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EVALUATION OF WOUND HEALING PROPERTY OF*CAESALPINIA BONDUCELLA* LEAVES, STEM BARK EXTRACTS AND THEIR PHYTOCONSTITUENTS

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Abstract:

The crude extracts of leaves, stem bark and their phytoconstituents were used for assessing thewound healing activity on rat models viz. incision, excision and dead space wounds.The significant wound healing activity was observed in animals treated with stem bark chloroform extract and its constituent SC2 [(Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate)] by decrease in the period of epithelialization (14.25 ± 0.48 and 15.25 ± 0.25 days), increase in the wound contraction rate (97.3 % and 90.4 %), increase in the tensile strength (680.5 ± 12.5 and 621.5 ± 8.5 g/cm^2), restoration the levels of hydroxyproline (4.54 ± 0.07 and 3.98 ± 0.07 mg/100 mg dry tissue), hexosamine (0.53 ± 0.02 and 0.51 ± 0.02 µg/100 mg dry tissue) and uronic acid content (0.22 ± 0.01 and 0.2 ± 0.01 µg/100 mg dry tissue). The result supported the traditional claim that stem bark chloroform extract and its isolated constituent Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate showed significant wound healing property.

Key Words: *Caesalpiniabonducella*, Phytoconstituents, Crude Extract, Incision Wound, Excision Wound & Dead Space Wound.

Introduction:

Caesalpiniabonducella (Family: Fabaceae) is a spinucent woody liana very sparsely distributed in the Western Ghats of Karnataka state, India. The medicinal property of *C. bonducella* was described as Rasa: Tikta (bitter), kashaya (astringent); Guna: Laghu (light), ruksha (dry), tikshna (sharp); Veerya: Ushna (hoGVipaka: Katu (pungent); Dosha: Pacifies tridosha(Williamson, 2002; Nazeerullah et al., 2012). The seeds, leaves and roots used in the treatment of tachycardia, bradycardia, tuberculosis, tympanitis, pain in the abdomen, fever, cold, cough and liver fluke in ruminants (Nazeerullah et al., 2012).Phytoconstituentsanaylsis of *C. bonducella* showed homoisoflavonoids, 6-Omethylcaesalpinianone, caesalpinianone, Hematoxylol, 6-O-acetylloganic acid, 4-O-acetyllo-ganic acid stereochenol A, and 2-O-a-d-glucosyloxy-4- methoxybenzenepropanoic acid from ethanolic extracts of stem bark (Ataa et al., 2009). The Brazillin and bonducin phytochemicals has been isolated from leaf (Watt et al., 1962). The Caesaldekarins F, G & C, (Lyder et al., 1998), Bonducellpins A, B, C & D (Peter et al., 1997), Diosgenin A - steroidal saponin were isolated and identified from the roots (Jain et al., 1997).The traditional practitioners residing in the vicinity of the Western Ghats of Karnataka using the plants of leaves and stem bark to cure liver disorders, diabetes and wounds. Therefore, phytochemical screening of the leaves and the stem bark of this plant forwound healing has not been worked out scientifically so far. In the view of the above, an attempt was made evaluate the wound healing potential of leaves and stem bark of *C. bonducella*and their phytoconstituents.

Methods and Materials:

Plant Collection and Preparation of Extract:

The leaves and stem bark of *C. bonducella* were collected from forest ranges of Bhadra Wild Life Sanctuary (1 Km from Kuvempu University) of the Western Ghats, Karnataka. The plant was authenticated by Prof V. Krishna, author of Flora of Davanagere District, Karnataka (Manjunatha et al., 2004)and Prof. Y.L Krishnamurthy, Department of Applied Botany, Kuvempu University, Shankaraghatta, Shivamogga, District, Karnataka comparing with the herbarium voucher specimen (KUAB.301) deposited at Kuvempu University herbaria. The leaves and stem bark materials were shade dried, powdered mechanically (sieve No. 10/44), stored in airtight containers. The powdered material (600 g) was refluxed successively with the solvents Chloroform (E-Mark Mumbai, India) and ethanol for 48 hr. All the extracts were filtered and concentrated in vacuum using rotary flash evaporator (Buchi, Flawil, Switzerland). Left over solvent was completely removed on water bath and finally dried in the desiccators. The crude extracts obtained from each of the solvents were labeled, weighed and the percentage of yield was recorded.

Quantitative Estimation Phenolics and Flavonoids by HPLC-UV Analysis:

Phenolic acids and flavonoids from various extracts of leaves and stem bark of *C. bonducella* were analyzed according to the method (Pradeepa et al., 2014).

Isolation and Characterization of Phytoconstituents from Leaves and Stem Bark of *C.Bonducella:*

The leaves and stem bark chloroform extract were subjected for Thin Layer Chromatography (TLC) using the solvent system petroleum ether, hexane and chloroform. The pure constituents were eluted by column chromatography (60×4 cm, 60–120 mesh, 200g silica gel) by gradient elution method using petroleum ether/chloroform/hexane in combination and the fractions were collected at the intervals of 5 min. The purity of the isolated compounds was monitored by TLC examination, based on single spot separation. These isolated pure compounds were subjected for structural analysis studies using spectroscopy and labelled as leaves chloroform (LC3), stem bark chloroform (SC2).

Wound Healing Activity:

a) Preparation of Ointments: Formulations of extract and its constituents were prepared to evaluate its efficacy, in comparison with povidone–iodine ointment. Paraffin wax was used as ointment base. Two types of topical formulations were prepared, 5% w/w (crude extracts ointment formulation) and 0.25% w/w (isolated constituents ointment formulation). The ointment base was mixed with extracts and isolated constituents, then stirred to get the homogeneous ointment preparation. The drug formulations were freshly prepared on every third day.

b) Experimental Animals: Wistar albino rats of either sex, weighing about 180–200 g were used for the study. The animals were housed at (25 ± 1) ºC, humidity 55-60% in the Department of Biotechnology, Kuvempu University, Karnataka, India. They were fed with a standard commercial pellet diet (Sai Durga feeds and foods, Bangalore) and water ad libitum during the experiment. The study was permitted by the Institutional Animal Ethical Committee (Reg. No. NCP/IAEC/CL/10/12/2010-11).

