In-silico Molecular and Dynamic Analysis of Biomarkers Against Cordycepin from *Cordyceps militaris* in Colorectal Cancer

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Abstract. Colorectal cancer (CRC) is notoriously known as the third most common cancer worldwide, and the fourth common cause of death caused by cancer with 700,000 deaths per year. CRC incidence rates were observed to be rising in developing countries including Malaysia, as it was reflected by increased prevalence of risk factors for CRC that are associated with westernization such as unhealthy diet, obesity and smoking prevalence. The fungus family Cordyceps spp. has long been explored in the Traditional Chinese Medicine (TCM) as food, tonic and folk medicine to treat diseases ranging from malaria to cancer. Cordycepin, an active component in *Cordyceps militaris* were shown to have the anticancer and antimetastatic effect related to its adenosine and its derivatives. In current study, cordycepin inhibitory property against several CRC biomarkers was explored *in-silico*. Molecular docking and dynamics study of cordycepin against 6 important CRC biomarkers, namely caspase-3, caspase-8, COX-2, IL-2 and IL-6 were performed and its affinity was compared with obatoclax, a Phase II clinical trial antitumor drug which induces apoptosis in cancer cells by functioning as an inhibitor for Bcl-2 family proteins. The result shows that cordycepin is able to act as inhibitors for the selected CRC biomarkers, with equivalent or higher affinity compared to obatoclax. The *in-silico* prediction study provides a screening platform for the development of anti-CRC drug based on the Cordyceps spp., and in addition, provides a protocol to minimize the laboratory toxicological hazard and promotes the application of green chemistry computing in the drug discovery research.

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INTRODUCTION

Colorectal cancer (CRC) has been acknowledge as the third most common cancer worldwide, only behind lung and breast cancer [1] and is the fourth common cause of death caused by cancer with 700,000 deaths per year [2]. The CRC incidence rates is rising worldwide, and the trend is visible especially in the developing countries including Malaysia [3]. This was reflected by the increased prevalence of risk factors for CRC that are associated with westernization such as unhealthy diet, obesity and smoking prevalence [4]. Most colon tumors develop via a multistep process involving a series of histological, morphological, and genetic changes that accumulate over time [5].

Since the old days, *Cordyceps militaris* has been widely used all over the world and highly prized for its medicinal properties [6]. Identified as a species of fungus in the family Cordycipitaceae, it is known to parasitized insects although it can be cultivated in a variety of media including silkworm pupae, rice or chemically synthesized media [7][8]. Cordyceps has long been associated with Traditional Chinese Medicine (TCM), where it has been associated with over-the-counter medicine/tonics which advertised as Chinese herbs with anti-aging, ‘‘pro-sexual”, anti-cancer and immune boosting effects [9]. However, cordyceps has officially classified as a drug in the Chinese Pharmacopoeia since 1964 [10]. Therefore, its effectiveness against respiratory, renal, liver, nervous system and cardiovascular diseases, and also tumours, aging, hypo-sexuality and hyperlipidemia has scientifically explored in the recent years [11][12].

Various pure compounds and extracts has been identified from cordyceps spp., and among the highly studied are of *Cordyceps militaris*, *Ophiocordyceps sinensis* and *Ophiocordyceps nutans*. The compounds elucidated from cordyceps spp. includes metabolites such as cordycepin, adenosine and hypoxanthine, and also various functional enzymes such as dismutase and proteases [13]. Differential adenosine (0.18% vs. 0.06%) and cordycepin (0.97% vs. 0.36%) contents between the fruiting body and the corpus highlight intriguing variations in Cordyceps spp., presenting a compelling subject for scientific inquiry [14]. Notably, Cordyceps spp. exhibit an extensive repertoire of biologically active compounds, including cordycepin, cordycepic acid, adenosine, exo-polysaccharides, vitamins, and enzymes, all of which hold immense potential for medicinal applications [14][15]. Of particular interest is cordycepin, also known as 3’-deoxyadenosine, derived from the ascomycetes fungus *C. militaris*. Its prominence as the primary active constituent, combined with its wide range of biological activities, renders it an attractive focal point for extensive investigation [16][17]. This comprehensive study aims to shed light on the pharmacological properties and potential therapeutic applications of cordycepin, offering valuable insights into the diverse and promising world of Cordyceps spp.

There are differences in adenosine (0.18 and 0.06 %) and cordycepin (0.97 and 0.36 %) contents between the fruiting body and the corpus, respectively [14]. Cordyceps spp. produce many biologically active compounds like cordycepin, cordycepic acid, adenosine, exo-polysaccharides, vitamins, enzymes etc. [14][15]. Out of these, cordycepin, i.e. 3’-deoxyadenosine isolated from ascomycetes fungus *C. militaris* is the main active constituent which is most widely studied for its medicinal value having a broad spectrum biological activity [16][17]. Therefore, a lot of approach has been taken to isolate cordycepin from cordyceps spp. including the genetic improvements for better cultivation yield [18] and the bioseparation processes to improve the cordycepin extraction yield [19][20].

