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Pharmacological investigations and chemical constituents of *Salsola kali* aerial parts

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Abstract

This study aimed to investigate the chemical constituents and *in vitro* pharmacological activity (antioxidant, antimicrobial, anti-inflammatory and cytotoxic activity) of the ethyl acetate (EtOAc) and methanol (MeOH) extracts of *Salsola kali* aerial parts. EtOAc extract showed potent activity against all tested pharmacological studies better than MeOH extract. It exhibited potent antioxidant activity for three tested mechanisms where, the total antioxidant capacities (TAC) were 873.27 and 302.5 mg AAE/g extract, reducing power was 223.62±3.46 & 213.02±1.99 mg AAE/g extract and DPPH scavenging activity with an IC_{50} of 41.52±2.09 and 80.64±4.53 µg/ml for EtOAc and MeOH extracts of *S. kali*, respectively. EtOAc and MeOH extracts displayed moderate to weak antibacterial activity against tested strains. EtOAc extract revealed the highest membrane stabilizing activity with IC_{50} of 1.68 mg/mL whereas, MeOH extract exhibited membrane stabilizing activity with IC_{50} of 2.60 mg/mL as compared to the drug reference. EtOAc extract showed potent cytotoxic activity against HepG2, A549 and MCF-7 with IC_{50} 24.7, 29.3, and 44.0 µg/mL respectively, while the MeOH extract showed slight activity against the three tested cell lines with IC_{50} between 180-291µg/mL. For the first time, salisoflavan, syringic acid, and tricin-7-O-glucopyranoside were isolated from this plant. LC-MS analysis of the EtOAc extract of *S. kali* led to the identification of 127 phytoconstituents.

Keywords: *Salsola kali*, phytochemical studies, LC-MS, cytotoxic, anti-inflammatory, antimicrobial, antioxidant.

INTRODUCTION

Medicinal plants are an excellent source for treating diseases. Most people rely on medicinal plants because they don't have the side effects that come from manufactured medications. Plant-based products are especially effective for the treatment and prevention of a wide range of human disorders, and most people around the world rely on traditional medicine, particularly plant-based medicines, for their primary healthcare [1]. Salsola is a well-known Chenopodiaceae family genus with over 200 species found in arid and semi-arid regions of the Middle East, Asia, Europe, and Africa [2,3]. Some Salsola plants are commonly used in folk medicine to cure hepatitis [4] or diseases caused by tapeworms and parasites [5], and they also exhibit substantial vasoconstrictive, hypertensive, and cardiac stimulant action [6], as well as the ability to operate as an allergic material [7-8]. *Salsola* species exhibit anti-inflammatory and antioxidant properties. Some Salsola species were utilized as diuretic, anti-hypertensive, anti-cancer, purgative, emollient, anti-ulcer, and anti-inflammatory products in Russia, China, and Bahrain [9,10]. Previous phytochemical investigations on the genus Salsola yielded alkaloids, saponins, sterols and their glucosides, comarinolignan, isoflavonoids, and flavonoids [11-14]. Due to a lack of data on the pharmacological potential and phytochemistry of *S. kali*, and in continuation of our research on Chenopodiaceae family [15-17], we concerned to investigate the chemical constituents of EtOAc by chromatographic methods and LC-MS technique. Moreover, to estimate the pharmacological activity of EtOAc and MeOH extracts such as antioxidant, antimicrobial, anti-inflammatory and cytotoxic activity.

MATERIALS AND METHODS

Materials

All chemicals and reagents used in this study were analytical grade and obtained from Sigma-Aldrich (USA). For LC-MS, HPLC grade acetonitrile and methanol were purchased from Thermo-Fisher Scientific (Waltham, MA, USA), while formic acid (98%), ammonium hydroxide, and ammonium formate were purchased from Sigma-Aldrich Co. (USA).

Cell Lines and Culture

MCF-7 cells (a human breast cancer cell line), HepG2 cells (a human hepatocellular carcinoma cell line), and A549 cells (a human lung cancer cell line) were used and collected from The American Type Culture (ATCC, Rockville, MD). The cells were cultured in RPMI-1640 media with 10% inactivated fetal calf serum and 50g/ml gentamycin. The cells were cultivated two to three times a week at 37^{o}C in a humidified environment with 5% CO_2.

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Plant Material and Authentication

Plant materials were collected in May 2020 from the northern coastal region (Egypt). This halophyte was identified by the Herbarium team of Desert Research Center where A voucher specimen (CAIH-1024-R) was placed at the Desert Research Center's Herbarium.

Methods

Extraction

Salsola kali aerial parts were air-dried and finely powdered (630 g) and extracted in a 5 L conical flask for 48 hours using methanol (MeOH) (95%, 3 L). The extraction process was repeated under the same conditions three times. All aqueous methanolic extracts were collected, filtered, and vacuum evaporated. The resulting methanol extract was suspended in water and worked up successively with *n*-hexane, EtOAc, and MeOH. The resulting extracts were evaporated in a vacuum to get 14, 3.4 and 31g, respectively.

Phytochemical studies

Phytochemical isolation

About 3.4 g of EtOAc extract was performed on silica gel (60-120 mesh) column chromatography 2.5×120 cm with *n*-hexane eluting solvent, by increasing polarity using EtOAc to 100% MeOH. Seventy-one fractions (200 ml) were afforded. Similar fractions (E8-E28) were collected and applied on TLC silica gel plates with eluting system hexane: EtOAc: MeOH (6:3:1) which afforded one major spot, compound (**1**) (18 mg). Fractions (E35-E65) were also collected and applied to TLC with the eluting system (EtOAc: MeOH) (7:1.5) afforded two major spots, compound **2** and **3** (16 and 21 mg), respectively. The purification of isolated compounds was done using methanol as an eluting agent on the Sephadex column (60 x 1.5 cm). Physical and chemical properties of isolated compounds were proven by spectroscopic analysis using UV, MS, ^{1}H-NMR, ^{13}C-NMR by comparing to other available data reported in the literature.

LC-ESI-TOF-MS Analysis of Ethyl acetate Extract

LC-ESI-TOF-MS analyses of the EtOAc were performed as described by [18] at Proteomics and Metabolomics Research Program (Children's Cancer Hospital-Cairo, Egypt). The sample was injected in both positive and negative modes. The apparatus descriptions, techniques, methods of sample analysis and its identification were described in [19] and open-source software [20] was used for the non-targeting, small molecule comprehensive analysis of the sample. As reference databases, ReSpect positive (2737 records) or ReSpect negative (1573 records) databases were employed depending on the acquisition technique.

Pharmacological investigations

Antioxidant assay

Total antioxidant capacity

The *S. kali* EtOAc and MeOH extracts' total antioxidant capacities were assessed using the phosphomolybdenum reduction potential (PRP) method detailed in [21]. For a 90-minute incubation period at 95°C, 0.3 ml of extract was combined with 3 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The method's foundation is the extracts' conversion of Mo (VI) to Mo (V), which is followed by the creation of a green phosphate/Mo (V) complex at an acidic pH. The reaction solution was cooled to room temperature before measuring the absorbance at 695 nm in comparison to a blank. The amount of ascorbic acid equivalents used to measure the extract's antioxidant activity.

