pH fall below 7 can be fatal. [31] Hatching cysts require a pH of 8–9, which is slightly alkaline. In this investigation, the seawater temperature was 28.3oC. *Artemia salina* cannot thrive at temperatures below 6°C or above 35°C in hatcheries. The optimal Artemia growth temperature varies from 26 to 31 degrees Celsius [32]. During the hatching process, a lamp is added to the artemia larvae as a light source to maintain the optimal water temperature and to stimulate the larvae to shed their egg shells. The actively moving larvae swim toward the light source due to their positive phototaxis features that attract light [33].

Artemia salina's digestive tract is a nonselective filter, which makes it easier for poisonous chemicals to enter the mouth. Phyto-chemicals from clove flower extract can interact with cell membrane targets and enzymes, resulting in death [34]. It is in agreement with the initial researches that demonstrate the eugenol component of clove extract acts as a larvicide against *Artemia salina* by damaging cell membranes or interfering with the metabolism of the larvae [35,36].

The Brine Shrimp Lethality Technique (BSLT) is a leading method for evaluating the presence of bioactive compounds in natural products, with results typically related to cytotoxic and anticancer activity [37]. Meyer et al. [17] initially elucidated this method, 48 hours after being soaked in brine, brine shrimp eggs will begin to hatch. Each plant extract is diluted in 20 ml of a mixture of methylene chloride and methanol to form a stock solution with a 10 mg/ml concentration (1:1). Then, aliquots of 500, 50, and 5 g/ml are transferred in triplicate from the stock solutions to the vials, and the solvent is allowed to evaporate. After evaporation, 5 ml of brine are added to each vial to generate 1000, 100, and 10 ppm concentrations. Each vial is filled with ten nauplii (30 shrimps per concentration). Lethal Concentrations at 50% Mortality (LC50) values are determined using the Finney computer program's number of survivors at each concentration.

As a preliminary anticancer screening test, BSLT method is insufficient for determining the mechanism of action of bioactive substances in plants and is not specific for anticancer activity. However the BSLT method generates data that can be supported by more specific bioassays after the active compounds tested are toxic to Artemia salina, indicating that they are likely potential candidates for anticancer research. Further evaluation to indicate cytotoxicity of *Syzygium aromaticum* can be conducted in vitro by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay [38], to consider the safety of the product application in the human model cells.

Molecular Docking

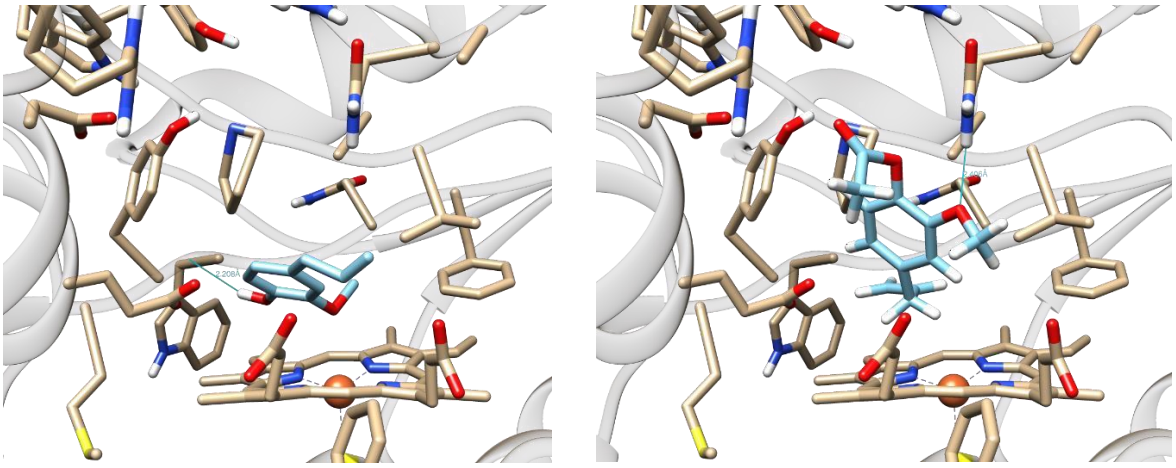
Clove essential oil includes many components, with eugenol, eugenol acetate, and caryophyllene constituting the majority. The three chemicals were analyzed in silico by molecular docking with nine proteins essential for cancer cell proliferation [7]. These three compounds can be evaluated by comparing their grid score to the original ligand's grid score (cognate). The outcomes are shown in Table 2. According to these findings, no substance can produce a higher grid score than cognate due to the clove essential oil's bioactive structure, including a small number of functional groups, particularly caryophyllene, which is rich in hydrophobic interactions. Other than the van der Waals interaction, there are few other possible interactions. In addition, caryophyllene produced the lowest grid score on nine target proteins compared to the other two chemicals.

