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Evaluation of the biological activity of plant extracts of Algerian *Solanum elaeagnifolium.*

Karima Mellouk[1]*, Nidhal Soualeh[2], Smaïl Meziani[3], Michel Linder[4], Boudjama Boughrara[1], Rachid Soulimani[2], and Belgacem Legseir[1].

[1]Organic Synthesis and Biocatalysis Laboratory, University of Annaba, 23000, Algeria.
[2]Laboratory of Neurotoxicology Alimentary and Bioactivity, Paul-Verlaine University, BP 4102, 57040 Metz, France.
[3]European Institute of antioxidants (IEA), 2 Avenue de la Forêt de Haye .TSA 40602, 54518 Vandoeuvre-Les-Nancy, France.
[4]National Polytechnic Institute of Lorraine, Engineering Laboratory of Biomolecules, 2 avenue de la ForêtDe Haye, BP 172, 54505 Vandoeuvre-Les-Nancy, France.

ABSTRACT

This study was conducted to evaluate the antioxidant activity of the fixed oil extracted from the seeds and the anti-inflammatory activity of the hydroethanolic extract (30%) of fruit and leaves of the plant Solanum elaeagnifolium. The results show that the fixed oil has a simple composition: palmitic acid 4.89%, Stearic Acid 2.90%, Oleic Acid 11.85%, Linoleic acid 78.65% and stearidonic acid 1.69%. This oil has proven good antioxidant activity in both tests: the total antioxidant power (PAOT®) and antioxidant activity in vitro. Fixed oil, fruit and leaf extracts exhibit cytotoxicity against mobile mononuclear cells of peripheral blood (PBMC) with IC50 values of 120 mg / mL, 80 mcg / mL and 200 mg / mL, respectively. The hydroethanolic fruit extract has more important inhibitory activity of TNF, NO and IL-6 (pro inflammatory) and IL10 (anti-inflammatory) than that obtained with the hydroethanolicextract of the leaves. This activity is good for concentrations confirmed 8µg / mL, 16µg / mL.

Keywords: fixed oil, hydroethanolic extracts, total antioxidant activity, anti-inflammatory activity, cytotoxicity.

Corresponding author

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INTRODUCTION

Solanaceae pass for one of the families who gathers the most dangerous species because of their richness in tropane alkaloids, thus, the number of species used in therapy is low [1]. Among the species in this family, there have been *Solanum elaeagnifolium*prevalent in some African countries, including Mediterranean countries (Algeria, Morocco and Tunisia.

This species includes several types of active substances such asmethyl benzoate kaempferol-3-(6'-coumaroyl-glucoside) which have a cytotoxic activity against the cell lines responsible for breast cancer (MCF7), and those responsible for cancer liver (HPG2) [2].

This Solanaceae was used by Native American in various preparation for curing sore throat and teeth [3]. The aim of this study is the evaluation of the biological activity of this species through the study of the antioxidant power of the fixed oil seeds and anti inflammatory activity of two hydro-alcoholic extracts (fruit and leaves) of this plant.

MANTERIALS AND METHODS

Chemicals:

Cell culture media (RPMI-1640 and DMEM-F12), L-Glutamine solution, penicillin-streptomycin solution, fetal bovine serum (FBS), Histopaque-1077 density gradient media, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Hanks'Balanced Salt solution (HBSS), Phosphate Buffered Saline (PBS), Hydrogen peroxide solution 30% (H_2O_2), lipopolysaccharides powder from Escherichia coli 0111:B4 (LPS), 2,7- dichlorofluorescein diacetate (DCFH-DA), trypsin-EDTA, 2-Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), pyrogallol, sodium azide solution, reduced gluthatione (GSH), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), sulfanilamide and naphthylethylenediaminedihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cytokines ELISA kits: interleukin 1β (IL-1b), interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα) and interleukin 10 (IL-10) were obtained from R&D Systems (Minneapolis, MN, USA).

Plant material:

Solanum eleaegnifolium was collected in the National Park of El Kala situated in the northeast of Algeria, during April 2015. The seeds were dried in the shade, crushed and stored in a glass bottle to protect from moisture and light.

Hydroethanolic extraction:

Dried plant material was extracted with 30% ethanol and shacked at room temperature for 16h. After a second extraction at 37°C for 14h, the macerate was filtered, evaporated using Rotary Evaporators Laborota 4000 (Heildoph, Germany) and lyophilized using a laboratory freeze dryer alpha 1-2 (Christ, Germany) for at least 24h.

Fixed oil extraction:

The extraction of the fixed oil (lipid fraction) have been performed according to Folsh protocol,10g of crushed fruit seed are macerated in 200mL chloroform-methanol (v/v 2:1) and stirred for 1h at room temperature and then the filtrate was washed with NaCl 0.9%. The organic phase containing the lipids was evaporated under vacuum in a rotary evaporator at 40°C [4].

Identification of the fatty acids:

Fatty acids composition of the fixed oil was determined by gas chromatography. The fatty acid methylesters (FAME) were obtained by transmethylation of lipid aliquots (100mg). According to Ackman (1998), the samples were dissolved with 1.5mL of hexane and 1.5 mL of boron trifluoride in methanol (8% w/v)

and heated at 100°C under nitrogen atmosphere for 1h. After cooling, the fatty acid methylesters were extracted into hexane under nitrogen atmosphere. FAME were analyzed by Gas Chromatography on Perichrom™ 2000 System (Saulx-les-Chartreux, France) equipped with a flame ionization detector (FID) and a silica capillary column (50x0,25mx0.5mm, BPX70 EMS, Australia Pty. Ltd.). Temperatures were set as follows: 2min initial period to 120°C, increasing to 40°C/min, the second stage at 180°C for 8min, flowing at 3°C/min to the final period to 220°C for 45min. The injection temperature is maintained at 230°C. The fatty acids were identified by comparing their retention time with the appropriate standards of plant and marine origin (PUFA) standards Supelco (Supelco Park, Bellefonte, PA 16823-0048 USA). Three tests were performed and the results for each fatty acid, are expressed in percentage with respect to total fatty acids identified [5].

