

Wound healing potential of *Rhodomyrtus tomentosa* and its bioactive compounds- rhodomyrtone

ABSTRACT

Aims: The present work was aimed to study the phytochemical composition of a crude ethanolic extract of *Rhodomyrtus tomentosa* [SERT], and the presence of rhodomyrtone and SERT's *in vitro* wound healing activity.

Introduction: *Rhodomyrtus tomentosa* is native plant to southern and southeastern Asia, India, east to southern China, Taiwan, Philippines, and south to Malaysia. In the traditional Vietnamese, Chinese and Malaysia, all its part, including leaves, roots, buds, and fruits have been used. A need for a new source of wound healing agent is the call for the investigation of the potential of *R. tomentosa* as the source of health-promoting agent, specifically as a natural wound healing agent.

Methodology: SERT was screen for its phytochemicals and the detection of rhodomyrtone using liquid chromatography–mass spectrometry, /Quadrupole time-of-flight [LC-MS/QTOF] analysis. Cell viability, cell proliferation, and migration assay were performed to examine the SERT effect's *in vitro* wound healing activity on human fibroblast cells [CRL-2522].

Results: The phytochemical study showed the presence of saponins, flavonoids, tannins and steroid in the crude ethanolic extract. The LC-MS analysis of crude ethanolic extract of SERT showed presents of rhodomyrtone which is one of the major compounds in the extract. SERT exhibit proliferative and migratory rate in human fibroblast cells [CRL 2522] in dose-dependent manner, which supports wound healing process. Its bioactive compounds presented wound healing activities at 0.325 up to 2.5 µg/mL.

Conclusion: Both SERT and rhodomyrtone portrayed *in vitro* wound healing activities. Further studies to elucidate the mechanism of action of SERT and rhodomyrtone is recommended.

Keywords: *Rhodomyrtus tomentosa*, Phytochemical Analysis, Rhodomyrtone, Migration, In vitro

1. INTRODUCTION

Rhodomyrtus tomentosa (Aiton) Hassk.) is a shrub of flowering plant and belongs to the family Myrtaceae. It originating from South- East Asia and is distributed in many countries like China, Taiwan, Philippines, Thailand, Indonesia, Vietnam, and Malaysia [1]. *R. tomentosa* was differently known according to the country. In Malaysia, it is known as “kemunting” or “karamunting”, “phruat” in Thailand, “harendong sabrang” in Indonesia and “downey rose myrtle” in most of the western countries [2]. Nearly 80% of the world’s population relies on traditional medicines for primary health care [3]. This is due to their role as a source of pharmacologically active compounds [4]. Back then, medicinal plants were used by people of ancient cultures without the knowledge of their active ingredients and this consist of phytochemicals that responsible for medicinal activity of plants which have protected human from various diseases [5]. *R. tomentosa* has often been used in traditional medicine to treat colic diarrhoea, dysentery, abscesses, haemorrhage, and gynecopathy [6]. Similar traditional usage as Thailand, in Bangladesh and Vietnam, while it is sold as an herbal supplementary product as vitamins in

America [7]. A review made by Hamid et al., stated that the tender leaves has been traditionally used to treat colic, dysentery, abscesses, sepsis and tuberculosis[8].

Phytochemicals can be categorized as primary and secondary metabolites.[9] It is well known that phytochemical compounds were responsible in delivering the medicinal properties of the plant[10]. The major constituents of phytochemical consist of carbohydrates, amino acids, proteins and chlorophylls, while, secondary metabolites generally consist of alkaloids, saponins, steroids, flavonoids and tannins [11]. Among other bioactive compounds present in the *R. tomentosa*, rhodomyrtone is a potentially great drug that offered pharmacology benefits specifically intended to promote the development and expansion of this chemical compounds for the new lead drug development [12]. Rhodomyrtone has been reported to have antimicrobial and anti-tumor activities as one of the active ingredients in *R. tomentosa* [13]. As rhodomyrtone is a potential antimicrobial drug, normal human cell toxicity has been examined and methods for detecting food safety have been developed; these studies are being examined elsewhere [14]. Ethnomedicinal activities of *R. tomentosa* proven by growing published data on its antioxidant [15][16][17], antimicrobial [18][19][20][21] and anti-inflammatory [21][22] activities.