c) Grouping of Animals: The excision, incision and dead space wound models were used to evaluate the wound healing activity of leaves, stem bark extracts and their isolated constituents of *C. bonducella*. The rats were divided into 8 groups, each containing six animals. For excision and incision wound models, 50 mg of formulated ointments were applied topically to each animal once a day. The animals of Group-I received ointment base (control). Groups-II: Leaves chloroform extract ointment (5 %w/w); Group-III: Leaves ethanol extract ointment (5 %w/w); Group-IV: Stem bark chloroform extract ointment (5 %w/w); Group-V: Stem bark ethanol extract ointment (5 %w/w); Group-VI: LC3 ointment (0.25%w/w); Group-VII: SC2 ointment (0.25%w/w); Group-VIII were treated with a 5%w/w povidone–iodine ointment. For dead space wound model, the rats were divided into 7 groups, each containing six animals. Group-I: Control animals were treated with vehicle (1% DMSO). Groups-II: Leaves chloroform extract (300 mg/kg b.wt.); Group-III: Leaves ethanol extract (300 mg/kg b.wt.); Group-IV: Stem bark chloroform extract (300 mg/kg b.wt.); Group-V: Stem bark ethanol extract (300 mg/kg b.wt.); Group-VI: LC3 (60 mg/kg b.wt.); Group-VII: SC2 (50 mg/kg b.wt.).

d) Excision Wound Model: The animals were anaesthetized prior to and during the creation of experimental wounds, with diethyl ether. Rats were then inflicted with excision wound as described (Morton& Malone, 1972). The dorsal fur of the animals was shaved with electric clipper and full thickness of excision wound of 500 mm^2 was created along the marking using toothed forceps, a surgical blade and pointed scissor. The entire wound was left open. All groups of animals were treated in the similar manner as mentioned above. The healing of wound was assessed by tracing the wound on 1^{st}, 8^{th} and 16^{th} post-wounding days using transparency paper and a marker, and the recorded wound areas were measured graphically.

e) Incision Wound Model: The test rats were anesthetized with diethyl ether prior to and during the creation of experimental wounds. The dorsal fur of the animals was shaved with electric clipper and two para-vertebra along incision of 6 cm length were made through the skin at a distance of about 1.5 cm from the midline on each side of the depilated back of the animals as described earlier (Ehrlich& Hunut, 1968). After incision, the parted skin was stitched together at intervals of one centimeter using surgical thread (No.000) and curved needle (No. 11). The wounds were then left undressed. All groups of animals were treated as described above. Sutures were removed on eighth post-wounding day and the treatment was continued. The skin breaking strength of healed wound was measured on the 10^{th} day by the method (Lee, 1968).

f) Dead Space Wound Model: Dead space wounds were created by subcutaneous implantation of sterile cylindrical grass pith (2.5 x 0.3 cm), on either side of the lumber region on ventral surface of each rat (Turner &Hebborn, 1965). On the 10^{th} post-wounding day, animals were sacrificed under diethyl ether anesthesia, and the granulation (wound) tissues formed on the grass piths were excised. The granulation tissue was dried at 60oC for 12 h in an oven to obtain a constant dry weight. Simultaneously, the dried tissue was hydrolyzed with 6N HCl (5.0 mL) for 24 h at 110 then neutralized (pH 7). The neutralized hydrolysate was used for biochemical estimations. The total collagen content of the granulation tissues was assessed by hydroxyproline index method (Neuman& Logan, 1950). Hexosamine was estimated by the method (Elson& Morgan, 1933). Uronic acid in the wound tissue was determined by thecarbazole method with some modifications (Bitter & Muir, 1962).

Statistical Analysis:

The statistical analysis was carried out using one way ANOVA followed by Tukey's*t*-test using ezANOVA (version 0.98) software. The difference in values at p= 0.01 was considered as statistically significant.

Results:

Yield of Crude Extracts:

The yield of chloroform (dark brown dry solid in nature) and ethanol crude (dark brown dry waxy in nature) extracts for 1 kg of the powdered stem bark plant material obtained from Soxhlet extraction was 16.83 g and 42.5 g. The yield of chloroform (dark green dry solid in nature) and ethanol crude (dark black dry solid in nature) extracts for 1 kg of the powdered leaves plant material obtained from Soxhlet extraction was 32.5 g and 87.2 g.

Quantitative Analysis for Phenolics and Flavonoids:

The quantitative estimation of phenolics and flavonoids contents in the leaves, stem bark and their calli extract has shown in the table 1.Among them, stem bark chloroform extract possess highest content of total phenolics (91.15 ± 1.0µg/mg of dry extract of Gallic acid equivalent, GAE) and flavonoids (104.1 ± 1.25µg/mg of dry extract of Quercetin equivalent, QE). Whereas, stem bark ethanol extract and stem calli ethanol extract possess moderate amount of phenolics (79.15 ± 0.3 and 88.45 ± 1.0µg/mg of dry extract GAE respectively) and flavonoids (67 ± 1.5 and 96.3 ± 2.7µg/mg of dry extract QE respectively) content. The leaves ethanol extract comprised comparatively less content of total phenolics (27.09 ± 0.02µg/mg of dry extract GAE) and flavonoids (12.33 ± 0.67µg/mg of dry extract QE).

Table 1: Estimation of total phenolic and flavonoid contents

Samples	Phenolic content (µg GAEs/mg extract)	Flavonoid content (µg QEs/mg extract)
CLC	42.20 ± 1.3	45.23 ± 1.2
CLE	27.09 ± 0.02	12.33 ± 0.67
CSC	91.15 ± 1.0	104.1 ± 1.25
CSE	79.15 ± 0.3	67 ± 1.5

Values expressed are means ± SE of three parallel measurements.

GAEs- Gallic acid equivalents; QEs- Quercetin equivalents; CLC- leaves chloroform extract; CLE- leaves ethanol extract; CSC- stem bark chloroform extract; CSE- stem bark ethanol extract.

HPLC–UV Analysis:

HPLC was employed to characterize the phenolic acids and flavonoids present in pharmacologically very active extract of leaves and stem bark (table 2). HPLC analysis of leaves chloroform extract revealed the presence of Gallic acid, ellagic acid and two unknown phenolic acids with retention time of 2.69 and 3.44 min respectively (fig. 1A). The HPLC analysis of stem bark chloroform extract showed the presence of Gallic acid, ellagic acid and two unknown phenolic acids with retention time of 1.09, and 4.18 min respectively (fig. 1B). The HPLC-UV spectral peaks of chloroform extract of leaves (fig. 1C) showed the peaks for presence of flavonoid compounds, Rutin, Quercetin, Myricetin, Kaempferol at 350 nm with the analysis of retention time of standard flavonoids. HPLC reports indicated that the stem bark chloroform extract showed peaks for the presence of flavonoid compounds, rutin, quercetin, and five unknown peaks (fig. 1D).