Biological activity:

Cell Culture and Treatment:

Peripheral Blood Mononuclear Cell (PBMC) isolation from mouse blood:

Female Swiss albino mice (CD1, Janvier Labs, France), aged 9 weeks (30–40g), were used in this study. They were housed 3 mice/cage and maintained under a reversed light/dark cycle (light on from 8:00 p.m. to 8:00 a.m.) at a constant temperature (22 ± 2°C) and a relative humidity of 55 ± 10%.

Peripheral blood from mice was collected in EDTA tubes (~ 1ml per mouse), pooled and diluted with Dulbecco's Phosphate Buffered Saline (PBS) (ratio 1:1). The diluted samples were subjected to density gradient separation on Hitopaque-1077 medium (ratio 1:1) and centrifuged at 500xg for 30min. After centrifugation, the PBMC layer was collected and washed with Hank's Balanced Salt Sodium (HBSS). PBMC were re-suspended in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine (200mM), 1% of mixture penicillin (100IU/ml) and streptomycin (100µg/ml).

Cell Proliferation and cytotoxicity Assay (MTT assay):

The MTT assay, a tetrazolium salt reduction reaction, is a colorimetric assaywhich provides sensitive measurements of cells viability based on the normal metabolic status of living cells, particularly that of the mitochondrion (Mosman, 1983). Cells (5×10^5cells/well in 96-well plates) were incubated at 37°C for 24h with different concentrations of *Solanum elaeagnifolium* extracts (0 - 500µg/ml). After treatment, the plates were incubated in the MTT solution (final concentration of 0.5mg/ml) for 3h. The dark-blue formazan crystals that formed in intact cells were dissolved with DMSO, and the absorbance at 540nm was measured with a spectrophotometer microplate reader MR96 A (Mindray, China). The results were expressed as the percentage of MTT reduction relative to the absorbances measured from untreated control cells [6-7-8].All assays were performed in quadruplicate. Therefore, the percentage of viability was ultimately calculated using the equation:

Viability (%) =100 (OD sample)/OD control (Mansroi et al., 2003).

Antioxidant activity in vitro:

To evaluate the antioxidant activity in cells in culture, peripheral blood mononuclear cells were employed. Cells were incubated in 6 well plates for 24h under the following conditions: 37°C, 5%CO_2/95% atmospheric air. *Solanum elaeagnifolium* extracts (fixed oil) were added to get final concentrations of 12 and 24µg/ml and plates were incubated for an additional hour. H_2O_2 solution (20mM) was used as external oxidant; and it was added only to half of wells of each vegetal extract in order to induce a possible pro-oxidant effect; and they were incubated for 2h. the cells homogenates and supernatants were stored at -80°C until analysis. Measurements of Lipid peroxidation products, enzymatic activities of Catalase (CAT) superoxide dismutase (SOD), glutathione peroxidase (GPx) and Reactive oxygen species (ROS) determination were applied to evaluate the antioxidant activity induced by Plant nom latin in PBMC [7].

Determination of lipid peroxidation products:

Lipid peroxidation was assayed by the measurement of organic compound, the malondialdehyde (MDA) according to the method of Ohkawa et al. (1979) [9]. Briefly, Cells were lysed by homogenization in ice-cold 1.15% KCl. 100µl of cell lysates were mixed with 0.2ml of 8.1% SDS, 1.5ml of 20% acetic acid (pH 3.5) and 1.5ml of 0.8% thiobarbituric acid and the mixture was adjusted to a final volume of 4 ml with distilled water. After heating to 95°C for 120min, 5ml of mixture of n-butanol and pyridine (15:1, v/v) was added to each sample and the mixture was shaken vigorously and centrifuged at 4000rpm for 10min. The supernatant fraction was collected and the absorbance measured against a reference blank of distilled water by a DU 720 spectrophotometer (Beckman Coulter, USA) at 532nm. The supernatant was collected and Lipid peroxidation was expressed in nmole/mg protein using the molar extinction coefficient for MDA (E532 = 1.56×105 $M^{-1}cm^{-1}$).

Measurement of SOD activity:

Superoxide dismutase (SOD) activity was determined according to the method described by Marklund and Marklund (1974) by assaying the auto-oxidation and illumination of pyrogallol at 440nm for 3min. One unit of SOD activity was calculated as the amount of protein that caused 50% pyrogallol autooxidation inhibition. The SOD activity is expressed as units per milligram of proteins [10].

Measurement of CAT activity

Catalase (CAT) activity was measured according to the method described by Aebi (1984) and Acuna et al. (2009) with modifications by assaying the hydrolysis of H_2O_2 and the resulting decrease was measured in by a DU 720 spectrophotometer (Beckman Coulter, USA) at wavelength absorbance 240nm over a 3min period at 25°C. The activity of CAT was calculated using the molar extinction coefficient (0.04$mM^{-1}cm^{-1}$). The results were expressed as micromole per minute per milligram protein [11-12].

