

**MORICANDIA ARVENSIS EXTRACT INHIBITS LIPID PEROXIDATION,  
ACETHYLCHOLINESTERASE ACTIVITY AS WELL AS MUTAGENICITY AND ROS  
PRODUCTION IN COLORECTAL CANCER CELLS**

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**ABSTRACT**

In this study, we investigate the potential of *Moricandia arvensis* chloroform leaf extract on acetylcholinesterase (AChE) activity, lipid peroxidation and DNA mutagenicity. Scavenging effect of reactive oxygen species (ROS) production in colorectal cells (BE) was also determined. The study of the chemical part of our extract shows a chemical profile containing squalene and fatty acids. Concerning the biological activities, the tested extract at the lowest dose decreased the acetylcholinesterase activity by 68.45% and inhibited strongly the lipoperoxidation induced by methyl methanesulfonate. Furthermore, chloroform leaf extract enhanced a significant antioxidant activity reaching 100%, in colorectal cancer cell line. A significant inhibition of mutagenicity induced by methyl methanesulfonate (76.32%) as well as 2-aminoanthracene (91%) in TA104 tested strain was also showed. This is the first study that shows the chemical composition of chloroform leaf extract and especially for this plant and its biological activities argue for its probable utilization in therapeutic or pharmaceutical arsenal.

**KEYWORDS:** ChlL extract; AChE; Lipid peroxidation; ROS; genotoxicity.

**1. INTRODUCTION**

Alzheimer's disease (AD) is a progressive brain disease more common in older people, with symptoms that worsen over time and can cause multiple damage affecting memory, emotions, language.<sup>[1]</sup> The brain of people with AD shows a progressive degeneration of nerve cells, especially those that make acetylcholine, a chemical substance considered important for learning and memory.<sup>[2]</sup> For this reason, patients who do not have enough of acetylcholine at the junction of neurons have problems with the transmission of messages from one neuron to another, and consequently loss of cognition and memory.<sup>[3]</sup> Thereby, the search for a drug that improves this situation is necessary to prevent acetylcholinesterase (AChE) from destroying acetylcholine.<sup>[4,3]</sup>

Reactive oxygen species (ROS) generated by various systems have been reported to play an important role in the pathogenesis of various diseases and can exacerbate their progression, including neurodegenerative disorders and cancer.<sup>[5]</sup> ROS can act on the cell, its components i.e., proteins, DNA and the lipid membrane causing enormous damage. Oxidative damage to lipids, i.e., lipid peroxidation, results in the production of several elevated markers of lipid,<sup>[6,7]</sup> reactive aldehydes including

malondialdehyde (MDA)<sup>[8]</sup> which has been detected in Alzheimer's disease (AD).<sup>[9]</sup> Significant studies presented by Casado et al. indicated on correlation between oxidative stress and stages of AD<sup>[10,11]</sup> as well as an increasing level of MDA with age.<sup>[11]</sup> Similarly, Padurariu et al.<sup>[12]</sup> took under consideration the high level of MDA in patients with AD<sup>[11]</sup> representing thus the most important causes of this pathogenesis.<sup>[11,7]</sup> Due to the fact that the brain is rich in easily peroxidizable fatty acids, these free radicals can effectively leads to the formation new, harmful structures.<sup>[11]</sup> Solutions to these problems are antioxidants, which counteract the negative impact of the reactive molecules. Unfortunately, the currently available drugs against AD do not exhibit activity toward these structures. Due to the fact that natural substances are extremely significant in new drug development, numerous studies are focused on substances which exhibit an important activities including antioxidants and anti-AD behaviors.<sup>[11]</sup> Several natural compounds with potent antioxidant properties,<sup>[13,7]</sup> such as flavonoids, phenolic acids, etc. are one of the most important classes of exogenous antioxidants that are present in human diet. Therefore, antioxidants from natural extracts are an important group in prevention and treatment of AD. Morillas-Ruiz et al.<sup>[14]</sup> in their study, determined that regular ingestion of

a functional drink, rich in antioxidant polyphenols from apples and lemons concentrate juice and green tea extracts (200 mL/person/day for 8 months) may decrease homocysteine plasmatic concentrations in Alzheimer's patients, especially in the moderate phase.<sup>[11]</sup>

