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Phytochemical Profiling, Cytotoxicity and Anti-inflammatory Potential of *Salvia hispanica* L. Seeds

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Abstract

Seeds of *Salvia hispanica* L. (chia seeds) have recently gained increasing attention as a functional food with numerous therapeutic benefits. The ethyl acetate and the methanol extracts of chia seeds were evaluated for their anti-inflammatory potential by measuring Nitric oxide production in LPS-stimulated RAW 264.7 cells and cytotoxic activity against HepG2, Paca II, HCT116, and HT29 cancer cell lines using MTT assay. The phytochemical composition was analyzed via GC-MS and UPLC-MS. Nineteen compounds were tentatively identified in the methanol extract via LC-MS and 10 compounds from the ethyl acetate extract by GC/MS. The ethyl acetate extract showed a remarkable cytotoxic effect against HepG2 and Paca-II with IC_{50} of 11 μg/mL and 87.7 μg/mL, respectively. The pre-treatment with both extracts significantly suppressed the NO production in LPS-stimulated RAW 264.7 cells. The results indicated that; *S. hispanica* L. seeds could be used as a potent anti-inflammatory and anticancer herbal medicine especially against hepatocellular carcinoma.

Keywords: *Salvia hispanica* L.; UPLC-ESI-MS; GC/MS; anticancer; anti-inflammatory

1. Introduction

Cancer has become the most prevalent and fatal illness nowadays. The majority of currently used cancer medicines have a huge side effect, and not all cancers respond to the treatment in the same way. As a result, novel approaches are crucially demanded. The use of medicinal plants for the prevention and the treatment of cancer has recently attracted increased attention, due to their wide spectrum of phytochemical components and minimal side effects. Additionally, the majority of the developing countries' population is still relying on herbal medications to cure their different maladies and pains [1, 2].

According to reports, inflammation is typically a vital immunological process caused in response to harmful stimuli such as physical agents, faulty immunological responses, toxins and invasive pathogenic bacteria, viruses, and fungi. The primary goal of an inflammatory reaction is to identify and get rid of dangerous substances; a secondary goal is to get rid of components of injured tissue, which leads to the healing of the affected tissues, organs, or systems [3–5]. Such important defense mechanism need to be regulated as the uncontrolled inflammation may lead to pathogenesis of life threatening diseases such as cancer [6].

Salvia hispanica L., or chia, an annual herbaceous plant, belongs to *Salvia* genus, family Lamiaceae, which includes approximately 900 species. Many published studies have demonstrated that different extracts of *Salvia* spp. or their derived phytochemicals exhibited a broad spectrum of pharmacological potential, including anti-

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inflammatory properties and cytotoxic activity against various cancer cells [7, 8].

Chia seeds have recently become one of the most well-known foods in the world due to its nutritional benefits and therapeutic uses [9, 10]. Chia seeds are rich source of dietary fibers, polyphenols as myricetin, rhamnetin, apigenin-7-O-β-D-glucoside, kaempferol-7-O-β-D-glucoside, quercetin, kaempferol, rosmarinic acid and chlorogenic acids and fatty acids as α- linolenic acid [11-14].

Therefore, the current study aimed to explore the anti-inflammatory and the cytotoxic potential of the ethyl acetate and the aqueous methanol extracts of chia seeds against three different carcinomas as well as identifying the phytochemical constituents in both extracts using two different techniques which are UPLC-ESI-MS and GLC-MS.

2. Materials and Methods

2.1. Plant material

The black chia seeds (*Salvia hispanica* L.) were purchased from a local market in Cairo, Egypt in February 2023. The seeds were authenticated by Mrs. Treaze Labib, a plant taxonomy expert at the Agriculture Ministry and the ex-director of Orman Garden. The dried seeds were ground to obtain a fine powder using an electric mortar and the resultant powder was then kept in dark, dry and clean container until extraction.

2.2. Chemicals and reagents

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12), Roswell Park Memorial Institute (RPMI 1640) medium (1% L-glutamine, sodium bicarbonate), Antibiotic-anti-mycotic mixture (10,000U/mL Potassium Penicillin, 10,000μg/mL Streptomycin Sulfate and 25μg/mL Amphotericin B), trypsin 0.15% and fetal bovine serum were purchased from Lonza, Spain. MTT salt (3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich. Dimethyl Sulfoxide (DMSO HPLC grade) was purchased from Loba-chemicals, India. The used solvents were of analytical grade that were obtained from El Nasr Pharmaceutical chemicals company (Kaliobeya, Egypt).

2.3. Extraction process

Two extracts were prepared via soaking 100 g of ground chia seeds in 500 mL solvent, ethyl acetate and 85% aqueous methanol, overnight at room temperature. The mixtures were then filtered and the filtrates were concentrated at low pressure and temperature using rotary evaporator (BUCHI, Germany). The extraction procedure was repeated five times producing two crude extracts with consistent weight which are 25.36 g of ethyl acetate extract and 7.00 g of 85% methanol extract. The two extracts were stored in dark and clean vials for further analyses.

2.4. Chromatographic separation and spectroscopic analysis

2.4.1. UPLC-ESI-MS analysis

Ultra-performance liquid chromatography equipped with negative ion electrospray ionization/triple quadrupole-linear ion trap-tandem mass spectrometer was used to separate and tentatively identify the phytochemicals in the seeds' crude methanolic extract. The sample (100 μg/mL) was dissolved in MeOH (HPLC grade), filtered through a membrane disc filter (0.2 μm) then (10 μL) were injected into XEVO TQD UPLC- ESI-MS apparatus (Waters, Milford, USA). The chromatographic separation was carried out through a reverse-phase RP-C18 column (ACQUITY UPLC— BEH C18 (1.7: 2.1 μm× 50 mm)). The mobile phase comprised water (A) and methanol (B) both filtered, degassed and acidified with formic acid (0.1%) at 0.2 mL/min flow rate. The single run took 32 min. The gradient elution was adjusted as follows: 0–2 min, 10%B; 2–5 min, 10%–30% B; 5–15 min, 30%–70% B; 15–25 min, 70%–90% B; 25–29 min, 90%–100% B; 29–32 min, 100%–10% B (washing and reconditioning the column).

