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Colorectal carcinoma cell targeting aromatherapy with *Teucrium ramosissimum* essential oil to sensitize TRAIL/Apo2L-induced HCT-116 cell death

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ABSTRACT

This report drives insights for the investigation of the underlying mechanisms of antitumor effects of *Teucrium ramosissimum* (TrS) essential oil (EO) that elicits colon tumor protection *via* activation of cell death machinery. A study of the aerial part phytocomplex was performed by FTIR spectra and GC/MS. *In vivo* colon carcinogenesis induced by LPS was carried out using mouse model. HCT-116 cells were coincubated with TrS EO and TRAIL-resistant cancer cells, and then cell lysates were assessed using Western blotting technique for death and decoy receptor expression. TrS essential oil potentiates TRAIL-mediated apoptosis cell death of HCT-116 as detected by PARP cleavage and caspase activation. Further data suggest that TrS up-regulates DR 5/4 expression, and down-regulates DcRs expression. Additionally, TrS potentiates apoptosis in TRAIL-resistant tumor cells through induction of MAPK signalling components, including ERK, p38 kinase, JNK, and activation of CHOP, and SP1, involved in DR5 expression. Moreover, Teucrium EO phytoconstituents mediate HCT-116 cells apoptosis by evoking cell cycle arrest at the G1 and G2/M phase through diminishing the expression of cyclin D1 acting as a potent multitargeted factors of inhibition of JAK/STAT oncogenic signaling pathway. These results demonstrate that TRAIL-induced apoptosis enhancing effect of TrS mediated through proto-oncogene expression in HCT-116. TrS administered intragastrically is able to prevent tumor of colon by stopping carcinogenesis process and impede tumor cell growth in *in vivo* analysis promoted by LPS. On the whole, our results revealed that TrS is an effective antitcancer agent through the induction of transcription factor and kinases, either are needed to trigger Apo2L receptors.

1. Introduction

Cancer is distinguished by unchecked proliferation cycle and blocking the cell cycle is an appropriate way for tumor management. Programmed cell death is a multistage mechanism that requires two main pathways to activate a cascade of events causing nuclear membrane and chromatin fragmentation [1]. DcRs (DcR1 and DcR2) co-expressed with DR4/DR5 on the cancer cell can reduce the transmission of the apoptotic signal and modulate the sensitivity of TRAIL/Apo2L [2]. Phytotherapy with medicinal plants or bioactif compounds was known for cancer treatment. Species of the Lamiceae family are principally shrubs and herbs with a perfumed essence and rich in biologically-active

Abbreviations: PBS, phosphate buffered saline; SDS-PAGE, SDS-Polyacrylamide gel electrophoresis; PBS-T, Phosphate-buffered saline-Tween; MAPKs, mitogen-activated protein kinases; FLICE, FADD-like interleukin-1b converting enzyme; MTT, tetrazolium salt 3-(4-5-dimethylthiozol- 2-yl)2-5-diphenyl-tetrazolium bromide; TRAIL, Tumor Necrosis factor inducing ligand; FTIR, Scanning electron micrograph; GC/MS, Gas Chromatography/Mass Spectrometry; DR 5/4, Death Receptors 5/4; DcRs, TRAIL decoy receptors.

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molecules of high value in medicine. Lamiacea genera, including *Plectranthus* sp., *Teucrium* sp., *Scutellaria* sp., *Melissa* sp., and *Thymus* sp., *Origanum* sp. induce anticancer potantial through the apoptosis pathway and influences angiogenesis [3]. Several herbs and plants are promising at stopping the cellular division [4]. The important biocompounds occuring in these genera are terpenic compounds and oxygenated terpenoids. The ethereal oils extracted from aromatic plants of the lamiaceae family contain secondary metabolites, including monoterpenes, sesquiterpenes, oxygenated monoterpenes, oxygenated sesquiterpenes, and phenols, among others [3] that have been used in clinical medicine as antiinflammatory, anticancer and antibacterial agents.

Several *Lamiaceae* species have been checked because of their extreme content of volatile oils, which are commonly used in cosmetics, medical preparations and perfumery [5]. *Teucrium* is one of the most genera distributed in the world. Notably, known medicinal *Teucrium* species have been used in traditional medicine, beverage industry, food and pharmacy. *Teucrium* species are extremely rich in several phytocomponents with crucial biological effects [6]. Phytotherapeutic effect of *Teucrium ramosissimum* phytocomplex and ontogenic feature of this specie was of considerable biological importance and that its aromatic parts were distillated to obtain essential oil.

As a part of our report on the analysis of medicinal and aromatic plants that developing spontaneously in the Southern Tunisia. We are now detailling the first researchs on the phytopharmacological activities of the volatile oil extracted from the upper parts of TrS. The paper sheds light on the anticancer potential of the TrS essential oil, specifically those related to molecular mode of action towards HCT-116 cell lines by monitoring PARP cleavage and caspase activation. Moreover, volatile oil mediated tumor apoptosis through death receptor up-regualtion, MAPK signaling molecules activation, esterase staining and the increase in the number of apoptotic cells in dose-dependent manner. Up to now, no researchs regarding the in vivo anticancer effect of TrS have been depicted.

2. Materials and methods

2.1. Plant materials

Fresh leaves, fruits and seeds of *T. ramosissimum* were gathered from the Orbata Mount location (inferior arid), the municipality of Zannouch (Gafsa, Tunisia) during the flowering stage. It is extended to the east by Bou Hedma mountain and to the west by the mount of Bou Ramli. The extent of occurrence in a latitudinal disposition was about 60 km and the culmination was at 1165 m above sea level. The flowering time takes place in Spring. Voucher specimen (1123) are stored in the herbarium of the National Institute of Agronomic of Tunisia.TrS, with phytocompounds as shown in Fig. 1**A** was extracted by hydrodistillation. To profile terpenic compounds of *Teucrium* essential oils, FTIR and GC/MS analysis were used.

2.2. Reagents

Essential oil of the fresh leaves of TrS (0.1 mg/ml). TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) was purshased from Pepro-Tech (Rocky Hill, NJ, USA). Tris, glycine, dimethyl sulfoxide (DMSO), MTT, Sodium dodecyl sulphate (SDS), NaCl, *Escherichia coli* 055:B5 (LPS), 5-Fluorouracil (5-FU) and antibody against β-actin were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). 2′, 7′-dichlorofluorescein diacetate (DCFDA) was purchased from Invitrogen. Streptomycin, Fetal bovine serum (FBS), Penicillin, Iscove's modified Dulbecco's medium (IMDM), Dulbecco's modified Eagle's medium (DMEM)/F12 medium, and Roswell Park Memorial Institute medium (RPMI-1 640) were purchased from Mediatech, Inc. (Herndon, VA, USA). Antibody against survivin was purchased from R&D Systems (Minneapolis, MN, USA). Antibody against X-chromosome-linked IAP (XIAP) was obtained from BD Biosciences (San Diego, CA, USA). Antibodies against FLICE/caspase-8 inhibitory protein (c-FLIP) and DcR1/2 were obtained from BD Biosciences and Imgenex (San Diego, CA, USA). Antibodies against B cell lymphoma extra large (Bcl-xL), cellular v-myc

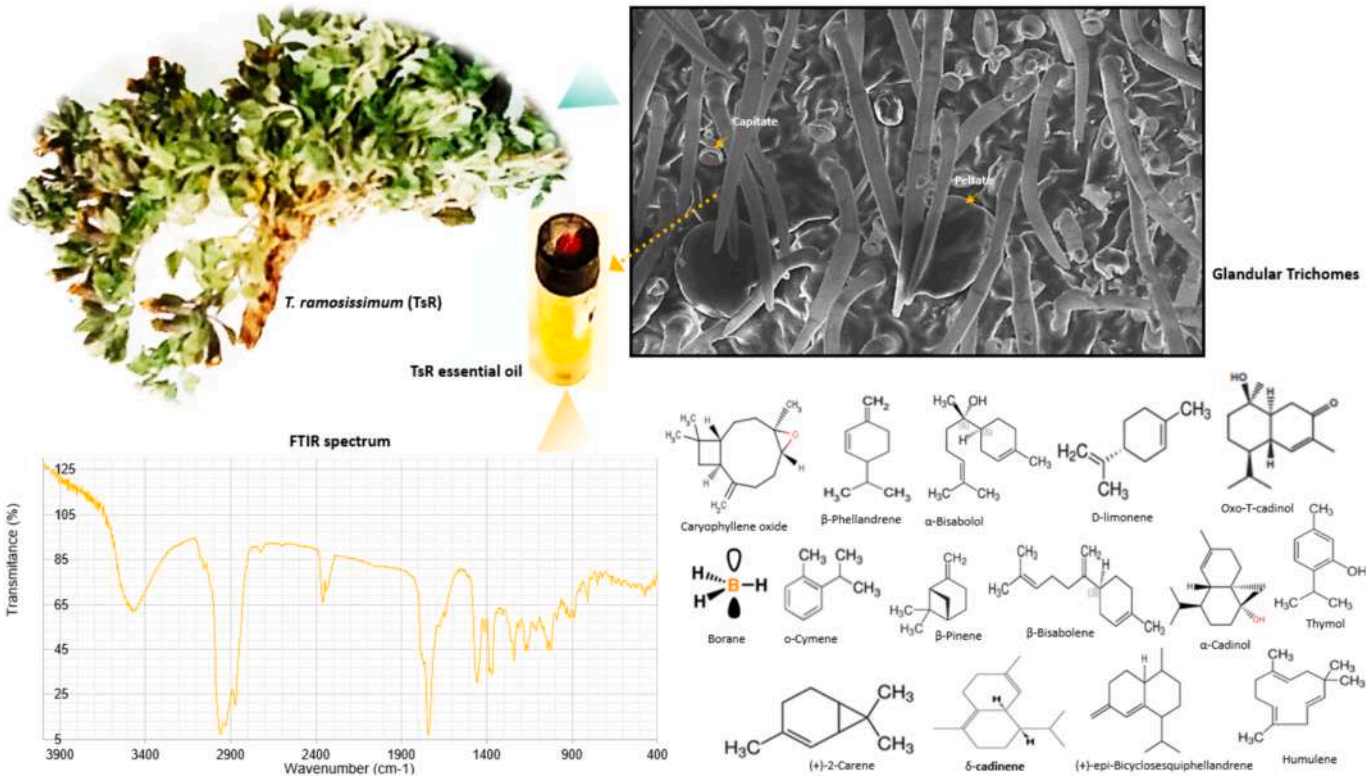

Fig. 1. Chemical structure of main compounds identified in *T. ramosissimum* essential oil.