Reducing power

The reducing capacity of EtOAc and MeOH extracts was monitored according to the method described in [22]. 1ml of prepared phosphate buffer (0.2 molL-1, pH 6.6) and 1ml potassium ferricyanide (10%) were added and mixed with an aliquot (1ml) of different concentrations of sample or ascorbic acid (standard). In blank tubes were prepared in a similar manner replacing potassium ferricyanide with ethanol/H_2O. All tubes were incubated at 50°C for 20 min. 2.5 ml of prepared Trichloroacetic acid (10%) was added to all tubes. All tubes were centrifuged at 3000 rpm for 10 min. 50µl upper layer from all tubes was taken, mixed with 50µl distilled water in wells. 10 µl ferric chloride (0.1%) was added to each well except sample blank wells. All wells were left to stand for 30 min at 25°C min (the test was carried out triplicate in each mixture using microtiter plate). The absorbance of blue ferrous complex was measured at 700 nm. The reducing power of the plant extracts was expressed in relation to the reducing power of ascorbic acid (mg of ascorbic acid equivalents per g extract).

DPPH free radical scavenging activity

The DPPH radical scavenging assay, which is described in [23], was used to evaluate the extracts' capacity to scavenge free radicals. The ability of the EtOAc and MeOH extracts to donate hydrogen atoms was assessed using the decolorization of a methanol solution containing 2, 2-diphenyl-1-picrylhydrazyl (DPPH). In methanol solution, DPPH creates a violet or purple color that fades to varying degrees of yellow in the presence of antioxidants. In order to combine different amounts of extract in methanol, 2.4 mL of 0.1 mM DPPH solution in methanol was produced. The reaction mixture was completely vortexed and kept at room temperature for 30 minutes in the dark. At 517 nm, the mixture's absorbance was determined spectrophotometrically. As a benchmark, ascorbic acid was employed. The following equation was used to compute the percentage of DPPH radical scavenging activity: The formula for % DPPH radical scavenging activity is (A0-A1)/A0×100. A1 is the absorbance of the extracts/standard, while A0 is the absorbance of the control. Then the percentage of inhibition was plotted against concentration, and the IC_{50} was determined from the graph. Three times at each concentration, the experiment was repeated.

Antimicrobial activity

Gram-positive bacteria strains included *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* RCMB 015 (1) NRRL B-543; Gram-negative bacteria strains included *Escherichia coli* ATCC 25922 and *Proteus vulgaris* RCMB 004 (1) ATCC 13315. Ketoconazole is the typical positive control drug for fungal strains, whereas Gentamycin is utilized for bacteria strains used for evaluation of the antimicrobial efficacy of EtOA and MeOH extracts using the diffusion agar technique at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University in Egypt [24,25]. The organisms were inoculated in nutritional broth and incubated overnight at 37°C in order to reduce the turbidity to 0.5 McFarland standards, yielding a final inoculum of 1.5 108 CFU/mL.

Agar plate grass culture was performed using standardized microbial culture broth. In DMSO, plant extracts at a concentration of 10 mg/mL were produced. With the help of a sterile cork-borer (6 mm), 6 mm wells were bored in the inoculation media. Each well received 100 L of extract. It was allowed to diffuse for around 30 minutes at room temperature before being cultured for 24 hours at 37 degrees Celsius (bacterial strains) and 7 days at 25 degrees Celsius (fungal strains). After incubation, the test compounds' antimicrobial activity was determined by looking at the plates for the development of a clear zone around the well. The observed zone of inhibition (ZOI) was measured in mm.

Red blood cell membrane stabilizing potentials

The anti-inflammatory activity of *S. kali* extracts was determined utilizing a membrane stabilizing technique for human red blood cells. This experiment was carried out using the procedure described in [26]. Human blood samples were collected in heparinized tubes and centrifuged three times for 10 minutes at 3000 rpm with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate-buffer (pH 7.4). Stock erythrocyte (RBC) suspension (0.50 mL) was combined with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (1, 2, 3, 4 mg/ml) or Diclofenac potassium (0.25, 0.5, 1, 2, 3, mg/ml). The control sample was 0.5 ml of RBCs mixed with hypotonic-buffered saline solution on its own. The mixtures were incubated at room temperature for 10 minutes before being centrifuged for 10 minutes at 3000 rpm and the absorbance of the supernatant was measured at 540 nm with an optima spectrophotometer. The test was performed three times. The following equation was used to compute the % inhibition of either hemolysis or membrane stabilization: % hemolysis inhibition = 100 x (OD_1-OD_2/OD_1) Where OD_1 represents the optical density of hypotonic-buffered saline solution alone (control) and OD_2 represents the optical density of the test sample in a hypotonic solution. The IC_{50} value was then obtained, which represents the concentration of medication or extract required to inhibit erythrocyte hemolysis by 50%.

Cytotoxicity Evaluation Using MTT Viability Assay

The tumor cell lines (MCF-7, HepG-2, and A-549) were suspended in media at a concentration of $5x10^4$ cells/well in Corning® 96-well tissue culture plates for antitumor tests, then incubated for 24 hours. The varying concentrations of the tested extracts EtOAc and MeOH were then added to 96-well plates (three repetitions) to achieve a total of twelve concentrations for each extract. As a control, six vehicle controls with media or 0.5% DMSO were run for each 96-well plate. The MTT test was used to assess the number of viable cells after 24 hours of incubation. In brief, the media in the 96 well plate was replaced with 100 µl of new culture RPMI 1640 medium without phenol red, followed by 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS) in each well, including the untreated controls. The 96 well plates were then incubated for 4 hours at 37°C and 5% CO_2. An 85 µl aliquot of the medium was taken from each well, and 50 l of DMSO was added to each well and carefully mixed with a pipette before incubating at 37°C for 10 minutes. The optical density was then measured at 590 nm with a microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells, and the viability percentage was calculated as [(ODt/ODc)]x100%, where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The survival curve of each tumor cell line after treatment with the specified substance is obtained by plotting the relationship between surviving cells and drug concentration. The 50% inhibitory concentration (IC_{50}), or the concentration required to elicit harmful effects in 50% of intact cells, was calculated using Graphpad Prism software (San Diego, CA, USA) using graphic plots of the dose-response curve for each concentration [27].

Statistical analysis

For statistical analysis, three replicates of each sample were employed, and the values are reported as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Phytochemical isolation

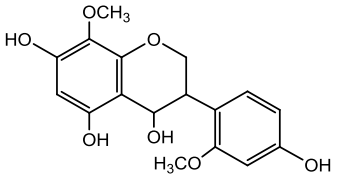
Literature revealed that EtOAc fraction is rich in semi-polar constituents, including sterols and terpenes. Also, is rich in coumarins, phenolic compounds and flavonoid aglycones and mono-glycosides and sometimes di-glycosides flavonoids. Some of these compounds were isolated by performing on silica gel columns using hexane: EtOAc to MeOH solvent systems with a gradual increase in polarity which led to isolation and identification of three major compounds with finally purification on sephadex LH-20 column. The structure elucidation of these compounds was proven with different spectroscopic apparatuses (MS-UV- NMR of ^{1}H and ^{13}C).

