



## OPEN Bioactive compounds and therapeutic potential of *Salvia fruticosa* Mill. leaves against microorganisms and osteosarcoma

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Many studies reported the cytotoxic effect of *Salvia fruticosa* Mill. (*S. fruticosa*) on different cancer cell lines. This study aims to investigate the methanolic extract of *S. fruticosa* leaves in terms of its polyphenolic content, antioxidant activity, antimicrobial activity against different microorganisms, and cytotoxic activities on the U2OS osteosarcoma cell line. Fourier Transform Infrared Spectroscopy was performed to detect the major functional groups in the extract. The extract's total carbohydrate content was found to be  $45.63 \pm 3.33$  mg glucose equivalent, the total phenolic content was  $170.68 \pm 6.52$  mg caffeic acid equivalent, and the total flavonoid content was  $44.71 \pm 5.67$  mg rutin equivalent per gram of the extract. Polyphenolic bioactive compounds of the extract were identified and quantified using Liquid chromatography-electrospray ionization-tandem mass spectrometry, demonstrating that naringenin is the most abundant polyphenolic compound, quantified at  $3137.22$   $\mu\text{g}$  of the standard per gram of the extract. The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity assay, revealing an  $\text{IC}_{50}$  of  $25.17 \pm 0.304$   $\mu\text{g}/\text{mL}$ , which demonstrates the extract's concentration-dependent activity. The extract also exhibited good antimicrobial activity against gram-positive *S. aureus* and *B. subtilis*, and the fungus *S. cerevisiae* with MIC values of 1.562 mg/mL, while the MIC for gram-negative *E. coli* was observed at 12.5 mg/mL. The extract showed a significant reduction in U2OS cell viability with an  $\text{IC}_{50}$  of  $77.58 \pm 3.47$   $\mu\text{g}/\text{mL}$  after 72 h of incubation, while it had no significant effect on the viability of human skin fibroblasts. These findings suggest that the methanolic extract of *S. fruticosa* leaves shows potent antioxidant activity due to its high content of polyphenolics; hence, the extract could be a potential treatment for different microbial infections and osteosarcoma.

**Keywords** *Salvia fruticosa*, Antimicrobial, Cytotoxicity, Osteosarcoma, U2OS, HSE, Phenolics, Flavonoids, DPPH

Osteosarcoma is a tumor of mesenchymal origin (i.e., the tissue consists of stromal cells that can produce bone-like tissues) characterized by uncontrolled production of immature osteoid tissue<sup>1,2</sup>. Worldwide, osteosarcoma is ranked the eighth highest childhood cancer at about 2.4% of tumors diagnosed, and is more predominant among those aged 10–14 years<sup>3</sup>. During adolescence, osteosarcoma typically develops in the metaphysis of long bones close to the growth plates, accounting for 10% of tumors. Adolescent osteosarcoma is commonly associated with metastasis, especially lung metastasis<sup>4,5</sup>. Worldwide, 2–4 per million females and 3–5 per million males suffer from osteosarcoma, and it affects African American and Indigenous African males<sup>3</sup>.

Poor osteosarcoma prognosis is indicated by metastasis development, particularly lung metastasis, and drug resistance. Additionally, most patients do not exhibit significant lung metastases at the time of initial assessment,

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despite the presence of micro-metastases. The overall survival of osteosarcoma patients can be improved by early identification of micro-metastases using newly developed sensitive diagnostic techniques<sup>6</sup>. So, it is highly recommended to conduct more research to develop more effective treatment strategies<sup>3</sup>.

Greek sage, *Salvia fruticosa* Mill., syn. *Salvia triloba* L., is an aromatic small shrub from the mint family (*Lamiaceae*). It is abundant in the Eastern Mediterranean region, particularly in the mountainous regions in Lebanon, Palestine, Jordan, Egypt (Sinai), Syria, Cyprus, Türkiye, and Greece (Crete). *Salvia fruticosa* (*S. fruticosa*) leaves are rich in essential oils, including 1,8-cineole (most abundant),  $\alpha$ - and  $\beta$ -thujone, and camphor, which are known for their antioxidant, antimicrobial, and anticancer activities. *S. fruticosa* leaves were used in folk medicine to treat common cold, diarrhea, toothaches, snake bites, enteritis, and sore throat. Essential oil of *S. fruticosa* possesses antimicrobial activity against a wide range of gram-positive and gram-negative bacteria. Furthermore, many studies revealed its anticancer activity against neuroblastoma, melanoma, prostate, colon, breast and ovarian cancers<sup>6–14</sup>.

The primary objective of this study is to provide novel insights into the bioactive compounds, including phenolics, flavonoids, and carbohydrates, present in *S. fruticosa* leaves. Specifically, this represents the first comprehensive investigation into the phenolic and flavonoid content extracted from *S. fruticosa* leaves using methanol and its targeted effects on both microorganisms and osteosarcoma cells. To achieve this, our study involved a clear sequence of investigative steps: first, chemical characterization and phytochemical analyses of the methanolic extract were conducted. Subsequently, we evaluated its biological activities, including its antimicrobial effect on both gram-positive and gram-negative microorganisms, as well as its impact on U2OS osteosarcoma cell viability, morphology, and migration. Additionally, the cytotoxic effect of the extract on human skin fibroblasts (HSF) was tested to assess its impact on normal human cells.

## Methods

### Plant material and extract preparation

*S. fruticosa* leaves were collected from Hebron city, Palestine, at the following geographical coordinates: 31°32'04.2"N 35°06'00.4"E in the Spring of 2022. Sample collection complies with relevant institutional, national, and international guidelines and legislations. Since the plant is not listed in the endangered or protected plants, no permission was needed for collection purposes. The plant morphological identification was performed by Dr. Rami Arafah at Palestine-Korea Biotechnology Center, Palestine Polytechnic University, Hebron by referring to the flora of Palestine, Sinai, and Syria (see supplementary Fig. S1 online)<sup>15</sup>. Additionally, molecular identification was performed using a BLAST search and comparison of the DNA sequence of the internal transcribed spacers (ITS) in the ribosomal DNA (rDNA) region (NCBI GenBank # PQ804261).

Leaves were washed with tap water, followed by distilled water, and then left to air-dry at room temperature ( $25 \pm 2$  °C). The dried sample was ground into fine powder. Five grams of ground leaves were macerated in 70% methanol (PIOCHEM, Giza, Egypt) and placed for 15 min at room temperature in the ultrasonicator (Branson 2510 sonicator, model 2510DTH, Hampton, NH, USA) to extract polyphenolic compounds<sup>16</sup>. This was followed by filtration using cheesecloth; then, the filtrate was left to air-dry until complete evaporation of methanol at room temperature. After complete drying, approximately 4 g of the extract was collected in an Eppendorf tube and stored at room temperature for chemical and phytochemical analyses. In contrast, for testing the biological activity, the extract was dissolved in dimethyl sulfoxide (DMSO) (SIGMA - ALDRICH, Steinheim, Germany) to prepare a 75 mg/mL stock solution and kept at -20 °C.

### Characterization of the chemical structure of *S. fruticosa* methanolic extract

FTIR was conducted to identify the major functional groups in the methanolic extract of *S. fruticosa*. Briefly, we combined a sample of the powdered *S. fruticosa* leaves with potassium bromide to form a 1.0 mm-sized pellet. Then the analysis was performed using a Nicolet 380 Thermogravimetric Analysis/Fourier Transform Infrared (TGA/FTIR) spectrometer over a range of wave numbers from 500 to 4000  $\text{cm}^{-1}$ <sup>17</sup>.

### Phytochemical analyses of *S. fruticosa* methanolic extract

#### Determination of total carbohydrate content (TCC)

The TCC in *S. fruticosa* extract was determined using the phenol-sulfuric acid method, with minor modification<sup>18</sup>. Briefly, the powdered *S. fruticosa* extract was dissolved in distilled water at a concentration of 1 mg/mL then 1 mL of the dissolved extract was added to 5 vol% phenol (Loba Chemie, Mumbai, India) and 5 mL concentrated sulfuric acid (Penta, Prague, Czech Republic) and left for 30 min in a water bath for cooling. At the end of the incubation period, a yellow-colored complex was formed and measured at 490 nm using Amersham Biosciences Ultrospec 3100 Pro UV/Visible Spectrophotometer. TCC quantification was determined in  $\mu\text{g}$  glucose equivalent/g based on Eq. (1), which was obtained from the standard glucose (PIOCHEM, Giza, Egypt) curve shown in supplementary Fig. S2A online.

$$\text{Absorbance} = 0.0051 \times \text{Glucose } (\mu\text{g/mL}) + 0.032 \quad (1)$$

#### Determination of total phenolic content (TPC)

The TPC in *S. fruticosa* extract was determined by NAWAH Scientific (9 Al-Asmarat St, El Mokattam, Cairo, Egypt) using Folin-Ciocalteu colorimetric assay<sup>19</sup>. The powdered *S. fruticosa* extract was dissolved in 99.8 vol% methanol (PIOCHEM, Giza, Egypt) at a concentration of 3.2 mg/mL. Then, 10  $\mu\text{L}$  of the dissolved extract was added to 100  $\mu\text{L}$  of Folin-Ciocalteu reagent (Loba Chemie, Mumbai, India) (diluted 1: 10 vol.) in a 96-well plate. Then, 80  $\mu\text{L}$  of 1 M sodium carbonate (El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt) was added and incubated in the dark at room temperature for 20 min. A blue-colored complex resulted at the end of the incubation period, which was measured at 630 nm using a microplate reader (FluoStar Omega, Otenberg,

Germany). TPC was expressed in  $\mu\text{g}$  caffeic acid equivalent/g based on Eq. (2) that was obtained from the standard caffeic acid (NAWAH Scientific, Cairo, Egypt) curve as shown in supplementary Fig. S2B online.

$$\text{Absorbance} = 0.0025 \times \text{Caffeic acid } (\mu\text{g/mL}) - 0.0967 \quad (2)$$