The parameters for MS analysis were conducted in the negative ion mode as follows: source temperature at 150ºC; capillary voltage, 3 kV; cone voltage set at 60 eV; cone gas flow at 50 L/h; desolvation temperature at 440ºC; and desolvation gas flow at 900 L/h. Mass spectra, in the ESI, were recorded between *m/z* 100 Da and 1000 Da. The chromatogram and MS spectra were processed using Masslynx 4.1 software. The tentative identification of secondary metabolites was carried out by comparing the retention time (R_t) of each peak and the corresponding molecular ion peak, together with the major fragments recorded in its spectra, with previously published data.

2.4.2. GLC-MS analysis

The ethyl acetate extract of chia seeds was subjected to gas liquid chromatography hyphenated with mass spectrometry (Trace GC-TSQ mass

spectrometer (Thermo Scientific, Austin, TX, USA) equipped with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness) in order to separate, identify and determine the percentage abundance of its phytochemicals. The column oven temperature was set at 50°C, then increased to 250 °C with a rate of 5°C /min, isothermal for 2 min and finally increased to 300°C and remained constant at 300°C for 2 min. The injector temperature was set at 270 °C while the MS transfer line temperature was set at 260°C. The carrier gas (helium) flows at a constant rate of 1 mL/min, and the solvent delay was 4 min. and One microliter of the test sample, diluted with methanol, was injected automatically using Auto sampler AS1300 coupled with GC in the split mode. EI mass spectra were recorded over the range of m/z 50 Da and 650 Da in full scan mode with ionization voltage of 70 eV. The ion source temperature was adjusted at 200 °C. The phytoconstituents were identified by comparing their retention times and mass spectra with those reported in WILEY 09 and NIST 14 libraries, as well as literature.

2.5. *In-vitro* evaluation of cytotoxic activity

2.5.1. Cell culture

Three human cancer cell lines; human colorectal carcinoma (HCT-116 and HT-29), pancreatic cancer cell line (Paca-II) and hepatocellular carcinoma (HepG2) in addition to human normal immortalized epithelial cell line (hTERT-RPE-1) cell line were initially obtained from ATCC (American type culture collection). All cell lines were kept in DMEM-F12 supplemented with fetal bovine serum (10%). Cells were cultured in a sterile area using a laminar flow cabinet bio-safety class II level (Baker, SG403INT, and Sanford, ME, USA) and were kept at 37 °C in 5% CO_2 and 95% humidity. Cells were sub-cultured using trypsin versene 0.15 % to get monolayer cultures.

2.5.2. Cytotoxic activity on cancer monolayers

The cytotoxic effect of the two chia seeds' extracts on the human cancer cell lines was assessed using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) test according to the method described by [15, 16] with some modifications. Briefly, after 24 h of seeding 2×10^4 cells/well of the various cell lines in 96 well plates, the medium was replaced by fresh medium and the cells were treated with different concentrations (0.78 -100 μg/mL) of the two extracts in triplicates for 48 h. Moreover, 100 μM doxorubicin as positive control and 100 μM DMSO (0.5 %) as negative control were also used. After the incubation period and the discharge of the cells' medium, MTT solution (100 μL) was added to each well then another incubation period for 1 h at 37 ˚C. The formed purple formazan crystals were finally dissolved in DMSO and the optical density was measured at 595 nm and a reference wavelength at 690 nm using microplate spectrophotometer (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA).

The cytotoxicity percentage was calculated according to the following formula:

Cytotoxicity % = [1- (AV_x / AV_{NC})] x 100 Where NC is the negative control's absorbance, Av is the average and X is the sample's absorbance.

The IC_{50} values were calculated using the concentration-response curve fit to the non-linear regression model using GraphPad Prism® v6.0 software.

The selectivity index (SI) was also calculated according to the following formula:

SI = IC_{50} of extract in the normal cell line / IC_{50} of the same extract in the cancer cell line. The higher the SI value, the higher the safety of the extract. Extracts possessing SI value > 2 are considered nontoxic to the normal cells [17, 18].

2.6. *In-vitro* evaluation of anti-inflammatory activity

2.6.1. Cell culture

The RAW 264.7 macrophage cell line was provided by the ATCC. The cells were cultured in RPMI 1640 medium supplemented with penicillin/streptomycin (1%) and heat inactivated fetal bovine serum (10%). The cells were maintained at 37 °C in a 5% CO_2 humidified incubator (Sheldon, TC2323, Cornelius, OR, USA) and were sub-cultured twice before the experiment.

2.6.2. Determination of NO generation

Briefly, RAW 264.7 macrophage cells were firstly grown in 96 well plates at a density of 1×10^5 cells/well for 24 h. Then, they were treated with different concentrations of the two chia seeds' extracts dissolved in DMSO (100, 50, 25, and 12.5 μg/mL) for 1 h then addition of 10 μg/mL Lipopolysaccharide (LPS) for 24 h. Finally, the amount of NO released from the treated and activated macrophages was quantified through mixing 50 μL of the culture supernatant in each well after being transferred to new plates with 50 μL of Griess reagent (sulfanilamide (1%) and naphthylethylenediamine

dihydrochloride (0.1%) in phosphoric acid (2.5%)) then plates were allowed to stand at room temperature for 15 min before measuring at 540 nm using a micro plate reader [19]. A sodium nitrite standard curve was used to calculate the amount of nitrite, as shown in equation:

Nitric Oxide inhibition (%) = $(A_{Control} - A_{Test}/A_{Control}) \times 100$

The cytotoxicity of the two extracts on the remaining RAW 264.7 cells in the plates was also estimated through treating the cells with each extract for 24 hours whether LPS is present or not, and the cell viability was measured according to the MTT method described earlier.

3. Statistical analysis

All statistical analyses were conducted using GraphPad Prism® v7.0 (GraphPad Software Inc., San Diego, CA, USA), through which IC50 values were calculated based on the concentration-response curve using a non-linear regression model and One-way ANOVA with Dunnet's posttest.