*Moricandia arvensis* (MA) named locally "Kromb ejmel" belongs to the crucifer family. It is a medicinal plant used in Tunisia in traditional food, by cooking leaves in a salt water. Then, the latter is removed, the leaves are mixed with a little oil and they are cooked again giving a flavor similar to that of "Mloukhia" which is a traditional dish very popular in Tunisia. The decoction of leaves and stems was employed in the treatment of syphilis and scurvy which is a disease resulting from a lack of vitamin C.<sup>[15]</sup> Previous studies found that plant extracts have a good effects in the treatment of AD using animal models.<sup>[16,17,18,19]</sup> But till now, there are scarce information conducted to find out the effect of MA extracts consumption on neuroprotection and especially in mice models. To fill the information gap, we designed this study to investigate whether consumption of MA extract would alleviate oxidative status and protect to cognitive decline in Specific pathogen free BALB/c mice, while taking into account that animal studies provide ample evidence for the neuroprotective potential of plant extracts and compounds.<sup>[20,21,19]</sup> In addition, in view of the strong relationship between the oxidizing effect and AD described by several authors and the important *in vitro* antioxidant and antigenotoxic activities found in our previous works<sup>[22,23,15,24]</sup> of our extracts from MA, we decided to deepen and confirm our antioxidant studies in the animal models and to search a possible Anti-AD effect.

Not only AD has been associated with ROS in many reports<sup>[25,26]</sup> but also the excess these free radicals has also been a source of mutagens that cause cancer,<sup>[27]</sup> genetic damage and mutagenesis.<sup>[28]</sup> Therefore natural antioxidants should be valuable candidates to inhibit formation of ROS explaining thus the increase of employment of antimutagens or anticancer agents using plants.<sup>[29,30]</sup> Various epidemiological studies have reported a strong relationship between the consumption of cruciferous vegetables and reduced cancer risk of the one part<sup>[31]</sup> and protection against mutagenesis and drug toxicities on the other hand.<sup>[26]</sup> In addition the high consumption of cruciferous vegetables attributes not only to the prevention of cellular oxidation and DNA damage<sup>[32]</sup> but also could have a potential to treat symptoms of AD.<sup>[33]</sup>

As far as we reported in our previous studies significant anticancer, antioxidant and antigenotoxic effects of our Chloroformic tested extract (ChIL extract),<sup>[22,23,15,24]</sup> we undertook to evaluate the same activities on other cellular models, as well as other ones, *in vivo*. In fact, it is a necessary step before considering further studies in the pharmaceutical field. Therefore the effect of ChIL

extract on proliferation of cancerous colorectal cell line (BE), ROS production, MMS and 2-AA induced mutagenicity were also investigated.

## 2. MATERIALS AND METHODS

### 2.1. Plant Material

Harvested leaves of MA were collected in the southern region of Tunisia and precisely in Gafsa (Oued Ghezran). MA subsp. *eu-arvensis* is a medicinal plant identified by Pr. M. Cheieb (Department of Botany, Faculty of Sciences, University of Sfax, Tunisia). A sample (M.a-12.05) has been preserved in our laboratory and also deposited in a herbarium accessible to the public for a future reference, at the faculty of pharmacy of Monastir. The leaves are dried at room temperature, ground and then stored in our laboratory for preparing extracts.

### 2.2. Preparation of Plant Extracts

The ChIL extract was prepared from the dried leaves of MA using soxhlet. After degreasing the plant with petroleum ether, the chloroformic solvent was used to obtain the various low polar molecules thereby forming the chloroform extract (ChIL). The extract was then concentrated to dryness and residue was kept at 4 ° C.

### 2.3. Fractionation and purification of the ChIL extract from the leaves of *Moricandia arvensis*

ChIL extract was fractionated by RP-18 Medium Pressure Liquid Chromatography (MPLC), eluted with MeOH: H<sub>2</sub>O with gradual increasing of the MeOH content (20:80 to 100:0) and 10 different fractions (15A-15J) were collected. The MA15F fraction (600 mg) was fractionated with Vacuum Liquid Chromatography (VLC) (silica), leading thus to the production of 9 fractions from 23A to 23I, eluted with a gradient of CH<sub>2</sub>Cl<sub>2</sub>:EtOAc (100:0 to 0:100). Sub-fraction MA23B (236 mg) was rechromatographed on Sephadex LH-20 using CH<sub>2</sub>Cl<sub>2</sub>:MeOH (60:40) as eluent solvent system to give five fractions (28A-28E). MA28C resulted sub-fraction (168 mg), followed a separation on (Solid-Phase Extraction) SPE (RP-18) (Water:MeOH) (100:0 to 0:100), leading to the formation of 37 fractions (MA33A to MA33 K1).