#### Determination of total flavonoid content (TFC)

The TFC in *S. fruticosa* extract was determined by NAWAH Scientific (9 Al-Asmarat St, El Mokattam, Cairo, Egypt) using the aluminum chloride method, with minor modification<sup>20</sup>. The powdered extract was dissolved in 99.8 vol% methanol (PIOCHEM, Giza, Egypt) at a concentration of 3.2 mg/mL then in a 96-well plate, 15  $\mu\text{L}$  of the dissolved extract was added to 175  $\mu\text{L}$  of methanol followed by 30  $\mu\text{L}$  of 1.25% aluminum chloride (Sigma - Aldrich, Steinheim, Germany) and finally 30  $\mu\text{L}$  of 0.125 M sodium acetate (Sigma - Aldrich, Steinheim, Germany) were added and incubated for 5 min. At the end of the incubation period, a yellow-colored complex was formed that was measured at 420 nm using a microplate reader (FluoStar Omega, Otenberg, Germany). TFC was measured in  $\mu\text{g}$  rutin equivalent/g based on Eq. (3) that was obtained from the standard rutin (NAWAH Scientific, Cairo, Egypt) curve as shown in supplementary Fig. S2C online.

$$\text{Absorbance} = 0.002 \times \text{Rutin } (\mu\text{g/mL}) - 0.014 \quad (3)$$

#### Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS)

The profiling of phenolic and flavonoid compounds in the extract of *S. fruticosa* was performed by the National Research Center (33 El Buhouth St, Dokki, Cairo, Egypt) using Liquid Chromatography coupled with Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS). An ExionLC AC system was utilized for the separation process, while detection was carried out using a SCIEX Triple Quad 5500 + MS/MS system equipped with electrospray ionization (ESI)<sup>21</sup>. The separation was conducted on ZORBAX SB-C18 column (4.6  $\times$  100 mm, 1.8  $\mu\text{m}$ ). The mobile phase consisted of two eluents: A (0.1% formic acid in water) and B (acetonitrile, LC/MS grade). The gradient elution was programmed as follows: 2% B from 0 to 1 min, transitioning from 2 to 60% B between 1 and 21 min, maintaining 60% B from 21 to 25 min, and returning to 2% B from 25.01 to 28 min. The flow rate was established at 0.8 mL/min, with an injection volume of 3  $\mu\text{L}$ . For the Multiple Reaction Monitoring (MRM) analysis of the selected polyphenols, both positive and negative ionization modes were employed in a single run, with the following parameters: curtain gas set at 25 psi, ion spray voltage at 4500 V for positive mode and  $-4500$  V for negative mode, source temperature at 400  $^{\circ}\text{C}$ , ion source gases 1 and 2 at 55 psi, declustering potential at 50, collision energy at 25, and a collision energy spread of 10. The obtained chromatograms for the sample extract and standards are shown in supplementary Fig. S3 online, and the concentration of each phenolic and flavonoid compound was measured using Eq. (4).

$$\text{Concentration} = \frac{A_s}{A_{st}} \times C_{st} \quad (4)$$

where  $A_s$  = Area of sample,  $A_{st}$  = Area of standard, and  $C_{st}$  = standard concentration.

### Examining the biological activities of *S. fruticosa* extract

#### Free radical scavenging test

The antioxidant activity of the extract was evaluated by NAWAH Scientific (9 Al-Asmarat St, El Mokattam, Cairo, Egypt) using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity according to previous literature using Trolox as a positive control<sup>22</sup>. Briefly, a serial dilution of *S. fruticosa* extract was prepared in methanol (PIOCHEM, Giza, Egypt) to obtain concentrations of 5, 10, 20, 40, and 80  $\mu\text{g/mL}$ . Then, 100  $\mu\text{L}$  of freshly prepared DPPH reagent (0.1 vol% in methanol) was added to 100  $\mu\text{L}$  of each extract concentration in a 96-well plate and incubated in the dark at room temperature for 30 min. The resulting yellow color intensity was measured at 540 nm using a microplate reader (FluoStar Omega, Otenberg, Germany) after blanking it with a mixture of DPPH and methanol without extract<sup>23,24</sup>. Absorbance values of the samples were evaluated against Trolox as a control. The ability of the extract to scavenge DPPH free radical was calculated using the following Eq. (5)<sup>22,24</sup>:

$$\text{DPPH scavenging effect } (\%) = \left( \frac{A_c - A_E}{A_c} \right) \times 100 \quad (5)$$

where  $A_c$  is the average absorbance of the control (DPPH + methanol without extract), and  $A_E$  is the average absorbance of the extract.

The extract's ability to scavenge 50% of the free radicals is known as  $\text{IC}_{50}$  and this was calculated by plotting logarithm extract concentrations against their scavenging activity percentage and applying a nonlinear regression analysis using Eq. (6) (log (inhibitor) vs. normalized response -variable slope) on GraphPad Prism 9 software after normalizing the data to a scale ranging from 0 to 100<sup>25</sup>:

$$Y = \frac{100}{\left( 1 + 10^{((\log \text{IC}_{50} - X) \times \text{HillSlope})} \right)} \quad (6)$$

where  $X$  = logarithmic concentration of the sample to be assessed (*S. fruticosa* extract and Trolox),  $Y$  = antioxidant activity percentage.

Statistically significant difference among the free radical scavenging effects of different extract concentrations on DPPH was analyzed using one-way ANOVA followed by Tukey's multiple comparisons test at a 95% confidence level<sup>25</sup>.

#### *Antimicrobial test for S. fruticosa extract*

*S. fruticosa* antimicrobial activity was evaluated using a micro dilution method as previously described in<sup>26</sup> with minor modifications<sup>27</sup> towards four microorganisms: three bacteria: gram-positive *Staphylococcus aureus* (*S. aureus*) ATCC6538, gram-positive *Bacillus subtilis* (*B. subtilis*) ATCC35854, and gram-negative *Escherichia coli* (*E. coli*) ATCC8739 and one fungus: *Saccharomyces cerevisiae* (*S. cerevisiae*) ATCC9763 obtained from the American Type Culture Collection (ATCC, Virginia, USA). The minimum inhibitory concentration (MIC) of each microorganism was determined using 96-well microdilution plates with a flat bottom. Briefly, the extract was dissolved in 100% DMSO, followed by a serial dilution from 25 to 0.049 mg/mL using Mueller-Hinton broth (MHB, Oxoid, United Kingdom) (for bacteria) and Sabouraud Dextrose broth (SDB, Himedia, Mumbai, India) (for fungi). The tested microorganisms were isolated on Tryptone Soya agar (Himedia, Maharashtra, India) (for bacteria) and Sabouraud Dextrose agar (Himedia, Mumbai, India) (for fungi) after incubation at 37 °C for 24 h. A suspension for each microorganism was adjusted with sterile saline to achieve a turbidity of 0.5 McFarland (approximately 10<sup>8</sup> CFU/mL). Each suspension was diluted with media (MHB and SDB) to achieve a concentration of 5 × 10<sup>5</sup> CFU/mL. A volume of 10 µL of each microbial suspension was inoculated with the extract serial dilution and the final inoculum was 10<sup>4</sup> CFU/well. Two positive and one negative control were applied. A serial dilution (from 50 to 0.098%) of DMSO was inoculated with the same concentration of each microorganism (10<sup>4</sup> CFU/well) and used as a positive control to ensure that the extract achieved antimicrobial activity, not the DMSO. The second positive control is MHB and SDB inoculated with only the tested microorganisms, while both MHB and SDB were incubated and used as negative controls. The microdilution plates for the four microorganisms were incubated at 37 °C for 24 h. The experiment was performed in triplicate, and the MIC was recorded as the lowest concentration that inhibited microbial growth.

#### *Cytotoxic effect of S. fruticosa extract*

**MTT cell viability assay** U2OS cells (kindly provided as gifts from Dr. Andreas Kakaroukas' Lab (Department of Biology, The American University in Cairo, Egypt)) were cultured in Dulbecco's Modified Eagle Medium (DMEM, 1X) with stable glutamine and high glucose (SERANA, Pessin, Germany) supplemented with 10% Fetal Bovine Serum (FBS) (SERANA, Pessin, Germany) and 5 vol% PEN-STREP (Lonza, Verviers, Belgium) and incubated at 37 °C and 5% CO<sub>2</sub> in humidified Hera cell CO<sub>2</sub> incubator (Thermo-Fisher, USA). Regular cell splitting was performed before reaching confluency by washing the cells with Phosphate-Buffered Saline (PBS, 1X) (CORNING, Manassas, USA) and detaching them using 0.05 vol% of 1X trypsin-EDTA (SERANA, Pessin, Germany). We used an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) to visualize the cells<sup>28,29</sup>.

U2OS cell viability after incubation with *S. fruticosa* methanolic extract was evaluated using the MTT assay<sup>30</sup>. Briefly, three experiments were conducted in which 5000 U2OS cells were incubated in a 96-well plate for 24 h at 37 °C in a CO<sub>2</sub> incubator. Then, the cells were incubated with serial dilutions of *S. fruticosa* extract (prepared in DMEM complete medium) at concentrations of 150, 75, 37.5, 18.75, and 9.375 µg/mL for 24, 48, and 72 h. Then, 100 µL of fresh medium containing 10 vol% MTT solution (5 mg/mL) (SERVA, Heidelberg, Germany) was added to each well and incubated for 3 h. After the incubation period, the medium was replaced with 100 µL of DMSO and incubated in the dark at room temperature for 10 min to solubilize the formed formazan crystals, which have a purple color whose intensity is directly proportional to the number of metabolically active cells. The intensity was measured spectrophotometrically at 570 nm using a SPECTROstar nano microplate reader (BMG LABTECH, Ortenberg, Germany) after blanking the device with DMSO. The viability of treated cells was expressed as a percentage of the viability of untreated cells (negative control). The highest concentration of the extract in the serial dilution (150 µg/mL) contained 0.2 vol% DMSO. Therefore, this percentage of DMSO was prepared using DMEM complete medium and used as a control to test its effect on U2OS cell viability. The significance of the obtained viability percentage data was confirmed using two-way ANOVA analysis followed by Tukey's multiple comparisons test at a 95% confidence level in GraphPad Prism 9 software. Nonlinear regression analysis on GraphPad Prism 9 software was performed to generate the dose-response curves for *S. fruticosa* extract after the three investigated time intervals using cell viability percentages obtained from the MTT assay plotted against logarithmic concentrations of *S. fruticosa* extract followed by applying Eq. (6) to calculate the IC<sub>50</sub> which is the treatment dose required to inhibit cell growth by 50%<sup>31</sup>.