myelocytomatosis viral oncogene homolog (c-Myc), DR4, inhibitor of apoptosis protein (cIAP1/2), myeloid cell leukemia (Mcl-1), cell cycle regulator protein (cyclin D1), c-jun N-terminal kinases (JNK1), caspases-3, −8, and −9, matrix metalloproteinase (MMP-9), extracellular signal-regulated kinase 2 (ERK2), the tumor suppressor gene (p53), poly-adenosine diphosphate ribose polymerase (PARP), Intercellular Adhesion Molecule 1 (ICAM1), B-cell lymphoma 2 (Bcl-2), specificity proteins 1 (SP1), phospho-protein kinase B (p-Akt), p38, C/EBP homologous protein (CHOP), C-X-C chemokine receptor type 4 (CXCR4), phosphor-c-jun N-terminal kinases (p-JNK1), phosphor-signal transducers and activators of transcription protein (pSTAT3 (Ser727)), signal transducers and activators of transcription protein (STAT-3), protein kinase B (Akt), and phosphor-extracellular signal-regulated kinases ½ (p-ERK1/2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against vascular endothelial growth factor (VEGF) was purshased from NeoMarkers (Fremont, CA, USA). Thermo Scientific. Anti-DR5 was purchased from ProSci, Inc. (Poway, CA).

2.3. Phytocomplex analysis with FTIR spectrometer

Extracted phytocompounds (phenols, amine, polysaccharides, aromatic compounds) were analysed by FTIR apparatus that was performed at room temperature in the range 4000–400 cm^{-1} with 4 cm^{-1} resolution by FTIR spectra (Shimadzu 8400s, France).

2.4. Cell lines

The human colon cancer cell lines (HCT116), human multiple myeloma (U266), human chronic myeloid leukemia cells (KBM-5), RAW 264.7 (mouse monocyte macrophage), human squamous cell carcinoma (SCC4) and human pancreatic carcinoma (Panc28) were obtained from the American Type Culture Collection (Manassas, VA, USA). HCT116, Panc-28, RAW 264.7, and SCC4 cells were cultured in DMEM with 10 % FBS, 100 U streptomycin and 100 U penicillin. KBM-5 cells were cultured in IMDM with 15 % FBS with 100 $U.mL^{-1}$ Penicillin and 100 μg. mL^{-1} Streptomycin. U266 were cultured in RPMI-1 640 with 10 % FBS, 100 μg/ml streptomycin and 100 U/ml penicillin. The above-mentioned cell lines have been tested for authentication by the authors in the laboratory of Experimental Therapeutics, the University of Texas M. D. Anderson Cancer Center, Houston, TX, USA. Tumor cell culture was done in flasks and kept in a humidified incubator (5 % CO_2 at 37 °C) (Thermo Fisher Scientific). *In vitro* assays were assessed in the exponentialy growing cells.

2.5. Antiproliferative effect of T. ramosissimum

To determine whether the essence of Teucrium induced antiproliferative effect towards cancer cells, we performed MTT assay. Briefly, tumor cells (5×10^2 per well) were plated in 96-well plates with increasing doses of TrS essence (0.5, 1, 5, or 10 pg/mL) for 1, 3, or 5 days. At the end of treatment, the solution of MTT was added to cancer cells. Next, cells were incubated at 37 °C for 2 h and the extraction buffer was then added. Optic density was determined at 570 nm using an MRX Revelation 96-well multiscanner (Dynex Technologies, Chantilly, VA, USA).

2.6. Cytotoxicity assays

Normal (RAW 264.7) and cancer cells, seeded in triplicate in 96-well plates, were pre-treated with different concentrations of TrS (0.5, 5, 10, 50 pg/mL) and incubated for different time points (24 h, 48 h and 72 h). Cell viability was assessed with the MTT assay.

Whether TrS enhances TRAIL/Apo2L-mediated cytotoxicity was analyzed by MTT assay. Tumor cells (5×10^3 per well) were incubated in triplicate in 96-well plates with diluted TrS volatile compounds (0, 10, 50 pg/ml) at 37 °C for 12 h. The essential oil was then removed and cells were exposed to TRAIL (25 ng/ml) at 37 °C for 24 h. Tumor cells were treated with the MTT solution, after which we add to the mixture the extraction buffer. The extraction buffer was considered as a blank and the vehicle was considered as control. The absorbance was read at 570 nm by an MRX Revelation 96-well multiscanner.

2.7. Alteration of the cell cycle progression by TrS

We also analyzed sub-G1 fraction of the cell cycle using Flow cytometry by propidium iodide staining to examine the change in the progression of cell cycle induced by TrS in colorectal cancer cells. Briefly, HCT-116 (1×10^6 cells/well) were treated with different doses of TrS (5—200 pg/ml) for 24 h. The DNA spin pellets were at maximum of 30′, washed and resuspended in PBS. Then, ethanol (700 μl, 100 %) was added while keeping it on vortex at very slow speed and put on –20 °C overnight. The following day, cancer cells were cleaned with PBS (Spin at max = 30′) decant, washed again 2 fold with PBS and resuspended in PI (0.5 ml). RNase A (5 μl) was added to stain cellular DNA and the mixture was stored for 15 min at RT.

2.8. Live/Dead assay

To measure apoptosis and count live and dead cells, we resort to Live/Dead assay. Treated or untreated HCT116 cells (1×10^6) prepared in 96-well plates and treated with various concentrations (0.1, 0.5 or 1 pg/ml) of Teucrium essential oils. After 24 h of exposure, the medium was removed. After which 20 μl of the solution composed of Ethidium homodimer + PBS + Calcein was added to each well, mixed properly and cells were incubated at 37 °C for 30 min in the dark to protect it from light. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon, Melville, NY, USA). Ethidium homodimer is fluorescent when bound to nucleic acids, and produces a bright red fluorescence in dead cells, and calcein, non fluorescent, is retained by live cells, in which it produces intense green fluorescence. The numbers of dead and live cells can be expressed in terms of percentages.

Whether TrS enhances TRAIL/Apo2L-mediated cell death was assessed by intracellular esterase activity. Briefly, HCT-116 cells (2×10^3) were pretreated with 0.5 pg/ml TrS for 4 h, the medium was withdrawn, then cells were exposed to TRAIL (25 ng/ml) for 24 h. Apoptosis was investigated by the esterase live/Dead assay.

2.9. HCT-116 colony-forming assay

To investigate whether the decline in HCT-116 single cell to grow into a colony in 6-well plates over 9 days was due to the presence of TrS and/or TRAIL, we assessed colony formation assay. HCT-116 cancer cells were harvested in 6-well plates and plated with TrS and/or TRAIL. The medium was changed, and colonies were formed and kept until the ninth day. We next fixed HCT-116 cells with clonogenic acid before 20 min incubation and stained colonies for 30 min with 0.5 % crystal violet dye, and then cancer cells were washed two fold to count colonies with blue color.

2.10. Western blot analysis

Western blotting technique is used for the analysis and identification of protein depend on their potential to fix to specific antibodies. Treated and untreated tumor cells were prepared in lysis buffer (20 mM, Tris (pH 7.4), 250 Mm NaCl, 2 Mm EDTA (pH 8.0), 0.1 % triton X-100, 0.01 μg/ml aprotinin, 0.005 μg/ml leupeptin, 0.4 M phenylmethylsulfonyl fluoride, and 4 Mm Na_3VO_4). Cancer cells were cotreated with stimuli (TrS and/or TRAIL), then prepared in lysis buffer for 4 °C. Lysates were centrifuged (1400 rpm/10 min) and the supernatants were collected and kept at – 80 °C. Thirty μg of lysates were resolved by 10 % SDS-PAGE and proteins were then transferred to nitrocellulose membranes where it bound, forming the blot. After that, membranes were probed overnight

with relevant primary antibodies at 4 °C: survivin (1:2000), pSTAT3 (Tyr^{705} and Ser^{727}) (1:2000), XIAP (1:3000), CXCR4 (1:5000), Bcl-xL (1:2000), c-FLIP (1:3000), VEGF (1:1000), β-actin (1:10,000), caspase-3 (1:2000), −8 (1:2000) and −9 (1:2000), c-Myc (1:2000), ICAM-1 (1:2000), p53 (1:3000), cIAP-1/2 (1:2000), Mcl-1 (1:3000), PARP (1:5000), cyclin D1 (1:3000), Bcl-2 (1:3000), MMP-9 (1:1000) and TRAF1 (1:2000). Then proteins are exposed to thin surface layer and detected with secondary antibodies (antirabbit IgG-HRP and antimouse IgG-HRP) (1:5000). Proteins were detected by Enhanced Chemiluminescence Reagent (GE Healthcare, Piscataway, NJ, USA).

2.11. Intracellular radical oxygen species (ROS) production in HCT-116

To quantify ROS production and to investigate its potent effect in TrS-induced apoptosis, HCT116 (1×10^6) cell lines were labeled with DCF-DA (20 μM) at 37 °C and then exposed for 1 h to 0,2 μg/ml TrS essential oil, after which we measured DCF formation by fluorescence-activated cell sorting (FACS) at 530 nm. Data on at least 10,000 cells at a flow rate of 250–300 cells/second were collected. The ROS mean fluorescence intensity (MFI) was determined.

2.12. T. ramosissimum protects mice from LPS-mediated colon inflammation and carcinogenesis

2.12.1. Mice model

Swiss albino mice (n = 48, male and female, 6-week-old, 22 ± 4 g weight) were housed in group cages (n = 6 mice/experimental group), at a room temperature of 18–25 ∘C. Animals, purchased from the Central Animal Laboratory (Sfax, Tunisia), were left for at least one week in a propylene cage (Tecniplast, Italy) for adaptation to laboratory conditions prior to their use in *in vivo* experimental strategy, maintained under 12 h light/dark schedule and a relative humidity of 55–65 % and provided with free access to tap water and food pellets *ad libitum*. Experimental process was performed according to the guidelines of the Ethic Committee of Gafsa University (G/A/SV/2016/001), Tunisia.

