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POTENTIAL ANALYSIS OF HERBAL ACTIVE COMPOUNDS AS IMMUNOTHERAPEUTIC AGENTS AGAINST CLTA-4 RECEPTOR THROUGH IN-SILICO

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ABSTRACT
Immunotherapy is a promising cancer treatment that enhances the body's immune system by targeting immune checkpoint receptors. One of these receptors, CTLA-4, suppresses the activity of T lymphocytes. Several active compounds derived from herbs, including astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrhizol, have demonstrated potential in anticancer immunotherapy. The objective of this study is to investigate the interaction between these active compounds and the CTLA-4 receptor using molecular docking simulation. This experimental research was conducted from January – June 2023 in the pharmaceutical chemistry laboratory at Al-Irsyad Cilacap University with methods including ligand and receptor preparation, blind molecular docking, RMSD validation, and visualization of the structure. Our findings indicate that all four active compounds can interact with the CTLA-4 receptor and inhibit it with bond energies of astragaloside IV -7.3, flindersine -5.7, n-butylidenephthalide -5.0, and xanthorrhizol -4.9, at RMSD 0. However, the interaction does not involve the same amino acid residues as the comparator ligand ipilimumab due to differences in bond area.

Keywords: Immunotherapeutic; herbal active compounds, CTLA-4; in-silico

1. INTRODUCTION

Immunotherapy has evolved into a potent clinical method for cancer treatment. The quantity of immunotherapy medicine approvals has increased, and a variety of treatments are currently under clinical and preclinical stage of development. Agents are employed in cancer immunotherapy to activate or increase the immune system's ability to fight cells with cancer through natural mechanisms (Riley et al., 2019). This technology has increased the chances of patient survival in cancer and higher therapeutic efficacy (Falzone et al., 2018; Waldman et al., 2020). When immune checkpoint inhibitors are stimulated, they dampen the immune response activated by cancer cells. Immune checkpoints function physiologically to maintain adequate immune responses and to protect the healthy tissue from immunological attacks (Lawrenti, 2018; Ong et al., 2018).

Cytotoxic T Lymphocyte Associated Protein 4 (CTLA-4) is a frequent immune checkpoint inhibitor receptor. Cancer can use the CTLA-4 receptor to grow. The receptor can bind to CD80 and CD86 on antigen-presenting cells (APCs), causing immunosuppression and inhibiting T lymphocyte function (Sobhani et al., 2021). Immunotherapy aims to suppress cancer proliferation by inhibiting or blocking CTLA-4 expression on T lymphocytes, limiting CTLA-4

interaction with CD80 and CD86. A decrease in the immunosuppressive response will increase the immune response of active T lymphocytes to inhibit tumor and cancer growth (Destiawan et al., 2021; Sugawara et al., 2021).

As an immunomodulator, Ipilimumab acts on the CTLA4 receptor in T cells (Usama et al., 2019). Ipilimumab is the first anti-CTLA-4 drug to bind and inhibit the interaction of CTLA-4 with its ligand and has been approved by the US FDA (Biorad, 2015; Lawrenti, 2018). However, as of September 30, 2020, there were 22,451 adverse events reported for ipilimumab. The most severe adverse events reported to the FDA database were myocarditis, pneumonitis, hypophysitis, and hepatitis (Jacob et al., 2021).

Indonesian people have used natural resources for medicinal purposes in their daily lives. Therapy of complicated and multivariate physiological imbalances, as well as several other health problems, including cancer, is carried out globally using compounds derived from medicinal plants (Banerjee et al., 2023; Kusnul, 2019). Active substances with potential as anti-cancer, specifically immunotherapy, include astragaloside IV from the Huang qi plant (*Astragalus membranaceus*), flindersine from the Ki Sampang plant (*Melicope denhamii*), n-butylidenephthalide from the female ginseng plant (*Angelica sinensis*), and xanthrorrhizol from the ginger plant (*Curcuma xanthorrhiza*) (Banerjee et al., 2023; Saputri et al., 2018). In silico research can be used to find more relevant and successful candidates with low side effects through the exploration of natural chemicals that have the potential to prevent cancer growth and reduce side effects (Amalina et al., 2020).