4. Results and Discussion

4.1. Phytochemical profiling of the major phytochemicals in *Salvia hispanica* L. seeds

Two different solvent systems were used to extract the bioactive phytochemicals that existed in *Salvia hispanica* L. seeds (black chia seeds) which are pure ethyl acetate and 85% aqueous methanol. UPLC-ESI-MS and GLC-MS are two powerful hyphenated tools, used in metabolic profiling, which were applied in the current study to identify the phytoconstituents in the methanol and the ethyl acetate extracts, respectively.

4.1.1 UPLC-ESI-MS analysis

The UPLC-ESI-MS was used to separate and tentatively identify the major phytochemicals in the crude methanol extract of chia seeds and the total ion chromatogram is displayed in **Figure 1.**

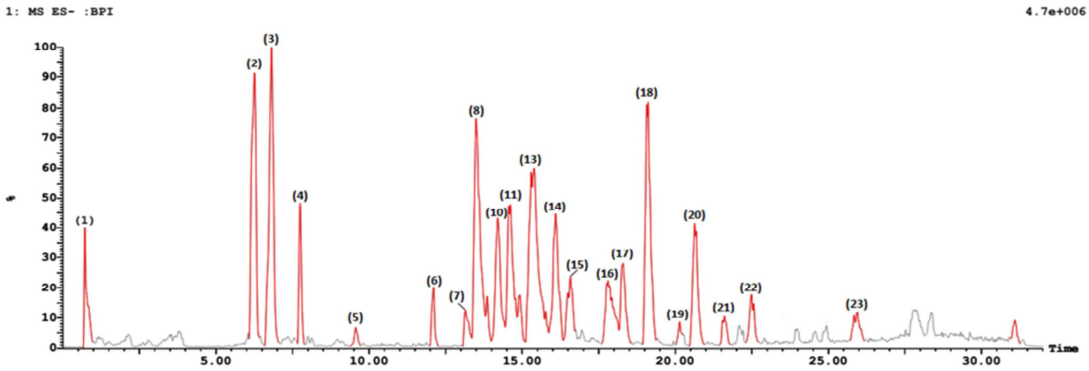

Figure 1: Total ion chromatogram (TIC) of 85% MeOH extract of *Salvia hispanica* L. seeds, peaks numbers indicates compounds tentatively identified in Table 1.

4.1.2. Compounds tentatively identified from 85% MeOH extract of *Salvia hispanica* L. seeds

Twenty-three compounds were chromatographically separated from the 85% methanol extract of chia seeds via UPLC. Nineteen compounds of them were tentatively identified via MS and only four compounds were unknown. The isolated compounds comprise different phytochemical classes including six flavonoids, three phenolic acid and phenolic acid derivative, four unsaturated fatty acids, one saturated fatty acid, one steroid, one sapogenin, one triterpenoid, one triterpene glycoside and one carbohydrate. The retention time, the molecular formula, the molecular weight, the MS fragmentation data and the type of these compounds are presented in **Table 1**. Compound 1, R_t 0.74 min, showed a molecular ion at m/*z* 503 $[M-H]^-$ which produced fragment ions at m/*z* 341 $[M-H-gly]^-$ due to loss of hexose unit (162 Da), 179 $[M-H-2gly]^-$ due to loss of another hexoside moiety and 161 $[M-H-H_2O-2gly]^-$ due to neutral loss of water molecule. This compound was identified as raffinose, a trisaccharide [*α*-D-Gal-(1→6)-*α*-D-Glc-(1→2)-*β*-D-Fru] commonly exists in higher plants [20]. Raffinose was previously reported in chia seeds [21]. Compound 2, R_t 6.26 min, exhibited a molecular ion $[M-H]^-$ at m/*z* 521 which

