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Antiangiogenesis Activity of Indonesian Local Black Garlic (*Allium Sativum* 'Solo): Experiments and Bibliometric Analysis

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A B S T R A C T

Cancer is still one of the leading causes of death in the world. Angiogenesis is the formation of new blood vessels, which plays a vital role in the growth of cancer cells. As a result, inhibition of proangiogenic factors such as vascular epidermal growth factor (VEGF) could be used as a cancer treatment strategy. Black garlic (BG) is processed fresh garlic (*Allium sativum* L.) for several days under controlled high temperature and humidity. The research aims to investigate the effect of Indonesian local BG on antiangiogenic activity through *in vivo* and *in silico* studies. The *in vivo* test was carried out by observing the formation of new blood vessels using the Chorio-Allantoic Membrane Assay (CAM). The *in silico* study was performed by docking analysis of BG bioactive compounds on the VEGF receptor (VEGFR). The results showed that ethanol extract, ethyl acetate fraction, and n-hexane fraction of BG inhibit new vessel blood formation, with n-hexane fraction having the highest efficacy. The molecular docking assay indicated some similarities in the amino acids involved in the interaction between the BG bioactive compounds and the native ligand in binding to VEGFR. This similarity may lead to interference with VEGFR resulting in angiogenesis inhibition. The study suggests that BG has the potential to be developed as an anticancer agent, which might be through the mechanism of inhibiting the angiogenesis process.

A R T I C L E I N F O

1. INTRODUCTION

Cancer is one of the primary causes of death and the major impediment to improving life expectancy in every country worldwide. Based on GLOBOCAN 2020 data, an estimated there were 19.3 million new cancer cases and about 10.0 million cancer deaths occurred in 2020 (Sung *et. al*, 2021).

Cancer is a non-communicable disease that results from uncontrolled cell or tissue proliferation. One of the characteristics of cancer during the carcinogenesis process of human tumors is induced angiogenesis. Angiogenesis plays a role in supporting the life of cancer cells through the growth of new blood vessels, particularly through invasion and metastasis (Hanahan & Weinberg, 2011).

VEGF is an important proangiogenic factor that regulates endothelial cell sprouting and proliferation during vasculogenesis. Tumor cells secrete VEGF, which promotes neovascularization resulting in cancer progression (Zhang *et al.*, 2019). Therefore, targeting the VEGF receptor signaling is considered a promising strategy for cancer treatment by inhibiting angiogenesis. (Abuzenadah *et al.*, 2022).

Black garlic (BG) is a form of aged garlic prepared from fresh garlic (*Allium sativum*) by a Millard reaction at high temperature (60–90°C) and humidity (70–90%) for several times (Ahmed & Wang, 2021). Bioactive compounds in BG are more potent than those in fresh garlic. Some of the bioactive compounds found in BG including DL-lactic acid; 5-hydroxymethyl-2-furfural; adenosine; uridine; (1S,3S)-1-methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid; (1R, 3S)-1-methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid; and 2-acetylpyrrole (Lu *et al.*, 2017).

Regarding anticancer activity, it has been reported that BG has a dose-dependent chemopreventive effect on several cancers both in vitro and in vivo. The BG ethanol extract has a cytotoxic effect on several human cancer cells, including AGS gastric cancer cells, A549 lung cancer cells, HepG2 liver cancer cells, MCF-7 breast cancer cells (Purev *et al.*, 2012), and HT29 colon cancer cells (Dong *et al.*, 2014). Furthermore, in SGC-7901 human gastric cancer cells, BG inhibited cell growth and caused apoptosis. BG has also been shown to inhibit motility and invasiveness in AGS. The hexane extract of BG induced apoptosis in U937 leukemia cells (Park *et al.*, 2014). However, the effect of BG on angiogenesis has yet to be reported.