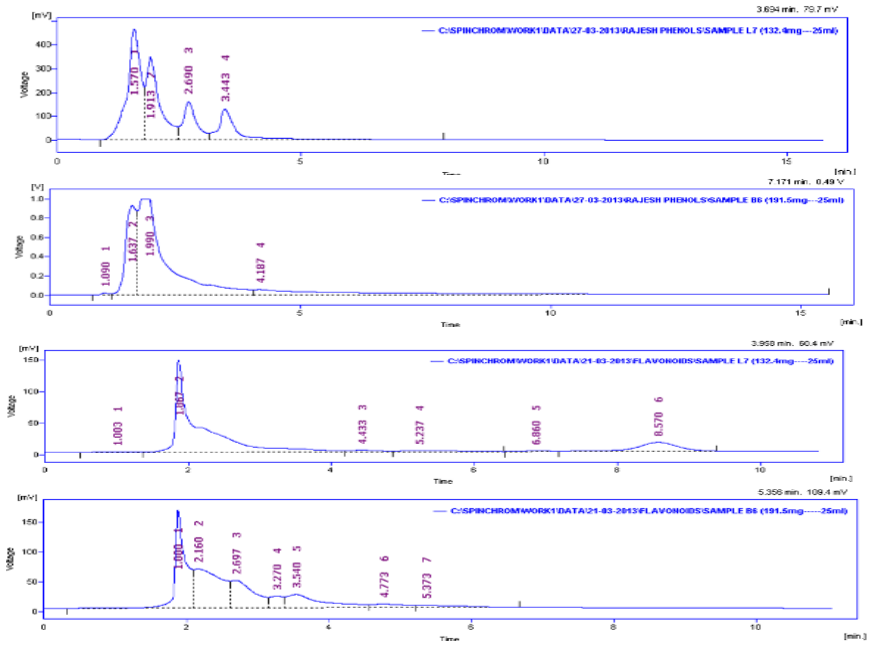


Figure 1: HPLC chromatogram of crude extract of *C. bonducella*. (A) phenolic acids of leaves chloroform extract. (B) phenolic acids of stem bark chloroform extract. (C) flavonoids of leaves chloroform extract. (D) flavonoids of stem bark chloroform extract.

Table 2: HPLC analysis of leaves and stem bark for phenolic acids and flavonoids

Phenolic acids and flavonoids		**CLC(mg/ g)**	**CSC(mg/ g)**
Phenolic acids	Gallic Acid	194.93	263.92
	Coumaric Acid	–	–
	Ellagic Acid	40.9	71.45
	Hydroxybenzoic Acid	–	–
	Vanillic Acid	–	–
Flavonoids	Rutin	265.33	109.9
	Quercetin	48.02	45.6
	Myricetin	2.72	4.32
	Kaempferol	66.42	9.20
	Luteolin	–	–

Note: CLC- leaves chloroform extract; CSC-stem bark chloroform extract.

The qualitative phytochemical screening revealed the presence of steroids and the melting point is 136°C. The IR spectra exhibits absorption bands at 2927 to 3018 cm^{-1} for C-H stretching and at 1458 and 1375 $cm^{-1}$for C-H bending frequencies. It does not showed any characteristic bands for the functional groups. This suggests that the compound may be a hydrocarbon (fig.2A). The ^{1}H-NMR spectra showed a doublet peak at δ 5.37 for two allylic protons at C-2, C-3 and a proton of ring. A multiple peak at δ 2 corresponds to hydroxyl group (-OH). The signals from δ 0.972 to 2.78for46 proton (fig.2B). The mass spectra showed a molecular ion peak at m/z 412.69. Based on the above data the tentative structure of the compound may be C_{29} $H_{48}O$ (fig.2C).

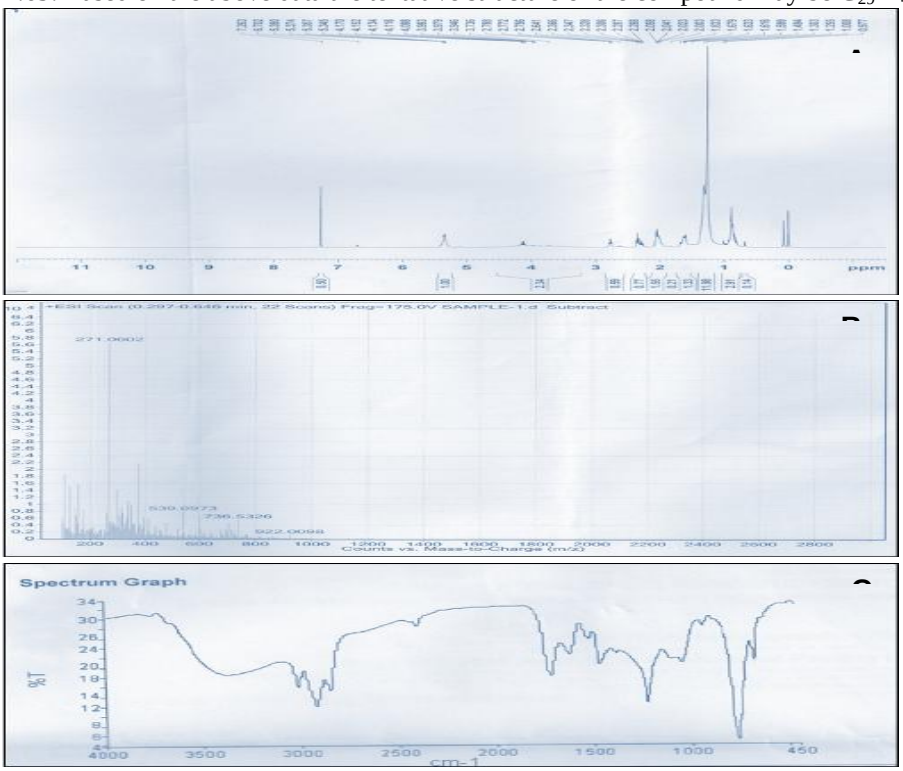
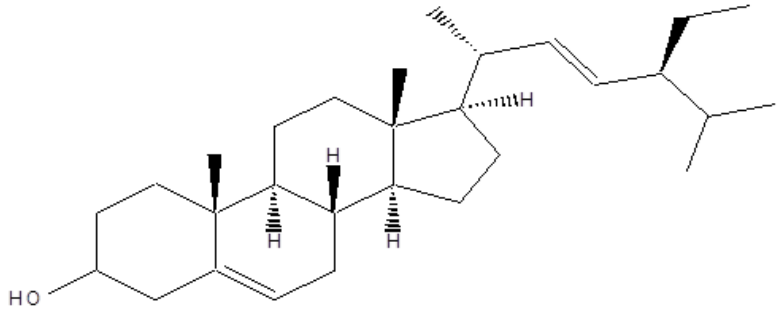