**Evaluating treatment cytotoxicity on HSF cells compared to U2OS cells** To ensure the safety of the extract on normal cells, an MTT assay was conducted by the Central Laboratory in the Faculty of Science at Mansoura University (60 El Gomhouria St, Mansoura 1, Dakahlia, Egypt) on the available normal cells of HSF (NAWAH Scientific) to compare its results to U2OS<sup>15</sup>. Briefly, two experiments were conducted in which HSF and U2OS cells were seeded in 96-well plates at a density of 5,000 cells per well and incubated for 24 h using complete DMEM media. Then, the medium was discarded and replaced with the obtained IC<sub>50</sub> of *S. fruticosa* extract and 4 µg/mL of cisplatin (as a reference), and the mixture was incubated at 37 °C in a CO<sub>2</sub> incubator for 72 h. After that, the treatments were discarded, and 100 µL of DMEM complete media containing 10% MTT solution (5 mg/mL) was added to each well and incubated for 3 h. After the incubation period, the media was replaced by 100 µL of DMSO and incubated in the dark at room temperature for 10 min, followed by measuring the optical density at 570 nm using a SPECTROstar nano microplate reader (BMG LABTECH, Ortenberg, Germany) after blanking the device with DMSO. The viability of treated cells was expressed as a percentage of the untreated cells (negative control). The significance of the data was analyzed using two-way ANOVA followed by Tukey's multiple comparisons test at a 95% confidence level in GraphPad Prism 9 software<sup>15,30</sup>.

**Treated U2OS cell morphology** U2OS cell morphology was observed under an Olympus IX70 inverted microscope at 20X magnification after treatment with *S. fruticosa* extract for 72 h. The morphological changes were assessed and compared with those of the untreated cells.

**Scratch wound healing assay** A scratch wound healing assay was performed to evaluate U2OS cell migration ability after treatment with *S. fruticosa* extract<sup>32</sup>. Briefly, U2OS cells were seeded at a density of 100,000 cells per well in a 24-well plate and incubated until reaching 80% confluency. Then, the media were replaced with 100  $\mu$ L of PBS. A scratch was made in the cell monolayer using a 200  $\mu$ L sterile tip. Then, 300  $\mu$ L of PBS were added to wash all the cell debris followed by adding the prepared treatment of *S. fruticosa* extract using DMEM complete media and incubation at 37 °C in a CO<sub>2</sub> incubator until almost complete closure of wounds in the untreated wells (negative control) which, in our study, was achieved after 48 h. During the incubation period, images were taken at 0, 12, 24, and 48 h of incubation with the treatments using an Olympus IX70 inverted microscope at 20X magnification. The wound area was measured using ImageJ 1.51j8 software, and the wound confluency percentage was calculated according to Eq. (7). The significance of the obtained data was analyzed using two-way ANOVA, followed by Tukey's multiple comparisons test at a 95% confidence level in GraphPad Prism 9 software<sup>32</sup>.

$$\text{Wound confluency \%} = \left( \frac{\text{Wound area at zero time} - \text{Wound area at X h}}{\text{Wound area at zero time}} \right) \times 100 \quad (7)$$

where X is the time point (interval).

**Statistical analysis** All the above experiments were conducted in triplicate, and data are presented as mean  $\pm$  standard deviation (SD) unless otherwise specified. All statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, CA, USA) as well as Microsoft Excel. A dose-response curve was generated after transforming and normalizing the obtained data using GraphPad Prism 9. ImageJ software was used to measure the wound area in the wound healing assay, and GraphPad Prism 9 was used to analyze the data. One-way or two-way ANOVA, followed by Dunnett's or Tukey's multiple comparisons tests, was used to determine the significant differences among different means. *P* values less than 0.05 were considered significant (\*\**p* < 0.01, \*\*\*\**p* < 0.0001).

## Results

### Extraction yield and chemical characteristics of *S. fruticosa* extract

An average yield of 0.144  $\pm$  0.008 g/g of the dry leaves was obtained from the ultrasonic extraction of *S. fruticosa* leaves in 70% methanol and the obtained FTIR spectrum of the extract is presented in Fig. 1.

### Phytochemical properties of *S. fruticosa* extract

#### Total carbohydrate, phenolic, and flavonoid content

TCC in *S. fruticosa* extract was estimated to be 45.63  $\pm$  3.33 mg glucose equivalent/g of the extract. In , TPC and TFC were estimated to be 170.68  $\pm$  6.52 mg caffeic acid equivalent and 44.71  $\pm$  5.67 mg rutin equivalent/g of the extract, respectively (see supplementary Fig. S2 online).

#### Polyphenolics and flavonoids in *S. fruticosa* extract

Sixteen polyphenolic compounds were detected and measured in *S. fruticosa* methanolic extract using LC-ESI-MS/MS analysis. *S. fruticosa* extract consisted of nine phenolic compounds: chlorogenic acid, gallic acid, caffeic acid, kaempferol, ferulic acid, methyl gallate, coumaric acid, syringic acid, and 3,4-Dihydroxybenzoic acid in 0.12 to 1900.97  $\mu$ g/g of the extract, whereas it contained seven flavonoids: rutin, hesperidin, quercetin, apigenin, luteolin, naringenin, and vanillin in 0.33 to 3137.22  $\mu$ g/g of the extract. The retention times, areas, and fragmentation chromatograms for both the extract (sample) and standards are presented in supplementary Fig. S3 and Tables S1 and S2 online.

### Biological activities of *S. fruticosa* extract

#### Antioxidant activity

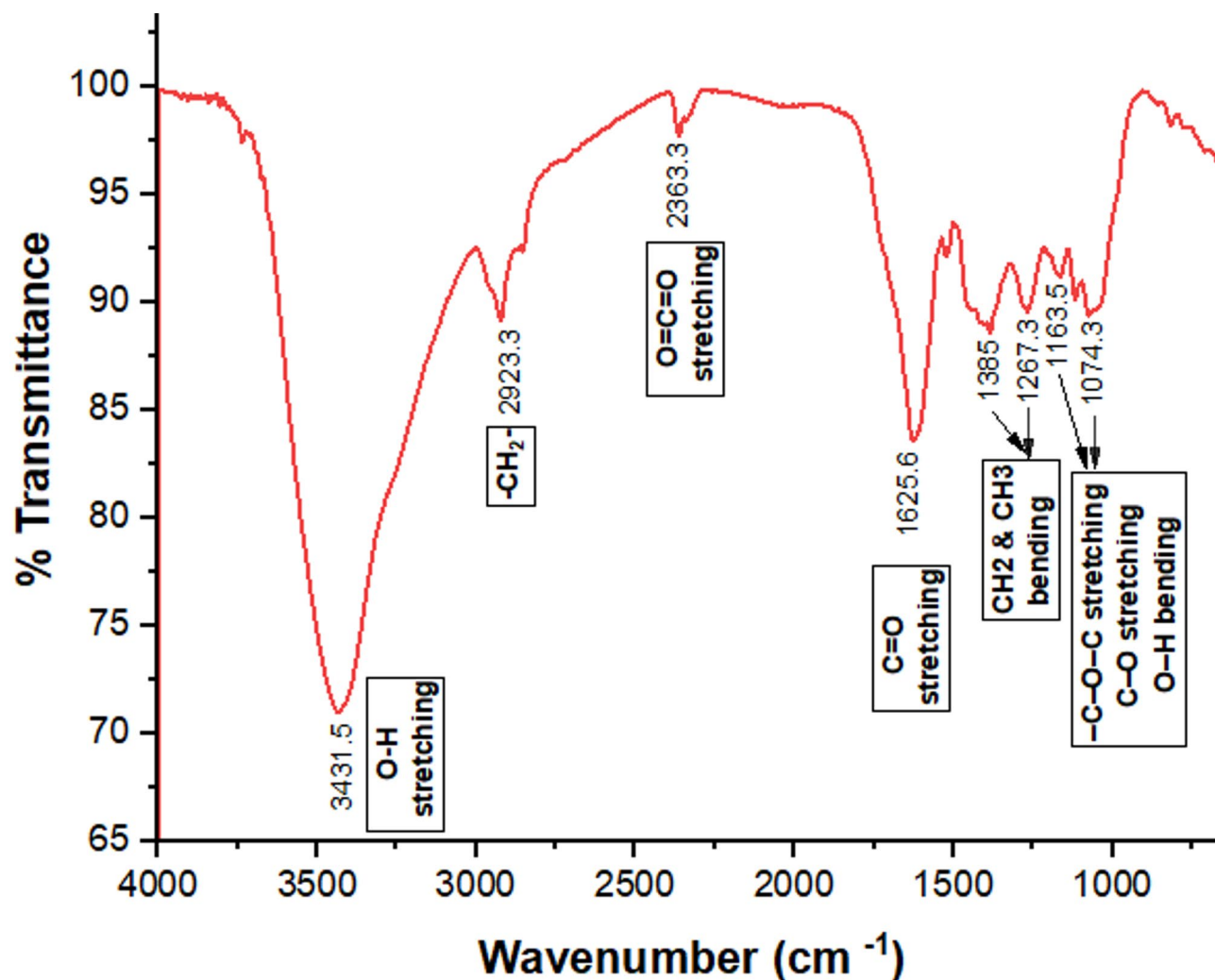
As shown in Fig. 2a, the extract exhibits concentration-dependent free radical scavenging activity, ranging from 7.19 to 84.23% at concentrations of 5 to 80  $\mu$ g/mL, respectively. Trolox also shows a concentration-dependent free radical scavenging activity ranging from 3.79 to 82.59% but at a lower concentration range (1.25 to 12.5  $\mu$ g/mL), as shown in Fig. 2b. The IC<sub>50</sub> of the extract and Trolox were determined using the extract dose-response curve, as shown in Fig. 2c and d. *S. fruticosa* extract showed an antioxidant capacity with an IC<sub>50</sub> of 25.17  $\pm$  0.304  $\mu$ g/mL of DPPH compared to Trolox (IC<sub>50</sub> = 6.352  $\mu$ g/mL). Tukey's multiple comparisons test results for the antioxidant activity of *S. fruticosa* extract and Trolox at various concentrations are summarized in supplementary Table S3 online.