An extensive efficacy study of compounds isolated from cordyceps spp. including *Cordyceps militaris* and its immunostimulatory and immunomodulatory potential, which eventually exhibit the anti-tumor properties has been explored [21]. The extract and its bioactive compounds have been associated with cytokine production such as interleukins (IL-1β, IL-2, IL-6, IL-8, IL-10 and IL-12), tumor necrosis factors (TNF-α), phagocytosis stimulation and the stimulation of inflammatory response via mitogen-activated protein kinase (MAPK) pathway [21]. The activation of innate immune system was shown to be responsible for the significant progress against malignant melanoma and non-small cell lung cancer [22]. However, its activation and resistance against CRC is yet to be visible [22], although some progress has been made [23]. The innate immune system biomarkers also been associated with the progression of CRC [24] and carcinogenesis such as interleukin-2 (IL-2), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), caspase-3 and caspase-8 which inhibits the apoptosis in the cancer cells [25][26].

In current study, the affinity of cordycepin, the metabolite that is highly abundant in cordyceps is studied in-silico against the crucial CRC targets of interleukin-2 (IL-2), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), caspase-3 and caspase-8. Here, molecular docking was performed and followed by molecular dynamics simulation for 10 ns to observe the stability of each cordycepin-protein complex. Obatoclax, the Phase II clinical trial antitumor drug which induces apoptosis in cancer cells by functioning as an inhibitor for Bcl-2 family proteins and also target the antiapoptotic protein [27] was employed as the control. The biomarkers targeted affected the immunomodulatory

response against carcinogenesis thus determine the severity of the CRC at each stage. Therefore, the inhibition of each studied biomarkers is crucial to dampen the growth of CRC in patients.

METHODOLOGY

Physicochemical Properties Analysis

The physicochemical property of cordycepin and obatoclax was analyzed by using Molinspiration web-server tool (https://www.molinspiration.com/), together with the bioactivity of both compounds. Molinspiration provides the parameters, such as log p, molecular weight (MW), and number of hydrogen bond donors (HBDs), hydrogen bond acceptors (HBAs), and rotatable bonds (Rot B). These physicochemical properties examined is also known as Lipinski's Rule of Five (RO5) used in the design of drug molecules for potential of drug-likeness and oral availability [28].

Molecular Docking

Each target structure IL-2 (PDB ID: 1Z92), IL-6 (1IL6), COX-2 (4COX), caspase-3 (1QX3) and caspase-8 (1IBC) were obtained from RCSB Protein Data Bank, respectively. The 3D structure of cordycepin and obatoclax were obtained from PubChem and minimized using Swiss-PdbViewer employing the the GROMOS 43B1 force field [29]. Molecular docking of the cordycepin and obatoclax on each target was performed by AutoDock Vina (The Scripps Research Institute, US). Hydrogen atoms, both polar and non-polar, were meticulously incorporated into each protein through the implementation of AutoDock Tools (http://mgltools.scripps.edu) [30]. A grid box comprising 40 x 40 x 40 points was meticulously positioned around the active binding site of each protein, as previously determined in a seminal study [26]. This strategic arrangement ensured comprehensive coverage of the protein epitope during the docking process. The protein targets were constrained in a rigid manner, whereas the bonds of cordycepin and obatoclax were allowed to be rotatable during the docking simulations. The molecular docking study between each protein biomarker and cordycepin/obatoclax was performed using AutoDock Vina, a powerful tool that integrates the benefits of knowledge-based potentials and empirical scoring functions. By harnessing information from both the conformational preferences of the receptor-ligand complexes and the experimental affinity measurements, AutoDock Vina provides a robust framework for accurate and comprehensive ligand-binding predictions [31]. The most favorable conformation of each protein-ligand complex was identified by selecting the complex with the lowest docked energy, signifying the most promising binding configuration. To elucidate the molecular interactions between each protein and cordycepin/obatoclax, including hydrogen bonding and bond lengths, comprehensive analyses were performed using PyMOL (ver. 1.7.0.0-1, Schrödinger LLC), Discovery Studio Visualizer (ver. 16.1.0.15350, BIOVIA), and VMD (ver. 1.9.3, University of Illinois). These sophisticated visualization and analytical tools collectively allowed for a thorough examination of the intricate binding patterns and structural characteristics at the atomic level.

Molecular Dynamics (MD) Simulation

The GROMACS 2020 software was employed to conduct Molecular Dynamics (MD) simulations of the complexes, utilizing the GROMOS54a7 force field [32][33]. The force field demonstrated consistency with a single cutoff [32]. Individual molecular dynamics simulations were conducted for each cordycepin-protein and obatoclax-protein complex. For the molecular dynamics (MD) simulations, each complex was solvated in a cubic box, with a distance of 1.2 nm maintained between the complex and each side of the solvated box. Additionally, sodium and chloride ions were added to neutralize the overall charge of the system [34]. After solvation, the complex underwent energy minimization using the steepest descent algorithm. The simulation was conducted at room temperature (300 K) and atmospheric pressure (1 bar) to replicate typical experimental conditions [34]. The NVT thermal equilibration was performed using a constrained structure and a GROMACS-specific velocity rescale thermostat. Subsequently, NPT pressure equilibration was applied, employing the same velocity-rescale temperature coupling, along with the Parrinello−Rahman pressure coupling. The system, which was fully equilibrated in terms of temperature and pressure, was utilized as the starting point for the MD production dynamic analysis after being employed as the minimization step for the complex. All simulations were conducted for for 10 ns using a 2 fs time step. The results then analysed using common GROMACS functions RMSD and RMSF, while the formation of the intermolecular hydrogen bonds

in the The fully temperature and pressure equilibrated system was treated as the minimization step for the complex and used as the initial configuration for the MD production dynamic analysis complex were analyzed using ‘gmx_hbond’ function. The 'gmx_pairdist' function was utilized to measure the intermolecular distance between each protein and its corresponding cordycepin/obatoclax ligand.