2.12.2. Experimental design

Animals, divided into 8 groups and treated orally with drugs for one week, were as follows: (1) saline-treated sham group, (2) LPS oral 10 μg/ml treated group, (3) TrS EO oral 20 μg/kg treated group, (4) TrS EO oral 50 μg/kg treated group, (5) 5-Fluorouracil oral 20 mg/ kg/day treated group, (6) both TrS EO and LPS oral (20 μg/kg and 10 μg/ml, respectively) treated group, (7) both TrS EO and LPS oral (50 μg/kg and 10 μg/ml, respectively) treated group, (8) both 5-FU and LPS oral (20 mg/ kg and 10 μg/ml, respectively) treated group. The drug 5-FU is taken as standard. EO solution was prepared by freshly diluting the Teucrium oily fraction in Tween 80 (2 %) vehicle. For group 6, 7 and 8, the treatments with drugs were given daily, 1 h before intragastrically administration of LPS. Treatment of mice was done once daily for 7 consecutive days. After one week of experimentation, animals were sacrificed and liver, kidney, colon, spleen and lung were removed and prepared for histological analysis using H&E staining. Examination of organ tissues were done with microscopy (Motic SFC-28 SERIES).

2.13. Statistical analysis

Data were analysed by GraphPad Prism 4.02. The values were reported as mean ± S.E.M and One-Way ANOVA and Tukey's test were used to compare the groups. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Phytochemical profile

The mass yield of the plant essence was 2 % at the flowering stage. As seen in Fig. 1, terpenic compounds identified in TrS EO using GC/MS analysis include monoterpenes and sesquiterpenes. Our results showed that the major compounds of *T. ramosissimum* were (+)-Epi-Bicyclo sesquiphellandrene, thymol, o-Cymene, β-Selinene, α-Cadinol, δ-Cadinene and α-Bisabolol. FTIR chromatogram (Fig. 1) of TrS aerial parts showed the identification of various bands. The bands at 3481 cm^{-1} and 3504 cm^{-1} would be related to O–H bending vibration. The bands at 2960 cm^{-1}, 2951 cm^{-1}, 2899 cm^{-1} and 2752 cm^{-1} correspond to CH_2, CH_3 and C–H stretching vibrations. The bands at 1674 cm^{-1}, 1635 cm^{-1} and 1618 cm^{-1} correspond to C = C and C = O stretching vibrations. The bands at 1360 cm^{-1} and 1335 cm^{-1} would be related to C–H stretching and to O–H bending vibrations, respectively. The band in the region 1741 cm^{-1} would be related to C = O stretching. The bands in the region 1456 cm^{-1} and 1367 cm^{-1} would be related to C–H bending (hydrocarbons, flavonoids). The bands between 1300 cm^{-1} and 1000 cm^{-1} would be related to C–C stretching.

3.2. TrS reduces the cancer cell proliferation

To evaluate whether TrS induced antitumoral effect, we assessed antiproliferative assay using MTT reagent for 1, 3, or 5 days. In response to TrS essential oil, HCT-116 cell proliferation displayed dose- and time-dependent decrease as compared to untreated control, as seen in Fig. 2**A**. The most effective acivity accured at 10 pg/mL for KBM5, SCC4, and U266 beween the first and the fifth days of incubation. Whereas, Panc28 cancer cells are less sensitive to TrS essential oil.

3.3. TrS and/or TRAIL mediated cytotoxicity in tumor cells

As seen in Fig. 2**Bi**, MTT assay did not show any significant difference ($P > 0.05$) in RAW 264.7 macrophage cell viability among the control and TrS-treated groups. In contrast, TrS induced cytotoxicity in HCT-116 in dose- and time- dependent manner as shown in Fig. 2**Bii, iii**. The combination of TrS and TRAIL mediated markedly decline of HCT-116 cell growth in dose-and time-dependent manner as compared to untreated control. In addition, we found that KBM5, U266, Panc28 and SCC4 cells were sensitive to high dose of the essence of Teucrium that increase its cytotoxicity (~81 %). Thus, it is noteworthy that *T. ramosissimum* sensitizes cell death (~81 %) induced by TRAIL/Apo2L (Fig. 2**Bvi**). In contrast, TRAIL was less effective towards some tumor cells, such as Panc28 and SCC4.

3.4. TrS induces cell cycle arrest through a p21- and p53-dependent pathway

To measure apoptosis and the ability of terpenic compounds of TrS to interfere with the HCT-116 cell cycle and inhibit its progression causing cell death, we used FACS analysis by PI staining of DNA. Fig. 2**C** depicted a dose-dependent increase of of the sub-G1 level upon TrS treatment. Interestingly, we observed that percentage of apoptotic cells was dropped to ~ 52.01 % by low dose of TrS in HCT-116. Cell cycle effects were clearly appeared at 50 pg/ml and 100 pg/ml. Pretreatment with 200 pg/ml of essential oil resulted in an increase of sub-G1 levels (~92.72 %). Moreover, we noticed that there was a high level of apoptosis as compared with that of the control cells (~45.37 %). TrS-treated HCT-116 cells induced G2/M phase arrest in dose-dependent manner. This was accompanied by a significant decrease in the G1 phase compared to the untreated cells.

3.5. TrS sensitizes TRAIL-mediated HCT-116 cytotoxicity

As showed in Fig. 3**Ai**, we observed that *T. ramosissimum* EO (1 pg/ml) mediates highly cell death (~100 %) after counting live and dead HCT-116 cells. Apoptosis of colon cancer cells by TrS essence was promoted by the presence of TRAIL/Apo2L. Coincubation of HCT-116 cells with either EO (0.5 pg/ml) and TRAIL mediated up to ~ 87 % cell death

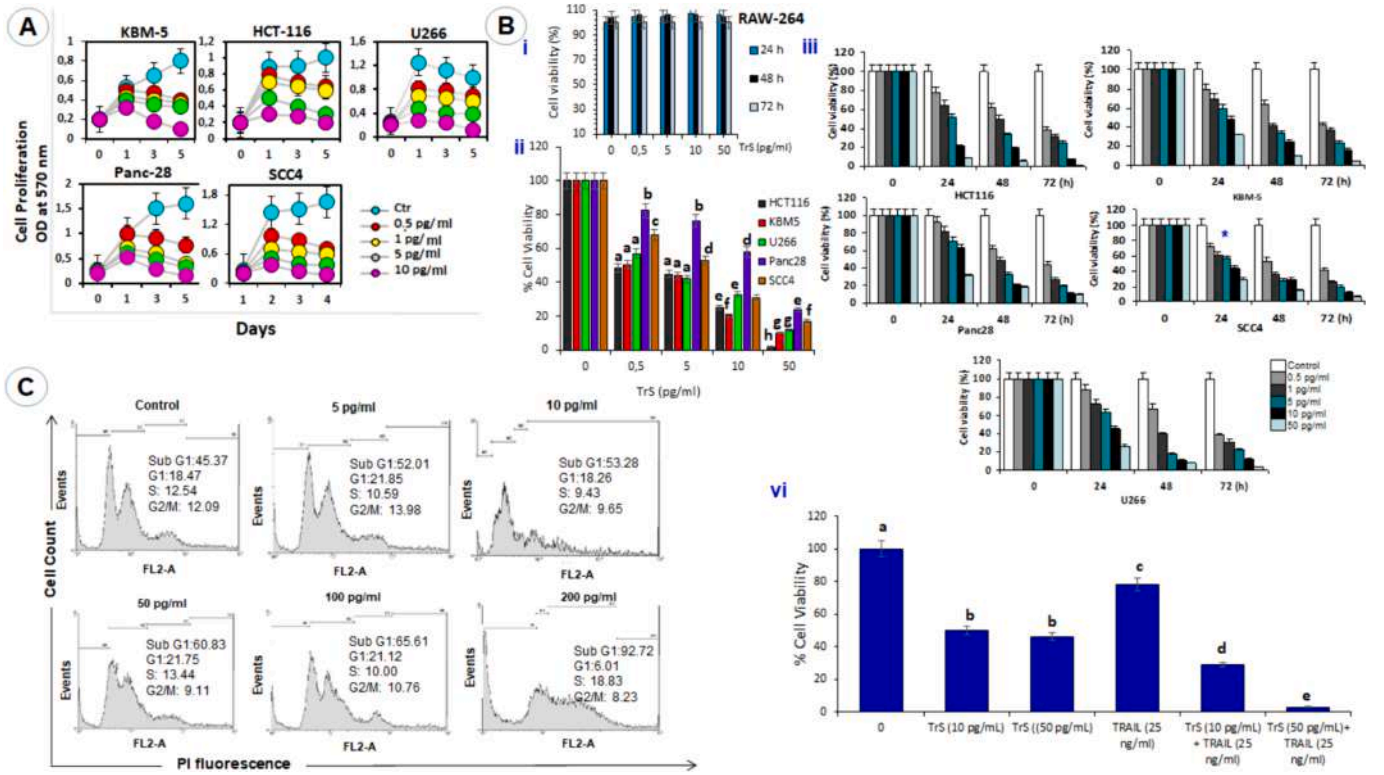

Fig. 2. **A**, Antiproliferative effect of *T. ramosissimum* for differents dose- and time-dependent manner. **B**, Cytotoxicity of normal and tumor cells induced by TrS for dose- (**i,ii**) and time- (**iii**) dependent manner. TrS enhances TRAIL-mediated cytotoxicity (**vi**). **C**, HCT116 cells (1×10^6 per well) were incubated with the indicated concentrations of TrS EO for 24 h and then stained with propidium iodide. The sub-G1 fraction was analyzed by flow cytometry. Means denoted by a different letter (a–e) show markedly differences between groups ($P < 0.05$).

while TRAIL alone was somewhat less effective and produced ~ 8 % cytotoxicity in colon cancer cells at 25 ng/ml, as shown in Fig. 3**Aii**.

3.6. TrS decreased HCT-116 clonogenicity

As seen in Fig. 3**Bi,** TrS mediates colony-forming ability reduction in a dose-dependent manner. At high dose (0.1 μg/ml), *T. ramosissimum* markedly declines the colony numbers over 90 % as compared to the untreated control. The percentage of colony formation has been reduced with increasing dose of essential oil. Whereas, TRAIL/Apo2L (25 ng/ml) was less effective against HCT-116 cells that were 70 % growth inhibited. Notably, potential inhibition of colony-forming was seen when colon cancer cells were coincubated with either TrS and TRAIL with 100 % reduction (Fig. 3**Bii**).