Compound (1): isolated as a colorless amorphous powder. The EIMS spectrum exhibited molecular ion peak at *m*/*z* 318 and the apparent molecular ion was observed at *m*/*z* 300 after loss of a water molecule. which corresponds to molecular formula ($C_{17}H_{18}O_6$). UV λ_{max} (nm): MeOH: 285, 210; (NaOMe): 265, 305, 355; (NaOAc): 264, 305, 355; (NaOAc/H_3BO_3): 253, 270,305 nm. ^{1}H-NMR (400 MHz $CDCl_3$): 3.98 (1H, dd, *J*= 9.5, 2.5 Hz, H-2_{ax}), 3.72 (1H, t, *J* =9.5 Hz, H-2_{eq}), 3.36 (1H, m, H-3), 5.42 (1H, d, *J* =7.5 Hz, H-4), 7.01 (1H, d, *J*=8.4 Hz, H-5), 6.44 (1H, d, *J*= 8.5 Hz, H-6), 6.29 (1H, d, *J*= 2.5 Hz, H-3'), 6.76 (1H, dd, *J*= 8.5, 2.5 Hz, H-5'), 7.45 (1H, d, *J*=8.5, H-6'), 3.82 (6H, s, OMe). ^{13}CNMR ($CDCl_3$): 67.4 (C-2), 40.7 (C-3), 76.9 (C-4), 119.0 (C-5), 105.4 (C-6), 154.1 (C-7), 136.2 (C-8), 155.5 (C-9), 113.1 (C-10), 123.7 (C-1'), 154.5(C-2'), 101.5 (C-3'),158.0 (C-4'), 108.4 (C-5'),133.1 (C-6'), 58.4 (C2'-OMe), 60.6 (C8-OMe). The physical and chemical data predicted the chemical structure of compound was to be Salisoflavan compared to data recorded in [28].

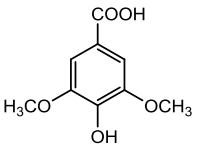
Compound (2): was isolated as a pale-yellow powder, showed R_f on silica gel TLC: 0.28 (BAW), 0.03 (15% AcOH). The EIMS spectrum exhibited molecular ion peak at *m/z* 198 $[M+H]^+$ with molecular formula $C_9H_{10}O_5$. UV λ_{max} (MeOH) nm: 245.^{1}H-NMR (400 MHz, MeOD) δ: 7.26 (2H, s, H-2, H-6), 3.84 (6H, s, OMe). ^{13}C-NMR (MeOD, 100 MHz) δ: 176.2 (C=O), 120.7 (C-1), 106.5 (C-2, C-6), 147.4 (C-3, C-5), 140.6 (C-4), 56.6 (OMe). The out-lined data found to fit well with that a symmetric trisubstituted phenolic acid of 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid)

Compound (3): was isolated as yellow amorphous powder, showed R_f on silica gel TLC: 0.37 (BAW), 0.04 (15% AcOH). UV λ_{max} (MeOH) nm: 246, 269, 300, 354; (NaOMe): 279, 278, 395. ($AlCl_3$): 275, 305, 365, 406; (NaOAc): 259, 277, 425, 416. The EIMS spectrum exhibited molecular ion peak at *m/z* 493 $[M+H]^+$ with molecular formula $C_{23}H_{24}O_{12}$. ^{1}H-NMR (400 MHz,

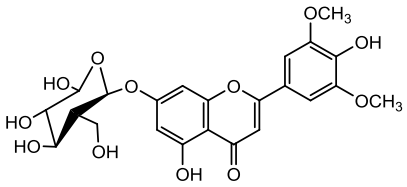
MeOD) δ: 7.14 (2H, s, H-2', H-6'), 6.84 (1H, br. s, H-8), 6.68 (1H, br. s, H-3), 6.43 (1H, d, *J*=1.8 Hz, H-6), 5.09 (1H, d, *J*=7.50 Hz, H-1" a numeric H), 3.82 (6H, s, -OMe at C-3' and C-5'), 3.42-4.03 (m, overlapped, glucosidic protons). ^{13}C-NMR (MeOD, 100 MHz)δ: 184.04 (C-4), 166.71 (C-2, C-7), 163.80 (C-9), 156.98 (C-5), 147.81 (C-3', C-5'), 141.84 (C-4'), 120.64 (C-1'), 104.61 (C-2', C-6'), 103.14 (C-3), 102.64 (C-10), 101.26 (C-1"), 101.16 (C-6), 96.55 (C-8), 79.02 (C-3"), 77.31 (C-5"), 72.90 (C-2'). The physical and chemical spectral data were accordance with those data reported in the literature [29] and predicted to the chemical structure of compound was to be Tricin-7-*O*-glucopyranoside.



1- Salisoflavan



2- Syringic acid



3- Tricin-7-O-glucopyranoside

Fig 1. Chemical structure of isolated compounds from *S. kali* EtOAc extract

Characterization of phytochemical constituents of EtOAc extract using LC-Ms

The identification of secondary metabolites in the aerial parts of *S. kali* was done using LC-ESI-TOF-MS system in positive and negative mode resulting in 127 compounds including phenolic, flavonoid, nitrogenous, terpenes and other related compounds. In this study, we discussed the identified nitrogenous, carboxylic, terpene and stilbene secondary metabolites, while the phenolic and flavonoid components discussed in part II [15].

Each component was identified primarily based on its mass spectrum, fragmentation patterns, and prior publications on the phytoconstituents of *S. kali* and other plants in the genus Salsola. Furthermore, based on the total peak area in the chromatogram, the relative percentages of the detected chemicals in the plant extracts were computed.

LC-MS analysis led to the identification of 59 nitrogenous, 9 carboxylic, 2 terpenes and 1 stilbene compound, representing about 52 % of the total content. The nitrogenous compound included purines, pyrimidine and indole alkaloids, amines, amino acids and their derivatives, thiazole, and fatty acids summarized in Table 1 and fig 2 according to their retention time (RT) and percentage %. α-Aminoisobutyrate (9.765%), histidine (2.321%) and tyrosine (2.045%) were identified as the major amino acids and their derivatives constituents, Thymidine-5'-monophosphate (2.962%), 6-Hydroxynicotinic acid (2.041%), trans-Zeatin-9-glucoside (1.474 %), S-Adenosyl-L-methionine (1.245%) and adenine (1.112%) were the major alkaloid and its related compounds. While, the major constituents of phenolic and carboxylic compounds were malic acid (9.065%), chlolic acid (3.921%) and rosmarin acid (1.245%).