The two main focuses of this research are to investigate the effect of ethanol extract, ethyl acetate fraction, and n-hexane fraction of BG on antiangiogenic activity by CAM assay and to investigate the influence of bioactive compounds in BG on VEGF receptor activity by docking approach. This study will present an overview of the prospects for using BG as a functional food product with anticancer effects (see **Figures 1** and **2**).
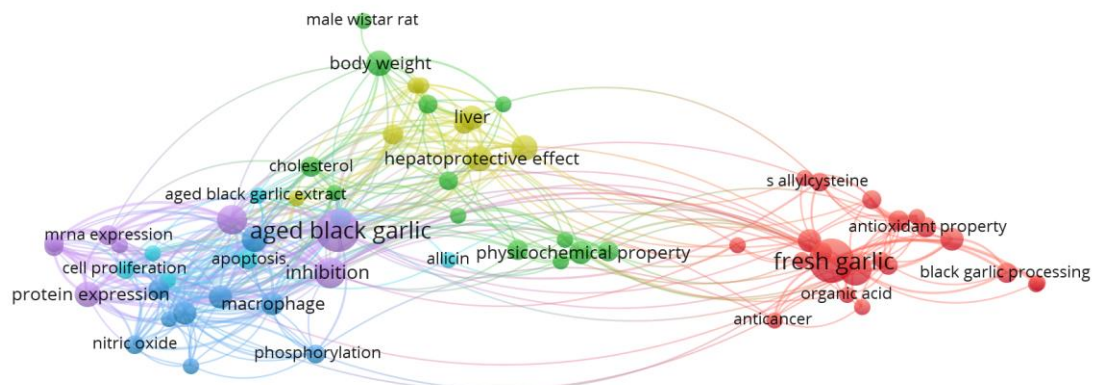
Figure 1. The co-word map network visualization of the progress of BG research is divided into six clusters, where there has been little research on BG's anticancer activity.
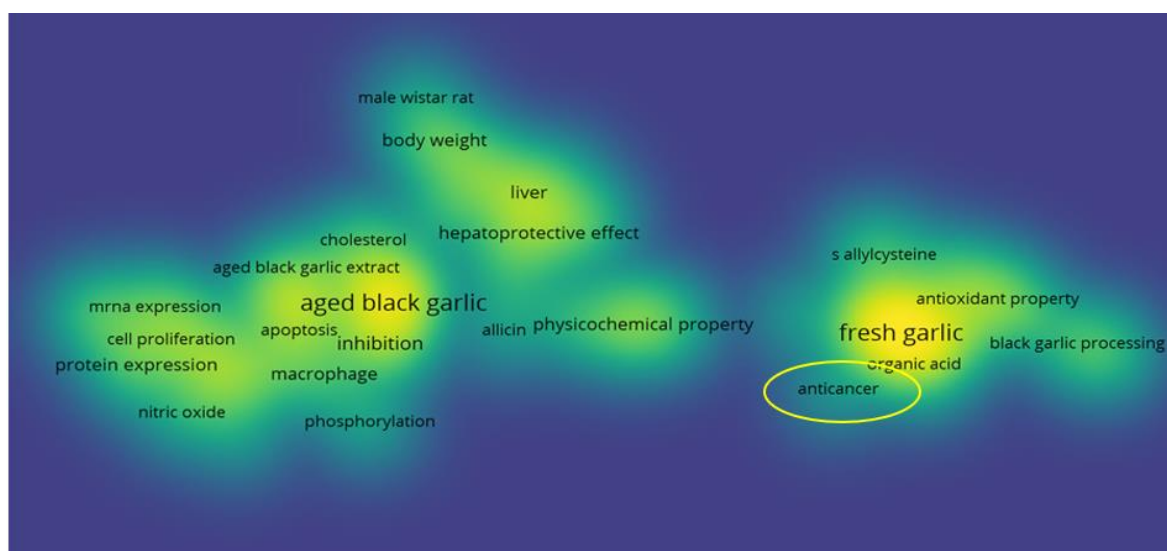
Figure 2. Co-word map density visualization of research developments regarding BG.

2. METHODS

2.1. Apparatus and Materials

The equipment used for in vivo testing were a freeze dryer, rotary evaporator, laminar airflow (LAF, Shimadzu SCB–1000 A), autoclave, mini drill, syringe, digital microscope, and Kitagawa egg incubator. The tools used for in silico testing were Laptop OS Windows 8, CPU Intel Core i5 3210M/5Hz, GPU NVIDIA GEFORCE GT 650M-2GB, memory 4GB DDR3 1600 MHz, Marvin sketch, Avogadro, Mopac2016, Autodock tools, Pymol Molecular Visualization, Pymol for Educational, Pyrx, and Biovia Discovery Studio.

