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Anti-aging Effect of Black Garlic Through Anti-senescence, Gelatinase Inhibition Mechanism, and Formulation of NLC Serum

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ABSTRACT

Aging associated with cellular senescence was responsible for the degradation of collagen and elastin by activation of matrix metalloproteinases (MMPs) that produced wrinkles. Black garlic is known to have an anti-aging potency on premature aging. This study aims to reveal the anti-aging potency of black garlic through anti-senescence and gelatinase inhibition mechanisms and its formulation of Nanostructured Lipid Carrier Serum. Black Garlic Extract (BGE) was macerated with ethanol 50% then heated with low temperature at 50°C. The extract obtained was profiled with Thin Layer Chromatography and antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The cytotoxic effect of BGE was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on Vero cells. Anti-senescence effect of BGE was conducted by SA-β-Gal assay. The inhibition of gelatinase activities was predicted by molecular docking using MOE2010 software. The preparation of Nanostructured Lipid Carrier (NLC) is done by High Shear Homogenization method, then the best formula continued to make NLC-BGE Serum. The BGE contained S-allyl cysteine as a major organosulfur compound. BGE showed non-toxic to Vero cells with IC_{50} >500 µg/mL. Furthermore, 50 µg/mL of BGE showed inhibits doxorubicin-induced senescence in Vero cells. BGE also appeared to have good affinity on inhibitory domains of MMP-1 (ΔG -7,754 kcal/mol) and MMP-2 (ΔG -9,130 kcal/mol). NLC-BGE serum formula has met nanoparticles criteria and showed good stability. Based on this study, BGE revealed anti-senescence and gelatinase inhibition that is considered to have high anti-aging properties and can be applied in the NLC-BGE serum formula.

Keywords: Black garlic; NLC; anti-aging; senescence; MMP

INTRODUCTION

Exposure to UV radiation may contribute to the production of reactive oxygen species (ROS), which in turn, induce MMP synthesis. The accumulation of senescent cells is characterized by the expression of senescence-associated secretory phenotype (SASP), which includes MMP-1, MMP-3, MMP-10, and MMP-12 (Rodriguez et al*.,* 2017). In 2020, Botox and filler treatment were the most popular treatments for skin aging, with the number of patients reaching 4.4 million and 3.4 million, respectively. These treatments are the second and third trend in skin care for 20- to 29-year-olds (ASPS, 2020). However, Botox and fillers are known to cause pain, inflammation, and facial nerve disorders, followed by high treatment costs. Therefore, exploration of the effective premature aging prevention with affordable cost and non-invasive approach is needed.

Treatment using rich antioxidant anti-aging agents provides a solution to premature aging due to internal factors, merely of oxidative stress caused by high ROS level (Gonzalez et al., 2022). ROS production may be enhanced by UV and visible light exposure through strong oxidants formation (Nakai and Tsuruta, 2021). Additionally, black garlic (*Allium sativum* L.) is known to have more antioxidant properties compared to fresh garlic after fermentation through Maillard reaction (Kang, 2016). Based on the fact that S-allylcysteine content in black garlic oil has effectively inhibited premature aging and prevented oxidative damage, black garlic might have the potential to be developed as an anti-aging agent through more efficient scavenging activity of ROS and free radicals than other natural ingredient agents (Choi et al., 2014). Moreover, this study aims to explore the antioxidant and antiaging effect through gelatinase inhibition and anti-senescence, which remains underexplored despite numerous present studies exploring general health benefits of black garlic. This study evaluates the efficacy of black garlic-based in mitigating aging-related pathologies at molecular level. Bridging this gap

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through well-designed in vitro study might offer valuable evidence regarding black garlic benefits in cosmeceuticals function.