### 2.4. Animal treatments

Specific pathogen free BALB/c mice (6-8-week-old, female, 18-20 g) obtained from the Pasteur Institute (Tunis, Tunisia) were selected for experiments. All protocols were carried out in accordance with French standard ethical guidelines for laboratory animals (agreement 75-178,5-16-2000) and all *in vivo* experiments were approved and followed by the Institutional Ethics Committee of the Faculty of Pharmacy (University of Monastir, Tunisia). During a week before the test, the animals were acclimated to the experimental conditions and were maintained with food (conventional chow) and water ad libitum. Mice placed in individual cages were housed in an air-conditioned room kept at ± 25 ° C at a relative humidity of 45 ± 10%, with a 12 h light-dark cycle.

The animals were treated as follows:

1. Animals were given 100  $\mu$ L of solvent (DMSO 10%), administrated intraperitoneally (IP), as negative control group (3 mice/negative control and each experience was repeated three times).
2. Concerning lipid peroxidation assay: animals of positive control group given a single dose of MMS (40 mg/kg), administrated IP, which compared to animals given MMS followed by three different doses of ChIL extract (12.5, 25, 75 and 150 mg/kg bw), administrated IP. Noting that the selected doses administered to mice are not lethal and did not increase body weight, feed intake and the size and shape of liver and kidney (3 mice/dose and each experience was repeated three times).
3. Concerning AChE activity: animals given different doses of ChIL (12.5, 25, 75 and 150 mg/kg bw) administrated IP (3 mice/dose and each experience was repeated three times).

After 24h of treatment, the animals were euthanized by cervical dislocation.

### 2.5. Serum preparation for studying AChE activity and lipid peroxidation effect

Blood was collected in heparin glass tubes and the plasma (supernatant) was recuperated by centrifugation at 3,500 rpm for 10 min. Mouse plasma was used then for studying lipid peroxidation assay and AChE activity.

### 2.6. Lipid peroxidation assay in vivo

MDA level is considered a reliable marker of membrane lipid peroxidation. In fact, this compound is a degradation product systematically induced in lipid peroxidation, indicating a radical attack of polyunsaturated lipids.<sup>[34]</sup> For the realization of this experiment, 0.4 mL from each mouse plasma was mixed with 1.5 mL of acetic acid (20%) and 1.5 mL of thiobarbituric acid (TBA) (0.8%). The mixture was brought to a final volume of 4.0 mL with distilled water and heated to 95°C for 120 min. After cooling on ice for 10 min, 5.0 mL of a mixture of n-butanol and pyridine (15:1 v/v) were added to each sample (analyzed in triplicate), and the mixture was shaken vigorously. The supernatant fraction was isolated after centrifugation (3,000 rpm, 20°C, 10 min) and the absorbance was measured. The MDA can react with TBA, leading thus to the formation of a pink pigment absorbed at 532 nm.<sup>[35]</sup> Induction of lipid peroxidation was expressed as equivalent of MDA (nM) and compared to negative control (NC: untreated animals with extract).

### 2.7. Acetylcholinesterase activity in vivo

The classic method of determining the AcChE activity<sup>[36]</sup> was adapted to our experimental conditions. Briefly, one mL of phosphate buffer (100 mM, pH=8), containing 0.26 mM of DTNB and 100  $\mu$ L of diluted plasma (1/200), was placed in a microcuvette which was incubated for 15 min at 30°C. The enzymatic reaction was started by the addition of the substrate

(acetylthiocholine (0.5 mM)) and monitored by the formation of yellow 5-thio-2-nitrobenzoate anions resulting from the reaction of DTNB with the thiocholine released by the enzymatic hydrolysis of substrate. Absorbance was measured at 405 nm using an M350 double Beam UV-VIS spectrophotometer «Camespec» (TovaTech, South Orange, New Jersey, USA), and read every 30 s during a 10 min (time period to verify the linearity of the reaction).

The enzymatic activity and the inhibition percentage were calculated as follows:

$$\text{Enzymatic activity } (\mu\text{moles/mL/min}) = \Delta \text{ absorbance/min} / 1.36 \times 10^4$$

$$\text{Inhibition percentage (\% of AcChE activity (\%))} = (E - S) / E * 100$$

Where E is the enzymatic activity without extract and S is the enzymatic activity in the presence of extract.