#### Antimicrobial activity

The results of the MIC assay (Table 1) indicate that the extract exhibits effective antimicrobial activity, with the best MIC values observed against *S. aureus*, *B. subtilis*, and *S. cerevisiae* at 1.562 mg/mL, compared to the MIC value for *E. coli*, which was 12.5 mg/mL.

#### U2OS cell viability after incubation with *S. fruticosa* extract and 0.2 vol% DMSO

As shown in Fig. 3a, *S. fruticosa* extract decreased U2OS cell viability in a dose- and time-dependent manner. A higher *S. fruticosa* extract concentration of 150  $\mu$ g/mL decreased U2OS viability by 32%, 50%, and 96% after



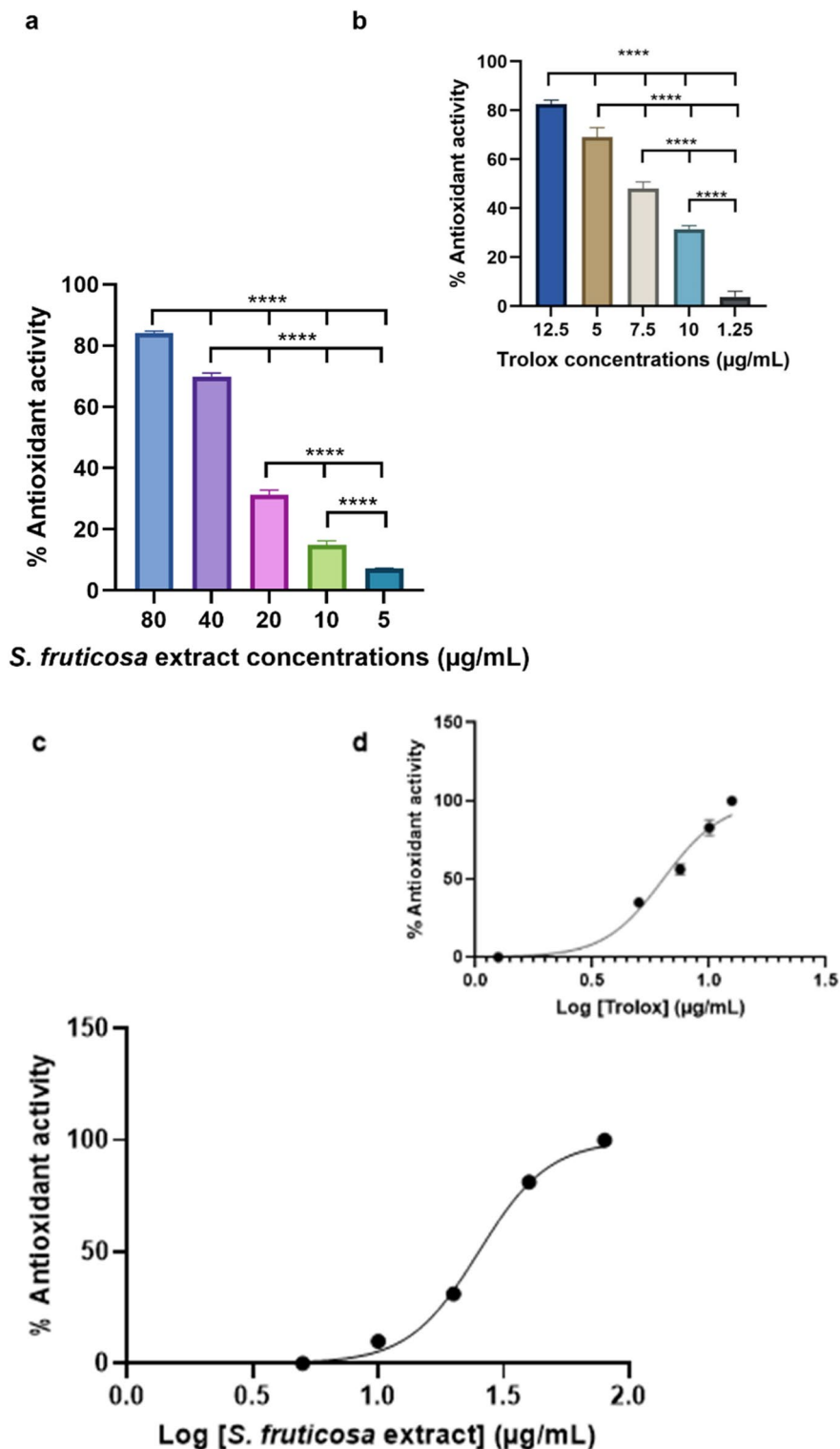
**Fig. 1.** FTIR spectrum of *Salvia fruticosa* extract.

24, 48, and 72 h, respectively, with  $p < 0.0001$ . Additionally, 75  $\mu\text{g}/\text{mL}$  of *S. fruticosa* extract had no significant effect on U2OS cell viability after 24 h of incubation, but decreased cell viability by 20% ( $p = 0.0033$ ) and 42% ( $p < 0.0001$ ) after 48 and 72 h, respectively. On the other hand, lower *S. fruticosa* extract concentrations showed no significant difference in cell viability between treated and untreated cells over the three investigated time intervals. A dose-response curve for the extract was generated using MTT data, as shown in Fig. 3b.  $\text{IC}_{50}$  values obtained after 24, 48, and 72 h had standard error of residual plots (Sy.x) values of 26.04, 22.88, and 7.92, respectively. The  $\text{IC}_{50}$  had coefficient of determination ( $R^2$ ) values of 0.6241, 0.6991, and 0.9581, respectively, and all Hillslope values were lower than one. The lowest recorded  $\text{IC}_{50}$  value was 77.58  $\mu\text{g}/\text{mL}$  after 72 h of incubation, followed by 80.85  $\mu\text{g}/\text{mL}$  after 48 h of incubation and 87.75  $\mu\text{g}/\text{mL}$  after 24 h of incubation with *S. fruticosa* extract. The  $\text{IC}_{50}$  obtained after 72 h of incubation had the lowest value, with the lowest Sy.x and the best  $R^2$  value (near 1), as summarized in supplementary Table S4 online.

To ensure that the cytotoxicity induced by different concentrations of *S. fruticosa* was due to its extract and not the DMSO used for its dissolving, we conducted an MTT assay to evaluate the effect of 0.2 vol% DMSO, which constituted the highest concentration of *S. fruticosa* extract (150  $\mu\text{g}/\text{mL}$ ), on cell viability after 24, 48, and 72 h of incubation with U2OS. As presented in Fig. 3c, a 0.2 vol% DMSO concentration had no significant effect on U2OS cell viability after incubation at the three time points.

#### Cytotoxicity of *S. fruticosa* extract on HSF cells compared to U2OS cells

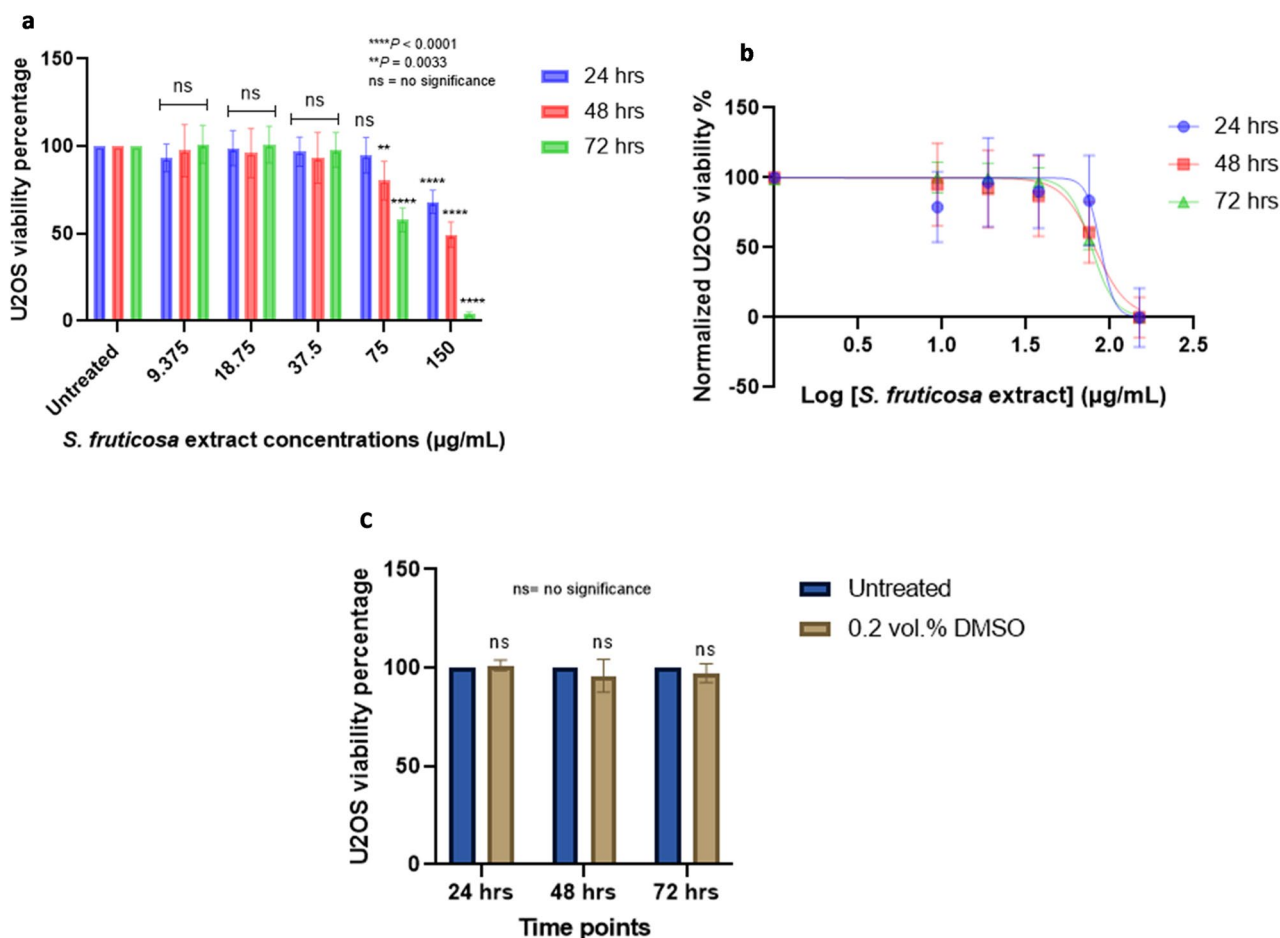
In our study, we used HSF as a model for normal cells on which we could test the cytotoxicity of *S. fruticosa* extract  $\text{IC}_{50}$  compared to U2OS cells to evaluate the possible side effects that may be caused by the extract on normal cells using the MTT assay and cisplatin as a reference<sup>15,30</sup>. As shown in Fig. 4, cisplatin significantly decreased the viability of U2OS and HSF cells by approximately  $27 \pm 4.08\%$  and  $32 \pm 4.08\%$ , respectively. In contrast, the extract decreased U2OS cell viability by nearly 19% but had no significant effect on HSF viability.