GPx Activity:

To determinate Glutathione peroxidase activity, the method of Flohe and Gunzler (1984) was used with modifications. Briefly, 0.2ml of the cells lysates were mixed with 0.4ml of phosphate buffer (0.4M, pH 7.0), 0.2ml EDTA (0.8mM), 0.1ml sodium azide (10mM), 0.1ml de reduced glutathione GSH (4mM) and 0.1ml de H_2O_2(30mM). The mixture was incubated for 10min in room temperature. After incubation, 0.5ml of TCA (5%) was added and the mixture was centrifuged for 10min at 1500rpm. 0.5ml of DTNB (0.04%) was added and the absorbance was measured against a reference blank of distilled water at 420nm. The GPx activity was expressed as nM of glutathione oxidized/min/mg of proteins [13].

Reactive oxygen species determination:

Intracellular amount of reactive oxygen species (ROS) was measured using a fluorometric assay with DCFHDA used extensively to monitor oxidation in biological and quantify intracellular ROS such as superoxide radical, hydroxyl radical and hydrogen peroxide. The non-fluorescent DCFH-DA was converted to the highly fluorescent 2, 7-dichlorofluorescein product (DCF) after diffusing in the cell membrane, hydrolyzation by intracellular esterases and oxidation by the ROS present in cells. Therefore, The DCF fluorescence intensity and reactive oxygen species were well proportioned (Le Bel et al., 1992). ROS production using DCFHDA was determinated by flow cytometry as previously described (Ardzivian et al., 2016). Briefly, Cells were incubated with DCFHDA (final concentration, 20µM) in HBSS for 30min at 37°C. Then, samples were analyzed using a FC500 flow cytometer (Beckman Coulter, USA) and the results are expressed as the ratio of intensity of fluorescence in treated cells to that of the corresponding fluorescence in the untreated cells [14-15].

Anti-inflammatory activity in vitro:

To investigate the anti-inflammatory effect of *Solanum elaeagnifolium* extracts from fruits and leaves, peripheral blood mononuclear cells PBMC were incubated in 6 well plated for 24h and treated with final concentrations of 20 and 40µg/ml and 8 and 16µg/ml respectively for leaves and fruits extracts of the *Solanum elaeagnifolium* for one additional hour and then incubated to a final concentration 1µg/ml of LPS for 4h.

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Homogenated cells and supernatants were stored at -80°C until analysis. Cytokines Production and Nitric oxide determination were used to evaluate anti-inflammatory effect of *Solanum elaeagnifolium*in PBMC.

Cytokines measurement:

Five mouse ELISA kits were used: interleukin 1β (IL-1b), interleukin 6 (IL-6), TNFα and interleukin 10 (IL-10) and its levels were assessed according to the manufacturer's protocol using a standard curve provided in each kit (R&D Systems, Minneapolis, MN, USA) and a microplate reader MR96 A (Mindray, China) at 450nm.

NO production

Nitric oxide (NO) production was determinated using the Griess reaction assay previously described by green et al, 1982 (Green et al., 1982; Drisch et al., 1998). Briefly, NO was measured by adding 100µl Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100µl of PBMC supernatant [16][17]. The resulting color was measured by a DU720 spectrophotometer (Beckman Coulter, USA) at 540nm. The absorbance values were compared to a standard sodium nitrite curve and the absorbance values were converted to corresponding nitrite concentrations (mM).

RESULTS AND DISCUSSION

Chemical composition of vegetable oil:

The seed oil of *Solanum elaeagnifolium* was extracted with a yield of 18.80% (w/w). Identification of fatty acids by GC / FID (Figure 1) revealed a single chemical composition.

In fact, five compounds were identified with as majority linoleic acid C18: 2n6 (78.65%), (table 1).