A potential carrier to be developed further is namely nanostructured lipid carrier (NLC). This system is known to increase solubility, bioavailability, and stability of hydrophobic and hydrophilic compounds. Lipid encapsulation in NLC is known to reduce the unpleasant aroma of the material (Baqeri et al., 2020). This feature potentially helps to reduce black garlic aroma as to increase public acceptance as well. The formula is also suitable for transdermal route as it can increase skin hydration and adhere to the outermost layer of the epidermis (Chauhan et al., 2020). Serum preparations were chosen as a delivery system because they can penetrate well into the deepest layers of the skin, so they are effectively preventing skin aging (Budiasih et al., 2018). Modification of the lipid matrix in protecting aqueous preparations from oxidation as well as allowing greater drug loading makes NLC suitable for serum production (Tofani et al., 2016; Rohmah et al., 2022). Thus, the black garlic NLC serum preparation is expected to have good bioavailability and protection from oxidative reactions to inhibit senescence and gelatinase activity in collagen degradation to which the causes of premature aging in skin cells.

MATERIALS AND METHODS

Materials

Black garlic (*Allium sativa* L.), aquadest, 96% ethanol (Brataco), silica gel 60 GF_{254} (Merck®), ninhydrin 0.1% (Merck®), butanol (Merck®), acetic acid (Merck®), MTT (Sigma®), Vero cell (CCL-81), DMSO (Merck®), Penicillin-Streptomycin 1% (Gibco®), EDTA trypsin 0.25% (Gibco®), FBS (Gibco®), DPPH (Smartlab®), ascorbic acid (Vitamin C, Sigma®), PFA 4% (Gibco®), X-gal dye (Sigma®), palm stearin, olive oil (Brataco), tween80 (Brataco), span80 (Brataco), aquabidest, sodium benzoate (Wuhan Youji®), xanthan gum (Deosen®), rotary evaporator, ELISA plate reader, Molecular Operating Environment (MOE) software 2010, ultraturrax (IKA), homogenizer (IKA), viscometer (Lamy Rheology), PSA (Malven), doxorubicine.

Extraction of Black Garlic

Black garlic was determined at Pharmacognosy Laboratory, Pharmaceutical Biology Department, Faculty of Pharmacy, UGM (No. 42.25.09/UN1/FFA.2/ BF/PT/2023). Black garlic was separated from the peel and sliced to smaller cuts. Ethanol 50% maceration was done for 2 hours (solid:liquid 1:10) and by digestion method extraction, heated at (50 ºC) for 90 minutes. The solvent was evaporated using rotary evaporator (Thach and Thuy, 2017).

Phytochemical screening

Phytochemicals such as S-allyl cysteine and other organosulfur were identified using thin layer chromatography. Butanol:acetic acid: aquadest (4:1:1) was used as mobile phase and ninhydrin 0.1% as dipping reagent to induce color reactions (Chen et al., 2015).

Antioxidant Activity of BGE

Free radical scavenging activity of BGE is assessed by the ability of scavenging free 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and turning it into a stable DPPH. The method is adapted from Choi et al. (2014) with brief modification. BGE was diluted to serial dilution of 7-700 µg/mL concentration using ethanol 96%. Ascorbic acid was diluted to make 1-100 µg/mL serial dilution. Each dilution pipetted to 96-well plate and added with DPPH dissolved in ethanol 96% (750 mM). The plate then incubated for 30 minutes in dark milieu. Absorbance of each solution then was read using ELISA plate reader at 517 nm (Choi et al., 2014).

Cytotoxic Effect of BGE in Vero Cells

Up to 80% confluent cells were harvested and seeded at 96-well plate. Cell then treated using BGE (1-500 µg/mL), incubated within 24 hours. Afterwards, medium is replaced with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and continued with incubation for 4 hours at 37°C and 5% CO_2. Then plate was added with 10% SDS in 0.01 N HCl solution, incubated in dark environment overnight to dissolve formazan salt formed in each well. Absorbance of cells to determine cell viability was measured using ELISA reader at 595 nm. In calculating 50% value of concentration to inhibit 50% cell proliferation, linear regression between concentration and % cell viability was made (Novitasari et al., 2018).