Molecular docking is a structure-based drug design approach that simulates molecular interactions and evaluates the contacts and affinities between ligands and receptors (Fan et al., 2019). Astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrhizol are compounds derived from alkaloids, saponins, terpenoids, and phthalides that can generally be found in various plants. These four compounds have the potential to inhibit cancer cell growth by reducing the risk of metastasis as well as inducing apoptosis in cancer (Banerjee et al., 2023; Saputri et al., 2018). This research has urgency in overcoming side effects and providing more effective cancer treatment through immunotherapy with astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrhizol compounds against CTLA-4 receptors determined using molecular docking to determine affinity and interactions that occur.

2. METHODS

This study is an experimental study conducted in January – June 2023 in the pharmaceutical chemistry laboratory at Al-Irsyad Cilacap University. This study uses in silico analysis methodology through blind molecular docking to predict the activity of molecules with selected receptors. This research method includes materials and tools, preparation stage, analysis stage, and interpretation stage.

2.1. Material and Tools

The 3-dimensional structures of astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrizhol were downloaded from https://pubchem.ncbi.nlm.nih.gov/. The structures of the CTLA-4 receptor and the comparator ligand ipilimumab (PDB ID: 6RP8) were downloaded from the PDB (protein data bank) website, https://www.rcsb.org/. This study used the computer with Windows 10 64-bit specifications equipped with PyRx, Discovery Studio, and PyMol programs. HDOCK server to perform protein-protein docking.

2.2. Preparation Stage

2.2.1. Ligands and Receptor Preparation

The 3-dimensional structures of astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrizhol compounds were prepared with the Pyrx application. The compound preparation was optimized through the open babel section until it was visible in pdbqt format. Preparation of the 3-dimensional structure of comparator ligand ipilimumab and Cytotoxic T Lymphocyte

Associated Protein 4 (CTLA-4) receptor was carried out by separating the two and adding polar hydrogen to the receptor using the Discovery Studio program.

2.3. Analysis Stage

2.3.1. Lipinski's Rule of Five Analysis

The compounds obtained can be examined in physicochemical tests with Lipinski's Rule of Five. The website to do the analysis is https://www.scfbioiitd.res.in/software/drugdesign/lipinski.jsp. The compounds examined are inputted in SDF or PDB format at normal pH (7) and then submitted.

2.3.2. Docking Ligand to Receptor

Docking the comparator ligand ipilimumab to CTLA-4 via protein-protein docking using the HDOCK server. When docking the test ligand to CTLA-4, PyRx-Autodock Vina software was used with a customized grid box on the active side of the receptor. Docking of molecules generates Gibbs free energy, which indicates the capacity of the ligand to bind to the receptor. The stronger the bond between the receptor and ligand, the lower the binding affinity value (Purwanto et al., 2021).

2.4. Interpretation Stage

2.4.1. RMSD Validation

The RMSD value is a validation parameter of the molecular docking method. The RMSD value reflects how much the surface interaction of the ligand in the structure varies before and after docking. In the docking simulation, the RMSD value ≤ 2.0 is used as a reference; if the RMSD value ≤ 2.0, it can proceed to the next stage; otherwise, it must be reconfigured (Nursanti et al., 2022).

2.4.2. Visualization of Molecular Docking

The visualization stage of the molecular docking process is used to determine the optimal configuration of the ligand with the receptor. The 3D visualization study was performed using the Discovery Studio application for the test ligand and Pymol for the comparison ligand docking. The parameters analyzed were amino acid residues and ligand-receptor interactions.