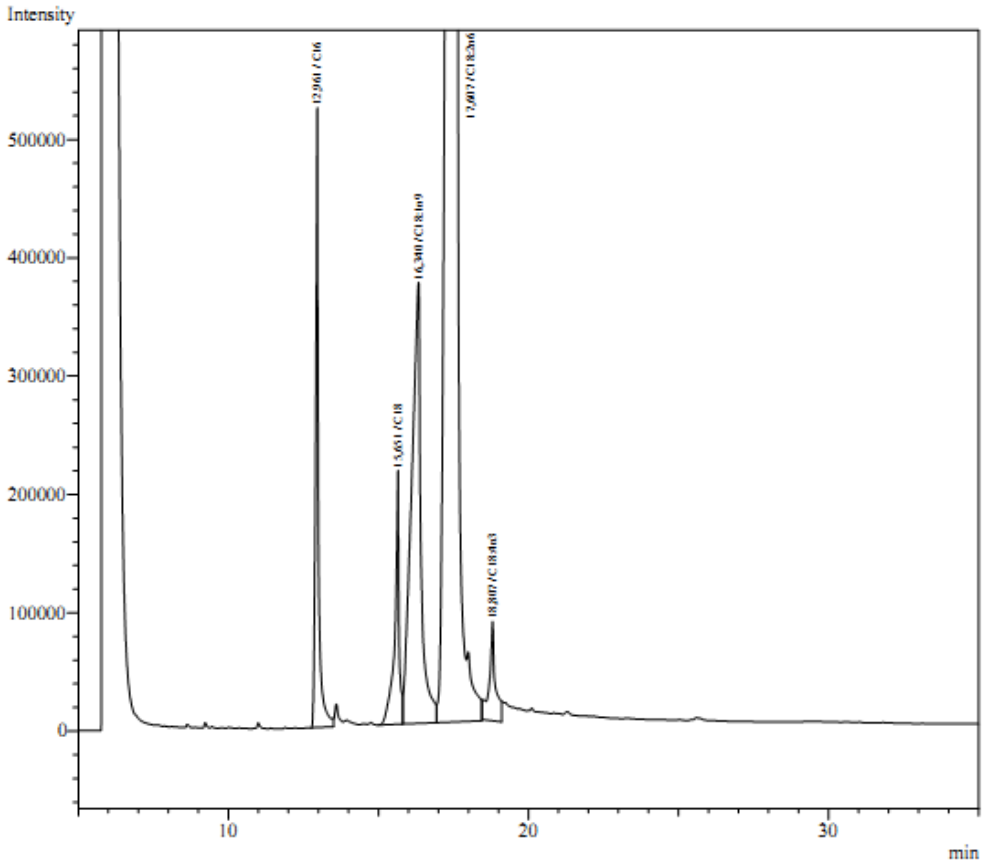

Figure 1: Chromatogram of the fatty oil of *Solanum elaeagnifolium*

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Table 1: Chemical composition of the fatty oil of *Solanum elaeagnifolium*

Peak#	Ret. Time	Area	Area %	Compd. Name
1	12,961	3425454	4,8922	Palmitic ac. C16
2	15,651	2037344	2,9097	Stearic ac. C18
3	16,340	8299673	11,8536	Oleic ac. C18:1n9
4	17,607	55071953	78,6537	Linoleic ac. C18:2n6
5	18,807	1183871	1,6908	Stearidonic ac. C18:4n3
Total		70018295	100,0000	

It turned out that the composition of vegetable oil extracted from *Solanum elaeagnifolium* harvested in Algeria is different from that harvested in Tunisia with twelve compounds [19].

Cytotoxicity:

Cell viability was assessed on cell lines (PBMC: peripheral blood mononuclear cells) by measuring the activity of a mitochondrial enzyme succinate dehydrogenase, using the MTT colorimetric test. The intensity of the color being proportional to the enzymatic activity of viable cells, absorbance was measured using a microplate reader (ELISA). The percentage of cell viability was determined relative to untreated cells.

The results of the evaluation of cytotoxicity of the plant (Figure 2) showed an increase in the percentage of viable cells in PBMC treated with certain fractions EHE fruits, leaves EHE and the fixed oil (500-250, 200-100 mcg/ml).

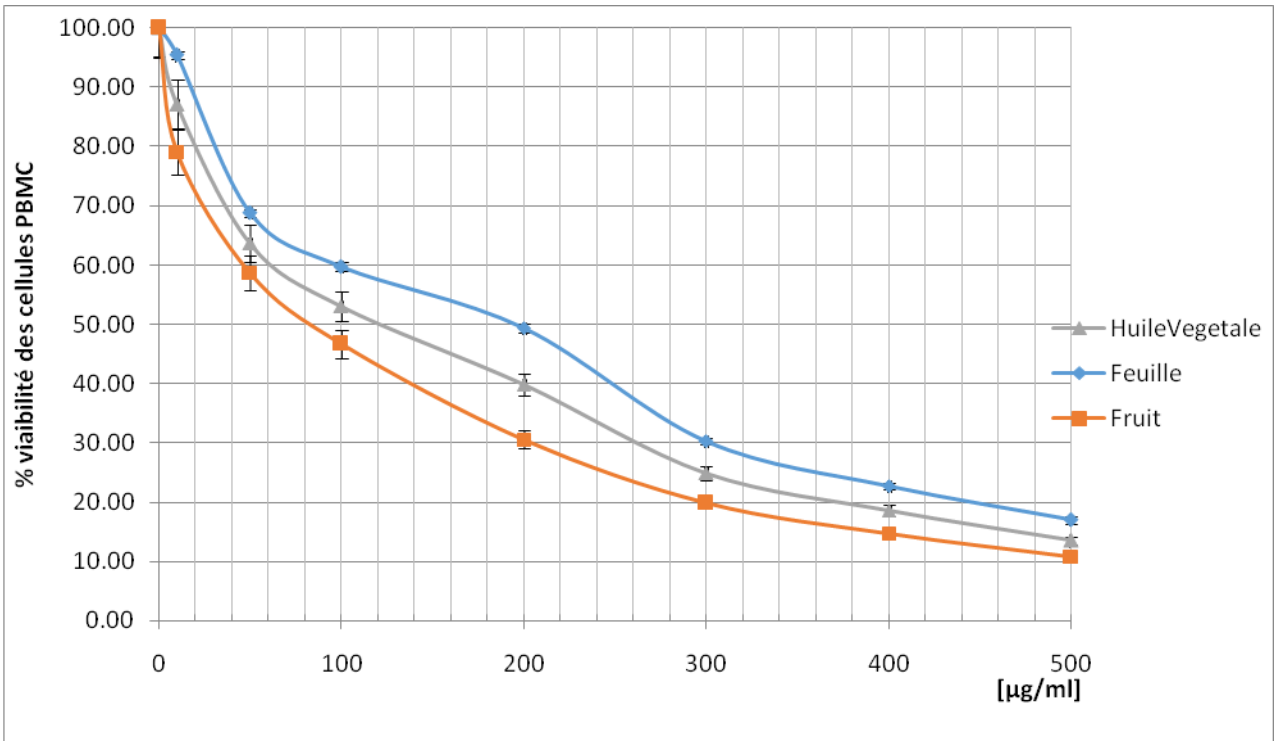

Figure 2: Percentage of cell viability PBMC according to the concentration of plant extracts

A major immuno-modulation was observed in PBMC treated with certain fractions HEE_{fruits} (200-100µg/ml; 80-10 ug/ml), HEE_{leaves} (200-100ug/ml; 50-10µg/ml) and oil fixed (200-120µg/ml, 120-50µg/ml, 50-5g/ml). These results indicate the potential toxicity which is characterized by the IC50 (50% to reduce cell viability) of each extract of the plant (HEE_{fruit}: 80µg/ml, HEEleaves: 200µg/ml, fixed oil: 120µg/ml).