was further divided into base peaks at m/z 359[M−H−hexoside moiety (162Da)]$^-$, 323, 197 and 161 correspond to the distinctive fragments of rosmarinic acid (an ester of caffeic acid and 3, 4-dihydroxyphenyl lactic acid). This compound was identified as rosmarinic acid hexoside [22] which was previously identified in chia seeds [23]. Compound 3, R_t 6.81 min, showed a deprotonated molecular ion at m/*z* 359 and MS fragment ions at m/*z* 197, 161 and 135 characteristic to rosmarinic acid [24]. Rosmarinic acid was reported as the major polyphenolic constituent in chia seeds that owed remarkable pharmacological potential as antioxidant, antimicrobial, antidiabetic, anti-inflammatory and hepatoprotective agent [25, 26]. Compound 4, R_t 7.75 min, possessed a parent ion at m/*z* 395 and a daughter ion at m/*z* 201. This compound was identified as hexacosanoic acid (cerotic acid) [27]. Compound 5, R_t 9.56 min, had a molecular ion $[M-H]^-$ peak at m/*z* 329 and fragment ions at m/*z* 229 produced due to the loss of 100 Da corresponding to the neutral loss of $(HO-CH=CH(CH_2)_3CH_3)$, an end group of oxylipin molecule, 211, 171 $[OOC(CH_2)_7CH-OH]^-$ and 137. This compound was proposed to be trihydroxy octadecenoic acid [28]. Compound 6, R_t 12.10 min, exhibited a molecular ion peak $[M-H]^-$ at m/*z* 311 and three base peaks at m/*z* 293$[M-H-H_2O]^-$, 275 $[M-H-2H_2O]^-$ and 223, the most intense product ion, formed due to *β*-fission of hydroxyl group and the subsequent loss of 2-hydroxy-butyraldehyde or 1- hydroxy-2-butanone (88 Da). Based on this MS data, this compound was identified as 15, 16-dihydroxy-9, 12-octadecadienoic acid [29]. Compound 7 and compound 8 eluted at R_t 13.14 and 13.50 min, respectively displayed the same parent ions at m/*z* 593 $[M-H]^-$ with fragment ions at m/*z* 473 $[M-H-120]^-$ resulting from the loss of 120 Da which is characteristic to *C*-glycosides and another ion at m/*z* 311 $[M-H-120-162]^-$ due to loss of a glucose residue (162 Da). These compounds were identified as saponarin and isosaponarin [28, 30]. Compound 9, R_t 13.85 min, exhibited a molecular ion at m/*z* 593 and base peaks at m/*z* 503 $[M-H-90]^-$, 473 $[M-H-120]^-$ which are the diagnostic fragment ions of vicenin-2 [31]. glucronoide [38]. Compound 20, R_t 20.63 min, displayed a deprotonated molecule at m/*z* 279 $[M-H]^-$ and a very weak fragment ion at m/*z* 255. This compound was recognized as linoelaidic or linoleic acid [43]. Compound 21, R_t 21.54 min, Compound 10, R_t 14.20 min, exhibited a precursor ion at m/*z* 563 and two fragment ions at m/*z* 503 $[M-H-60]^-$ and 473 $[M-H-90]^-$ which are the characteristic fragment of *C*- pentoside-*C*- hexoside derivative of apigenin. According to this MS results, this compound was either recognized as schaftoside or isoschaftoside [32, 33]. Compound 11, R_t 14.61 min, was identified as stigmasteryl glucoside as it possessed a molecular ion peak at m/*z* 595 $[M-2H+Na]^-$ and a product ion peak at m/*z* 395 corresponding to the dehydrated ion of free sterol, where the m/z for free sterol and steryl glucoside are 412 Da and 574 Da, respectively [34, 35]. Both Δ^5-avenasterol and Δ^7-avenasterol were previously detected in chia seeds [36, 37]. Compound 12, R_t 14.78 min, showed a molecule ion at m/*z* 639 $[M-H]^-$ and a product ion at m/*z* 477 $[M-H-162]^-$ formed due to loss of a hexoside moiety (162 Da) in addition to three other fragment ion at m/*z* 315$[M-H-162-162]^-$, 255 and 151 characteristic to isorhamnetin aglycone. This compound was identified as isorhamnetin dihexoside [38]. Compound 16 appeared at R_t 17.76 with a molecular ion peak at m/*z* 599 $[M-H]^-$ and fragment peaks at m/*z* 447 $[M-H-152]^-$produced as a result of loss of galloyl residue (152 Da), 285$[M-H-152-162]^-$formed due to the hexosyl moiety's loss (162 Da) and 255. Based on this spectral data, this compound was identified as kaempferol-*O*- galloylhexoside [39]. Compound 18, R_t 19.07 min, showed a molecule ion at m/*z* 455 and a base peak at m/*z* 377 which are characteristic peaks to oleanolic (OA) or ursolic (UA) or betulinic acids (BA) [40, 41]. Both OA and BA were tentatively identified in the aerial parts of *S. hispanica* [41]. The three triterpenoids were previously separated from *Salvia* sp. and exhibited numerous therapeutic activities where UA and OA showed anticancer, antimicrobial, anti-inflammatory, hypoglycemic, hypolipidemic, antiulcer and hepatoprotective activities while BA possessed anticancer, anti-inflammatory and antimalarial potential [42]**.** Compound 19, R_t 20.14 min, showed a molecule ion at m/*z* 339 in addition to fragment ions at m/*z* 163 and 119 which are characteristic fragments of *p*-coumaric acid. This compound was identified as *p*-coumaric acid exhibited a molecular ion peak at m/*z* 471 $[M-H]^-$ and a fragment ion peak at m/*z* 397. This compound was identified as hederagenin [44]. Compound 22, R_t 22.49 min, was identified as oleic acid as it possessed a molecular ion peak at m/*z* 281 [45]. According to

[46], chia seeds are rich source of polyunsaturated fatty acids mainly linolenic acid, linoleic acid and oleic acid. Compound 23, R_t 25.84 min, was identified as periandrin III as it exhibited a parent ion peak at m/*z* 822 $[M-2H]^-$ and a daughter peak at m/*z* 762 corresponding to loss of one molecule of carbon dioxide (44 Da) and another water molecule (18 Da) [47]. Rosmarinic acid, rosmarinic acid hexoside, oleanolic acid/ ursolic acid/ betulinic acid and isosaponarin represent the dominant secondary metabolites in the methanol extract of chia seeds based on LC-MS analysis.

Table 1: Compounds tentatively identified in *Salvia hispanica* seeds' 85% methanolic extract by UPLC-ESI-(-ve)-MS

Peak no.	RT (min)	MW	Molecular Formula	$[M-H]^-$ (m/*z*)	Fragments (m/*z*)	Tentative Identification	Type
1.	0.74	504	$C_{18}H_{32}O_{16}$	503	341, 179, 161	Raffinose	Carbohydrate
2.	6.26	522	$C_{24}H_{26}O_{13}$	521	359, 323, 197, 161	Rosmarinic acid hexoside	Phenolic acid derivative
3.	6.81	360	$C_{18}H_{16}O_8$	359	197, 161, 135	Rosmarinic acid	Phenolic acid
4.	7.75	397	$C_{26}H_{52}O_2$	395(M-2H)	201	Hexacosanic acid	Saturated fatty acid
5.	9.56	330	$C_{18}H_{34}O_5$	329	229, 211, 171, 137	Trihydoxyoctadecenoic acid	linoleic acid-derived oxylipins (Monounsaturated fatty acid)
6.	12.10	312	$C_{18}H_{32}O_4$	311	293, 275, 223	15,16-dihydroxy-9,12-octadecadienoic acid	Diunsaturated fatty acid
7.	13.14	594	$C_{27}H_{30}O_{15}$	593	473, 311, 153	Isovitexin-7-*O*-glucoside (saponarin)	Flavonoid
8.	13.50	594	$C_{27}H_{30}O_{15}$	593	473, 311	Isovitexin 4'-*O*-glucoside (Isosaponarin)	Flavonoid
9.	13.85	594	$C_{27}H_{30}O_{15}$	593	503, 473	Apigenin 6,8-di-*C*-glucoside (Vicenin-2)	Flavonoid
10.	14.20	564	$C_{26}H_{28}O_{14}$	563	503, 473	Apigenin-*C*-hexoside-*C*-pentoside (Schaftoside or isoschaftoside)	Flavonoid
11.	14.61	597	$C_{35}H_{58}O_6Na$	595	395	Stigmasteryl glucoside (Δ^5-avenasteryl glucoside or Δ^7-avenasteryl glucoside)	Steroid
12.	14.78	640	$C_{28}H_{32}O_{17}$	639	477, 315, 255, 151	Isorhamnetin dihexoside	Flavonoid
13.	15.39	572	----	571	504, 295, 277	Unknown	----
14.	16.09	541	----	540	480, 452, 297, 255	Unknown	----
15.	16.49	567	----	566	506, 483, 297, 255	Unknown	----
16.	17.76	600	$C_{28}H_{24}O_{15}$	599	447, 285, 255	Kaempferol-*O*-galloylhexoside	Flavonoid
17.	18.29	569	----	568	508, 480, 283	Unknown	----
18.	19.07	456	$C_{30}H_{48}O_3$	455	377, 277, 255	Oleanolic acid or ursolic acid or betulinic acid	Triterpenoid
19.	20.14	340	$C_{15}H_{16}O_9$	339	163, 119	*p*-coumaric acid glucronoide	Phenolic acid derivative
20.	20.63	280	$C_{18}H_{32}O_2$	279	255	Linoelaidic acid or Linoleic acid	Polyunsaturated fatty acid (omega-6 fatty acid)
21.	21.54	472	$C_{30}H_{48}O_4$	471	397	Hederagenin	Sapogenin
22.	22.49	282	$C_{18}H_{34}O_2$	281	116	Oleic acid	Polyunsaturated fatty acid
23.	25.84	824	$C_{42}H_{64}O_{16}$	822 (M-2H)	762	Periandrin III	Triterpene glycoside

