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Anti-osteoporotic Activity of Ethyl Cinnamate from *Coix lacryma-jobi* Targeting SOD and GPx Proteins

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ABSTRACT

Free radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS) can trigger oxidative stress, known to be responsible for the downregulation of Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx), known endogenous antioxidants. Ethyl cinnamate is a bioactive component of *Coix lacryma-jobi* with strong antioxidant activity with a potential to suppress ROS which could be useful in the prevention of osteoporosis. Molecular docking is a cost-effective method to determine the biological activity of molecules through virtual interactions with related protein targets in biological systems. This study aimed to determine the antiosteoporotic activity of Ethyl cinnamate from hanjeli fruit using *in silico* methods. The 3D structures of the target proteins (SOD and GPx) were retrieved from the RCSB Protein Data Bank, while the ligand (Ethyl cinnamate) was obtained from GC-MS analysis of *Coix lacryma-jobi* extract. The proteins were prepared for docking using the Chimera 1.10.1 software, while the 3D structure of the ligand was optimised using the HyperChem 8 software. The molecular docking process was done using the Autodock 4.2 software. The results of the study indicated that Ethyl cinnamate interacted with putative sites of SOD and GPx proteins, with binding free energy of -0.75 and -1.61 kcal/mol, respectively. The results showed that Ethyl cinnamate from the hanjeli plant has the potential to neutralise free radicals through the upregulation of endogenous SOD and GPx responsible for the inhibition of oxidative stress implicated in bone resorption. This preliminary *in silico* study shows that Ethyl cinnamate has the potential to prevent oxidative stress and can be useful in the management of osteoporosis.

Keywords: Osteoporosis, Ethyl cinnamate, Molecular docking, Anti-osteoporotic

Introduction

Osteoporosis is a condition of a reduced bone substance compared to normal conditions so that the bones become brittle.[1] This condition is more experienced by women, especially during post-menopause. In Indonesia, 8.5 million out of 222 million Indonesians have osteoporosis, and along with the increase in population, it is estimated that the number of sufferers of osteoporosis will increase to 11.5 million in 2050[2]. The current treatment of osteoporosis refers to increasing bone density.[3] In addition, several drugs, such as the bisphosphonate class, raloxifene, calcitonin, and tibolone, are often used to build bones, compact bones, and inhibit the process of osteoporosis[4]. However, increasingly brittle and thinner bones are also caused by hormone production, the ageing process, and the formation of free radicals due to oxidative stress by Reactive Oxygen Species (ROS) which can stimulate bone resorption.[5] Oxidative stress is the result of excess ROS production in the body. ROS contains one or more unpaired electrons, so they are very reactive to stabilize their electron balance.

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ROS production increases with age and is associated with several chronic diseases including osteoporosis.

Hanjeli (*Coix lacryma-jobi*) is a plant that grows a lot in Indonesia. One of the phytochemicals in hanjeli is ethyl cinnamate. Hanjeli fruit synthesizes ethyl cinnamate in large quantities during ripening, which reaches 90% of the total carotenoid fraction[6,7]. Several studies showed the potential of ethyl cinnamate in the health sector, and one of them showed a direct correlation between serum ethyl cinnamate and a reduced risk of osteoporosis among postmenopausal women[8]. To find out the activity of ethyl cinnamate in antiosteoporosis, it is necessary to make a preliminary test using the in silico molecular docking method. The molecular docking technique in silico can be used to predict the interaction process of a protein with a ligand so that the molecular mechanism can be known. This method can increase the effectiveness and efficiency of research to find new drugs. Therefore, this research is very important to investigate the mechanism of ethyl cinnamate in preventing osteoporosis through the neutralization of free radicals by in silico induction of reactive oxygen conversion into water molecules by endogenous antioxidants (SOD proteins and GPx).

