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DNA-Binding and Cytotoxic activities of Supercritical-CO_2 extracts of *Ganoderma lucidum* (Curt.:Fr.) P. Karst. collected from the Wild of Bukidnon Province, Philippines

Elumba Zeus S.[1*], Teves Franco G.[2] and Madamba Ma. Reina Suzette B.[2]
[1]Department of Biology, College of Arts and Sciences, Central Mindanao University, Musuan, Bukidnon, PHILIPPINES
[2]Dept. of Biological Sci., College of Sci. and Mathematics, Mindanao State University-Iligan Institute of Technology, Iligan City, PHILIPPINES

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Abstract

DNA-binding and cytotoxic natural products are of current interest for the discovery of new anticancer and/or antitumor drugs. Mushrooms in the wild such as Ganoderma lucidum (Curt.:Fr.) P. Karst. is a promising source of such bioactive natural products, thus it must be investigated for potential bioprospecting. In the present study, the bioactive compounds from the three supercritical-CO_2 (SC-CO_2) extracts of G. lucidum (Curt.:Fr.) P. Karst (GL1, GL2 and GL3) collected in wild of Bukidnon Province, Philippines were tested in their ability to bind to genomic DNA in vitro and their cytotoxicity in vivo using biomolecular-chemical screening and brine shrimp lethality assay (BSLA), respectively. Results revealed that the extracts from 10 (GL1) and 30 (GL3) MPa extraction pressures contained compounds that exhibit binding affinity towards Salmon sperm DNA as indicated by their Rf_2/Rf_1 ratios below 1. For BSLA, the LC_{50} (24h) values ranged from 31.62-89.12 µg/ml, indicate significant cytotoxic activity. Based on the results, the wild G. lucidum (Curt.:Fr.) P. Karst used could be a potential source of DNA-binding and cytotoxic compounds that can be exploited for the development of therapeutically-useful drugs for tumors and/or cancers.

Keywords: DNA-binding, cytotoxicity, Ganoderma lucidum, biomolecular-chemical screening, Brine shrimp lethality assay.

Introduction

Natural products from a wide range of plants, fungi and microorganisms are of significant interest because of the effects they can exert on other organisms. Some of these important biological activities are altering cellular processes by binding to genomic DNA and killing tumor cells by direct cytotoxicity. DNA-binding compounds are of exceptional importance in producing new effective drugs[1]. Today, DNA is the pharmacological target of most anticancer drugs. Over 60% of the clinical anticancer drugs introduced in 2002 are natural products or natural products derivatives, and most exert their effects by acting on genomic DNA[2]. On the other hand, the cytotoxicity of certain compounds may suggest its potential antitumor activity. In fact, cytotoxicity was found out to be consistent with antitumor activity[3].

The importance of these functional biological activities had led to the development of simple yet reliable assays such as biomolecular-chemical screening and brine shrimp lethality assay for detecting DNA-binding and cytotoxic activity, respectively[4,5]. These assays had been proven to be useful in natural products research for they can establish information on the biological activities of active principles from plants, fungi and microorganisms.

Interestingly, fungi represent an immense source of bioactive substances with wide array of medicinal properties that made them very potent natural supplements in cancer therapy[6]. *Ganoderma* is a genus of higher basidiomycetes and known as a prolific producer of novel mycochemicals[7]. *G. lucidum* (Curt.:Fr.) P. Karst. (reishi, Ling Zhi) in particular has been an economic species particularly in the Far East countries (China, Japan, Korea, etc.), for over 4000 years. The virtues of *G. lucidum* (Curt.:Fr.) P. Karst. extract handed down from generation to generation include as a "cancer cure" and a symbol of happy augury, good fortune, good health, longevity, and even immortality[8]. It has also been reported to have a number of pharmacological effects including antimutagenic[9], antioxidative[10], antifungal[11], antibacterial and antiviral[12] activities. Previous studies reported the presence of approximately 400 different bioactive compounds with anticancer or antitumor activities which mainly include triterpenoids and polysaccharides[13-15].