Anti-senescence Effect of BGE

Cells that are 80% confluent treated with BGE and incubated 24 hours, the media was removed and washed with PBS 2x. Then a fixation buffer was added. Added 1-2 mL X-gal solution then incubated for 72 hours. Cells were observed under a microscope and % senescent cell was calculated (Novitasari et al., 2018). As senescence inducer, doxorubicine was used at a concentration of 50 nM.

Gelatinase Activity Inhibition in Silico Model

Native ligand complexed with protein target (MMP) was downloaded from PDB. The docking process was carried out using default MOE 2010: placement Triangle Matcher, rescoring London dG, and refinement forcefield. Compounds were visualized using MOE 2010, the energy Gibbs (ΔG) expresses the affinity of the ligand-receptor interaction (Lestari and Utomo, 2022).

NLC-BGE Serum Formulation

Nanostructured Lipid Carrier (NLC) formulation of BGE was carried out using high shear homogenization method including lipid phase melting process at 50°C temperature. Water phase containing tween80, span80, and 1/3 of water were homogenized 30 minutes at 750 rpm. The rest of the water was heated (50°C), then homogenized with Ultraturrax for 15 minutes at 15,000 rpm (Suryawijaya et al., 2022). NLC-BGE serum formulation was performed by stirring NLC, xanthan gum, and preservative at 750 rpm. The formula was then stored for 2 months to test stability by turbidity parameter.

Data Analysis

IC_{50} value was calculated to obtain antioxidant and cytotoxic activity. Senescence effect was determined by ImageJ software then analyzed using analytical statistic SPSS with ANOVA method of 95% confidence level (Jenie et al., 2021). Molecular docking method was identified valid when root mean square distances (RMSD) <2Å. The lowest MMP docking score was chosen and compared to MMP-compound complex. Visualization is done in 3D to identify MMP and target compound (Lestari and Utomo, 2022). The characteristic particle of NLC BGE was identified by particle size analyzer (PSA). Turbidity before and after is centrifuged at 5,000 rpm for 10 minutes and compared to each in order to obtain NLC stability.

RESULTS

Extraction of BGE

BGE is obtained by black garlic maceration at 50^0C for 90 minutes with 50% Ethanol (solid:liquid 1:10). The macerate was thickened using rotary evaporator, then obtained 1,524 g BGE with a yield of 30.30% (w/w). The Optimum extraction of polyphenol, flavonoid, and organosulfur on BG were done with higher water composition of solvent in ratio of 50:50 water/ethanol (Thach and Thuy, 2018; Riwanti et al., 2021). BG extraction using several methods in previous studies has also shown different yields. Maceration using 70% ethanol showed yields 49.58%; 34.5382%; 21.8645% (Rumaseuw et al., 2021). Although differs from other studies, extraction using 50% ethanol with heat modification showed yield extract as good as 70% ethanol. This low heat modification (digestion method) applied to lower the viscosity of solvent to which extracted compounds is enhanced (Abubakar and Haque, 2020).

Phytochemical Identification by TLC

BGE (35,000 ppm) was spotted on a silica60 plate and eluted with butanol: acetic acid: aquadest (4:1:1) as mobile phase. After elution, the plate was dipped into ninhydrin reagent to identify organosulfur. Red violet spot obtained were identified as SAC at Rf 0.37. SAC was a major organosulfur that are enhanced during the production process of BG by both heat and fermentation (Park et al., 2014). This process has caused BG to have higher SAC compound compared to regular garlic. The result showed that SAC is present and visualized as red violet spot after dipped onto ninhydrin. The color changing happened as amino chains of SAC and other organosulfur compounds formed cyclic when ninhydrin is added. The color intensity also depends on the different number of amino chains contained in every organosulfur (Basak et al., 2005).