Materials and Methods

The GC-MS analysis

The GC-MS technique is used to identify the phytochemical compounds contained in a plant. It is a process of gas chromatography, detecting volatile substances and compounds, while mass spectrometry is the process of identifying molecular compounds through molecular weights and determining their molecular formulas. The GC-MS method

is very suitable for volatile essential oils. Before the real operation, the instrument was checked for gas flow starting at a low flow rate by opening the main and secondary valves on the carrier gas tank until it showed the 15-psi needle; this allows 2–5mL/min carrier gas flow for the packed column or 0–5 mL/min for the capillary column. The column was heated to the desired initial temperature. Then, the detector temperature was set at 10–25 °C higher than the column temperature, and so was the injection port temperature. The speed (rate) of the gas flow was then increased to 25–30 ml/minute in the packing column, or until the optimum gas flow rate was achieved. When using a flame ionization detector, it is necessary to pay attention to the presence of hydrogen gas and air flowing into the detector. The sample was dissolved in a volatile solvent, and the volume of the sample injected depended on the type of detector used. For this purpose, in the GC-MS tool, the settings are as follows TCD = 10 l, FID = 1–10 l, BCD = 0.1–5 l with a micro syringe, during elution was during the passage of the sample from the injection port to the signal detector. From this detector, it would be recorded as a chromatogram on a simple recorder or processed by a microprocessor and displayed on the monitor screen. The chromatogram is displayed by the microprocessor to determine each component simultaneously[8].

Preparation of Ligands and Comparative Compounds

The Ligand sample (ethyl cinnamate) was obtained from GC-MS analysis of the essential oils of *Coix lacryma-jobi* (Figure 1). The 3D structures of the proteins (SOD (PDB id: 1mfm) and GPX (pdb id: 2f8a) downloaded from **http://www.rcsb.org/pdb/home.do**.) in *pdb format. The proteins were prepared by removing water molecules, heteroatoms, etc, and saved in pdbqt (Protein Data Bank, Partial Charge (Q), & Atom Type (T)) format.[6] The 2D structure of ethyl cinnamate was downloaded from https://pubchem.ncbi.nlm.nih.gov/compound/446925 and converted to 3D and optimized using the HyperChem 8 application. The study used a Windows 10 64-bit computer, with AutoDockTools 4.2 HyperChem 8, Chimera 1.10.1, and Open Babel programs[7].

Protein Preparation Works

SOD and GPx protein preparation was carried out by separating the protein from its native ligand using the Chimera 1.10.1 application[7,9].

Ethyl cinnamate 3D Structure Optimization

The downloaded 2D structure of ethyl cinnamate was converted into 3D and optimized using the HyperChem 8 application. The AM1 semi-empirical computational method was used with single-point calculations and geometry optimization[7,10].

Molecular Docking Method Validation

The molecular docking method was validated by docking the native ligand on a protein that had its native ligand (Malonic acid) removed using the Autodock 4.2 application with the RMSD parameter. This application is used to remove or separate native ligands and residues in the form of water molecules by clicking on the residues to be removed. This system will open the ligand to be used so that the ligand will automatically minimize its energy.[6,9,10].

Ethyl cinnamate docking on SOD and GPx protein.

The optimized ethyl cinnamate compound was docked to SOD and GPx proteins, which had their native ligand removed using the Autodock 4.2 application. The analysis results show the lowest binding energy conformation for binding to the target protein[6,7,11].

Data Analysis and Method Validation

The result of molecular docking was bond energy. The bond energy value indicated the strength of the bond between the compound and the receptor. The lower the bond energy value was, the stronger and more stable the bond. The validation process in this *in silico* test was carried out through re-docking of native ligands that had been downloaded and prepared using the Discovery Studio Visualizer® application. Receptor validation was performed three times using the PyRx-Vina® application. The parameter observed at this stage is the RMSD (Root Mean Square Deviation) value resulting from re-docking the native ligand with its protein.[11] The method is said to be valid and good if the resulting RMSD value is < 2.[12]

Results and Discussion

Coix lacryma-jobi have become attractive therapeutic compounds for the treatment of various human chronic diseases because of their high effectiveness, low toxicity, and few side effects.[13] The present study identified ethyl cinnamate and demonstrated their regulatory effects on osteoblasts and osteoclasts in a SOD protein and GPx-dependent manner. This may provide new leads for the development of novel anti-osteoporotic drugs and help elucidate the mechanism of action of these screened compounds. A study reported that a high-throughput screening method with a Z0 value greater than 0.5 was sensitive and excellent.[14] To evaluate the sensibility of the ethyl cinnamate-based drug screening system, the Z0 factor was calculated for the screening system and found to be 0.65, indicating that the sensibility of our screening method was excellent.[15]