G. lucidum (Curt.:Fr.) P. Karst. is widely grown on a commercial scale due to high demands for its products. The successful cultivation of this valuable mushroom was achieved in the early 1970's and since 1980 the production of *G. lucidum* (Curt.:Fr.) P. Karst. has developed rapidly, particularly in China[8]. But since then, it seems that *G. lucidum* (Curt.:Fr.) P. Karst. in the wild became largely untapped because they are overshadowed by cultivated and other commercialized mushroom species. There are qualitative and quantitative differences in the chemical composition of *G. lucidum* (Curt.:Fr.) P. Karst. products depending on the strain, origin,

extracting process, and cultivation conditions[15]. Also fungi that grow in the wild are said to synthesize chemical compounds with stronger bioactivities that would serve as competitive weapons against their environmental competitors[16]. These chemical compounds could have important functional biological activities that can be exploited for the discovery of lead compounds for drug development. It is in this context that this present work was done in order to determine the DNA-binding and cytotoxic activities of the supercritical-CO_2 (SC-CO_2) extracts of *G. lucidum* (Curt.:Fr.) P. Karst. found in the wild ecosystems of Bukidnon Province, Philippines, employing the methods mentioned previously. To obtain a characteristic chemical fingerprint of the extracts, Fourier transform-infra red spectroscopy (FTIR) was carried out.

Material and Methods

Mushroom samples: Basidiocarps (fruit bodies) were used in this study because between 80 and 85% of all medicinal mushroom products are derived from basidiocarps[17]. Fresh basidiocarps of *G. lucidum* (Curt.:Fr.) P. Karst. (figure- 1) were collected from the secondary forests of Bukidnon Province, Philippines. Samples from the fields were placed in the sterile plastic cellophane and were brought to the laboratory. The identification of the samples were confirmed and authenticated through differentiating characteristics as described by previous study[18], and by comparing with voucher specimens of previous collections deposited in the Molecular Biology and Biotechnology and at the Natural Science Museum, both at Mindanao State University-Iligan Institute of Technology, Iligan City, Philippines.

Sample preparation and supercritical-CO_2 extraction: *G. lucidum* (Curt.:Fr.) P. Karst. basidiocarps were cut into pieces (≈1x1 mm) and air-dried to achieve the <14% moisture content as required for the SC-CO_2 extraction. The cut and dried basidiocarps were pulverized using a mechanical grinder. The pulverized basidiocarps were contained in the white cloth bag and then in the SC-CO_2 metal sample cartridge and subjected to non-modified SC-CO_2 extraction using a supercritical fluid extractor (Akico™). After stabilization for 5 minutes, extraction was followed using three (3) pressure levels; 10 MPa, 20 MPa and 30 MPa in 40°C water bath for 2h/pressure level and a CO_2 flow rate of 0.5 ml/minute, and the resulting extracts were labeled GL1, GL2, and GL3, respectively. The extracts were collected in 5 ml test tubes, sealed with laboratory film, wrapped with aluminum foil and stored in the refrigerator at 4°C until use. These extracts were solvent-free.

DNA-binding activity: The biomolecular-chemical screening by two-dimensional thin layer chromatography (2D TLC)[4,19] was adopted with slight modifications to determine the DNA-binding activity of the components of the extracts. Homogenized Salmon sperm DNA purchased from Chemline Scientific Enterprises (11 mg/ml) was used for the assay. DNA fragments are between 300-3000 base pairs in size as revealed by gel electrophoresis. For working solution, 181µl of DNA sample was withdrawn from the stock and diluted with 819µl sterile distilled water to have a concentration of 2 mg/ml. The DNA was denatured by heating at 95°C for 10 minutes and cooling on ice immediately after. This was stored at -20°C until its use in the assay. For 2D TLC, the TLC plates (TLC silica gel 60 F_{254} Merck) were cut into a 6.5 cm x 6.5 cm dimension. The extracts were spotted at one corner of the TLC plate using a non-heparinized capillary tube with 1 cm distance from the base and the side of the plate. Two plates were prepared for each extract, the measuring plate and the reference plate. For the first development, GL2 and GL3 were run in a solvent system constituting chloroform: ethyl acetate: methanol (55+35+10) and GL1 was run in a solvent system constituting chloroform: methanol (95+5). The separations produced in the first development were viewed under UV light at 254 nm. Interaction with DNA was analyzed in the second dimension in the solvent system constituting chloroform: methanol: glacial acetic acid (47.5+47.5+5). DNA was spotted in the measuring plate above the separations using non-heparinized capillary tube (5 µg DNA/cm) before the second development/chromatographic step. The solvent systems used for first and second developments were determined by several trials. Detection of DNA-binding was achieved by viewing the plates under UV light at 254 nm. Changes in the Rf (retention factor) values indicate an interaction between separated spot (ligand) and DNA and were expressed by the Rf_2/Rf_1 ratio, in which Rf_1 represents the Rf value without DNA (reference plate), and Rf_2 represents the Rf value with DNA (measuring plate). Rf_2/Rf_1 ratios below 1 indicate that DNA-binding occurs[4]. To eliminate the dilemma in classifying Rf_2/Rf_1 ratios that reached 1 if rounded off, the ratios that fall below 1 were categorized as to whether they had strong or weak affinity to DNA. Separated spots with Rf_2/Rf_1 ratios < 0.85 are considered moderate to strong DNA-binders and those spots with Rf_2/Rf_1 ratios > 0.85 are considered weak DNA-binders[20]. Figure- 2 shows a schematic depiction of the method.