### 2.8. Cell culture and cell viability assay

The human colon cancer cell line (BE) were maintained in DMEM (Dulbecco's modified Eagle's medium) medium supplemented with 10% fetal bovine serum and 1% L-glutamine (from Sigma Cell Culture, Courtaboeuf, France), in humidified incubator at 37°C and an atmosphere enriched with 5% CO<sub>2</sub>. To control the impact of the tested concentrations of ChIL extract (200, 400, 600 or 800  $\mu$ g/mL) on cell viability, we estimated viability of BE cells by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. Cells were seeded in 96-well microtiterplates; 24 h later, the test samples were added in serial dilutions before incubating the plates for 48 h. Cells were washed once before adding 10  $\mu$ L of PBS containing 5 mg/mL MTT in 100  $\mu$ L of medium. After two hours of incubation at 37°C, the medium was discarded, and the formazan blue formed in the cells was dissolved by adding 100  $\mu$ L DMSO. Negative control without the tested extract was prepared in the same manner. Optical density (OD) was measured at 570 nm. Data were obtained from triplicate wells. Cell cytotoxicity was calculated as follows:

$$\text{Cell cytotoxicity (\%)} = (1 - (\text{OD of treated cells} / \text{OD of control cells})) \times 100.$$

### 2.9. Measurement of ROS production in vitro

According to the ROS production method,<sup>[37]</sup> BE cells ( $5 \times 10^4$  cells/well) were seeded in white 96-well, flat-bottom microplates. After 24 h of incubation, different concentrations of ChIL extract (200 400 and 600  $\mu$ g/mL) were added for two time of incubation (2 h and 6 h). The cells were triggered by adding a mixture of lucigenin (50  $\mu$ M) and NADPH (4 mM), and chemiluminescence was monitored every minute during a 45 min observation period using a microplate luminometer reader. The

inhibition percentage of ROS production was calculated as follows:

The inhibition percentage of ROS production (%) =  $(1 - (\text{OD of treated cells} / \text{OD of control cells}) \times 100)$ .

### 2.10. Bacterial Tester Strain and S9 fraction

TA104 (*hisG428*, *rfa*,  $\Delta$ *uvrB*, *pKM101*) strain of *Salmonella typhimurium* (*S.typhimurium*) was kindly supplied by Dr. I. Felzen, Universidade do Estado do Rio de Janeiro (UERJ, Brazil). This strain contains an ochre mutation AT base pairs at the *hisG428* mutant site which can be reverted by mutagens that cause oxidative damage.<sup>[38]</sup> The S9 fractions were prepared from livers of rats according to the method described by Maron and Ames.<sup>[39]</sup>

### 2.11. Mutagenicity assay in vitro

Ames assay and precisely TA104 strain<sup>[39]</sup> is used to examine whether ChIL extract is capable of inducing a mutation through oxidative damage. After 48 h of incubation, the revertant (R) bacterial colonies of each plate were counted and compared to the number of spontaneous revertants (SR) of negative control (NC: untreated strain with tested extract) and the number of revertants of positive controls (RPC) (the direct mutagen: MMS and the indirect mutagen: 2-AA). The extract was considered mutagenic if the number of revertants of ChIL extract (RChIL) per plate was at least doubled in strain, over the SR frequency of the NC.

### 2.12. Antimutagenicity assay in vitro

A modified procedure<sup>[23]</sup> was employed to determine the effect of ChIL extract on MMS and 2-AA induced mutagenicity. After incubation of plates at 37°C for 48 h, the R bacterial colonies were counted and the inhibition percentage of mutagenicity (%) was calculated by the following formula:

Inhibition percentage of mutagenicity (%) =  $[1 - (\text{RChIL} - \text{SR}) / (\text{RPC} - \text{SR})] \times 100$ .

Where: RChIL is the number of revertants on ChIL extract plates,

RPC is the number of revertants on positive mutagen control plates

SR is the number of spontaneous revertants on negative control plates.

### 2.13. Statistical analysis

All data were expressed as mean ( $\pm$  SD) and compared using a Student's *t*-test. Statistical significance was assigned at *p* values < 0.05. All data were analyzed using SPSS 11.0 software (SPSS INC; Illinois, USA).

## 1. RESULTS

### 1.1. Identification of purified compounds

Fractions MA33A1, MA33B1 and MA33C1 (60 mg) were gathered and rechromatographed on preparative plates RP-18 using MeOH:H<sub>2</sub>O (95: 5) as eluent solvent

system (Fig. 1) to obtain the pure compound MA39A named squalene (SQ) (4 mg) identified by Nuclear Magnetic Resonance (NMR) (Fig. 1, Table 1) and two mixtures of fatty acids (FA) such as MA40I (4 mg) (containing palmitic acid (Fig. 1) and eicostrienoic acid (Fig. 1)), and MA40J (4 mg) (containing palmitic acid and stearic acid (Fig. 1)). The identification of these FA was made by mass spectrometry (electron impact).