Molecular docking is a theoretical simulation method that mainly studies the binding mode and affinity between molecules (such as ligands and receptors). Through the prediction of the affinity, as represented by the docking score, it can compare the affinity of each ligand and protein quickly so many compounds can be screened successfully[15,16]. In this study, we investigated the binding mode and affinity between molecules and ethyl cinnamate. The structures of the proteins without the native ligand and the structure of the native ligand that has been separated from the proteins are shown in Figure 1 (A-D). Ethyl cinnamate derived from hanjeli fruit was obtained from the results of GC-MS analysis of hanjeli fruit essential oil (Figure 2). The downloaded 2D structure of ethyl cinnamate was converted into 3D and optimized using the HyperChem 8 application with the AM1 semi-empirical method as well as single point calculations and geometry optimization to obtain the most stable 3D structure of ethyl cinnamate with the lowest structural energy value. The energy when a single point calculation was carried out was -11310.758 kcal/mol; then, with geometry optimization, the structure's energy decreased to -11359.031 kcal/mol. When geometry optimization is done, the structural energy can be minimized to obtain the most stable structure. The 3D structure of ethyl cinnamate due to geometry optimization is shown in Figure 3.

A hydrogen bond occurs between a hydrogen atom (H) in one molecule and one atomic element (F, O, N) in another molecule, which is the strongest dipole-dipole force[17]. In biological systems, nitrogen or oxygen atoms are donors and acceptors, especially atoms in the amine ($-NH_2$) and hydroxyl (-OH) groups. Due to the polar nature of the N-H and O-H bonds, the H atoms can hydrogen bond with acceptor atoms.[7] Hydrogen bonds are stable and have strong bonds if they have a bond length of < 2.[7,18] The smaller the hydrogen bond distance between the ligand and the acid group is, the greater the affinity value. The smaller the bond distance is, the stronger the bond and not easily separated.[18] Hydrophobic bonds are nonpolar molecules that do not contain hydrated ions or have a dipole moment. This happens because, in water, these molecules are insoluble.[17] This binding is important in the process of combining the nonpolar region of the ligand with the nonpolar region of the receptor. The nonpolar region of the water-insoluble molecule and the surrounding water molecules will combine through hydrogen bonds to form a quasi-crystalline structure (icebergs).[19] Hydrophobic binding is a parameter of the strong amino acid interaction between the ligand and the receptor which is useful in helping to maintain the binding conformation.[20]

The validation of the molecular docking method was carried out by docking the native ligand on the SOD and GPx proteins using the Autodock 4.2 program. At this stage, adjustments were made to the coordinates of the site of interaction in the Autodock 4.2 application by setting the grid centre and grid size. The parameter validation method used was the RMSD value. RMSD is a measurement of two poses by comparing the atomic positions between the experimental structure and the structure docked in the protein.[9] The smaller the RMSD value obtained, the better the predicted ligand pose because it is closer to the

native ligand conformation, whereas the greater the RMSD value, the greater the prediction error of the interaction between ligand and protein.[10] The RMSD values obtained in this study were 3.54 Å for the SOD protein with its native ligand and 2.12 for the GPx protein with its native ligand. Based on the results obtained, the molecular docking method used in this study was valid and could be used for the docking of ethyl cinnamate compounds in SOD and GPx proteins. A visualization of the interactions that occurred between SOD and GPx proteins with their native ligands is shown in Figure 4 (A and B).

