ORIGINAL ARTICLE
DOI: 10.4274/tjps.galenos.2024.34734

Insecticidal and Bactericidal activities of *Cassia Nigricans* and Molecular Docking analysis on Insect Acetylcholinesterase

Natural Insecticides and Bactericides, Molecular Docking

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24.07.2023
12.01.2024
19.01.2024

Abstract
Objectives: This study focused on phytochemicals, insecticidal, and bactericidal activities of *Cassia nigricans*, as well as the molecular docking analysis for acetylcholinesterase inhibitor (AChEI) as a promising natural insecticide.
Material and methods: The leaves of *C. nigricans* were extracted successively with n-hexane, acetone, and methanol. Silica gel column chromatography of the methanol extract yielded compound 1. The insecticidal properties of the extracts and compound 1 were evaluated by contact toxicity against *Sitophilus zeamais*. The bactericidal activity was achieved by photodynamic inactivation of faecal coliforms and faecal enterococci in water using extracts and compound 1 as natural photosensitizers. Compound 1 was analyzed for physicochemical and pharmacokinetic parameters and molecular docking against an AChE protein (6XYU).
Results: Compound 1 was characterized as emodin (1,3,8-trihydroxy-6-methylanthracene-9,10-dione) using 1D-2D-^{1}H-^{13}C NMR and MS methods. The insecticidal properties showed that emodin exhibited the highest toxicity with an LC_{50} = 5.00 mg/mL compared to all extracts. The *n*-hexane extract showed the highest insecticidal activity (LC_{50} = 177.48 mg/mL) compared to the methanol (LC_{50} = 195.08 mg/mL) and acetone (LC_{50} = 374.14 mg/mL) extracts. Complete inhibition of faecal enterococci by photosensitization was observed after 60 minutes of light exposure of emodin treated water at all concentrations used (1-5 mg/mL) and 120 minutes for faecal coliforms under the same conditions. Based on the docking score, the binding energy of emodin (-6.38 kcal/mol) is close to that of the marketed insecticide pirimiphos-methyl (-6.25 kcal/mol). In addition, emodin was subjected to insecticide probability prediction and absorption, distribution, metabolism, excretion, and toxicity (ADMET) analysis and was found to be satisfactory as a natural insecticide. Emodin could be a promising candidate for insecticidal pest control.
Keywords: *Cassia nigricans*, Insecticidal, Bactericidal, Molecular docking.

INTRODUCTION

Several methods are used to control stored grain insect pests: smoking, heating, and synthetic chemicals.[1] Synthetic insecticides have drawbacks and their high cost limits their accessibility to farmers. To minimise post-harvest losses, plants containing alkaloids, terpenoids, and anthraquinones are used as natural insecticides and are of purely ecological interest as they are not harmful to the environment.[2] Some compounds can act as insecticides by inhibiting insect acetylcholinesterase (AChE) and preventing the breakdown of acetylcholine, the accumulation of which causes insect death. Currently, many compounds such as the organophosphates used as insecticides for pest control exert their effects as AChE inhibitors.[3] Numerous models are used for AChE inhibition as part of the study of the insecticidal effect, such as the molecular docking analysis against insect AChE.[4]

Some natural compounds used to protect stored foodstuffs can have a bactericidal effect.[5] The bactericidal effect may be due to the photodynamic inactivation of microorganisms by the photosensitizing effect of plants or compounds in the presence of light.[6] This photosensitizing effect is directly linked to the presence of substances that generate singlet oxygen, which can damage microorganisms present in the environment.[7] The alkaloids, coumarins, and anthraquinones present in plants are responsible for photosensitizing and bactericidal activities.[8]

Cassia nigricans Vahl (Caesalpinaceae) has been previously studied for its antimicrobial, insecticidal, analgesic, anti-inflammatory, and antiplasmodial activities.[9]
This work presents for the first time the insecticidal activity by contact toxicity against *S. zeamais*, the photosensitized inactivation of faecal coliforms (FC) and faecal enterococci (FE) by the leaves of *C. nigricans* as well as the molecular docking of the isolated anthraquinone against *Drosophila melanogaster* acetylcholinesterase (DmAChE) and analysis of its ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties.