Antioxidant Activity by DPPH Assay

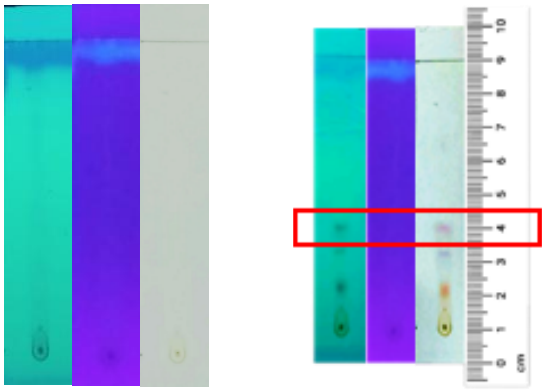
Antioxidant activity assay is conducted by DPPH method which showed the activity of sample treated in free radical of 1,1-diphenyl-2-picrylhydrazyl (DPPH). If the sample consist of radical scavenger (RAS) activity, free radical DPPH will be neutralized by donor electron of antioxidant compounds to a stable form (Singh et al., 2021). IC_{50} value is assessed to show which concentration of treatment is able to reduce 50% free radical DPPH. IC_{50} of BGE was 488 µg/mL, while compounds with IC_{50} value >100 µg/mL are considered to have less potent radical scavenging antioxidant activity. This reveals that BGE antioxidant properties were obtained through an enzymatic mechanism involving interactions with antioxidant enzymes (Flieger et al., 2021). Cysteine in SAC is known to induce glutathione production in Balb/c mice. Meanwhile, SAC is able to inhibit prooxidant enzymes production such as NO, NADPH oxidase, and COX which supports cytoprotective and anti-aging effects (Gonzalez et al., 2012).

Cytotoxic Activity to Normal Cells

To ensure extract safety, cytotoxicity test was carried out in Vero cells, normal epithelial

(a) **(b)**

Figure 1. Black Garlic and Black Garlic Extract (BGE). (a) Black garlic; (b) Thickened extract of black garlic.



(a) **(b)**

Figure 2. Phytochemical profile of BGE. Visualization under UV_{254}, UV_{365} and visual before (a) and after (b) ninhydrin treatment.

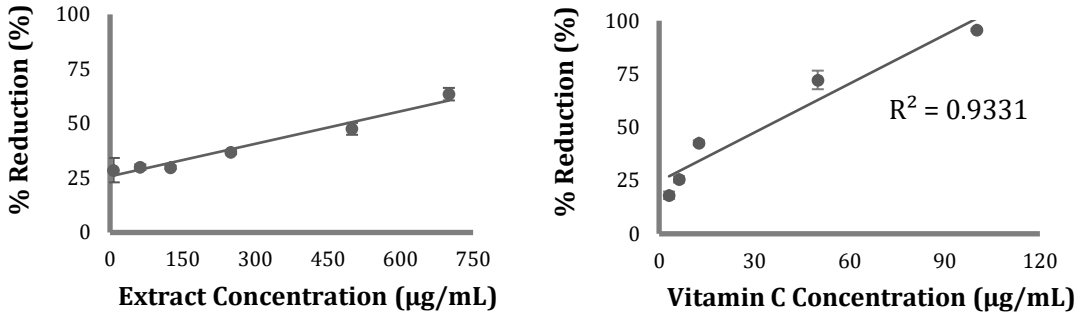
Table I. NLC formula and BlincSerum formula

Components	F1	F2	F3	Components	Percentage
Extract	0.1%	0.1%	0.1%	NLC BGE*	70%
Palm stearin	0.8%	0.8%	0.8%	Xanthan gum	0.7%
Olive oil	0.3%	0.3%	0.3%	Preservative	0.1%
Tween 80	1.3%	0.9%	0.6%	Fragrance	0.01%
Span 80	3.0%	3.5%	3.7%	Aquabidest	29.3%
		(a)			(b)

tissue cells representation. BGE cytotoxic effect examined by MTT assay with IC_{50} as a parameter in showing at which concentration a sample is able to inhibit 50% of live cells. Cell viability profile after BGE showed IC_{50} of 1,100 µg/mL hence considered non-toxic to normal cells. The cell viability almost static to a concentration of 1,100 µg/mL. These results indicate that BGE is predicted to be safe for skin tissue.