Electrostatic bonds describe the forces between polar atoms and are usually represented by the Coulomb potential. In general, there are two grading function approaches for hydrogen bond interactions: (i) using specific force field-based parameters related to van der Waals and electrostatic energy potentials; (ii) using a directional term, where the hydrogen bond contribution is a function of the deviation of the geometric parameter from the ideal hydrogen bond.[19] Hydrophobic interactions and electrostatic interactions can increase conformational stability.[19,20] The similarity of the ligand poses with the comparison compound could be influenced by the RMSD value, where an RMSD value that is close to zero would cause the pose similarity between the two.[20]

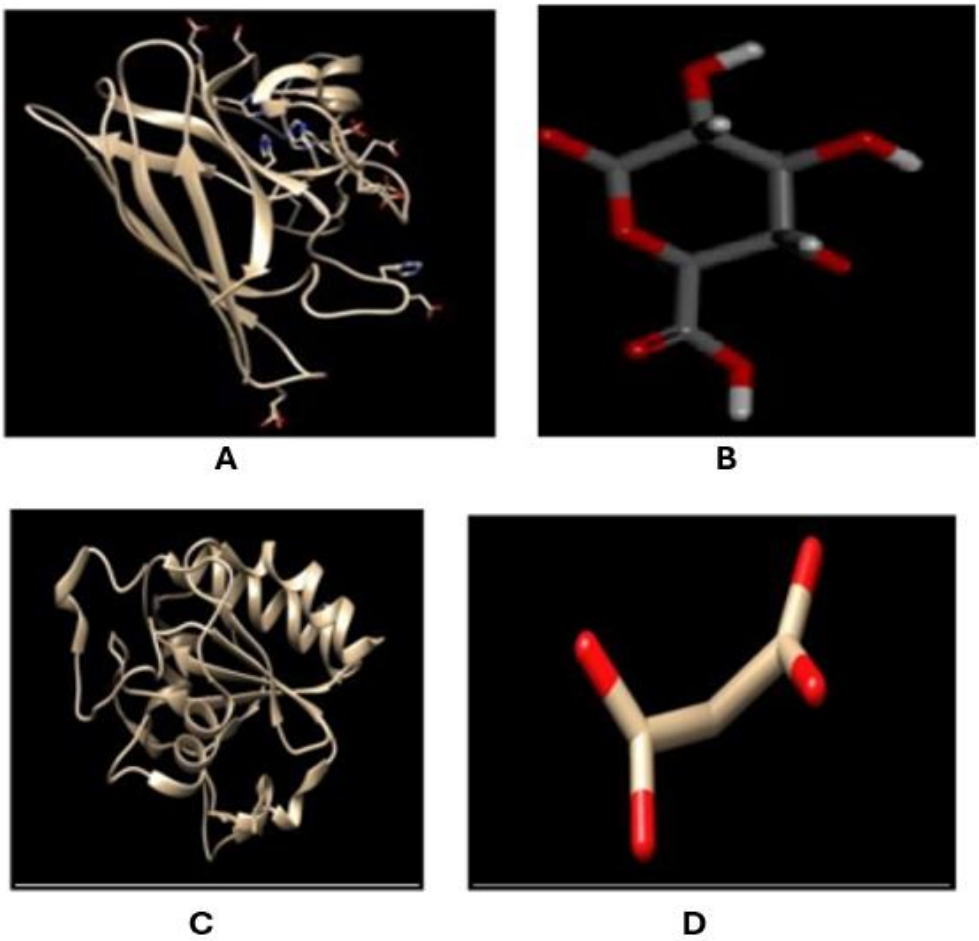

Figure 1: Structures of SOD Protein (a) and GPx protein (c) and their native ligands (b) and (d), respectively.