EXPERIMENTAL

Plant material

The leaves of *C. nigricans* were collected in September 2021 in Dobem, Chad. A voucher specimen has been deposited in the Cameroun National Her barium.

Extraction and isolation

Dried and ground *C. nigricans* leaves (1 kg) were extracted with 4L *n*-hexane for 48 h and concentrated to give the *n*-hexane extract (HE). The residues were successively extracted with acetone and methanol following the same procedure used previously to give the acetone extract (AE) and methanol extract (ME) respectively. Column chromatography (CC) of ME (40 g) on silica gel (60-120 Mesh) using a gradient of increasing polarity of n-hexane/EtOAc (1-100% EtOAC) yielded 83 fractions. Fractions 39-54, eluted with the Hexane/EtOAc (3/1) system formed a precipitate which was purified by recrystallisation with 5% methanol/EtOAc yielding compound 1 (1 g).^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz using TMS as reference (spectrometer Bruker AM-400 Darmstadt, Germany Company). Analytical TLC plates on silica gel 60 F254 TLC (Merck, Germany) were used for the TLC profile.

Obtaining and rearing the insects

S. zeamais strains were collected from infested maize grains from Booster Soumian Entreprise, an agropastoral and quality control company in Chad. Mass rearing was carried out with adult insects collected from already infested maize grains from the Booster Soumian Entreprise rearing site. The insects were reared in the dark on white maize grains in a chamber (temperature 28±2°C, relative humidity 65±5%).[10]

Contact toxicity test

Contact toxicity test was performed using Ndomo et al.[9] Five concentrations of crude extracts (25, 50, 100, 200, and 400 mg/ml) and compound 1 (5, 12.5, 25, 50, and 100 mg/ml) were prepared. 1 ml of each dose was added to 40 g of clean and undamaged maize grains. After evaporation of the solvent, each was infested with a batch of 20 two-day-old unsexed adult insects. The marketed insecticide pirimiphos-methyl was used as a positive control. The number of dead insects was estimated daily for 3 days. Abbott's (1925) formula was used to calculate the corrected insect mortality rate.[11]

$$\% \text{ Mortality} = \frac{\text{Number of dead insects}}{\text{Total number of insects}} \times 100$$

$$Mc(\%) = (Mt - Mo) / (100 - Mo)$$

Mc: corrected mortality (%); Mt : mortality in the treated batch (%) ; Mo: mortality in untreated control (%)
LC_{50} values were determined by Probit analysis.[12]

Microorganisms

The microorganisms used for the photosensitization tests were FC and FE from an open well for consumption by the population of Dobem, Chad. This water contained 25.10^2 CFU FC/100ml and 15.10^2 CFU FE/100ml.

Photosensitized inactivation of bacteria

Five concentrations of 1, 2, 3, 4, and 5 mg/ml of ME and compound 1 were used for photosensitization experiments using the method of Sunda et al.[13] A batch of treated water samples (with ME and compound 1) and another batch of untreated water samples (blank) were exposed to light (UV lamp, brand B-100 AP, emitting between 320 and 400 nm, with a maximum at 365 nm). The lamp was placed 15 cm from the water samples for 0, 30, 45, 60, 120, and 180 minutes. A batch of treated and untreated samples were kept in the dark. For each data set, the standard error was calculated (mean ± SD).

Bacteriological analysis

Bacteriological analysis was performed by culture on rapid *E. Coli* and Bile Esculin agar media for FC and FE. After the photosensitization experiments, 1 ml of water from the samples was inoculated into the culture medium and the number of colonies formed after 24 h of incubation at a temperature of 44.5 °C was counted.[12] The number of germs in colony forming units (CFU) was determined in the water samples before and after the application of the photosensitizer.