### 1.2. Lipid peroxidation assay in vivo

The effects of ChIL extract on lipid peroxidation were summarized in Fig. 2. After IP administration of the tested extract, the level of MDA decreased markedly with four tested doses 12.5, 25, 75 and 150 mg/kg bw (corresponding to 310, 370, 420 and 450 nM of MDA respectively) compared to the positive control MMS (860 nM). After IP administration of 12.5 mg/kg of ChIL extract the level of MDA (310 nM) was almost similar to that found with untreated animals NC (300 nM).

### 1.3. AChE activity in vivo

Inhibition of AChE activity in the plasma of mice treated by different concentrations of extract was reported in Fig. 3. ChIL extract decreases the AChE activity in a dose independent manner. Indeed, 68.54% of the enzymatic activity was inhibited after the injection of the lower dose of ChIL extract (12.5 mg/kg bw).

### 1.4. Cell culture and cell viability assay

The potential cytotoxic effect of ChIL extract on a human colorectal cell line (BE) was investigated. Fig. 4 showed that ChIL extract was able to induce cytotoxicity in BE cells in a dose dependent manner. After 48h of incubation, the lowest concentration (200  $\mu$ g/mL) was able to reduce cell proliferation by 76.84%. This antiproliferative effect increases by 95.21% when colorectal cells were incubated with the highest concentration (800  $\mu$ g/mL).

### 1.5. Measurement of ROS production in vitro

To examine whether ChIL extract could prevent ROS production, we set up an *in vitro* method with lucigenin-amplified chemiluminescence to measure the extracellular ROS production in BE cells. The results summarized in Fig. 5 showed that ChIL extract has a potent antioxidant activity in a dose and time dependent manner. Indeed, at the lowest concentration 200  $\mu$ g/mL, the extract inhibits lucigenin to induce ROS production by 48.21%, after 2 h of incubation. This effect increases when increasing the amount of extract reaching thus 65.17% at a concentration of 600  $\mu$ g/mL. After 6 h of incubation, the antioxidant effect increases with the different tested concentrations to reach a value of 100% at both 400 and 600  $\mu$ g/mL.

### 1.6. Mutagenicity and Antimutagenicity tests in vitro

Results of induced histidine (*his*) reversion mutations, in the presence of the tested extract with and without metabolic activation are presented in Table 2. ChIL extract did not induce increase of the R number in

TA104 strain, as well as with or without S9 metabolic system.

Doses of 0.65 µg/plate of MMS and 5 µg/plate of 2-AA were chosen for the antimutagenicity studies, since these doses were not toxic and induced 3455±38 (MMS), and 2141±27 (2-AA) R in *S. thyphimurium* TA104 (Table 3). The addition of ChL extract to TA104 strain was

effective in reducing the mutagenicity caused by the direct mutagen MMS by 76.32% (at the highest tested dose of 50 µg/plate). Similarly, ChL extract exhibited significantly an important inhibition activity against indirect mutagen 2-AA induced mutagenicity, in a dose dependant manner. The highest inhibition percentage obtained was 91%, when 50 µg/plate of extract was added to the *S. thyphimurium* TA104 assay system.