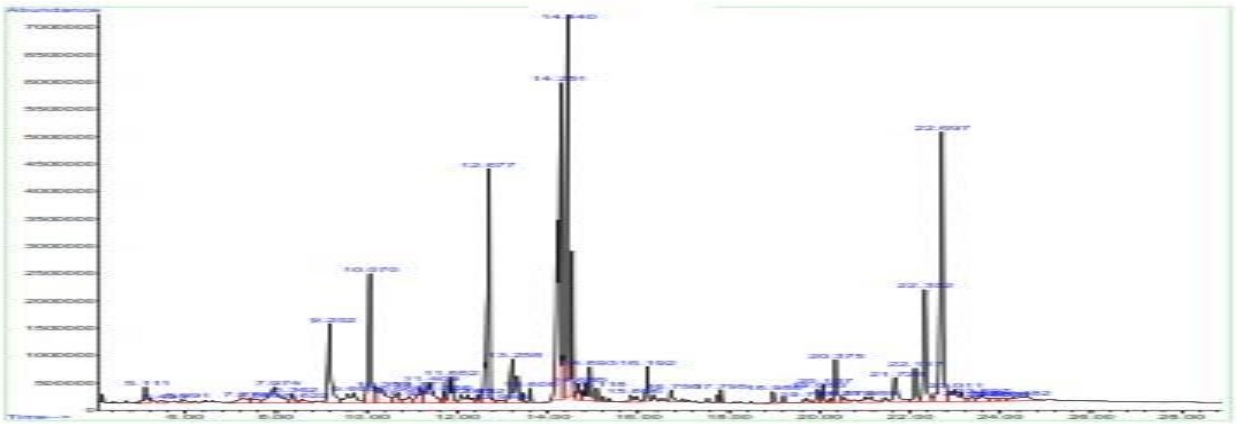

Figure 2: GC-MS chromatogram and library of Hanjeli (*Coix lacryma-jobi*) fruit essential oil

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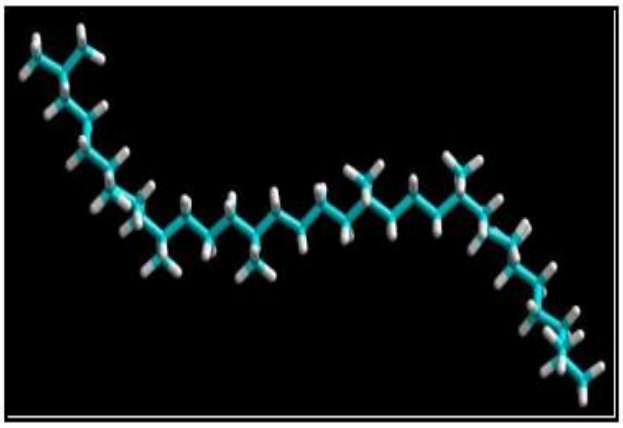

Figure 3. Results of 3D Structure Optimization of Ethyl Cinnamate Compounds

The optimized ethyl cinnamate compound was docked to SOD and GPx proteins using the Autodock 4.2 program at the same coordinates as those used when conducting method validation. The docking process resulted in ten conformations of ethyl cinnamate binding to SOD and GPx proteins. Of the ten conformations, one with the lowest bond energy value was selected. Bond energy indicates the affinity of ethyl cinnamate for proteins. The smaller the bond energy value obtained, the more stable the bond that occurred between ethyl cinnamate and SOD and GPx proteins. The expected conformation of the docking result was the conformation with the lowest bond energy value, which was on the active side of the protein, or the coordinates of the binding sites that were the same as the prearranged native ligand. The lowest binding energy values were obtained between ethyl cinnamate and SOD protein and GPx (-0.75 and -1.61 kcal/mol, respectively).

Compounds could be said to not meet if there was more than one criterion that deviates. The condition for the value of LogP (XLogP3) is -0.4-5. The larger or the more positive the log P value is, the more hydrophobic is the molecule. If hydrophobicity is high, the level of toxicity will also be high because it will be retained longer in the lipid bilayer or the base of the cell membrane structure and distributed more widely in the body so that the selectivity of binding to the target enzyme is reduced.[21] Molar refractivity that does not meet the requirements would cause nonpolar compounds to be unable to form momentum so that they cannot bind to receptors, and their polar nature prevents the excretion of metabolic residues of the compound.[22] This shows that ethyl cinnamate can interact with the active site of the two proteins. Therefore, based on the results of this study, it can be predicted that ethyl cinnamate has activity as an anti-osteoporosis agent because it has an affinity for SOD and GPx proteins. A visualization of the interaction between ethyl cinnamate and SOD and GPX proteins is shown in Figure 4 (C and D). The interaction between ethyl cinnamate and the two proteins can induce the neutralisation of free radicals so that bone resorption can be prevented.