Figure 2: Compound –LC3. (A) IR spectrum; (B) ^{1}H-NMR spectrum and (C) Mass spectrum

Spectral Characteristics of Compound - LC3:

The LC3 compound isolated from the leaves chloroform extract of *C. bonducella*.The elevates Compound-1 were collected from column chromatography of chloroform extract of bark yielded compound showed positive results in Liebermann-Burchard's test, Salkowaski reaction tests and identified the presence of diterpenes. Careful interpretation and mechanistic investigations using IR, NMR and Mass spectral studies of isolated compound Compound-1 was identified as Sitosterol (Fig.3), from the chloroform extract of stem bark of *C. bonducella*gave proof for the structure of isolated compounds. Sitosterol is one of several phytosterols with chemical structures similar to that of cholesterol. Sitosterols are white, waxy powders with a characteristic odor. They are hydrophobic and soluble in alcohol. Molecular formula is $C_{29}H_{48}O$ and IUPAC Name17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahy-dro-1H cyclopenta [a] phenanthren-3-ol.

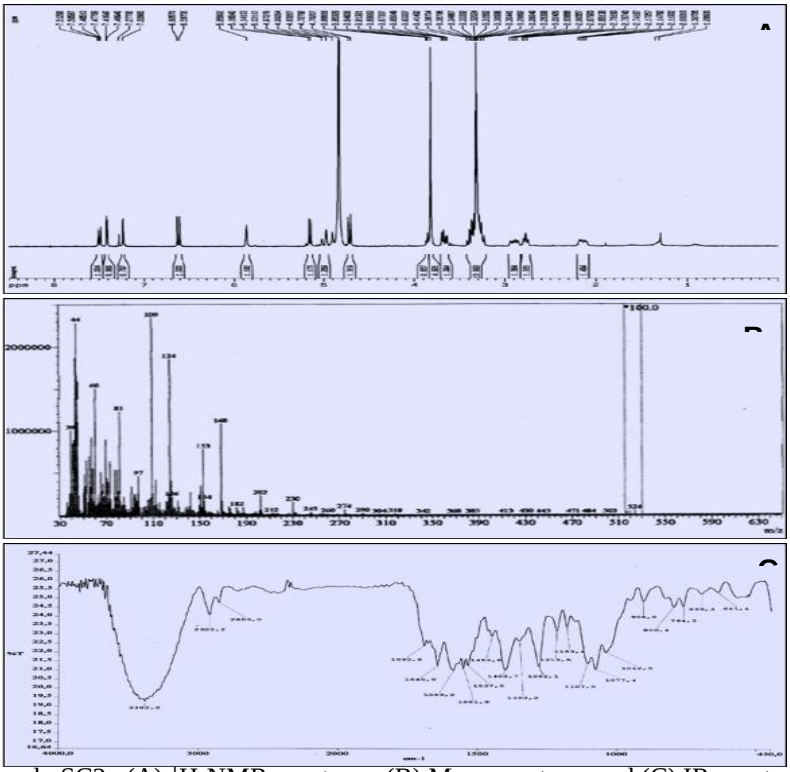


(8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-((*E*,2*R*,5*S*)-5-ethyl-6-methylhept-3-en-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-10,13-dimethyl-1*H*-cyclopenta[*a*]phenanthren-3-ol

Figure 3: Structure of Compound LC3: β-Sitosterol

Spectral Characteristics of Compound - SC2:

SC2 compoundisolated from the stem bark chloroform extract of *C.bonducella*. The qualitative phytochemical screening revealed the presence of triterpenoids. IR spectra showed a strong absorbance frequency at 3392cm^{-1} observed for the –OH groups, Strong absorbance frequency at 2920cm^{-1} observed for the alkyl (-CH) groups and strong absorbance frequency at 1692 cm^{-1} observed for the ester (-COO) group (fig.4A). ^{1}HNMR spetra showed the singlet and multiplet appeared at the region of 7.2-7.5 δ value is confirmed the presence of three aromatic protons. The singlets appeared at the region of 5.8 and 6.5 δ value is for two –OH groups. The two doublets for –CH=CH appeared at the region of 4.7 to 5.2 δ values. The singlet for –OCH_3 group appeared at the region of 3.8 δ values. The peaks appeared at the region of 3.3-3.4 δ value is for –CH_2 (methylene) protons. ^{13}CNMR spectra showed the peak appeared at 176 δ value confirms the presence of aldehyde carbon. The peak appeared at 169 δ value confirms the presence of C–OH carbon. The peaks appeared at 118-146 δ value confirms the presence of aromatic ring carbons. The peaks appeared at 71-100 δ value confirms the presence of –CH=CH carbons. The peak appeared at 57 δ value confirms the presence of –OCH_3 group. The peak appeared at 37-42 δ value confirms the presence of aliphatic (-CH) carbons (fig.4B). The Mass spectra peak observed at 274 confirms the molecular weight of the compound (fig.4C).



Figur4: Compound –SC2. (A) ^{1}H-NMR spectrum; (B) Mass spectrum and (C) IR spectrum on the above obtained spectral data, the compound can be predicted as the Methyl (4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate (fig.5).

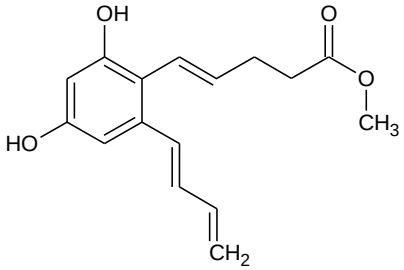


Figure 5: Compound- SC2: Methyl (4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxy phenyl}pent-4-enoate

The three different wound models *viz.*, excision, incision and dead space wound models have been used to screen the healing property of different crude extracts of leaves and stem bark, and their isolated compounds. The commercial ointment povidone iodine has used as the reference standard to assess the healing effect of the various extracts and the constituents.

