Experimental and computational study of biological activities of alkaloids isolated from *Peganum harmala* seeds

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Abstract

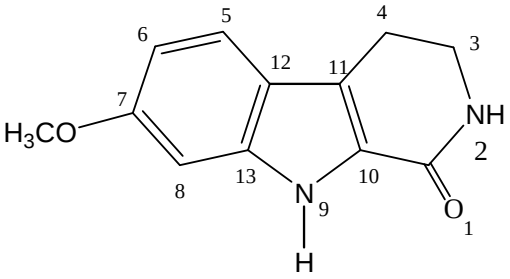
Anticancer compounds from natural products were screened using tumor cell lines. In the present study, we investigated the antiproliferative activity of four compounds isolated from the alkaloidic fraction of *Peganum harmala*. Compounds (1-4) were assayed *in vitro* for their effect on thymidine incorporation using Jurkat leukaemia cell line at concentration of 5, 10, 25, 50 and 100 µg/ml. Their cytotoxicity was also evaluated at the concentrations indicated above on several murine cell lines. Results showed that vasicinone, harmine and harmalacidine inhibited the proliferation of Jurkat, clone E6-1 cell line with significant cytotoxic effect. No noticeable effect of peganine (compund 3) on thymidine incorporation was observed. On the other hand, DFT calculation has been used to analyze the electronic and geometric characteristics The HOMO, LUMO and gap energies E_g were also deduced for the stable structure for each compound. These results will be correlated with the experimental values.

Keywords: Vasicinone, Harmine, Peganine, Harmalacidine, Cytotoxicity and antiproliferative activities, DFT study, energies LUMO, HOMO, Gap

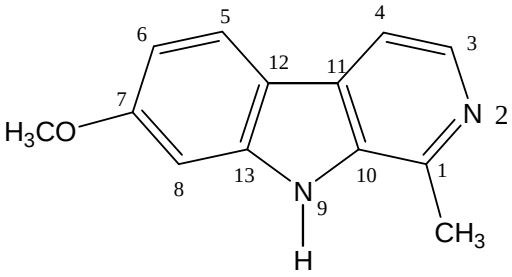
1. Introduction

The large projections of anti-cancer chemotherapy were obtained thanks to the discovery of drugs of new structures, and new mechanisms of action. The vegetable world may constitute a potential source of drugs or models of molecules for new drugs. The spontaneous medicinal plants in the Moroccan Saharian areas would constitute a potential source of new anti-cancer materials. *Peganum harmala* is among the most typical plants and most interesting of the central Sahara in Morocco. Several biological activities of *P. harmala* have been described in literature. These include antibacterial, antifungal and antiviral activities [1, 2, 3, 4], central nervous system, analgesic and vasorelaxant effects [5, 6, 7]. Within the research framework on the new anti-cancer compounds, we were interested in studying the anti-cancer activity of *Peganum harmala*.
We showed in a previous work, that aqueous extract of *P. harmala* had an antimitotic activity. Total alkaloids of *Peganum* have cytotoxic activity on cancerous cell lines and an inhibiting activity of the synthesis of DNA. The study of the antitumor activity showed that the aqueous and alcaloïdic extract of *P.*

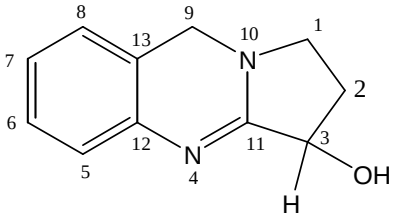
harmala presented an antitumor activity [8, 9, 10, 11]. The results obtained with total alkaloids of *P. harmala* encouraged us to seek in the alkaloidic extract the active principle (s) responsible (s) for this cytotoxic and antitumor activity. The chemical study of *P. harmala* revealed 4 pure products (compounds 1-4). The compounds were identified as alkaloids: the harmalacidine (1) the harmine (2), the peganine (3) and the vasicinone (4) [12], and tested against cancer cell lines for antiproliferative and cytotoxic activity [13].
In the present paper, four compound (pure alkaloids) of *P. harmala* seed were tested for the cytotoxic and antiproliferating activity against several murine tumor cell lines. The chemical structures of the studied molecules are given in Figure 1. On the other hand, recently, density functional theory has been used to analyze the electronic and geometric characteristics [14]. Furthermore, it is considered a very useful technique to analyze the experimental data [15]. The electronic properties of studied molecules such as the highest occupied molecule orbital (HOMO), the lowest unoccupied molecule orbital (LUMO), Gap energy difference between E_{HOMO} and E_{LUMO} and dipole moments have been achieved the appropriate correlation.