The material for in vivo study were Black Garlic One Clove Sindoro 171 Temanggung, Indonesia, fertile chicken eggs (Elba), bFGF (Sigma), 10mM pH 7,4 phosphate buffer saline (PBS) from Sigma), 0.8% dimethyl sulfoxide (DMSO) form Merck, 0.1% NaCl (Merck), 96% ethanol (Merck), ethyl acetate (Merck), n-hexana (Merck), and paper disc (Oxoid). The compounds contained in black garlic for docking were DL-lactic acid; 5-hydroxymethyl-2-furfural; adenosine; uridine; (1S,3S)-1-methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid; (1R, 3S)-1-methyl-1,2,3,4-tetrahydro-b-car-boline-3-carboxylic acid; and 2-acetyl pyrrole (Lu *et al.,* 2017).

2.2. Preparation of Black Garlic Extract

BG is put into the freeze-dry to remove the moisture content. A total of 500 g freeze-dry black garlic was macerated using 1,5 L ethanol 96% (1:3) for 3 x 24 hours at room temperature. The filtrate was collected and concentrated using a vacuum rotary evaporator. The black garlic ethanol extract was then fractionated using ethyl acetate (1:1) to obtain the ethyl acetate fraction. The remaining ethanol fraction was then fractionated using n-hexane (1:1) to obtain the n-hexane fraction. Each fraction was concentrated by a vacuum rotary evaporator.

A stock solution of each black garlic extract was prepared by dissolving 10 mg of the extract in 10 mL of 0.8% DMSO. Then the solution was diluted with ultrapure water (aquabidest) to a concentration of 20, 40, and 80 µg/mL. A stock solution of bFGF (50 ng/µL) was prepared from 25µg of bFGF dissolved in 500 µL of 10 mM PBS pH 7.4. Then the solution was diluted with 10 mM PBS pH 7.4 to obtain 1 ng/µL bFGF.

2.3. Antiangiogenic Activity Test

The tools used in in vivo testing were sterilized by autoclave. One-day-old eggs are cleaned of contaminants with 70% alcohol and then incubated at 37ºC. When the eggs

are 3-4 days old, candling is carried out to determine the position of an embryo and the air cavity. The eggshell is at the poles where there is an air cavity, and a needle is used to create a small hole above the embryo. Suck air through the polar holes with a suction pipette until the embryo shifts and the air cavity seems to be filled.

The hole was then closed again with paraffin. On the top of the embryo that still has space, mark a 1x1 cm^2 square on the surface of the shell using a pencil. The eggs are then returned to the incubator. After 8-9 days, the eggshells are cleaned using 70% ethanol, and the 1x1 cm^2 square image is opened with a mini drill and scalpel as a hole to insert the sample. Samples of BG extract and other reagents (**Table 2**) were placed on a paper disc using a micropipette. Then the paper disc is inserted into the egg through the hole. This procedure is performed at the LAF.

Then the hole is closed again using paraffin and returned to the incubator. When the eggs reach the age of 12 days, they are stored in the refrigerator/freezer at <10°C for 24 hours. The eggs are opened at 13 days by splitting the eggshell into two sections then the egg contents are removed carefully so that the CAM attached to the eggshell is not wasted and damaged. The CAM section containing the paper disc was washed with a saline solution and fixed using a 1:1 mixture of methanol and acetone. Then the newly formed blood vessels around the paper disc can be observed macroscopically (West *et al.,* 2001).

2.4   Data Analysis

Thin blood vessels that come out of the main blood vessels (existing vessels) are counted as new blood vessels. The angiogenic response of each treatment was calculated using the following Eq. (1):

$$Relative\ angiogenic\ response\ (RAS) = \frac{a}{b} \times 100\% \qquad (1)$$

where *a* is the number of new blood vessels treated with the sample, and *b* is the number of new blood vessels in bFGF group.