Molecular Mechanics/Poisson-Boltzmann Surface Analysis (MM/PBSA)

The binding free energy between each protein and both cordycepin and obatoclax was computed using the 'g_mmpbsa' function in GROMACS, which employs the MM/PBSA method [35]. A selection of 21 snapshots, extracted from the trajectories within the 1-10 ns simulation timeframe, was used for the MMPBSA binding energy calculations.

RESULTS & DISCUSSIONS

Molinspiration Physicochemical and Bioactivity

Molinspiration offers a comprehensive online platform for ADME-Tox prediction, which involves calculating essential molecular properties, including logP, polar surface area, and the number of hydrogen bond donors and acceptors. These properties are utilized to determine Lipinski's Rule of Five (RO5). Molinspiration further facilitates the analysis by providing its own prediction for the bioactivity score of crucial drug targets, such as GPCR ligands, kinase inhibitors, ion channel modulators, and nuclear receptors [36]. The evaluation of Molinspiration parameters revealed no violations for the chosen ligands, namely cordycepin from Cordyceps militaris, indicating its similarity with the drug candidate obatoclax. Table 1 shows the 2D and 3D structure of each cordycepin and obatoclax. While cordycepin contains adenosine group and hydroxyl group which increase the polarity of the structure, obatoclax has more cyclic carbon ring and lesser polar groups. Therefore, the octanol-water coefficient of obatoclax is significantly higher compared to cordycepin, which eventually will affect its absorption and permeation into tissues. The Total Polar Surface Area (TPSA) value of cordycepin and obatoclax are both lower than 140 Å, suggesting its higher absorption and promising oral bioavailability [36]. While both molecules obey the criteria for Lipinski RO5 as in Table 2(a), the molecular weight and volume of cordycepin is significantly lower than obatoclax, which increase its absorption rate [37]. Table 2(b) shows the prediction of bioavailability for both compounds. Based on the findings, cordycepin exhibits superior characteristics as a GPCR ligand, ion channel modulator, kinase inhibitor, and enzyme inhibitor compared to obatoclax [38].

TABLE 1. The 2D and 3D structures, along with the SMILES format, of cordycepin from C. militaris and obatoclax.

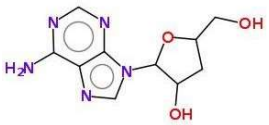
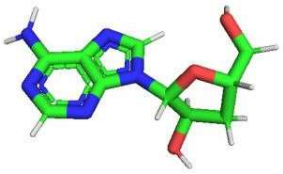
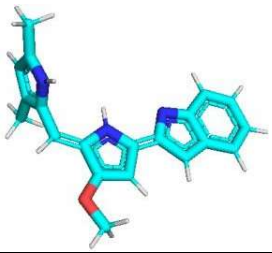
Compounds	2D	3D	SMILES
Cordycepin			C1C(OC(C1O)N2C=NC3=C(N=CN=C32)N)CO
Obatoclax			CC1=CC(=C(N1)C=C2C(=CC(=C3C=C4C=CC=CC4=N3)N2)OC)C.CS(=O)(=O)O

TABLE 2(a). Molinspiration calculation of cordycepin and obatoclax

Compounds	Formula	Molecular weight	H-donor	H-acceptor	Mi LogP	Rot. Bond	N viol	TPSA	Vol.	N atom
Cordycepin	$C_{10}H_{13}N_5O_3$	251.25	4	8	-0.90	2	0	119.32	210.49	18
Obatoclax	$C_{21}H_{23}N_3O_4S$	317.39	2	4	4.58	2	0	53.71	295.33	24

TABLE 2(b). Molinspiration calculation of drug-likeness score for cordycepin and obatoclax

Compounds	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
Cordycepin	0.83	0.41	0.76	-1.65	-0.20	1.30
Obatoclax	-0.19	-0.41	0.32	-0.21	-0.47	-0.22

AutoDock Vina Docking

AutoDock Vina docking is an automated docking approach and considered one of the most important tools for structure-based drug design. It allows prediction of ligand binding poses and also provides an estimate of how well small molecules fit in the binding site of a protein [39]. Lower docking binding energy indicates tighter binding of the ligand against the targeted protein [40]. The lowest binding energy score of each cordycepin and obatoclax is recorded in Table 3. It shows that the docking of both compounds against COX-2 gave the lowest Vina binding energy value compared to other complexes, and overall, obatoclax gave lowest score compared to cordycepin. Figure 1 and Figure 2 shows the interaction of cordycepin and obatoclax on each target biomarker. The docking result from Table 3 shows reasonably low Vina binding energy score of both compounds, while both Figure 1 and 2 recorded multiple non-covalent interaction. The conventional hydrogen bond between polar atoms on both protein's amino acids residues and compound's atom contribute the most to the number of non-covalent interaction in each complexes. In addition, the pi-related interactions such as pi-pi stacking, pi- alkyl and also pi-amide interactions also majorly contributed to the stable docking position of each complex, due to the existence of carbon rings in each compound. However, the protein-ligand complex stability observed from the docking results are not conclusive, as after 2 ns of MD simulation obatoclax was observed to dissociates from its original docking position on caspase 8 (data not shown). Therefore, 10 ns MD simulation was performed on each protein-ligand complex.