Healing of Wounds:

a) Wound Contraction: Percentage of wound contraction in different group of animals on day 1st, 8th and 16th is presented in table 4. In the control group animals wound contraction rate was 72.38% (fig. 6 A, B, C) on post wound day 16. Animals treated with leaves chloroform extract showed 96% of wound contraction (fig. 6 A1, B1, C1). The leaves ethanol extract moderately improved wound healing with the wound contraction rate of 84 % (fig. 7 A, B, C). Better healing and complete wound closure has observed on 16th day of post wounding in stem bark chloroform extract (97.3%) (fig. 7 A1, B1, C1).

Table 4: Rate of excision wound contraction

Groups	**Treatment**	**Drug formulations**	**Wound contraction in excised wounds (%)**		
			1 day	8 day	16 day
Crude extracts					
Group II	CLC + Wound	5% Ointment	21.5	51.25	96
Group III	CLE+ Wound	5% Ointment	19.5	46	84
Group IV	CSC+ Wound	5% Ointment	21.3	58.0	97.3
Group V	CSE+ Wound	5% Ointment	17.5	50	91.2
Isolated phytoconstituents					
Group VI	LC3+ Wound	0.25% Ointment	18.3	40.0	75.1
Group VII	SC2+ Wound	0.25% Ointment	20.2	49.6	90.4
Standard ointment (Positive control)					
Group VIII	Povidone iodine ointment + Wound	5% Ointment	22.3	56.2	97.15

Values are the mean ± S.E.M of six rats. Symbols represent statistical significance. * P<0.05, **P<0.01, as compared to control rat group. CLC- Leaves chloroform extract; CLE- Leaves ethanol extract; CSC- Stem bark chloroform extract; CSE- Stem bark ethanol extract. LC3: β Sitosterol; SC2: Methyl (4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate.

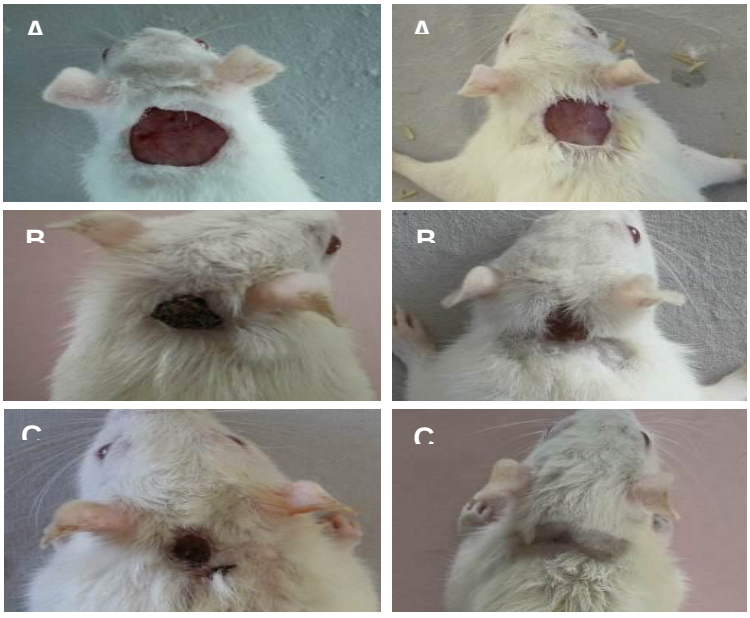


Figure 6: Excision wound contraction on 1st, 8th and 16th day in rat model treated with leaf chloroform extract.(A, B, C) Control animal; (A1, B1, C1), Leaves chloroform extract treated.

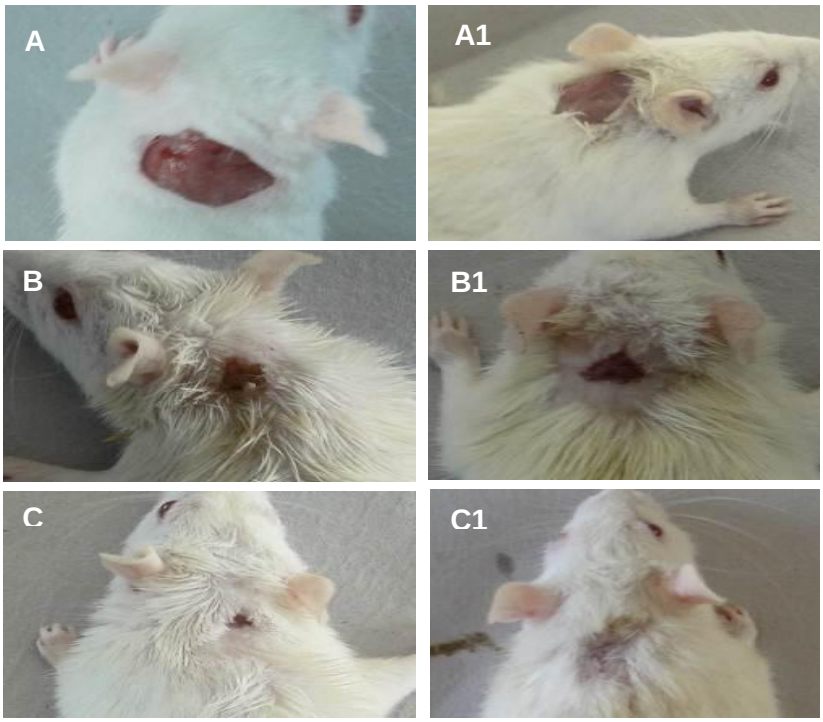


Figure 7: Excision wound contraction on 1st, 8th and 16th day in rat model treated with leaf ethanol extract and stem bark chloroform extract. (A, B, C) Leaves ethanol extract treated; (A1, B1, C1) Stem bark chloroform extract treated.

Stem bark ethanol extract treated animals showed 91.2 % wound contraction (fig. 8 A, B, C). The compound LC3 showed least effect (fig. 8 A1, B, C1) on rate of wound area reduction with 75.1 %. SC2: (90.4 %) treated animals (fig. 9 A, B, C) have shown remarkable wound healing activity when compared with the standard povidone iodine ointment (97.12% wound contraction) (fig. 9 A1, B1, C1).