Cytotoxic activity: Cytotoxic activity of the extracts was determined through BSLA[5, 21] with slight modifications. Brine shrimp eggs (*Artemia salina* Leach) were purchased from a local pet shop. The eggs were hatched in a small tank with two unequal-sized chambers filled with boiled and filtered sea water. One chamber was covered with aluminum foil and fully aerated. After 48 h incubation at room temperature and under illumination (18 W bulb), the resulting nauplii (larvae) were attracted to the other side of the tank with a light source and collected with Pasteur pipette. Samples for testing were prepared by initially dissolving 20 mg of each extract in 2 ml dimethyl sulfoxide (DMSO) to increase the solubility of the extracts and further diluted with sea water to produce the required concentrations. Appropriate amounts (200-, 20-, or 2-µL for 1000, 100, and 10 µg/mL, respectively) were transferred to vials. Ten 24-hour old nauplii were transferred to each sample vial, and each vial was filled with boiled, filtered sea water to make a final volume of 5 ml. Tests for each concentration were done in triplicates. A control experiment

containing 200-, 20- or 2- µL DMSO in 5 mL boiled, filtered sea water and ten nauplii was also performed in triplicates for each concentration. The vials were placed uncovered under 18 W fluorescent bulb for 24 h after which the survivors were counted and the percentage mortality at each vial and control was determined using the formula:

$$\% \text{ mortality} = (\text{no. of dead nauplii / initial no. of live nauplii}) \times 100$$

Graphical method of Probit analysis was used to calculate LC_{50} (concentration at which lethality to brine shrimps represents 50%)[22]. Extracts with LC_{50} values <100 µg/ml were considered significant[23,24].

Fourier transform infra-red spectroscopy: Spectrum 100 Fourier-transform infrared (FT-IR) spectrometer (Perkin-Elmer) was used for the functional group elucidation of extracts. Each sample was applied to the attenuated total reflectance (ATR) sample well and scanned at room temperature with a range of 4000-550 cm^{-1}. The resulting spectra were analyzed and functional groups were assigned to the functional group region, between 1300 cm^{-1} to 4000 cm^{-1}.

Figure- 1
***G. lucidum* (Curt.:Fr.) P. Karst. basidiocarps collected in Bukidnon Province, Philippines**