The amount of inhibition of each concentration of garlic ethanol fraction on the angiogenesis response can be determined by the following Eq. (2):

$$inhibition\ percentage = 100\% - RAS \qquad (2)$$

2.5.   In Silico Procedure

This study used VEGF target protein molecules with the codes 2P2I (Arianingrum *et al.,* 2019), 3HNG, and 3VHE (Asthana *et al.,* 2015). These receptors were downloaded from the RCSB PDB (Protein Data Bank) on the https://www.rcsb.org/ page. There are 7 other compounds contained in black garlic as ligands (Lu *et al.,* 2017). Bevacizumab was used as a positive control. The structure of the compound is drawn using Marvin's sketch. Avogadro used visualization and editing of molecules, while optimizing molecular geometry used Mopac2016 with the PM7 semi-empirical method.

The Autodock tool was used to create separate ligand and receptor files. Pymol Molecular Visualization was used to visualize the docking results of the original ligand with the docking ligand and Pymol for education to calculate the Root Mean Square Deviation (RMSD) value. The docking process was carried out using Pyrx and the visualization of the interaction of the ligand with the receptor was used by Biovia Discovery Studio for the docking process.

2.6. Bibliometric Analysis

To support the experiment, we added bibliometric analysis. Detailed information for this method is explained in the literature (Al Husaeni & Nandiyanto, 2022).

3. RESULTS AND DISCUSSION
3.1. Bibliometric analysis

We performed a bibliometric analysis with Vosviewer and Publish or Perish (PoP) (Al Husaeni & Nandiyanto, 2022) on a database

source from Pub Med with the title word "black garlic" from 2011 to 2023 (12 years) to determine the number of articles regarding with BG. We found 116 out of 123 articles related to BG, totaling 5.55 authors/papers.

The number of publications fluctuated in these years but tended to rise from 2016 to 2018, then fall until 2020 before increasing again from 2021 to 2023. Through network visualization, the research development map on BG is divided into 6 clusters. Cluster 1 consists of 21 items, cluster 2 consists of 13 items, cluster 3 consists of 9 items, cluster 4 consists of 8 items, cluster 5 consists of 8 items, and cluster 6 consists of 7 items. Anticancer is one of the items in cluster 1. However, angiogenesis is not identified in any of the clusters. The VOSviewer density map demonstrates that there is still minimal research on BG-related anticancer, as shown by a density map with a small diameter and faded yellow color (**Figure 2**).

3.2. Extraction of Black Garlic

This study used BG obtained from Temanggung, Wonosobo, Central Java Province, Indonesia. In the study, we used 500 kg of freeze-dried black garlic as a sample. All secondary metabolites will be expected to dissolve during the ethanol maceration process. The fractionation technique is used to separate compounds based on polarity. Nonpolar compounds are predicted to dissolve in an n-hexane solvent, while semipolar compounds are expected to dissolve in an ethyl acetate solvent. **Table 1** contains data from the extraction and partitioning of BG.

The yields of ethanol extract, ethyl acetate fraction, and n-hexane fraction of BG in the present research were 31.69; 36.00; and 8.00%, respectively. According to Atun *et al.* (2020), the ethanol extract and hexane fraction of BG contains many phenolic and flavonoid compounds.

3.3. Antiangiogenesis Activity of Black Garlic

The CAM assay was used in the study to investigate antiangiogenesis activity. The approach has been widely utilized to explore the basic principles of blood vessel development, such as endothelial cell migration, proliferation, and differentiation. CAM is well adapted to studying the pro- or anti-angiogenic effects of compound natural and pharmaceutical stimuli (Laschke *et al.*, 2022). CAM is easier to work with than other media, such as rabbit corneas (Ribatti *et al.*, 2000). Furthermore, the CAM membrane is a highly vascularized area with numerous blood vessels that are easily observed in line with the growth process of the chicken embryo (Ribatti, 2016).

We used the basic Fibroblast Growth Factor (bFGF) as an angiogenic inducer in this study, which makes it easier to observe changes in the development of new vascular blood. The bFGF has a mechanism by attaching to particular receptors on blood vessel endothelial cells and triggering growth signals. Endothelial cells produce protease enzymes, then proliferate and migrate, eventually forming a new extracellular matrix. The newly generated blood vessels in the last step are structurally stabilized so blood can circulate (Ribatti *et al.*, 2001). The dose of bFGF used for each treatment is 10ng.