Table 1: List of the identified N-compounds, carboxylic, terpene and stilbene constituents of EtOAc extract of *S. kali* by LC – ESI-TOF–MS in both positive and negative modes.

No	RT (min)	%*	Adduct ion	Molecular Formula, Precursor *m/z*	Compounds Identity**
1	1.08	0.011	$[M+H]^+$	$C_6H_{14}N_2O$ (131.12)	N-Acetylputrescine
2	1.08	9.065	$[M-H]^-$	$C_4H_6O_5$ (133.01)	D-(+)-Malic acid
3	1.09	0.133	$[M-H]^-$	$C_4H_4N_2O_2$ (111.07)	Uracil
4	1.11	2.962	$[M-H]^-$	$C_{10}H_{15}N_2O_8P$ (321.07)	Thymidine-5'-monophosphate
5	1.13	0.525	$[M-H]^-$	$C_7H_{12}O_6$ (191.09)	D-(-)-Quinic acid
6	1.16	0.496	$[M-H]^-$	$C_{10}H_{12}N_4O_5$ (267.07)	Inosine
7	1.17	0.687	$[M-H]^-$	$C_5H_7NO_3$ (128.05)	L-5-Oxoproline
8	1.18	0.380	$[M+H]^+$	$C_6H_6N_2O$ (122.95)	Nicotinamide
9	1.19	9.765	$[M+H]^+$	$C_4H_9NO_2$ (104.01)	α-Aminoisobutyrate
10	1.26	0.169	$[M-H]^-$	$C_5H_6O_4$ (129.01)	Citraconic acid
11	1.28	2.321	$[M+H]^+$	$C_6H_9N_3O_2$ (156.04)	Histidine
12	1.28	0.046	$[M-H]^-$	$C_7H_{12}O_5$ (175.06)	2-Isopropylmalic acid
13	1.29	2.041	$[M+H]^+$	$C_6H_5NO_3$ (140.06)	6-Hydroxynicotinic acid
14	1.29	0.017	$[M+H]^+$	$C_5H_9N_3$ (112.04)	Histamine
15	1.29	0.379	$[M-H]^-$	$C_9H_{14}N_2O_{12}P_2$ (403.08)	Uridine 5'-diphosphate
16	1.32	0.266	$[M-H]^-$	$C_9H_{13}N_2O_8P$ (307.12)	2'-Deoxyuridine-5'-monophosphate
17	1.38	0.705	$[M+H]^+$	$C_9H_{11}NO_2$ (166.08)	L-(-)-Phenylalanine
18	1.38	0.600	$[M+H]^+$	$C_{10}H_{13}NO_2$ (180.10)	L-beta-Homophenylalanine
19	1.38	0.674	$[M+H]^+$	$C_5H_9NO_2$ (116.06)	L-Proline
20	1.40	0.072	$[M-H]^-$	$C_{10}H_{13}N_4O_9P$ (363.09)	Xanthosine-5'-monophosphate
21	1.41	1.112	$[M+H]^+$	$C_5H_5N_5$ (136.06)	Adenine
22	1.44	0.161	$[M+H]^+$	$C_7H_7NO_2$ (138.09)	Trigonelline
23	1.46	0.352	$[M+H]^+$	$C_{10}H_{17}N$ (152.05)	Amantadine
24	1.47	0.230	$[M+H]^+$	C_9H_7NO (146.05)	3-Formylindole
25	1.51	0.034	$[M+H]^+$	$C_{13}H_{22}O_3$ (227.13)	methyl dihydrojasmonate
26	1.56	0.551	$[M+H]^+$	$C_6H_7N_5$ (150.09)	3-Methyladenine
27	1.58	0.038	$[M+H]^+$	$C_{10}H_9NO_3$ (192.13)	5-Hydroxyindoleacetic acid
28	1.72	0.125	$[M-H]^-$	$C_9H_{12}N_2O_6$ (243.01)	Uridine
29	1.81	0.179	$[M-H]^-$	$C_6H_{14}N_2O_3$ (161.04)	DL-5-Hydroxylysine
30	1.86	0.432	$[M+H]^+$	$C_{10}H_{13}N_5O_4$ (268.14)	Adenosine
31	1.88	0.965	$[M+H]^+$	$C_5H_7NO_3$ (130.04)	L-5-Oxoproline
32	1.88	0.027	$[M-H]^-$	$C_4H_8N_2O_3$ (131.08)	N-Glycylglycine

33	1.94	0.085	$[M+H]^+$	$C_5H_{11}N$ (86.02)	Piperidine
34	2.22	0.745	$[M-H]^-$	$C_8H_9NO_4$ (182.04)	4-Pyridoxate
35	2.28	0.061	$[M-H]^-$	$C_9H_{14}N_4O_3$ (225.12)	Carnosine
36	2.56	0.025	$[M+H]^+$	$C_{10}H_{13}N_5O$ (220.11)	trans-Zeatin
37	2.80	0.026	$[M+H]^+$	$C_{10}H_{14}N_5O_6P$ (332.37)	2'-Deoxyadenosine 5'-monophosphate
38	3.03	1.125	$[M+H]^+$	$C_{15}H_{22}N_6O_5S$ (399.03)	S-Adenosyl-L-methionine
39	3.68	1.245	$[M-H]^-$	$C_{18}H_{16}O_8$ (359.14)	rosmarinic acid
40	3.70	0.061	$[M-H]^-$	$C_{21}H_{24}O_9$ (419.15)	E-Isorhapontin
41	4.22	0.116	$[M-H]^-$	$C_7H_{15}NO_2$ (144.04)	L-beta-Homoisoleucine
42	4.51	2.045	$[M+H]^+$	$C_9H_{11}NO_3$ (182.07)	Tyrosine
43	4.80	0.030	$[M+H]^+$	$C_7H_8N_2$ (121.06)	Benzamidine
44	4.81	0.041	$[M-H]^-$	$C_{11}H_{20}N_2O_6$ (275.62)	L-Saccharopine
45	4.90	0.132	$[M+H]^+$	$C_{10}H_{12}N_2O_3$ (209.31)	Kynurenine
46	4.97	0.767	$[M+H]^+$	$C_9H_{17}NO_5$ (220.08)	Pantothenate
47	5.02	0.978	$[M+H]^+$	$C_{20}H_{38}N_6O_4$ (427.15)	Leupeptin hemisulfate salt
48	5.07	0.026	$[M+H]^+$	$C_{10}H_{16}$ (137.09)	Sabinene
49	5.09	0.119	$[M+H]^+$	C_6H_9NOS (144.07)	4-Methyl-5-thiazoleethanol
50	5.52	0.0273	$[M-H]^-$	$C_5H_4N_4O_3$ (169.07)	Uric acid
51	5.34	3.921	$[M+H]^+$	$C_{24}H_{40}O_5$ (409.10)	Cholic acid
52	5.67	0.017	$[M+H]^+$	$C_{10}H_{16}$ (137.05)	Gamma-terpinene
53	5.71	0.030	$[M-H]^-$	$C_{10}H_{14}N_5O_8P$ (362.09)	Guanosine 5'-monophosphate
54	5.90	0.161	$[M+H]^+$	$C_{18}H_{29}NO_3$ (308.18)	Dihydrocapsaicin
55	6.29	0.127	$[M-H]^-$	$C_9H_{12}N_3O_7P$ (304.08)	Cytidine-3',5'-cyclicmonophosphate
56	7.17	0.328	$[M-H]^-$	$C_{19}H_{22}O_6$ (345.04)	Gibberelin A3
57	7.34	0.022	$[M+H]^+$	$C_{17}H_{20}N_4O_6$ (377.24)	(-)-Riboflavin
58	7.55	0.030	$[M+H]^+$	$C_7H_{14}N_2O_4S$ (223.08)	DL-Cystathionine
59	7.70	1.474	$[M+H]^+$	$C_{16}H_{23}N_5O_6$ (382.09)	trans-Zeatin-9-glucoside
60	8.53	0.010	$[M+H]^+$	$C_6H_5N_5O$ (164.05)	Pterine
61	8.68	0.089	$[M-H]^-$	$C_5H_4N_4O_2$ (151.04)	Xanthine
62	9.41	0.726	$[M+H]^+$	$C_{15}H_{21}N_5O_5$ (352.07)	trans-Zeatin riboside
63	10.06	0.127	$[M+H]^+$	$C_{17}H_{21}N_4O_9P$ (457.18)	Riboflavin-5'-monophosphate
64	10.25	0.056	$[M-H]^-$	$C_{10}H_{16}N_4O_3$ (239.09)	L-Homocarnosine
65	10.33	0.139	$[M-H]^-$	$C_8H_{18}N_2O_6S_2$ (301.08)	PIPES (N- piperazine)
66	12.13	0.253	$[M+H]^+$	$C_{15}H_{21}N_5O_4$ (336.07)	N-6-(delta-2-Isopentenyl)adenosinehemihydrate
67	13.37	0.183	$[M+H]^+$	$C7H11NO_3$ (158.14)	N-Tigloylglycine
68	14.31	0.314	$[M+H]^+$	$C_9H_{15}N_3O_{11}P_2$ (404.28)	Cytidine-5'-diphosphate
69	14.61	0.042	$[M+H]^+$	$C_9H_{13}N_3O_4$ (228.13)	2'-Deoxycytidine
70	19.16	0.613	$[M-H]^-$	$C_{18}H_{30}O_2$ (277.21)	gamma-Linolenic acid
71	27.02	0.215	$[M+H]^+$	$C_5H_{11}NO_2$ (118.08)	Glycine-Betaine