Molecular docking and dynamics analysis

Molecular Operating Environment (MOE, 2013) software[14] was used to dock a protein of *Dm*AChE against compound 1 and two marketed insecticides into the protein's active site. The crystal structure of *Dm*AChE (PID: 6XYU, resolution: 2.51 Å) was downloaded from the Protein Data Bank (https://www.rcsb.org/). The water molecules and heteroatoms of protein were removed. Compound 1 and the two marketed insecticides: emodin (PubChem CID 3220), cypermethrin (PubChem CID 91691), and pirimiphos-methyl (PubChem CID 34526) were collected from the chemical database (https://pubchem.ncbi.nlm.nih.gov/), inputted into the MOE program

and subjected to 3D protonation and energy minimization. The MMFF94X force field was used to minimize ligands and the protein structure. After docking, the best and top conformation was determined based on S-score and interacting residues.[15] The prediction of physicochemical, pharmacokinetic, and ADMET properties of compound 1 was performed using the SwissADME web tool.[16]

Statistical analysis

Experiments were performed in triplicate and mean values were obtained. All the statistical analyses were carried out using SPSS version 21.0. Data on corrected mortality were subjected to analysis of variance using the Waller Duncan's Test.

RESULTS

Phytochemical studies

Extraction of *C. nigricans* leaves with HE, AE, and ME gave yields of 28.6 g (2.9%), 36.5 g (3.9%), and 95.3 g (11.2%) of crude extracts respectively.

Characterization of the isolated compound

Chromatographic fractionation of ME yielded compound 1 which the structure (Figure 1) was established by spectral data and by comparison with literature data.[17]

Compound 1: Orange needles; melting point 260-263°C. Solubility in DMSO; HRESIMS at *m/z* 270.3 (calculated for $C_{15}H_{10}O_5$). ESI-MS (70 ev): *m/z* (rel. Int.) 271.3 (5), 226.5 (10), 224.4 (15), and 222.6 (20). ^{1}H-NMR (500 MHz, DMSO-d_6) and ^{13}C-NMR (125 MHz, DMSO-d_6) (Table 1).

Mortality Activity against S. zeamais

A time and concentration dependent increase in the percentage mortality of adult *S. zeamais* was observed for all extracts and emodin (Table 2). The highest dose (400 mg/mL) caused 95.7, 22.2, and 55.7% mortality of insects on the second day of exposure for the HE, AE and ME respectively. The insecticidal activity of emodin was found to be higher than that of the HE, which was the most active of the extracts tested. Similarly, the activity of emodin (LC_{50}, 5 mg/mL) was higher than that of the positive control, Pirimiphos-methyl (LC_{50}, 1.25 mg/mL) (Table 3).

Photosensitizer inactivation of bacteria

Results of the light exposure of the water samples treated and untreated with ME and emodin showed complete inactivation of FC after 3 hours of exposure for the ME treatment and 2 hours for the emodin treatment (Table 4). Complete inhibition of EF was observed after 2 hours of light exposure in water treated with ME and after 1 hour of treatment with emodin for all concentrations used. No inactivation of FC et FE in the water was observed after 3 hours of light exposure in the untreated water samples and for all treated water samples exposed to darkness. An increase in photodisinfection efficiency was observed as a function of concentration and irradiation time. (Figures 2-3).

Molecular docking

A molecular docking analysis of emodin was performed to study its binding to *Dm*ACHE and then compare it to marketed insecticides used to control pests of crops, fruit trees, and ornamental plants. The crystal structure of *Dm*AChE was used as a model to study insecticidal potential due to the unavailability of that from *S. zeamais* in the PDB database. The binding energy of emodin (-6.38 kcal/mol) is close to that of pirimiphos-methyl (-6.25 kcal/mol) and lower than that of cypermethrin (5.52 kcal/mol) (Table 5). Emodin has two types of interaction bonds (pi-pi) with residues TRP83 and TYR370, similar to those of tacrine-derived insecticides used as ligands.[18] Emodin and pirimiphos-methyl have a common amino acid TRP83 residue for binding in the 6XYU active site (Table 5).

ADMET analysis

Evaluation of the pharmacokinetic parameters of emodin and other marketed insecticides enabled us to assess the insecticide-likeness as well as intestinal absorption and brain permeation, key toxicokinetic parameters that determine its toxicity, including neurotoxicity. The insecticide-likeliness of emodin was evaluated based on Tice's rule of five which helps to identify herbicides and insecticides.[19] The pharmacokinetic properties (Table 6) show that all ligands have a number of hydrogen bond donors ≤ 2 and hydrogen bond acceptors between 1 and 8. The molecular weight of these ligands is between 150 and 500 g/mol, and the CLogP values are between 0 and 5. The number of rotable bonds for all ligands is < 12. The same is true for the bioavailability radar of the predicted physicochemical descriptors and pharmacokinetic properties (Figure 4).