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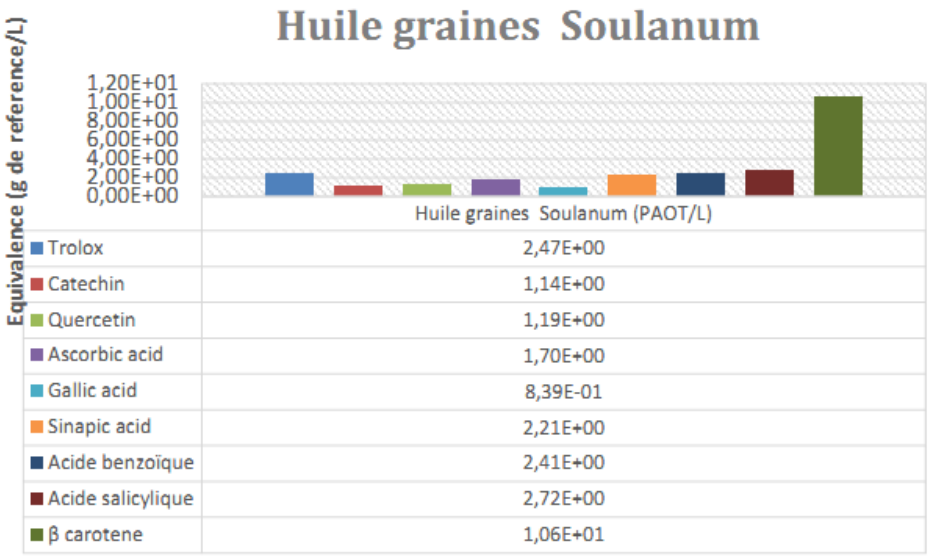
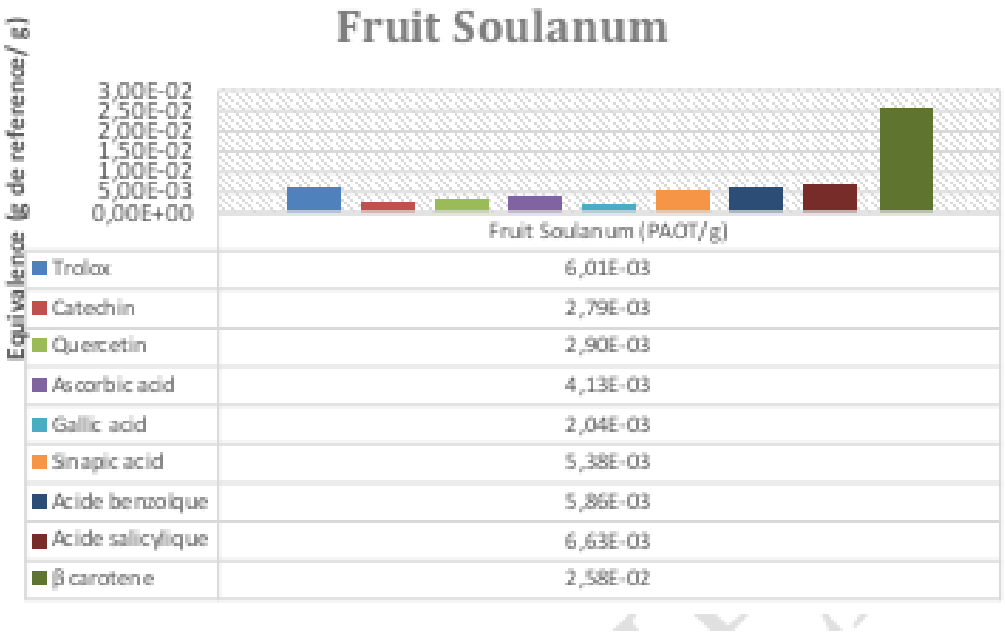
Total anti oxidant activity by the method PAOT/POT®:

In this study, the power or the antioxidant capacity of the fixed oil and hydroethanolics extracts from the fruit and leaf was determined by the PAOT/POT® technology (Total Antioxidant Power / Power Total Oxidant). Theresultsobtained are shown in figure 3:





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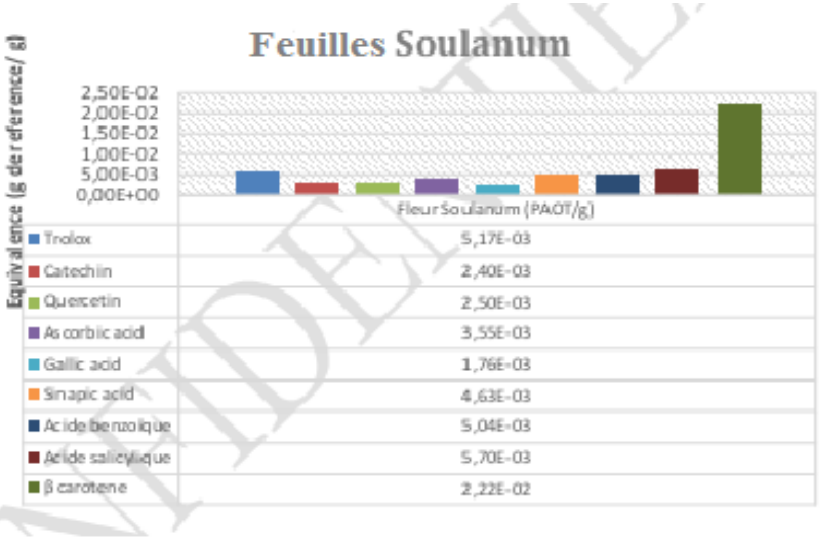

