RESEARCH PAPER

Mechanistic Insights into the Wound Healing Properties of Guaianolide Sesquiterpenoid isolated from the plant, *Eupatorium glandulosum*: Cell migration, *in silico* docking and qPCR analysis

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ABSTRACT

Introduction: The leaf juice of *Eupatorium glandulosum* H.B & K, has traditionally been used to treat cuts and wounds by the tribal communities in the Nilgiris district, Tamilnadu, India. The current research focuses on to the evaluation of the wound healing potential of the fresh and dry plant methanolic extracts, the bioactive compound, Guaianolide sesquiterpenoid, isolated from the ethyl acetate fraction of the methanolic extract and the mechanistic insights into the wound healing properties of the bioactive compound.

Materials and methods: The study reports on the viability and the migratory potential of the fresh and dry plant methanolic extracts on mouse NIH3T3 fibroblast and the compound, Guaianolide sesquiterpenoid on human HaCaT keratinocytes cell lines. Guaianolide sesquiterpenoid was further evaluated for *in silico* docking and qPCR analysis to obtain insights into the mechanism of wound healing properties.

Results: The extracts show significant viability. At the concentration of 250, 125 and 62.5 µg/ml, the fresh plant methanolic extracts show a wound closure of 98.97, 92.61% and 85.64% at 12h, respectively and 100.00% in all the concentrations at 48h. Guaianolide sesquiterpenoid, isolated from the ethyl acetate fraction of the fresh plant methanolic extract, shows a wound closure of 99% at 24h. Molecular docking studies reveal that the bioactive compound interacts with the wound healing targets, TNF-α, IL-12, IL-18, GM-CSF, MMP-2, MMP-9 and IL-1β and shows an affinity score of -8.8, -5.9, -9, -6.9, -7.2, -7.5, -6.4 kcal/mole, respectively. Based on its high binding affinity to IL-18, TNF-α and MMP-9, these three target proteins were evaluated for gene expression analysis.

Discussion: The fresh plant methanolic extracts show significant wound closure and the compound, Guaianolide sesquiterpenoid, isolated from the extract shows significant binding affinity towards TNF-α, IL-18 and MMP-9. The qPCR analysis reveal early cytokine expressions.

Conclusion: Guaianolide sesquiterpenoid shows significant migratory potential and hence a possible drug/lead.

Keywords: *Eupatorium glandulosum,* migration, Guaianolide sesquiterpenoid, molecular docking, qPCR.

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1. INTRODUCTION

Wound healing is an intricate, dynamic and multi-phased process of regeneration of the broken tissues [1]. It involves highly programmed overlapping phases, namely hemostasis, inflammation, proliferation and remodelling which must take place in a proper sequence at a specific time and in a regulated manner [2, 3]. Any interruption, aberrance, or prolongation in these four phases leads to problems. Today wounds are treated using antibiotics systemically or antiseptics topically. These treatments only keeps the wound area sterile

to prevent further infection but do not actively participate in the process of physiological and molecular repair processes [4 & 5].

In tissue regeneration, macrophages, keratinocytes, fibroblasts and other immune cells in the skin are activated to initiate the process. Generally, fibroblasts help in converting epithelial cells to mesenchymal cells, migration and collagen deposition. Progression of repair mechanism mainly depends on the migration of fibroblasts [6]. The crude extracts of the Indian traditional plant, *Ficus trijuja* [7], Iranian traditional plant, *Punica granatum* [8], Ethiopian traditional plant, *Vernonia auriculifera* [9] and the Asian traditional plant, *Calotropis procera* [10] have been scientifically reported for its regeneration property. Traditionally, only the crude extracts are evaluated for tissue regeneration [11]. The bioactive compounds present in the plant extracts are, therefore, hopeful alternates for wound healing. Plants based bioactive compounds form the basis for several drugs currently in use.

The plant, *Eupatorium glandulosum* (Syn- *Ageratina adenophora*)**,** is a perennial plant and grows up to 2 m tall [12]. Traditionally, the juice of the plant has been used by the Badagar, Irular and Toda tribes of the Nilgiris, Tamilnadu, India, in the treatment of cuts and wounds [13]. A wide variety of phytochemicals isolated from this plant have shown an impressive range of medicinal uses [14]. The current investigation focuses on the *in vitro*, *in silico* and mRNA expression analysis of the wound healing targets, IL-18, TNF-α and MMP-9, to access the regeneration property of the bioactive compound, Guaianolide sesquiterpenoid, isolated from *Eupatorium glandulosum.*

2. MATERIALS AND METHODS

2.2. Plant extraction and isolation

The plant was collected from the Nilgiris district, TamilNadu, India, identified at Government Arts College, Udhagamandalam and deposited at JSS College of Pharmacy, Ooty with the specimen No JSSCPO/15/20-21.

The dry (1kg) and the fresh plant (2kg) were extracted by the maceration process using methanol (7000 ml) for 3 days with regular shaking. The plant extracts filtered and evaporated at 40^0 C under reduced pressure resulted with dry weight of 56g and 83.5g, respectively.

The fresh plant methanolic extract concentrate (80g) was dissolved in distilled water. The solution was successively fractionated using a series of solvents, namely petroleum ether, chloroform, ethyl acetate, and methanol using a separating funnel [13]. The residue of the ethyl acetate fraction was taken in a column and eluted using ethanol: benzene (10:90 to 100:0) to obtain Fr. B1fractions [1].

The Fr. B1was further fractionated using methanol: water in the ratio of 70:30. The two peaks, Fr. B1B and Fr. B1A obtained with the retention time of 5.362 and 3.246 min, respectively were collected one by one from the HPLC outlet (Fig. 1). On analysis a single spot was observed on the preparative TLC plate for fraction the Fr. B1B. The spot was removed and dissolved in ethanol and filtered to yield a compound with Rf value of 0.94 [1]. The yield was 22.2mg.

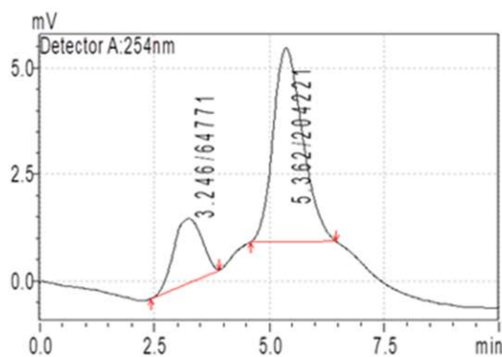

Fig.1. HPLC Analysis of Fr. B1 [1]

2.2. Preliminary phytochemical evaluation

The fresh and dry plant methanolic extracts were subjected to phytochemical analysis [1].

