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Chemical composition, docking simulations and burn wound healing effect of *Micromeria fruticosa* extract and its isolated flavonoidal compound

Naglaa G Shehab[1,2]*, Eman Abu-Gharbieh[3,4] and Ihab M Almasri[5]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt
[2]Department of Clinical Pharmacy and Pharmacotherapeutics, Dubai Pharmacy College, Dubai, UAE
[3]Department of Clinical Sciences, College of Medicine, University of Sharjah, Sharjah, United Arab Emirates
[4]Sharjah Institute for Medical Research, University of Sharjah, Sharjah, United Arab Emirates
[5]Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, Al Azhar University, Gaza, Palestine

Abstract:The study aimed to investigate the constituents of the ethanolic extract of *Micromeria fruticosa* and evaluate its antimicrobial and burn healing activities and the isolated compound, rutin. The plant was extracted with ethanol and the active constituents were isolated. The antimicrobial activities of the extract and the isolated compounds were assessed. The burn healing potentiality was evaluated in a second-degree burn model on rats. Five compounds were isolated and identified namely, oleanolic acid 3-O-β-D-glucopyranoside, apigenin, tectochrysin, 7,4' dihydroxyflavone7-rhamnoglucoside, and rutin. Noticeable antimicrobial activities of the extract, fractions and rutin, were obtained. These effects could be attributed to the isolated flavonoids and triterpenes compounds. The topical application of the extract or rutin significantly reduced the wound size and improved the skin histology. The molecular docking simulations predicted potential inhibitory interaction between rutin and the active site of IKKβ that could be responsible for blocking NF-kB activation; this could explain the possible mechanism by which rutin enhances the burn wounds healing process. Ethanolic extract, fractions and isolated compound, rutin of *M. fruticosa* exhibited significant antimicrobial activities. The plant extract and rutin demonstrated high potentialities to heal burns.

Keywords: Burn healing, docking simulation, *Micromeria fruticosa,* rutin, IKKβ

INTRODUCTION

Burns are among the major challenging conditions in emergency medicine. Burns outcomes range from physical impairments to psychological consequences, all of which are associated with high medical care costs (Mohammadi *et al.*, 2008). Management of major burns requires, in most cases, hospitalisation, multiple medications, surgical procedures and a prolonged rehabilitation stage (Mohammadi, Amini *et al.*, 2008). Wound healing in thermal burns is a complicated process mediated by activating inflammatory cells, macrophages and neutrophils that produce pro-inflammatory cytokines such as tumour necrosis factor (TNF-α) and interleukin-I (Kimura *et al.*, 2008). Though the inflammatory response is fundamental to the healing process, aberrant levels of inflammatory mediators and persistent inflammation can be damaging and lead to fibrosis, hypertrophic scars, keloids and even multiple organ failure (Portou *et al.*, 2015, Ward and Till, 1990). In the literature, NF-κB is known as one of the important molecular targets in inflammation (Yamamoto and Gaynor, 2001). NF-κB is reported to be activated after burn injury (Carter *et al.*, 2014). Therefore, there is a great necessity to develop treatments that can control inflammation in burn wounds, reduce the risk of systemic inflammatory response syndrome (SIRS) and prevent excessive scar formation while maintaining the ability to fight infection.

Recently, there has been evidence of the potential applications of topical natural products to enhance both acute and chronic wound healing. However, only a few plant-derived chemicals have been evaluated in human trials, such as *Aloe vera* (Hekmatpou *et al*., 2019) and Avena sp (Sabadotto *et al*., 2014). Therefore, many potential natural products are being studied to overcome the side effects associated with chemotherapeutic agents (Hai *et al.*, 2019).

Micromeria fruticosa (L) Druce ssp *serpyllifolia* belongs to the family *Labiatae,* one of the largest and most distinguished families of flowering plants with around 220 genera and around 4000 species worldwide (Naghibi *et al.*, 2005). *Micromeria* species are reported to have many *in-vitro* and *in-vivo* pharmacological activities. The aerial parts of *Micromeria* species were investigated for *in vitro* antimicrobial and antiseptic effects. The studies showed that the genus Micromeria exhibited significant antimicrobial activities against bacteria and fungus, and the extracts could help treat skin infections (Azab, 2016, Duru *et al.*, 2004).

Previously, Abu-Gharbieh *et al.* reported that *Micromeria fruticosa* (L) had significant anti-inflammatory,

**Corresponding author:* e-mail: naglaa@dpc.edu

antitumor, antioxidant, gastro and hepatoprotective activities (Abu-Gharbieh and Ahmed, 2016, Abu-Gharbieh *et al.*, 2013, Shehab and Abu-Gharbieh, 2012), and it was able to ameliorate the antioxidant enzymes including glutathione peroxidase and catalase (Abu-Gharbieh and Ahmed, 2016). Similarly, Abu-Gharbieh *et al*. proved that the anti-inflammatory activity of the plant extract was attributed to its ability to inhibit the neutrophilic release of myeloperoxidase enzyme (MPO) and other inflammatory mediators (Abu-Gharbieh *et al.*, 2016). Therefore, several *Micromeria* species have medicinal values. However, thus far, no scientific report could be found regarding the wound healing activity of the plant, despite there being a plentiful ethnobotanical claim for this property.

Most of the chemical studies of *M. fruticosa* were carried out to investigate its volatile constituents (Azab, 2016). High-Performance Liquid Chromatography (HPLC) analysis of the methanolic extract was carried on, and rutin and quercitrin were found to be the main flavonoidal glycosidal compounds (Abu-Gharbieh and Ahmed, 2016). Rutin has been shown to possess many desirable therapeutic properties, particularly antioxidant, anti-inflammatory and wound healing (Gao *et al.*, 2013, La Casa *et al.*, 2000). It is proposed that, in burn wound management, early antioxidant therapy would strengthen the cellular antioxidant defence mechanisms, decrease free oxygen radicals mediated delay in healing, and promote the wound's healing process. Oxygen-free radicals have been associated with various pathological processes, including burn-induced organ damage (Ward and Till, 1990).

Accordingly, this study aimed to investigate the constituents of the ethanolic extract of *M. fruticosa* and evaluate its antimicrobial and burn healing activities and its major isolated compound, rutin.