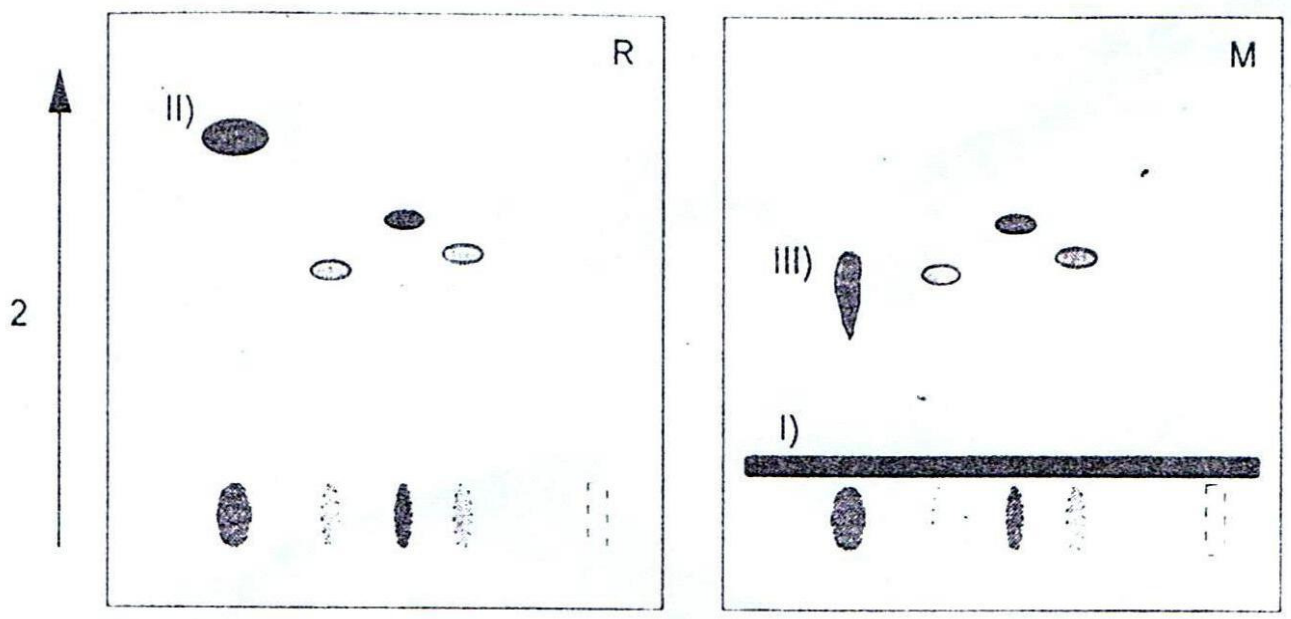

Figure- 2
Schematic depiction of 2D TLC analysis; M: measuring plate, R: reference plate, I: spotted DNA, II: chromatogram without DNA interaction, III: chromatogram with DNA interaction. Numbering of the arrows shows the sequence of TLC development[4]

Table- 1
DNA-binding behavior of natural products in the *G. lucidum* (Curt.:Fr.) P. Karst. SC-CO_2 extracts analyzed by 2D TLC-binding study

G. lucidum extracts	Number of separated spots in first development (1-D)	1-D TLC Rf value	2-D TLC Rf value		
			without DNA (Rf_1)	with DNA (Rf_2)	Rf_2/Rf_1 ratio[a]
GL1	6	0.20	*	*	-
		0.26	0.42	0.42	**1.00**
		0.34	0.66	0.66	**1.00**
		0.58	0.78	0.76	**0.97**
		0.66	0.96	0.90	**0.94**
		0.96	0.96	0.90	**0.94**
GL2	5	0.42	0.45	0.45	**1.00**
		0.52	0.62	0.62	**1.00**
		0.60	0.66	0.66	**1.00**
		0.70	0.75	0.75	**1.00**
		0.86	0.93	0.93	**1.00**
GL3	5	0.46	0.52	0.48	**0.92**
		0.63	0.62	0.62	**1.00**
		0.71	0.72	0.70	**0.97**
		0.83	0.84	0.80	**0.95**
		0.95	0.86	0.76	**0.88**

[a] Changes in Rf-values are expressed by the Rf_2/Rf_1-ratio in which Rf_1 represents the Rf-value without, and Rf_2 represents the Rf value with DNA. Rf_2/Rf_1 ratios below 1 indicate DNA-binding activity, *Spots were lost after the second development.

Table- 2
Results of BSLA on SC-CO_2 extracts of *G. lucidum* (Curt.:Fr.) P. Karst

Extracts	% mortality at different concentrations*			LC_{50}, 24 h
	10 µg/ml	100 µg/ml	1000 µg/ml	µg/ml
GL1	03.33	16.67	100.00	89.12
GL2	36.67	40.00	100.00	31.62
GL3	30.00	33.33	100.00	39.81
Negative control (DMSO)	00.00	00.00	00.00	00.00