Table 2. Grid-score results and hydrogen bond interactions.

Protein	Ligand	Grid-score	H-Bond number	Residue (H-Bond length, Å)
Estrogen receptor-α	Eugenol	-29.758898	2	Lys145 (2.455); Ile82 (2.637)
	Eugenol acetate	-34.403477	0	
	Caryophyllene	-18.799114	0	
	Tamoxifen	**-66.970085**	1	Glu49 (1.954); Arg90 (2.046)
MMP-2	Eugenol	-41.468246	0	
	Eugenol acetate	-43.793243	1	Ser151 (2.388)
	Caryophyllene	-34.205738	0	
	SC-74020	**-109.474319**	4	Leu83 (2.017); Ala84 (2.441); His120 (3.054); Glu121 (2.421)
PARP	Eugenol	-31.103073	1	Ser243 (2.205)
	Eugenol acetate	-36.013237	1	Ser203 (2.585)
	Caryophyllene	-32.597618	0	
	FR257517	**-63.837357**	0	
CDK2	Eugenol	-36.910698	2	Asp74 (1.845); Lys77 (1.815)
	Eugenol acetate	-41.676758	1	Lys33 (2.136)
	Caryophyllene	-40.535130	0	
	Roscovitine	**-71.813713**	2	Leu71 (2.572; 1.992)
Chk1 kinase	Eugenol	-38.836273	3	Glu44 (1.504); Asn48 (2.658); Asp137 (1.801)
	Eugenol acetate	-39.099670	1	Asp137 (2.447)

	Caryophyllene	-35.456528	0	
	Cpd. 54	**-84.449051**	5	Glu74 (1.844); Cys76 (1.793); Glu80 (1.634); Glu123 (2.297); Asp137 (1.966)
NO synthase	Eugenol	-41.503204	1	Tyr291 (2.208)
	Eugenol acetate	-42.120934	1	Gln181 (2.406)
	Caryophyllene	-35.420425	0	
	AR-C95791	**-61.271812**	2	Tyr265 (2.129); Glu295 (1.937)
Human Cytochrome P450	Eugenol	-29.011292	0	
	Eugenol acetate	-32.352654	0	
	Caryophyllene	-31.890057	0	
CYP17A1	**Abiraterone**	**-61.819771**	1	Asn172 (1.864)
BCL-2	Eugenol	-35.300983	0	
	Eugenol acetate	-36.267906	0	
	Caryophyllene	-28.939451	0	
	ABT-199	**-79.199051**	1	Asn82 (2.186)
Cyclin A	Eugenol	-29.981400	2	Lys34 (2.082); Asp141 (2.623)
	Eugenol acetate	-31.090176	0	
	Caryophyllene	-25.151398	0	
	AZD5438	**-60.700954**	4	Lys34 (2.228); Leu79 (2.197); Leu79 (2.333); Asp82 (1.946)

Eugenol acetate has the closest grid score to AR-C95791 (-42.12 vs. -61.27) in the NO synthase enzyme compared to other ligands and targets. This molecule can maintain hydrogen bond contacts between oxygen in methyl ether and Gln181 residue when examined from the docking stance. The fragment allyl points to Heme (Figure 3a). Eugenol has a comparable grid score (-41.5). However, his docking position is the opposite. The phenolic group in eugenol points to heme, and the phenolic OH group interacts with the Tyr291 residue's backbone (Figure 3b). Although not as effective as its homolog, eugenol can inhibit the production of NO by this enzyme [36]. Eugenol may also be created as a NO synthase inhibitor by including multiple functional groups to enhance the number of contacts through a series of chemical modifications.