MATERIALS AND METHODS

Materials and apparatus for the chromatographic technique

The UV absorption spectra were measured by Shimadzu 1700 spectrophotometer (Kyoto, Japan). Electrothermal 9100 equipment was used to determine the melting points. Nuclear magnetic resonance (NMR) spectra were measured in dimethyl sulfoxide (DMSO) or deuterated methanol (CD_3OD) or deuterochloroform ($CDCl_3$); ^{1}H-NMR and ^{13}C-NMR spectra were measured at 400 and 100 MHz respectively using JEOL GX-400 spectrometer with the chemical shifts (δ ppm) expressed relative to TMS as an internal standard. Pre-coated plates of silica gel 60 F_{254} (Fluka, Switzerland) were used. Chromatograms were visualised using ultraviolet (UV) light (254 and 366 nm) pre and post-exposure to ammonia vapour and by spraying with *p*-anisaldehyde reagent. Columns chromatography were performed over silica gel 60 GF (Merck, Darmstadt, Germany) for vacuum liquid chromatography (VLC), silica gel 100C_{18}-Reversed Phase (Fluka, Switzerland) and Sephadex LH-20 (Sigma-Aldrich, United States).

Plant material

The aerial parts of *M. fruticosa* were collected during the flowering stage in September 2015 in Nablus, Palestine. The plant was previously authenticated by Professor Hassnaa Hosny, Department of Botany, Faculty of Science-Cairo University, Egypt. Voucher specimens were preserved at the Herbarium of Dubai Pharmacy College (Reference # 015.09.08).

Drugs and chemicals

The used solvents were of analytical grades and purchased from Fisher Scientific (UK). Sterile dimethyl sulphoxide (DMSO), gentamicin and fluconazole from Sigma-Aldrich, United States, and MEBO® ointment was obtained from Julphar Company, Gulf Pharmaceutical Industry, Ras Al Khaimah, UAE.

Extraction and fractionation

The fresh plant (1.5kg) was air-dried and powdered to yield 900g of *M. fruticosa*. Cold maceration in 70% ethanol (5Lx2) was used to extract the plant exhaustively. Ethanol was reported previously by Abu-Gharbieh *et al.* to be a suitable solvent to extract most of the plant's active constituents (Abu-Gharbieh and Ahmed, 2016). Later, the solvent was evaporated under reduced pressure at 50°C, giving 302g residue. Then, 200 grams were suspended in 150mL of water and consecutively fractionated with n-hexane, chloroform, and n-butanol saturated with water to yield 40, 5 and 25 Grams, respectively. A portion from the dried extract of *M. fruticosa* was completely dissolved in a sterile DMSO for topical application.

Isolation of the constituents of the chloroform fraction

A precisely measured amount of the chloroform extract (3.0g) was fractionated by the Sephadex LH-20 column (37 X 3.2 cm). Elution was accomplished with methanol-water (80:20 v/v). Fractions, each of 20mL, were collected and monitored by TLC using system A; chloroform-methanol 8.8: 1.2. Spots were set by visualisation under UV_{365} nm pre- and post-exposure to ammonia vapour and by spraying with *p*-anisaldehyde. Similar fractions were combined to yield five collective fractions (F1-F5). According to the weight of the fraction and the number of spots, fractions F1 and F4 were assigned for additional isolation.
F1: (0.5 g; 2 spots, R*f* values 0.50 and 0.43, system A), compound 1 was obtained upon recrystallisation from methanol.

F4: (0.2 g; 3 spots, R*f* values 0.40, 0.23 and 0.15, system A), compound 2 was obtained following rechromatography on a Sephadex LH-20 column using, methanol: Water 9: 1 (v/v) as eluent.

Isolation of the constituents of the n-butanol fraction

VLC on a silica gel 60 GF column (13 X 4.5cm) was used to fractionate the *n*-butanol residue (15g); gradient elution was used with chloroform-ethyl acetate and ethyl acetate-methanol mixtures. Fractions, 300mL each, were collected and checked by TLC (system A; system B: ethyl acetate-acetic acid-formic acid-water, 10:1.1:1.1:0.5 v/v/v/v). Spots were seen under UV_{365} nm lamp pre-and post- ammonia vapour exposure and spraying with *p*-anisaldehyde. Similar fractions were combined to yield 14 collective fractions (P1-P14). According to the weight of the fraction and the number of spots, fractions P 9 and P 10 were selected for additional isolation.

P 9: (0.8g; 3 spots, R*f* values 0.75, 0.54 and 0.35, system B) was applied on sephadex LH-20 column (37X 3.2 cm) using methanol: water 8: 2% (v/v) as eluent, resulted in isolation of compounds 3 and 4.

P 10: (5.0g; 3 spots, R*f* values 0.46, 0.34 and 0.28, system B) was applied on a sephadex LH-20 column (37 X 3.2 cm) using methanol: water 8: 2 (v/v) as eluent, resulted in isolation of compound 5.

Molecular docking simulations

The chemical structures of the compounds isolated from the ethanolic extract of *M. fruticosa* were sketched in MarvinSketch 16.10.24 (MarvinSketch, calculation module developed by ChemAxon, 2014) and saved in MDL molfile format. Afterwards, a group of energetically accessible conformers was generated using OMEGA software (OMEGA 2.5.1.4, 2013). The generated conformers were saved in SDF format. The 3D coordinates of human IKKβ (PDB codes: 4KIK, resolution; 2.83 Å) was collected from the RCSB Protein Data Bank. DS Visualizer 2.0 (Accelrsy Inc.) templates for protein residues was used to add the hydrogen atoms to the proteins. The docking study was performed in the presence of explicit water molecules. The natural components were docked into the binding site of the target protein using FRED software within the OEDocking suite (OEDOCKING 3.2.0.2, 2015). The protein structure and ligands conformers were treated as rigid entities during docking simulations. The top-scoring poses were optimised and assigned a final score using Chemgauss 4.

Biological study

Microorganisms

Three bacterial strains *(Staphylococcus aureus* RMTCC 3161; *Escherichia coli* RMTCC 2682 and *Pseudomonas aeruginosa* RMTCC 1687) and a fungus *(Candida albicans* RMTCC 5122) were kindly provided by Rashid Hospital (Dubai-United Arab Emirates) to screen the antimicrobial activity. Different media were used to cultivate the microorganisms: nutrient agar (*S. aureus* and *P. aeruginosa),* MacConkey agar (*E. coli)* and Sabouraud dextrose agar (*C. albicans*).