2.3. *In vitro* cytotoxicity assay by SRB

The viability of the two extracts (fresh and dry plant methanolic extracts) and the compound, Guaianolide sesquiterpenoid were evaluated on Mouse NIH3T3 fibroblasts and Human HaCaT

keratinocyte cell lines using the colorimetric assay, Sulforhodamide B (SRB) [15]. NIH3T3 mouse fibroblast and HaCaT human keratinocytes were provided by Dr. Subba Rao V Madhunapantula, Dept of Biochemistry, JSS Medical College, Mysuru. Initially, $1*10^4$ Mouse NIH3T3 fibroblasts cells and Human HaCaT keratinocyte cells were added to 200µL of DMEM present in each well and incubated at 37^0 C with 5% CO_2 until the cells reached 60-70% confluency. The cells were then treated with 7.81, 15.625, 31.25, 62.5, 125, 250, and 500µg/ml of the plant extracts of various concentrations (100, 200,400, 800, 1600 and 3200µM) of the bioactive compound and incubated for 24h. DMSO and Cisplatin (100µM) were used as control and standard group, respectively. After 24h, trichloroacetic acid (50µl of 50%) was slowly dispensed into well with medium and the plate was incubated for 1h at 4^0 C. The plate was washed with tap water and dried to remove the excessive water. SRB (100 µl) was added to each well and incubated for 30 min. Acetic acid (1%) was added to each well and dried. Finally, 100 µl (10 mM) of tris base was added and the absorbance level was measured at 510 nm. The % cell viability was calculated using the following formula,

"Cell viability (%) = Absorbance of the test sample/absorbance of the control*100".

2.4. Wound scratch test assay

The migratory potential of the plant extracts and Guaianolide sesquiterpenoid on Mouse NIH 3T3 fibroblasts and Human HaCaT keratinocyte cell lines were evaluated by scratch assay [1]. $1* 10^5$ of Mouse NIH3T3 fibroblasts cells/well and $0.5* 10^6$ of HaCaT cells/well were kept for incubation at 37^0 C for 24 h. After reaching 80% monolayer, a 0.7mm width scratch was created using10µl sterile pipette. The monolayer was washed with phosphate-buffered saline to remove the debris. The fresh and dry plant methanolic extract and the Guaianolide sesquiterpenoid were serially diluted with the medium. The extracts at 62.5, 125 and 250 µg/ml concentration and the bioactive compound at 800, 1600 and 3200 µM were kept for incubation at 37^0 C with 5% carbon dioxide for 12 h. DMSO and Itraconazole (5µg/ml) were used as control group and the standard, respectively. Photomicrographs were recorded at 0, 24 and 48h to analyse the migration of fibroblasts. The artificial scratch area was calculated using imageJ. The following formula was used to calculate the wound closure,

"% Wound closure=Wound area at initial time-Wound at n time/Wound area at initial time*100".

2.5. Molecular docking studies

Three dimensional structures of the wound healing targets, TNF-α (PDB ID-2AZ5), IL-12 (PDB ID-6WDP), IL-18 (PDB-4R6U), GM-CSF (PDB ID-5D71), MMP-2 (PDB ID-1HOV) MMP-9 (PDB ID-4H1Q) and IL-1β (PDB ID-4GAF), were downloaded from the Protein Data Bank (PDB: http:// www. rcsb. org/ pdb) [1]. The co-crystals, heteroatoms and water molecules were removed from the target proteins, the missing residues were added using PyRx (intergrated with Autodock) and Discovery Studio Visualizer was used to perform docking. Energy was minimized by using Swiss-PDB Viewer force field. The derived target proteins were stored as PDBQT files.

The structure of Guaianolide sesquiterpenoid was drawn in MarvinSketch© software and converted to PDBQT module of PyRx version 0.9. The interaction between the compound with various targets was evaluated utilizing PyRx version 0.9. The bioactive compound was docked against the various targets using Autodock vina programme [16].

2.6. Prediction of ADMET properties

The physicochemical properties and toxicity property of Guaianolide sesquiterpenoid were analysed using http://www.swiss adme. ch/ and https:preadmit.webservice.bmdrc.org/toxicity/ server, respectively [17].

2.7. Quantitative-polymerase chain reaction (qPCR)

TNF-α, IL-18, and MMP-9 mRNA expression on human HaCaT keratinocyte cell lines were measured using real-time qPCR. The primers of wound healing targets, IL-18, TNF-α and MMP-9 and the reference gene β-actin are shown in Table 1.

Table 1 Reverse and Forward primer sequences for qPCR analysis [1]

S.No	Target	Primer Sequence
1	IL-18	Forward primer CAGTCAGCAAGGAATTGTCTC Reverse primer GAGGAAGCGATCTGGAAGG

2	TNF-α	Forward primer CCTTCCTGATCGTGGCAG Reverse primer GCTTGAGGGTTTGCTACAAC
3	MMP-9	Forward primer GCCACTACTGTGCCTTTGAGTC Reverse primer CCCTCAGAGAATCGCCAGTACT
4	β-actin	Forward primer TGGTGGTACCACCATGTACC Reverse primer AGGGGCCGGACTCATCGTACT

The amplification of the DNA in real-time PCR was analysed using DyNAmo Flash SYBR Green QPCR Kit 500 Reaction. The complementary DNA was synthesized using the Verso cDNA Synthesis Kit. The HaCaT cell lines were treated with concentrations of 800, 1600, and 3200 µM of Guaianolide sesquiterpenoid. The total RNA from HaCaT cells were extracted at different time points (1, 8, and 24 h) utilizing the Trizol method and quantified using NanoDrop spectrophotometer, Denovix. The complementary DNA was synthesised by reverse transcriptase enzyme using 50ng of RNA as template strand. cDNA synthesis was carried out using the Takara Prime Script 1st strand cDNA synthesis kit. cDNA (50ng) was added with 10pmol each of reverse and forward primer to make the final volume to 20µl. Initially denaturation was performed at 95 °C for 10min followed by 40 cycles at 95° C for 30sec. The forward and reverse primer annealing of TNF-α, IL-18, and MMP-9 primers were performed at temperature of 60°C, 60°C and 66°C, respectively. The expression levels of TNF-α, IL-18, and MMP-9 mRNA were measured using Qiagen machine. To ensure accuracy, gene expression was normalized to β-actin mRNA, serving as the internal reference. During the elongation of each PCR cycle fluorescence signals were analysed and the melting curve was created. The relative gene expression levels were determined using the $2^{\wedge-\Delta\Delta Ct}$ method [18]. The average Ct values were calculated by averaging the Ct values of replicates of each sample.

The delta Ct (ΔCt) values were calculated using the formula,

"ΔCt = Ct (gene of interest) – Ct (housekeeping gene)"

The delta-delta Ct (ΔΔCt) values were calculated using the formula,

"ΔΔCt = ΔCt (Sample) – ΔCt (Control average)"

The fold gene expression was calculated using the formula,

"Fold gene expression = 2^-(ΔΔCt)"

2.8. Statistical Analysis

The *in vitro* studies were carried out in triplicate and the results presented in mean±standard error mean. All the statistical calculations were made using the Graph pad prism version 9. A *P-value* lesser than 0.05 was considered as significant.