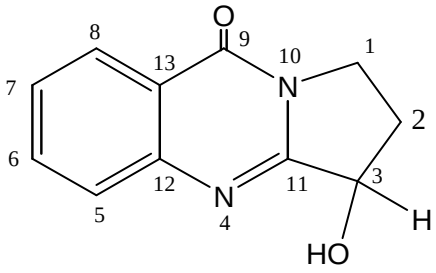
Harmalacidine : 1



Harmine : 2



Peganine : 3



Vasicinone : 4

Fig. 1. Structures of compounds **1-4** isolated from *Peganum harmala*.
Harmalacidine **(1)**, Harmine **(2)**, Peganine **(3)**, **and** Vasicinone **(4)**.

2. Experimental details

2.1. Plant materiel
Dried seed of *P. harmala* were collected from the Figuig region of eastern Morocco, in July and August 2000 and authenticated by Dr Bennabidine, botanist in (Ecole Forestière de Salé). A voucher specimen is preserved in the herbarium of our Institute.

2.2. Tested materiel
Total alkaloids were recovered from a methanolic extract of *Peganum harmala* seeds as described previously (yield: 1.2 %) [8, 9, 13].
Four products (1-4) **(**Fig.1) were isolated by Chromatography from the alkaloidic fraction. They were identified by spectroscopic techniques (Nuclear Magnetic Resonance, and mass spectrometry) as alkaloids: harmalacidine (1), harmine (2), peganine (3) and vasicinone (4). The yield of the four compounds was **52 %, 10 %**, **4 %** and **1.3 %** respectively. The isolation and characterization of the four natural compounds are described in detail in a previous work [12].

2.3. Tumoral cell lines

Cytotoxicity was evaluated on four cell-lines: **(i)** Med-mek Carcinoma, a cell-line obtained from a hepatocarcinoma induced by diethylnitrosamine administration to a Wistar rat. **(ii)** UCP-med Carcinoma obtained from a hepatocarcinoma on cirrhosis induced by carbon tetrachloride administration to a Wistar rat. **(iii)** UCP-med Sarcoma obtained from a fibrosarcoma induced by administration of 3-methylcholanthrene to a Wistar rat [10] and **(iv)** Sp2/O-Ag14 a myeloma cell line [16].

2.4. Cytotoxicity assays

In vitro cytotoxicity assay was carried out using trypan blue assay. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fœtal calf serum, 2% L-glutamine and 5 mg/l of each of the antibiotics gentamicin, penicillin, and streptomycin to prevent bacterial and mycoplasmal contamination. Cells were maintained in a 5% CO_2 incubator at 37°C. They were grown to confluence for 2 to 3 days and adjusted to 10^5 cells/ml with fresh medium. Initial cultures of 10 ml were prepared in 25 cm^2 flasks before application of treatments.

Alkaloids were solubilized in 0.2 % DMSO and applied to the cell culture at final concentrations of 10, 20, 40, 60, 80 and 100 µg/ml. Alkaloid solutions were adjusted so the same volume of DMSO was added to each culture. Controls received the same amount of DMSO. An additional normal control was carried out with an equivalent volume of culture medium instated of DMSO.

All treatments were done in five replicates. The cultures were maintained under normal incubation conditions and were examined daily under an inverted microscope. Cell counts were made on 0.1 ml samples taken from cultures after vigorous shaking to monitor cell viability using the Trypan Blue colorimetric method [17]. Statistical analysis of the data was analysed using Student's t-test and the values were expressed as mean ± SD, n=5. The dose effect plotted line which permits the determination of IC_{50} was analysed using the program MICROPHARM [18].

2.5. Antiproliferative assay

The cancerous cellular line used is the line Jurkat, E6-1 clone. This line was established by Dr A. Weiss in 1984 by cloning the Jurkat-FHCRC line.

This cell line was cultured in RPMI with 10% fetal calf Serum, L-glutamine (0.1%) and antibiotics (gentamicin 0.1% - Penicillin /streptomycin 0.1%). Cells were cultured in round bottomed plates. Each well contained 200 µl of the test compounds (5 - 10 - 25 - 50 and 100 µg/ml). Plates were incubated for 48 h at 37°C with 5% CO_2 supply and 5% RH.