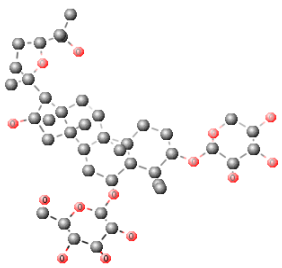
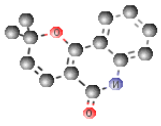
3. RESULTS AND DISCUSSION

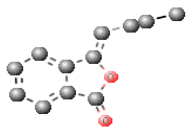
3.1. Ligands and Receptor Preparation

3.1.1. Test Ligands Preparation

The structures of the active herbal compounds astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrizhol were prepared in 3-dimensional form. The results of the preparation of active herbal compounds are shown in **Table 1.**

Table 1. Test Ligands 3D Structure

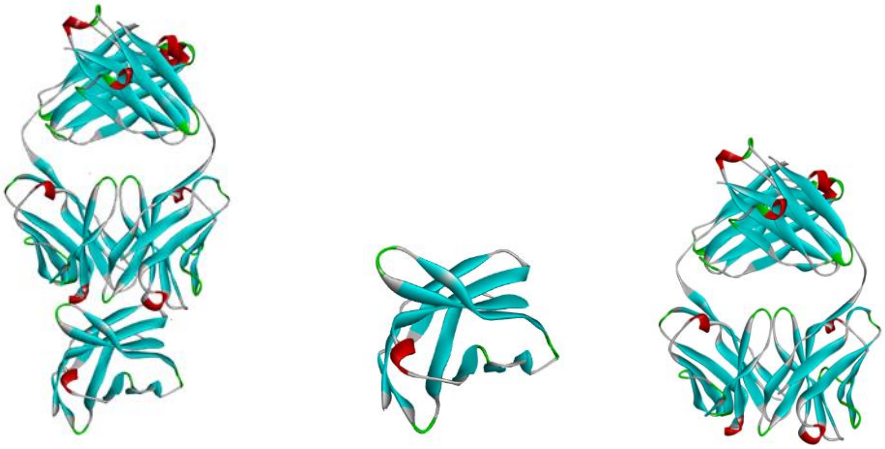
Test Ligands	CID	3D Structure
Astragaloside IV ($C_{41}H_{68}O_{14}$)	13943297	
Flindersine ($C_{14}H_{13}O_2$)	68230	

N-Butylidenephthalide ($C_{12}H_{12}O_2$)	642376	
Xanthorrhizol ($C_{15}H_{22}O$)	93135	

3.1.2. Comparator Ligand and Receptor Preparation

The structure of the ipilimumab-CTLA4 complex was downloaded through the PDB on the crystal structure with the code 6RP8. The structure with code 6RP8 has the classification of immune system and homo sapiens organism. This structure has three protein sub-units with ligands bound by X-ray diffraction results at a large enough resolution distance (2.60 Å). The 6RP8 identify structure was chosen because the CTLA-4 receptor already binds to a therapeutic ligand, ipilimumab, which was the first anti CTLA-4 drug developed.

The Cytotoxic T Lymphocyte Associated Protein 4 (CTLA-4) receptor structure is separated from the comparative ligand ipilimumab by removing the solvent, preferably water, so that the ligand and receptor do not interfere during the docking process. In the CTLA-4 receptor that has been separated, hydrogen atoms are added to adjust the docking atmosphere to approach the atmosphere in the body at pH 7 (Harir, 2022; Sari et al., 2020). Then, each structure of the comparator ligand and receptor was stored separately (PDB format). The preparation of ipilimumab and CTLA-4 is shown in **Figure 1.**



(a) (b) (c)

Figure 1. (a) Complex CTLA4-Ipilimumab,
(b) CTLA-4 Receptor, (c) Comparator Ipilimumab

3.2. Analysis Stage

3.2.1. Lipinski's Rule of Five Analysis

To evaluate the absorption character of a substance in the body, a ligand that has been produced in three-dimensional structure is tested physicochemically using Lipinski's Rule of Five. According to Lipinski's rule, the physicochemical prerequisites of a molecule are five: molecular weight < 500 Da, number of hydrogen bond donors < 5, total hydrogen bond acceptors < 10, log P value < 5, and molar refractivity in its range of 40-130 (Lipinski, 2004).

Molecular weights greater than 500 g/mol cannot diffuse through the cell membrane. The H donor and H acceptor parameters illustrate how the greater the hydrogen bonding capacity, the greater the energy required for the sorption process. Negative log P values are also

undesirable because molecules cannot cross the lipid bilayer membrane (Rukmono et al., 2019). The polarizability of a pharmaceutical molecule is measured by its molar refractivity. In general, Lipinski's rule characterizes a substance's ability to infiltrate the cell membrane through passive diffusion (Alfathin et al., 2021).