TABLE 3. Autodock Vina binding energy docking score

Proteino	PDB ID	Ligand	Vina binding energy score (kcal/mol)
Caspase 3	1QX3	Cordycepin	-5.2
		Obatoclax	-7.7
Caspase 8	1IBC	Cordycepin	-5.2
		Obatoclax	-6.6
COX 2	4COX	Cordycepin	-7.7
		Obatoclax	-9.8
IL-2	1Z92	Cordycepin	-5.1
		Obatoclax	-6.8
IL-6	1IL6	Cordycepin	-6.2
		Obatoclax	-7.0

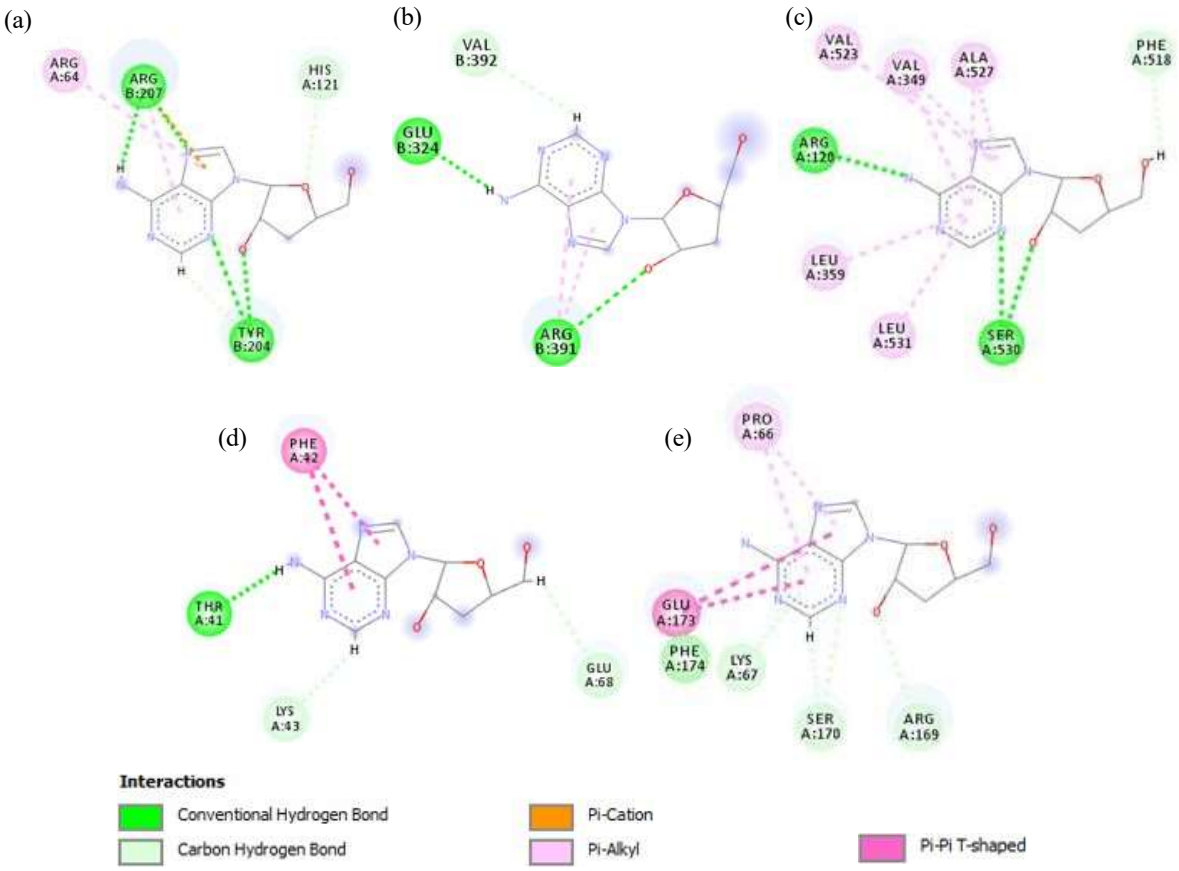


FIGURE 1. 2D representation of cordycepin docking against (a) caspase 3 (b) caspase 8 (c) COX-2 (d) interleukin-2 (e) interleukin-6. The type of interaction between the residues were labelled.