We used six groups in this study, three control groups and three sample groups. Each group's treatment was repeated three times. The control group consisted of PBS solvent, bFGF, and DMSO solvent. The sample group consisted of ethanol extract, ethyl acetate fraction, and n-hexane fraction of BG with concentrations of 20, 40, and 80 µg/mL. CAM macroscopic results and the average number of new blood vessels in each group are shown in **Figure 3** and **Table 2**. **Figure 4** represents the percentage of antiangiogenic.

**Table 1.** The result of extract and fraction of BG.

Material Weight	Concentrated Extract Weight	Yield (%)
500 g BG	Ethanol extract 158.454 g	31.69
250 mg ethanol extract	Ethyl acetate fraction 90 mg	36.00
250 mg ethanol extract	n-hexane fraction 20.01 mg	8.00
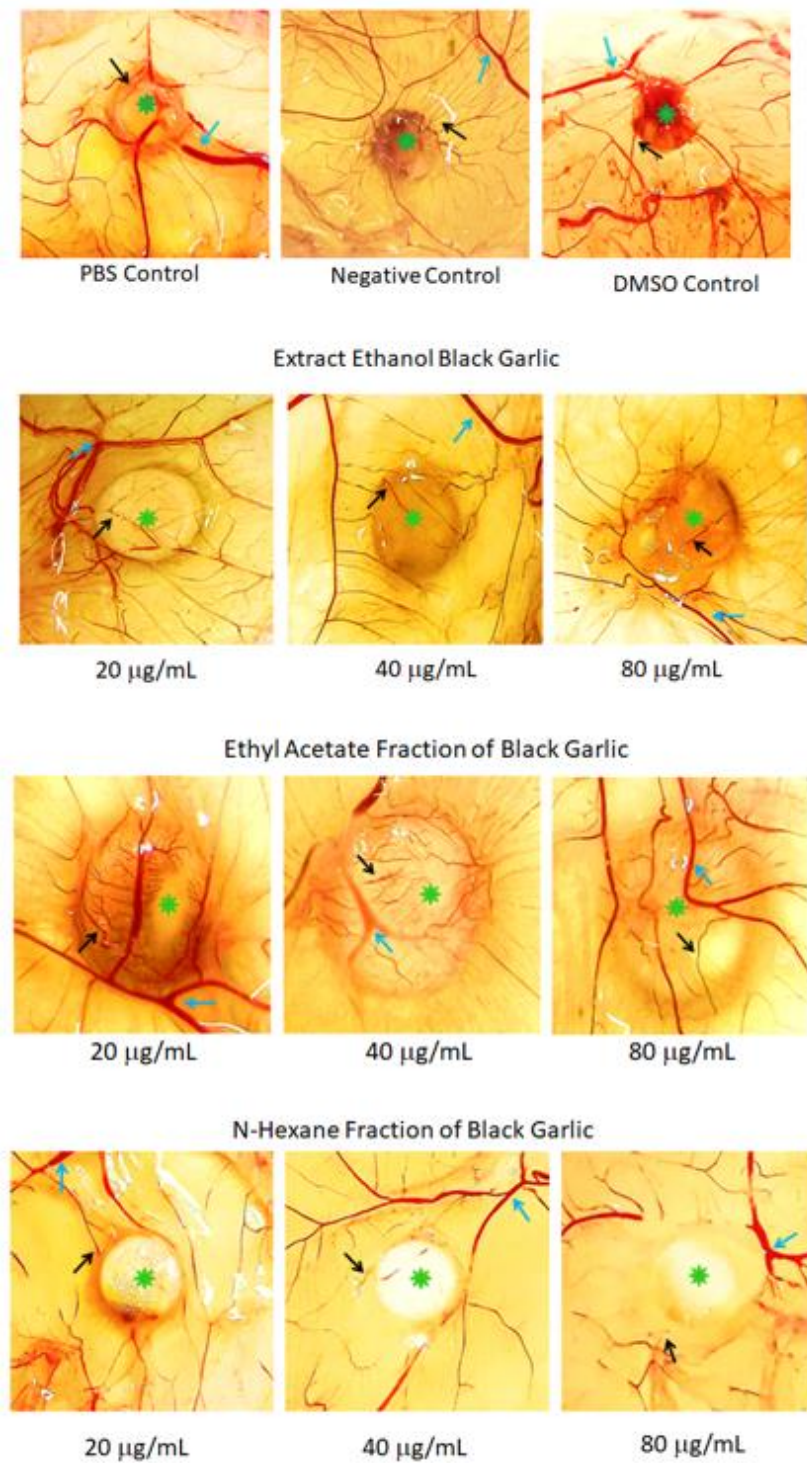
Figure 3. CAM macroscopic observation of ethanol extract, ethyl acetate fraction, and n-hexane fraction of BG. The green star indicated paper disk, the black arrow indicated main blood vessels and the blue arrow indicated new blood vessels.