Antimicrobial activity

The ethanolic extract of *M. fruticosa* and its fractions, chloroform and *n*-butanol (375µg/mL, each) and the isolated compound 5 (140µg/mL) were subjected for *in vitro* antimicrobial activity screening. The agar diffusion technique was used (Lorian, 2005). The samples were solubilised in sterile DMSO, and the DMSO was used as a control. Both samples and DMSO were discharged separately into a hole of 1.0cm in diameter. After incubation, the diameters of the growth inhibition zones were measured in mm. The results obtained by the test compounds were compared with the control drugs: broad-spectrum antibacterial ofloxacin (30µg) and antifungal fluconazole (30µg). All analyses were tested in triplicate.

Experimental animals

Animal investigations were carried out based on the ethical standards for the proper care and use of laboratory animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals., 2011) and under aseptic conditions and the approval of the Ethical Research Committee at Dubai Pharmacy College, Dubai, UAE (Reference # 015.11.22). The skin irritation study was carried out on nine albino rabbits, weighing approximately 2.2kg, for both doses of the crude ethanolic extract of *M. fruticosa* as well the isolated compound 5. Meanwhile, the burn healing activity was evaluated on male Wistar rats weighing 240±5g. Standard hygienic conditions (temperature 22±2 ^{O}C, relative humidity 50-60%, with day/night lighting cycle) were kept during the experiment. The animals received a balanced diet and were supplied with water ad libitum. They were accommodated for seven days before the experiment.

Skin irritation study

The dorsal side of the animal was carefully shaved before the experiment. The back of the animal was marked for the topical application of 4 concentrations of the ethanolic extract (50, 75, 100 and 125mg/mL sterile DMSO), and two concentrations for rutin (20 and 40mg/mL sterile DMSO) and the positive control (sterile DMSO). The treated sites were covered by gauzes, and the back of the animal was wrapped with a non-occlusive bandage. After 24, 48 and 72 hours, signs of inflammation of the skin were observed and scored as follows: 0: No erythema, no edema; 1: very slight erythema, very slight edema; 2: well-defined erythema with slight edema; 3: moderate to severe erythema with moderate edema and 4: Severe erythema with severe edema. Two different animals for each dose were used, and the average of the two scores was recorded as an index of skin irritation (Ananth *et al.*, 2010).

Burn model and experimental protocol

During the experiment, one animal was maintained per cage. Each rat was initially anaesthetised by

intramuscular injection of ketamine (60mg/kg) and xylazine (10mg/kg). His back hair was shaved and cleaned with povidone-iodine solution, then wiped with sterile water before induction of experimental burn injuries. The burn was made by holding a stainless-steel stamp of 6.9 cm^2 surface area and weight of 150 g (previously heated to 100°C) placed over the skin for 60 seconds without applying pressure. Approximately 10 % of the total body surface (TBSA) was burned in this experiment.

This heat exposure caused a uniform second-degree burn on the back of the skin. Postoperative pain was treated with diclofenac injection given intramuscularly (10 mg/kg) once daily for two successive days (Rasool *et al.*, 2014). After 24 hours, the animals were assigned randomly into six groups of six animals each. Group 1 was the control group without the topical agent; group 2 was treated with sterile DMSO; group 3 was treated with the well-known reference drug "MEBO®", which is a natural ointment composed from β-sitosterol, sesame oil, and beeswax. One millilitre of *M. fruticosa* ethanolic extract solution in concentrations of 50 and 75mg/mL sterile DMSO was applied to the wounds for animals in groups 4 and 5.

Similarly, one millilitre of the isolated compound no. 5 solution (rutin) at 20mg/mL sterile DMSO was applied to the wounds of animals in group 6. The wounds were washed with normal saline before dressing for all groups, then covered once daily by a similar thin layer of the extract or rutin or with standard MEBO in each group. After 72 hours, the entire group wounds were kept open after dressing. The area of the lesions was measured on days 1, 3, 5, 11, 13 and 15 after the burn injury to assess the rate of wound healing. On each experimental day, the wound area was measured by a ruler and recorded in square millimetres and photographed. Percentage wound contraction at each time point was calculated using the following formula: Percentage wound contraction = (initial wound area current area)/initial wound area X 100. Measurements were made daily by the same examiner by using a ruler and by tracing paper (Tavares Pereira Ddos *et al.*, 2012).

Histological evaluation

Two animals from each group were sacrificed with cervical dislocation at the end of the treatment period. For histological studies, samples from the skin tissue were taken with a small excision containing part of the burned area. Tissues were fixed in 10% formalin, and paraffin-embedded sections (5-µm thick) were cut and stained with hematoxylin and eosin. The light microscope was used to assess the pathological changes, e.g., re-epithelialisation, crusting blistering, spongiosis, granulation tissue and collagen matrix organisation, inflammation, congestion and edema.

STATISTICAL ANALYSIS

The obtained results were expressed as mean ± SEM. Two-way Analysis of Variance (ANOVA) followed by Bonferroni's test was used to compare between different groups. GraphPad Prism (Version 8, San Diego, CA, USA) was used for statistical analyses.The results were considered statistically significant when the probability was less than 0.05 ($p < 0.05$).

RESULTS

Isolation of chloroform and n-butanol fractions constituents

Isolated compounds

Fig. 1 shows the chemical structures of the isolated compounds

Compound 1 (Oleanolic acid 3-O-β-D-glucopyranoside); $C_{36}H_{58}O_8$; white powder; 15 mg; soluble in chloroform-methanol mixture; R_f : 0.43 (mobile phase A); m. p. 309 ^{0}C; ^{1}HNMR, (400 MHz, CD_3OD): δ_H 0.79, 0.89, 0.90, 0.93, 0.98, 1.01, 1.20 (21 H, 7s , CH_3), 3.27 (1 H, dd, *J*= 8.0, 2.4 Hz, H-3), 3.46 (1 H, dd, *J*=12, 2.2 Hz, H-18), 3.33-3.67 (m of sugars moieties), 4. 9 (1 H, d, *J* =7.8 Hz, H-1 Glc), 5.27 (1 H, t, *J* = 3.6 Hz, H-12).