* Relative percentages of the identified compounds in the plant extracts were calculated based on the total peak area in the chromatogram; ** Tentative identity

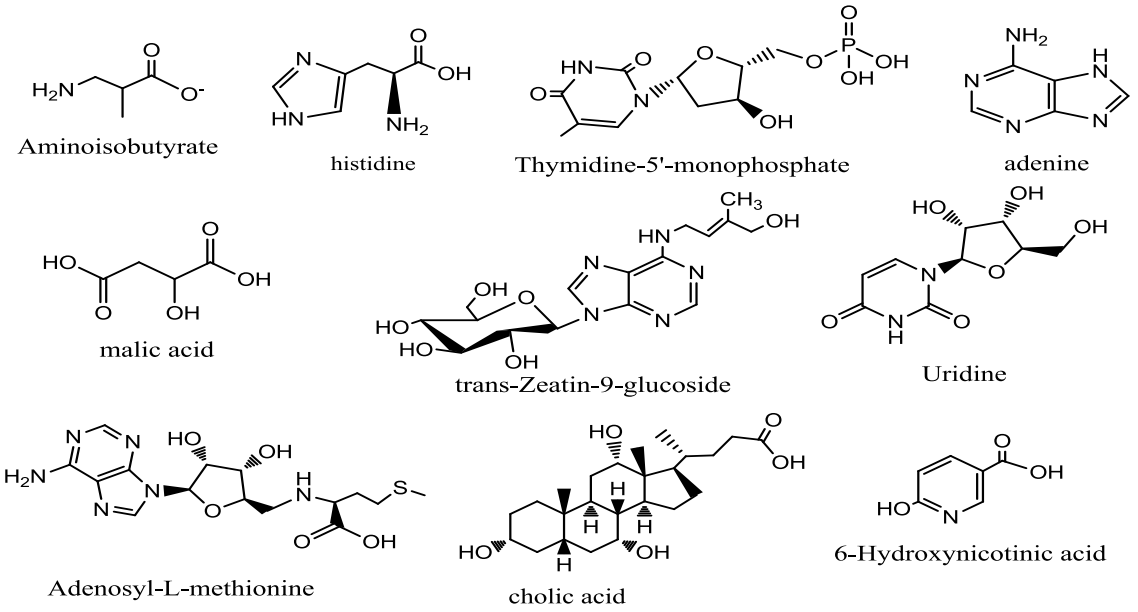


Fig 2. Chemical structure of major identified compounds from EtOAc of *S. kali* by LC–ESI-TOF–MS in both positive and negative mode.

Antioxidant activity

Total antioxidant capacity

The basic idea underlying the phosphomolybdenum assay for assessing antioxidant capacity is the reduction of Mo (VI) to Mo (V) by plant extracts containing antioxidant chemicals. The total antioxidant capacity in EtOAc and MeOH extracts of *S. kali* was determined using the linear regression equation (y= 0.0008x + 0.0045, r^2= 0.9862, Fig. 3). The total antioxidant capacities of EtOAc and MeOH extracts of *S. kali* were 873.27±17.7 and 302.5±15.9 mg/g plant extract in AAE, respectively (Table 2).

Reducing power

Reducing power of plant extracts of *S. kali* was expressed in relation to the reducing power of ascorbic acid. Both extracts exhibited promising reducing power with 223.62±3.46 & 213.02±1.99 mg AAE/g extract for EtOAc and MeOH extracts, respectively (Table 2); (y= 0.0723x, r^2= 0.968, Fig. 4). An increase in the absorbance of the sample solution is an indicator of the reducing power of the sample [30]. The extracts showed noticeable iron-reducing power that is comparable to the reducing power of ascorbic acid (Fig. 5). The presence of antioxidants in the extract can reduce the oxidized form of iron (Fe^{3+}) to its reduced form (Fe^{2+}) in the reducing power assay by donating an electron. As a result, the presence of reductants (antioxidants) in *S. kali* extracts is thought to trigger the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form.

DPPH free radical scavenging activity

DPPH free radical scavenging activity was used to evaluate the possible antioxidant principles present in the extracts by its radical scavenging capacity measurement. The percent DPPH radical scavenging by the plant extracts illustrated in Fig. (5). The EtOAc extract showed scavenging activity with an IC_{50} value of 41.52±2.09 µg/ml while MeOH extract showed scavenging activity with an IC_{50} value of 80.64±4.53 µg/ml as compared with ascorbic acid (IC_{50}= 5.97±0.8) (Table 2).