A wound is a break in the skin, the first line of defense against infection. Wound healing is a complex and dynamic process, consisting of hemostasis, inflammation, proliferation, and maturation. Any disruption in the overlapping process resulting to failure for skin restoration. Plant with antioxidant, anti-microbial and anti-inflammatory exhibit potential for wound healing agent [23] [24]. Most of the commercially available wound care products are generally expensive, which impose a significant financial burden on the patient and the healthcare system [25]. Therefore, there is a need for alternative and cost-effective therapies in wound management. In this study, the phytochemical properties of *R. tomentosa* were analyzed, detection of rhodomyrtone were performed using LC-MS MS/QTOF and *in vitro* wound healing potential of *R. tomentosa* crude leaves ethanolic extract and rhodomyrtone been assessed.

2. MATERIAL AND METHODS

2.1 Collection and Identification of Plant materials

Rhodomyrtus tomentosa leaves were collected from Bukit Batu Putih, Negeri Sembilan, Malaysia and authenticated at the Forest Research Institute Malaysia (FRIM) with the voucher identification number of PID 050319-05. Leaves were washed with water and oven dried (40^o C) for three days [26]. Dried leaves were ground in a mechanical blender into powder and stored at -20^o C until use.

2.2 Ethanolic Extraction of *Rhodomyrtus tomentosa* leaves

For ethanolic extraction, powdered leaves of *Rhodomyrtus tomentosa* [200 g : 1000 mL] were mixed with ethanol [95%] and place on orbital shaker 100 rpm for 7 days. The mixture was filtered [Whatman No. 1, UK] and filtrate was collected and evaporated using a rotary evaporator at 40°C. Crude yield of the *Rhodomyrtus tomentosa* ethanolic extract [SERT] was calculated as follow.

$$\text{Extraction yield [\%]}: \quad [\text{Weight of extract [g]} / \text{Weight of dried plant material [g]}] \times 100$$

2.3 Preliminary Phytochemical Analysis

The sample was dissolved in various solvents and the preliminary phytochemical tests were carried out using Harborne [27]. About 10 mg of extract mixed with 5mL of ammoniacalchloroform and 2.5mL of chloroform. After filtration, the supernatant was shaken with drops of 0.5M sulfuric acid. The appearance of a creamy precipitate indicated the presence of alkaloids. 10mg extract vigorously shaken with 1mL ethyl ether and 3mL 2N hydrochloride solution (HCl). Precipitate formation indicated the presence of saponins. 5 mg of extract was dissolved in 10 mL of 70 percent ethanol. The sample was then diluted 1: 2 (v/v) with sterile distilled water. Following that, three drops of a 10% (w/v) ferric chloride solution were added. The presence of tannins was indicated by the presence of a blue to black precipitate. About 5mg of the extract was dissolved in 5mL of absolute ethanol and treated with a few drops of concentrated HCl and 0.2 g of magnesium ribbon. The occurrence of a pink-red color was indicative of the flavonoids. Steroids and terpenoids were detected using the Liebermann-Burchard reaction. A solution containing 5mg of extract dissolved in chloroform was

filtered. The filtrate (2 mL) was added to 2mL of acetic anhydride and 50% concentrated sulfuric acid. A blue-green ring indicated the presence of steroids while a red color indicated the presence of terpenoids.

2.4 Standardize Compound of SERT

The detection of rhodomyrtone as was as described by Rofiee et al.,[28]Rhodomyrtone in the SERT were detected using LC-MS-QTOF. About 1 mg/mL of *Rhodomyrtus tomentosa* ethanolic extract were reconstituted in the mobile phase. The sample was injected into LC-MS/QTOF [model 6520 Agilent Technologies, SA, USA] using a ZORBAX Eclipse Plus C18 column [100 mm x 2.1 mm x 1.8 μ m, Agilent Technologies, SA, USA] that was maintained at 40°C. The flow rate was 0.25 mL/min with a linear gradient of solvent A [water containing 0.1 percent formic acid] and solvent B [acetonitrile containing 0.1 percent formic acid] over a 36-minute period from 5% to 95% of mobile phase [B].Each analysis took a total of 48 minutes to complete. The electrospray ionisation [ESI] source was set to 4000 V Cap, 65 V skimmer, and 125 V fragmentor. The nitrogen nebulizer was set to 45 psig and the nitrogen drying gas flow rate was set to 12 L/min. The temperature of the drying gas was maintained at 35°C. The data collection range for positive ESI mode was 100–1000 m/z. Two reference masses, 121.0509 m/z [C₅H₄N₄] and 922.0098 m/z [C₁₈H₁₈O₆N₃P₃F₂₄], were continuously injected to monitor and ensure accurate mass detection of the compounds. The MS data were processed and analysed using Agilent Mass Hunter Qualitative Analysis B.05.00 software [Agilent Technologies, Santa Clara, CA, USA]. Rhodomyrtone's chromatographic profiles were analysed using the precise mass data identified.