3. RESULTS AND DISCUSSION

3.1. Results

The leaf juice of the plant, *Eupatorium glandulosum,* has been utilised to treat wounds by the communities of the Nilgiri district, Tamilnadu, India [13]. Crude extracts of this plant have been reported for *in vivo* tissue repairing [19, 20 & 21]. The traditional reports, however, have not been studied by *in vitro* biological assays related to wound healing activity. In addition, the bioactive compound responsible for the activity also needs to be isolated and evaluated.

The present study is focussed on the migratory potential of the dry and fresh plant methanolic extracts and the bioactive compound, Guaianolide sesquiterpenoid, in addition to the molecular docking approaches and the mRNA expression of the wound healing targets, IL-18, TNF-α and MMP-9 to obtain mechanistic insights into the wound healing potential of Guaianolide sesquiterpenoid.

3.1. Plant extraction and yield

The aerial parts of the dry plant and fresh plant were successively extracted using methanol. The yield of the dry plant methanolic extracts was 5.6% and the fresh plant methanolic extract was 4.1%.

3.2. Preliminary phytochemical evaluation

The dry and fresh plant methanolic extracts were evaluated for phytochemical analysis. The extracts showed the presence of alkaloids, carbohydrates,

glycosides, flavonoids, tannins, triterpenoids along with fats and fixed oils (Table.2).

Table 2: Qualitative phytochemical analysis of dry and fresh plant methanolic extracts

S.No	Tests	Dry plant Methanolic Extract	Fresh plant Methanolic Extract
1	Alkaloids		
	a. Dragendorff's test	+	+
	b. Wagner's test	+	+
	c. Mayer's test	+	+
	d. Hager's test	+	+
2	Carbohydrates		
	a. Molisch test	++	++
	b. Fehlings test	++	++
	c. Salkowski test	+	+
	d. Iodine test	++	+
3	Glycosides		
	a. Legal's test	+	+
	b. Borntrager's test	++	+
4	Flavonoids		
	a. Ferric chloride test	+++	++
	b. Shinoda test	+	+
5	Tannins and Phenolic Compounds		
	Ferric chloride test	+++	+++
	Braymer's test	++	++
6	Triterpenoids	+	++
7	Saponin test	-	-
8	Fats and Fixed oils		
	a. Spot test	++	+++
	b. Saponification	-	-

Based on the characterization, the molecular weight and the molecular formula of the isolated compound (reddish amorphous powder) was determined as a 360.41 (m/z 359 $[M - H]^{+}$) and $C_{20} H_{24} O_6$, respectively. The IR pattern exhibits absorbance at 2943.8, 1219.16, 3313.11 and 1419.42, indicating C-H stretching (alkyl), C-O, OH and C=O, respectively. ^{1}H-NMR spectrum (400MHz, $CDCL_3$) reveals a single proton singlet at δH 4.8 and 7.2 indicating the presence of OH and CH groups, respectively. The six proton singlets and the three proton singlets at δH 1.8 and 2.3 indicate the presence of methyl group and two proton singlet δH 3.8 indicate the presence of methylene group. Three proton doublet and single proton doublet at δH 2.2 and 3.2 reveal the presence of methyl and methylene, respectively. Single proton quartet at δH 3.8, 7.1 and 3.4 reveals the presence of CH group at C_{10}, C_{11} and C_{17}, respectively. The two proton triplet at δH 4.3 reveals the presence of methylene group at C_8. Single proton multiplet at δH 4.1 and 7.5 indicate the presence of CH group at C_{13} and C_9, respectively. ^{13}C-NMR spectrum (126 MHz, DMSO) signals at δC 181.86 shows the presence of carbon in ketone carbon and signals at 173.58 and 168.50 reveal the presence of carbon in ester carbonyl in the lactone ring and α, β-unsaturated ester carbonyl, respectively. The peak at 156.63 indicates the presence of conjugated double bond at in the carbonyl group and signals at 148.92 and 148.39 indicate the presence of in δC in the aromatic ring. Peaks at 78.62, 56.72 and 31.13 indicate the presence of oxygenated carbon, methoxy carbon (O-CH) and aliphatic methylene carbon, respectively. Signals at 20.90, 18.37 and 14.63 indicate the presence carbon in methyl groups ($-CH_3$) in the

aliphatic region. LC-MS/MS analysis revealed that the exact mass of the bioactive compound is 360.41; LCMS/MS (negative mode) m/z 359; MS/MS fragments: 360.41/320.17/161.17/110.1. The compound was identified as Guaianolide sesquiterpenoid [22] (Fig. 2a & b).

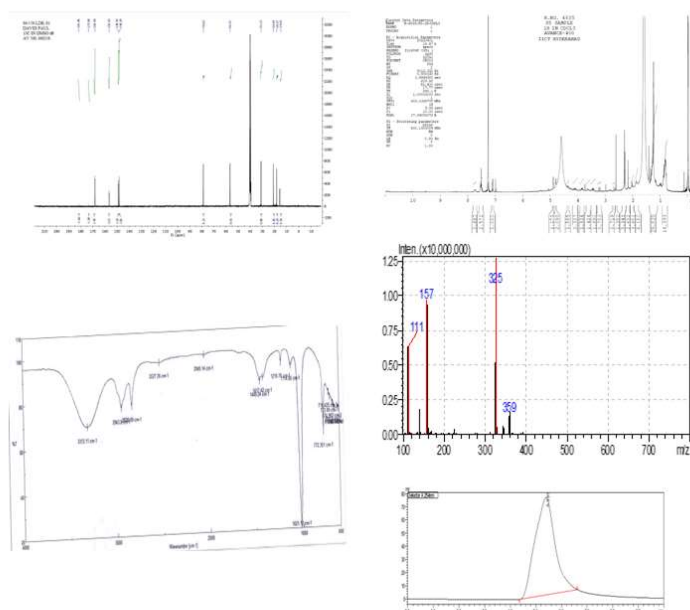

Fig.2a. $_{13}$C-NMR, $_{1}$H-NMR, FTIR, LC-MS/MS and HPLC of Guaianolide sesquiterpenoid

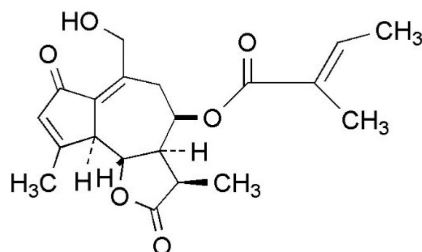

Fig.2b. Structure of Guaianolide sesquiterpenoid

3.3. *In vitro* cytotoxicity assay by SRB

In vitro studies of the extracts, evaluated in NIH3T3 cell lines, are presented in Table 3 and Fig. 3. The results reveal that the extracts show greater cell viability at lower concentrations. The cytotoxicity of the dry plant extracts is lower than the fresh plant extracts. The results thus indicate that higher extract concentrations should be avoided to treat wounds.