(a) (b)

Figure 3: *Docking pose* eugenol (a) and (b) eugenol acetate on the active site of the NO synthase enzyme

4. Conclusion

Clove flower extract was determined to be moderately toxic to *Artemia salina* in the BSLT test with an LC50 value of 227.1 g/ml based on probit analysis. Hence it has the potential to be developed as an anticancer medicine. In addition, eugenol acetate is close to its cognate enzyme nitric oxide synthase based on its proximity to the grid-score value (3E7G). Therefore, if in vitro test results indicate that cancer cell proliferation is inhibited, the most likely mechanism is the suppression of nitric oxide synthase.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Clove Flower Extract (*Syzygium aromaticum*) Has Anti-Cancer Potential Effect Analized by Molecular Docking and *Brine Shrimp Lethality Test* (BSLT)

Table 1. Crystal structures used

No	Access code PDB	Description	Resolution	Reference (DOI)
1	2JF9	Estrogen receptor alpha LBD in complex with a tamoxifen-specific peptide antagonist	2.10 Å	10.1074/jbc.M611424200
2	1HOV	Solution structure of a catalytic domain of MMP-2 complexed with SC-74020	NMR	10.1016/s0167-4838(02)00307-2
3	1UK0	Crystal structure of the catalytic domainof human poly(ADP-ribose) polymerase with a novel inhibitor	3.00 Å	10.1016/s0014-5793(03)01362-0
4	2A4L	Human cyclin-dependent kinase 2 in complex with roscovitine	2.40 Å	10.1111/j.1432-1033.1997.0518a.x
5	2R0U	Crystal Structure of Chek1 in Complex with Inhibitor 54	1.90 Å	10.1016/j.bmcl.2007.09.007
6	2X7F	Crystal structure of the kinase domain of human Traf2- and Nck-interacting Kinase with Wee1Chk1 Inhibitor	2.80 Å	-
7	3E7G	Structure of human INOSOX with inhibitor AR-C95791	2.20 Å	10.1038/nchembio.115
8	3RUK	Human Cytochrome P450 CYP17A1 in complex with Abiraterone	2.60 Å	10.1038/nature10743
9	4LXD	Bcl_2-Navitoclax Analog (without Thiophenyl) Complex	1.90 Å	10.1038/nm.3048
10	6GUE	CDK2/CyclinA in complex with AZD5438	1.99 Å	10.1016/j.chembiol.2018.10.015

Table 2. Grid-score results and hydrogen bond interactions.

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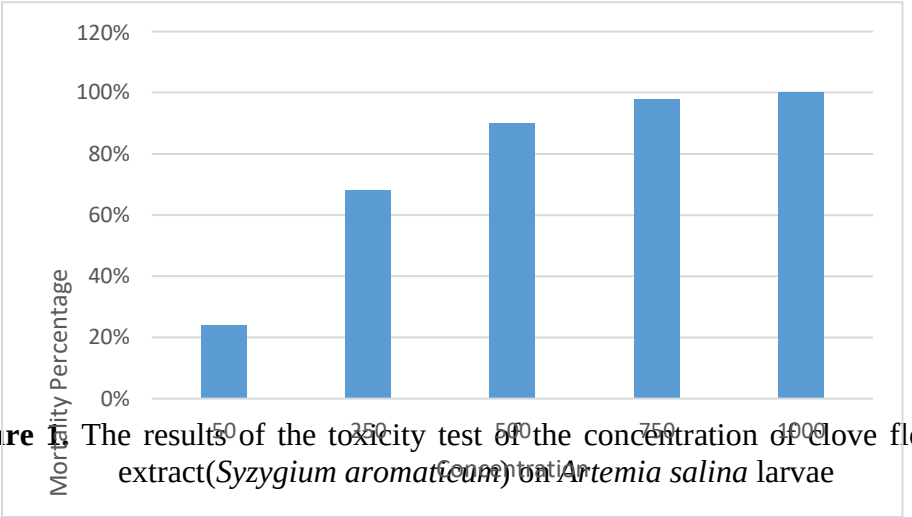


Figure 1. The results of the toxicity test of the concentration of clove flower extract(*Syzygium aromaticum*) on *Artemia salina* larvae