Figure 3: Total antioxidant activity expressed in grams of reference antioxidant per liter or gram of product

The total antioxidant power (PAOT®) a 1gr of hydroéthanolics extracts (fruit and leaf) of *Solanum eleaegnifolium* is equivalent to 22.2mg of β-carotene (α-tocopherol) and 3.35mg of vitamin C (ascorbic acid) from 5.17mg to 2.50mg of trolox and quercetin.

In parallel, the total antioxidant power (PAOT®) of Solanum seed oil of 1Litre is equivalent to 10.6gr of β-carotene and 1.7gr of vitamin C (ascorbic acid), 2.47gr of trolox and 0.83gr ofgallic acid.

Antioxidant Activity in vitro of vegetable oil:

The antioxidant activity was evaluated in spleen cells treated with the vegetable oil, by measuring the rates of enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) using a spectrophotometer UV/Vis [20]. Lipid peroxidation was evaluated by measuring the production of malondialdehyde (MDA) and the mean intensity of fluorescence emitted by the fluorochrome oxidized (DCFHDA) is proportional to the amount of reactive oxygen species (ROS) produced in intracellular, was quantified by flow cytometry.

For the evaluation of this activity was chosen concentration 120µg/mL (IC50 vegetable oil) so that the concentration used in vegetable oil is 24 mcg/ml (IC50/5) and 12µg/ml (IC50/10).

Table 2: Analysis of changes in enzymatic and nonenzymatic parameters at splenocytes

Average	ROS (mean fluorescence intensity)	MDA (µmol MDA/mg proteins) λ= 546nm	SOD activity (USOD/mg proteins) λ= 440nm	CAT activity (µmol H_2O_2/min/mg proteins) λ= 240nm	GPx (nmol GSH/min/mg proteins)
Control (untreated cells)	0,331	0,057	6,647	156,436	18,494
Fixedoil 12	0,618	0,054	8,469	203,953	17,910
Fixedoil 24	0,551	0,054	8,506	171,498	15,835
Fixedoil 12+ H_2O_2	59,135	2,089	30,811	2258,638	25,383
Fixedoil 24+ H_2O_2	41,425	1,602	22,847	2209,351	26,896
H_2O_2	75,400	2,858	33,434	3422,628	24,761

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It is noted a decrease in splenocytes treated with both concentrations of the fixed oil (24µg/ml, 12µg/ml), indicating a decrease in pro-oxidant activity induced by hydrogen peroxide (H_2O_2). These concentrations induced increased CAT levels in splenocytes, meaning an increase in antioxidant activity. Finally, an increased rate of GPx was observed following treatment of cells with concentrations of vegetable oil (12µg/ml, 24µg/ml), showing an increase in antioxidant activity.

Anti-inflammatory activity:

The anti-inflammatory activity of the plant was evaluated on PBMC by measuring levels ofproi-nflammatory markers: nitric oxide (NO), tumor necrosis factor(TNFα), interleukins (IL-6), and anti-inflammatory marker (IL-10) using a microplate reader (ELISA). In addition, the concentrations of HEE were used (fruit and leaves) IC50/5, IC50/10 for each sample (Table3):

Table 3: values used of hydroethanolicextract concentrations of fruits and leaves