In vitro studies of Guaianolide sesquiterpenoid, evaluated in HaCaT keratinocyte cells, are shown in Table 4 and Fig. 4. The compound shows significant cell viability. Lower extract concentrations of the compound show more number of viable cells compared to higher concentrations. The compound shows 90 to 100% viability in all the concentrations.

Table 3: % cell viability of dry and fresh plant methanolic extract in fibroblast cell lines

S.No	Concentrations (µg/ml)	Percentage cell viability	
		Dry plant methanol extract	Fresh plant methanol extract
1	Cisplatin	26.45±1.41	26.45±1.41
2	7.810	104.76±5.03	95.46±4.46
3	15.62	98.30±2.18	86.81±2.36
4	31.25	93.81±6.65	84.96±3.38
5	62.50	90.65±5.31	80.46±3.39

6	125	84.15±5.30	70.47±4.16
7	250	72.24±1.59	64.12±2.06
8	500	59.97±1.52	54.55±3.77

mean±SEM, n=3, ****p<0.05, ***p<0.05, **p<0.05, *p<0.05 vs. the control

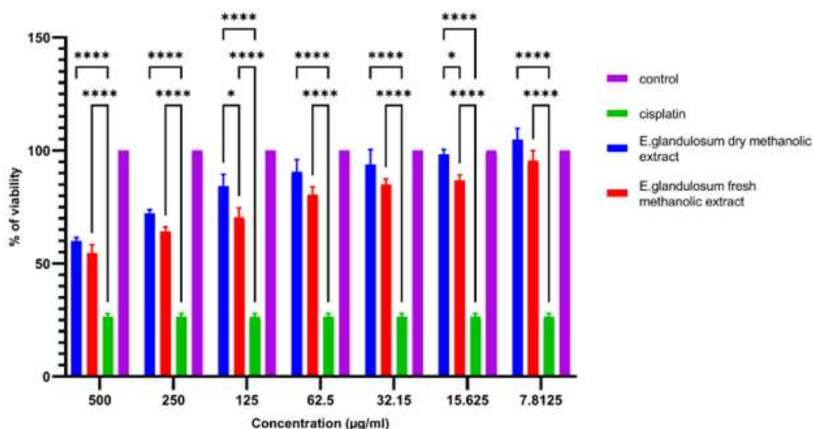

Fig.3. % of cell viability of fresh and dry plant methanolic extract in NIH3T3 cell lines

The effect of different concentrations from 7.81 to 500µg/ml of extract on Mouse fibroblasts were plotted to represent cell viability. 100µM of Cisplatin and untreated cells represent as reference and control, respectively. The results are presented in mean±standard error mean with the significant p-value of <0.05. The data represent three replicates

Table 4: Percentage of cell viability of Guaianolide sesquiterpenoid in HaCaT cell lines

S.No	Concentrations (µM)	Percentage of cell viability
1	3200	90.27±3.75
2	1600	97.70±4.24
3	800	99.91±1.34
4	400	99.77±5.67
5	200	102.07±2.24
6	100	101.28±0.66
7	Cisplatin	24.53±0.00

mean±SEM, n=3, *P < 0.05, **P < 0.05, ***P < 0.05, ****P < 0.05 vs. the control

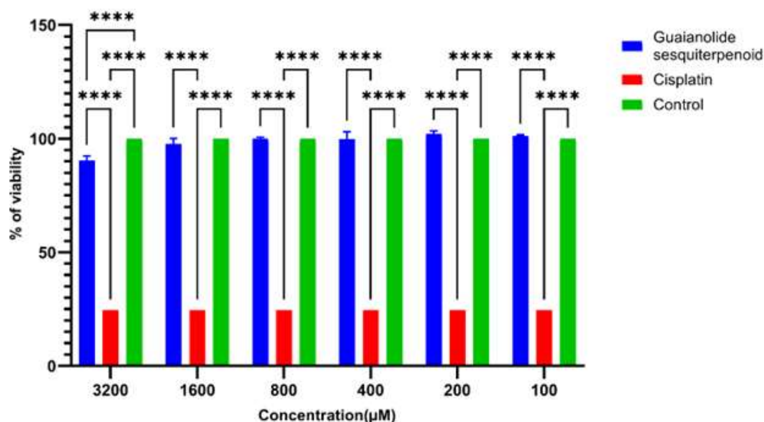

Fig.4. % of cell viability of Guaianolide sesquiterpenoid in HaCaT cell lines

The effect of different concentrations from 100 to 3200Mm of Guaianolide sesquiterpenoid on Human keratinocytes were plotted to represent cell viability. 100µM of Cisplatin and untreated cells represent as reference and control, respectively. The results are presented in mean±standard error mean with significant p-value of <0.05. The data represent three replicates

3.4. Wound scratch assay

The evaluated percentage of wound closure of the dry and the fresh plant methanolic extracts against concentrations at 24 and 48 h are shown in Table 5 & 6 and Fig. 5 a, b & 6a, b. The results indicate that the dry plant methanolic extract at the concentrations of 250, 125 and 62.5 µg/ml show a wound closure of 67.11, 98.80 and 76.55% at 48h, respectively and the fresh plant methanolic extract at the concentrations of 125, 62.5 and 31.25µg/ml show 100 % wound closure at 48h in all concentrations. The percentage wound closure of Guaianolide sesquiterpenoid against concentrations is shown in Table 7 and Fig. 7a, b. The results reveal that Guaianolide sesquiterpenoid at 3200, 1600 and 800 µM concentrations, show a wound closure of 37.8, 43.9 and 36.8 % at 12h, 45.9, 63.78 and 45.7 % at 18h, respectively and 99% at 24h.

Table 5: Percentage wound closure of the dry plant methanolic extract

S.No	Concentrations (µg/ml)	% wound closure	
		Dry plant methanolic extract	
		24h	48h
1	Control	34.85	50.74
2	VC	37.54	46.15
3	62.5	48.30	76.55
4	125	77.66	98.80
5	250	42.86	67.11
6	Itraconazole	5.00	7.28

The effect of different concentration from 62.5 to 250 µg/ml of dry plant extract on % of migration was evaluated by calculating the difference in the wound area at 0 time with 24 and 48h. Cont and VC represent Control and Vehicle control, respectively. The data represent three replicates