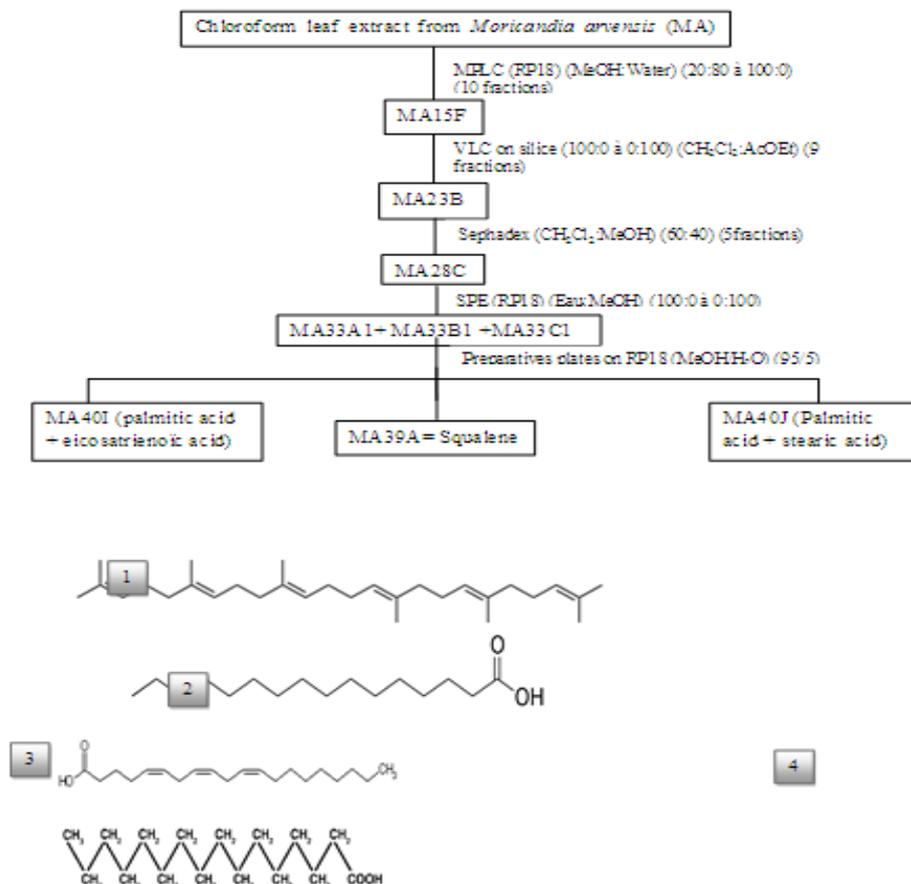


Fig. 1: Purification of MA40I, MA39A et MA40J obtained from the fractionation of chloroform leaf extract. (a): Squalene; (b): Palmitic acid; (c): Eicosatrienoic acid; (d): Stearic acid.

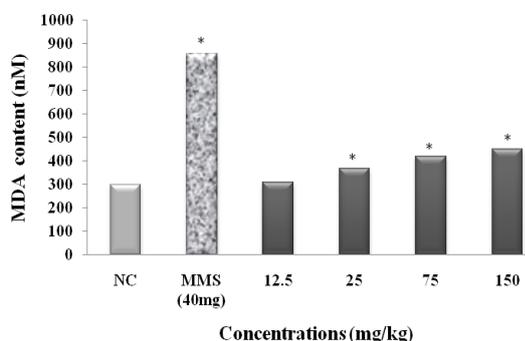


Fig. 2: Plasma levels of malonaldehyde (MDA) in mouse bone marrow after an increasing intraperitoneal (IP) doses injection of ChL extract. Animals treated by MMS (40 mg/kg) alone were used as a control for lipid peroxidation induction. NC: untreated animals used as negative control. Results are represented by the means ± SD of n = 3. (\*) p <

0.05 means a significant difference between the untreated and treated animals.

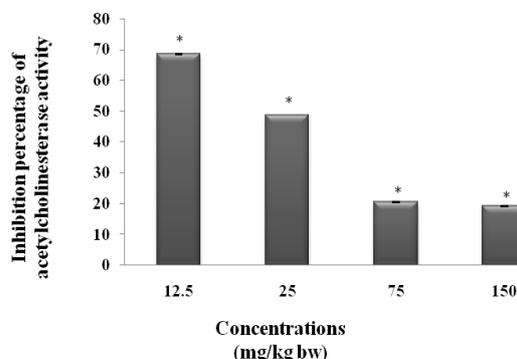
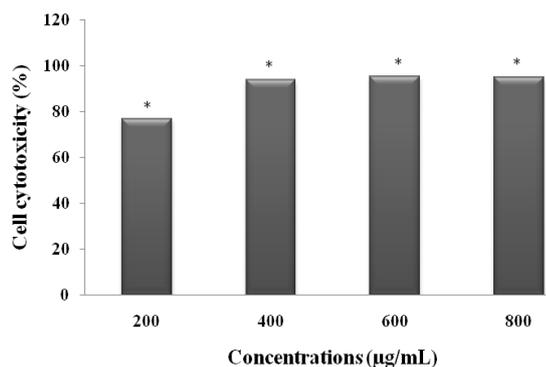
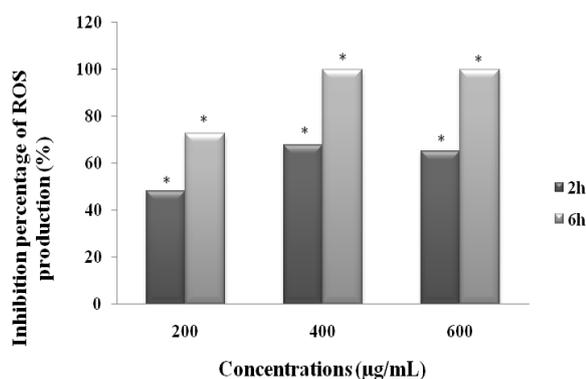


Fig. 3: Inhibition percentage of acetylcholinesterase activity by ChL extract. (\*) p < 0.05 means a significant difference between the untreated and treated animals.