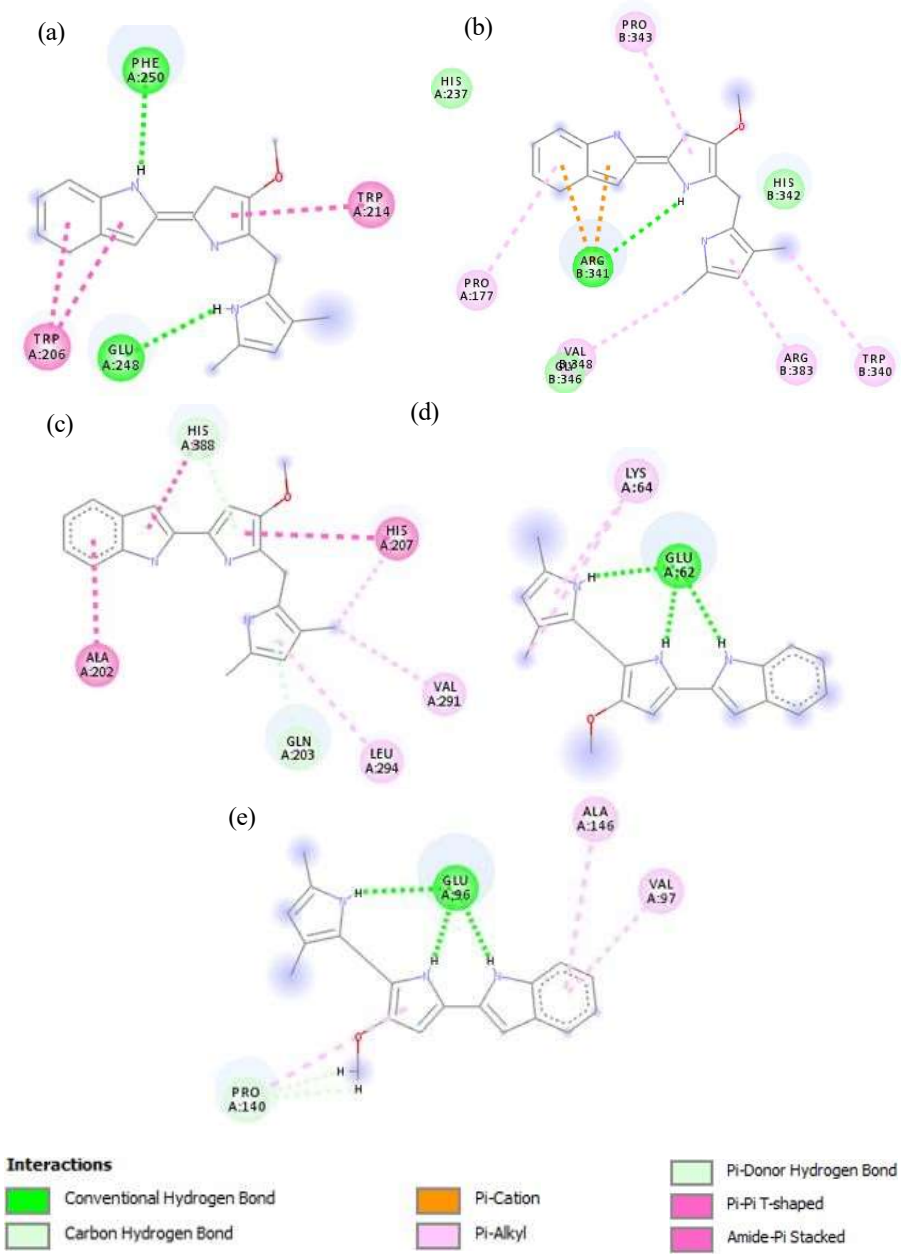


FIGURE 2. 2D representation of obatoclax docking against (a) caspase 3 (b) Caspase-8 (c) COX-2 (d) interleukin-2 (e) interleukin-6. The type of interaction between the residues were labelled. The interaction between obatoclax and caspase 8 was later dissociates after 4 ns of simulation.

Ten-nanoseconds MD simulation

Each docked complex was undergone 10 ns MD simulation to examine the stability of each docked complex on the targeted biomarker proteins. Although the simulation time were short, each protein backbones was shown to stabilized within the simulated time. As Figure 3 implies, the Root Means Square Deviation (RMSD) of C-α backbone of each complex shows that each protein has stabilized after 2 ns of simulation, although caspase-8 slightly fluctuates in comparison with other biomarkers. Here, it is important to note that although the caspase 8 backbone initially had

a higher fluctuation, cordycepin was managed to hold into the docking site position for the whole duration after 2 ns, thus given a stable complex. In addition, all protein complexes with cordycepin were shown to be stabilized with low fluctuations during the 10 ns compared to obatoclax. This observation is contributed by high number of conventional hydrogen bond formation between the biomarker and cordycepin ligand, as observable in Figure 4. The caspase 3 and cordycepin complex formed the most number of intermolecular hydrogen bond with average of 6 hydrogen bonds formed compared with obatoclax averaged of 2 hydrogen bonds. The pattern was similarly observed for caspase 8 and COX-2, while in interleukin 2 and interleukin 6, obatoclax form slightly higher intermolecular hydrogen bonds compared to cordycepin for the whole 10 ns duration. The stable hydrogen form is contributed by the Glu residues available in each biomarker, where the –OH functional group in Glu (residue Glu62 in interleukin 2 and Glu96 in interleukin 6) formed interactions with –NH rich obatoclax thus stabilized the position of the ligand.