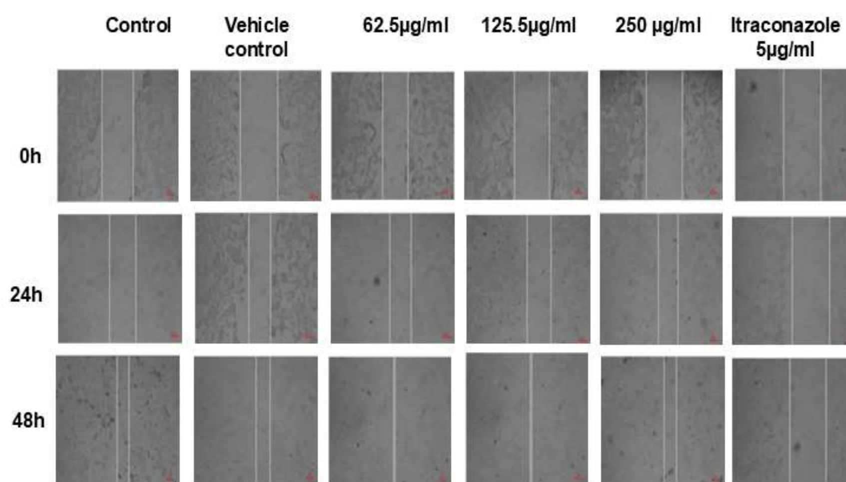

Fig. 5a: *In vitro* migration assay of dry plant methanolic extract

The effect of different concentrations from 62.5 to 250 of dry plant extract on the % of migration evaluated by calculating the difference in the wound area at 0 time with 24 and 48h. Cont and VC represent Control and Vehicle control, respectively. The data represent three replicates

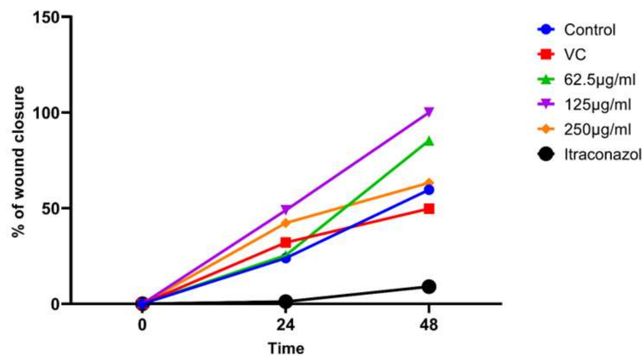

Fig.5b. Percentage of wound closure of dry plant methanolic extract

Table 6. Percentage wound closure of fresh plant methanolic extract

S.No	Concentrations (µg/ml)	% wound closure	
		Fresh plant methanolic extract	
		24h	48h
1	Control	34.85	50.74
2	VC	37.54	46.15
3	31.25	85.64	100.00
4	62.5	92.61	100.00
5	125	98.97	100.00
6	Itraconazole	5.00	7.28

The effect of different concentrations from 31.25 to 125 µg/ml of fresh plant extract on the % of migration was evaluated by calculating the difference in the wound area at 0 time with 24 and 48h. Cont and VC represent Control and Vehicle control, respectively. The data represent three replicates

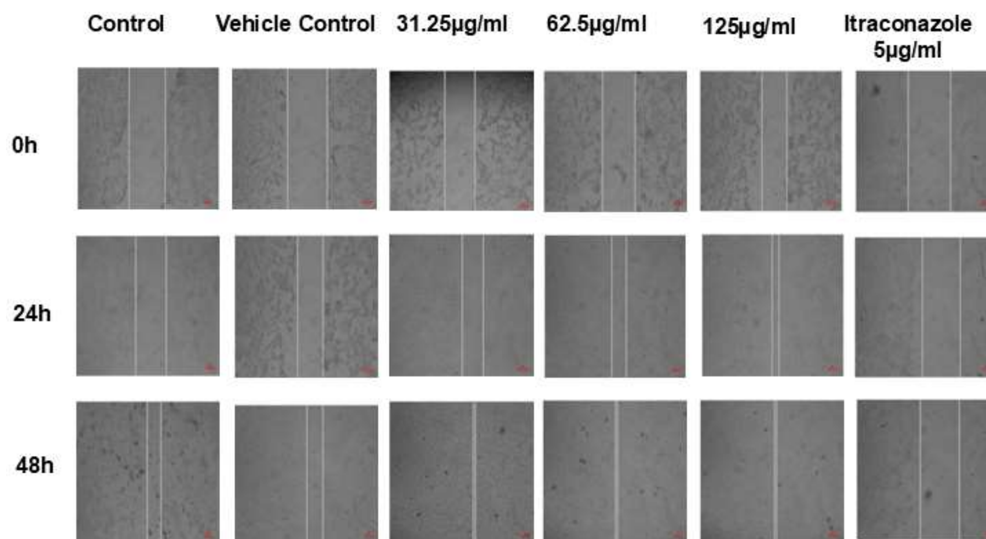

Fig. 6a: *In vitro* migration assay of fresh plant methanolic extract

The effect of different concentrations from 31.25 to 125µg/ml of fresh plant extract on the % of migration was evaluated by calculating the difference in wound area at 0 time with 24 and 48h. Cont and VC represent Control and Vehicle control, respectively. The data represent three replicate

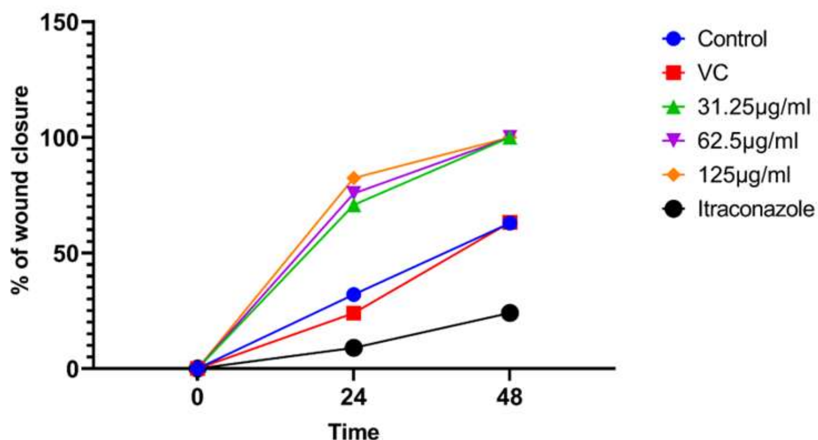

Fig.6b. Percentage of wound closure of fresh plant methanolic extract

Table 7: Percentage wound closure of Guaianolide sesquiterpenoid in HaCaT cell lines

S.No	Concentrations (µM)	% wound closure of Guaianolide sesquiterpenoid		
		12h	18h	24h
1	Control	17.5	25.4	34.2
2	VC	15.33	27.52	36.18
3	800	36.8	45.7	99.9
4	1600	43.9	63.78	99.71
5	3200	37.8	45.9	99.9
6	Itraconazole	21.8	33.65	41.36

The effect of different concentrations from 800 to 3200 µM of Guaianolide sesquiterpenoid on the % of migration was evaluated by calculating the difference in the wound area at 0 time with 12, 18 and 24h. Cont and VC represent Control and Vehicle control, respectively. The data represent three replicates