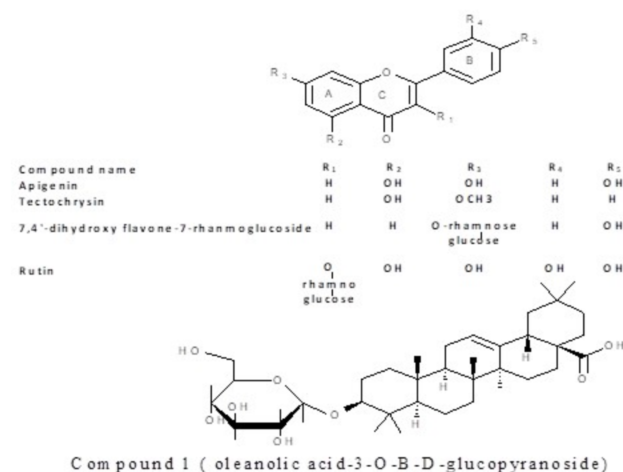

Fig. 1: Chemical structures of the isolated compounds of *Micromeria fruticosa*

^{13}CNMR, (100 MHz, CD_3OD): δ_C 38.4 (C-1), 26.5(C-2), 80.0 (C-3), 38.8 (C-4), 55.4 (C-5), 18.1 (C-6), 32.0 (C-7), 39.4 (C-8), 47.8 (C-9), 37.2 (C-10), 23.7 (C-11), 124.5 (C-12), 145.0 (C-13), 41.9 (C-14), 27.8 (C-15), 23.6 (C-16), 47.5 (C-17), 42.0 (C-18), 46.9 (C-19), 31.9 (C-20), 34.7 (C-21), 32.9 (C-22), 28.7 (C-23), 16.3 (C-24), 15.4 (C-25), 17.4 (C-26), 26.5 (C-27), 180.4 (C-28), 32.9 (C-29), 23.8 (C-30), 105.9 (C-1'), 74.9 (C-2'), 78.3 (C-3'), 71.9 (C-4'), 78.0 (C-5'), 67.7 (C-6').

Compound 2 (Apigenin); $C_{15}H_{10}O_5$; yellow powder; 20 mg; soluble in methanol; R_f: 0.23 (mobile phase A); m.p. 345 ^{0}C; brown in visible light, dull brown under UV_{365nm},

and under UV_{365nm}/NH_3; UV λ_{max} nm: CH_3OH, 267, 296 sh, 336; CH_3ONa, 276, 324, 392; $AlCl_3$, 276, 321,348, 384; $AlCl_3/HCl$, 276, 299,340, 381; NaOAc, 274, 301, 376; $NaOAc/H_3BO_3$, 269, 302 sh, 338; ^{1}HNMR, (400 MHz, DMSO):δ_H 6.19 (1H, d, *J*=2.5 Hz H-6), 6.46 (1H, s, H-3), 6.66 (1H, d, *J*=2.5 Hz, H-8), 6.90 (2H, d, *J*=8.4 Hz, H-3', H-5'), 7.71 (2H, d, *J*=8.4 Hz ,H-2', H-6').

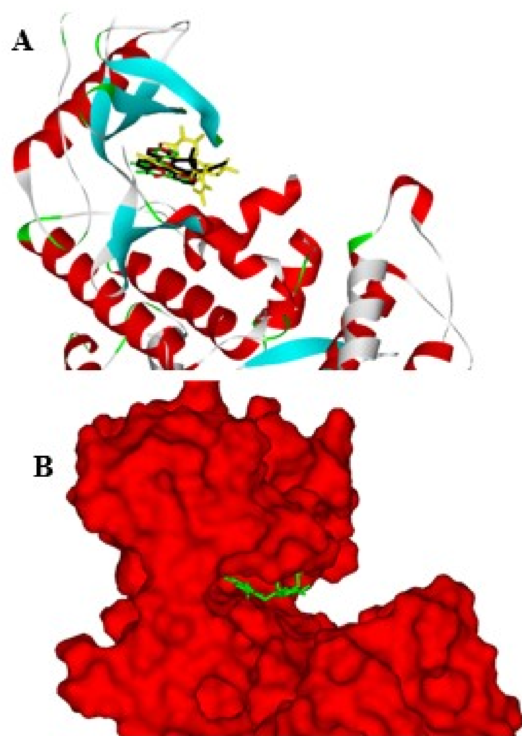

Fig. 2: (A) Ribbon diagram of the ATP-binding pocket of in kinase domain of IKKβ occupied by the co-crystallised ligand inhibitor (PDB code: KSA, black) overlaid with the docked poses of: Rutin (yellow); Apigenin (red); Tectochrysin (green). (B) Rutin docked in the pocket of IKKβ. The structure of IKKβ is depicted as a solvent-accessible surface model.

Compound 3 (Tectochrysin); $C_{16}H_{12}O_4$; yellow crystalline powder; 10 mg; soluble in methanol; R_f : 0.54 (mobile phase B); m.p.168; pale yellow in visible light, dull brown under UV_{365} nm, and under UV_{365} nm/NH_3; UV λ_{max} nm: CH_3OH, 247 sh, 268, 303 sh; CH_3ONa, 245, 271; $AlCl_3$, 252, 279, 330, 380; $AlCl_3/HCl$, 252, 279, 330, 380; NaOAc, 268, 308; $NaOAc/H_3BO_3$, 268, 308; ^{1}HNMR, (400 MHz, CD_3OD): δ_H 3.79 (3H, s, OCH_3),6.48 (1H, d, *J*=2.0 Hz, H-6), 6.72 (1H, s, H-3), 6.73 (1H, d, *J*=2.0 Hz, H-8), 7.51 (3H, m, H-3', H-4', H-5'), 7.78 (2H, d, *J*=8.4 Hz ,H-2', H-6').

^{13}C NMR (100 MHz, CD_3OD): δ_C 163.4 (C-2), 108.2 (C-3), 176.4 (C-4), 156.6 (C-5), 98.5 (C-6), 150.6 (C-7), 108.2 (C-8), 156.6 (C-9), 111.3 (C-10), 131.1 (C-1'), 126.2 (C-2'), 128.5 (C-3'), 131.4 (C-4'), 128.5 (C-5'), 126.2 (C-6'), 51.9 (OCH_3).