Extract	Concentration (µg/ml)
Leaf	40 and 20
Fruit	16 and 8

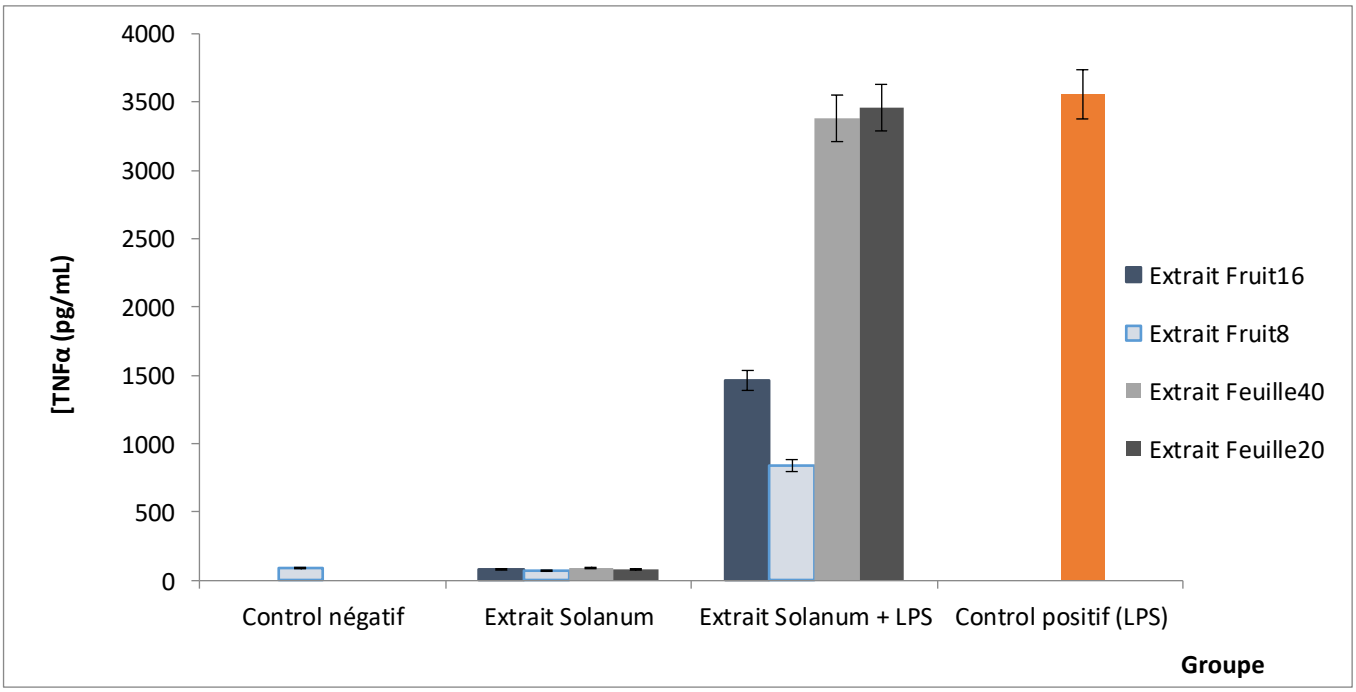

Figure 4: Production of pro-inflammatory TNFα

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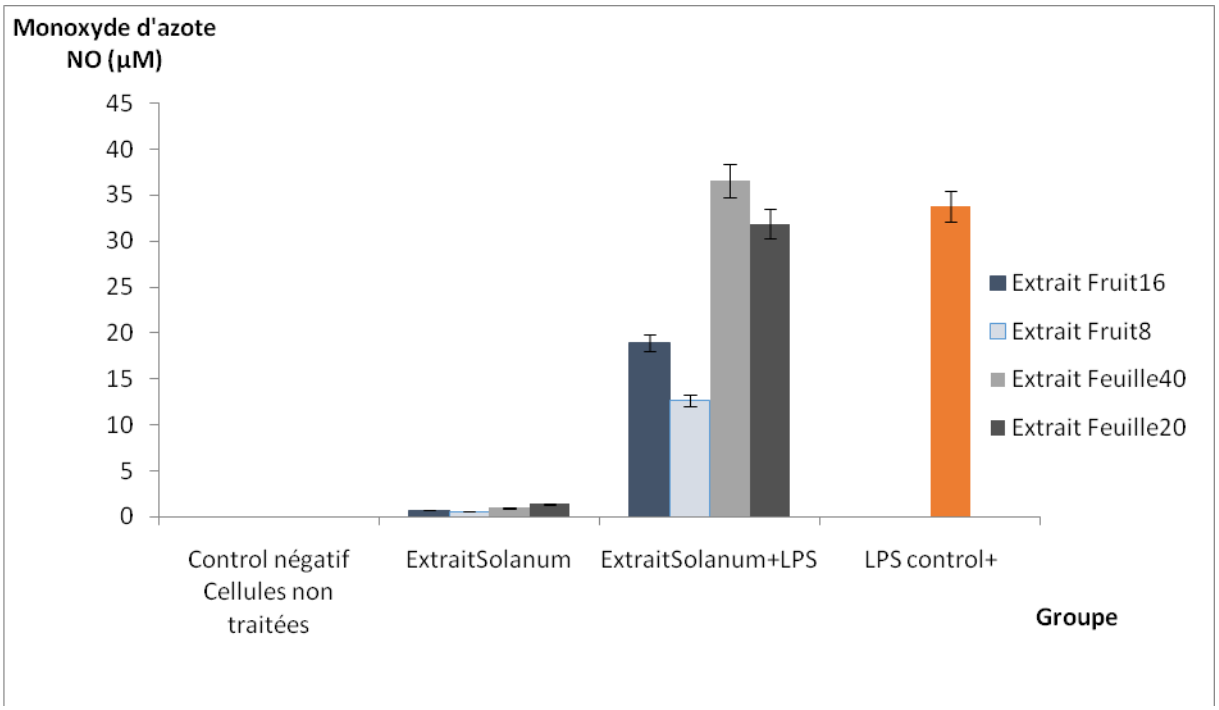