3.7. Teucrium volatile oil induced ROS level generation in HCT-116 human colorectal cancer cells

Whether TrS volatile fractions induce ROS production investigated by FACS analysis. As seen in Fig. 3**C**, we noted a detection of fluorescence signals in HCT-116 cells exposed to 0.2 μg/ml TrS. Furthermore, a significant ($P < 0.05$) increase in ROS levels was shown in HCT-116 cells treated with TrS volatile oil compared to the control.

3.8. T. ramosissimum and/or TRAIL/Apo2L-induced caspase activation and PARP cleavage

In vitro study revealed that the incubation of tumor cells with increased concentration (0.1 or 0.2 μg/ml) of TrS markedly induced 5-fold activation of caspase cascades in human HCT-116 cells (Fig. 3**D**). Therefore, we found that cotreatment of HCT-116 cells with TrS (0.1 and 0.2 pg/ml) or TRAIL (25 ng/ml) enhances the activation of caspase 9 (3-fold) and caspase 8 (4-fold) and the proteolysis of PARP in dose- (Fig. 3**Ei**) and time-dependent manner (Fig. 3**Eiii**). Colon cancer cells coincubated with *T. ramosissimum* and TRAIL sensitizes the cleavage of PARP that occured at least 2-fold (Fig. 3**Ei,ii**), thus suggesting TrS potentiates the antitumor potential of Apo/2L that HCT116 undergo apoptosis.

3.9. TrS upmodulates death receptors DR4 and DR5

T. ramosissimum essence potentiates TRAIL-mediated cell death via DRs in HCT-116 cells. Fig. 4**A** shows that increased DR4 and DR5 protein expression in human HCT-116 cells in a dose-and time-dependent applications, contributes to TrS-induced apoptosis (Fig. 4**Ai, iii**). Thus, treatment of HCT-116 cells with TrS (0.05 μg/ml) and TRAIL (25 ng/ml) for 24 h appears to increase the level of DR5 in HCT-116 and other cell lines (Fig. 4**Bi, ii**). The results show that TrS induces apoptotic cell death of HCT-116 through the intrinsic and the extrinsic pathway (Fig. 7).

3.10. TrS potentiated HCT-116 cells to TRAIL/Apo2L by down-modulating MAPK and Akt pathways

We assessed the mechanism by which TrS-induced apoptosis further and the involvement of ERK1/2, JNK, and p38 MAPK in the up-modulation of death receptots. Therefore, TrS dose (Fig. 5**Ai**) – and time (Fig. 5**Aii**)-dependently up-regulates the ERK1/2 prosurvival signaling pathway, p38, Akt and JNK at least in HCT-116 cells.

3.11. TrS up-modulates transcription factors (CHOP bZip and Sp1)

As seen in Fig. 5**B**, TrS EO (100 pg/ml) markedly mediated the up-modulation of Sp1 and CHOP bZip transcription factors in HCT-116 cancer cells in dose- (Fig. 5**Bi**) and time-dependent manner (Fig. 5**Bii**).

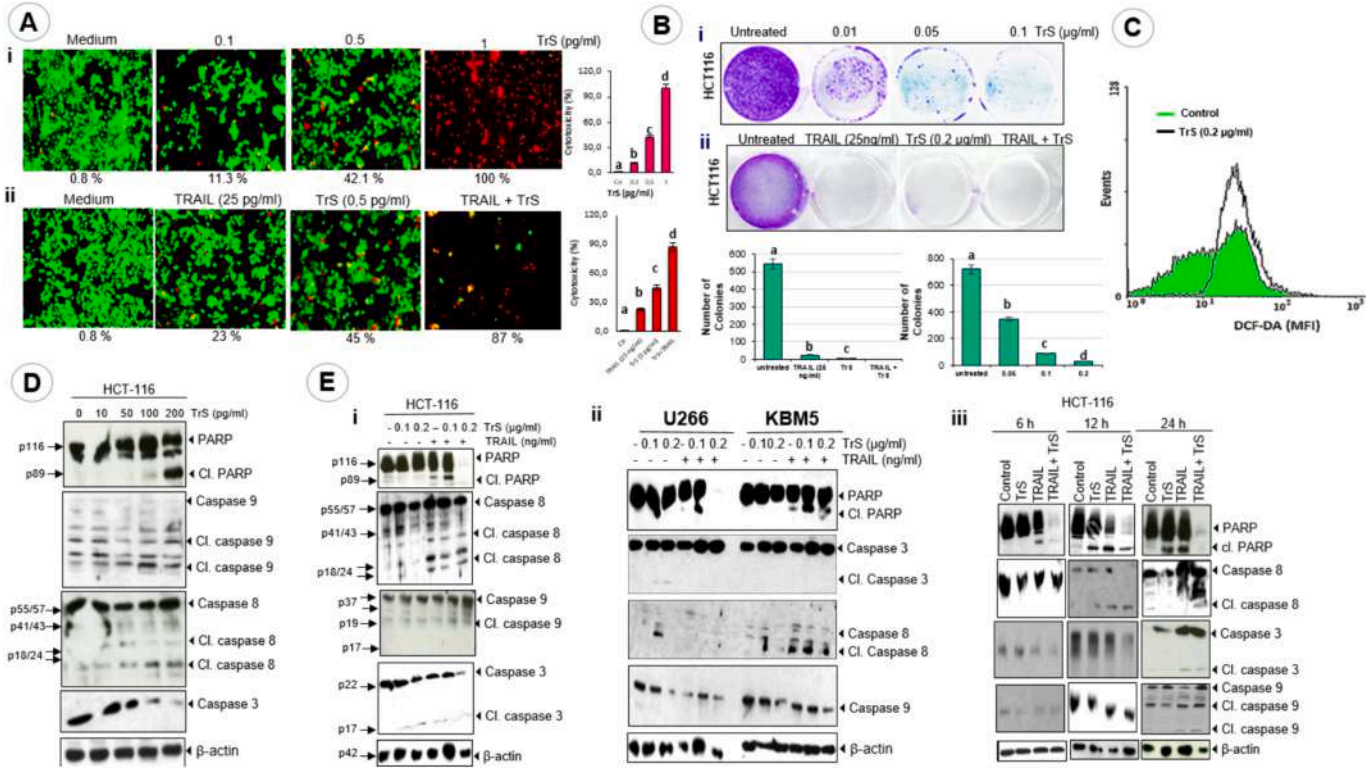

Fig. 3. *T. ramosissimum* volatile oil-potentiates TRAIL mediates HCT-116 cell death. **A**, HCT-116 cell death induced by the combination of TrS and TRAIL using the live/dead assay. **B**, The clonogenic effect of TrS (0.2 µg/ml) and TRAIL (25 ng/ml). **C**, HCT-116 cells (1×10^6 cells/well) were labeled with DCFDA, exposed to indicated doses of Teucrium volatile oil for 1 h, then examined for ROS production. These are representative results of three independent experiments. **D**, *T. ramosissimum* induced caspase activation and PARP cleavage in dose-dependent manner in HCT-116. **E,** *T. ramosissimum* enhances TRAIL-mediated the activation of caspases and the cleavage of PARP in dose-dependent manner in HCT-116 **(i)** and U266 and KBM5 **(ii)** cancer cells. Means denoted by a different letter (a–d) show markedly differences between groups ($P < 0.05$).

3.12. TrS represses the phosphorylation of STAT3 (ser^{727})

For dose-dependent investigation, Fig. 5**C** shows that TrS induced dose-dependent inactivation of STAT-3 in HCT-116 cells; and the maximum inhibition of STAT-3 phosphorylation has been occured at 100 pg/ml.

3.13. Either Teucrium volatile oil and TRAIL/Apo2L dose- and time-dependently supresses pro-oncogene products in HCT-116

TrS treatment decreased cell survival and activated tumor suppressor p53 in dose (Fig. 6**Ai**)-and time (Fig. 6**Aii**)-dependent manner. Because c-FLIP, especially c-$FLIP_L$, plays crucial function in TRAIL sensitivity regulation in HCT-116 cells after chemotherapy [7], we investigated whether TrS down-regulated c-FLIP alone or in presence of TRAIL/Apo2L. DRs binding to TRAIL/Apo2L, thereby leads to down-regulation of pro-oncogene products through inhibition of cIAP1/2, ICAM1, surviving, Mcl1, cFLIP, Bcl2, CXCR4, c-myc, Bcl-xL activities and VEGF secretion in dose (Fig. 6**Bi,ii,iii**)-and time (Fig. 6**Bvi**)-dependent manner.

3.14. Macroscopic and histomorphological overview of inflamed colon and therapeutic potential of TrS

First, we analyze the safety of high and low dose of TrS oil. No toxicity was detected for mice treated with essential oil of teucrium diluted in Tween solution. No behavioural change was observed neither in LPS-treated group nor in mice treated with comparator control (5-FU) or up to 50 µg/Kg TrS treatment dose. Otherwise, mice treated with TrS (Fig. 8**Ai)** showed high food intake and water consumption (Fig. 8**Aii, iii**), and gained weight during 7 days of intragastrically treatment (Fig. 8**Avi**). Next, we performed macroscopic analysis in animals treated with TrS, LPS, 5-FU alone or in combination. In this study, macroscopic analysis showed that intragastrically administration of LPS to animals markedly mediates few fecal pellet production (Fig. 8**Bi**), colon injury (Fig. 8**Bii**) and shortening (Fig. 8**Biii, vi**), and decreases lung, heart, spleen (Fig. 8**Ci**) liver and kidney (Fig. 8**Cii**) weight. Whereas, oral administration of TrS to mice markedly reduces the colon shortening and attenuated organ inflammation. Further, our histopathologic studies revealed the presence of high numbers of inflammatory cells, like leukocytes and neutrophils in the lung of LPS-induced group (Fig. 9**A**). Moreover, tumor cells and the infiltration of inflammatory cell were detected in the spleen (Fig. 9**B**), liver (Fig. 9**C**) and kidney (Fig. 9**D**) of mice treated with LPS and LPS + 5-FU, respectively. Indeed, no microscopical damage of organs was shown in TrS-treated mice. Overall, TrS exhibited potential therapeutic anticancer drug and inhibited the effect of LPS-induced carcinogenesis. Normal colon architecture was found in normal group, TrS and 5-FU-treated group (Fig. 10**b, c**). In contrast, LPS-treated mice mediated colon tissue lesions (Fig. 10**d,** 11**,** 12). Our findings clearly indicate that colon injury and inflammation has been reduced in TrS-treated mice (Fig. 10**e, f**).