Anti-senescence Effect of BGE

The SA-β-Gal assay aims to evaluate the anti-senescence effect of BGE on normal (Vero) cells with a treatment dose of 50 µg/mL by using Doxo 50 nM as a senescence inducer. We see that Doxo significantly increase senescent cells on Vero ($p<0.05$) shown by the green color of the cells' appearance, which conveys that treatment with Doxo is appropriate for inducing senescence in normal cells. We found that BGE in a single treatment at a concentration of 50 µg/mL does not cause senescence in Vero cells compared to cells without treatment. Furthermore, based on ANOVA, the combination of 50 µg/mL BGE and Doxo 50 nM decreased senescent cells significantly ($p<0.05$). This piece of evidence indicated that SAC compounds in BGE significantly reduce cellular senescence in normal cells.



(a) (b)

Figure 3. RAS activity is analyzed using DPPH assay based on % reduction after BGE treatment (7-700 µg/mL concentration). (a) IC_{50} of BGE 488 µg/mL. (b) IC_{50} of Vitamin C 33 µg/mL.

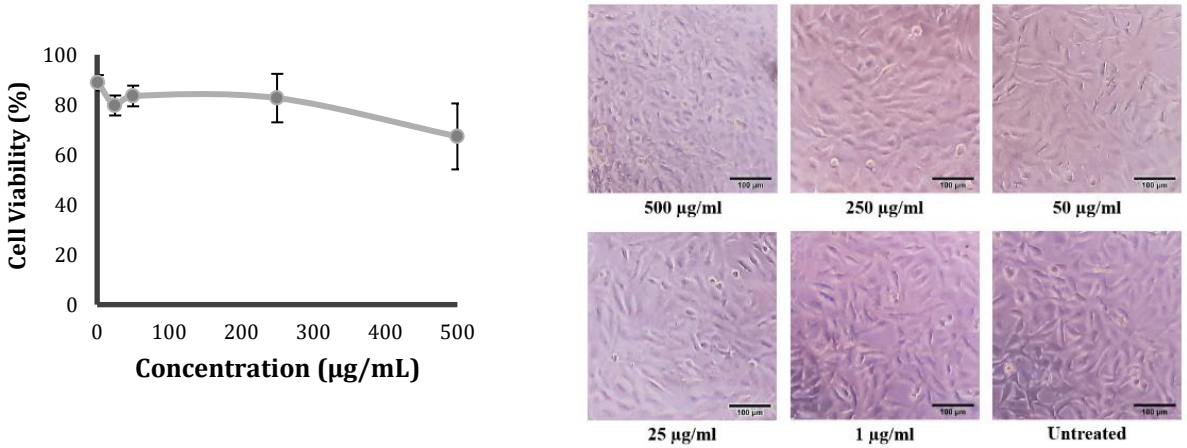


Figure 4. BGE cytotoxic effect towards normal cells. BGE toxicity effect calculated using MTT assay. Vero cells were treated with BGE concentration of 1-500 µg/mL, then IC_{50} 1,100 µg/mL is obtained. Photo was taken under microscope 100x magnification.