**Fig. 4:** Cytotoxic effect of ChlL extract on colorectal cancer (BE) cells. Cells were treated with different concentrations of ChlL extract for 48 h, and cytotoxic effects were determined by MTT assay. The values are represented as the percentage of cell cytotoxicity. Values represent mean  $\pm$  SE of three independent experiments. (\*)  $p < 0.05$  means a significant difference between the untreated and treated cells.



**Fig. 5:** Effect of ChlL extract from MA on the inhibition of lucigenin-induced ROS production in human cancerous colorectal cells (BE). The values are represented as the percentage of ROS production inhibition. Data represent the mean  $\pm$  SE of 3 experiments, each conducted in triplicate. (\*)  $p < 0.05$  means a significant difference between the untreated and treated cells.

**Table 1:** NMR data of MA39A compound (squalene).

N°	$^1\text{H } \delta$ (ppm)	$^{13}\text{C } \delta$ (ppm)
1,24	1,66	25,7
2,23	-	131,2
3,22	5,10	124,4
4,21	nd	26,8
5, 9, 16, 20	nd	39,7
6, 19	-	135,1
7, 11, 14, 18	5,10	124,3
8, 17	nd	26,7
10, 15	-	134,9
12, 13	nd	28,3
Me-2,23	1,58 (s,18H)	17,7
Me-6, 10, 15, 19		16,0

The different spectral data was compared with literature data (58) allowed us to confirm that MA39A is squalene.

**Table 2:** Mutagenic effect of chloroform leaf extract (ChlL) from MA in *Salmonella typhimurium* TA104 assay system in the presence and absence of exogenous metabolic activation system (S9).

	Dose $\mu\text{g}/\text{plate}$	Nb of R/plate	Nb of R/plate
		-S9	+S9
ChlL	100	450 $\pm$ 15	499 $\pm$ 8
	250	516 $\pm$ 12	510 $\pm$ 8
	500	544 $\pm$ 14	560 $\pm$ 3
SR	-	477 $\pm$ 12	744 $\pm$ 18
PC(MMS)	0.65	3400 $\pm$ 31*	-
PC (2-AA)	5	-	2130 $\pm$ 23*

Positive control (PC): TA104/-S9, methyl-methane-sulfonate (MMS) (0.65  $\mu\text{g}/\text{plate}$ ); TA104/+S9, 2-aminoanthracene (2-AA) (5  $\mu\text{g}/\text{plate}$ ). SR: Spontaneous revertants. ChlL extract (Chloroform leaf extract). (\*)  $p < 0.05$  means a significant difference between the revertants of negative control (SR) and the revertants of ChlL extract (RChlL).

**Table 3:** Effect of MA extracts on the mutagenicity induced by MMS and 2-AA in the *Salmonella typhimurium* TA104 assay system.

	Effect of ChlL extract on the mutagenicity induced by MMS			Effect of ChlL extract on the mutagenicity induced by 2-AA	
	Doses $\mu\text{g}/\text{plate}$	Nb of R/plate	IP (%)	Nb of R/plate	IP (%)
ChlL	5	3029 $\pm$ 41*	14.5	760 $\pm$ 25*	83
	10	2255 $\pm$ 15*	51.5	670 $\pm$ 19*	88
	50	1227 $\pm$ 3*	76.32	628 $\pm$ 23*	91
SR	-	532 $\pm$ 15	-	420 $\pm$ 10	-
SR+S9	-	-	-	470 $\pm$ 9	-
MMS	0.65	3455 $\pm$ 38	-	-	-
2-AA	5	-	-	2141 $\pm$ 27	-

Positive control (PC): TA104/-S9, methyl-methane-sulfonate (MMS) (0.65  $\mu\text{g}/\text{plate}$ ); TA104/+S9, 2-aminoanthracene (2-AA) (5  $\mu\text{g}/\text{plate}$ ). SR: Spontaneous revertants. SR+S9: Spontaneous revertants induced after metabolic activation with S9. Nb of R: number of revertants. ChlL extract (Chloroform leaf extract). (\*)  $p < 0.05$  means a significant difference between the revertants of positive control (RPC) and the revertants of ChlL extract (RChlL).