DISCUSSION

The high yield of the ME (11.2%) compared to the HE (2.9%) and AE (3.9%) could be due to the presence of many more polar compounds in the leaves of *C. nigricans*. Terpenoids, flavonoids, anthraquinones, and quinones were detected in all extracts. However, coumarins, glycosides, alkaloids, and tannins were not detected in the HE compared to AE and ME. These results are similar to previous work on the plant.[9] Compound 1 was identified as emodin, a known compound, by MS and 1D and 2D NMR, and its structure was confirmed by literature data.[17] The analytical TLCs carried out on the three extracts: HE, AE, and ME showed that only the ME had a spot (R_f = 0.56) corresponding to emodin. The yield of emodin obtained from the ME was 1g (1.04%).

Insect mortality tests showed that HE, AE, and ME were active against *S. zeamais*, a most destructive insect pest

of stored maize. Results are similar to previous studies reported on the insecticidal activity of *C. nigricans* extracts on mosquito larvae (*Anopheles gambiaea*) and whitefly (*Bemisia tabaci*).[20,21] Emodin showed very high toxicity against *S. zeamais* compared to all extracts. The lowest concentration (100 mg/mL) necessary to obtain 100% insect mortality on the first day of exposure was recorded with emodin. The HE (LC_{50} of 177.48 mg/mL) was more active than the ME (LC_{50} = 195.08 mg/L) and the AE (LC_{50} = 374.14 mg/mL). The efficacy of HE and ME against *S. zeamais* could be due to the presence of terpenoids and phenolic compounds, respectively, which are highly toxic to insects.[22] Previous reports have shown that emodin inhibits AChE and Glutathione S-transferase activities in insects, resulting in their death.[23] Previous studies have shown that emodin may be useful as a new natural larviciding agent against mosquitoes.[24] Our study revealed for the first time the insecticidal activity of the leaf extract and emodin from *C. nigricans* against *S. zeamais*.

The minimum concentration of emodin resulting in complete inactivation is 4 mg/mL for FC and 3 mg/mL for FE for an irradiation time of 30 minutes (Table 4). This means that FE (Gram+) are more sensitive to the ME than FC (Gram-). These results are similar to previous studies showing that emodin under visible light was more likely to penetrate the intracellular environment of Gram-positive bacteria permeable to bioactive compounds, thus enhancing the local killing effect on the bacteria. In contrast to Gram-positive bacteria, Gram-negative bacteria are more resistant to the photodynamic effect due to the different surface structures of bacterial cells.[25,26] The photosensitising activity of ME may be due to a combination of several factors: photosensitiser, sunlight, and oxygen.[6] This photoreactivity is mainly due to the presence of photoactivatable molecules (anthraquinones and quinones) which are natural dyes capable of storing light energy that is then transferred to stable oxygen to generate singlet oxygen.[8] When emodin is exposed to light, visible light photons are excited and transfer energy to the oxygen molecules as they return to their ground state, generating reactive oxygen species with cytotoxic properties that cause irreversible damage to cell membranes, DNA, and proteins in bacterial cells.[25]

Energy score results showed that the lowest values were obtained with emodin and pirimiphos-methyl which is an insecticide used as an AChE inhibitor. Results indicate that all ligands are non-violent and conform to the rules of Tice, Hao, and Clark.[18,27,28] The ADMET evaluation showed that emodin and the selected insecticides predicted high intestinal absorption but were not expected to penetrate the brain (Figure 5). All ligands showed no inhibition of the human ether-a-go-go gene growth enzyme (hERG) (Table 6). Acute oral toxicity was higher for emodin (2.01 mol/kg) than for the marketed insecticides pirimiphos-methyl (3.10 mol/kg) and cypermethrin (3.19 mol/kg). The aqueous solubility of emodin is -3.91, that of pirimiphos-methyl -3.16, and that of cypermethrin -6.24. MD results revealed that emodin created a high affinity pi-pi bond with TRP83 in the *Dm*AChE active site (distance: 3.63 Å) similar to that created by ZAI (iodobenzyltacrine) with TRP83.[18] Additionally, emodin establishes a pi-pi bond with the same TYR370 at a distance of 3.84 Å (Figure 6). *In silico* molecular docking studies provide more detailed information on the interactions between emodin and *Dm*AChE (6XYU). The labeled insecticides pirimiphos-methyl and cypermethrin give an idea of the possibility of using emodin as a promising insecticide. The study clearly shows that emodin is promising in terms of binding affinity and pharmacokinetic properties. These results are consistent with in vitro studies on the inhibition of human AChE, which showed inhibitory activity of emodin with an IC_{50} = 15.215.21 ± 3.52 µM.[29]