2.5 Cell Culture Maintenance.

Human fibroblast cells [HDF] acquired from American Type Culture Collection [BJ, ATCC® CRL -2522™] Manassas, VA, USA. The cell line was thawed and well maintained. Cells were cultured and maintained in high glucose Dulbecco's Modified Eagle Medium [DMEM] premixed with 10% fetal bovine serum and antibiotics [streptomycin 100 μ g/mL and penicillin 100 U/mL] in a humidified 5% CO₂ incubator at 37°C [29]

2.6 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl Tetrazolium Bromide [MTT] Assays.

HDF cells were seeded into 96-well plate at a density of 1×10^4 [in 100 μ L of DMEM medium] per well and grown for 24hrs.The medium was replaced with different concentrations of SERT of 15.62, 31.25, 62.5, 125, 250, and 500 μ g/mL, and the plates were incubated for 24 h. 100 μ L of 5mg/mL MTT reagent was then added to each of the wells and incubated for another 4h. The purple formazan formed was solubilized by adding 100 μ L dimethyl sulfoxide to all the wells including control [without any treatment] and then swirled gently to mix well on agitator for 10 min. Microplate reader was used to read absorbance at 570nm [30]. The percentage cell viability [CV] was calculated as follow:

$$CV[\%] = [\text{Absorbance of test sample} / \text{Absorbance of control}] \times 100 \quad [30]$$

Accordingly, graph of percentage of cell viability against concentrations was plotted. Experiments were performed in triplicate and the data were presented as mean \pm SD [$n = 3$].

2.7 Migration Assay

The migration capabilities of HDF cells were assessed using scratch wound assay, which measures the expansion of a cell population on surfaces as described by Fronza et al. [31]. The cells were seeded into 12-well tissue culture dishes in DMEM containing 10% FBS [fetal bovine serum] and 1% penicillin and streptomycin. After the cells had formed a confluent cell monolayer, a linear wound was generated in the monolayer using a sterile 100 μ L plastic pipette tip. Any cellular debris was removed by washing the wells with phosphate buffer saline [PBS]. The medium [DMEM] used consisted of 30 μ g/mL allantoin [as positive control], SERT [15.62, 31.25, 62.5 μ g/mL] and rhodomyrtone [0.625 μ g/mL, 1.25 μ g/mL, 2.5 μ g/mL and 10 μ g/mL]. The cells were then incubated for 24 h at 37 °C with 5% CO₂. The scratched cell layers incubated under the different conditions were then photographed to estimate relative cell migration. The data was analyzed using IMAGE J and the experiment was performed in triplicate. Allantoin was used as a positive control and help for wound healing, remover of necrotic tissue and promoter of epithelial stimulation and it has been used in pharmaceutical preparations for more than 70 years [32]. The rate of migration refers to speed of cell migration within certain time duration[33].

2.8 Statistical Analysis.

The result was expressed as a mean \pm SD, and the significance of results was analyzed using GraphPad Prism software [GraphPad Prism 8, USA]. Results obtained were compared with control and treated groups using One way ANOVA. Differences between the groups were considered as statistically significant at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus control group.