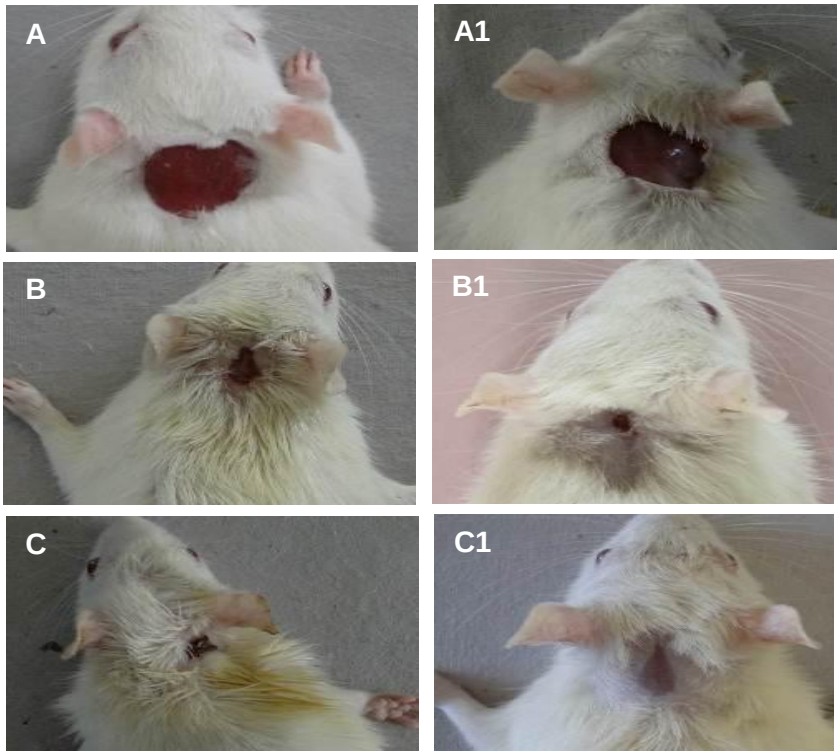


Figure 8: Excision wound contraction on 1st, 8th and 16th day in rat model treated with stem bark ethanol extract and isolated compound LC3. (A, B, C) Stem bark ethanol extract treated; (A1, B1, C1) LC3 treated.

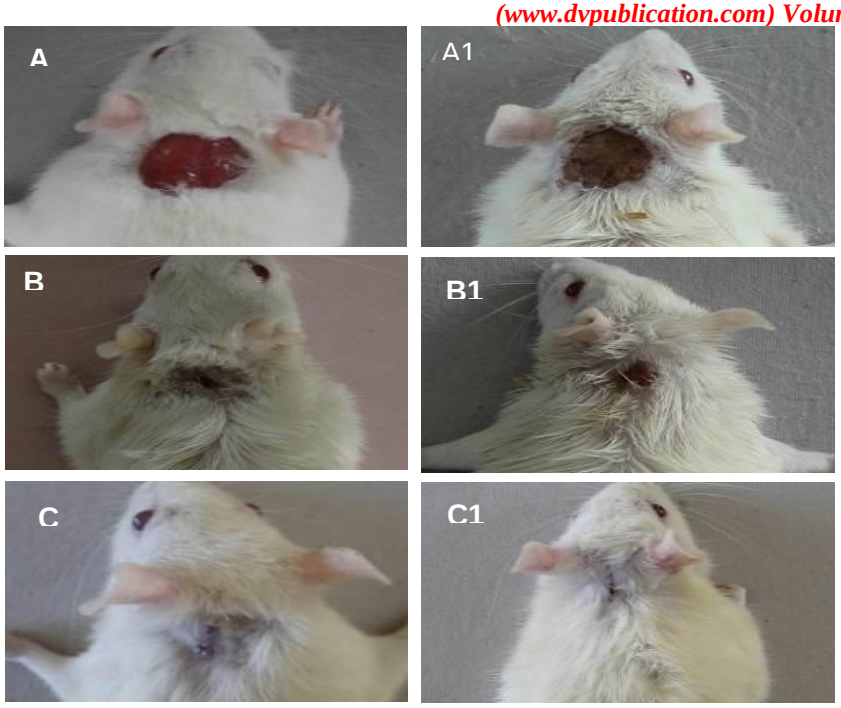


Figure 9: Excision wound contraction on 1st, 8th and 16th day in rat model treated with isolated compound SC2 and standard Povidone iodine. (A, B, C) SC2 treated; (A1, B1, C1) Povidone iodine treated.

b) Epithelialization Time: The period of epithelialization wounds treated with extracts and isolated compounds are shown in the fig. 10. It was observed that stem bark chloroform extract significantly reduced epithelialization time (14.25 ± 0.48 days) of wounds as compared to control animals (21.75 ± 0.25 days). It was found that the wound healing promoting activity of stem bark chloroform extract was more effective than the effect of standard drug povidone iodine ointment (14.5 ± 0.29 days). Faster rate of epithelialization was observed in the wounds treated with SC2: (Methyl (4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl} pent-4-enoate) (15.25 ± 0.25 days) Whereas, moderate and least wound healing activity was observed in the animals treated with the compound LC3: (β - Sitosterol) (17.5 ± 0.87 days) and leaves chloroform extract (16.75 ± 0.48 days) respectively.