Table 2. The number of new blood vessels from the control and the sample group.

Group	Number of New Blood Vessels
PBS control	19.00±2.64
Negative control (1ng/µL bFGF)	26.00±1.00
DMSO control (1ng/µL bFGF+DMSO)	23.33±2.51
BG Ethanol extract 20 µg/mL + 1ng/µL bFGF	17.33±3.80
BG Ethanol extract 40 µg/mL + 1ng/µL bFGF	14.00±1.70
BG Ethanol extract 80 µg/mL + 1ng/µL bFGF	7.00 ±2.00
BG Ethyl acetate fraction 20 µg/mL + 1ng/µL bFGF	21.67±4.50
BG Ethyl acetate fraction 40 µg/mL + 1ng/µL bFGF	19.67±4.72
BG Ethyl acetate fraction 80 µg/mL + 1ng/µL bFGF	16.00±3.46
BG n-hexana fraction 20 µg/mL + 1ng/µL bFGF	12.00±1.00
BG n-hexana fraction 40 µg/mL + 1ng/µL bFGF	8.33±1.53
BG n-hexana fraction 80 µg/mL + 1ng/µL bFGF	4.33±2.52
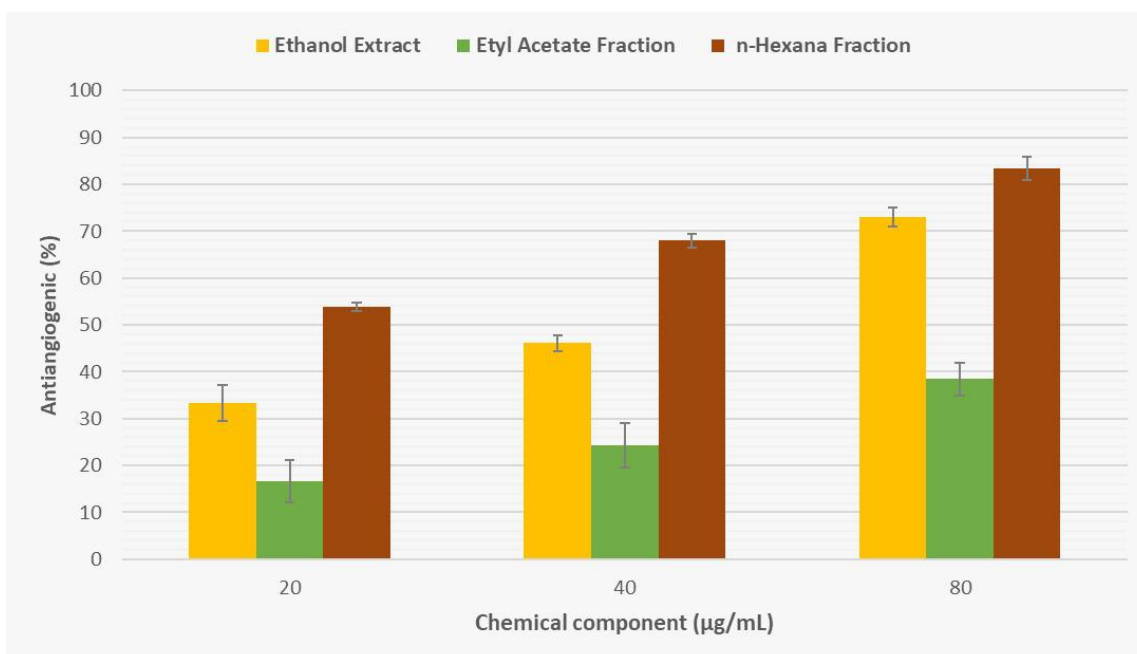
Figure 4. The antiangiogenic using ethanol extract, ethyl acetate fraction, and n-hexane fraction of BG.