3. RESULTS AND DISCUSSIONS

3.1 Phytochemicals Screening of SERT

The secondary metabolites screening showed the presence of saponins, flavonoids, tannins, phenolics and steroids in the crude ethanolic extract of *Rhodomyrtus tomentosa* [Table 1]. However, terpenoids and alkaloids were not detected in the extracts. The phytochemical analysis is not in agreement with previous study that showed present of terpenoid but absent of flavonoid in the ethanol extract of *Rhodomyrtus tomentosa* [5]. This can be justified by different type of solvent extraction and method extraction. In previous study, present of saponin from ginseng able to accelerate neurovascularization [34], while flavanoids have been proven to enhance synthesis of collagen and increase rate of wound contraction [35][36]. While tannins and phenolics contribute to action as anti-inflammatory, antioxidant and been claimed responsible in enhancing wound healing [37].

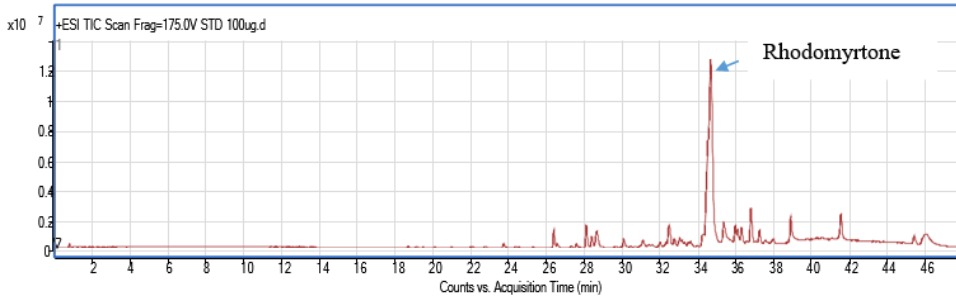
Table 1. Secondary metabolites of SERT extract.

Test		
Saponins	2+ (white foam)	Where, -: indicates Absence, 1+: weak colour, 2+ mild colour
Flavonoids	2+ (pink red)	
Terp)enoids	-	
Steroids	1+(blue green ring)	
Tannins	2+ (blue black)	
Alkaloids	-	
Phenolics	2+(blue black)	

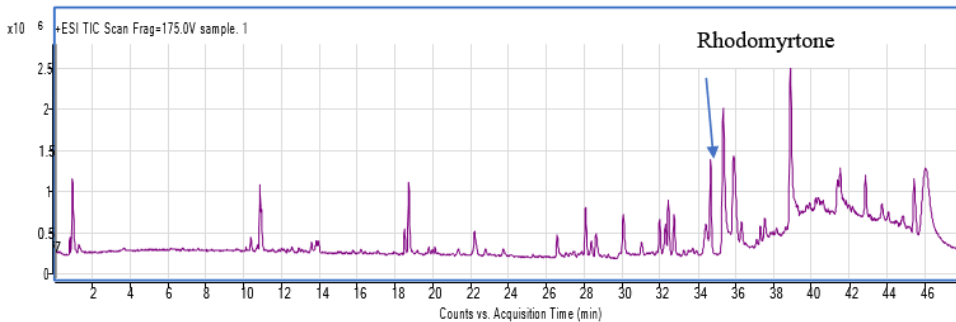
3.2 Detection of Rhodomyrtone in SERT

The total ion chromatograms [TICs] of SERT and rhodomyrtone standard in positive ion mode are shown in Figure 1.0. Rhodomyrtone is one of the major compounds identified with highest peak which belonged to acylphloroglucinols group. The extracted ion chromatograms [EICs] $M/Z = 443.5$ [calculated formula $C_{26}H_{34}O_6$] were confirm by comparing the EICs of the standard and based on the accurate mass data identified. Rhodomyrtone first reported by [38] and in 2013, by HPLC technique liposomal encapsulated rhodomyrtone were established for anti-acne drug [39]. In the same year, the researcher developed a concise synthesis of the rhodomyrtone and isomeric acylphloroglucinols rhodomyrtosone B which displayed antibacterial activity [40]. Further research on rhodomyrtone proved the antibacterial properties and clinical management in Acne Vulgaris [41]. Recently, it was discovered that rhodomyrtone did not exhibit any toxicity in invertebrate or vertebrate models [42].

Total ion chromatogram (TIC) of the Standard



Total ion chromatogram (TIC) of the sample



Extracted ion chromatogram (EIC) of Rhodomyrton (M/Z = 443.5) from the sample.