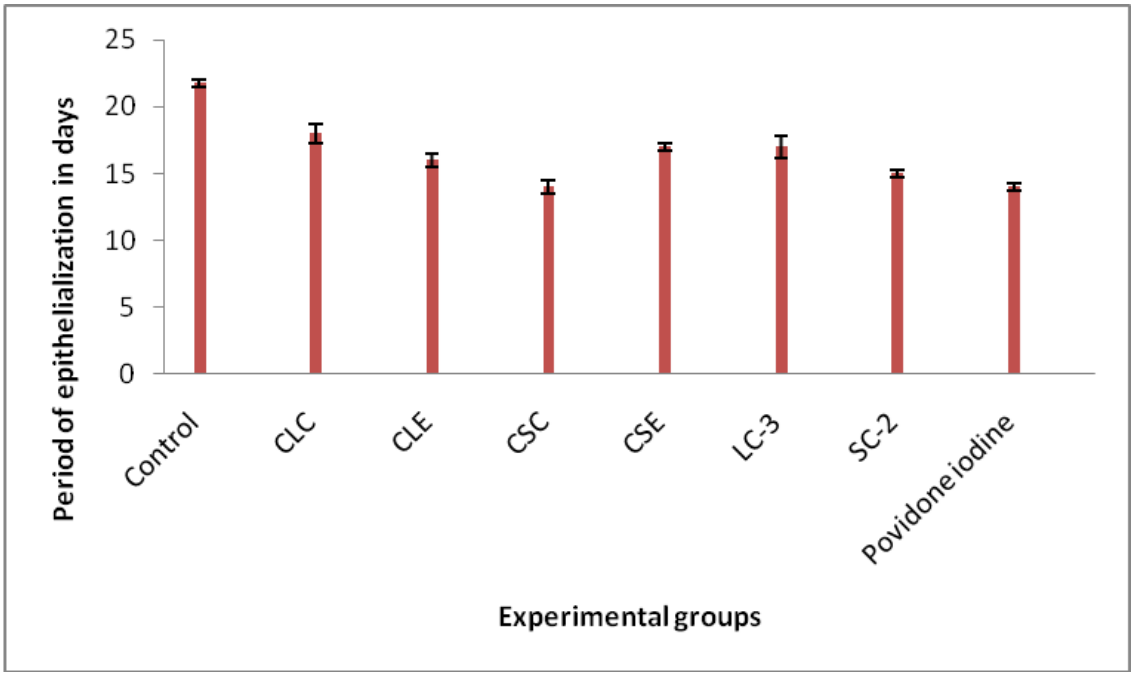


Figure 10: Period of epithelialization in control and different extracts of leaf and stem bark, and isolated phytoconstituents treated wounds. CLC (Leaves chloroform extract), CLE (Leaves ethanol extract), CSC (Stem bark chloroform extract), CSE (Stem bark ethanol extract), LC3: β Sitosterol; SC2: methyl(4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate.

c) Measurement of Tensile Strength: The tensile strength of incision wound rat model has been conducted for all the crude extracts and isolated constituents represented by the instrument. Among the crude extracts, the stem bark chloroform extract and leaves ethanol extract showed significant increase in the tensile strength on 10^{th} post wounding day (680.5 ± 12.5 and 671.5 ± 23.5 g respectively) as compared to control animals (476±12 g). The skin breaking strength of the animals treated with leaves chloroform extract showed moderate elevation (605 ± 14 g). Whereas, poor tensile strength (589.5 ± 21.5 g) has been found in stem bark ethanol extract treated wounds **(fig. 11)**. Among the isolated constituents, significant increase in skin breaking strength was noticed in the animals treated with the compound SC2: (Methyl (4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate) (621.5 ± 8.5) followed by LC3: (β Sitosterol) (524 ± 12 g). The animals treated with standard reference drug povidone iodine also showed significant increase in the tensile strength of the healed incision wound (710.5 ± 10.5 g).

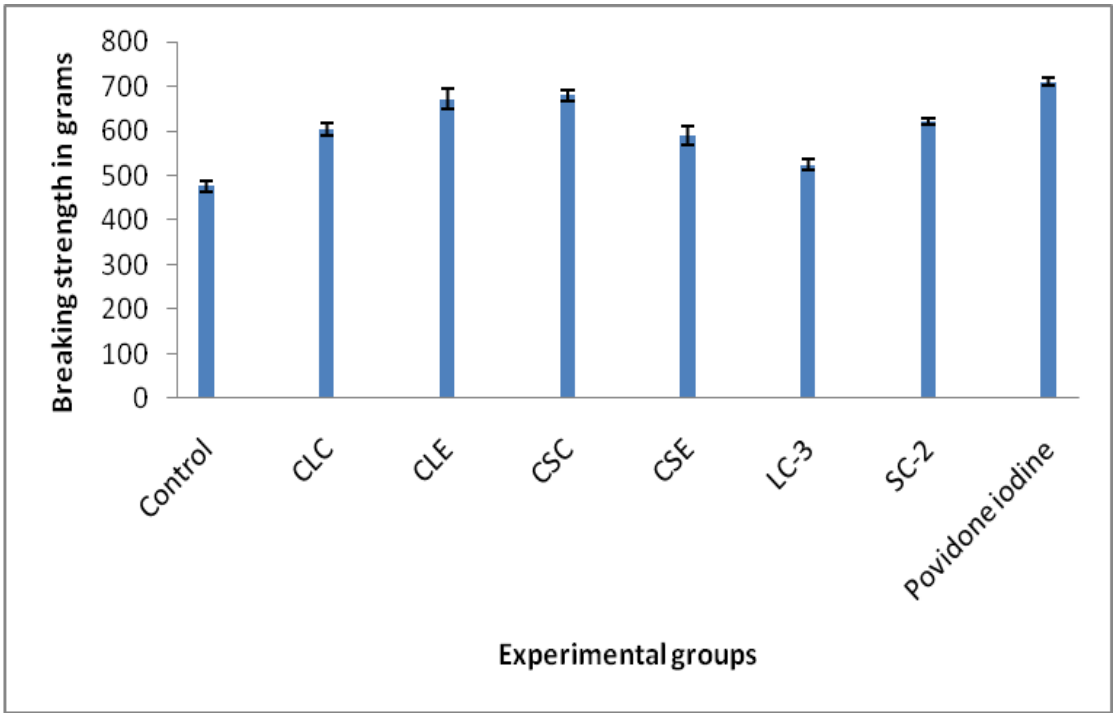


Figure 11: Tensile strength of incision model of rats treated with different extracts of leaf and stembark, and isolated phytoconstituents. CLC (Leaves chloroform extract), CLE (Leaves ethanol extract), CSC (Stem bark chloroform extract), CSE (Stem bark ethanol extract), LC3: β Sitosterol; SC2: methyl(4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate.

d) Estimation of Hydroxyproline, Hexosamine and Uronic Acid: The result estimation of hydroxyproline, uronic acid and hexosamine of the wound tissue of experimental and control animals are presented in the fig. 12A, B & C respectively. It revealed that the stem bark chloroform extract and leaves ethanol extract were significantly increased the hydroxyproline (4.54 ± 0.07 and 4.29 ± 0.13 mg/100 mg dry tissue), hexosamine (0.53 ± 0.02 and 0.55 ± 0.03 µg /100 mg dry tissue) and uronic acid (0.22 ± 0.01 and 0.19 ± 0.01 µg /100 mg dry tissue) content of the wound tissue. In control animals the values of hydroxyproline, uronic acid and hexosamine of the wound tissue were 3.04 ± 0.07 mg/100 mg dry tissue, 0.11 ± 0.01 and 0.35 ± 0.02µg /100 mg dry tissue respectively. The healing effect was moderate in animals treated with stem bark ethanol extract and least effect was observed in leaves chloroform extract treated animals. Among the compounds evaluated, the SC2: (Methyl(4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pe-nt-4-enoate) isolated from the stem bark chloroform extract have shown highest hydroxyl proline (3.98 ± 0.07 mg/100 mg dry tissue respectively), hexosamine (0.51 ± 0.02 µg/100 mg dry tissue) and uronic acid content (0.2 ± 0.01µg/100 mg dry tissue respectively. The healing potency of LC3: (β- Sitosterol) in elevating the levels of hydroxyl proline, hexosamine and uronic acid contents of wound tissue during healing process was moderate.