4.1.3. GLC-MS analysisGC/MS analysis of the ethyl acetate extract of chia seeds revealed the identification of 10 compounds constituting 99.33% of the total composition of the extract. These compounds, listed in Table 2, were identified by comparing their retention time, mass spectra with reported data and library search. The GLC-MS chromatogram was displayed in Figure 2. The major constituents in the extract, based on the peak area percentage, were methyl linolenate (39.67%), α-linolenic acid (19.24%), β-sitosterol (11.95%), methyl palmitate (6.26%) and palmitic Acid (6.03%), respectively. The chemical structure of these compounds as well as their MS fragmentation patterns were shown in Figure 3. The percentage of unsaturated fatty acids reached 62.79%, and the percentage of unsaturated phytosterols were 18.66% while that of the saturated fatty acids were only 18.15%.

Table 2: Chemical constituents of the ethyl acetate extract of chia seeds by GC-MS

No.	Rt.	Area %	Compounds	Molecular Formula	Molecular weight
1	25.63	6.26	Methyl palmitate	$C_{17}H_{34}O_2$	270
2	26.42	6.03	Palmitic acid	$C_{16}H_{32}O_2$	256
3	28.72	39.67	Methyl linolenate	$C_{19}H_{32}O_2$	292
4	28.82	3.88	Methyl octadecenoate	$C_{19}H_{36}O_2$	296
5	29.38	2.72	Methyl stearate	$C_{19}H_{38}O_2$	298
6	29.53	19.24	α-Linolenic acid (Omega-3 fatty acid)	$C_{18}H_{30}O_2$	278
7	30.08	2.87	Stearic acid	$C_{18}H_{36}O_2$	284
8	43.91	3.34	γ-sitosterol (Clionasterol)	$C_{29}H_{50}O$	414
9	44.21	3.37	Stigmasterol	$C_{29}H_{48}O$	412
10	44.73	11.95	β-sitosterol	$C_{29}H_{50}O$	414
% Saturated fatty acids (SFA)					18.15
% Unsaturated fatty acids (USFA)					62.79
% Unsaturated phytosterol					18.66