As a result of the antioxidant assays for evaluating antioxidant activity for the two plant extracts, the EtOAc extract of *S. kali* showed higher total antioxidant capacity, greater reducing power and higher degree of free radicals scavenging activity than that of the MeOH extract, suggesting the presence of effective antioxidant compounds in EtOAc extract of *S. kali* more than found in MeOH extract. The possible antioxidant activity of EtOAc extract may be due to the presence of phenolic compounds such as flavonoids and phenolic acids which are characterized by their antioxidant activity [31,32]. Because the phenolic hydroxyl group is the antioxidant ability core of phenolic acids, the number and position of phenolic hydroxyl groups are strongly connected to their antioxidant activity [33]. Furthermore, the methoxy and carboxylic acid groups have a significant impact on phenolic acid antioxidant activity [34,35]. Additionally, nitrogenous compounds like alkaloids were reported to be among the abundant compounds in the EtOAc extract based on our results of the identification of secondary metabolites in EtOAc extract of *S. kali* using LC-ESI-TOF-MS system. Many studies demonstrated the potent role of these compounds in the antioxidant activity. For example, Thawabteh *et al*. [36] noted that alkaloids have antioxidant qualities and can prevent a number of degenerative diseases by catching free radicals or binding to catalysts involved in various oxidation processes that occur within the human body. *S. kali* is considered a halophyte plant, so the limited antioxidant activity of MeOH extract of this plant may be due to the accumulation of alkaline salts presence of polar extracts which made a protection layer or barrier around the compounds with the chelating complex of metals and salts by decreasing free electron on oxygen atom of hydroxyl group that will be coupled to free radical, so led to increasing of free radicals and decreasing scavenging groups of the extract led to decreasing of its probability to have promising antioxidant activity.

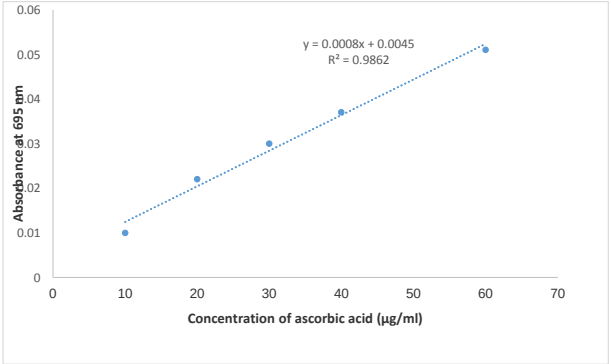


Fig 3. Standard calibration curve of ascorbic acid for total antioxidant capacity determination.

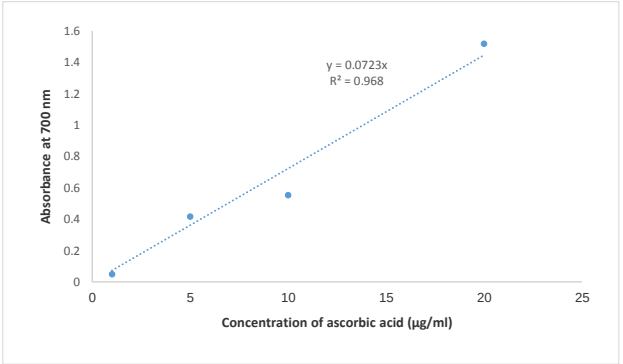


Fig 4. Standard calibration curve of ascorbic acid for iron reducing power capacity determination.

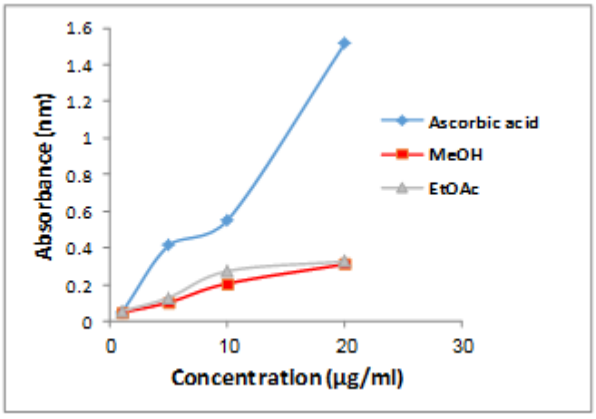


Fig 5. Ferric reducing antioxidant power of EtOAc and MeOH extracts of *S. kali* along with ascorbic acid at different concentrations.

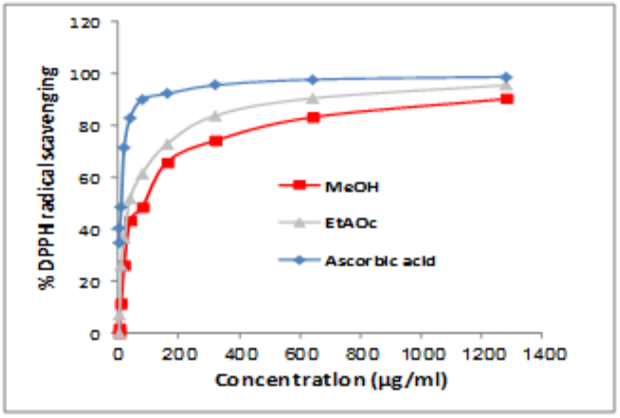


Fig 6. % DPPH radical scavenging activities of EtOAc and MeOH extracts of *S. kali*.

Table 2. DPPH radical scavenging activity and total antioxidant capacity of EtOAc and MeOH extracts of *S. kali*.

Sample	Total antioxidant capacity (mg AAE /g extract)	Reducing power (mg AAE /g extract)	DPPH radical scavenging activity IC_{50} value (µg/mL)
EtOAc	873.27±17.70	223.62±3.46	41.52±2.09
MeOH	302.5±15.90	213.02±1.99	80.64±4.53
Ascorbic acid	-	-	5.97±0.8

Results are expressed as mean ± standard deviation (n = 3). Ascorbic acid equivalent (AAE).

Antimicrobial activity

Results are given in table 3 illustrated mean zone of inhibition in mm produced on a range of pathogenic microorganisms. The antimicrobial activity of EtOAc and MeOH extracts of *S. kali* was evaluated by diffusion agar technique. The two tested extracts showed no antifungal activity against the tested strains in comparison with ketoconazole reference drug. EtOAc and MeOH extracts of *S. kali* showed moderate antibacterial activity against *Staphylococcus aureus* with 11 & 13mm inhibition zone respectively, on the other hand, EtOAc extract of *S. kali* showed moderate antibacterial activity against *Bacillus subtilis* with 14 mm inhibition zone while MeOH extract showed no antibacterial activity against the same strain as compared to gentamycin reference drug. Furthermore, EtOAc and MeOH extracts of *S. kali* showed weak antibacterial activity against *Proteus vulgaris* with 7 & 3mm inhibition zone respectively and showed no antibacterial activity against *Escherichia coli* as compared to gentamycin reference drug. The concentration of phenolics present in the *S. kali* EtOAc extract appears to affect the antibacterial activity of the extract. This remarkable correspond was demonstrated in [37]. It was observed that bacterial species exhibited different sensitivities towards the different concentrations of phenolic compounds. Furthermore, nitrogenous compounds are secondary metabolites found in the EtOAc extract and are known to possess a wide range of therapeutic uses, such as antimicrobial agent [38]. It has believed that the nitrogen atom is responsible for the bioactivity of this class [36]. Moreover, the antibacterial mechanism of alkaloidal phytochemicals against various infectious diseases is through efflux pump inhibition [39].