4. Discussion

For centuries, scientists have been looking for new effective methods to control tumor progression through different approaches [8]). A large variety of herbs and plants and their bioactive molecules, including phenolic compounds, flavonoids and terpenes have potent anticarcinogenic, free radical scavenging and antimutagenic activities [9]. Naturally occurring flavonoids have functional antitumor effects and are well

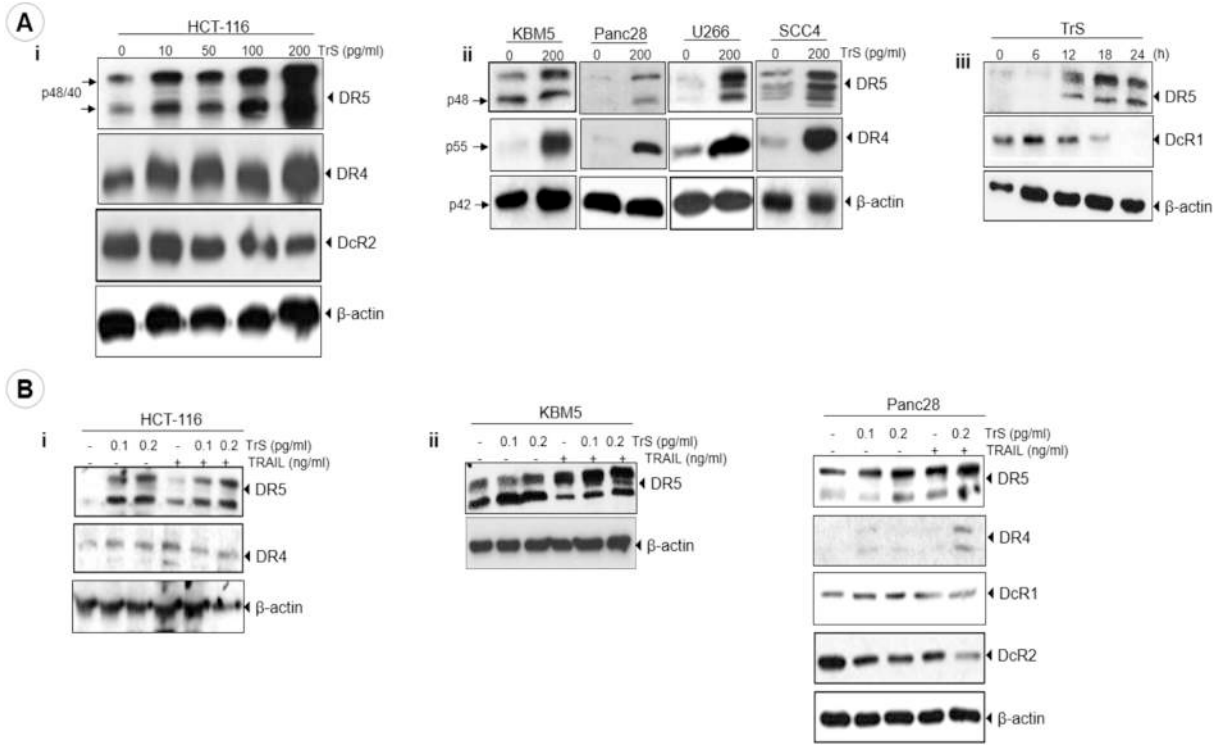

Fig. 4. **A,** Up-modulation of DRs (DR4 and DR5) and down-regulation of DcRs by TrS EO in dose (**i,ii**)- and time (**iii**)-dependent manner in HCT-116 and other tumor cells. Cancer cells (1×10^6 cells/ well) were treated with various concentrations of TrS for 24 h or treated with 0.05 μg/ml *Teucrium* EO for the indicated durations. Whole-cell lysates were subjected to WB for relevant antibodies. **B,** *T. ramosissimum* EO potentiates TRAIL-mediated up-regulation of death receptors and down-regulation of DcRs expression in dose- (i,ii) dependent manner in cancer cells.

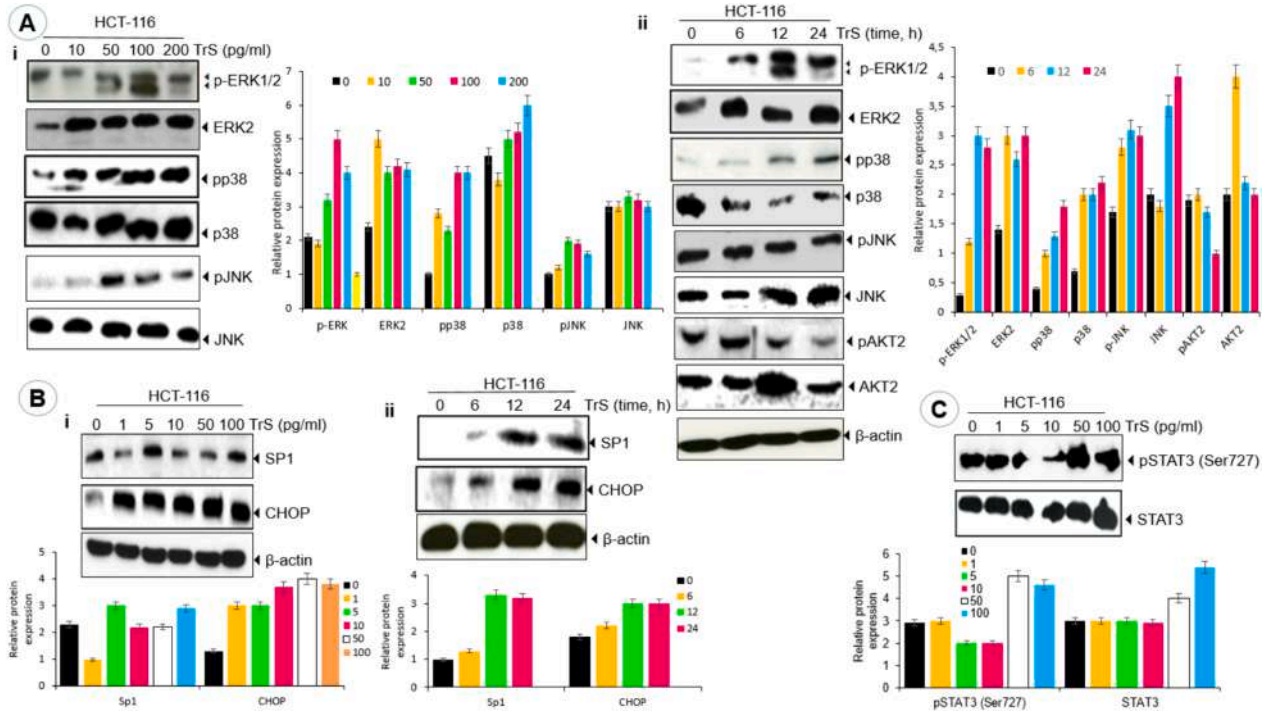

Fig. 5. DR up-regulation is ERK1/2-, p38 MAPK- and JNK-dependent. **A,** TrS induces the upregulation of p38 MAPK, JNK and ERK2 levels. HCT-116 cells (1×10^6 cells/well) were incubated with TrS volatile oil at the indicated concentrations for 24 h and the indicated times. Whole-cell lysates were subjected to Western blotting analysis using relevant antibodies. **B.** TrS induces upregulation in transcription factors (Sp1 and CHOP bZip) in dose- and time-dependent manner. **C.** TrS represses STAT3 (ser^{727}) phosphorylation. These are representative results of three independent experiments.