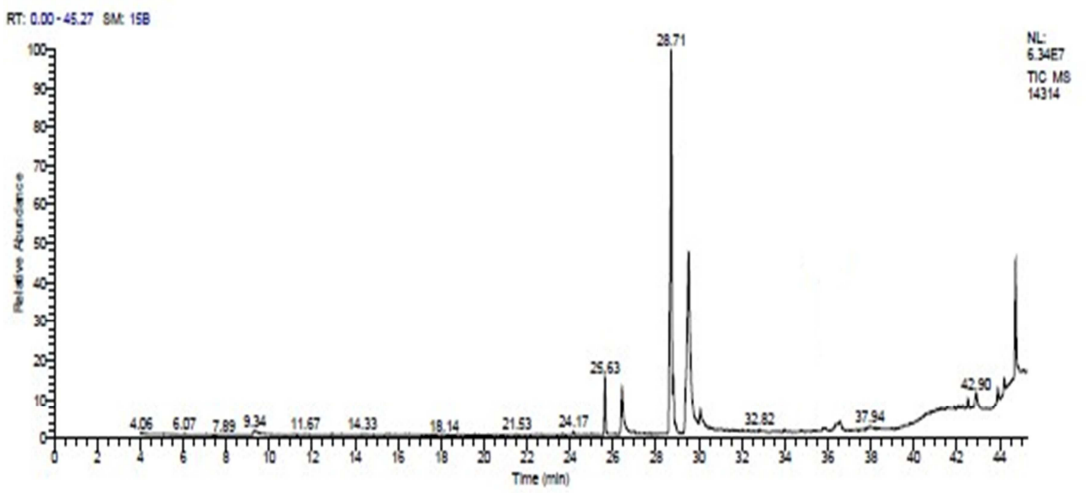

Figure 2: GLC-MS chromatogram of the ethyl acetate extract of chia seeds

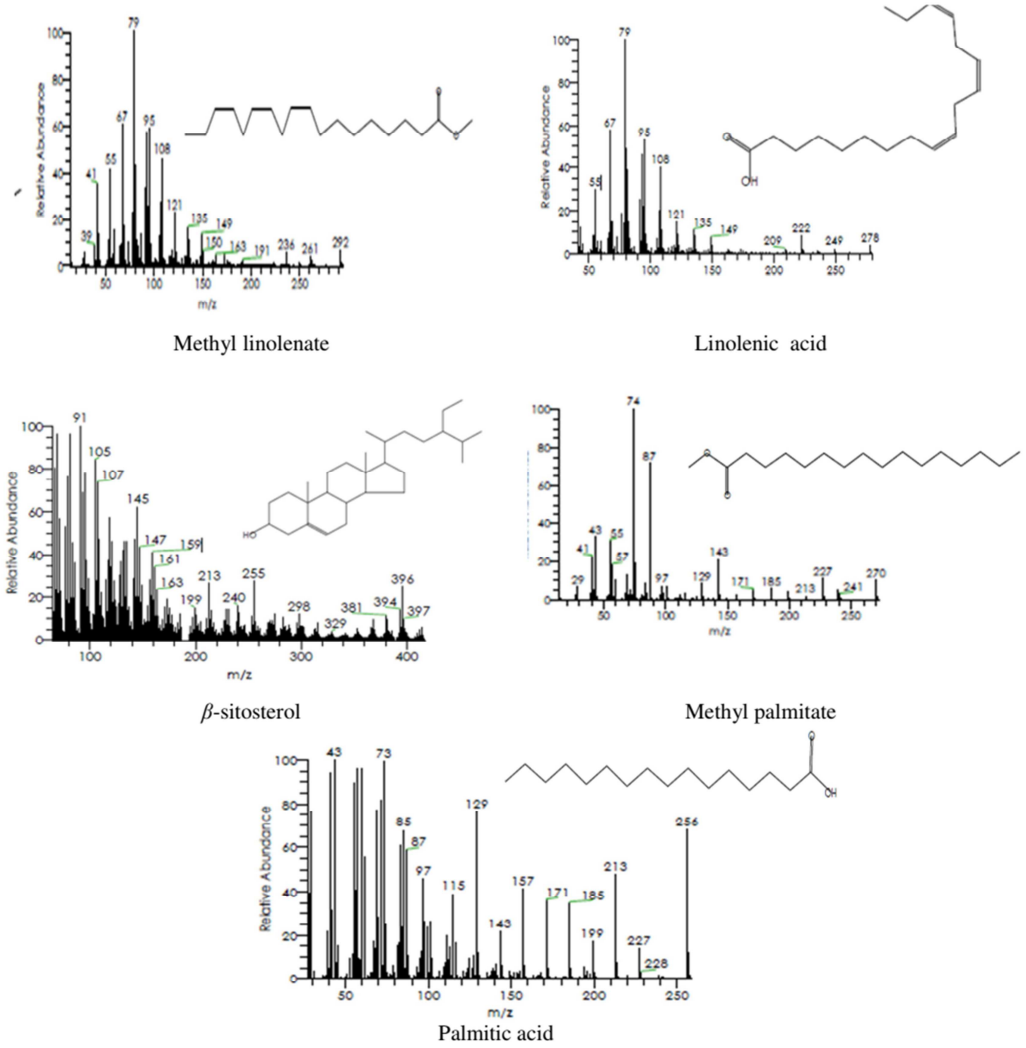

Figure 3: MS spectra and chemical structures of the major chemical constituents of the ethyl acetate extract of chia seeds

4.2. *In-vitro* evaluation of cytotoxic activity

The cytotoxic potential of the ethyl acetate as well as the methanol extracts of the black chia seeds was estimated against HepG2, Paca II, HCT116 and HT29 cancer cell lines using MTT assay.

The two extracts exhibited no cytotoxic potential against RPE-1 cell line and variable anticancer response against the three tested cancer cell lines as shown in **Figure 4**.

The ethyl acetate extract of the chia seeds showed potent cytotoxic effect against HepG2 as well as Paca-II cell lines with IC_{50} of 11 μg/mL and 87.7 μg/mL and cytotoxicity percentage at 100 μg/mL of 98% and 56.2%, respectively. It also exhibited significant cytotoxic potential against HepG2 cells (**Figure 5**) in comparison to doxorubicin at the same concentration. Moreover, it appeared to be more selective to hepatocellular carcinoma (SI ≥ 16.05) than pancreatic cancer cells (SI ≥2.01)**.**

On the other hand, the methanol extract exhibited moderate cytotoxic effect against Paca-II (cytotoxicity % = 65.3%) and HepG2 (cytotoxicity % = 56.7%) cells with an IC_{50} of 82 μg/mL and 34

μg/mL, respectively and SI of ≥1.3 and ≥3.18, respectively.

Moreover, both extracts exhibited no cytotoxic potential against the colon cancer cell lines (HCT116 and HT29). The reported cytotoxic activity of chia seeds against colorectal cancer was attributed to its wealth with fibers, indigestible by the human body, and minerals especially calcium which aid in the prevention of constipation and formation of adenomatous polyps which are the main risk factors of development of colorectal cancer [48] which were not included in our study.

Based on the United States National Cancer Institute (NCI) considerations, a crude extract is assumed to be a promising anticancer agent if its IC_{50} value ranges between 30 and 40 μg/mL and consequently both extracts could be a promising source for the development of anticancer drug against HepG2 especially the ethyl acetate extract regarding their IC_{50} values as mentioned above. Furthermore, other published reports considered the extract to be effective against cancer proliferation if its IC_{50} value is up to 100 μg/mL and hence both extracts could be effective against both HepG2 and Paca-II cancer cell lines [49].

In addition, the SI, also referred to as a therapeutic index, is a crucial indicator in deciding which extract/s will proceed further in laboratory and clinical trials. The relationship between the extract's potency (pharmacology) along with its safety (toxicology) is thought to be quantitative. Extracts with a SI value of two or greater than two are thought to have good selectivity against particular cancer cells and to have good chance to develop novel, highly selective anticancer drugs [50]. As a result, both extracts possessed high selectivity toward HepG2 cells.

Many published papers reported the potent efficacy of chia seeds against proliferation of several cancer cell lines such as liver, pancreatic and colorectal cancer. Such significant activity was attributed to the richness of the chia seeds with omega-3 fatty acids, phenolics, phytosterols, tocopherols and fibers [12]. Tocopherols, omega-3 unsaturated fatty acid and phytosterols can reduce the formation of bad cholesterol and saturated fats within the body and hence can suppress the incidence of obesity as well as nonalcoholic fatty liver and type II diabetes which are the main threats of liver and pancreatic cancers' development [51, 52].