Table 3. Antimicrobial effect of EtOAc and MeOH extracts of *S. kali* on a range of pathogenic microorganisms.

Tested microorganisms	Inhibitions zones (mm)		
	EtOAc	MeOH	Control
Fungi			Ketoconazole
Aspergillus fumigatus (RCMB 002008)	NA	NA	17
Candida albicans RCMB 005003 (1) ATCC 10231	NA	NA	20
Gram-positive bacteria			Gentamycin
Staphylococcus aureus ATCC 25923	11	13	24
Bacillus subtilis RCMB 015 (1) NRRL B-543	14	NA	26
Gram-negatvie bacteria			Gentamycin
Escherichia coli ATCC 25922	NA	NA	30
Proteus vulgaris RCMB 004 (1) ATCC 13315	7	3	25

Anti-inflammatory activity

Anti-inflammatory activity of EtOAc and MeOH extracts of *S. kali* was investigated using stabilization of red blood cell membrane lysing technique. The percentage membrane stability exhibited by EtOAc and MeOH extracts was concentration dependent and compared favorably with those of standard drugs, Diclofenac potassium (table 4). It was noted that, EtOAc extract exhibited the highest membrane stabilizing activity by IC_{50} = 1.68 mg/mL and the lowest was found in MeOH extract by IC_{50} = 2.60 mg/mL compared to that of diclofenac potassium (IC_{50} = 0.83 mg/mL). Suggesting that, EtOAc extract contained more principles that protected the erythrocyte membranes against induced lyses therefore could be regarded as a natural source of membrane stabilizers and may be capable of providing an alternative remedy for the management of inflammatory. The extract exhibited membrane stabilization effect by inhibiting hypotonicity induced lysis of erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes [40] Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release [41]. Although the precise method through which the extract maintains the membrane is still unclear, hypotonicity induced hemolysis may result from cell shrinkage brought on by osmotic fluid and electrolyte loss within the cells. The processes that could increase or accelerate the efflux of these intracellular components may be inhibited by the extract [42,43]. On the basis of the findings of the present study it can be assumed that the plant extract contained principles that protected the erythrocyte membranes effectively and may have potential anti-inflammatory activity. This activity may be explained by the phytochemicals found in the extracts, for instance the phenolic compounds. Phenolic compounds are known to possess anti-inflammatory activity [44]. Furthermore, nitrogenous compounds are secondary metabolites found in the EtOAc extract and previously demonstrated to have an anti-inflammatory activity [45].

Table 4. Effect of EtOAc and MeOH extracts of *S. kali* on red blood cell membrane stabilizing.

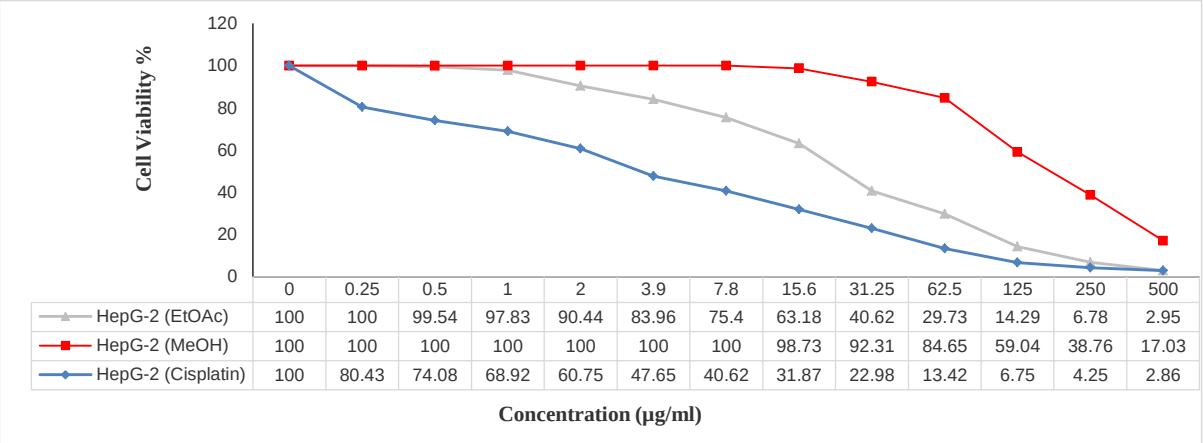
Samples	Concentration (mg/mL)	Inhibition of Haemolysis (%)	IC_{50} (mg/mL)
EtOAc	1	38.37±4.93	1.68
	2	55.81±0.55	
	3	59.30±2.20	
	4	61.24±3.29	
MeOH	1	32.17±2.74	2.60
	2	47.67±4.93	
	3	54.65±3.29	
	4	60.47±2.74	
Diclofenac potassium	0.25	22.87±2.31	0.83
	0.5	42.40±1.84	
	1	63.00±2.12	
	2	85.85±2.19	
	3	92.75±3.46	

Values are expressed as mean ± standard deviation.