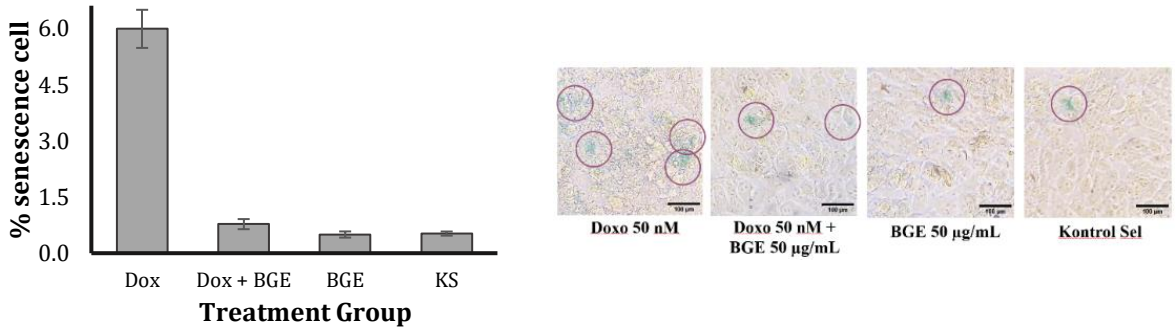
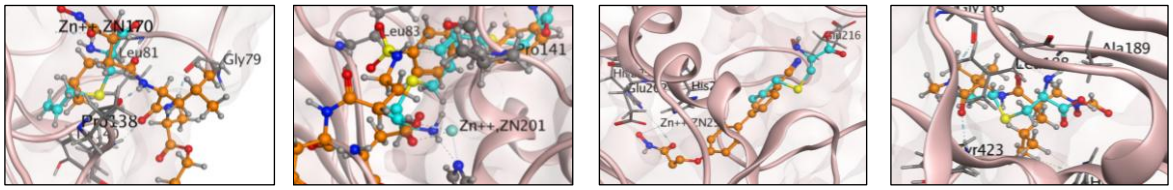


Figure 5. Administration of BGE significantly reduced the percentage of cell senescence in vero cells induced by doxorubicin. In addition, administration of BGE was not significantly different from control cells indicating the safety level of the extract.

Gelatinase Activity Inhibition Model with Molecular Docking

The validation of docking method was performed by observing the RMSD score of the PDB target protein with its native ligand. In this study, the RMSD value between target protein PDB and native ligand had less than 2 Å. The docking score of SAC was compared to the native ligand for each type of MMP as presented in Table I. While the visualization of SAC interaction



(a) (b) (c) (d)

Figure 6. Ligand interaction (orange) and S-allylcystein (blue) with MMP protein visualized in 3D. (a) MMP1, (b) MMP2, (c) MMP3, (d) MMP9. Ligand RMSD value as follows 1.83; 1.94; 1.16; and 1.43 shows valid docking (RMSD < 2Å).

Table II. Docking Score of *S-allylcystein* (SAC)

ΔG (kcal/mol)	MMP1	MMP2	MMP3	MMP9
Ligand	-8,457	-8,927	-13,377	-10,928
SAC	-7,754	-9,130	-8,088	-8,911

with the native ligand for each type of MMP (MMP1, MMP2, MMP3, and MMP9) is displayed in Figure 6. The visualization of interactions was carried out using MOE program. It is known that SAC has good affinity for the inhibitory domains of MMP1 and MMP2 in terms of negativity and similarity of docking scores towards the ligand.

NLC-BGE Formulation and NLC-BGE Serum

NLC BGE formulation is made with high-shear homogenization (HSH) using ultraturrax 15,000 rpm for 15 minutes. HSH method is used as it could break down surfactant, lipid, and water particles above lipid melting point temperature hence forming an emulsion system (Babazadeh et al., 2017). The formula of NLC BGE contains BGE extract, palm stearin and olive oil as lipid phase, also tween80 and span80 as surfactant. NLC BGE formulation is based on particle characterization and stability testing. Characterization of NLC BGE is included by assessing particle size and polydispersity index with particle size analyzer (PSA). The following results of NLC particle size is shown in Table II. The three formulas have met nano-size range of 53.61-82.10 nm (<100 nm), F2 is considered most stable formula in terms of significant differences absence ($p < 0.05$) after 5,000 rpm centrifugation for 10 minutes (Figure 7). This is supported by the smallest PdI value of 0.50 which showed homogenity of particles (PdI<0.7).