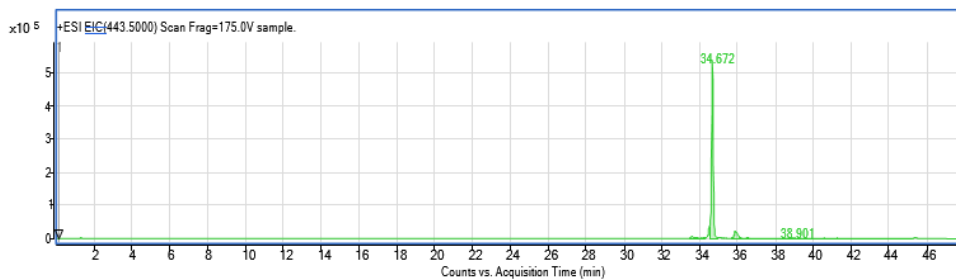


Fig. 1. - LC/MS Q-TOF total ion chromatogram [TIC] of SERT and rhodomyrton
3.3 Cell viability of SERT and rhodomyrton

Cell viability assay was performed to determine non toxic concentration of the SERT to be used in the proliferation and migration assay. Based on Figure 2, concentration that showed the highest percentage of cell viability within the range were selected for the migration assay were 15.62, 31.25, 61.5 and 125 $\mu\text{g}/\text{mL}$. While for the rhodomyrton standard from the cell viability results portray the best range were 0.32, 0.62, 1.25 and 2.5 $\mu\text{g}/\text{mL}$. In any study, utilization of medicinal plants require a basic screening to know the safe and non toxic dose before any experiment been carried out [43]. Based on above results, the non toxic dose for SERT was from 3.90 $\mu\text{g}/\text{mL}$ up to 250 $\mu\text{g}/\text{mL}$, on the other hand, rhodomyrton non toxic dose ranging from 0.325 $\mu\text{g}/\text{mL}$ to 2.5 $\mu\text{g}/\text{mL}$. In another study, cell viability of *T. ferdinandiana* fruits and seedcoats extract of intestinal and hepatic cells range was higher than this study, which were between 3650 and 14400 $\mu\text{g}/\text{mL}$ and its abundant compound ellagic acid varied from 1190 to 2390 $\mu\text{g}/\text{mL}$ [44]. **The divergent findings could be explained by the cell line used, the extraction method used, or the plant part tested.**

A

B

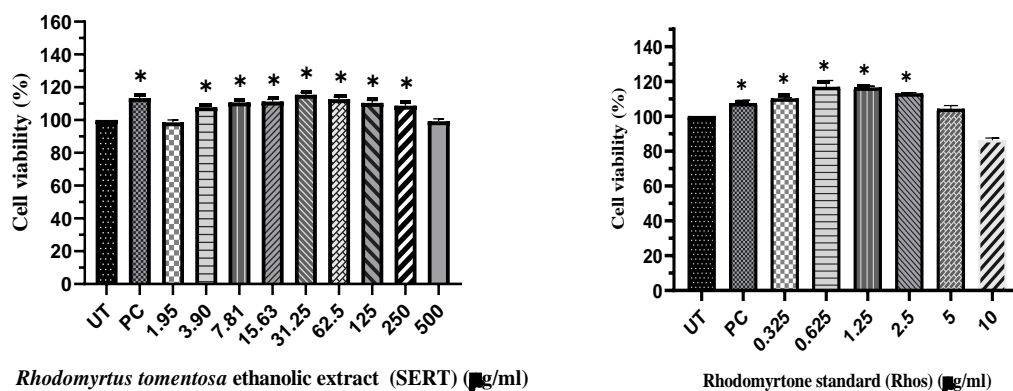


Fig. 2.. Cell viability [%] of human dermal fibroblast [CRL-2522] treated A) with *Rhodomyrtus tomentosa* ethanolic extract at [1.95, 3.90, 7.81, 15.62, 31.25, 62.50, 125, 250, 500 µg/mL] and allantoin 30 µg/mL as positive control [PC] for 24h at 37°C, and B) with rhodomyrtone standard [0.325, 0.625, 1.25, 2.5, 5, 10 µg/mL] and allantoin 30 µg/mL as positive control [PC] for 24h at 37°C. *Significant differences from untreated, as normal control at P < 0.001. Bars represent the mean ± S.E.M. of three experiments.