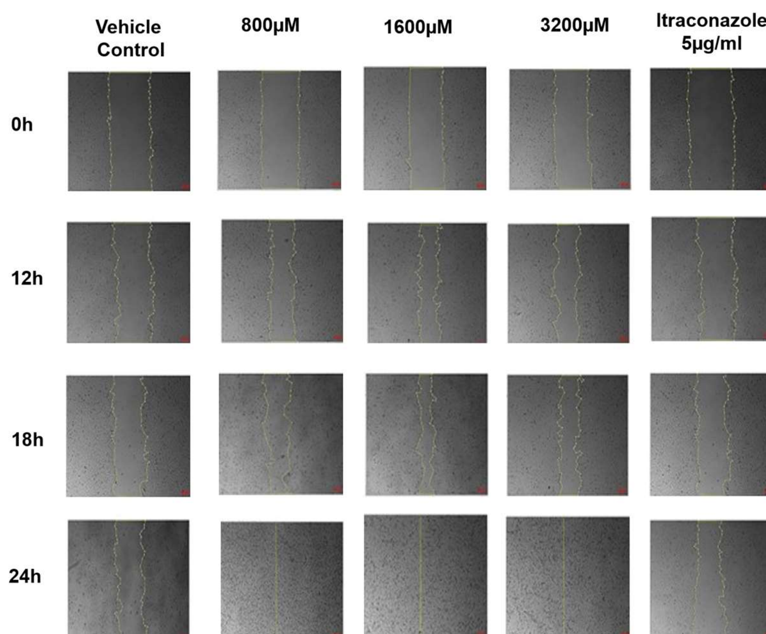

Fig.7a. *In vitro* migration assay of Guaianolide sesquiterpenoid in HaCaT cell lines

The effect of different concentrations from 800 to 3200µM of Guaianolide sesquiterpenoid on keratinocytes were plotted to represent cell viability. 5µg/ml of Itraconazole and untreated cells represent as a reference and control, respectively. The data represent three replicates.

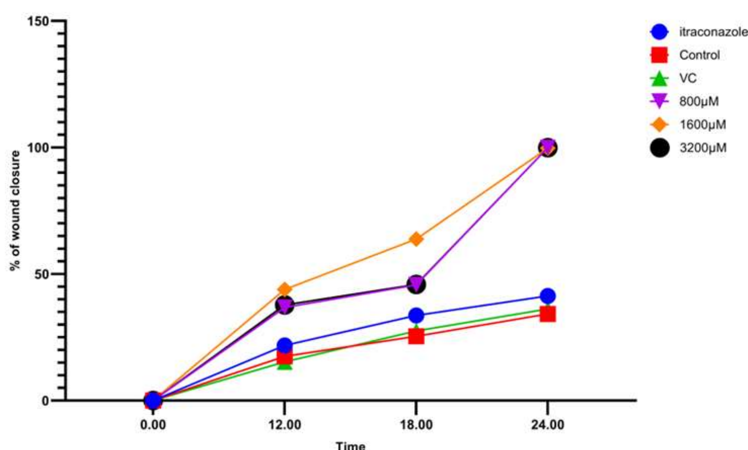

Fig.7b. % wound closure of the Guaianolide sesquiterpenoid in HaCaT cell lines

3.5. *In silico* docking

The interaction between Guaianolide sesquiterpenoid and the wound healing targets, TNF-α, IL-1β, IL-12, IL-18, GM-CSF, MMP-2, and MMP-9 was evaluated using molecular docking studies and the results obtained are shown in Table 8. The results reveal that IL-18 has the strongest binding with the compound followed by TNF- α and MMP-9 as visualized using the BIOVIA Discovery Studio Visualizer**.**

Table 8: Docking scores of Guaianolide sesquiterpenoid with wound healing targets

S.No	Target	Resolution (A^0)	Docking score (kcal/mol)
1	TNF-α	2.1	-8.8
2	IL-12	2.01	-5.9
3	IL-18	2.80	-9
4	GM-CSF	2.25	-6.9
5	MMP-2		-7.2
6	MMP-9	1.59 A^0	-7.5
7	IL-1β	2.15 A^0	-6.4

3.7. Physicochemical properties and toxicity study of Guaianolide sesquiterpenoid

The physicochemical properties and toxicity of Guaianolide sesquiterpenoid are presented in Table 9 & 10. The results reveal that the bioactive compound possesses good GI absorption property and hence a higher bioavailability. The total polar surface area of the compound, 89.90, indicates significant oral absorption and membrane permeation. The bioactive compound does not act as a substrate for the isoenzyme, CYP2D6, but inhibits protein-protein interaction and enhances drug metabolism in normal rate [23 & 24]. The compound is mutagenic to several strain of Salmonella species and carcinogenic to both mouse and rat. The compound shows low aquatic toxicity and does not produce cardiotoxic effects.

Table 9: Physicochemical properties of Guaianolide sesquiterpenoid

MW	RB	TBSA	XLOGP3	GI absorption	BBB permeant	CYP2D6 Inhibitor	Lipinski violations	PAINSalerts	Synthetic access
366.45	5	89.90	11.45	High	No	No	0	0	4.91

Table 10: Toxicity study of Guaianolide sesquiterpenoid

Algae test	Ames test	Carcino Mouse	Carcino Rat	Daphnia -at	hERG inhibition	TA100-10RLI	TA100-NA	TA1 35-10RLI	TA1535 - NA
0.0599984	Mutagen	Positive	Positive	0.187118	Low risk	Negative	Negative	Negative	Negative

3.8. Effect of Guaianolide sesquiterpenoid on the mRNA expression of TNF-α, IL-18 and MMP-9

Based on the docking score three targets, namely TNF-α, IL-18 and MMP-9, were selected and the effect of Guaianolide sesquiterpenoid on the mRNA expression of these targets were evaluated using $2^{\wedge-\Delta\Delta Ct}$ method. The findings are shown in Fig 8, 9 &10. The data reveal that the expression initially increases at 1h and reduces at 18h and at 24h.

The bioactive compound stimulates the expression of IL-18 in all phases of wound healing. Initial increase of IL-18 helps in binding with its receptor, IL-18R1 which activates macrophages, interferon-gamma (IFN-γ) and caspase 3 [25]. Activation of macrophages leads to phagocytosis and inflammatory cytokine production. The expression of IL-18 at 18h was less when compared to 1h. The detectable expression at 18h stimulates IFN-γ to bridge the innate and adaptive immunity which plays a major role in inflammation and apoptotic tissue remodeling. Sustained release of IL-18 activates Caspase 3 to stimulate the apoptosis in both the phases of inflammation and tissue remodeling [26].