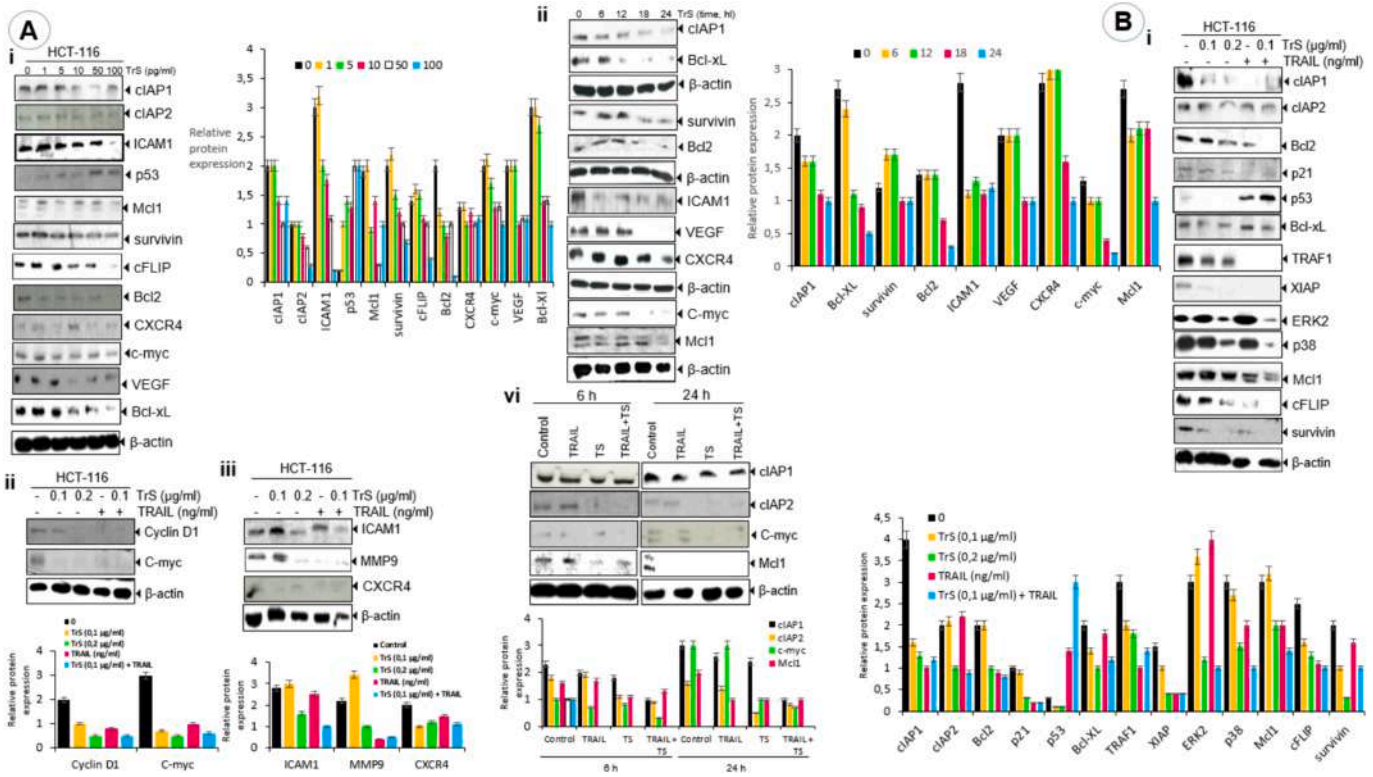

Fig. 6. A, TrS mediates the up-regulation of antiapoptotic, proliferative, metastatic gene product levels in dose (i)- and time (ii) −dependent manner. **B.** TrS enhances sensitizes TRAIL-induced change in antiapoptotic, proliferative, metastatic gene product levels in dose (i,ii,iii)- and time (vi) −dependent manner. Colon cancer (HCT-116) cells (1×10^6 cells/well) incubated with Teucrium volatile oil at the indicated concentrations for 24 h or treated with either Teucrium volatile essential oil and TRAIL for the indicated times (bottom). Whole-cell lysates were subjected to Western blotting analysis using relevant antibodies. The same blots were stripped and reprobed with β-actin antibodies to verify equal protein loading. These are representative results of three independent experiments.

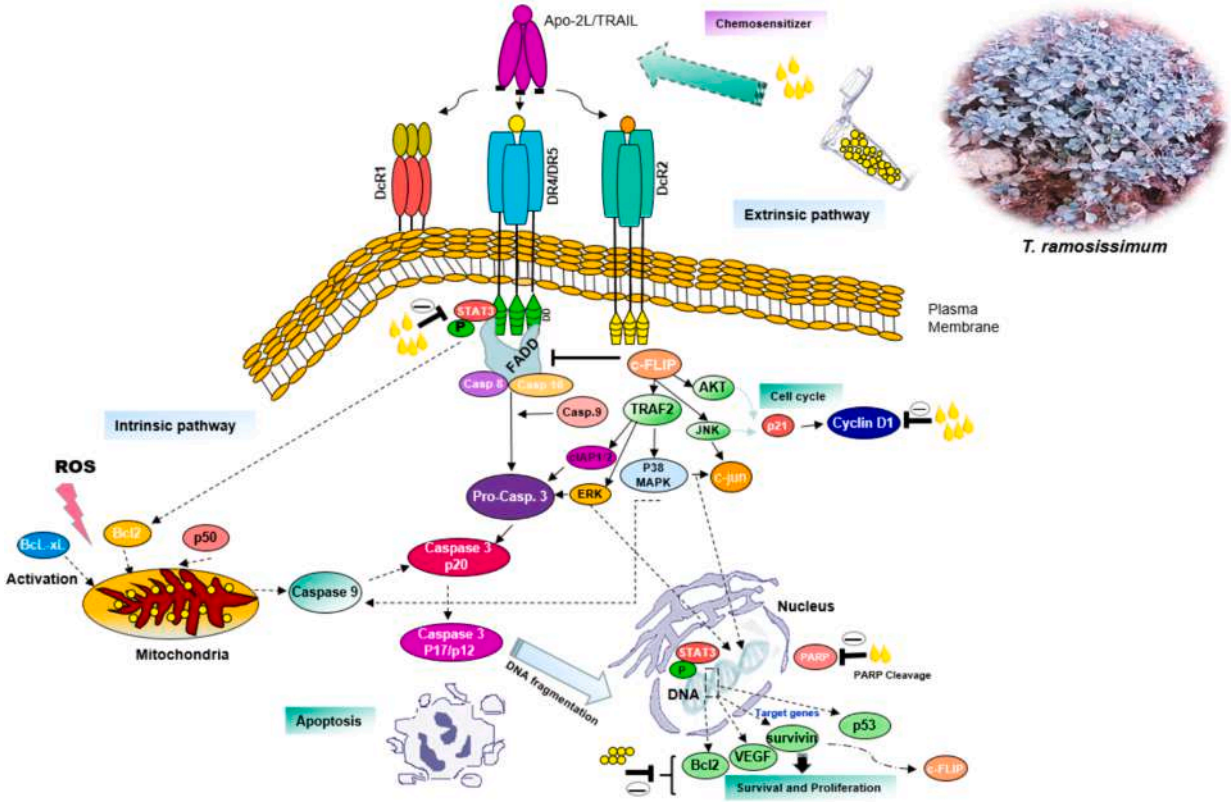

Fig. 7. *In vitro* anticancer process overview of TrS.

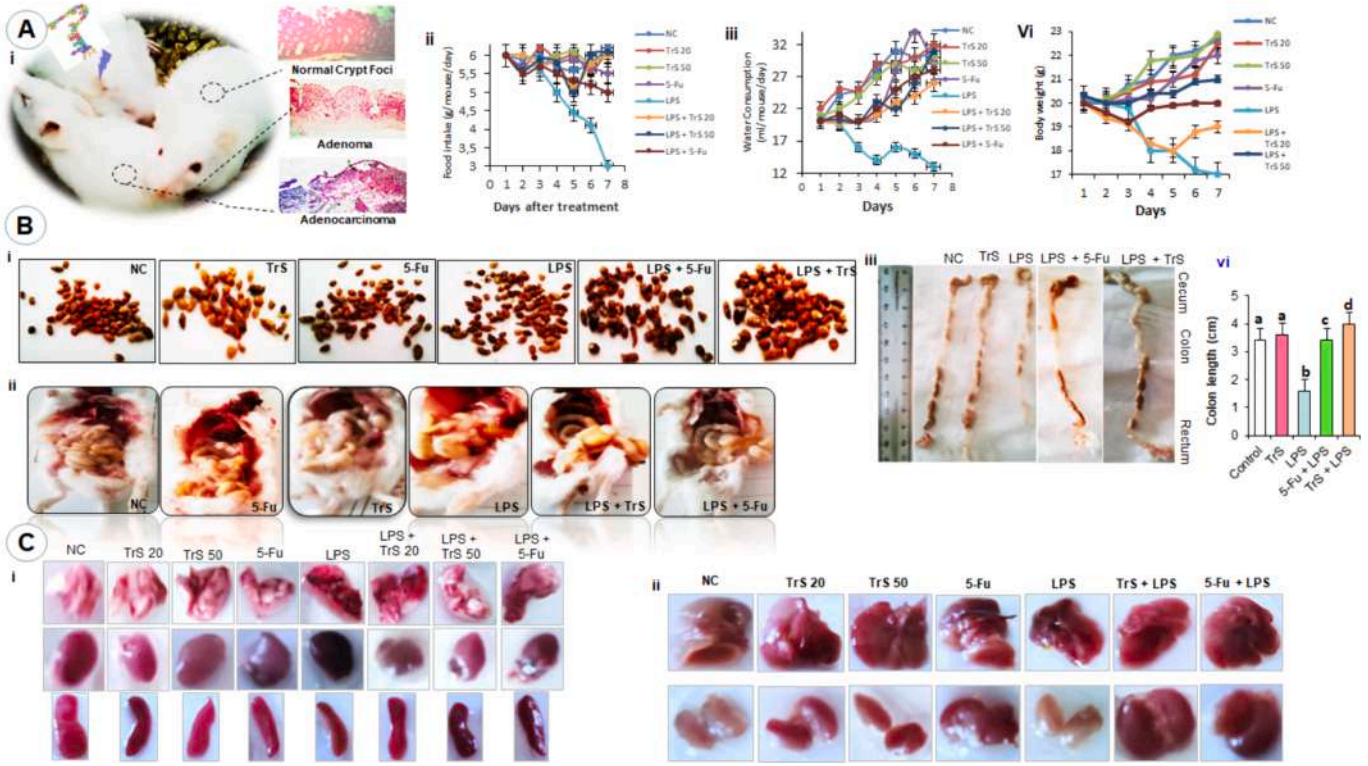

Fig. 8. A. Experimental set-up (i), food intake (ii), water consumption (iii) and body weight (vi). **B.** Macroscopic view of mice feces **(i)**, digestive system **(ii)**, colorectal parts **(iii)** and colon length **(vi)** of different groups. **C.** Macroscopical overview of mice lung (upper panel), heart (middle panel) and spleen (lower pane) (i) and liver (upper panel), and kidney (lower pane) (ii) treated with Teucrium essential oil, LPS or both LPS and essential oil. Means denoted by a different letter (a–d) show markedly differences between groups ($P < 0.05$).