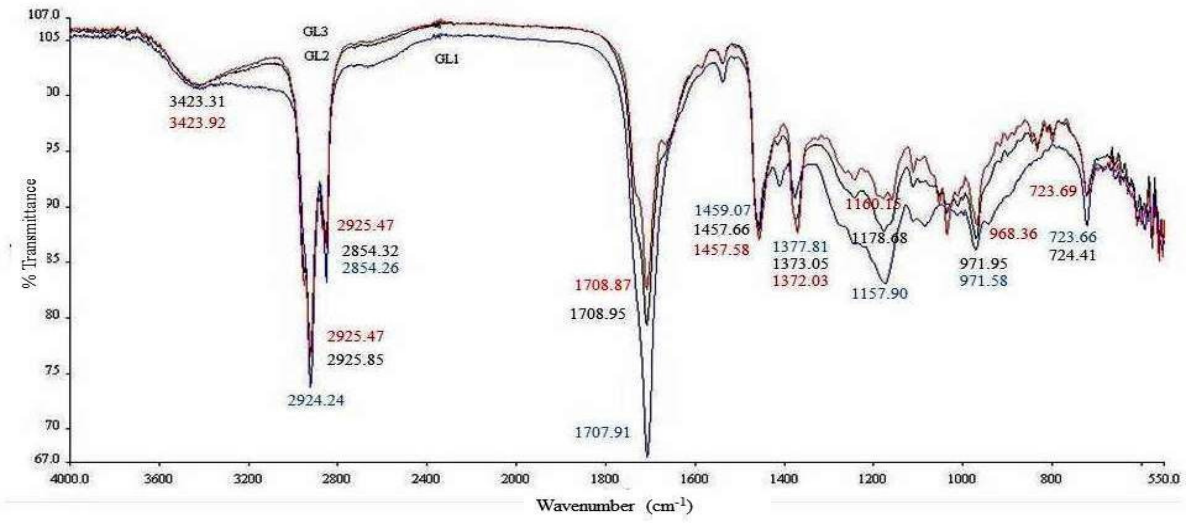

Figure- 2
FTIR spectra of the extracts: GL1 extracted at 10 MPa, GL2 extracted at 20 MPa, and GL3 extracted at 30 MPa. Peaks or spectra appearing at different band wavelength (in % transmittance) represent specific functional groups identified by their wave number (see text for the functional groups identified)

Results and Discussions

The basidiocarps of *G. lucidum* (Curt.:Fr.) P. Karst. collected in the wild ecosystems of Bukidnon Province, Philippines were subjected to non-modified SC-CO_2 extraction under three pressure levels (10 MPa, 20 MPa and 30 MPa), and the chemical components of the extracts were tested for DNA-binding and cytotoxic activities. Results revealed that the extracts from 10 MPa (GL1) and 30 MPa (GL2) applied pressures contained compounds with DNA-binding activity as shown by their Rf_2/Rf_1 ratios below 1 (Table- 1). The one dimensional TLC had successfully separated the chemical components of the extracts, however, some of which did not show affinity towards DNA such as in the extract from 20 MPa (GL2). Probably the 20 MPa extraction pressure selectively extracted compounds from *G. lucidum* (Curt.:Fr.) P. Karst. that naturally do not have DNA-binding activity. The observed DNA-binding compounds/ separated spots were considered as weak DNA-binders based on the criteria set by previous study[20]. However, this result is of great significance since DNA-binding compounds are of current importance for the discovery of new drugs or lead compounds. The fact that many anticancer, antibiotic, and antiviral drugs exert their primary biological effects by reversibly interacting with nucleic acids, findings of the present study is helpful for potential bioprospecting. Studies show that the mechanism of action of antitumor compounds is through DNA-binding. A good example is the Irofulven (MGI 114), a unique cytotoxic agent derived from the sesquiterpene mushroom metabolite illudin S. Irofulven undergoes rapid cellular uptake and covalently binds to DNA and protein targets and the binding interferes with DNA replication and cell division of tumor cells leading to tumor-specific apoptotic cell death[25]. Its is already known that fungi are sources of potent metabolites capable of penetrating cell membranes and interfering with particular signal transduction pathways linked to processes such as inflammation, cell differentiation and survival, carcinogenesis and metastasis[6]. Although the molecular mechanism of DNA-binding have not been investigated in the present study, the baseline information that DNA-binding compounds are present in *G. lucidum* (Curt.:Fr.) P. Karst. SC-CO_2 extracts as revealed by biomolecular-chemical screening is of great importance for this would serve as an impetus for further investigations on the DNA-binding compounds from this mushroom species and their DNA-binding mechanisms.