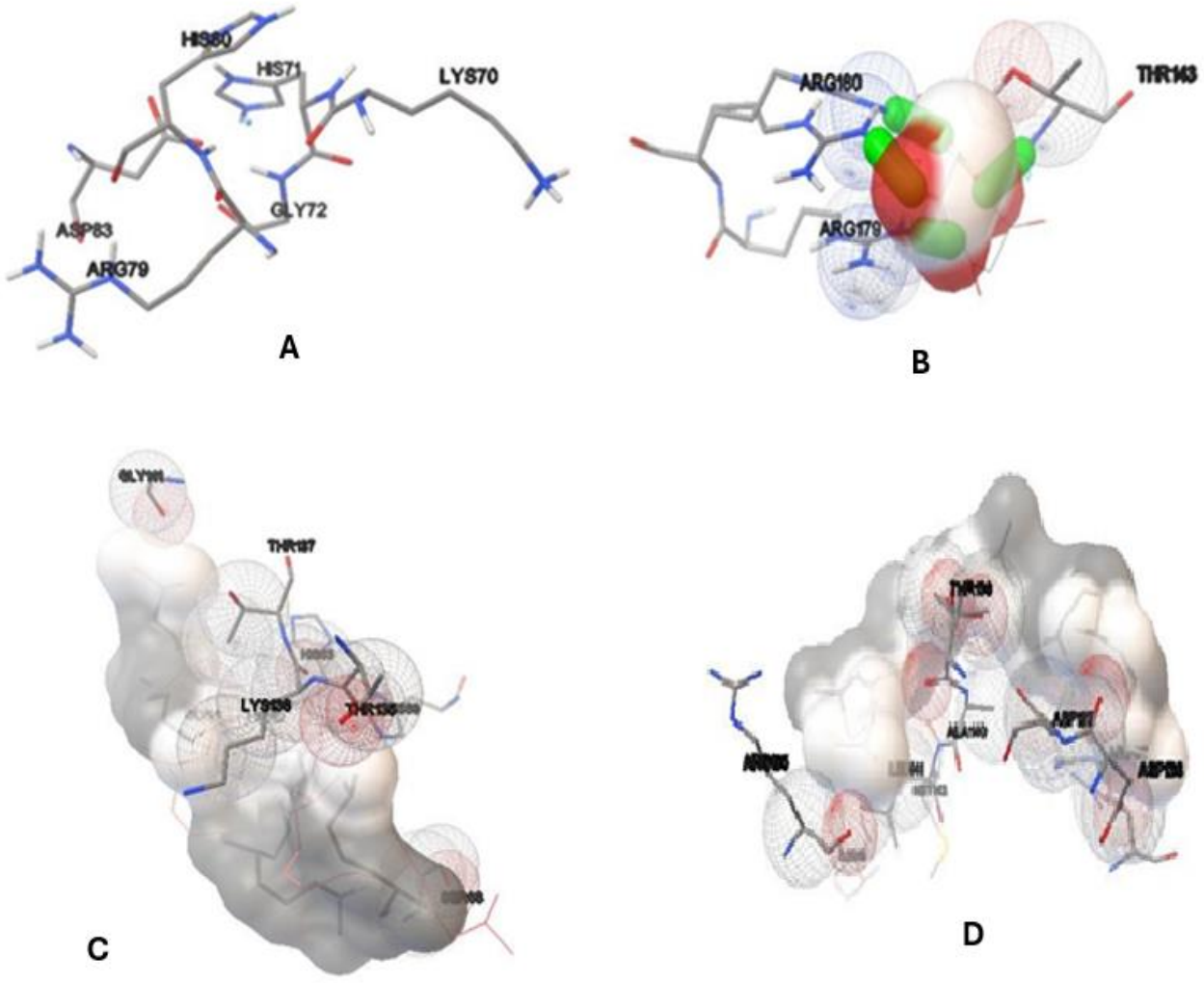

Figure 4: Visualization of the interactions between the native ligands with SOD (A) and GPx (B), and Ethyl Cinnamate with SOD (C) and GPx (D) proteins

Conclusion

Ethyl cinnamate has potential as a molecular anti-osteoporosis agent because it has an affinity for SOD and GPx proteins with binding energy values of -0.75 and -1.61 kcal/mol, respectively, hence it can induce SOD and GPx proteins to neutralise free radicals. *In vitro* and *in vivo* tests need to be carried out to determine the ability and effectiveness of ethyl cinnamate as an anti-osteoporosis agent.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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