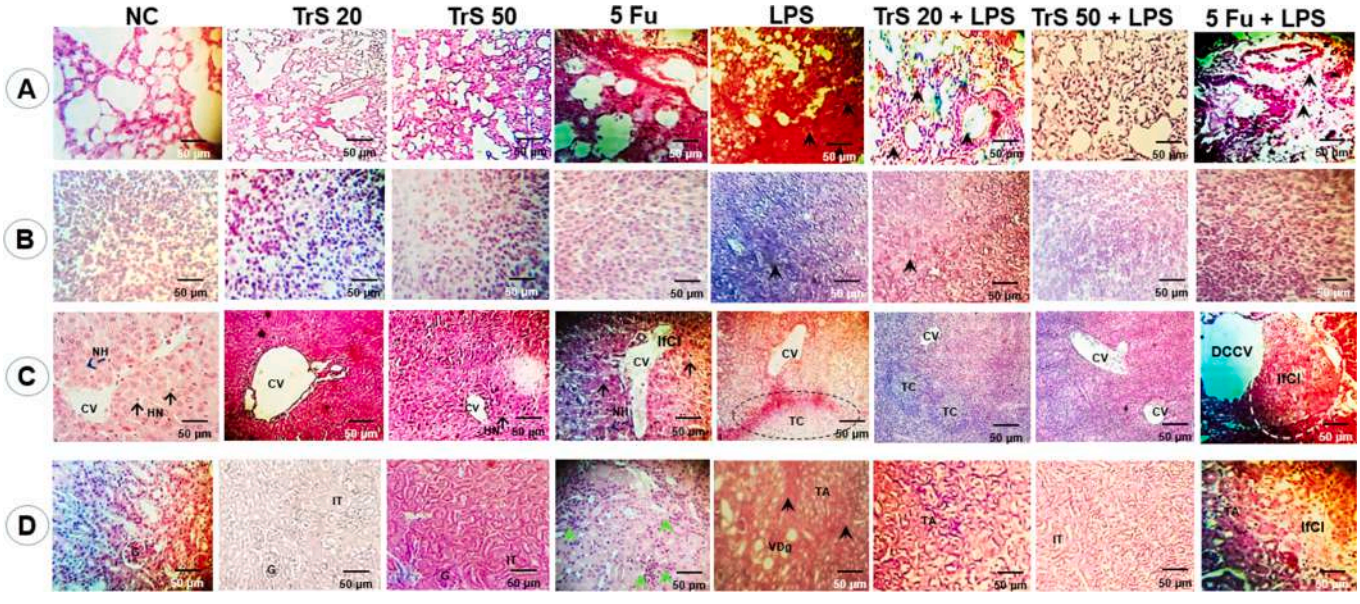

Fig. 9. Histological sections of lung (A), spleen (B), liver (C) and kidney (D) of different groups. Black arrows indicate tumor cells, blue and green arrows indicate inflammatory cells. CV: central Vein; DCCV: dilated and congested central vein; HN: hepatocyte nuclei; NH: normal hepatocyte; G: glomerulus; IT: interstitial tubule; IfCI: inflammatory cellular infiltration; TA: tubular atrophy; VDg: vacuolar degeneration; TC: tumor cells. Six mice were analyzed in each group. (H&E staining, Magnification × 10 and × 40).

investigated in preclinical research. Based in current research, the biological effect and metabolic impact of a set of new 3′,4′,5′-trimethoxy flavonoid salicylate derivatives were detected in the HCT-116 [10]. Some essential oils are considered as preventive and chain breaking antioxidants and possess anti-inflammatory effect, which may be due not only to their free radical scavenger activities but also to their interactions with signaling cascades involving regulatory transcription factors and cytokines, and on the pro-inflammatory genes expression [11]. The major components of TrS volatile oil that are able to prevent animals against colon adenocarcinoma are δ-cadinene, thymol, oxo-T-

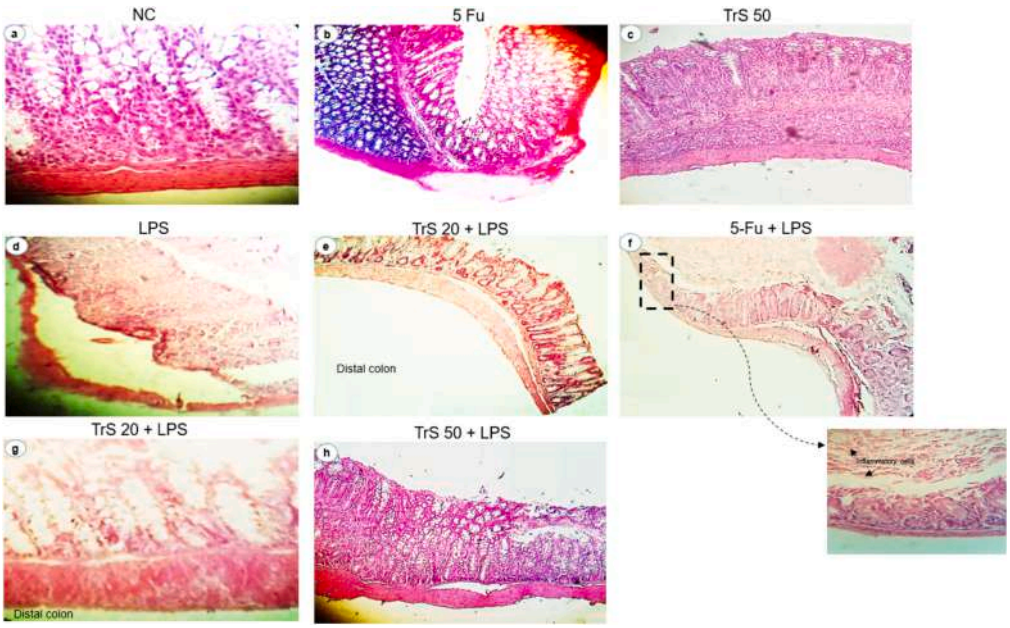

Fig. 10. Histological sections of colon of different groups.

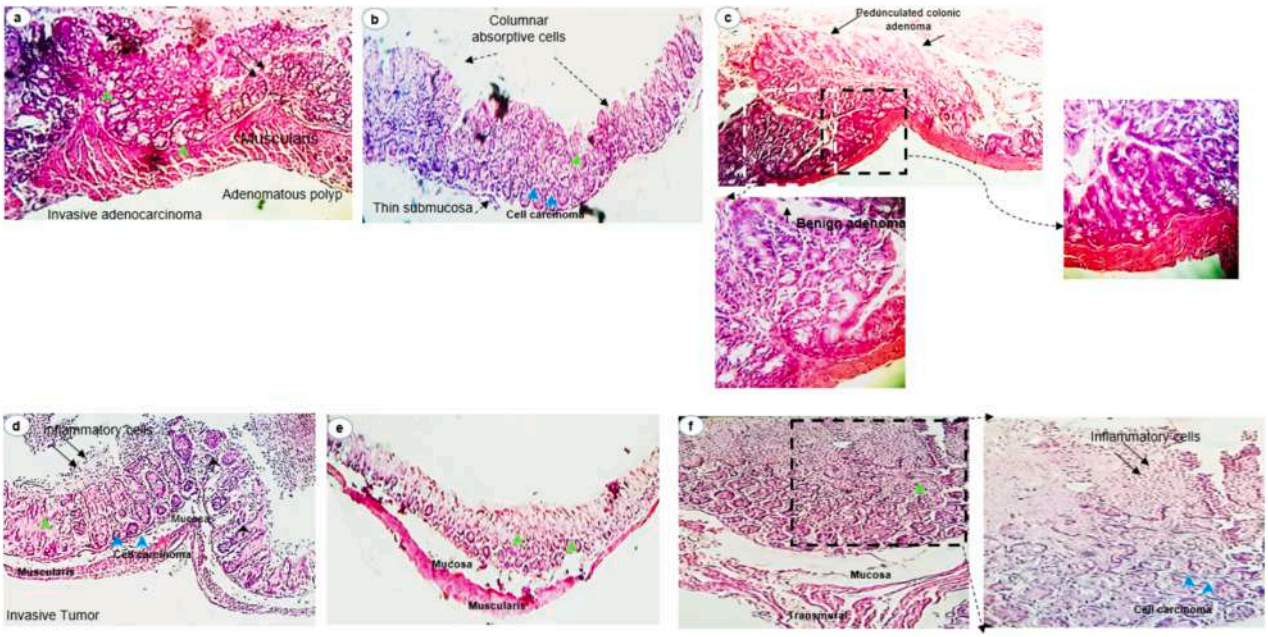

Fig. 11. Photomicrograph showing colon colorectal adenocarcinoma in LPS-treated groups. Microadenoma (a); invasive adenocarcinoma (b,e,f); inflammatory cells (c,g,i); pedunculated colonic (d) and tubular (h) adenoma. Black arrows-inflammatory cell infiltration within mucosa and pink arrows-inflammatory cell infiltration within submucosa; blue arrow-cancer cells, green arrows-crypt and goblet cell loss (H&E staining, Magnification × 40).

cadinol, α-Bisabolol, humulene, o-Cymene, (+-epi-Bicyclosesquiphellandrene, and β-eudesmol. Another report showed the isolation of oxo-T-cadinol, teucmosin $[\alpha]_D^{20} + 6.5°$ (*c* 0.30, $CHCl_3$), homalomenol C, oplopanone, 1β,4β,7α-trihydroxy-8,9-eudesmene $[\alpha]_D^{20} + 6.2°$ (*c* 0.22, $CHCl_3$), 1β,4β,7α-trihydroxyeudesmane, 1β,4β,6β-trihydroxyeudesmane, and 4α-hydroxy-homalomenol C $[\alpha]_D^{20} + 2°$ (*c* 0.25, $CHCl_3$) from the upper parts of *T. ramosissimum*. Moreover, most of sesquiterpenes and trinorsesquiterpenoids isolated from the ethanolic extract of *T. ramosissimum* aerial parts, including homalomenol C, oxo-T-cadinol, 1β,4β,6β-trihydroxyeudesmane and 4β-hydroxy-11,12,13-trinor-5-eudesmen-1,7-dione exhibited *in vitro* antiplasmodial activities against *P. falciparum* with median inhibitory concentration values (IC50) ranging from 1.2 to 5.0 μg/mL [12]. Additionally, Hachicha et al. [13] suggested that germacradien-4-*α*-1-ol, *δ*-Cadinene, were the major phytoconstituents identified in the Teucrium essential oil. Flavonol glycosides, as the main flavonoids extracted from the genus Teucrium are characteristic of this taxon whereas the diterpenes, neo-clerodane are regarded as taxonomic markers [14]. Based on the data in the literature, β-Sesquiphellandrene might be the most bioactive terpenic compounds [15] in *T. ramosissimum* with the greatest potential for clinical antitumoral strategies on the human colorectal cancer. The sesquiterpenoid alcohol isolated from the aerial parts of *Teucrium ramosissimum*, known as β-eudesmol inhibited superoxide production, proliferation, adhesion and migration of human lung (A549), colon