The study showed that the number of blood vessels with the DMSO treatment (1ng/mL bFGF and DMSO) was not significantly different from the bFGF treatment (1 ng/mL). This demonstrates that the DMSO solvent does not affect blood vessel development. DMSO at or above 0.1% can change the morphology of the capillary network. In this study, the stock sample solution was dissolved in 0.8% DMSO and

subsequently diluted with distilled water until the final DMSO concentration was less than 0.06%, which did not influence the number of blood vessels.

In this study, bFGF increased the number of blood vessels by 37%. We discovered that BG ethanol extract at 20, 40, and 80 g/mL effectively reduced angiogenesis (33.24; 46.15; and 73.08%) in a dose-dependent manner. The ethyl acetate fraction suppressed angiogenesis significantly, but its efficacy was lower than that of the ethanol extract (16.65; 24.35; and 38.46%). The n-hexane fraction at the same concentration showed the highest angiogenesis activity (53.85; 67.96; and 83.35%) compared to the ethanol extract and n-hexane fraction. This study concludes that black garlic has the potential to inhibit angiogenesis.

So far, research on BG's angiogenesis activity has been quite restricted. Several chemicals in fresh garlic (FG) have been reported to suppress angiogenesis, including Alliin (Mousa & Mousa, 2005), diallyl trisulfide (DATS) (Xiao *et al.*, 2006), and Allicin (diallyl-thiosulfinate) (Sela *et al.*, 2008).

Alliin significantly inhibits the secretion of fibroblast growth factor-2 (FGF2) and VEGF in the CAM model (Mousa & Mousa, 2005). Diallyl trisulfide (DATS) influences the angiogenesis properties of endothelial cells (EC) (Xiao *et al.*, 2006), and Allicin (diallyl-thiosulfinate) suppresses VEGF and bFGF-induced blood vessel formation (Sela *et al.*, 2008).

The Alliin content of BG decreases during the preparation process, whereas the quantities of flavonoids, pyruvate, alkaloids, organic acids, and phenols increase (Ryu & Kang, 2017; Ahmed & Wang, 2021). Alkaloids and flavonoids may be present in the BG ethanol extract, ethyl acetate, and n-hexane fractions. DATS was also discovered in BG (Ahmed and Wang, 2021). The compound might be able to inhibit angiogenesis caused by BG. According to Xiao *et al.* (2006), DAS inhibits Akt phosphorylation and suppresses

the expression of vascular endothelial growth factor (VEGF) receptors on cell membranes (endothelial cells).

Several studies have found a synergistic relationship between antioxidants and anti-angiogenesis and anticancer (Mousa & Mousa, 2005; Ahmed & Wang, 2021). Lu *et al.* (2017) discovered DL-lactic acid; 5-hydroxymethyl-2-furfural; adenosine; uridine; (1S,3S)-1-methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid; (1R, 3S)-1-methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid; and 2-acetylpyrrole ABCD in BG with high antioxidant activity. Therefore, in this work, we performed a docking test to investigate how it affected VEGFR.

3.4. In Silico Study

The results of energy calculations between the ligand and the receptor are shown in **Table 3**. In this study, we used bevacizumab as a positive control. This drug can inhibit angiogenesis. However, bevacizumab can cause severe intraocular inflammation (Li *et al.*, 2014). Therefore, for effective anti-angiogenesis treatment, it is necessary to discover potential VEGFR inhibitors from natural products using computer-assisted approaches.

In this study we used PDB ID: 2P2I (Arianingrum, 2019), 3VHE, and 3HNG (Asthana, 2015). The docking approach was validated by comparing the RMSD score of the target protein PDB with its natural ligand. The RMSD score between the target protein PDBs and the natural ligand in this study was less than 2 angstroms (**Table 3**). The data indicated that the protocol was acceptable because the validation conditions were completed, and molecular docking could then be performed (Yusuf *et al.*, 2008).

These findings indicate that Bevacizumab has the lowest binding energy. Based on the results of molecular docking studies, the active compounds contained in black garlic extract that have low binding energy are (1R,3S)-1-methyl-1,2,3,4-tetrahydro-b-car-boline-3-carboxylic acid and (1S,3S)-1-

methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid.