Compound 4 (7,4' dihydroxyflavone-7-rhamnoglucoside); $C_{27}H_{30}O_{13}$; yellow powder; 10 mg; soluble in methanol; R_f : 0.35 (mobile phase B); m.p. 345^{0}C; brown in visible light, yellowish green under UV_{365nm}, and under UV_{365nm}/NH_3; UV λ_{max} nm: CH_3OH, 255, 311, 325; CH_3ONa, 251sh, 294,304, 385; $AlCl_3$, 255, 311, 327; $AlCl_3/HCl$, 255, 311, 327; NaOAc, 255, 311, 386 sh; $NaOAc/H_3BO_3$, 255, 311, 328; ^{1}HNMR, (400 MHz, CD_3OD): 6.43(1H, s, H-3), 6.68 (1H, d, *J*=2.5 Hz, H-8), 6.72 (1H, dd, *J*=8.7, 2.2 Hz, H-6), 7.02 (2H, dd, *J*=8.7, 2.2 Hz, H-3', H-5'), 7.71 (2H, d, *J*=8.4 Hz, H-2', H-6'), 7.86 (1H, d, *J*=8.7 Hz, H-5), 3.33-3.67 (m of sugar moieties), 4.75 (1H, d, *J*=1.5 Hz, Rham), 1.20 (3H, d, *J*= 6 Hz, CH_3-Rham), 5.20 (1H, d, *J*=7.8 Hz, H-1 Glu). ^{13}C NMR (100 MHz, CD_3OD): δ_C 162.4 (C-2), 104.2 (C-3), 176.3 (C-4), 126.6 (C-5), 114.8 (C-6), 162.6 (C-7), 102.2 (C-8), 157.6 (C-9), 116.3 (C-10), 121.1 (C-1'), 128.2 (C-2'), 115.5 (C-3'), 160.4 (C-4'), 115.5 (C-5'), 128.2 (C-6'), 101.2 (C-1''), 73.8 (C-2''), 76.8 (C-3''), 72.5 (C-4''), 75.8 (C-5''), 67.1 (C-6''), 102.3 (C-1'''), 70.8 (C-2'''), 70.0 (C-3'''), 71.4 (C-4'''), 69.1 (C-5'''), 18.1 (C-6''').

Compound 5 (rutin); $C_{27}H_{30}O_{16}$; yellow powder; 2.0 g; soluble in methanol; R_f : 0.28 (mobile phase B); m.p. 245; brown in visible light, dull brown under UV_{365}nm, and yellow under UV_{365}nm/NH_3; UV λ_{max} nm: CH_3OH, 262, 264 sh, 301 sh, 357; CH_3ONa, 275, 325, 410; $AlCl_3$, 271, 300sh, 429; $AlCl_3/HCl$, 268, 297, 359 sh, 401; NaOAc, 270, 324, 391; $NaOAc/H_3BO_3$, 260, 301, 384; ^{1}HNMR, (400 MHz, CD_3OD): δ_H 6.10 (1H, d, *J*=2 Hz, H-6), 6.30 (1H, d, *J*=2 Hz, H-8), 6.77 (1H, d, *J*=8.4 Hz, H-5'), 7.56 (2H, d, *J*=8.4 Hz ,H-2', H-6'); 3.32-3.66 (12H, m of sugar moieties), 4.42(1H, d, *J*=1.5 Hz, Rham), 1.06 (3H, d, *J* = 6 Hz, CH_3-Rham), 5.1 (1H, d, *J*=7.8 Hz, H-1 Glu).

The triterpenoidal nature of compound 1 was confirmed with a positive result with Salkoweski test .^{1}H- and ^{13}C–NMR spectra revealed that compound 1 was pentacyclic triterpene. Compound 1 was recognised as Oleanolic acid 3-O-β-D-glucopyranoside from its physical properties and its spectral data (^{1}HNMR and ^{13}CNMR) (Sha *et al.*, 2008).

The ^{1}HNMR spectra of compounds 2, 4 and 5 exhibited flavonoid pattern and were showed signals for H-3 in the range at δ_H 6.42-6.72 suggested that they belong to the flavone skeleton that was confirmed by ^{13}CNMR spectra. The physical properties and the spectroscopic spectra (UV and ^{1}H–NMR) of compound 2, apigenin was identified. The ^{1}HNMR and ^{13}C spectra for Compound 3 showed multiplet signals at δ_H 7.51 indicated H-3', H-4', H-5' and a methoxy group signal at δ_H 3.79 which appeared in ^{13}CNMR at δ_C51.9, suggesting that there is no substitution in the B ring of the flavonoidand the methoxy group attached to position 7. Compound 3 was identified as tectochrysin (Sha, Yan *et al.*, 2008).

Table 1: FRED docking results of natural compounds isolated from *M. fruticosa* on IKKβ

Rank	Natural Compound	Dock Score*	HB scoring
1	Apigenin	-13.81	-2.92
2	Tectochrysin	-13.08	-0.93
3	7,4' dihydroxyflavone-7-rhamnoglucoside	-11.94	-6.89
4	Rutin	-9.67	-6.97
5	Oleanolic acid glycoside	-5.14	-5.29

*FRED Chemgauss 4 score

Table 2: Antimicrobial activity of the ethanolic extract, fractions and isolated rutin of *M. fruticosa* (zone of inhibition, mm)

Tested sample	*S. aureus*	*P. aeruginosa*	*E. coli*	*C. albicans*
Ethanolic extract	9.0±0.1	26.0±2.3	26.1±1.5	5.0±0.9
Chloroform fraction	17.0±0.9	26.1±2.1	27.5±2.3	8.0±0.6
n-Butanol fraction	15.1±1.1	24.4±1.7	22.2±1.7	Negative
Rutin	8.0±0.4	20.0±1.8	17.3±0.8	Negative
Ofloxacin	20.0±2.1	18.0±2.1	20.2±1.1	Negative
Fluconazole	Negative	Negative	Negative	25±2.1

Values do not include the diameter of the cork borer

Table 3: Average wound size in animals, values are expressed as the mean ± SEM (mm)

Group	Treatment	Days after burn injury						
		1	3	5	7	11	13	15
1	Control	27.06±2.1	31.1±5.3	29.73±1.1	23.16±5.5	22.5±1.7	22.4±2.5	22.4±0.8
2	DMSO	30.43±2.8	33.5±3.2	31.7±0.9	26.45±1.6	24.5±1.9	22.1±2.6	22±1.9
3	MEBO	29.63±0.9	26.23±1.7	21.3±1.3*	17.6±1.5**	11.7±2.4**	6.03±1.3**	0 **
4	*M. fruticosa* extract 50mg/ml	29.03±1.3	28.96±0.6	24.16±1.1	19.5±1.9**	10.83±0.5**	5.3±1.7**	0.43±0.7**
5	*M. fruticosa* extract 75mg/ml	27.83±1.8	22.93±2.1	20.63±1.6*	18±2.1**	7.3±0.9**	1.46±2.5**	0**
6	Rutin 20mg/ml	29.33±0.9	29.73±4.3	27.76±2.6	16.8 ±1.3**	10.46±1.6**	4.66±2.8**	0**

* $p< 0.05$; ** $p<0.01$; determined by two-way ANOVA compared with control

Compound 4 showed a sugar moiety which was identified as rutinoside from the ^{1}HNMR and ^{13}CNMR data. Compound 4 was identified as 7,4' dihydroxyflavone7-rhamnoglucoside (Mabry *et al.*, 2012). The structure of compound 5 was identified as rutin from its physical properties and different spectral data (UV, ^{1}HNMR) and by comparing it with authentic rutin (Sintayehu *et al.*, 2012).