NLC-BGE serum was prepared with the best NLC-BGE (F2), which added with various excipients to obtain the desired viscosity. The excipients are xanthan gum as a gelling agent, preservative as an antimicrobial, fragrance to cover the odor of tween80 and span80, and distilled water as a solvent. Physical properties of NLC-BGE serum presented in Table III.

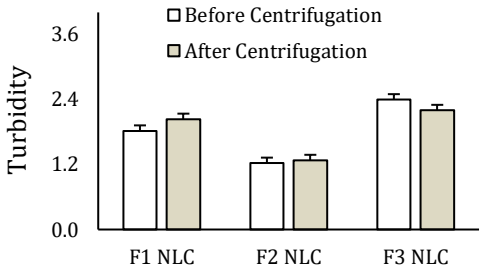


Figure 7. Turbidity stability of NLC BGE. Result shows no significant turbidity of F2 after centrifugation (*p value<0.05)*, shows F2 formula is the most stable.

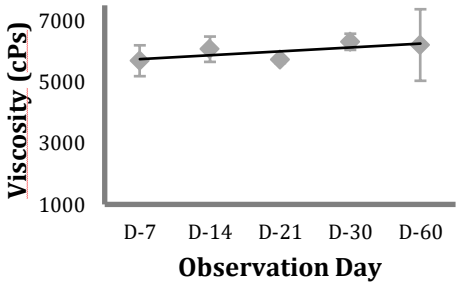


Figure 8. NLC-BGE Serum Viscosity Stability. Serum was stable for 2 months storage in room temperature with viscosity mean of 5,996 cPs.

Viscosity parameter can used to determine stability of the preparation with NLC-BGE serum is considered to have a stable viscosity in 2 months of storage with the average of 5,996 ± 297 cPs.

DISCUSSION

This research reveals that BGE has antioxidant properties were obtained through an

Table III. Particle Characterization of NLC BGE

Formula	Particle Size	PdI	HLB
F1	65.40 nm	0.53	11.87
F2	82.10 nm	0.51	12.92
F3	53.61 nm	0.90	13.50

Table IV. NLC-BGE Serum Formula and Evaluation

Physical Properties		pH
Visual	Opaque	6.52
Color	Beige	
Odor	Acceptable	
Texture	Less Thick	

**nanostructured lipid carrier black garlic extract*

enzymatic mechanism involving interactions with antioxidant enzymes. This experiment also aims to explore BGE anti-aging potency through anti-senescence mechanism and gelatinase activity inhibition in normal cell model (Vero) which formulated in NLC serum. To ensure extract safety, a cytotoxicity test was carried out in Vero cells, normal epithelial tissue cells to represent normal cells. The results of BGE cytotoxicity showed that BGE was not toxic and cell viability not decreased up to a concentration of 1,100 µg/mL. These results indicate that BGE is predicted to be safe for skin tissue as well. The anti-senescence effect shows that BGE in a single treatment at a concentration of 50 µg/mL does not cause senescence in Vero cells compared to cells without treatment, but interestingly, after being combined with doxorubicin at a 50 nM concentration, BGE at a 50 µg/mL is able to reduce senescence occurrence significantly in Vero cells ($p < 0.05$). Doxorubicin was chosen as a senescence inducer model because it is a chemotherapy drug and bearing the ability to induce aging with the help of enzymes in mitochondria that can convert ROS through redox reactions that lead to DNA damage (Cappetta et al., 2017). Based on that, SAC compounds have cytoprotective activity that supports tissue repair thereby preventing senescence.