Tritiated thymidine [3H]-TDR in 10 ml of phosphate buffer/saline was added to each well for 5 h. The cell were harvested and deposited on a glass fibber filter using Shatron multiple cell harvester. The filters were dried for 24 h at room temperature and transferred to miniscintillation vials. To each vial 10 ml of scintillation fluid was added. Radioactivity counting was performed by liquid scintillation spectrometer and tritiated thymidine incorporations. Measures were after one day.

Three separated sets of control Jurkat, clone E6-1 containing the compound solvant were used in each assay. Data was obtained as counts/minute and the mean of each triplicate was calculated.

For each product tested, we determined after 48 hours of treatment the concentration of a substance inhibiting 50% (IC_{50}) of the thymidine tritiated incorporation of the cells of the line Jurkat, E6-1 clone. IC_{50} values were obtained from the dose-response curve analysed with the MICROPHARM software [18].

2.6. Computational details

The quantum calculations were performed using the Gaussian 03 program [19]. The geometry of the studied compound was evaluated using the DFT level of the three-parameter compound functional of Becke (B3LYP**)** [20]. The 6-31G* basis set was used for all atoms. The geometry structure was optimized under no constraint. We have also examined HOMO and LUMO levels; the energy gap was evaluated as the difference between the HOMO and LUMO due to the MO energies [21-22].

3. Results and discussion

Alkaloidic fraction, 2 β-carbolines alkaloids : harmalacidine (1) and harmine (2) and 2 quinazolines alkaloids : peganine (3) and vasicinone (4) (Fig. 1) were extracted from *Peganum harmala.* They were tested for cytotoxicity on a panel of murine tumor cell lines. The results are summarised in table 1.

Table 1 Cytotoxicity of compounds 1-4 isolated from *Peganum harmala* seeds on 4 cell-lines

Cell lines	IC_{50} (µg/ml)*		
	1	**2**	**3**
	4		
Med-mek Carcinoma	17.72 ± 0.05	14.40 ± 0.03	52.24 ± 0.04
	25.32 ± 0.08		
UCP-med Carcinoma	28.93 ± 0.02	18.39 ± 0.03	> 100
UCP-med Sarcoma	59.97 ± 0.05		
Sp2/o-Ag14	17.60 ± 0.03	6.48 ± 0.05	52.36 ± 0.05
	64.79 ± 0.04		
	7.96 ± 0.04	2.43 ± 0.14	> 100
	19.20 ± 0.04		

Data shown are mean ± SD of five independent experiments.
*** Cytotoxicity as IC_{50} for each cell line, is the concentration of compound that inhibits of 50% the cell multiplication after 48 hours of treatment.**