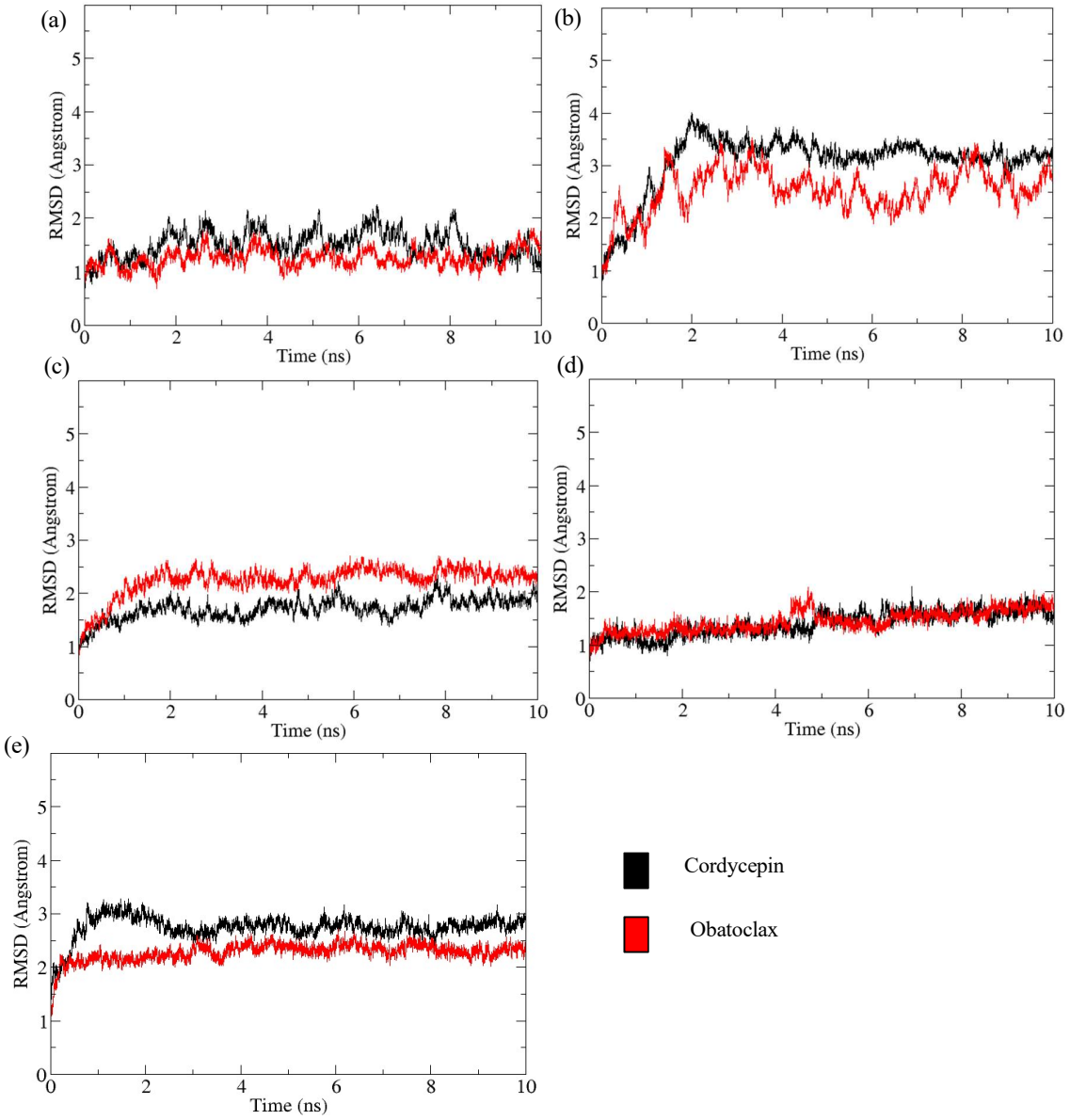


FIGURE 3. C-α RMSD of (a) caspase 3, (b) caspase 8, (c) COX-2, (d) interleukin-2 and (e) interleukin-6 complexes with cordycepin and obatoclax for 10 ns MD duration.