The lower the binding energy level, the stronger the interaction between the ligand and receptor. The interaction that occurs is the interaction between VEGFR (2PI, 3HVE, and 3HNG) and the active compounds in black garlic. Even though the active compound in BG has a higher energy than the original ligand and Bevacizumab, the docking results show that there are similar amino acids in the interaction (**Figure 5**).

The similarity of amino acids involved in the interaction between ligand and receptor causes inhibition of the activity of the VEGFR which is responsible for angiogenesis. Inhibition of VEGFR activity causes inhibition of angiogenesis activity. These docking results show that the active compounds in BG have the potential to inhibit angiogenesis.

Table 3. The binding activity of ligand and receptor.

Ligand	Binding Affinity		
	2P2I	3HVE	3HNG
	RMSD: 1.041	RMSD: 0.859	RMSD: 0.554
Native Ligand	-12.3	-11.8	-10.7
Bevacizumab	-10.1	-10.6	-8.8
(1R,3S)-1-methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid	-7.5	-8.8	-7.5
(1S,3S)-1-methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid	-7.4	-8.2	-7.4
Adenosine	-6.8	-7	-7.2
Uridine	-6.2	-6.9	-6.8
2-acetylpyrrole	-5.1	-5.1	-5
5-hydroxymethyl-2-furfural	-5.1	-4.9	-4.8
DL-lactic acid	-3.8	-3.6	-3.6
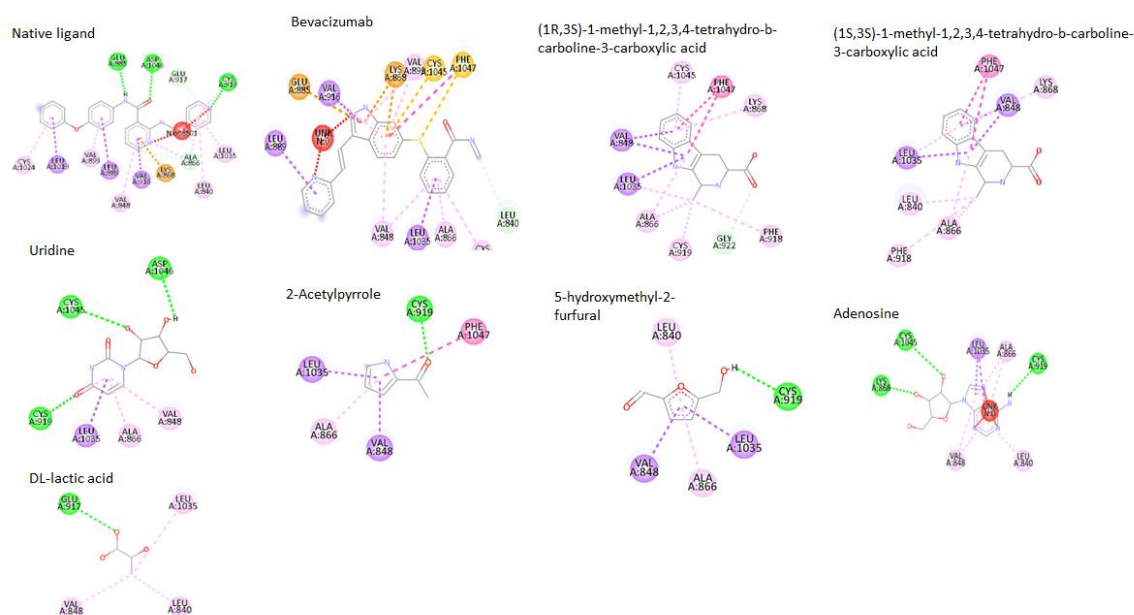
Figure 5. Visualization of the interaction of compounds in black garlic with 2P2I receptors.

4. CONCLUSION

The results showed that the ethanol extract, ethyl acetate fraction, and n-hexane BG fraction had antiangiogenesis activity. BG bioactive chemicals can interfere with the binding of VEGF receptors with other ligands, inhibiting their pro-angiogenic activity. This study provides information that BG has the potential to be developed as an antiangiogenesis drug for cancer treatment.

5. ACKNOWLEDGMENT

6. AUTHORS' NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. The authors confirmed that the paper was free of plagiarism.

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