Computational docking simulations

To further explore the potential of natural products isolated from *M. fruticosa* to inhibit NF-κB activation, by targeting the upstream activation of inhibitory kappa B (IκB) kinase β (IKKβ), the phytochemicals were docked to IKKβ. Interestingly, all the identified natural compounds were successfully fitted within the ATP-binding pocket in the kinase domain (KD) of IKKβ (PDB 4KIK), as shown in table 1. fig. 2 shows the highest-

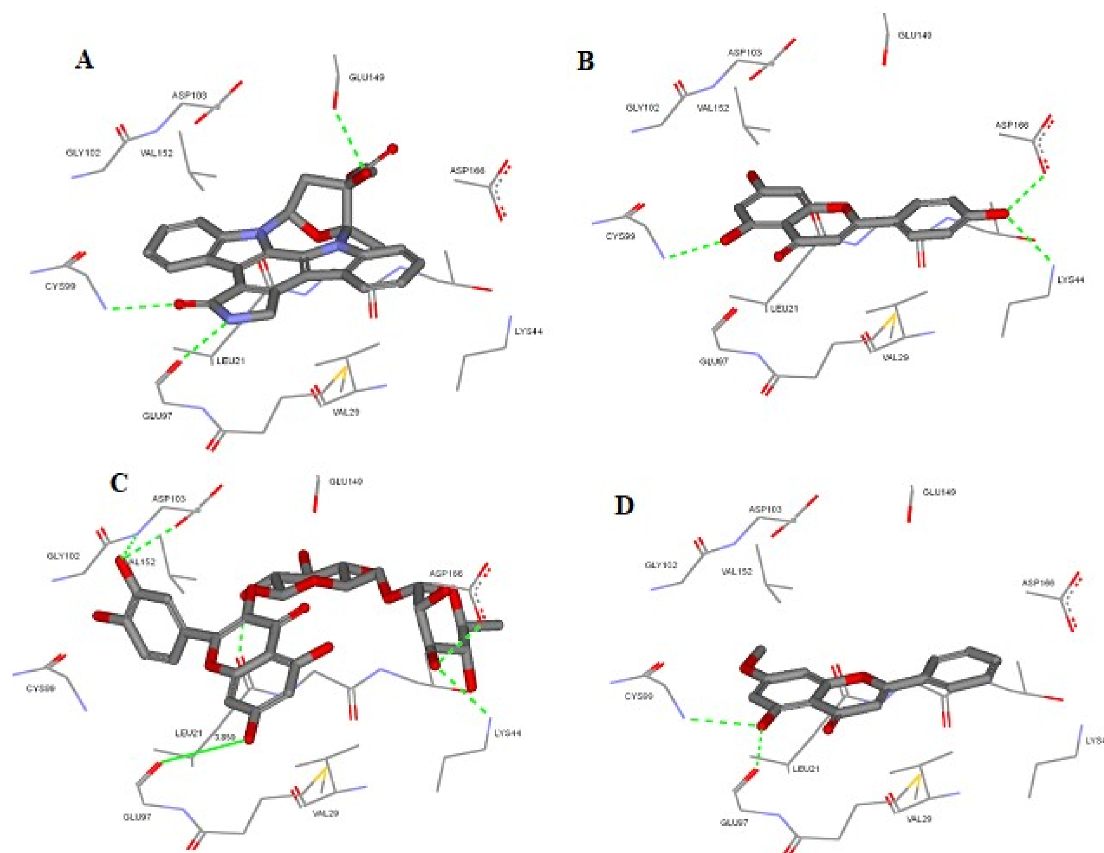

Fig. 3: Detailed view of co-crystallized staurosporine analog inhibitor and the corresponding interacting amino acids within the ATP-binding active site of IKKβ (A, PDB code: 4KIK) in comparison to the top-scoring docked poses of: (B) Apigenin; (C) Rutin; (D) Tectochrysin. H-bonds are indicated as green lines.

ranking docked poses of these compounds within the active site of the target protein. The alignment of natural hits and co-crystallised known inhibitor (PDB code: KSA), showed a good overlay and potentially mimicked the molecular interactions of the inhibitor (fig. 2A and 3). fig. 3A shows that the co-crystallised inhibitor binds to KD at the hinge loop connecting N- and C- lobes, a region that recognises the adenine in ATP.

Similarly, most of the isolated natural phytochemicals were able to form hydrogen bonds with the hinge residues, including Glu97 and Cys99 (fig. 3). It is well recognised that this hinge region of KD was critical for hydrogen-bonding interactions with inhibitors (Veerappan *et al.*, 2017). Furthermore, several of the compounds formed additional hydrogen bonds with the residues in the KD. For example, rutin forms a network of hydrogen bonding interactions with a hinge region, N-lobe, and C-lobe within ATP pocket. The hydroxyl group at position 7 of the chromone ring forms a hydrogen bond with Glu97 carbonyl oxygen at the hinge region of the active site, and the hydroxyl groups at position 3` of the phenyl ring make strong hydrogen bonds with Asp103 at the N-lobe, as shown in fig. 3C. Furthermore, the sugar moieties are hydrogen-bonded to Lue21, Lys44 and Asp166. All these multiple interactions greatly stabilise the rutin-IKKβ binding complex. Similarly, apigenin forms hydrogen bonds with Lue21, Lys44 and Asp166; oleanolic acid glycoside is hydrogen-bonded to Lue21 and Asp103, and dihydroxyflavones is bonded to Lys44 and Asp103. Noteworthy, The ability of apigenin to inhibit the NF-κB pathway by blocking IKK is reported in the literature (Shukla *et al.*, 2015).

Finally, several potential hydrophobic interactions are found between the natural compounds and Lue21, Val29, Met96, Val152 and Asp166.