Cytotoxic activity

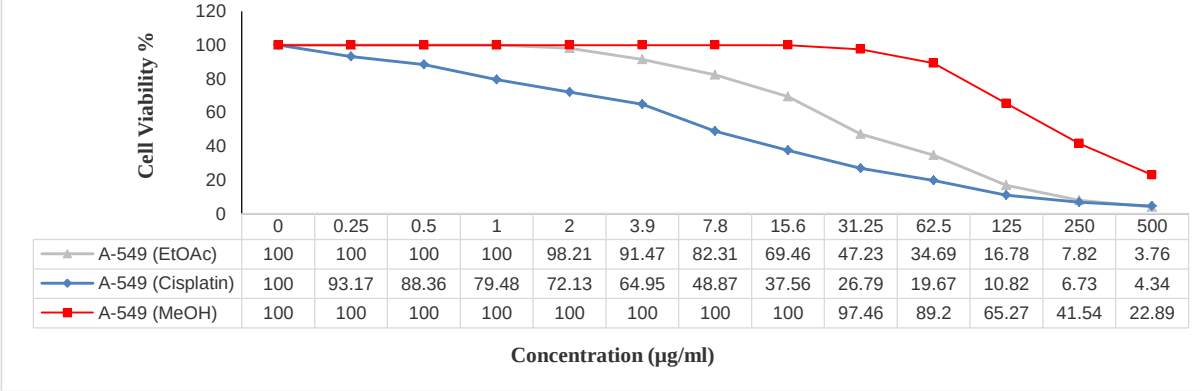
The outlined results of the cytotoxic effect of EtOAc and MeOH extracts of *S. kali* against the tumor cell lines (MCF-7, HepG2 & A549) were illustrated in table 5 and figures 5 & 6. The EtOAc extract showed the highest *in vitro* cytotoxic effect against HepG2, A549 and MCF-7 with IC_{50} 24.7, 29.3, and 44.0 µg/mL respectively, while the MeOH extract showed slight effect against the three tested cell lines with IC_{50} between 180-291µg/mL as compared to the drug reference, Cisplatin. Our results revealed diversity among various cell lines when exposed to various EtOAc and MeOH concentrations of *S. kali* extracts. HepG2 cells demonstrated the greatest reduction in cell viability in this circumstance, followed by A549 and MCF-7. The results attained are consistent with those mentioned in [46]. They summarized that, depending on the extract concentration and cell type, the plant extract significantly decreased the viability of several cell lines. EtOAc extract from *S. kali* has a strong cytotoxic impact, which may generally be linked to the extract's existence, nature, and composition. Based on our results of the identification of secondary metabolites in EtOAc extract of *S. kali* using the LC-ESI-TOF-MS system, among the constituents present in the extract were flavonoids. Several studies have found that flavonoids have growth-inhibitory effects on several types of cancer cells, which are mediated by different molecular targets and function via different metabolic pathways [47-49]. Flavonoids have the ability to quickly bind to cell membranes, penetrate *in vitro* grown cells, and regulate cellular metabolic activities [50-51]. Many studies have shown that flavonoids contribute to anti-carcinogenic activity by reducing oxidative damage, inactivating carcinogens, inhibiting proliferation, promoting differentiation, inducing cell cycle arrest and apoptosis, impairing tumor angiogenesis, and suppressing metastasis [52-57]. Other components in the S. kali EtOAc extract with higher specific activity, on the other hand, may work in an

additive or synergistic manner, eventually becoming substantial in the possible cytotoxic effect.



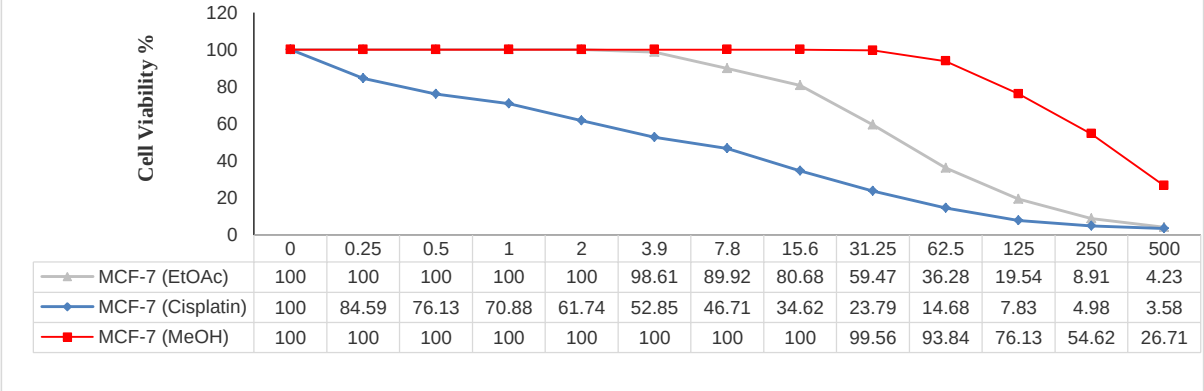
	0	0.25	0.5	1	2	3.9	7.8	15.6	31.25	62.5	125	250	500
HepG-2 (EtOAc)	100	100	99.54	97.83	90.44	83.96	75.4	63.18	40.62	29.73	14.29	6.78	2.95
HepG-2 (MeOH)	100	100	100	100	100	100	100	98.73	92.31	84.65	59.04	38.76	17.03
HepG-2 (Cisplatin)	100	80.43	74.08	68.92	60.75	47.65	40.62	31.87	22.98	13.42	6.75	4.25	2.86

Fig 7. Cytotoxic effect of EtOAc and MeOH extracts of *S. kali* on HepG2 cancer cell lines using MTT assay.



	0	0.25	0.5	1	2	3.9	7.8	15.6	31.25	62.5	125	250	500
A-549 (EtOAc)	100	100	100	100	98.21	91.47	82.31	69.46	47.23	34.69	16.78	7.82	3.76
A-549 (Cisplatin)	100	93.17	88.36	79.48	72.13	64.95	48.87	37.56	26.79	19.67	10.82	6.73	4.34
A-549 (MeOH)	100	100	100	100	100	100	100	100	97.46	89.2	65.27	41.54	22.89

Fig 8. Cytotoxic effect of EtOAc and MeOH extracts of *S. kali* on A-549 cancer cell lines using MTT assay.



	0	0.25	0.5	1	2	3.9	7.8	15.6	31.25	62.5	125	250	500
MCF-7 (EtOAc)	100	100	100	100	100	98.61	89.92	80.68	59.47	36.28	19.54	8.91	4.23
MCF-7 (Cisplatin)	100	84.59	76.13	70.88	61.74	52.85	46.71	34.62	23.79	14.68	7.83	4.98	3.58
MCF-7 (MeOH)	100	100	100	100	100	100	100	100	99.56	93.84	76.13	54.62	26.71

Fig 9. Cytotoxic effect of EtOAc and MeOH extracts of *S. kali* on MCF-7 cancer cell lines using MTT assay.

Table 5. IC_{50} values of EtOAc and MeOH extracts of *S. kali* against A549, HepG2 & MCF-7.

Cell lines	IC_{50} values (µg/mL)		
	EtOAc	MeOH	Cisplatin
A549	29.3 ± 2.03	205.4 ± 5.98	7.53± 0.51
HepG2	24.7 ± 1.72	180.7 ± 5.19	3.68± 0.24
MCF-7	44.0 ± 3.14	291.4 ± 8.76	5.69± 0.37

The results are represented as mean ± standard deviation.

CONCLUSION

The ethyl acetate and methanol extracts of *S. kali* were performed to phytochemical and pharmacological investigations i.e. antioxidant, antimicrobial, anti-inflammatory and cytotoxic activity for the first time. Our study revealed the EtOAc extract exhibited a promising nature thereby source for cure more diseases as it showed

potent antioxidant, anti-inflammatory and cytotoxic activity, also it was rich with different types of phytoconstituents i.e. (phenolic, flavonoid, alkaloids, N-compounds … etc) which may be related to its pharmacological activity.

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Nil.

Conflict of Interest: No conflict of interest associated with this work.

List of abbreviations
AAE= ascorbic acid equivalent; CE = chloroform extract; SD = standard deviation; **MCF-7** = human breast cancer cell line; **HepG-2** = human Hepatocellular carcinoma; **A-549** = human Lung Carcinoma; MTT assay = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay method; **PI =** propidium iodide

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