CONCLUSION

Extracts of *C. nigricans* as well as emodin isolated from the ME of the leaves showed toxicity against *S. zeamais* and can be used as natural insecticides for the protection of stored products. Additionally, ME showed bactericidal activity due to the presence of photoactivatable molecules, including emodin, a photoreaction site that can lead to the inactivation of FC and FE present in polluted waters. Molecular docking confirmed the binding positions of emodin in the active centre of AChEI. Emodin does not passively cross the BBB, but is passively absorbed from the HIA, and could be a promising natural insecticide for pest control.

ACKNOWLEDGEMENTS

The authors thank the Laboratory of Natural and Bioactive Substances, Faculty of Science, University of *** for their material support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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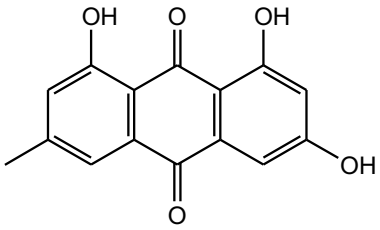
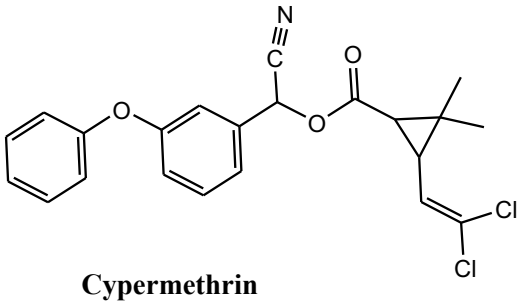
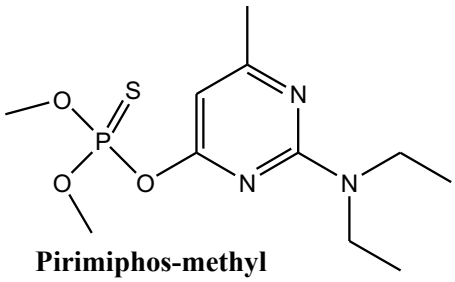


Emodin



Cypermethrin



Pirimiphos-methyl

Figure 1. Structures of isolated compound and drugs

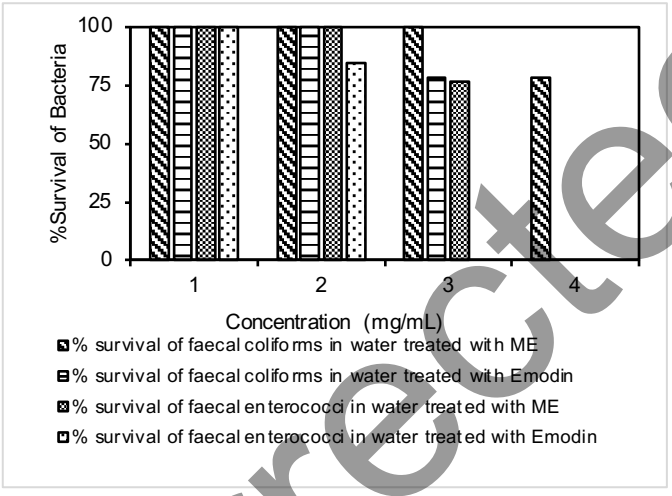


Figure 2. Bacterial survival (%) as a function of photosensitizer concentration. Irradiation time: 0.5h.