By comparing the IC_{50} of the various products tested in the 4 cellular lines, we can say that:
The Sp2/O-Ag14 line is the most sensitive of the lines studied to the various products tested. The IC_{50} varies between 2.43 and 19.20 µg/ml and 4 out of the 5 products tested have an IC_{50} lower than 20 µg/ml.
The line UCP-med Carcinoma is the least sensitive to the various products tested is compared to the 3 other lines used in our study. Indeed, the value of the IC_{50} varies between 13.83 and 59.97 µg/ml.
Among the 4 isolated pure products, the peganine (3) is the less active. Indeed, it has no effect neither on incorporation of the thymidine tritiated in the line Jurkat, clone E6-1 (the IC_{50} is higher than 100 µg/ml) nor on the growth of the cells Sp2/O-Ag14 and UCP-med Carcinoma (the IC_{50} is higher than 100 µg/ml). The IC_{50} in the 2 other lines remains higher than 50 µg/ml.
The harmine (2) was the most active compound in this study. Its IC_{50} varies in the 4 lines between 2.43 and 18.39 µg/ml. Moreover, this alkaloid presented the lower IC_{50} among all the products tested of *P. harmala* (2.43 ± 0.14 µg/ml in the Sp2/O-Ag14 line). The harmine should be tested *in vivo* to confirm these important results of *in vitro*. In the same way for the harmalacidine, the IC_{50} in the the 4 lines varies between 7.96 – 28.93 µg/ml.
Our results are in agreement with those of the literature. Indeed, harmine showed potent cytotoxic activity against KB, A549, CAKI-1, 1A9 and HEL cells with ED_{50} values of 2.2, 2.4, 1.9, 1.6 and 1.9 µg/ml. In addition, harmine exhibited cytotoxic effects in three drug-resistant cell lines including KB-7d, KB-VIN and KB-CPT [23].
According to Ayoub et al (1994) [24] the harmalol (an alkaloid of *P. harmala*) inhibits the proliferation of the carcinogenic cellular line Leukaemia K 562 with a concentration of 10 µg/ml by inhibiting the DNA synthesis and the cellular division.
Also work of Jin (1990) [25] showed that the harmaline (an alkaloid of *Peganum harmala)* in comparison with 5-fluorouracil (5-FU) had a cytotoxic activity *in vitro* on a cellular line SO-Rb50 from the human retinoblastoma with a concentration of 12 µg/ml and a 72 hours incubation, the rate of inhibition is of 96.6 ± 1.98% for the harmaline and 93.5 ± 0.90% for the 5-FU. Their respective IC_{50} are 1.437 µg/ml and 0.321 µg/ml. *In vivo* the same alkaloid appeared inactive on the same line. This inactivity was attributed to the existence of the blood barrier retinal `` fair retinal barrier' '. It should be pointed out that these studies could be completed by an *in vivo* study to withdraw valuable conclusions.
Moreover, we examined the effect of compounds (1-4) isolated from *Peganum harmala* seeds on the incorporation of the thymidine tritiated in the DNA of the cells from the Jurkat, E6-1 clone cell line.
The effect of the 4 pure alkaloids of *P. harmala* on the incorporation of the thymidine by the cells Jurkat, E6-1 clone is summarised in table 2. For each product, we determined the concentration which inhibits the

incorporation of the thymidine tritiated of 50% (IC_{50}) after 48 hours treatment. Table 2, however, presents the IC_{50} for each product in the line Jurkat, E6-1 clone.

Table 2 Effect of compounds 1-4 isolated from *Peganum harmala* seeds on the Jurkat, E6-1 clone proliferation

Cell lines	IC_{50} (µg/ml)*			
	1	**2**	**3**	**4**
Jurkat, E6-1 clone	27.10 ± 0.02	46.57 ± 0.02	> 100	8.60 ± 0.04

Data shown are mean ± SD of three independent experiments.

*** IC_{50} is the concentration of a substance inhibiting 50% (CI_{50}) incorporation of the thymidine tritiated of the cells of the line Jurkat, E6-1 clone analyzed by means of a nonlinear technique of regression using software MICROPHARM after 48 hours of treatment.**

For the vasicinone, the effect is depending on the amount during the first 24 hours contact with the product, and then we noticed a light increase in the incorporation of the thymidine with concentrations of 5 - 10 and 25 µg/ml from 24 to 48 hours. The IC_{50} calculated after 48 hours of treatment was of 8.60 ± 0.04 µg/ml.

With the harmine we noticed an effect which depends on the amount and the time. Indeed for concentrations of 5, 10, 25 and 50 µg/ml, the percentage of incorporation is always higher than 60% from 0 to 48 hours. With the higher concentration 100 µg/ml, the effect depends on time and reached a maximum at 48 hours. The IC_{50} calculated after 48 hours of treatment was of 46.57 ± 0.02 µg/ml.

The peganine seems to be less active compared to the total alkaloids of *P. harmala* and with the other pure alkaloids (compounds 1, 2 and 4) ; with 4 concentrations 5, 10, 25 and 50 µg/ml. The tritiated percentage of incorporation of the thymidine remained always higher than 50% during all the period of experimentation. For the higher concentration 100 µg/ml, the effect reaches 70% of inhibition in the first 24 hours then decreases by 24 to 48 hours. The IC_{50} calculated after 48 hours of treatment was higher than 100 µg/m.

The harmalacidine with concentrations of 5, 10 and 25 µg/ml shows a moderate effect on incorporation of the thymidine which is always higher than 60%. On the other hand with the concentration of 100 µg/ml, a 100% inhibition is reached at 48 hours. The IC_{50} calculated after 48 hours of treatment was of 27.10 ± 0.02 µg/ml.