Biological studies

Antimicrobial activity

Table 2 demonstrates the antimicrobial activity results and show that chloroform fraction, the ethanolic extract and the isolated compound, rutin, exhibit different antimicrobial activity against the test microorganisms. Furthermore, the antibacterial activities of the tested compounds were found to have variable inhibition zones ranging from 20.0±1.8 to 26.1±2.1mm in diameter against *P. aeruginosa*. All the test extracts, and the isolated

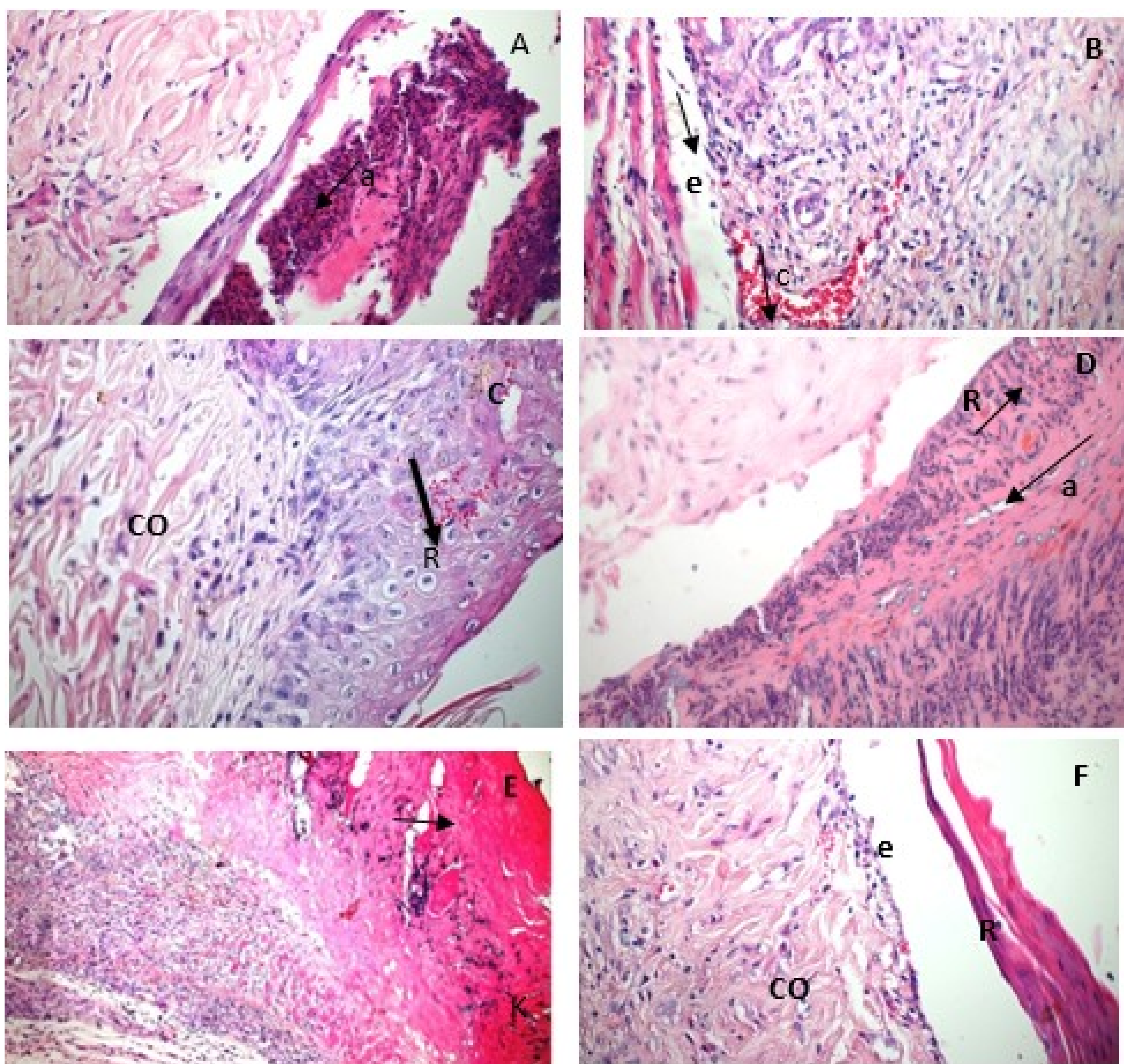

Fig. 4: (A); skin burn of control positive group reveling gross inflammation of epidermal layer (arrow), H&E 200 (B): skin burn of DMSO treated group showing few re-epithelization of edematous epidermal layer and congested blood vessels (e):edema, H&E 200, (C): skin burn of MEBO treated group showing marked re-epithelization, remodeling of epidermal cells and collagen deposition in the dermal layer(R):re-epithelization, (CO): congestion. H&E 400. (D) and (E): Skin burn of M. Fruticosa extract treated group at low and high doses, respectively showing enhanced re-epithelization of epidermis and angiogenesis (R): Re-epithelization, (a) angiogenesis. H&E 200. (F): skin burn of rutin treated group showing enhancement of healing proses with keratin formation, attempts of free epithelization and formation more collagen with less inflammation (K): Keratin, (R):re-epithelization, (CO) collagen, (e):edema. H&E 400.

compound, rutin, showed antibacterial activity against *E. coli* with an inhibition zone ranging from 17.3±0.8 to 27.5±2.3mm.

Wound healing

The skin irritation test on rabbits revealed that *M. fruticosa* extract at 50 and 75mg/mL concentrations and rutin at 20mg/mL were non-irritant, with a primary irritation index of 0.5 and 0.4, respectively. The healing pattern of the wound was studied by observing and measuring the wound area. The wound size decreased significantly ($p<0.05$) starting from day 3 for groups treated with MEBO and the highest extract dose. The wound size significantly reduced for all treated groups on day seven and beyond. On day 15, complete healing was achieved by MEBO, crude ethanolic extract at the highest dose and rutin. Interpretation of wound size revealed a more prominent wound closure in a dose-related manner in groups treated with the plant extract, as shown in table 3.

Histopathological analysis

Histological analysis revealed that the test and control wounds showed different degrees of inflammation that is essential in the healing process. Gross inflammation with necrosis of epidermal cells was seen in the control group (without drugs, fig. 4A), and few re-epithelialisation of the edematous epidermal layer and congested blood vessels was seen with the DMSO treated group (fig. 4 B). The reference drug (MEBO) enhanced skin healing process with marked re-epithelialisation, remodelling of epidermal cells and collagen deposition as shown in Fig. 4 C. On the other hand, skin treated with M. fruticosa extract at concentration of 50 mg/mL showed marked attempts of epidermal healing with enhanced re-epithelialisation and angiogenesis with large numbers of fibroblasts proliferation (fig. 4D). Similarly, skin treated with the plant extract at higher concentration (75 mg / mL) or rutin showed marked enhancement of healing process with keratin formation, re-epithelialisation and attempts of remodelling of skin appendages and more collagen with less inflammatory cells' infiltration (fig. 4 E and F, respectively).