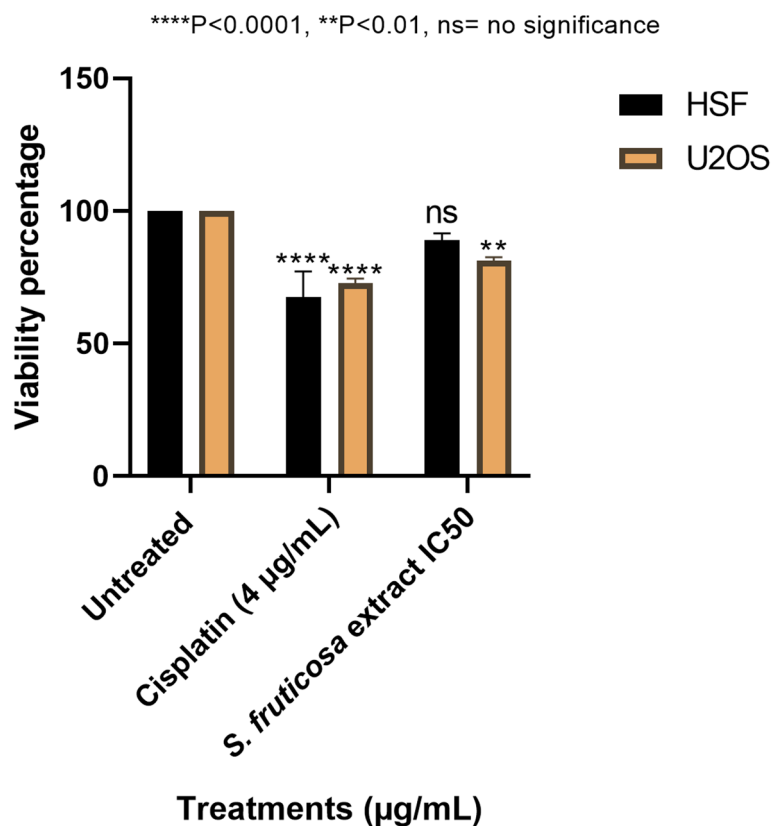
**Fig. 2.** Antioxidant activity of *Salvia fruticosa* extract using DPPH free radical scavenging activity assay. Antioxidant activity of *S. fruticosa* extract (**a**) and Trolox as a positive control (**b**). Dose-response curves against DPPH free radical for *S. fruticosa* extract (**c**) and Trolox (**d**). Data are representative of three experiments, and significance analysis was done using one-way ANOVA followed by Tukey's multiple comparisons test, at a 95% confidence level on GraphPad Prism 9 (\*\*\*\* $p < 0.0001$ ) (see supplementary Table S3 online). Data from dose-response curves represent the results of three experiments, and analysis was performed using nonlinear regression analysis in GraphPad Prism 9.  $IC_{50}$  values are shown as mean  $\pm$  SD.

	Concentrations (mg/mL)	25	12.5	6.25	3.125	1.562	0.781	0.391	0.195	0.098	0.049	
Microorganisms	<i>S. aureus</i> ATCC6538	-	-	-	-	-	+	+	+	+	+	
	<i>B. subtilis</i> ATCC35854	-	-	-	-	-	+	+	+	+	+	
	<i>E. coli</i> ATCC8739	-	-	+	+	+	+	+	+	+	+	
	<i>S. cerevisiae</i> ATCC9763	-	-	-	-	-	+	+	+	+	+	
	-ve C	-										
	+ve C	+										

**Table 1.** Antimicrobial activity of *Salvia fruticosa* extract using minimum inhibitory concentration assay by microdilution method. The experiment was done in triplicate. -ve C = negative control (broth only), +ve C = positive control (broth with microorganism), (-) = no growth, (+) = growth.



**Fig. 3.** Effect of *Salvia fruticosa* extract and 0.2 vol% DMSO on U2OS cell viability after 24, 48, and 72 h of incubation using MTT assay. **(a)** U2OS cell viability after incubation with *S. fruticosa* extract. The extract decreased viability in a dose and time-dependent manner. The highest decrease in cell viability (by 96%) was achieved after incubation with 150  $\mu\text{g/mL}$  of the extract for 72 h. **(b)** *S. fruticosa* extract dose-response curves. The obtained dose-response curves had a negative slope, indicating a decrease in cell viability with increasing extract concentration. The obtained  $\text{IC}_{50}$  values were  $87.75 \pm 4.02 \mu\text{g/mL}$  ( $R^2 = 0.6241$ ),  $80.85 \pm 10.65 \mu\text{g/mL}$  ( $R^2 = 0.6991$ ), and  $77.58 \pm 3.47 \mu\text{g/mL}$  ( $R^2 = 0.9581$ ) after 24, 48, and 72 h, respectively. **(c)** U2OS cell viability after incubation with 0.2 vol% DMSO, which constituted the highest concentration of the extract. No significant change in cell viability was observed over the three time points. All U2OS viability comparisons were made between the treated and untreated cells; data are representative of three experiments and significance analysis was done using two-way ANOVA followed by Tukey's multiple comparisons test, except for the 0.2 vol% DMSO viability data analysis which was done using one-way ANOVA followed by Dunnett's multiple comparisons test, at a 95% confidence level on GraphPad Prism 9 (\*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ , ns = no significance). Data from dose-response curves represent the results of three experiments, and analysis was performed using nonlinear regression analysis in GraphPad Prism 9.  $\text{IC}_{50}$  values are shown as mean  $\pm$  SD.



**Fig. 4.** Effect of *Salvia fruticosa* extract on the viability of HSF compared to U2OS cells after 72 h of incubation. The extract decreased U2OS cell viability by approximately 19% while it had no significant effect on HSF. On the other hand, 4 µg/mL of cisplatin showed a significant decrease in both U2OS and HSF by  $32 \pm 4.08\%$  and  $27 \pm 4.08\%$ , respectively. Comparisons were made between the treated and untreated cells; data are representative of three independent experiments, and significance analysis was done using two-way ANOVA followed by Tukey's multiple comparisons test at a 95% confidence level on GraphPad Prism 9 (\*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ , ns = no significance).

#### *U2OS cell morphology after incubation with S. fruticosa extract*

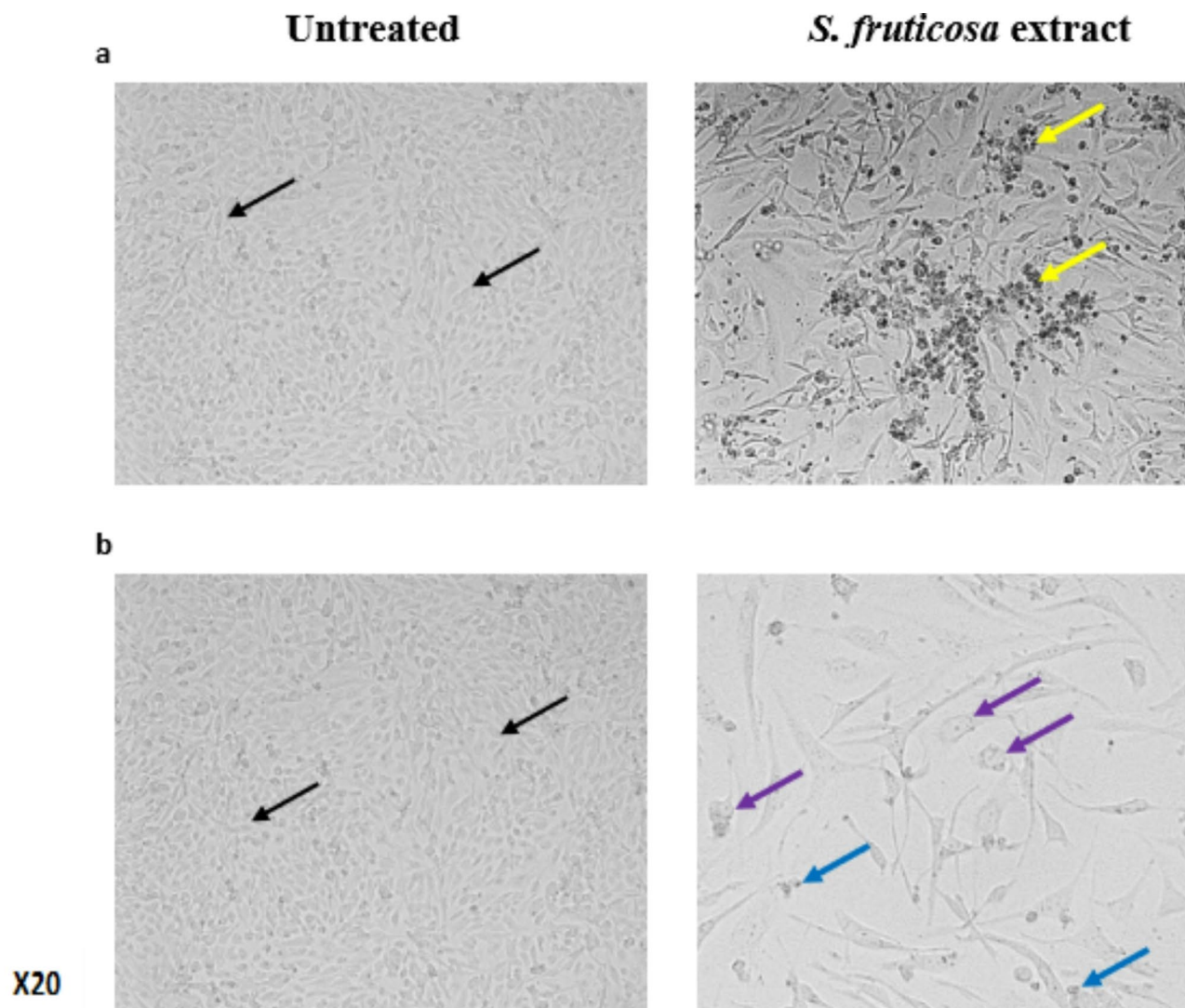
As shown in Fig. 5a, a noticeable number of dead cells resulted after incubation with the extract. The cells lost their epithelial morphology and floated in the medium compared to the untreated cells (labeled by the black arrows). After washing cells with PBS to remove dead cells and debris (labeled by the yellow arrows), other images were taken to examine cell morphology as shown in Fig. 5b. The surviving cells were elongated, compared to the untreated cells due to the large space between cells caused by the induction of cell death by the extract, making the living cells stretch to reach each other. Additionally, the treatment altered the morphological characteristics of U2OS cells compared to those of the untreated cells. Treated cells with the extract showed morphological changes where cells shrank (with a spherical shape) and exhibited chromatin condensation with a characteristic necklace shape (labeled by the blue arrows) and blebbing (labeled by the purple arrows) instead of being elliptical with wider center and tapering ends as the untreated cells<sup>33,34</sup>.