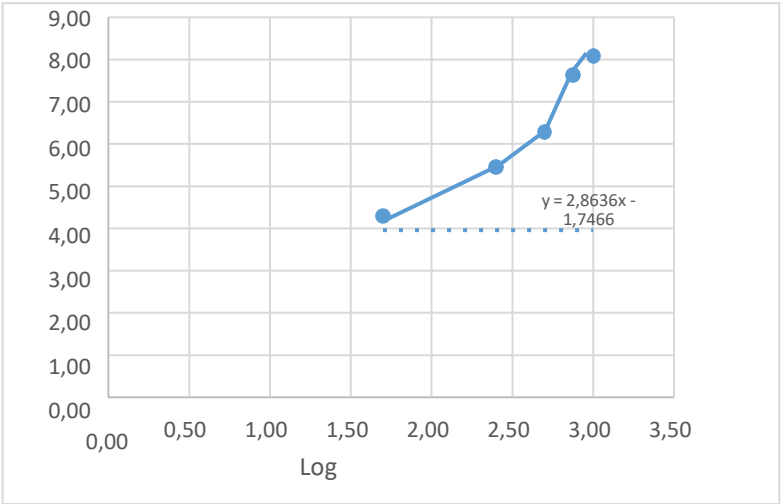
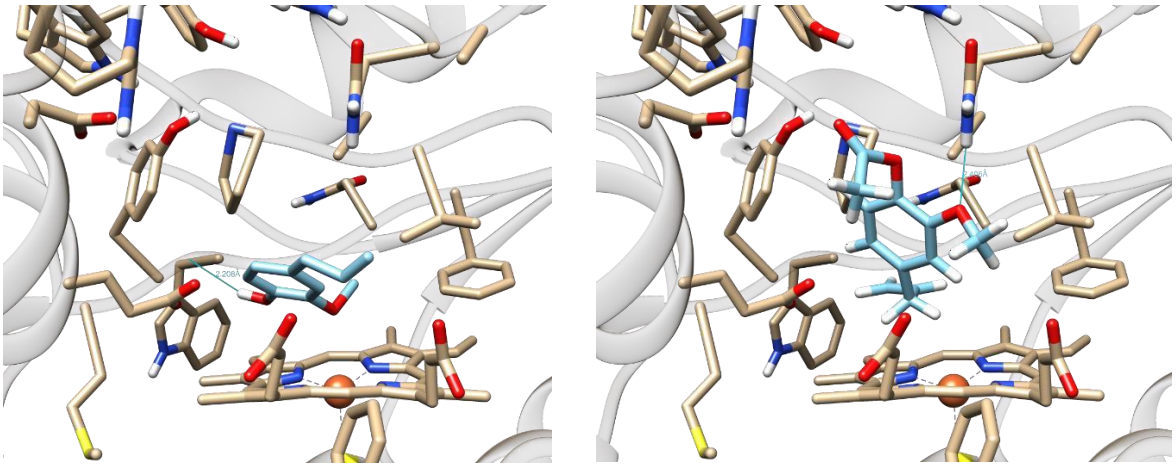


Figure 2. Graph of correlation of log concentration of clove flower extract (*Syzygium aromaticum*) with a probit mortality rate of *Artemia salina* larvae



(a) (b)

Figure 3: *Docking pose* eugenol (a) and (b) eugenol acetate on the active site of the NO synthase enzyme

Galley Proofs

There are no active galley proofs.

Manuscripts History

Journal	MS ID	Title & Authors	Last Submitted	Actions
Veterinary Medicine International	9761812	Phylogenetic Analysis and Antibiotics Resistance of Listeria Monocytogenes Contaminating Chicken Meat in Surabaya, Indonesia Eduardus Bimo Aksono, Katty Hendriana Priscilia Riwu, A. T. Soelih Estoepangestie, and herinda pertiwi	2/23/2020 1:15:34 PM	▸ View Published
Veterinary Medicine International	5113742	Clove Flower Extract (Syzygium aromaticum) Has Anticancer Potential Effect Analyzed by Molecular Docking and Brine Shrimp Lethality Test (BSLT) herinda pertiwi, Eduardus Bimo Aksono, Kautsar Ul Haq, Aprilia Cahya Latifah, and	9/1/2022 7:16:06 AM	▸ View Published