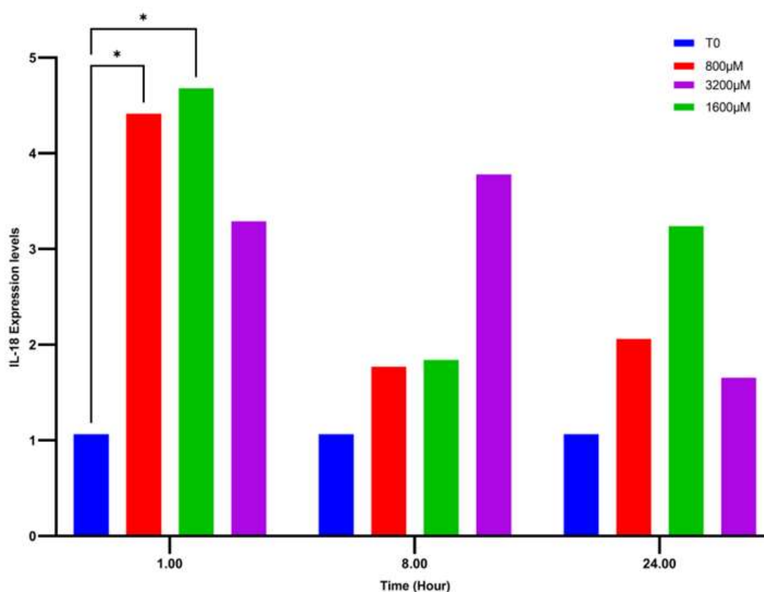

Fig. 8: Effect of Guaianolide sesquiterpenoid on mRNA expression of IL-18

The bar graph represents the IL-18 mRNA expression on HaCaT cells. The HaCaT cells were exposed to different concentrations, 800, 1600 and 3200 µM, of Guaianolide sesquiterpenoid and the expression was measured at 1, 8 and 24h period. β-actin was used as the internal control gene. *$p <0.05$ compared to T_0.

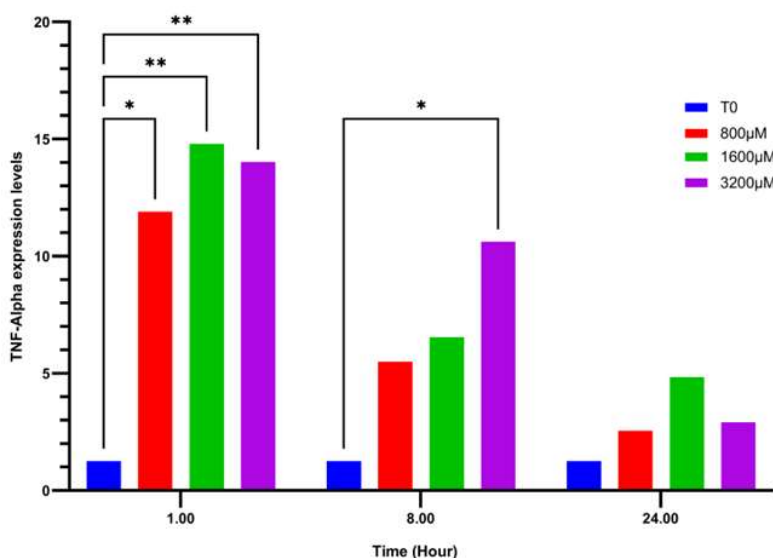

Fig. 9: Effect of Guaianolide sesquiterpenoid on mRNA expression of TNF-α

The bar graph represents the TNF-α mRNA expression on HaCaT cells. The HaCaT cells were exposed to different concentrations, 800, 1600 and 3200 µM, of Guaianolide sesquiterpenoid and the expression was measured at 1,8 and 24h period. β-actin was used as internal control gene. *$p <0.05$ compared toT_0.

The bioactive compound stimulates the expression of TNF-α in all the phases of wound healing. Initial increase of TNF-α, helps in recruiting lymphocytes and macrophages and upregulating adhesion molecules. The expression of TNF-α at 18h was less compared to 1h and the expression helps in proliferation and tissue repair [27]. The decreased expression at 24h stimulates apoptotic signaling by caspase-3 activation and remodelling by indirectly promoting MMP [28 & 29].

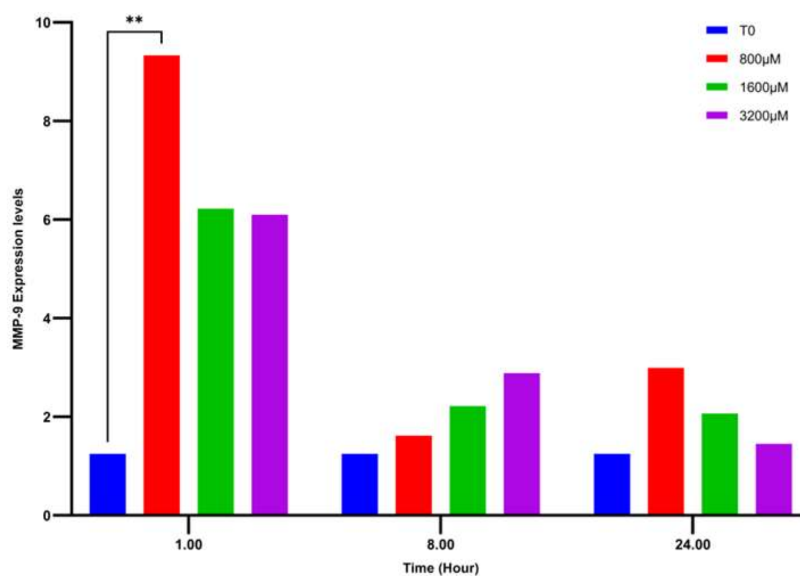

Fig. 10: Effect of Guaianolide sesquiterpenoid on mRNA expression of MMP-9

The bar graph represents the MMP-9 mRNA expression on HaCaT cells. The cells were exposed to different concentrations, 800, 1600 and 3200 µM, of Guaianolide sesquiterpenoid and the expression was measured at 1, 8 and 24h period. β-actin was used as internal control gene. $*p < 0.05$ compared to T_0.

The bioactive compound stimulates the expression of MMP-9 in all the phases of wound healing. A proper wound healing requires a controlled activity of MMPs at all stages. Initial increase is primarily helpful in breaking cell-cell adhesion molecules that are helpful in keratinocyte migration. During the later phase, it activates TGF-β to initiate angiogenesis, reepithelialization and tissue remodeling [30, 31 & 32]. Trace detection at 24h indicates the weak transcriptional regulation of MMP-9, that reduces the ECM degradation [33, 34].

4. Discussion

The leaf homogenate of the plant, *Eupatorium glandulosum*, has been utilized for skin regeneration by the communities in the Nilgiri district, Tamilnadu, India [13]. The crude extracts of this plant are known to promote tissue damage [19, 20 & 21]. Scientific data is required to claim its traditional value by *in vitro* biological assays relevant to wound healing. Our study was, therefore, focused on the bioguided fractionation of the plant extract to determine and evaluate the bioactive compound responsible for the wound healing activity. Plant based bioactive compounds form the basis for several drugs currently in use and in development process. These compounds, elaborated within living systems are known to have the ability to mask and fine tune the reactivity of their labile functional groups so that a small molecule can retain its kinetic stability needed for it to reach and specifically inhibit biological targets either by a covalent mechanism or by employing the exquisite structural complementarity between the small molecule and its biological target [5]. Subjecting plant based bioactive compounds as leads for structure-activity relationships, target-based drug discovery followed by animal studies and clinical trials has a tremendous potential for developing newer drugs for diseases including wound healing. Plant bioactive compound exhibit more drug likeliness compared to synthetic molecules, thus making them good candidates for drug development. Further, several plant based bioactive compounds have been successfully screened for wound healing activity. Betulin and Borneol have been approved by USFDA as a wound healing drugs [5].