#### *Effect of S. fruticosa extract on U2OS cell migration*

The percentage of wound closure or confluency was determined after 12, 24, and 48 h of incubation with the treatments until almost complete closure of wounds in the untreated wells (negative control). After 12 and 24 h of incubation, *S. fruticosa* extract showed no significant difference in the percentage of wound confluency compared to the control (untreated cells). After 48 h of incubation, the extract showed a significant difference in the percentage of wound confluency ( $57 \pm 8.39\%$ ) compared to the control's wound closure percentage, which was  $93 \pm 8.39\%$  as shown in Fig. 6.

## Discussion

The main aim of this study is to extract bioactive compounds including polyphenols from *S. fruticosa* leaves and test their antimicrobial effect on two gram-positive (*S. aureus* ATCC6538 and *B. subtilis* ATCC35854), one gram-negative bacteria (*E. coli* ATCC8739), and one fungus (*S. cerevisiae* ATCC9763) as well as their cytotoxic effect on U2OS cells, as a model for bone cancer. Modern ultrasonic extraction using 70% methanol was performed on ground *S. fruticosa* leaves with an average yield of  $0.144 \pm 0.008$  g/g of the dry leaves, followed by chemical characterization of the extract using FTIR. The obtained FTIR spectrum of *S. fruticosa* extract (Fig. 1) showed

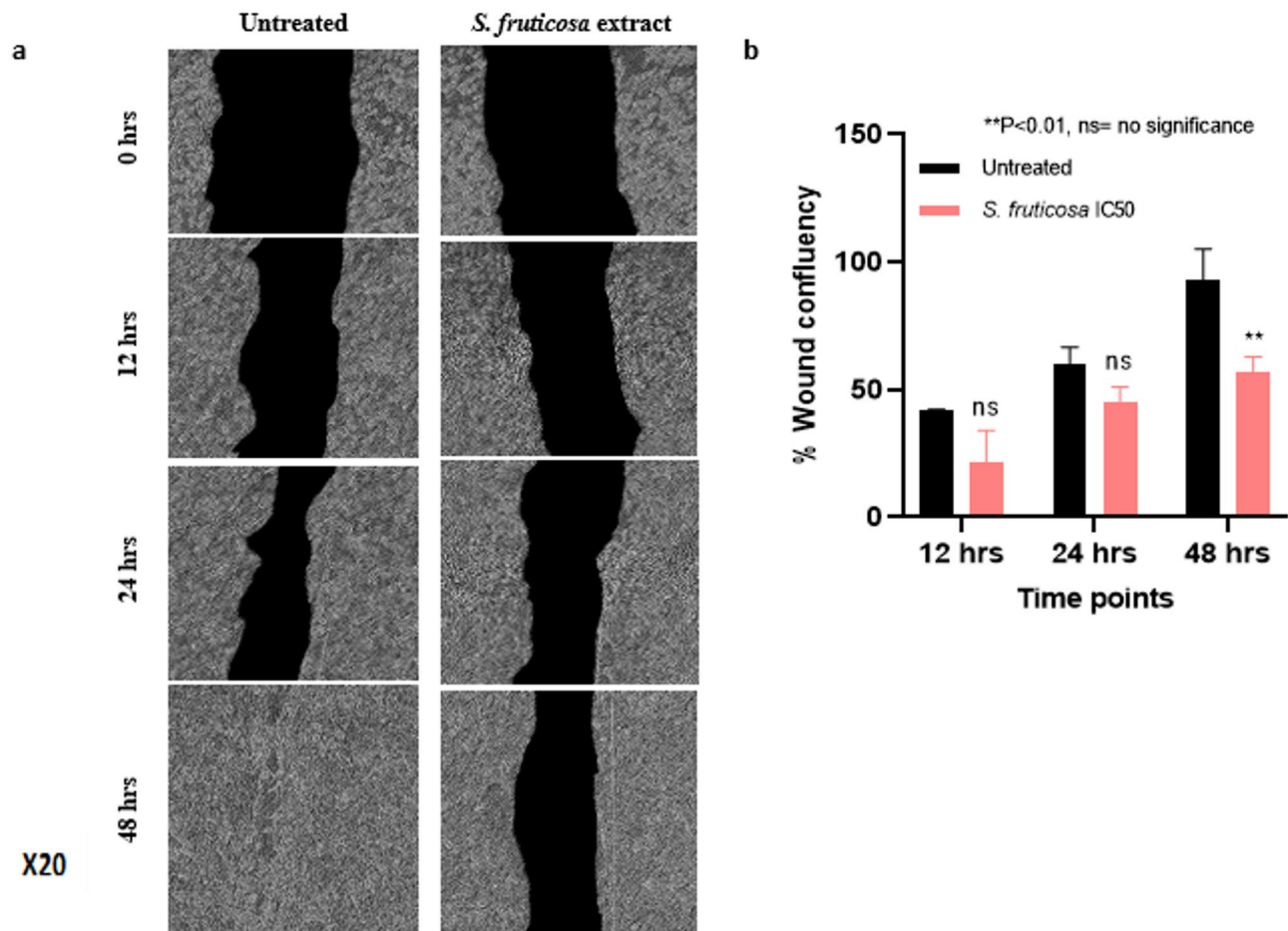


**Fig. 5.** Effect of *Salvia fruticosa* extract on U2OS osteosarcoma cell morphology. (a) Untreated and treated U2OS cells before washing with PBS. Untreated U2OS cells showed elliptical morphology and 100% confluency (labeled by the black arrows). Treated cells with the extract  $IC_{50}$  showed several dead cells and cell debris (labeled by the yellow arrows). (b) U2OS cell morphology after washing with PBS to remove dead cells and cell debris. Untreated U2OS cells showed an elliptical shape and 100% confluency (labeled by the black arrows). U2OS cells that were treated with the extract  $IC_{50}$  showed cell shrinkage & chromatin condensation (labeled by the purple arrows) and blebbing (labeled by the blue arrows). Images were captured using an inverted microscope with a 20X magnification lens.

bands at wavelengths 3431.5, 2923.3, 2363.3, and 1625.6 that correspond to O-H stretching in alcohol, water, and polyphenols, aliphatic stretching ( $-CH_2-$ ) in fats,  $O=C=O$  stretching in carbon dioxide, and  $C=O$  stretching in amide 1, respectively. Additional bands also appear at 1385 and  $1267.3\text{ cm}^{-1}$  which correspond to the respective bending modes of vibration of  $CH_2$  and  $CH_3$  in fatty acids, proteins, and phosphate-bearing compounds, along with bands at 1163.5 and  $1074.3\text{ cm}^{-1}$  arising from the stretching vibration of  $-C-O-C$  glycoside ring bond,  $C-O$  stretching in  $COOH$ , and  $O-H$  bending in carbohydrates and polysaccharides<sup>35,36</sup>.

Numerous studies have demonstrated the anticancer effects of polysaccharides on various cell lines. They have been reported to induce apoptosis, DNA damage, disruption of mitochondrial membrane, and cell cycle arrest to kill cancer cells and prevent metastasis<sup>37–40</sup>. Additionally, polysaccharides were tested against a wide range of microorganisms for their antimicrobial activities, including increasing cell membrane permeability, inhibiting microorganism attachment to the host, or blocking membrane transport of nutrients essential for energy production<sup>41–44</sup>. Therefore, the phenol-sulfuric acid method was conducted to determine TCC in *S. fruticosa* extract, which was estimated to be  $45.63 \pm 3.33$  mg glucose equivalent/g of the extract.

Sage is considered a rich source of phenolics that have been proven to have antimicrobial and anticancer activities. TPC in *S. fruticosa* extract was measured using folin-ciocalteu assay and detected spectrophotometrically with an estimated value of  $170.68 \pm 6.52$  mg caffeic acid equivalent/g of the extract. The measured percentage of



**Fig. 6.** Effect of *Salvia fruticosa* extract on U2OS osteosarcoma cell migration and wound closure over 12, 24, and 48 h of incubation. **(a)** Comparisons between treated and untreated cells revealed no significant difference in the wound closure between 12 and 24 h of incubation while the extract inhibited cell migration, with almost complete wound closure in untreated cells after 48 h. These results were supported by the statistical analysis in **(b)** where the extract's IC<sub>50</sub> concentration showed no significant decrease in wound closure percentage compared to the control (untreated cells) at the 12- and 24-hour time points. However, a significant decrease of nearly 43 ± 8.39% in wound closure was observed after 48 h. This suggests the extract's potential to inhibit cell metastasis. Images were captured using an inverted microscope with a 20X magnification lens. Data represents three independent experiments, and significance analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test at a 95% confidence level in GraphPad Prism 9 (\*\**p* < 0.01, ns = no significance).

total phenolic content in *S. fruticosa*, compared to that previously reported in *Salvia* species, is summarized in Table 2. According to our results, *S. fruticosa* is considered the second highest *Salvia* extract rich in phenolics with 17.07 ± 0.65%, after *S. africana* (35.06 ± 1.49%), compared to other 31 *Salvia* species with reported phenolic content percentages ranging from 0.7 to 35.06%<sup>56–63</sup>. This suggests that *S. fruticosa* extract may possess antimicrobial and anticancer properties, derived from its high content of phenolics, which have been previously reported to inhibit microbial growth and cancer progression.