3.4 Cell proliferation of SERT and rhodomyrtone

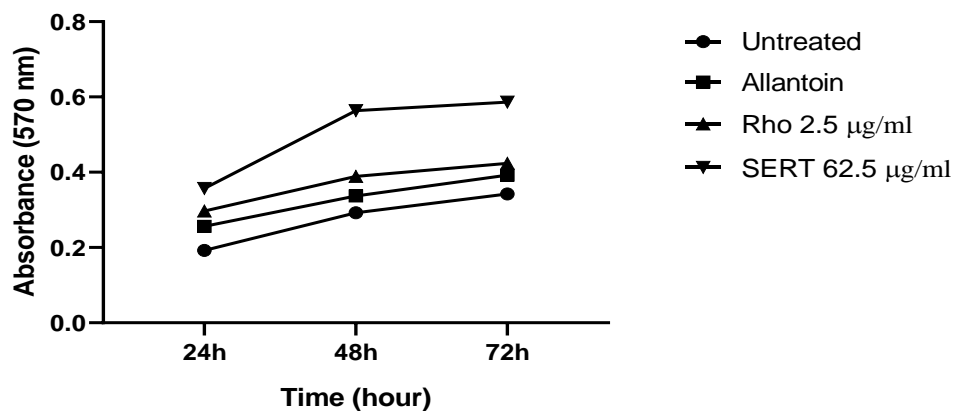


Fig. 3. – Cell proliferation of human dermal fibroblast [CRL-2522] treated with 62.5 µg/mL of *Rhodomyrtus tomentosa* ethanolic extract, 2.5 µg/mL of rhodomyrtone standard and allantoin 30 µg/mL as positive control [PC] for 24, 48 and 72h at 37°C.

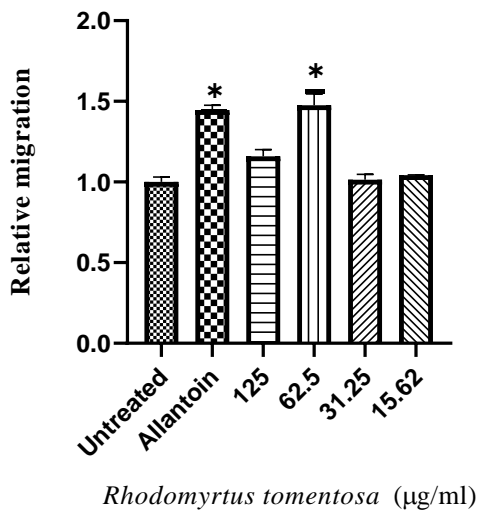
Cell proliferation was performed utilising the Microculture Tetrazolium Test [MTT] assay, where the reduction of tetrazolium salts represents the number of viable cells. Based on Figure 3 the graphical representation of cell proliferation after treatments with 62.5 µg/mL SERT in comparison with positive control [allantoin 30 µg/mL] at 24, 48 and 72h. Rhodomyrtone standard showed similar pattern with SERT to increase cell proliferation after treatment. Only one representative for the best concentration that showed increased cell proliferation was chosen for both SERT and rhodomyrtone standard. Ability of cell to proliferate play a role whereby, it shows the bioactive compounds in the SERT and the specific compound responsible to accelerate the wound healing process. Obtained results were in contrast with finding by Chorachoo et al.,[45] that showed anti proliferative effect at 2–32 µg/mL of rhodomyrtone. A possible explanation is that the cells used in their study were keratinocytes, whereas the cells used in this study were fibroblasts. In another study, human fibroblast cell proliferation significantly increased when treated with *Scrophularia striata* at 10, 50 and 100 µg/mL after 48h and with at 50, 100 and 200 µg/ml after 72h [46].

3.5 Migration assay of SERT and rhodomyrtone

Normally, the cells migrate towards empty spaces. Study by Chen claimed that the rate of migration is not significantly affected by the cell confluency [47]. The results of the migration test indicate that the standardized extract of *R. tomentosa* increased the rate of cell migration compared to untreated control but similar rate as allantoin. An increase in the rate of migration with increasing time may conclude that the treatment contains a component that indirectly influences cell

migration. A higher rate of cell migration during the first hours of treatment that decreases with time may be due to the presence of a compound that directly influences the rate of cell migration [3].