The dry and fresh plant leave methanolic extracts confirmed the presence of carbohydrates, glycosides, alkaloids, triterpenoids and tannins along with fats and fixed oils. Guaianolide sesquiterpenoid, isolated using bioguided fractionation from the fresh plant methanolic extract and has been previously reported in the plant, *Cichorium intybus* L [35], *Cichorium glandulosum* [36] and *Achillea millefolium* L [37]. Guaianolides, a large group of sesquiterpene lactone [38] and its IUPAC name is (E)-(3R,3aR,4R,9aS,9bR,Z)-6-(hydroxymethyl)-3,9-dimethyl-2,7-dioxo-2,3,3a,4,5,7,9a,9b-octahydroazuleno[4,5-b]furan-4-yl)2-methylbut-2 enoate contains 5, 7, 5-ring system known for its wide range of biological activity like anti-inflammatory [35, 37], anti-neuroinflammation [36], antihyperglycemic and anticancer activity [39, 40]. But the present study is the first that revealed the compounds potential for wound healing. During the healing process many skin cells namely, macrophages, fibroblasts, keratinocytes and other immune cells are activated to initiate the healing process [41]. Among them fibroblasts and keratinocytes are generally abundant and play a predominant role in epithelial-mesenchymal transition, migration and collagen deposition. Generally, progression of healing depends on the migration of fibroblast and keratinocytes. Based on this, *in vitro* scratch assays are employed to evaluate the migration of fibroblast and keratinocytes [42, 43]. The compound showed significant cell viability and migration. The migration dynamics of the bioactive compound on HaCaT cells was, therefore, evaluated in three different time points, 12, 18 and 24h. The speed of migration is significantly low from 0h to 18h due to the traumatic wounding caused during scratch. It not only removes the cells from the wound area but also generate significant shear stress extending from the wound edge into the surrounding monolayer. These shear stress disrupt the cell-cell and cell-matrix interactions during the early stage of wound healing. The keratinocytes need to reestablish prior to cell migration. Later the wound closure was significant and shows complete wound closure [44]. The compound was screened against multiple wound healing mediators, TNF-α, IL-1β, IL-12, IL-18, GM-CSF, MMP-2 and MMP-9 using *in silico* docking. Based on

the docking score, IL-18, TNF-α and MMP-9 were further evaluated for mRNA expression. Initially IL-18, TNF-α and MMP-9 expressions were higher at all concentrations which indicates that the bioactive compound plays a critical role in preliminary stage in tissue repair. The bioactive compound stimulates the expression of IL-18 rapidly from keratinocytes during the early phase and gradually decreases towards the progression of wound closure. Early induction activates neighbouring keratinocytes and immune cells. Activation of keratinocytes leads to reorganisation, formation of lamellipodia and coordinating collective migration [45]. Initial activation helps in early inflammation by activating macrophages, interferon-gamma (IFN-γ) and caspase 3 [46, 47]. A gradual decrease at later stage shows IL-18 helps in transition from inflammatory to proliferative phase and stimulates efficient wound healing.

The bioactive compound initially increases the TNF-α, is the first and predominant cytokines released immediately after injury. It also helps in chemotaxis, proliferation of keratinocytes and fibroblasts, recruiting immune cell, initiating collective migration and the synthesis and degradation of ECM proteins. TNF-α also helps in acute wound response [47]. Transient expression at the later stage helps in keratinocyte migration, expression of adhesion molecules and resolution of inflammation [48].

The bioactive compound rapidly increasing MMP-9 at the initial stage to degrade basement membrane components, detach keratinocytes from the wound edge and helps in keratinocyte migration. Decline of MMP-9 at the later stage indicates that once wound area is narrow and reepithelialization is about to complete, the expression of MMP-9 is no longer beneficial [49, 50].

When keratinocytes are treated with the bioactive compound IL-18 and TNF-α increase during the early phase of wound healing. These cytokines activate keratinocytes and immune cells, promote cell polarization, lamellipodia formation and collective migration. During this period TNF-α induces MMP-9 which degrades the basement membrane and extracellular matrix components and helps in detaching keratinocytes from the wound edge and enhancing the migration towards the scratched area. As wound closure progresses, the expression of IL-18, TNF-α and MMP-9 declines to prevent prolonged elevation of these markers that delay healing and maintain excessive inflammation.

5. Conclusion

The current investigation evaluates the mechanistic insights into the wound healing potential of the bioactive compound, Guaianolide sesquiterpenoid, isolated from *Eupatorium glandulosum*, the traditional medicinal plant used in the Nilgiri district, Tamilnadu, India through a comprehensive bioguided fractionation, *in vitro* and qPCR study. The methanolic extract that showed greater wound healing activity was subjected to fractionation. The ethyl acetate fraction yielded the bioactive compound, Guaianolide sesquiterpenoid. The well characterised compound shows significant cell migration and viability properties thus revealing its active role in tissue regeneration. *In silico* docking and mRNA expression studies reveal that the compound has a significant role in modulating inflammatory and remodelling mediators, namely TNF-α, IL-18, and MMP-9. IL-18 plays a dual function, namely inflammation and apoptotic tissue remodeling at early phase and later phase, respectively thus revealing its critical role in coordinating immune responses and enabling proper resolution of inflammation. TNF-α may help in recruiting immune cells and contribute to the inflammation, proliferation and apoptosis leading to tissue remodelling. MMP-9 plays various roles like extracellular matrix (ECM) remodelling, angiogenesis and reepithelialization. The present study thus reveals that Guaianolide sesquiterpenoid is a promising natural product for wound healing as it targets early inflammatory and remodelling pathways. Further comprehensive *in vivo* evaluations are required to evaluate its therapeutic potential and mechanism of action and develop the compound as a drug.

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8. Authors contribution statement

Dr. RS contributed to identifying the problem, designing and developing the concept and

methodology and also contributed to performing experiments like isolation, *In vitro*, *in silico* and qPCR analysis, in addition to collecting the resources and writing the manuscript.

Dr. M.J.N.C contributed to design, concept formation and developing the methodology and research design and manuscript editing.

Dr. M.JN contributed to thorough editing of the article

9. Conflict of Interest

The authors declare no conflict of interest regarding the contents of the present manuscript.

10. Abbreviation

PDB-Protein data bank
TNF-Tumor necrosis factor
IL - Interleukin
MMP-2- Matrix metalloprotease-2
qPCR- Quantitative polymerase chain reaction

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