Figure 5: Production of pro-inflammatory NO

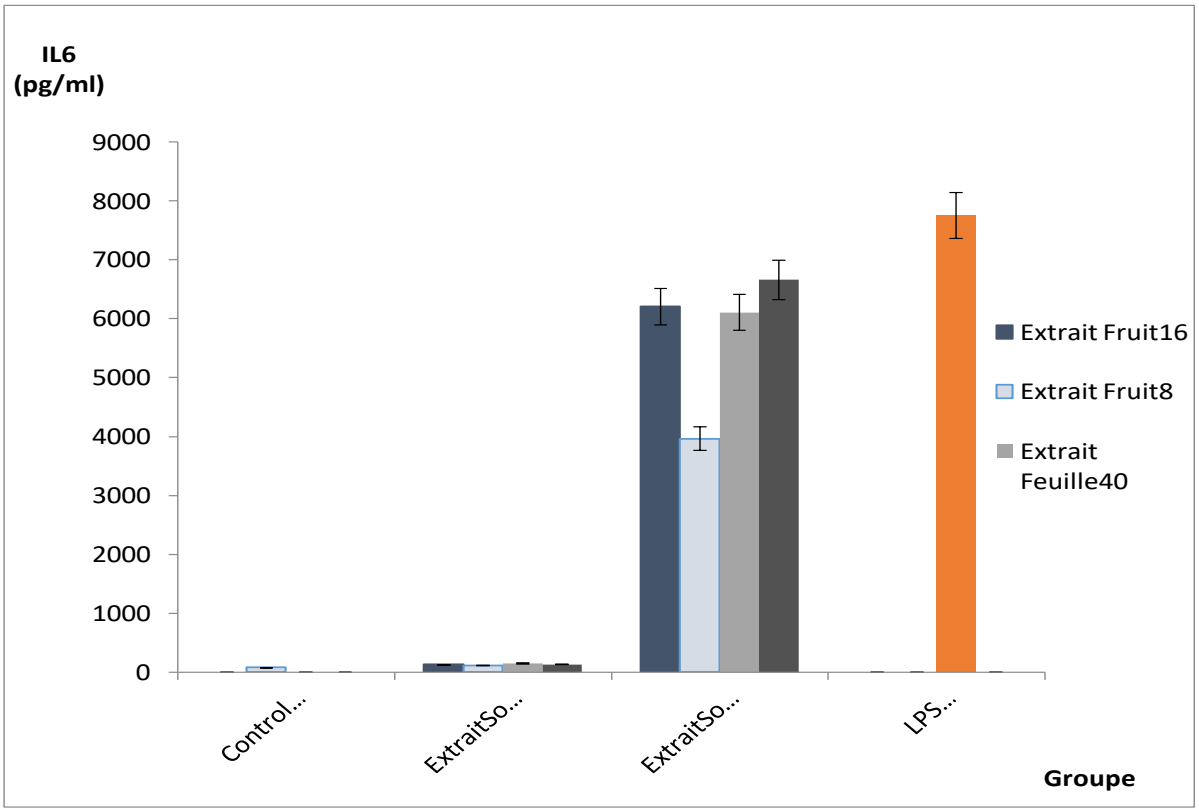

Figure 6: Production of pro-inflammatory IL6

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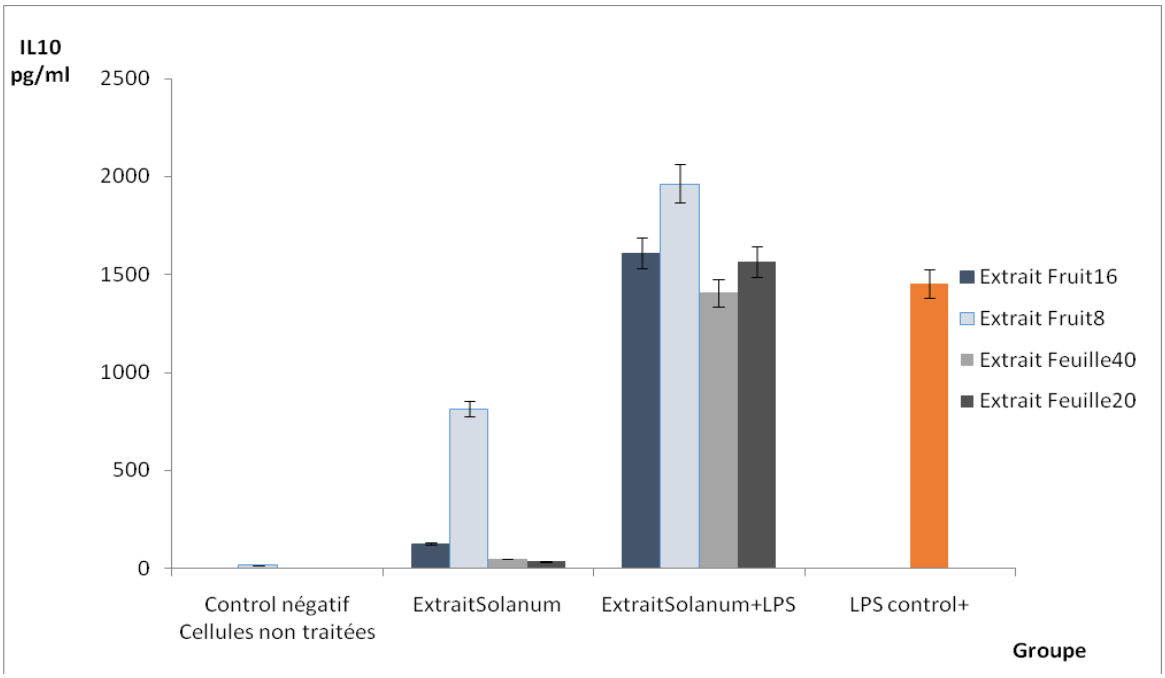

Figure 7: Production of anti-inflammatory IL10

The significant reduction in NO levels, TNF alpha and IL6 (inhibition of the production of pro-inflammatory), was observed following the induction of inflammation in the PBM by lipopolysaccharides (LPS) in cells treated with the plant extracts: HEE_{fruit} (8µg/ml, 16µg/ml), HEE_{leaves} (40µg/ml, 20µg/ml). The results showed a significant decrease in pro-inflammatory activities (Figures 4, 5 and 6) and an increase in anti-inflammatory activities corresponding to an overproduction of anti-inflammatory IL-10 (Figure 7) HEE_{fruits} at 8µg/ml.

CONCLUSION

The study of the chemical composition of vegetable oil extracted from the fruits of *Solanum elaeagnifolium*, revealed its richness in linoleic acid and good antioxidant activity. The evaluation of the in vitro biological activity of plant extracts revealed that the fruit of this plant has a significant anti-inflammatory activity concentration 8µg/ml.

The results of this study allow us supposing that the fruit of *Solanum elaeagnifolium* could be a potential source of natural antioxidant and anti-inflammatory.

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