Flavonoids have been reported to exhibit antimicrobial activity by suppressing nucleic acid synthesis, the functionality of the cytoplasmic membrane, and energy metabolism, in addition to their strong anticancer effects arising from their antioxidant, cell cycle-arresting, and induction of apoptosis and autophagy activities. They also inhibit cell proliferation and metastasis<sup>64–70</sup>. Therefore, we investigated the content of total flavonoids in *S. fruticosa* extract spectrophotometrically using the aluminum chloride method, and the estimated flavonoids were 44.71 ± 5.67 mg rutin equivalent/g of the extract. The percentage of total flavonoid content in *S. fruticosa* compared to the previously reported data in other 25 *Salvia* species is shown in Table 2. *S. fruticosa* contained 4.47 ± 0.57% of total flavonoids, which is lower than the highest reported flavonoid content in *S. hierosolymitana* by approximately 47.6% and ranked 6th in the highest content of flavonoids (after *S. hierosolymitana*, *S. eigii*, *S. viridis*, *S. reuteriana*, and *S. hypoleuca*) compared to other species with reported flavonoid content percentages ranging from 0.12 to 52.06%<sup>56,57,59–63,71</sup>. This indicates that *S. fruticosa* methanolic extract could also have good antimicrobial and anticancer properties due to its flavonoid content.

<i>Salvia</i> species	Total Phenolic content (%)	Total flavonoid content (%)	IC <sub>50</sub> (µg/mL DPPH)
<i>S. fruticosa</i> (current study)	17.07 ± 0.65%	4.47 ± 0.57%	25.17 ± 0.304
<i>S. tomentosa</i> Miller	4.93 ± 0.2%	3.89 ± 0.17%	30 ± 0.00
<i>S. fruticosa</i>	7.05 ± 0.13%	0.31 ± 0.009%	NA
<i>S. glutinosa</i>	3.77 ± 0.07%	3.18 ± 0.024%	80.42 ± 0.95
<i>S. nemorosa</i>	6.48 ± 0.01%	0.88 ± 0.004%	40.22 ± 1.13
<i>S. officinalis</i>	8.04 ± 0.10%	0.37 ± 0.002%	29.69 ± 1.32
<i>S. pratensis</i>	6.49 ± 0.01%	0.90 ± 0.007%	NA
<i>S. sclarea</i>	3.55 ± 0.04%	0.75 ± 0.002%	190.74 ± 5.7
<i>S. verticillate</i>	12.44 ± 0.01%	0.39 ± 0.003%	10 ± 0.00
<i>S. eigii</i>	2.50 ± 0.31%	52.06 ± 0.62%	248 ± 1.28
<i>S. hierosolymitana</i>	2.73 ± 0.09%	77.08 ± 0.53%	184 ± 12.2
<i>S. viridis</i>	1.85 ± 0.03%	31.14 ± 0.44%	234 ± 4.93
<i>S. Africana</i>	35.06 ± 1.49%	NA	6.6 ± 0.7
<i>S. Mexicana</i>	15.89 ± 3.8%	NA	10 ± 1.1
<i>S. xanthocheila</i>	1.25 ± 0.12%	0.22 ± 0.02%	457.00 ± 41.62
<i>S. limbate</i>	1.3 ± 0.07%	3.01 ± 0.252%	557.40 ± 12.73
<i>S. aegyptiaca</i>	1.38 ± 0.02%	NA	330.4 ± 11.06
<i>S. aethiopsis</i>	1.41 ± 0.09%	NA	237.37 ± 8.05
<i>S. syriaca</i>	1.81 ± 0.04%	4.05 ± 0.08%	315.1 ± 5.71
<i>S. Eremophila</i>	1.89 ± 0.10%	NA	114.57 ± 11.5
<i>S. santolinifolia</i>	2.02 ± 0.09%	NA	117.34 ± 4.07
<i>S. hypoleuca</i>	2.03 ± 0.05%	5.316 ± 0.195%	197.23 ± 6.86
<i>S. atropatana</i>	2.57 ± 0.004%	0.7 ± 0.04%	89.47 ± 5.97
<i>S. scabiosifolia</i>	5.942 ± 0.71%	0.33 ± 0.04%	NA
<i>S. ringens</i>	4.85 ± 0.57%	0.12 ± 0.06%	NA
<i>S. nutans</i>	3.05 ± 0.24%	0.205 ± 0.04%	NA
<i>S. ahendica</i>	0.7 ± 0.04%	0.32 ± 0.03%	16.2 ± 0.3
<i>S. hydrangea</i>	2.19 ± 0.01%	1.15 ± 0.01%	5.3 ± 0.1
<i>S. chloroleuca</i>	1.11 ± 0.15%	0.66 ± 0.1%	16.7 ± 1.0
<i>S. ceratophylla</i>	3.27 ± 0.17%	2.7 ± 0.1%	5.5 ± 0.1
<i>S. macrosiphon</i>	1.64 ± 0.01%	0.24 ± 0.09%	14.7 ± 0.1
<i>S. reuteriana</i>	7.19 ± 0.065%	8.25 ± 0.22%	86.76 ± 0.3

**Table 2.** Comparison of total phenolics, total flavonoids, and antioxidant activity (represented by DPPH IC<sub>50</sub>) of *Salvia fruticosa* (current study) to previously reported *Salvia* species. Three independent experiments were conducted, and results are presented as mean ± SD, NA = Not Tested<sup>56–63,71</sup>.

As the previous analyses indicated that *S. fruticosa* extract is rich in phenolics and flavonoids, we identified the major compounds from the two classes that are present in the extract using LC-ESI-MS/MS technique. As shown in Table 3, *S. fruticosa* extract constitutes eight phenolic compounds: chlorogenic acid, gallic acid, caffeic acid, kaempferol, ferulic acid, methyl gallate, coumaric acid, and 3,4-Dihydroxybenzoic acid while it comprises seven flavonoids: rutin, hesperidin, quercetin, apigenin, luteolin, naringenin, and vanillin in a concentration range from 0.12 to 3137.22 µg/g. According to previous studies, the detected phenolics and flavonoids were reported to be potential antimicrobial and anticancer agents<sup>72–111</sup>.

The concentrations of specific phenolics and flavonoids detected in *S. fruticosa* extract compared to extracts from other *Salvia* species are also reported in Table 3. Naringenin is the most abundant polyphenolic compound present in *S. fruticosa* methanolic extract, with a concentration of 3137.22 µg/g. It has also been reported in four other *Salvia* species, including *S. verticillata*, *S. viridis*, *S. eigii*, and *S. hierosolymitana*, in the range of 36.25–418.59 µg/g. Chlorogenic acid was also detected in *S. fruticosa* extract at 1900.97 µg/g, ranking it third among *Salvia* species rich in chlorogenic acid, after *S. viridis* and *S. officinalis*, with reported chlorogenic acid concentrations ranging from 159 to 6619.93 µg/g in eleven *Salvia* species. Caffeic acid and luteolin were also reported to have respective concentrations of 654.91 and 532.34 µg/g of *S. fruticosa* extract, rendering *S. fruticosa* the second *Salvia* rich in caffeic acid and luteolin after *S. tomentosa* Miller. As for 3,4-dihydroxybenzoic acid, it was detected at 179.23 µg/g of the *S. fruticosa* extract. However, this has not been reported in other *Salvia* species. Apigenin was also quantified in *S. fruticosa* extract at 170.80 µg/g, compared to the previously reported concentrations in six *Salvia* species, which ranged from 13.48 to 208 µg/g. Other phenolics and flavonoids, namely p-coumaric, syringic, ferulic acids, quercetin, gallic acid, and rutin were measured at 40.14, 34.22, 21.43, 2.59, 2.24, and 0.33 µg/g of *S. fruticosa* extract, respectively and these are the lowest reported concentrations for these compounds in *Salvia* species. Hesperidin was detected at 6.45 µg/g of *S. fruticosa* extract, while its

Compound	<i>S. fruticosa</i> (current study)	<i>S. tomentosa</i> Miller	<i>S. glutinosa</i>	<i>S. nemorosa</i>	<i>S. officinalis</i>	<i>S. pratensis</i>	<i>S. sclarea</i>	<i>S. verticillata</i>	<i>S. africana</i>	<i>S. mexicana</i>	<i>S. eigii</i>	<i>S. hierosolymitana</i>	<i>S. viridis</i>
<b>Phenolic compounds (µg/g dry extract)</b>													
Caffeic acid	654.91	2318	300	300	400	600	500	4100	0.0023	0.0009	32.97	260.79	453.03
Chlorogenic acid	1900.97	159	300	1900	2100	1600	1200	1500	NA	NA	173.75	201.19	6619.93
Gallic acid	2.24	22	NA	NA	6.67	NA	NA	NA	NA	NA	100.09	84.15	27.36
Kaempferol	2.31	623	NA	NA	NA	NA	NA	350.45	NA	NA	NA	NA	NA
p-Coumaric acid	40.14	2227	1200	1600	11600	600	2900	8400	NA	NA	NA	NA	NA
Ferulic acid	21.43	355	ND	ND	60.45	ND	ND	2200	ND	ND	NA	NA	NA
Ellagic acid	ND	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Methyl gallate	0.12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3,4-Dihydroxybenzoic acid	179.23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cinnamic acid	ND	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Syringic acid	34.22	NA	NA	NA	128.7	NA	NA	NA	NA	NA	NA	NA	NA
<b>Flavonoids (µg/g dry extract)</b>													
Rutin	0.33	591	ND	ND	75.28	76.24	ND	ND	NA	NA	54.49	3.71	100.89
Hesperidin	6.45	1002	NA	NA	NA	NA	NA	NA	ND	0.0005	142.74	95.69	298.38
Quercetin	2.59	538	ND	ND	ND	ND	ND	59.02	NA	NA	6.10	6.91	7.10
Catechin	ND	123	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Apigenin	170.80	208	ND	ND	13.48	ND	83.68	ND	NA	NA	111.31	26.08	39.37
Luteolin	532.34	899	ND	ND	27.51	25.62	163.54	31.48	NA	NA	NA	NA	NA
Naringenin	3137.22	NA	NA	NA	NA	NA	NA	36.25	NA	NA	154.02	418.59	67.77
Vanillin	13.23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Daidzein	ND	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Myricetin	ND	1001	NA	NA	NA	NA	NA	75.6	NA	NA	NA	NA	NA

**Table 3.** Reported concentrations of phenolics and flavonoids in different *Salvia* species compared to our liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI- MS/MS) study on *Salvia fruticosa* extract. Concentrations are expressed in µg of the standard per gram of the dry extract, NA = Not tested, ND = Tested but not detected<sup>57,61,62,112–114</sup>.