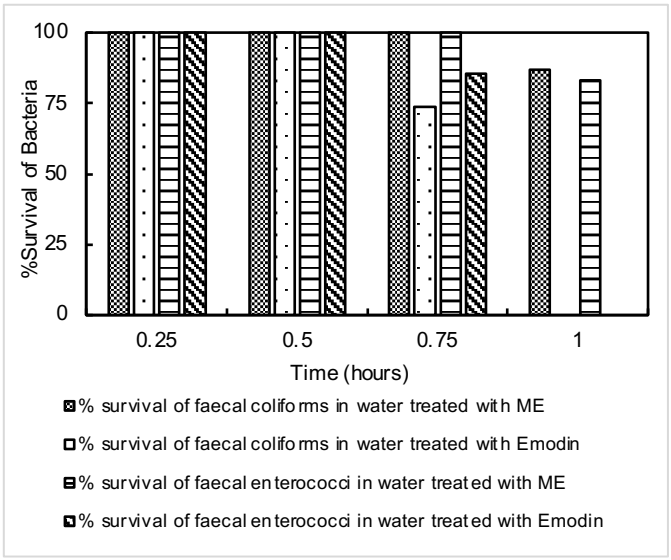


Figure 3. Bacteria survival (%) as a function of irradiation time. Concentration of sensitizer: 1mg/mL

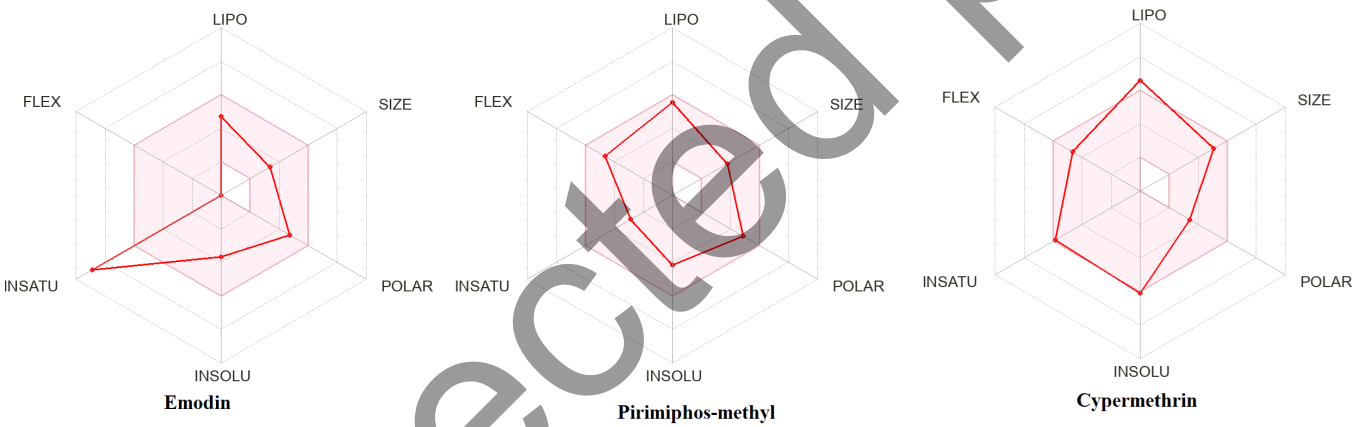


Figure 4. Bioavailability radar plot of drugs and emodin

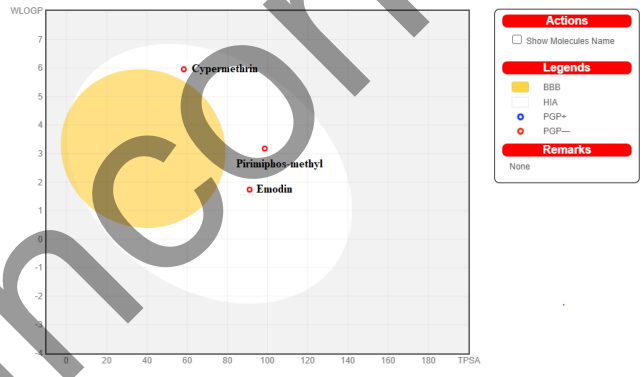


Figure 5. Egan BOILED-Egg plot prediction model for intestine and brain permeation. The white region is the physicochemical space of compounds predicted to exhibit high intestinal absorption, and the yellow region is the physicochemical space of compounds predicted to permeate the brain. tPSA: Topological polar surface area; WLogP: LogP value calculated according to the Wildman–Crippen method.