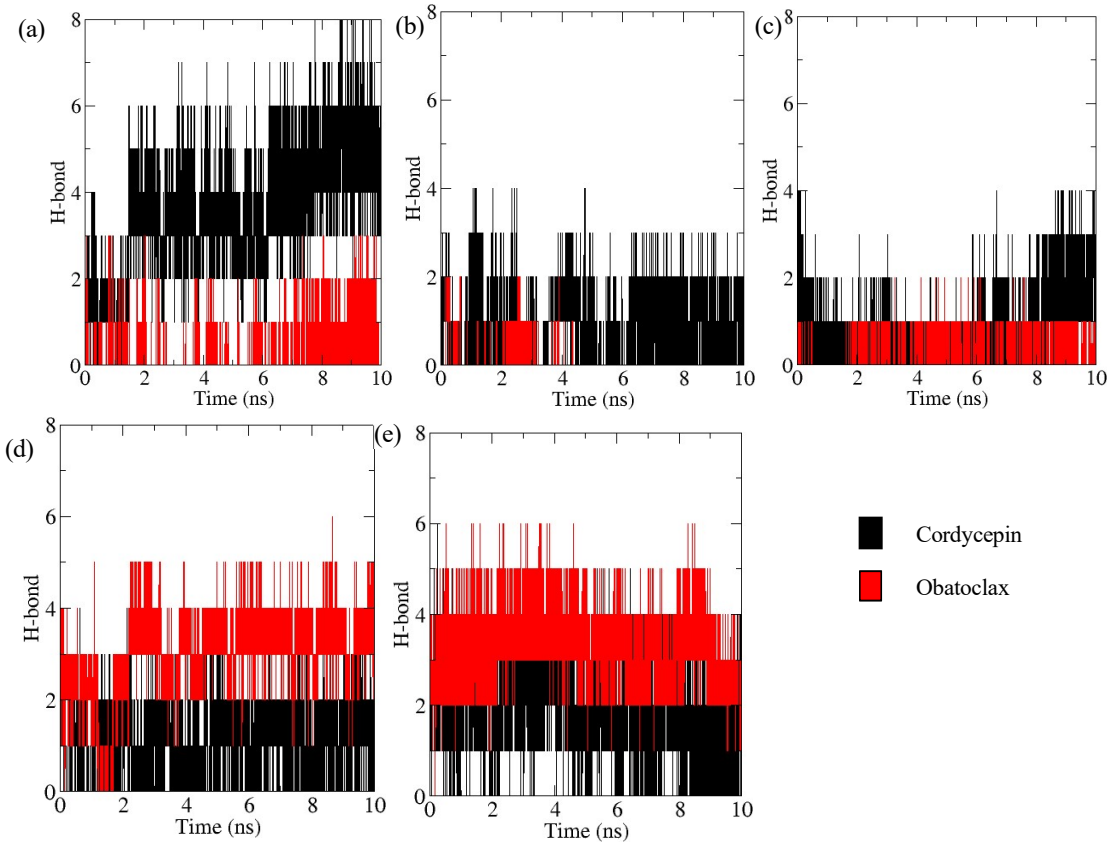


FIGURE 4. Hydrogen bond formation of (a) caspase 3, (b) caspase 8, (c) COX-2, (d) interleukin-2 and (e) interleukin-6 with cordycepin and obatoclax for 10 ns MD duration.

MM/PBSA Binding Energy

The MM/PBSA method has gained broad acceptance and is recognized as a dependable free energy simulation approach for elucidating protein-ligand binding interactions [41]. The free energy of a state, as described by Kollman and co-workers which is an integral part of this method is shown as [42];

$$\Delta G = \Delta E_{MM} + \Delta G_{PBSA} - T\Delta S_{MM} \qquad (1)$$

The method involves calculating the average free energy ΔG, which comprises the average molecular mechanics free energy ΔE_{MM}, the solvation free energy ΔG_{PBSA}, and $T\Delta S_{MM}$, representing the solute configuration entropy. The solute configuration entropy can be determined either through quasi-harmonic analysis of the trajectory or by normal mode is calculated using the numerical solution of the Poisson-Boltzmann equation, while the estimation of the nonpolar free energy is accomplished through a simple surface area term [43]. In the study, the complex binding energy was calculated at each 0.25 ns interval to closely monitored its changes for the last 5 ns from the overall 10 ns MD duration. The result shows that for all complexes, cordycepin formed a tighter complex with each protein compared to obatoclax, as per visualized in Figure 5. In addition, the stability of the protein-obatoclax complex also varies among the targeted proteins. Overall, cordycepin was shown to tightly bound to COX-2 enzyme (ave: -68.118 +/- 12.724 kJ/mol), followed by prozyme caspase 3 (ave: -52.691 +/- 15.886 kJ/mol) and caspase 8 (ave: -33.515 +/- 7.155 kJ/mol). The stable binding of cordycepin on COX-2 will inhibit the pro-inflammatory effect of the prostaglandins production cascade [44], while inhibition of prozyme caspase 3 and caspase 8 will silencing both caspases which function as the apoptosis regulators in the sequential "cell-death" mechanism of cells [45][46]. Eventually, the inhibition of both prozymes will promote the apoptosis or cell-death of cancerous cells in CRC treatment.