Analysis of the Lipinski test showed that the compounds flindersine, n-butylidenephthalide, and xanthorrhizol fit the criteria. The astragaloside IV compound, on the other hand, does not fit the Lipinski criteria because it has a mass or molecular weight of 784 (> 500 Da), hydrogen bond acceptor of 14 (> 10), and MR of 182.72 (> 130). The results of Lipinski's five-rule test are shown in **Table 2.**

Table 2. Lipinski's Rule of Five Analysis

Test Ligands	Parameters					Qualification
	Mass	H Bond Donors	H Bond Acceptors	Log P Value	Molar Refractivity	
Astragaloside IV	784	1	14	1,94	182,72	Not Qualified
Flindersine	227	1	3	2,71	66,29	Qualified
N-Butylidenephthalide	118	0	2	2,99	54,74	Qualified
Xanthorrhizol	218	1	1	4,6	69,92	Qualified

3.2.2. Docking Ligand to Receptor

PyRx-Autodock software was used to tether the test ligands astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrizhol. The grid box was determined to ensure that the docking molecule corresponds to the active site of the receptor used (Hasan et al., 2022). Determination of the grid box on the molecular docking of astragaloside IV, flindersine, n-butylidenephthalide, and xanthrorrhizol compounds to the Cytotoxic T Lymphocyte Associated Protein 4 (CTLA-4) receptor was adjusted at the center coordinate x = -2.9520; y = -2.3046; and z = -90.4265 and in dimensions (Angstrom) x = 39.7028; y = 25.0000; and z = 48.7110.

The molecular docking process of active herbal compounds to CTLA-4 receptors is carried out through PyRx-Autodock. After the molecular docking is completed, the affinity energy is obtained. The molecular docking performed on the CTLA-4 receptor, both the test ligand and comparator ligand, resulted in affinity energy displayed in **Table 3**.

Table 3. Docking Affinity Energy

Ligands	Affinity Energy (kcal/mol)
Ipilimumab	-314,38
Astragaloside IV	-7,3
Flindersine	-5,7
N-Butylidenephthalide	-5,0
Xanthorrhizol	-4,9

When the test and comparison ligands are molecularly tethered to the CTLA-4 receptor, various bonding conformations are formed, from which one has the lowest binding energy value is chosen. The ligand is more stable and the reaction is more spontaneous when the free energy of Gibbs is negative. According to the given data, all of ligand-CTLA-4 receptor interactions have an affinity energy of < 0. If the Gibbs index value is < 0, more spontaneous binding occurs (Sinurat et al., 2021).

The binding results for the comparison ligand were significantly different from the test ligand as this is a protein-protein binding, The docking of the ipilimumab comparator ligand to the CTLA-4 receptor through the HDOCK server is necessary because the ligand and receptor molecules are protein forms, thus requiring the use of a program capable of performing protein-protein docking. However, it was stated that since the score has not been calibrated with experimental data, it cannot be considered as the true binding affinity (Yan et al., 2020).

3.3. Interpretation Stage

3.3.1. RMSD Validation

Validation of the RMSD of the ligand against the receptor was performed as a parameter of the deviation of the docking result pose distance compared to the 3D pose of the target ligand (PDB code structure) calculated and visualized using an application for molecular graphics visualization. The value is said to be qualified because it is ≤ 2,0 Å (Nursanti et al., 2022). The RMSD value obtained for all test ligands astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrizhol was 0, while the RMSD of the comparator ligand ipilimumab was 1.34. The following RMSD validation values of the comparator ligand and test ligand against the CTLA-4 receptor are shown in **Table 4.**