	Salvia Species	<i>S. fruticosa</i> (current study)	<i>S. fruticosa</i>	<i>S. officinalis</i>	<i>S. lavandulifolia</i>	<i>S. sclarea</i>
Microorganisms	<i>S. aureus</i> ATCC6538	1.562	0.2–3.42	2.87–12.5	2.31	3.42
	<i>B. subtilis</i> ATCC35854	1.562	NA	NA	NA	NA
	<i>E. coli</i> ATCC8739	12.5	0.0024–0.625	NA	NA	NA
	<i>S. cerevisiae</i> ATCC9763	1.562	NA	NA	NA	NA

**Table 4.** Comparison of the antimicrobial activity of *Salvia fruticosa* extract of the current study to other reported *Salvia* species using the minimum inhibitory concentration assay. MIC values are expressed in mg/mL, NA = Not tested<sup>123–128</sup>.

concentration in five *Salvia* species ranged from 0.0005 to 1002 µg/g. Vanillin and methyl gallate were quantified at 13.23 and 0.12 µg/g of *S. fruticosa* extract, respectively, but were not reported in other *Salvia* species. On the other hand, ellagic acid, cinnamic acid, catechin, daidzein, and myricetin were not detected in *S. fruticosa* methanolic extract<sup>58,61,62,112–114</sup>.

As the correlation between total phenolic and flavonoid content and antioxidant activity is not yet confirmed, a DPPH free radical scavenging activity assay was performed to determine the antioxidant activity of the extract<sup>23,24,115</sup>. Since a lower IC<sub>50</sub> value indicates a higher ability of the extract to scavenge free radicals and consequently potent antioxidant activity, our extract showed a good antioxidant capacity with an IC<sub>50</sub> of 25.17 ± 0.304 µg/mL of DPPH (Fig. 2). Thus, *S. fruticosa* extract ranked the 9th most potent antioxidant—after *S. hydrangea*, *S. ceratophylla*, *S. africana*, *S. verticillata*, *S. mexicana*, *S. macrosiphon*, *S. ahendica*, *S. chloroleuca*—among the determined IC<sub>50</sub> of 26 *Salvia* species, ranging from 5.3 to 557.4 µg/mL as summarized in Table 2<sup>56–60,116–119</sup>. This indicates that the extract can scavenge free radicals due to its electron- and/or hydrogen atom-donating ability and consequently could have the ability to inhibit the initiation and/or progression of free radical-mediated chain reaction and thus inhibit cancer progression through preventing DNA damage, decreasing of free radical-mediated chain reaction and thus inhibiting cancer mutagenesis and reducing abnormal cell division progression through preventing DNA damage, decreasing mutagenesis and reducing abnormal cell division<sup>120–122</sup>.

As the extract is a rich source of carbohydrates, phenolics, and flavonoids that have been demonstrated to exhibit good antimicrobial activity, the antimicrobial activity of the methanolic extract of *S. fruticosa* was examined against microorganisms causing foodborne diseases and human infections, including *S. aureus*, *B. subtilis*, *E. coli*, and *S. cerevisiae*. *S. aureus* and *E. coli* are known for their multidrug resistance and association with nosocomial infections<sup>26</sup>. So, the need to find better antimicrobial alternatives is urgent. MIC by the microdilution method was performed to evaluate the antimicrobial activity of *S. fruticosa* extract. The MIC values were 1.562 mg/mL for *S. aureus*, *B. subtilis*, and *S. cerevisiae*, and 12.5 mg/mL for *E. coli*. Previous studies were conducted to evaluate the antimicrobial activities of different species of *Salvia* on similar strains of *S. aureus* and *E. coli*, and a comparison between the results of the current study and the previous ones is summarized in Table 4<sup>123–128</sup>. The methanolic extract exhibited good antimicrobial activity against *S. aureus* (MIC = 1.562 mg/mL) within the reported antimicrobial range of *S. fruticosa* evaluated in previous studies (MIC 0.2–3.42 mg/mL). The methanolic extract also exhibited better antimicrobial activity against *S. aureus* compared to other extracts of *S. officinalis*, *S. lavandulifolia*, and *S. sclarea* (MIC ranging from 2.31 to 12.5 mg/mL). Additionally, *S. fruticosa* water and ethanolic extracts were also reported to exhibit higher antimicrobial activity against *E. coli* with MICs ranging from 0.0024 to 0.625 mg/mL relative to *S. fruticosa* methanolic extract in the current study (MIC = 12.5 mg/mL)<sup>123–128</sup>. These findings suggest the potential antimicrobial activity of the methanolic extract of *S. fruticosa* against gram-positive, gram-negative bacteria, and fungi, likely due to its substantial content of carbohydrates, phenolics, and flavonoids.

Being rich in polyphenolic compounds, which exhibit potent antioxidant activity and can potentially act as anticancer agents. *S. fruticosa* methanolic extract was investigated for its cytotoxicity on U2OS osteosarcoma cells using the MTT assay. The extract inhibited U2OS cell proliferation with an IC<sub>50</sub> of 87.75 ± 4.02, 80.85 ± 10.65, and 77.58 ± 3.47 µg/mL after 24, 48, and 72 h of incubation, respectively, as shown in Fig. 3 and supplementary Table S3 online. To ensure that the cytotoxicity was due to the extract itself and not due to DMSO that was used to dissolve the extract, another MTT assay was performed on 0.2 vol% DMSO constituting the highest concentration of the extract and there was no significant effect on U2OS viability as illustrated in Fig. 3c. In a previous study, it was reported that the acetone extract of *S. fruticosa* leaves decreased U2OS cell viability but with a lower IC<sub>50</sub> (30.21 ± 1.18 µg/mL) than the methanolic extract after 48 h of incubation which indicates that the acetone extract of *S. fruticosa* had a higher cytotoxicity effect on U2OS cells than the methanolic extract<sup>15</sup>.

In our study, we aimed to investigate the cytotoxic effect of the extract compared to cisplatin (as a reference) on normal human cells versus cancerous ones, with the goal of evaluating potential side effects. So, we used HSF as a model for normal cells<sup>15</sup>. Fortunately, the extract exhibited no significant effect on HSF, while decreasing U2OS viability by approximately 19% compared to 4 µg/mL of cisplatin, which inhibited both U2OS and HSF by 32 ± 4.08% and 27 ± 4.08%, respectively, as shown in Fig. 4, indicating the potential use of the extract as a treatment for osteosarcoma without affecting normal body cells.

Additionally, we assessed the effect of *S. fruticosa* extract on U2OS cell morphology using a 20X magnification lens of the inverted microscope. The extract showed a change in U2OS cell morphology from being elliptical with a wider center and tapering ends to becoming shrunk and exhibiting chromatin condensation with a characteristic necklace shape and blebbing as illustrated in Fig. 5<sup>33,34</sup>.

Finally, in our study, we evaluated the effect of the extract on U2OS cell migration, an indicator of metastasis, by performing a scratch wound healing assay. After 48 h of incubation, the *S. fruticosa* extract showed a significant difference in the percentage of wound confluency,  $57 \pm 8.39\%$  compared to the control at  $93 \pm 8.39\%$ , indicating that the treatment may affect the genetic markers involved in cell proliferation, migration, and possibly metastasis, as shown in Fig. 6.

While this study provides valuable insights into the bioactive compounds and therapeutic potential of *S. fruticosa* methanolic extract, it is important to acknowledge certain limitations. Primarily, the current investigation is based on in vitro experiments, which, although crucial for initial screening and mechanistic understanding, may not fully capture the complex biological interactions occurring in vivo. Therefore, the observed antimicrobial and cytotoxic effects require further validation using animal models to evaluate efficacy, pharmacokinetics, and potential toxicity in a living system. Additionally, the scratch wound healing assay was performed without prior cell starvation, meaning the results reflect a combined effect of both cell proliferation and migration. Future research should aim to isolate and characterize the individual bioactive compounds responsible for the observed activities, moving beyond the crude extract to identify specific lead compounds for drug development. Moreover, a deeper investigation into the molecular mechanisms underlying the extract's effects on osteosarcoma cells, such as its influence on signaling pathways, cell cycle progression, and apoptosis, would enhance our understanding. Finally, developing formulations more suitable for in vivo applications would be a critical step toward translating these promising in vitro findings into clinical relevance.

## Conclusion

*S. fruticosa* leaves are rich sources of phenolic and flavonoid compounds, which have been previously reported to possess strong antimicrobial and anticancer effects. The methanolic extract exhibited good antimicrobial activity against both gram-positive and gram-negative bacteria, as well as fungi. It also demonstrated a powerful free radical scavenging activity. It showed inhibition of U2OS osteosarcoma cell migration while having no significant effect on normal HSF cells which indicated that the methanolic extract of *S. fruticosa* leaves could be a future potential treatment of different microbial infections in addition to osteosarcoma because of its high cytotoxicity and potential inhibition of U2OS cell metastasis while maintaining safety on normal cells.

## Data availability

Data is provided within the manuscript or supplementary information files.

Received: 17 September 2024; Accepted: 11 August 2025

Published online: 23 October 2025

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## Acknowledgements

This study was supported by the American University in Cairo Internal Faculty Research Grant awarded to A.A. and the Graduate Student Research Grant awarded to B.H.A.

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Study conception: A.A. Study design: A.A., B.H.A., M.M.H.E., and R.A. Data collection: B.H.A. Data analysis: B.H.A., H.L.E., M.M.H.E., and A.A. Manuscript writing: B.H.A. Manuscript review and editing: M.M.H.E., R.A., and A.A.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-15727-w>.

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