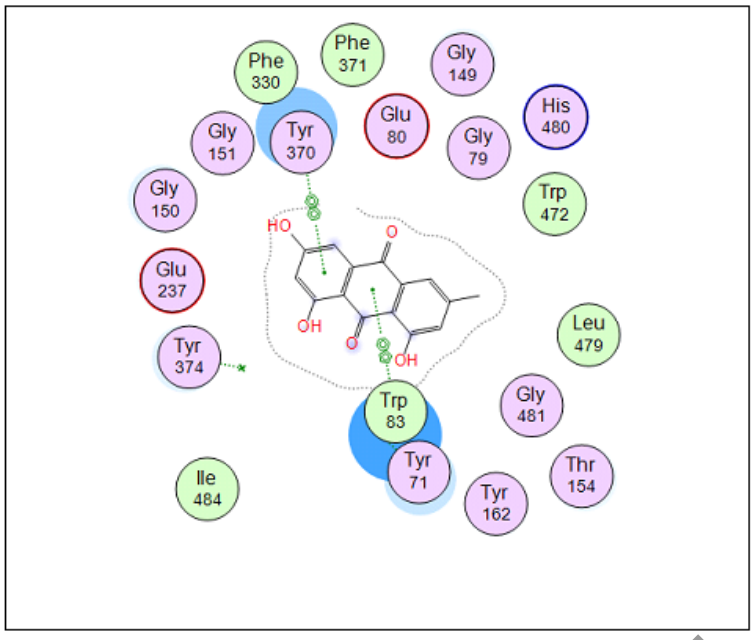


Figure 6. 2D visualization of the best pose of Emodin_6XYU for docking using MOE.

Table 1 Spectra data of Compound 1 in DMSO-d6

N°	1 δ_C en ppm	1 DEPT-135	Published [16] δ_C en ppm	1 H-Multiplicity
1	166.0	C	166.03	
2	108.3	CH	108.39	1Hd (2.4)
3	161.8	C	161.87	
4	109.2	CH	109.43	1Hd (2.4)
5	120.8	CH	124.59	1Hs
6	148.6	C	148.71	
7	124.5	CH	120.93	1Hs
8	164.8	C	164.90	
9	190.0	C	190.19	
10	181.6	C	181.85	
4a	135.4	C	135.58	
8a	113.6	C	113.85	
9a	109.2	C	109.22	
10a	133.1	C	133.29	
Ar-	21.9	CH_3	21.96	3Hs
1-	–			1Hs
3-	–			1Hs
8-	–			1

Table 2 Effect of the extracts and emodin on mortality of *S. zeamais*

Samples	**Conc. (mg/mL)**	**Mean % mortality ± S.E at 24 to 72 h post**		
		24h	48h	72h
HE	25.0	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
	50.0	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
	100.0	0.0±0.0[a]	12.6±0.4[b]	17.4±0.5[b]
	200.0	12.3±0.3[b]	32.3±0.3[c]	50.3±0.3[c]
	400.0	95.7±0.2[c]	100.0±0.0[d]	100.0±0.0[d]
AE	25.0	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
	50.0	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
	100.0	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
	200.0	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
	400.0	22.2±0.9[b]	42.3±0.2[b]	58.3±0.3$_b$
ME	25.0	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
	50.0	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
	100.0	0.0±0.0[a]	0.0±0.0[a]	13.6±0.4[b]
	200.0	0.0±0.0[a]	17.6±0.6[b]	32.6±0.4[c]
	400.0	55.7±0.5[b]	76.6±0.3[c]	100.0±0.0[d]
Emodin	5.0	18.0 ± 0.4[a]	24.1 ± 0.6[a]	58.3 ± 0.5[a]
	12.5	24.6±0.6[a]	38.6±0.8[a]	63.5±0.9[a]
	25.0	38.6±0.4[b]	52.9±0.7[b]	73.0±0.5[b]
	50.0	70.7±0.6[c]	85.5±0.4[c]	100.0±0.0[c]
	100.0	100.0±0.0[d]	100.0±0.0[d]	100.0±0.0[c]
Control	0.0	0.00.0[a]	0.0±0.0[a]	0.0±0.0[a]

Each value is a mean of + standard error of three replicates. Mean followed by the same letter in a column is not significantly different (P<0.05) from each other using the new Duncan multiple range test.