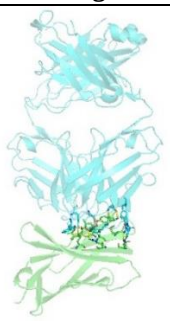
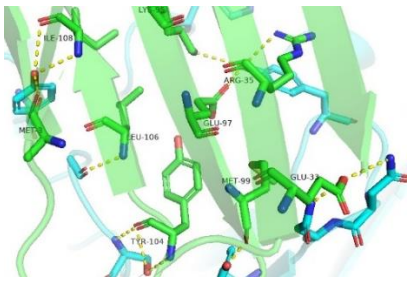
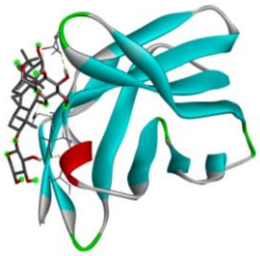
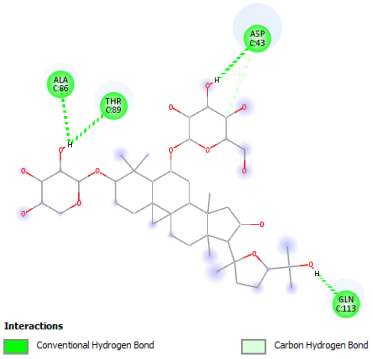
Table 4. RMSD Validation

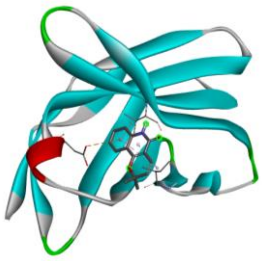
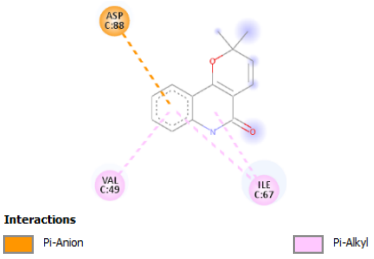
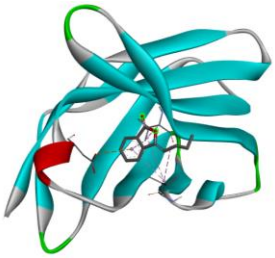
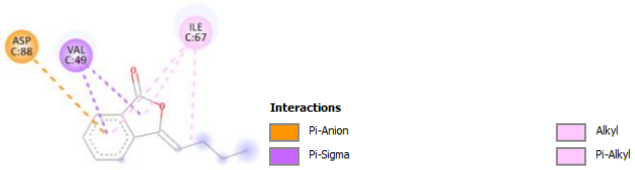
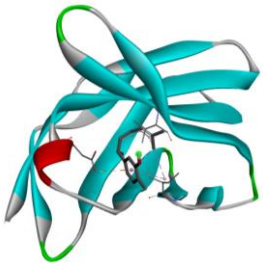
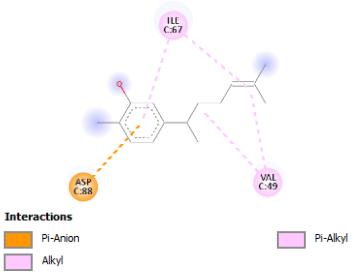
Ligands	RMSD Value (Å)	Qualification (≤ 2,0 Å)
Ipilimumab	1,34	Qualified
Astragaloside IV	0	Qualified
Flindersine	0	Qualified
N-Butylidenephthalide	0	Qualified
Xanthorrhizol	0	Qualified

3.3.2. Visualization of Molecular Docking

Visualization of molecular docking aims to see the similarity of amino acid residues and ligand-receptor interactions in test ligands with comparator ligands. Visualization of the test ligands of the active herbal compounds astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrizhol using the Discovery Studio program and can be seen the bonds that occur in 2D. The following visualization results of ligand molecular docking to CTLA-4 receptors are shown in **Table 5.**

Table 5. Visualization of Molecular Docking

Ligands	Binding Site	Ligand-Receptor Interaction
Ipilimumab		
Astragaloside IV		

Flindersine		
N-Butylidenephthalide		
Xanthorrhizol		

Based on the visualization table above, the docking regions and ligand-receptor interactions produced are different. The interactions that occur are conventional hydrogen bonds, carbon-hydrogen, alkyl, pi-alkyl, pi-sigma, pi-anion, and unbound bonds. The chemical bonds that occur show the characteristics of the ligand in binding to receptors in the body.