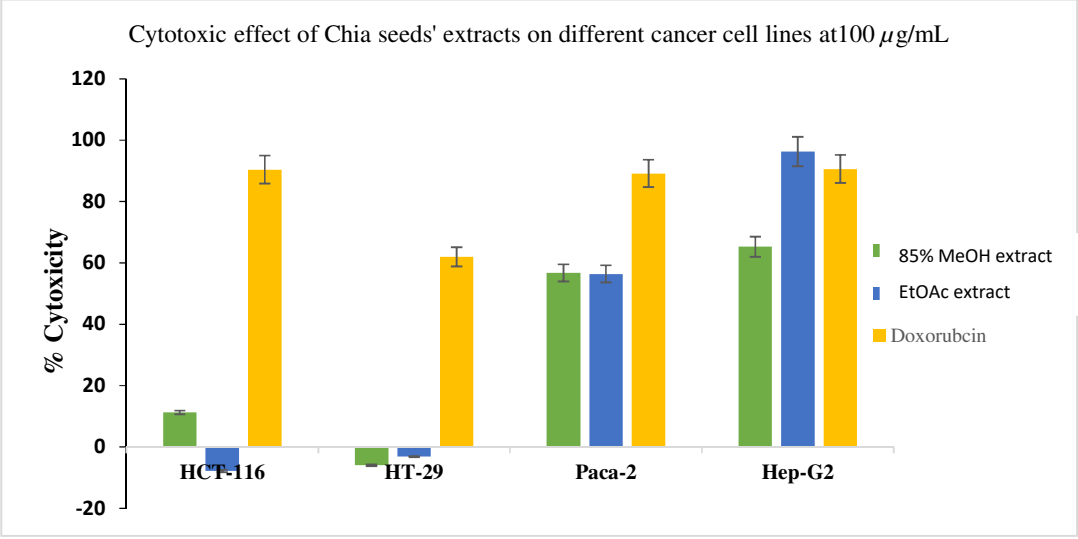

Figure 4: Average % cytotoxicity of two plant extracts on HCT-116, HT-29, Paca-2 and Hep-G2cell lines. Cell viability was determined after 48hr and evaluated by MTT assay. Values are expressed as mean ± SD, n = 3 at a concentration of 100μg/mL.

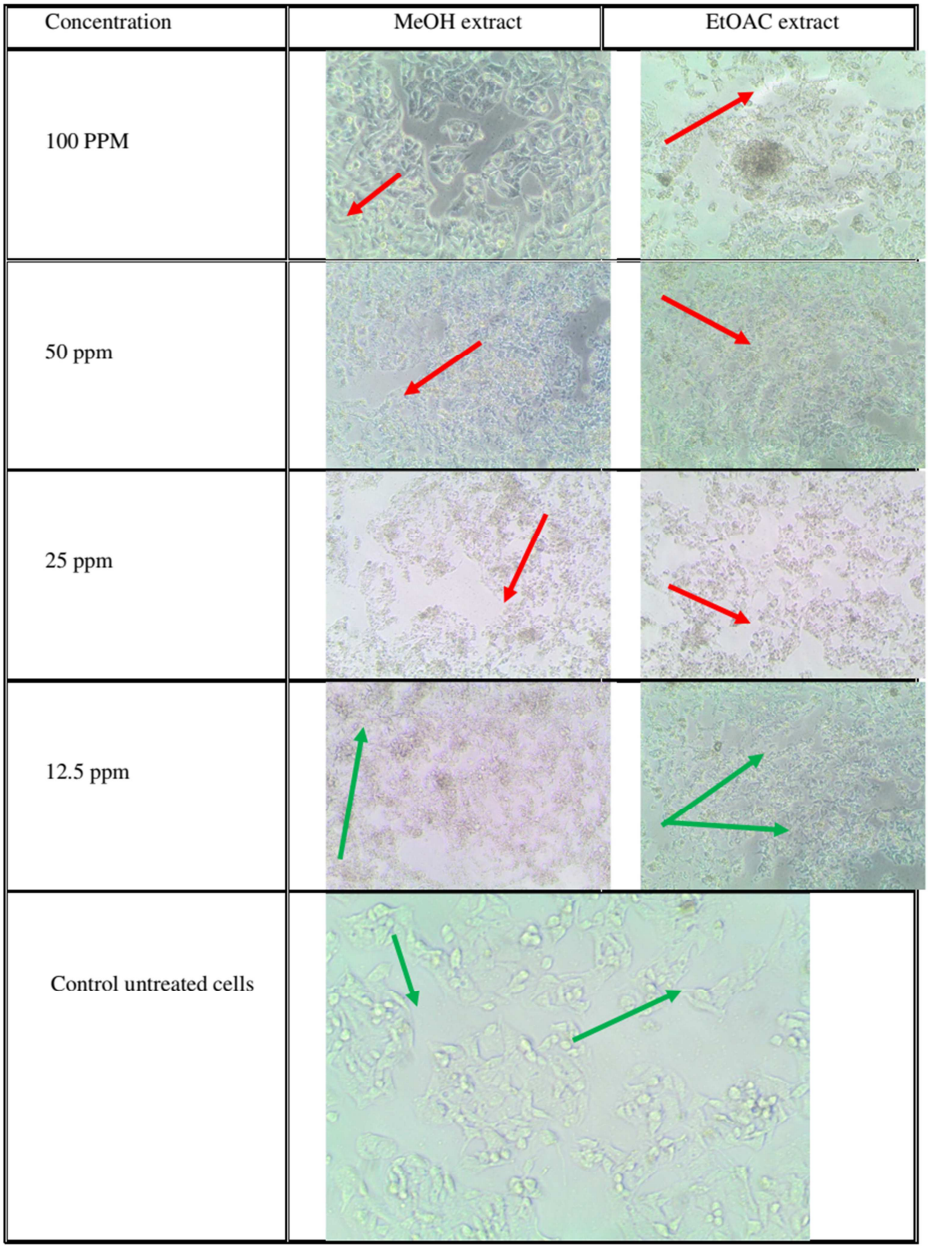

Figure 5: images of HepG2 cell line cultivated as 2D monolayers after 48 h with the ethyl acetate and the methanol extracts of Chia seeds at different concentrations (100, 50, 25 and 12.5ppm). **Green arrows** refer to normal epithelial-like cells which were mostly observed; spindle-shaped cells were sporadically found in less confluent places. While **red arrows** refer to dead cells, cell viability was determined by MTT assay. Control untreated cells were cultured in plain media. Using cellsens software and an Olympus CKX41 inverted phase-contrast microscope, imaging was done manually.