Inhibition of gelatinase activity was carried out by molecular docking with MOE software to predict the inhibitory interaction of SAC against MMP-1, MMP-2, MMP-3, and MMP-9 proteins. MMP protein is an essential protein of protease group involved in E CM degradation and supports skin structure (Shin et al., 2019). In the skin, excessively activated MMPs can degrade collagen and elastin, causing a decrease in skin strength and elasticity or skin aging (Rodriguez et al., 2017). SAC's docking score against MMP-1 (-7,754 kcal/mol) shows similar affinity to native ligand (-8,457 kcal/mol) beside SAC's docking score against MMP-2 (-9,130 kcal/mol). MMP-1 (collagenase 1) plays role on initiating degradation of collagen type 1 and 3, MMP3 (stromelysin 1) which contributes in continued collagen type 1 degradation and activation of MMP9, while MMP9 (gelatinase B) further degrades collagen fragments produced by collagen degradation types 1 and 3 and collagen degradation type 4. MMP2 plays a role in the degradation of type 4 collagen (Pittayapruek et al., 2016). MMP-2 and MMP-9 also play a role in elastin degradation with the help of their fibronectin-like modules (Van Doren, 2015). The docking score value shows inhibitory interaction against MMP2 in terms of lowering Gibbs energy compared to ligands. While SAC affinity for MMP3 and MMP9 shows a less good inhibitory interaction in terms of greater Gibbs energy.

BGE formulated in lipid-based NLC formula preparation. The NLC system is a lipid-based drug delivery system with improved stability and loading capacity so as to allow drugs to be better dispersed (Sharma et al., 2018). This system also developed encapsulation, protection, and transportation of both polar and nonpolar bioactive (de Souza Simões et al., 2017). This characteristic makes NLC a suitable system for serum production and is expected to increase the effectiveness of the formula. In the NLC formulation, palm stearin is used as a solid lipid and olive oil as a liquid lipid. Solid lipids play a role in reducing molecular diffusion in the emulsion system so that the chemical stability of the active substance increases, while liquid lipids function to increase the entrapment efficiency of the active substance (Helgason et al., 2009). The selection of tween80 and span80 surfactants is based on the classification of nonionic surfactants that are less toxic to biological systems (How et al., 2013). When particles stick to the skin surface,

lipids will reduce air loss at the surface and increase skin hydration (Ebtavanny et al., 2018). NLC particles have size of 10-500 nm, with homogenity parameter is seen from the polydispersity index (PdI) value <0.7. Meanwhile, NLC stability is seen from the formation of separating phase or creaming which resulting in increased turbidity (Sharma et al., 2018; Danaei et al., 2018; Fathi et al., 2018). The three formulas have met the nano-size range of 53.61-82.10 nm, with F2 being the most stable preparation in terms of significant differences absence ($p < 0.05$) after centrifugation 5,000 rpm for 10 minutes. This is supported by the smallest PdI value of 0.50. Then continued into the serum formula with adding xanthan gum (gelling agent) as a hydrocolloid to increase viscosity. The viscosity parameter was then used to assess the stability of the formula. NLC-BGE Serum has a stable viscosity during 2 months of storage with an average of 5,996 ± 297 cPs. Serum has a low viscosity and will form a thin film on the surface of the skin without giving an oily impression (Budiasih et al., 2018). In this way, BlincSerum is expected to be able to penetrate better into the deepest skin.

CONCLUSION

Based on research conducted, black garlic organosulfur content is detected using TLC showed the presence of S-allylcystein (SAC). Black garlic extract considered to be non toxic to normal cells, besides the ability to inhibit the incidence of senescence based on in vitro model and inhibition gelatinase activity of MMP proteins based on in silico model. These anti-senescence effect and MMPs inhibition play a significant role as anti-aging effect of black garlic. The formulation of NLC serum F2 showed great particles characteristic and stability over 2 months. Additionally, in order to assess the potency of NLC-BGE serum as an anti-aging serum in overcoming premature aging, further research is needed.

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ABBREVIATION

BG : Black garlic
BGE : Black garlic extract
DPPH : 1,1-diphenyl-2-picrylhydrazyl (radical compound)
ECM : Extracellular matrix
MMP : Matrix metalloproteinase
MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NLC : Nanostructured Lipid Carrier
SAC : S-Allyl-Cysteine
TLC : Thin layer chromatography

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