Hydrogen bonds have the power to bind to receptors and can also be unbonded after bonding and the chemical reactions involved. Conventional hydrogen bonding is a subset of hydrogen bonding. The value of hydrogen bond donors and acceptors is related to the biological activity of a drug molecule. Carbon hydrogen bond interaction is considered as a weaker hydrogen bond where the donor is a polarized carbon atom. Carbon atoms are considered donors if they are in acetylene groups or adjacent to oxygen or nitrogen atoms (Gómez-Jeria et al., 2020).

Unbound interactions are events between atoms that are not connected by covalent bonds (Waidyasooriya et al., 2017). Pi-anion interactions are referred to as favorable non-covalent contact bonds between electron-deficient aromatic systems (π-acids) and anions (Schottel et al., 2008). Hydrophobic bonds that play a role in stabilizing drug-receptor complexes include alkyl, pi-alkyl, and pi-sigma bonds (Gómez-Jeria et al., 2020).

Hydrophobic bonding is the mechanism of connecting non-polar sections of drug molecules with the non-polar areas of biological receptors, increasing entropy and resulting in a decrease in free energy, which stabilizes the drug-receptor complex (Rollando, 2017; Siswandono, 2016). The docking of compound molecules to receptors results in affinity energy, chemical bonds and amino acid residues. The amino acid residues in ligand-receptor interactions are shown in **Table 6**.

Table 6. Ligand-Receptor Interaction

Ligands	Amino Acid Residues		Ligand-Receptor Interaction
Ipilimumab	TYR	104	Hydrogen Bond
	ARG	35	Unbound Interaction
	MET	3	Hydrogen Bond
	MET	99	Hydrogen Bond
	GLU	33	Hydrogen Bond
	GLU	97	Hydrogen Bond
	LEU	106	Hydrogen Bond
	ILE	108	Hydrogen Bond
	LYS	95	Hydrogen Bond
Astragaloside IV	ALA	86	Hydrogen Bond
	THR	89	Hydrogen Bond
	ASP	43	Hydrogen Bond and Carbon Hydrogen Bond
	GLN	113	Hydrogen Bond
Flindersine	ASP	88	Pi-Anion Bond
	VAL	49	Pi-Alkyl Bond
	ILE	67	Pi-Alkyl Bond
N-Butylidenephthalide	ASP	88	Pi-Anion Bond
	VAL	49	Pi-Alkyl Bond
	ILE	67	Pi-Alkyl Bond
Xanthorrhizol	ASP	88	Pi-Anion Bond
	VAL	49	Pi-Alkyl Bond
	ILE	67	Pi-Alkyl Bond

Table 6 reveals that when astragaloside IV, flindersine, n-butylidenephthalide, and xanthrorrhizol test ligands bind with CTLA-4 receptors, they do not yield the same amino acid residues as when ipilimumab ligands interact with CTLA-4 receptors. The amino acid interaction allows for connection between the substance that binds and its receptor, resulting in inhibitory activity. The binding site is the region of protein that binds to the ligand and affects the protein's conformation and function. Binding sites provide the amino acid residues which have an important role in creating interactions among macromolecules and ligands (Sari et al., 2020).

The results of the molecular docking study of the test ligands astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrhizol against the CTLA-4 receptor demonstrate that the test ligand compounds bind to the same binding sites. The interaction of amino acid residues are different than ipilimumab. However, the binding site of CTLA-4 enclosed by the active compound. This suggests that the active compounds can bind to the CTLA-4 receptor. However, it remains unknown if the various binding sites will inhibit CTLA-4 interaction with CD80 and CD86 just like the comparator ligand ipilimumab, that enhancing the immune system's potential to suppress cancer progression.

4. CONCLUSION

The active herbal compounds astragaloside IV, flindersine, n-butylidenephthalide, and xanthorrhizol showed interaction with CTLA-4 receptor. Molecular docking towards CTLA-4 receptors creates different bond energies with the highest result in astragaloside IV compound, which is -7.3 kcal/mol. The interaction that occurs between the active compounds of herbal plants and CTLA-4 receptor does not produce the same amino acid residue similarity with the comparator ligand ipilimumab due to differences in binding area.

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6. CONFLICT OF INTEREST

All authors declared that there was no conflict of interest.

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