4.3. *In-vitro* evaluation of anti-inflammatory activity

Nitric oxide (NO), chemical mediator, plays a critical role in the pathogenesis of inflammation related disorders. The anti-inflammatory potential of the two chia seeds' extracts was assessed through measuring the quantity of nitrite ions (NO_2^-), stable metabolite of NO released from LPS-activated RAW 264.7 macrophages, using the Griess reagent.

Results in **Figure 6** showed that LPS alone markedly induced NO production. However, the pre-treatment with the two test extracts significantly

suppressed the NO production in LPS-stimulated RAW 264.7 cells especially the ethyl acetate extract. Moreover, no significant difference was observed in the anti-inflammatory response of the two extracts in the LPS-activated RAW 264.7 macrophages.

Furthermore, the cell viability was not affected by the two understudying extracts as indicated by the MTT reduction assay and consequently, it is clearly concluded that the anti-inflammatory potential in the LPS-stimulated RAW 264.7 macrophages was not because of the loss of cell viability but due to the anti-inflammatory action of the tested extracts (**Figure 7**). Such anti-inflammatory potential could be attributed to the bioactive phytoconstituents existed in both extracts.

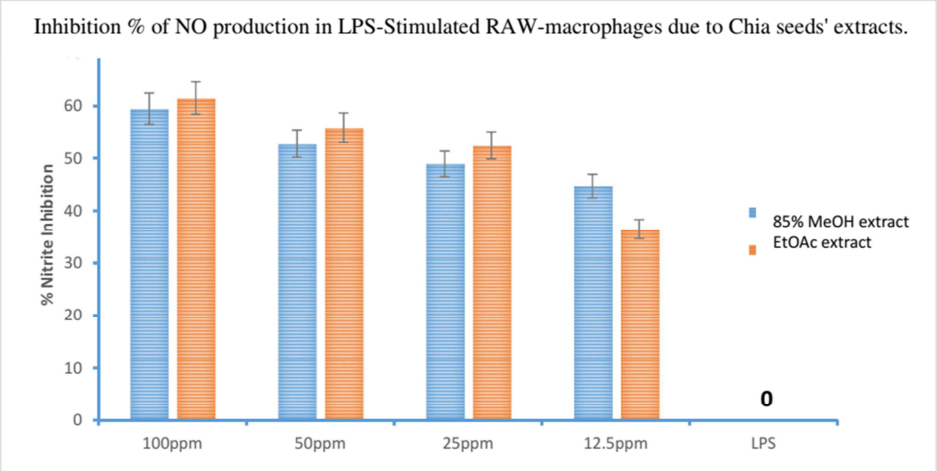

Figure 6: The effect of the two chia seeds' extracts on the inhibition % of nitric oxide production in the LPS-stimulated RAW264.7 macrophages. Values are expressed as means ±SD (n=3), p<0.05.

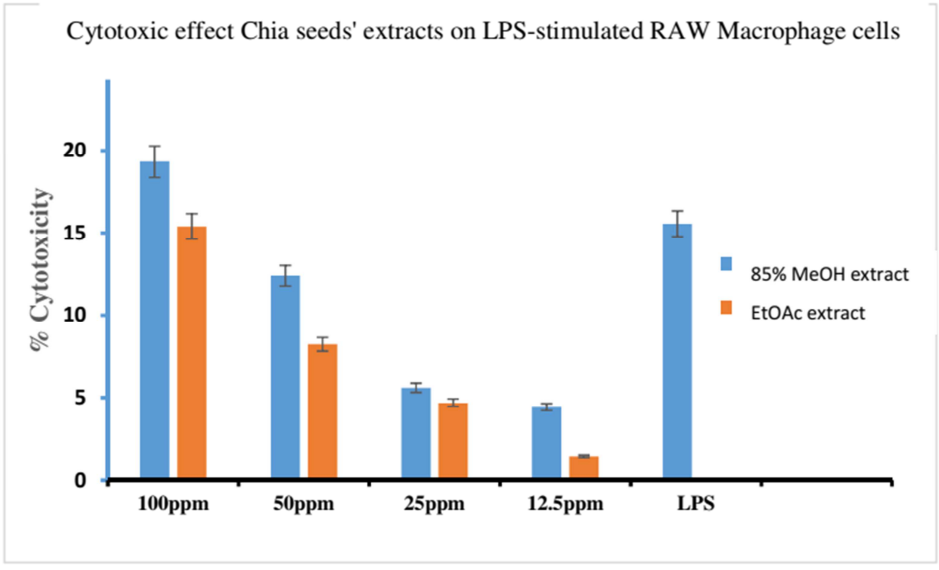

Figure 7: Effect of the two chia seeds' extracts on the viability of RAW-264.7 macrophages at concentrations (100-12.5 μg/mL). Cells were treated with each extract for 24 h and the cell viability was assessed using MTT assay. Values are expressed as means ±SD (n=3), p<0.05.

5. Conclusion

Both the ethyl acetate and the methanol extracts of black chia seeds exhibited significant anti-inflammatory potential in the LPS-activated RAW 264.7 macrophages and potent antitumor potential against HepG2 and Paca II cell lines. The ethyl acetate extract of chia seeds showed a remarkable cytotoxic potential and high selectivity toward HepG2 cells and hence it could be considered as a potent highly selective anti- hepatocellular carcinoma candidate. Moreover, the methanol extract of chia seeds is rich with diverse bioactive phytoconstituents. Rosmarinic acid and its glycosidic derivative represented the major phytochemicals in the methanol extract of chia seeds based on UPLC-ESI-MS analysis. Also, the ethyl acetate extract of chia seeds comprised saturated, unsaturated fatty acids in addition to phytosterols according to GLC-MS analysis. Chia seeds could represent a good opportunity to find novel and equipotent alternatives to be applied in the food and drug industries.

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7. Conflict of Interest

The authors declare that they have no conflict of interest.

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