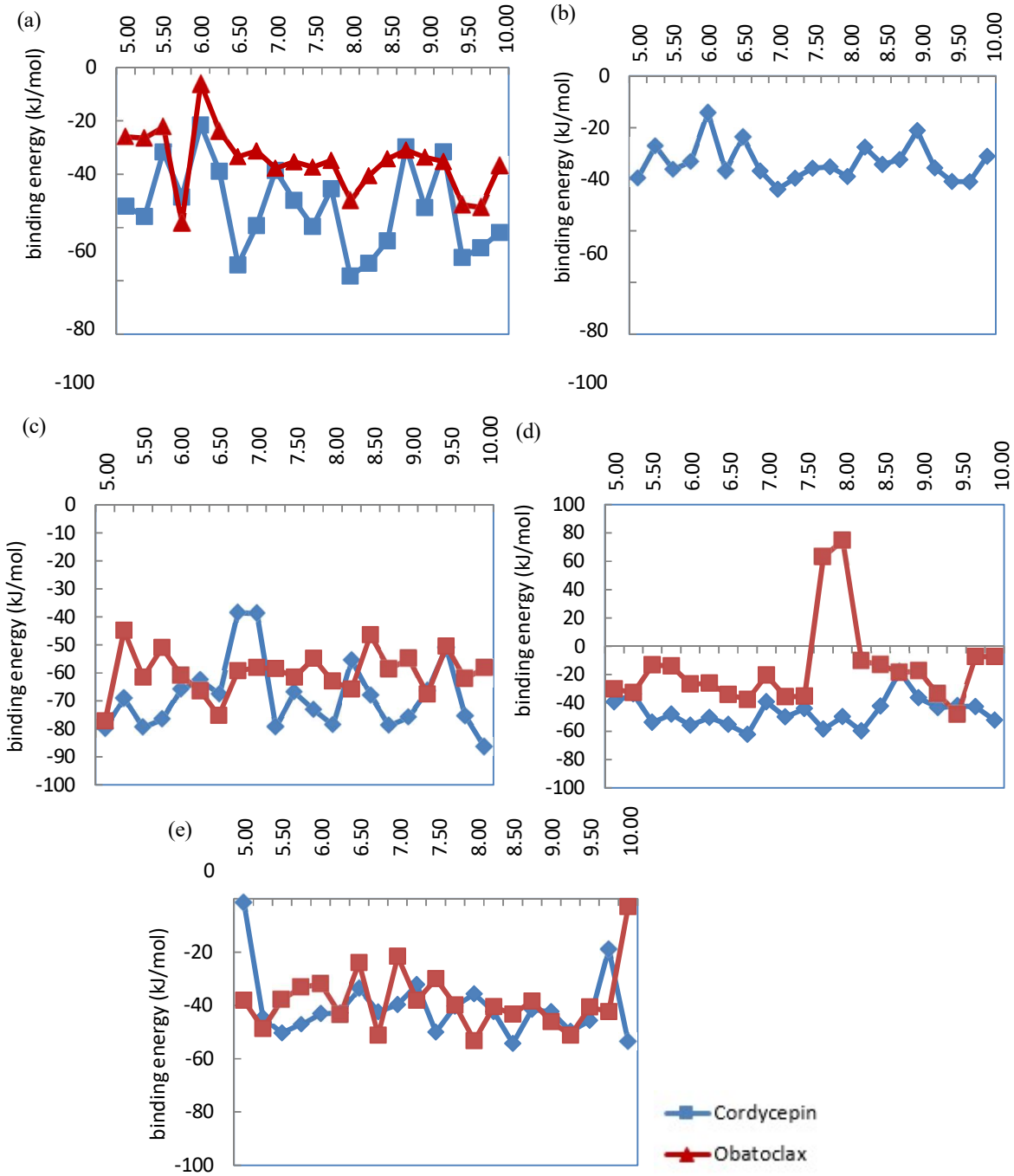


FIGURE 5. Total energy binding value of each complex with (a) caspase 3 (b) caspase 8 (c) COX-2 (d) Interleukin-2 (e) Interleukin-6 for the last 5 ns of simulation as per calculated using the MM/PBSA method. The interaction between obatoclax and caspase 8 was later dissociates after 4 ns of simulation, thus no data obtained for caspase 8 and Obatoclax complex.

CONCLUSION

Cordyceps spp. contains a wealth of bioactive compounds with numerous potential applications, particularly in the field of medicine. One such bioactive compound, cordycepin, is found in high concentrations in *Cordyceps militaris*, a highly sought-after species known for its traditional medicinal uses. Given its prominence, the potential of cordycepin in combating colorectal cancer (CRC), a major type of cancer, was investigated.

In this study, computational analyses were conducted to predict the ADME properties of cordycepin and compare them with those of obatoclax, a drug undergoing phase-2 trials, known for its high potential against CRC. Furthermore, molecular docking studies of cordycepin were employed to elucidate its probable molecular mechanism of action on specific targets that are biomarkers and targets for CRC. Our computational findings revealed favorable oral absorption, human albumin protein binding, and adherence to the Lipinski Rule of 5, suggesting that cordycepin could be stable in pharmaceutical formulations.

The molecular docking study indicated that cordycepin effectively bound to the active site of each biomarker. Molecular dynamics simulations further supported this observation by demonstrating the stability of the fundamental structure of the bestpose ligand-protein complexes throughout the simulation runs. The MM/PBSA analysis revealed varying stability of cordycepin on each docked protein, with the most stable binding observed on COX-2 and the prozyme caspases.

In conclusion, cordycepin exhibits promising potential as a therapeutic agent for CRC, with its favorable ADME properties and specific interactions with relevant biomarkers. As a next step, it is essential to explore the molecular mechanisms of action of both cordycepin and obatoclax in in-vitro and in-vivo settings, particularly focusing on IL-2, IL-6, COX-2, Caspase-3, and Caspase-8.

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