The lines Sp2/O-Ag14 and Jurkat, E6-1 clone are both of lymphocyte origin. A comparison of the IC_{50} after 48 hours of treatment with the compounds (1-4) show that the peganine (3) has no effect neither on the incorporation of the thymidine tritiated in the Jurkat line, E6-1 clone nor on the cell multiplication in the Sp2/O-Ag14 line: the IC_{50} is higher than 100 µg/ml. The same its effect on the 3 other types of cancer are not very important: the IC_{50} remains higher than 50 µg/ml.

Evaluation of proliferation, cytotoxicity and differentiation of harmine and harmaline on HL60 cells, alone or in combination with ATRA (all-trans retinoic acid), a drug for treatment of APL patients and G-CSF (Granulocyt-Colony Stimulating Factor), a cytokine which enhances the efficacy of antileukemic therapy showed that harmine and harmaline reduced proliferation in dose and time dependent manner [26].

In addition, some harmine derivatives showed high *in vitro* cytotoxicity and cell death via apoptosis. *In vitro* studies have shown that β-carbolines such as harmine were highly cytotoxic and significantly inhibited tumor cell growth with apoptotic effect [27, 28, 29]. Further mechanistic studies indicated that some β-carbolines could inhibit DNA synthesis and intercalate into the DNA helix and were inhibitors of DNA topoisomerase I and II [30, 31]. Moreover, many β-carbolines such as harmine were potent and specific inhibitors of cyclin-dependent kinases (CDKs) with most compounds inhibiting CDK2 and CDK 5 to the same extent [32].

Many naturally occurring alkaloids (e.g., camptothecin, vincristine and ellipticine) are potent anticancer agents. These alkaloids kill tumor cells by different biochemical modes of action, such as inhibition of apoptosis, inhibition of topoisomerase I and II and inhibition of microtubule formation. The molecular mechanisms for the antitumor activity of β-carbolines are not fully defined, but induction of apoptosis, inhibition of DNA topoisomerase I and II9–14 and CDKs have been implicated [32].

Mechanism of the cytotoxic activity of *P. harmala* seeds extract was studied by Sobhani et al. [33]. Results has shown that β-carbolines could intercalate into DNA. In theory, this effect may cause inhibition of DNA topoisomerases and results in cytotoxicity.

DNA relaxation assay was used to determine the extent of DNA topoisomerase I inhibition by *P. harmala* extract and its β-carboline alkaloids. It was found that *P. harmala* seeds extract do inhibit human DNA topoisomerase I and based on the results of HPTLC analysis, it appears that the biological activity of the extract can be explained by its β-carboline content. The most active compound was harmine with IC50 value of 13.5 ± 1.7 µg/ml.

4. Theoretical results

It was shown from experimental results that the investigated compounds have interesting biological activities but no giving further insight into the correlations between these experimental properties and the electronic ones such as the highest occupied molecule orbital (HOMO), the lowest unoccupied molecule orbital (LUMO), Gap energy difference between E_{HOMO} and E_{LUMO} and dipole moments. Thus the density functional theory was applied to study correlation with experimental results. The optimized geometry structures of the studied compounds are illustrated in **Fig 2.** The optimization process (the global minimum energy information of the materials is achieved) was fully done using B3LYP/ 6-31G* calculation.