Results of cytotoxic activity of *G. lucidum* (Curt.:Fr.) P. Karst.SC-CO_2 extracts through BSLA are shown in Table- 2. The LC_{50} values after 24h ranged from 31.62 to 89.12 µg/ml, and are considered significant. Mortality has already been observed at lowest concentration (10 µg/ml) and a 100% mortality at highest concentration (1000 µg/ml). GL2 gave the lowest LC_{50}, thus the most potent. This means that it will only take 31.62 µg/ml of the extract to kill half of the total individuals of the test organism. DMSO which act as negative control showed no toxicity to brine shrimps nauplii (LC_{50} 24h = 0 µg/ml). The cytotoxicity of a certain compound or natural product will not always suggest their outright toxicity, but it may also suggest their potential antitumor or anticancer activities. The observed significant cytotoxic effects is may be due to triterpenes or ganoderic acid, a bitter-taste chemical class that present naturally in *Ganoderma* species, and ganoderma alcohols. Triterpenes are considered as potential anticancer agents due to their direct cytotoxicity against tumor cells[26,27]. Previous reports also demonstrated the cytotoxic effects of ganoderic alcohols such as the ganoderiol F on Meth-A and LLC tumor cell lines and mouse Lewis lung carcinoma[28, 29].The findings of the previous studies as well as of the current one are promising since there is a positive correlation between brine shrimp toxicity and human tumor cell lines cytotoxicity[23].

G. lucidum (Curt.:Fr.) P. Karst.possess a high variety of bioactive compounds such as more than 120 different triterpenes, polysaccharides, proteins and other bioactive compounds[13,14,15,30]. The antitumor and other pharmacological effects of *G. lucidum* (Curt.:Fr.) P. Karst. are due to these bioactive compounds. Furthermore, the previously reported biological activities of *G. lucidum* (Curt.:Fr.) P. Karst. extracts are associated with DNA-binding, suggestive that this mushroom species is able to synthesize DNA-binding compounds.

The three extracts showed the same pattern in the IR spectra (figure- 3). FTIR analysis revealed that the extracts possessed several functional groups. These characteristic functional groups are NH_2 stretch of amines or OH stretch for alcohol (3412-3424 cm^{-1}), C = O carbonyl stretching or C = O stretching of saturated aliphatic esters (1707-1741 cm^{-1}), asymmetric and symmetric stretches of aliphatic CH_2 (2923-2925 cm^{-1} and 2853-2854 cm^{-1}), asymmetric and symmetric bending of aliphatic CH_3 (1457-1460 cm^{-1} and 1372-1377 cm^{-1}), and CH_2 rocking vibration for straight chain hydrocarbons of seven or more carbon atoms (722-724 cm^{-1}), which may indicate the presence of polysaccharides, proteins and fatty acids in the extracts. These organic compounds together with fiber are the main components of *G. lucidum* (Curt.:Fr.) P. Karst.[8].

Collectively, the observed DNA-binding and cytotoxic activities of the SC-CO_2 extracts of the wild type *G. lucidum* (Curt.:Fr.) P. Karst. are suggestive of their antitumor/anticancer activities. These results support previous studies on commercially cultivated *G. lucidum* (Curt.:Fr.) P. Karst. and also corroborated the claim on the anticancer and/or antitumor properties of this medicinal mushroom species. Furthermore, findings of the present study suggest a rich reserve of bioactive molecules in wild type mushroom species. This information could drive the local and scientific communities to tap the wild type mushroom species for bioprospecting and eventually drug development.

Conclusion

The SC-CO_2 extracts of *G. lucidum* (Curt.:Fr.) P. Karst. collected from the wild ecosystems of Bukidnon Province,

Philippines showed *in vitro* DNA-binding to Salmon sperm DNA and significant *in vivo* cytotoxicity to brine shrimp nauplii (*Artemia salina* Leach). These biological activities are attributed to the active components of *G. lucidum* (Curt.:Fr.) P. Karst. which are mainly proteins, carbohydrates and fatty acids. These findings suggest the potential of this wild mushroom species as drug sources particularly for tumors and cancers. Further investigation should be performed on the complete identification of compounds responsible for the reported biological activities in the study. The mechanism of action that mounts these observed biological activities especially the DNA-binding should also be investigated.

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