A.



B.

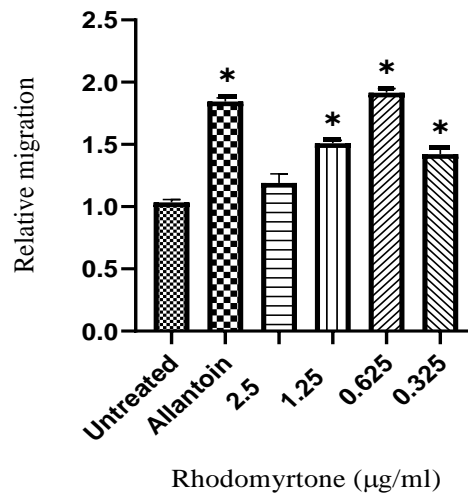
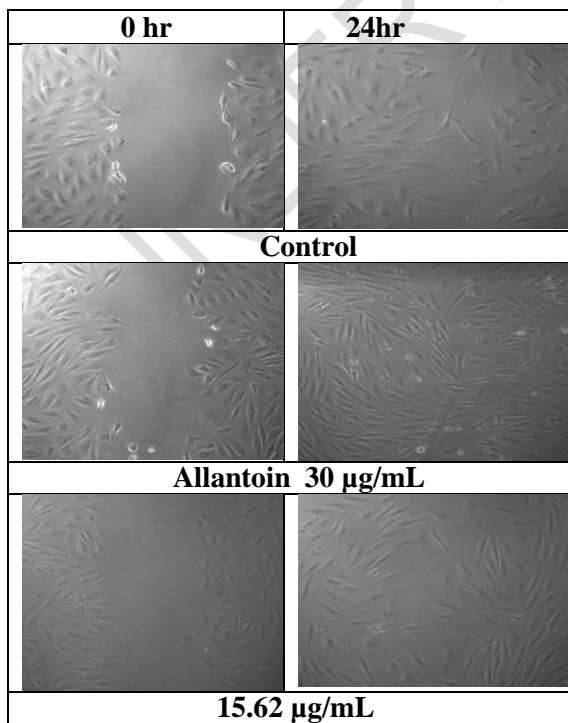
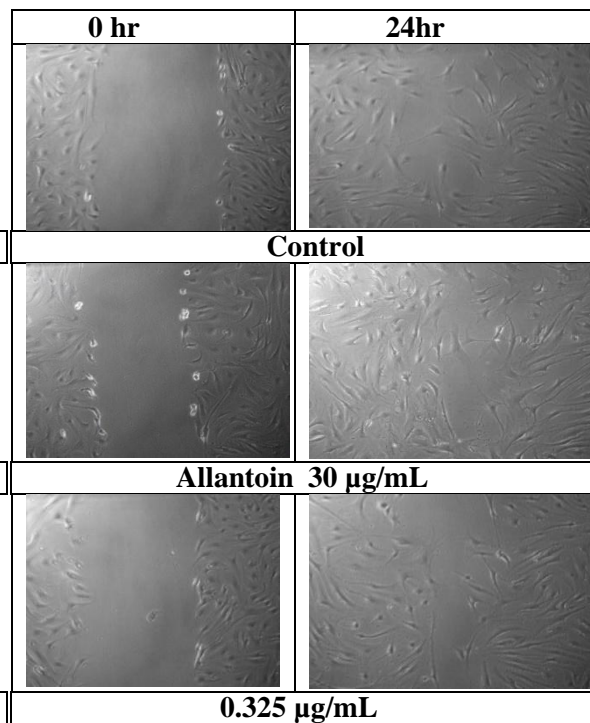


Fig.4. Relative migration of human dermal fibroblast [CRL-2522] treated A) with *Rhodomirtus tomentosa* extract at [15.62, 31.25, 62.50, 125 $\mu\text{g}/\text{mL}$] and allantoin 30 $\mu\text{g}/\text{mL}$ as positive control [PC] for 24h at 37 $^{\circ}\text{C}$, and B with rhodomirtone standard [0.325, 0.625, 1.25, 2.5, 5, 10 $\mu\text{g}/\text{mL}$] and allantoin 30 $\mu\text{g}/\text{mL}$ as positive control [PC] for 24h at 37 $^{\circ}\text{C}$. **Significant differences from untreated, as normal control at $P < 0.001$. Bars represent the mean \pm S.E.M. of three experiments.