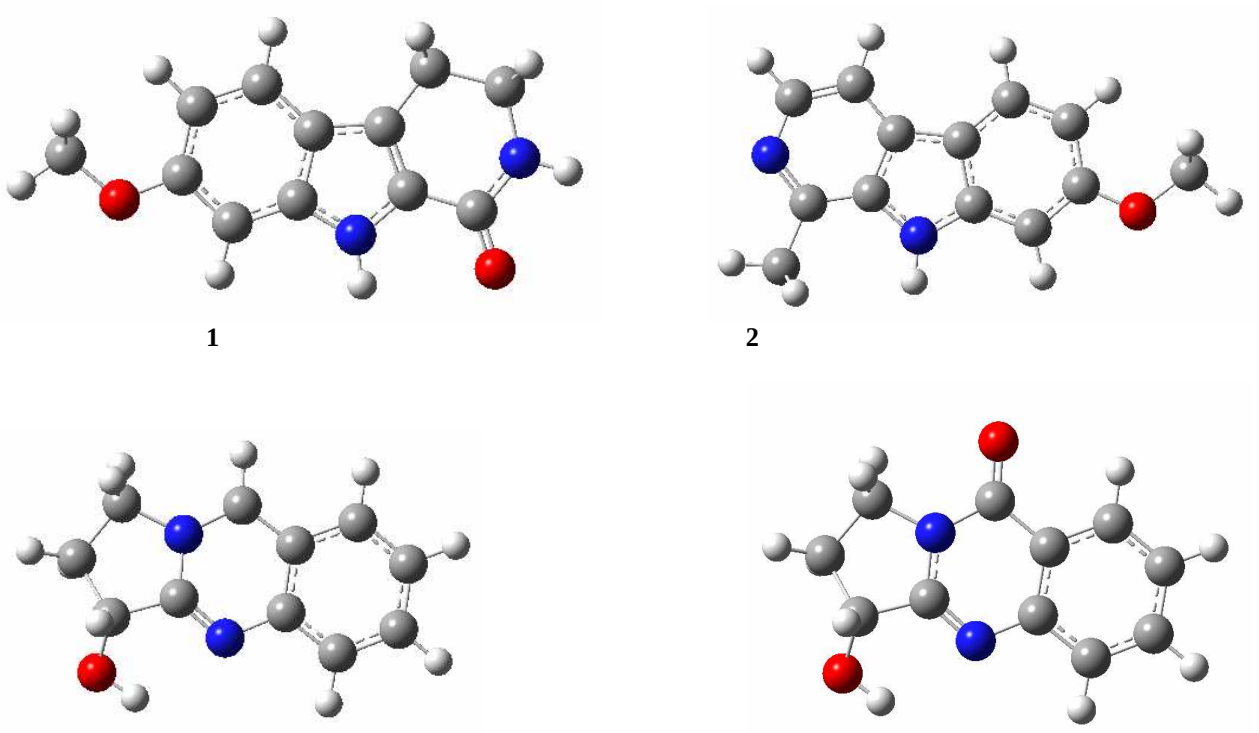


Fig. 2. Optimized structures of studied molecules obtained by B3LYP/6-31G* level.

According to the structures of the 4 studied molecules, one can compare only the similar structures presenting the same skeleton namely: **2**, **3** on the one hand and **3**, **4** on the other hand. For this purpose, we have calculated the HOMO and LUMO energies, Gap energy difference between E_{HOMO} and E_{LUMO}, dipole moments and total energies. The obtained results are presented in table 3.

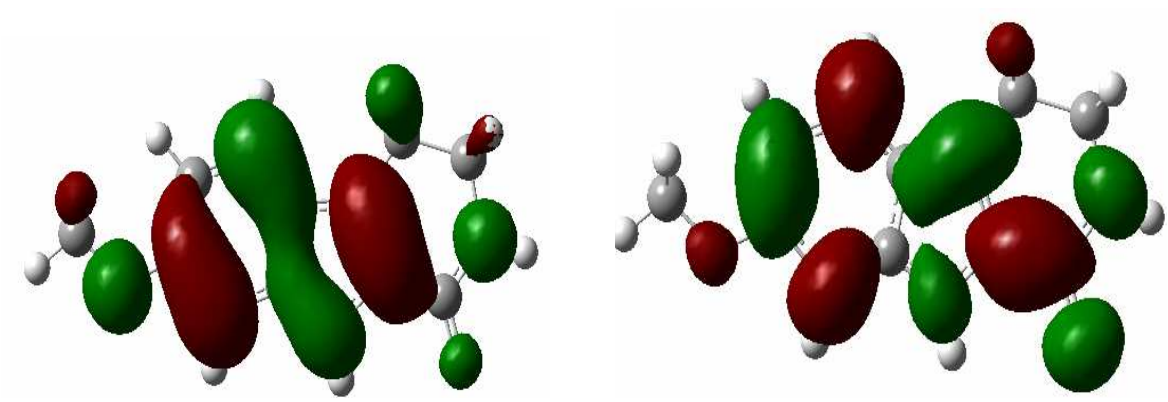
It is known that the value of E_{HOMO} is often associated with the electron donating ability of inhibitor molecule, higher values of E_{HOMO} is an indication of the greater ease of donating electrons to the unoccupied d orbital of the receptor. The value of E_{LUMO} is related to the ability of the molecule to accept electrons, lower values of E_{LUMO} shows the receptor would accept electrons. Consequently, the value of ΔE_{gap} provides a measure for the stability of the formed complex on the metal surface. The lower value of ΔE has, the higher stability is for the formed complex. The value of ΔE for **1** and **2** are 4.3, 4.7 eV respectively; the values of dipolar moment are 3.92 and 3.49; the values of total energies are -724 and -687. Let us recall from experimental results that the compound **2** has the best cytotoxicity. We can thus conclude that The