DISCUSSION

Burn healing is a complicated process of dermal and epidermal tissues regeneration associated with tissue inflammation (Martin and Leibovich, 2005). Acute inflammation in burns is mediated mainly via granulocytes or polymorphonuclear leukocytes (PMN) and excessive generation of free radicals (Edgar *et al.*, 2011). It is well known that inflammation and prolonged edema could aggravate pain and delay wound healing, especially in the absence of infection (Zhao *et al*., 2016). Wound infection in burns is another complicating factor that interferes with wound healing. Many Gram-positive and -negative bacteria and fungi are associated with wound infection (Bowler *et al.*, 2001). Microorganisms are known to delay wound healing at lower levels before tissue invasion via toxin secretion, either directly from viable cells (exotoxins) or because of cell lysis (endotoxins) (Ovington, 2003). Endotoxins are released by various microorganisms that reduce the proliferative capacity of fibroblasts and epithelial cells, causing collagen and fibrin lysis and growth factors degradation (Hsieh *et al*., 2017).

Micromeria fruticosa Druceis used widely in many Mediterranean countries as a herbal infusion and medicinal agent. The plant extract's phenolic composition, total flavonoid, and phenolic contents were also reported in our lab (Abu-Gharbieh and Ahmed, 2016). The plant was found to be rich in both phenolic acids and flavonoids. Ferulic acid, catechin, chrysin, and catechol were the most predominant phenolic acids, while quercitrin and rutin were the major flavonoids. In the present study, we explored the constituents of *M. fruticosa* extract and evaluated its antimicrobial and burn healing activities. For the first time, five compounds were isolated from the plant: one triterpenoidal saponin glycoside and four flavonoids (three flavones and one flavonol), of which rutin was isolated in large amounts (2.0g), and this encouraged us to carry out more biological investigation on rutin. Antimicrobial studies revealed that ethanolic extract, fractions; chloroform and *n*-butanol; of *M.fruticosa* and its isolated compound rutin exhibited noticeable antibacterial activities, particularly against *P. aeruginosa,* which is the main cause for burn infection. The antipseudomonal activity of the test extracts could be ascribed to their isolated compounds (triterpenoidal glycoside and apigenin from chloroform and tectochrysin, 7,4' dihydroxyflavone 7-rhamnoglucoside and rutin from n-butanol) through individual action or in a synergistic way (Dawid-Pać, 2013). For the burn healing activity, the crude ethanolic extract and rutin were found to be freely soluble in distilled water and sterile DMSO. Sterile DMSO is preferred as a solvent over water for topical preparation due to its ability to dissolve all active constituents and enhance skin permeability (Kolb *et al.*, 1967). On the other hand, water was not chosen to avoid excessive wetting that may enhance infections. A simple topical solution was selected to apply directly to the burning surface rather than a complicated formula (such as creams) to eliminate any interfering factors and elucidate the plant effectiveness as a simple home remedy (Visuthikosol *et al.*, 1995).

Results of *in-vivo* burn healing activity showed that wound size significantly reduced starting from day 7 in all treated groups with $p<0.01$. Rutin is a bioflavonoid that is found in many plants. It has potent anti-inflammatory and antioxidant properties (Babu and Krishnakumari, 2005). It also helps the body to produce more collagen and prevents venous edema and damages to the skin (Umar *et al.*, 2012). Moreover, rutin can empower blood circulation and capillaries, which is important in speeding the burn's healing process and preventing infection (Bhandary *et al.*, 2012). The ethanolic extract in high dose (75 mg / mL) showed a rapid reduction of wound size compared to the isolated compound rutin. In fact, this is due to the parent extract containing many constituents besides rutin, which results in synergistic activity and enhances healing. It was reported that oleanane-type triterpene glycosides and apigenin exhibited a marked anti-inflammatory activity in the TPA-induced inflammation in the mouse ear.

Several studies in the literature highlighted NF-κB as one of the important molecular targets in inflammation (Yamamoto and Gaynor, 2001). Moreover, the anti-inflammatory activities of many phytochemicals appear to be mediated by their common ability to block NF-κB activation (Shah and Amini-Nik, 2017). Accordingly, we explored the potential of natural products isolated from

*M. fruticos*a to block NF-κB pathway by inhibiting IKKβ using docking simulations. Phosphorylation of IκB by IKKβ triggers the degradation of IκB and subsequent activation and migration of NF-κB to the nucleus, where it promotes the transcription of its target gene in response to the pro-inflammatory stimuli (Leung *et al.*, 2013). Using molecular docking, we demonstrated potential interactions between the isolated natural compounds and the active site of IKKβ in a manner similar to the interactions with co-crystallised inhibitor. Several important hydrogen bonding interactions with key amino acids within the kinase active site were found, e.g., hinge residues, Glu97, and Cys99 in addition to Lue21, Lys44, Asp103, and Asp166. The interacting number of chemical bonds and binding patterns of the isolated phytochemicals within IKKβ active site could be partially responsible for inhibiting the target protein and blocking NF-kB activation. Moreover, rutin was the compound with the highest HB scoring energy, as shown in table 1, which gives a clue about its potential beneficial activity, along with the other isolated compounds, in controlling inflammation in burn wounds and preventing excess scar formation, while maintaining the ability to fight infection.

Although the molecular docking simulations predicted the possible mechanism by which rutin enhances the burn wound healing process by interacting with the active site of IKKβ, quantitative analyses, such as genes and proteins expression profiles, are needed to confirm the inhibitory action of rutin on IKKβl/NF-kB signalling pathway.

CONCLUSION

The findings of this study revealed that the ethanolic extract of the aerial part of *M. fruticosa* and its isolated compound rutin exhibit potent antibacterial and burns healing activities. Potential synergism between the bioactive compounds in the plant, including flavonoid (particularly rutin) and triterpenes oleanolic acid glucoside could explain the multiple pharmacological profiles of the plant. This study is the first to examine rutin's antimicrobial and burn wound healing effect.

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