A



B



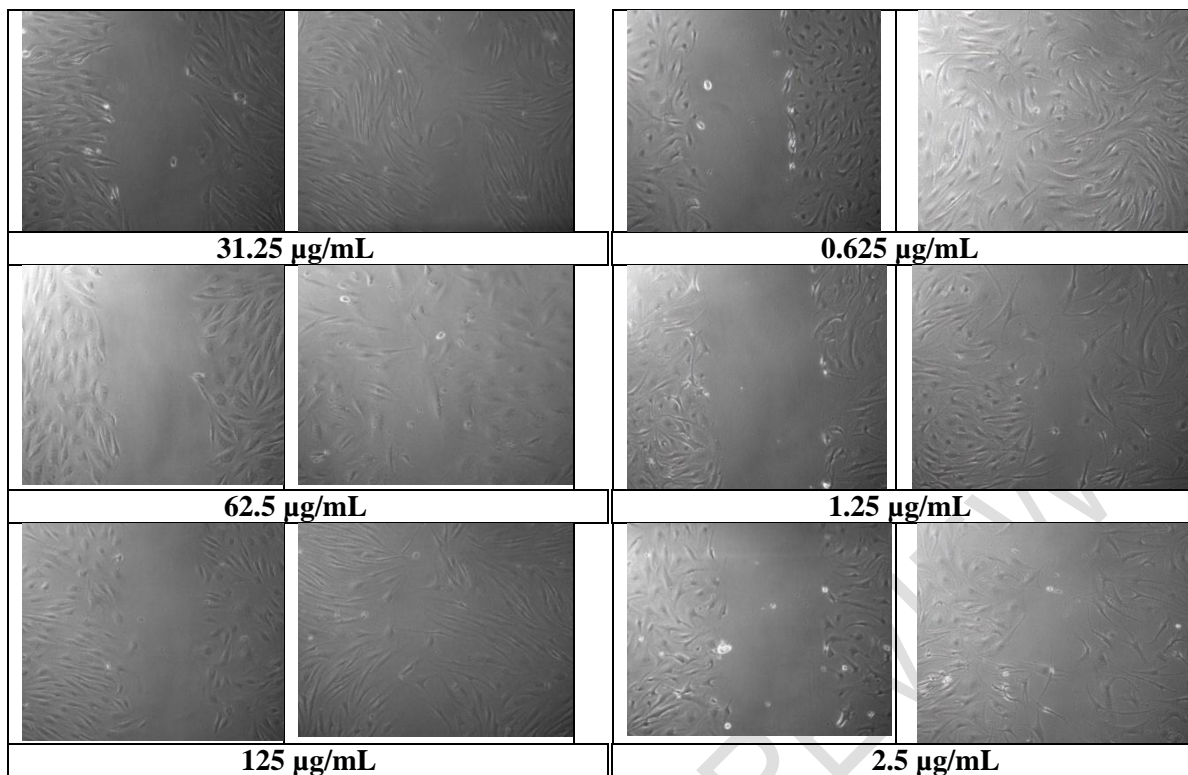


Fig.5. Images of the scratch area of human dermal fibroblast [CRL-2522] treated, A) with *Rhodomyrtus tomentosa* extract at [15.62, 31.25, 62.50, 125 µg/mL] and allantoin 30 µg/mL as positive control [PC] at 0h and 24h at 37°C, and B) with rhodomyrtone standard [0.325, 0.625, 1.25, 2.5, 5, 10 µg/mL] and allantoin 30 µg/mL as positive control [PC] at 0h and 24h at 37°C.

4. CONCLUSION

Both the crude ethanolic extract of *Rhodomyrtus tomentosa* [SERT] and rhodomyrtone were found to be capable of promoting wound healing via stimulation of human dermal fibroblasts. The mechanism of action of SERT in wound healing is unclear and further studies are needed to elucidate the mechanism of action of SERT and rhodomyrtone in wound healing process.

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