values of Gap energy and total energy are proportional to the cytotoxicity whereas the values of dipolar moment are inversely proportional. The same results are obtained by comparing **3** and **4**: the decrease of the energy difference between the frontier molecule orbital and the total energy on one hand and the increase of the dipolar moment while passing from **4** to **3** means that cytotoxicity of **4** is better than that of **3**. From these theoretical calculations carried out for studied molecules **1, 2, 3** and **4**, it can be concluded that the electronic properties are correlated with their experimental results.

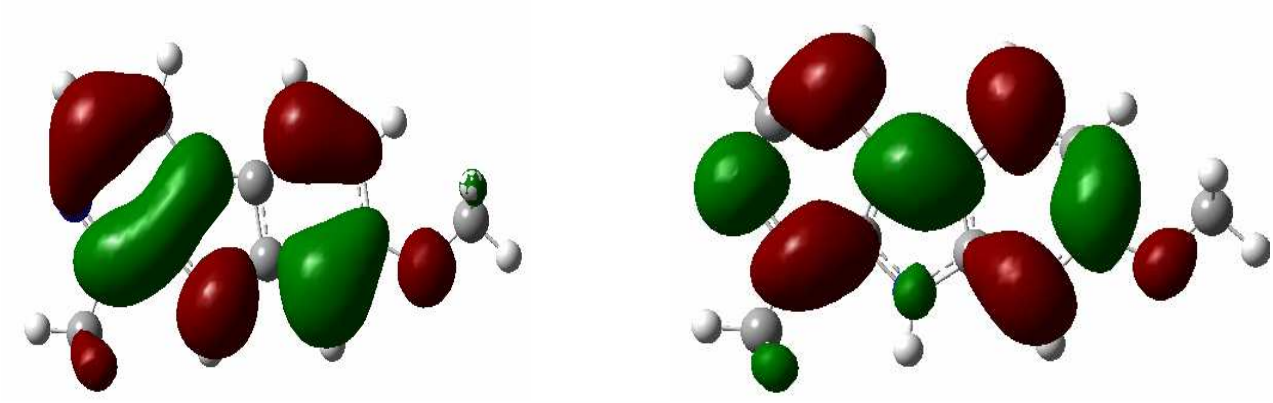
Table 3 Calculated HOMO (eV), LUMO (eV), ΔE_{gap} (=LUMO-HOMO) (eV), Dipole moments (Debye) and total energies (u.a.)

	1	**2**	**3**	**4**
HOMO (eV)	-5.1922	-5.5372	-3.5687	-6.0733
LUMO (eV)	-0.8008	-0.8237	-0.5731	-0.9617
GAP (eV)	4.391	4.713	2.995	5.111
Dipole Moments (Debye)	3.9239	3.4922	3.2786	2.503
Totale energies (u.a.)	-724.46452	-687.34969	-610.48850	-609.96304

On the other hand, it is important to examine the HOMO and the LUMO for these molecules because the relative ordering of occupied and virtual orbital provides a reasonable qualitative indication of electronic properties and the ability of electron hole transport. In general, as plotted in figure 4, the HOMO possesses an antibonding character between the consecutive subunits; whereas the LUMO generally shows a bonding character between the subunits.