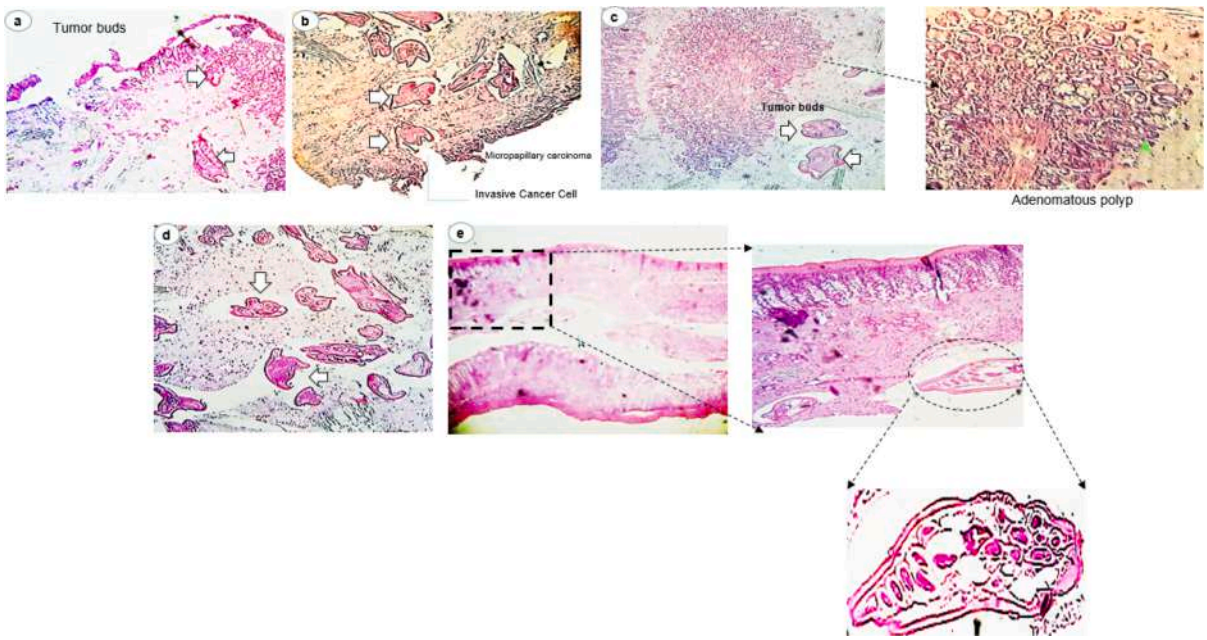

Fig. 12. Macroscopical analysis of tumor buds at the invasive front. White arrows indicate cell clusters; blue arrows indicate tumor cells; green arrows indicate crypt and goblet cell loss (H&E staining, Magnification a, b, c, f × 10; d, e × 40).

(HT29 and Caco-2) and K562 tumor cells [16,17]. Further results suggested that extracts from many species of the Lamiacea family show cytotoxic effects against colon, lung, prostate, and breast tumor cells [3]. Moreover, phenolic-enriched extracts from *T. ramosissimum* had a potent antimutagenic effects against benzo[a]pyrene, sodium azide, 4-nitro-o-phenylenediamine and aflatoxin B1. It decreased the genotoxicity induced by nitrofurantoin and aflatoxin B (1) and possess antioxidant activities by enzymatic (xanthine/xanthine oxidase assay) and non enzymatic (DPPH, NBT/riboflavine and ABTS assays) system [18] and antibacterial effect. In addition, *T. ramosissimum* leaves protected against DNA damage in human lymphoblast cell K562 [16]. Further, leaf extracts from *T. ramosissimum* and a sesquiterpene, β-eudesmol protected gastric mucosa from ethanol-induced ulcerogenesis [19]. Flavonoids isolated from chloroformic extracts of *T. ramosissimum* were cirsimaritin, 4′,7-dimethoxy apigenin and genkwanin exhibited higher free radical scavenging effects using the RP, TEAC, FRAP and CUPRAC assays [18]. Moreover, naringenin, apigenin-7-glucoside and genkwanin isolated from *T. ramosissimum* affect macrophage functions by modulating their nitric oxide (NO) release and lysosomal enzyme activity and enhanced the cytotoxic activity of the T lymphocyte (CTL) discarded from splenocytes and the killing activity of natural killer (NK) cells [20].

Data obtained concur with other researchers who reported that the extracts and essential oil of *Teucrium* species and their bioactive components showed antiproliferative effects on HCT-116, multiple signaling pathways, and altered the expression of genes and proteins involved in cancer development, cell cycle, apoptosis, angiogenesis, metastasis [21,22–23]. The fundamental mode of action, signaling pathways and phytotherapeutic targets of volatile oil and its active molecules for anticancer effeciency have been explored. Additionally, treatment with *Teucrium alopecurus* aerial flowering part essential oil enriched in T-Muurolol, d-limonene, β-phellandrene, (+)-epi-Bicyclosesquiphellandrene, α-Bisabolol, and α-Cadinol was detected to induce apoptosis via generation of the intrinsic and extrinsic pathway in colon cancer (HCT-116) cell lines [23], as well as NF-kB inhibition [22].

Botanicals depict a distinguished source in the search for anticancer treatment as they possess unique chemical structures and mixture of components that may be efficient against cancer with few side effects or a minimal toxicity profile compared to standard anticancer therapy [8]. Indeed, *T. ramosissimum* specie is able to block human colon tumor cells at differents phases, like G0/G1, G1/S, G1, G2/M, and S stage and inhibit their growth and proliferation, also down-modulate anti-apoptotic genes. Guesmi et al. [24] found that p53 as a tumor suppressor protein dependent manner increase in both time and dose in HCT-116 cells. Cell cycle arrest is one of the results of the activation of p53. These process have potential antitumor effects.

Here again, *in vitro* experiment showed that Coincubation of tumor cells with either the highest concentration (0.1 or 0.2 μg/ml) of TrS or TRAIL/Apo2L (25 ng/ml) alone markedly induced activation of caspase cascades (caspases 3, 8, and 9), as mediators of apoptosis, and cleavage of the caspase 3 substrate (PARP), at least in human HCT-116 cells, specifically those related to molecular mode of action towards colon tumor cell lines. Further, tumor cells cotreated with teucrium and TRAIL enhanced the proteolysis of PARP for at least f5-fold. Apoptotic signaling occurs through the activation of multiple caspases [25]. The activation often take place continuously in a cascade, with an initiator procaspase being activated first, which then cleaves and activates executioner procaspases [26]. Once activated, caspase-9 then activates further "downstreamcaspases" and can itself engender a caspase cascade: Caspase-9 directly fused and triggers caspase-3 and −7. Caspase-3 then activates caspases-2 and −6 in cell extracts, followed later by the generation of caspases-8 and −10 [27]. During apoptotic cell death, PARP-1 plays two opposite roles: its stimulation leads to poly(ADP-ribose) synthesis, whereas caspase activation causes PARP-1 cleavage and inactivation [28]. Accordingly, PARP-1 can also promote tissue survival by shifting the balance of cell death programs between autophagy and necrosis [29]. Cleavage of PARP-1 promotes apoptosis by preventing DNA repair-induced survival and by blocking energy depletion-induced necrosis. After cleavage, PARP-1 loses the nick-sensor function and is inactive towards DNA damage. Accordingly, the consumption of NAD + is avoided [28]. Further, PARP-1 cleavage is an event that results in the formation of two products at a ratio of 1:1 [30]. Specific PARP-1 fragments appearance will not only help us understand the involvement of specific cell death proteases in particular processes, but also different types of cell death [29]. The appearance of the 89 kDa fragment has been considered as a hallmark for apoptosis [30].

Our findings indicated that neither TrS alone nor TRAIL alone highly increased the DR expression and decreased the expression of decoy receptors (DcR1, DcR2), thus promoting apoptosis in HCT-116. TRAIL

decoy receptor family, including TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and osteoprotegrin have a critical role in driving TRAIL resistance [31]. Incubation of colon tumor cells with either TRAIL or TrS enhanced DRs upregulation. Thus, these data suggest that ROS is required for TRAIL receptor induction by TrS volatile oil. The inhibition in cell growth was also correlated with ROS generation and JNK activation [32]. In the current study, Western Blot Analysis suggested that TrS induced MAPKs activation at as little as 12 and 24 h of treatment and evidenced its upregulation between 0.1 and 0.2 μg/ml. Upmodulation of the p38 MAPK indicating path induces caspase-9 release [3]. In addition, TrS essential oil is able to act as a good inhibitors of the STAT's upstream gene, and therefore must be activated before STAT can be activated as well [33], while induced loss of Akt level that occurs at 12 h time point, and decreased the phosphorylation of pSTAT3 (Ser727). Interestingly, transcription factor specificity proteins (Sp), including Sp1, Sp3, and Sp4, are known to regulate survivin and are required for survival of tumor cells [32].

In the current reports, terpenic compounds of TrS essence are able to interfere with the cell cycle stages and biochemical and molecular pathways of cancer cell apoptosis, and induce oncogenes expression (MMP-9, survivin, XIAP, c-Myc) when combined with TRAIL/Apo2L. MMP-9 pro-oncogene mediates the sequence of a pro-angiogenic product and the release of VEGF during carcinogenesis [3]. Treatment of HCT-116 with combined drugs inhibit the apoptosis genes (IAP), mainly cIAP1/2 and Bcl2 that can block apoptosis. Effectively, resistance to TRAIL molecule has become one of the significant issues associated with the failure of TRAIL in treating colorectal cancer (CRC) [31]. *In vivo* experiment showed that essential oil of TrS given prior to LPS exposure protected animals from inflammation and colon tumorigenesis.

5. Conclusion

In this report, the molecular mechanism of the anticancer effects of *T. ramosissimum* essential oil towards HCT-116 cell lines is well investigated. Cell death apoptosis was mediated through DRs expression and MAPKs induction. Herbs essence may be used as protective phytcomplex towards colorectal tumorigenesis in mouse model, ethnophytotherapy as a traditional wound healing and good food preservatives.

CRediT authorship contribution statement

Fatma Guesmi: Writing – original draft. **Wiem Tahri:** Writing – review & editing. **Amel Mehrez:** Writing – review & editing. **Taha Barkaoui:** Writing – review & editing. **Sahdeo Prasad:** Writing – review & editing. **Angelo Maria Giuffrè:** Writing – review & editing. **Ahmed Landoulsi:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analyzed during this study are included in the article.

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Authors' contributions

Fatma Guesmi and Sahdeo Prasad conceived and designed the research and developed the methods. Performed experiments and data analysis were done by Fatma Guesmi. The first draft of the manuscript was written by Fatma Guesmi. Angelo Maria Giuffrè managed: software, visualization, supervision. All authors read and approved to the last versions of the manuscript.

Ethics approval and consent to participate

The current work was confirmed and approved by the Research Ethics Committees (Pasteur Institute, Tunisia) with ethical approval number: No. LNSP/Pro 152012. All human cancer cell lines were authenticated before use.

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