## 2. DISCUSSION

Numerous scientists showed correlation between free radicals essentially levels of MDA and advanced in AD.<sup>[11,40]</sup> Our present study showed that ChlL extract inhibits MDA formation, preventing thus lipid peroxidation *in vivo*, the neurotoxicity and thus AD. Besides polyphenols that are most known for their

significant antioxidant effects,<sup>[41]</sup> the purification of ChL extract leads to the formation of SQ and FA e.g palmitic acid, stearic acid and eicosatrienoic acid. Therefore, it was described that SQ was an effective quencher of singlet oxygen and a potent inhibitor of lipid peroxidation.<sup>[42]</sup> Likewise, FA can act as antioxidants through their capacity on scavenging OH<sup>•</sup> and O<sub>2</sub><sup>•-</sup> radicals using their COOH group.<sup>[43]</sup> Extract from *Sargassum thunbergii* contained FA, i.e palmitic acid, stearic acid and cis-5,8,11,14,17-eicosanoic acid, could contribute to reducing free radicals levels<sup>[44]</sup> as well as 8,11,14-eicosatrienoic acid.<sup>[45]</sup>

Extensive evidence has shown that lipid peroxidation was an important mechanism of neurodegeneration in AD and ROS were responsible for toxicity to neurons.<sup>[46]</sup> By rapid hydrolysis of acetylcholine, AChE is capable of inducing the transmission of nerve impulses in the cholinergic synapses. Indeed, inhibition of AChE serves as the best strategy for the treatment of AD.<sup>[3]</sup> Different drugs are currently available e.g donepezil and rivastigmine but unfortunately these compounds have other adverse effects, that's why the researcher all over the world are actively engaged in the screening of natural products as AChE inhibitors.<sup>[47]</sup> For this reason, we tried to carry out this activity and we have demonstrated that our extract can be an excellent proficient for anti-AD drugs. Several studies have shown that the natural extracts have an important anti-AChE activity e.g *Piper sarmetosum*<sup>[3]</sup> *S. officinalis*<sup>[2]</sup> and *Ginkgo*.<sup>[48]</sup> Polyphenols which are rich in our extract<sup>[15]</sup> and the abundant glucosinolates in cruciferous family are a group of compounds reported to have AChE inhibitory activity.<sup>[49,50,51]</sup> Taking into account that oxidative stress including lipoperoxidation are among the main causes of the formation of the AD,<sup>[1]</sup> ChL extract may inhibit AChE activity through its antioxidant effect. Siti-Hawa et al.<sup>[52]</sup> showed a significant positive correlation between anticholinesterase effect and antiradical activity of *Canarium Odontophyllum* extract.

This proven antioxidant activity *in vivo*, has been also confirmed *in vitro*; in fact ChL extract exhibited a potent cytotoxic effect and inhibited lucigenin to induce ROS production in colorectal cell lines, justifying thus its probable classification as an anticancer agent, but further research on its mechanism of action should be clarified. These activities can be attributed to SQ that has been described as chemiopreventive and antitumor agent against colorectal cancerous cells.<sup>[53,54]</sup> Likewise, crucifers contain many bioactive components i.e. polyphenols<sup>[15]</sup> minerals, vitamins, an indole derivatives and glucosinolates<sup>[51]</sup> which are among the most-studied bioactive compounds associated with cancer protection.<sup>[55]</sup>

Based on these important biological activities, it is important to know if ChL extract has a mutagenic effect and if it is able to prevent DNA against mutagens induced ROS. We showed that the extract was not

mutagenic, either alone or in the presence of S9 and exhibited a significant antimutagenic effect. Considering that the TA104 strain is special to detect mutations caused by free radicals,<sup>[38]</sup> the extract may inhibit directly ROS induced by MMS and indirectly ROS formed during the process of microsomal enzyme activation. The significant antimutagenic activity is congruent with its strong antioxidant capacity; a relationship that has been widely demonstrated by other studies for other extracts.<sup>[56]</sup> In fact, ChL extract was enriched with phenolic, steroid and iridoids; molecules which has been described as antimutagens.<sup>[22,23,57]</sup>

### 3. CONCLUSION

The fact that ChL extract is not mutagenic, has significant anti-AChE, antimutagenic and high antioxidant activities (*in vivo* and *in vitro*), it may be used in the treatment of anti-AD. In addition, the excellent cytotoxic and ROS scavenger effects prove its eventual use against colon cancer. Our results, suggest that consumption of ChL extract containing SQ and FA as a dietary supplement has the potential to provide health benefits via alleviations of radical-induced mutagenicity cancer and AD. The above observations may warrant further study of the molecular mechanisms involved and the purification of other molecules responsible for different activities.

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### Conflict of interest

The authors declare that they have no conflict of interest.

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