1



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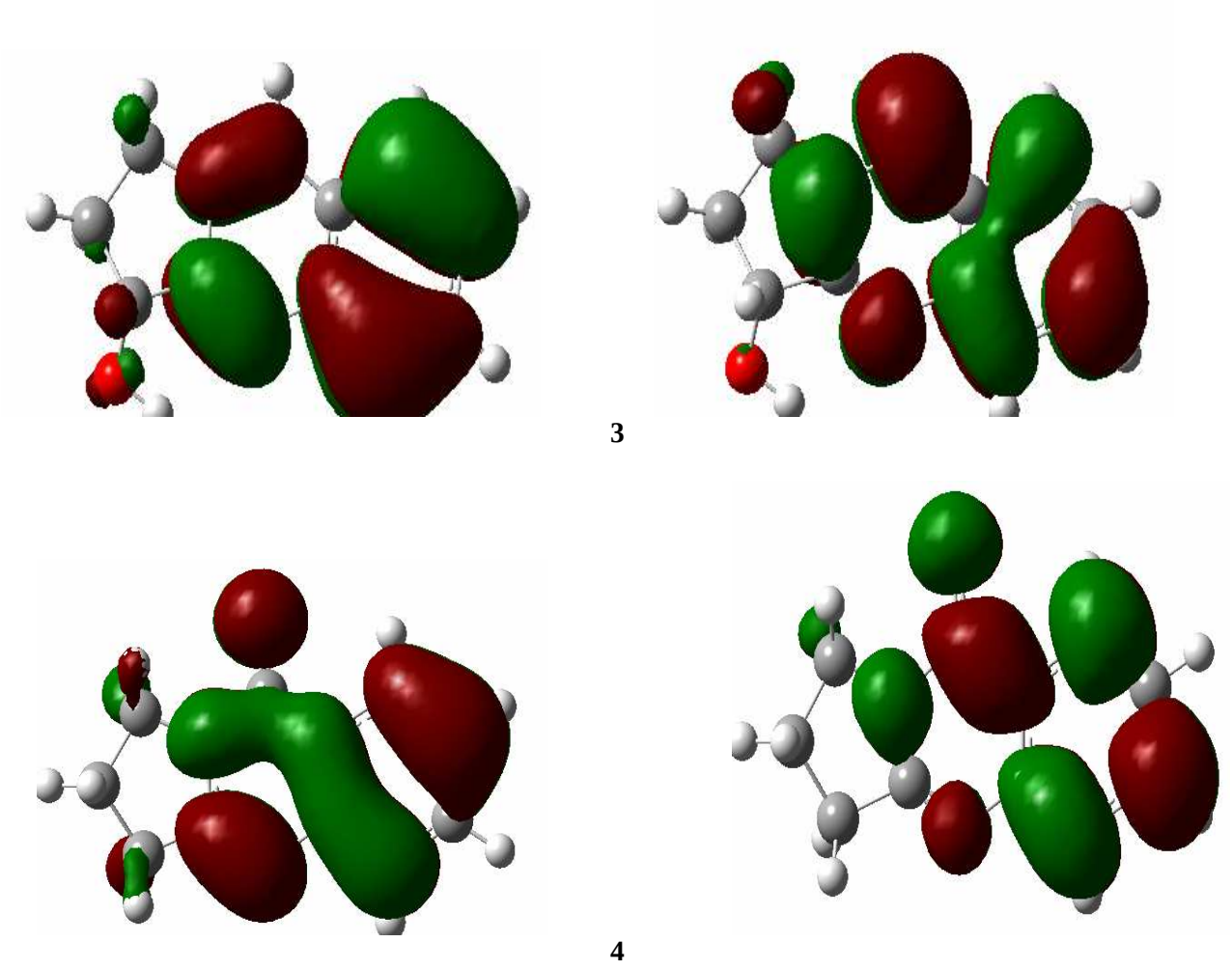

Fig. 4. Schematic representation of HOMO molecular orbital of studied molecules

Conclusion

1. The study of the activities cytotoxic and antiproliferative of *P. harmala* on experimental models of cancer allowed us to show that the compounds (1,2,4) isolated from the alcaloidic fraction have an *in vitro* cytotoxic activity on carcinogenic cellular lines and an inhibitory activity of the synthesis of DNA. The obtained results showed that the activity may depend on of the product tested and the cellular line considered. The 3 pure alkaloids of *P. harmala* contrary to the peganine seem to act on the Jurkat line, E6-1 clone by inhibiting the synthesis of the DNA and also the cellular division;
2. The active extracts of *P. harmala* studied *in vitro* on the cellular lines, must be tested *in vivo* for the antitumor effect. In the same way, the inactive extracts *in vitro* should be also tested because there are products which are inactive *in vitro* but have an antitumor activity *in vivo*. It is the case of the immunostimulateurs;
3. From The theoretical calculations carried out for the studied molecules **1, 2, 3** and **4**, it can be concluded that the electronic properties are correlated well with their experimental results.

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