The significant wound healing activity was observed in the animals treated with the stem bark chloroform extract and its constituent SC2: ((Methyl (4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl} pent-4-enoate). Considerable decrease in the period of epithelialization, increase in the wound contraction rate, increase in the tensile strength and restoration in the levels hydroxyproline, hexosamine and uronic acid content of the wound tissue were observed in these groups of animals. Whereas, least wound healing activity has observe in the animals treated with the leaves ethanol extract.

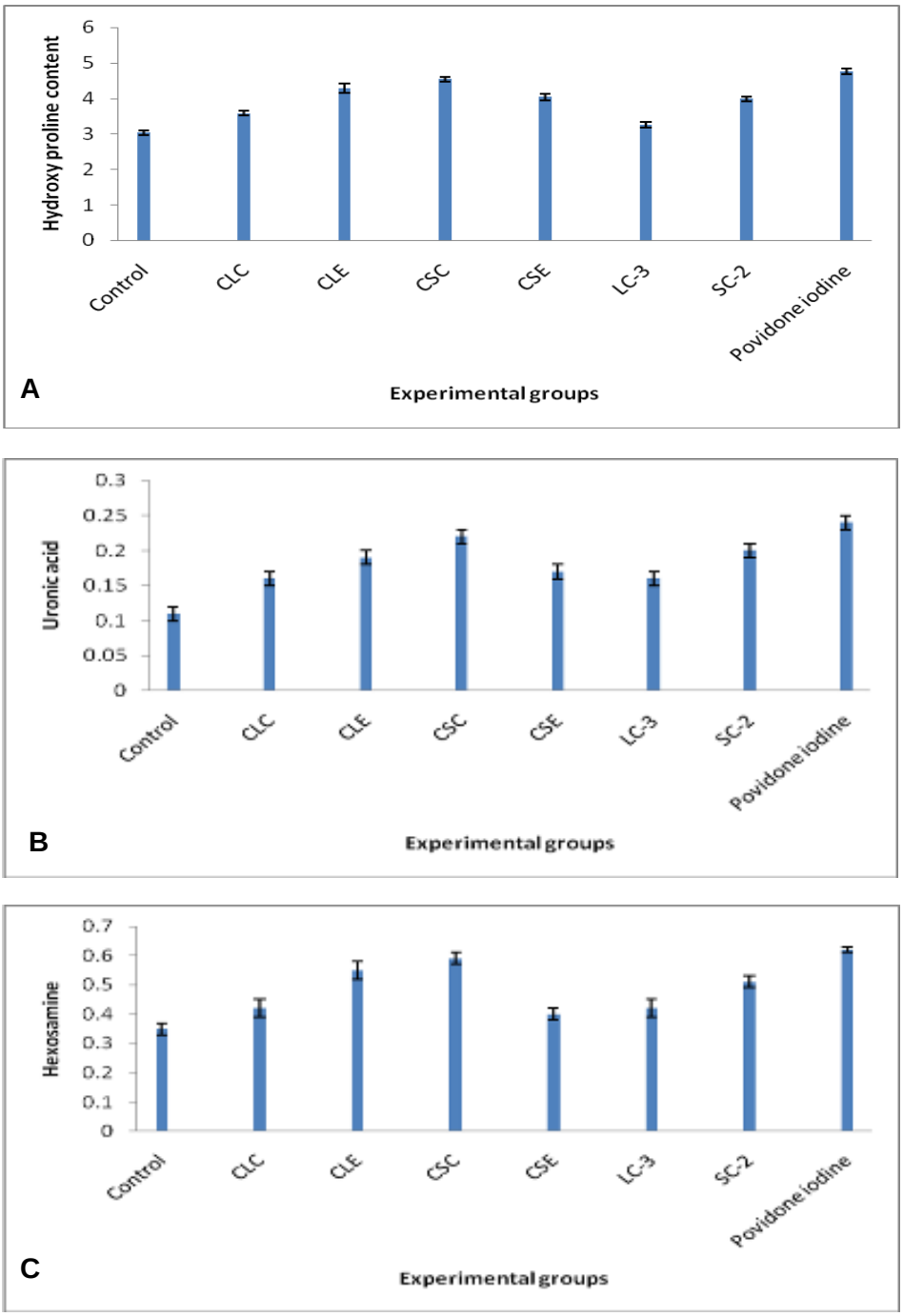


Figure 12: Hydroxyproline, Uronic acid and Hexosamine content of wound healing tissue of rats treated with different extracts of leaf, stembark and their isolated phytoconstituents.(A) Hydroxyproline content of the wound healing tissue of rats; (B) Uronic acid content of the wound healing tissue of rats ; (C) Hexosamine content of the wound healing tissue of rats treated different extracts of leaf and stembark, and isolated phytoconstituents. CLC (Leaves chloroform extract), CLE (Leaves ethanol extract), CSC (Stem bark chloroform extract), CSE (Stem bark ethanol extract), LC3: β-Sitosterol; SC2: methyl(4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxy-phenyl} pent-4-enoate.

e) Histopathology of Wound Tissue: Histological study of the granulation tissue provides further evidence on the wound healing efficacy of the extracts and the constituents. The section of the granulation tissue of the untreated animals showed monocytes and fibroblasts. Incomplete healing was evidenced with lesser epithelialization, fibrosis and collagen formation. The sections of the granulation tissues of the animals treated with stem bark chloroform extract showed complete epithelialization, fibrosis and collagen formation. Whereas, high deposition of collagen and significant reduction of macrophages infiltration has been noticed in histology of wound section treated with SC2: (Methyl (4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate).

The histological studies of the granulation tissue of the control group of the animals is showed in fig. 13A. Lesser epithelialization and collagen formation, with accumulation of macrophages indicated incomplete healing of the wound in control animals. In case of leaves chloroform extract treated animal groups, moderate collagen deposition and macrophages were observed (fig. 13B). Whereas, the animals administered with leaves ethanol extract, the healing activity was comparatively less evidence with less collagen deposition and more number of macrophages (fig. 13C). The sections of granulation tissue obtained from the stem bark chloroform extract treated animals showed significant increase in collagen deposition, few macrophages and more fibroblasts (fig. 13D). The stem bark ethanol extract showed less moderate effect of the granulation tissue in wound healing (fig. 13E). Wound tissue section of LC3: (β - Sitosterol) treated group showed moderate deposition of collagen content and reduction in the number of macrophages infiltration has observed (fig. 13F).

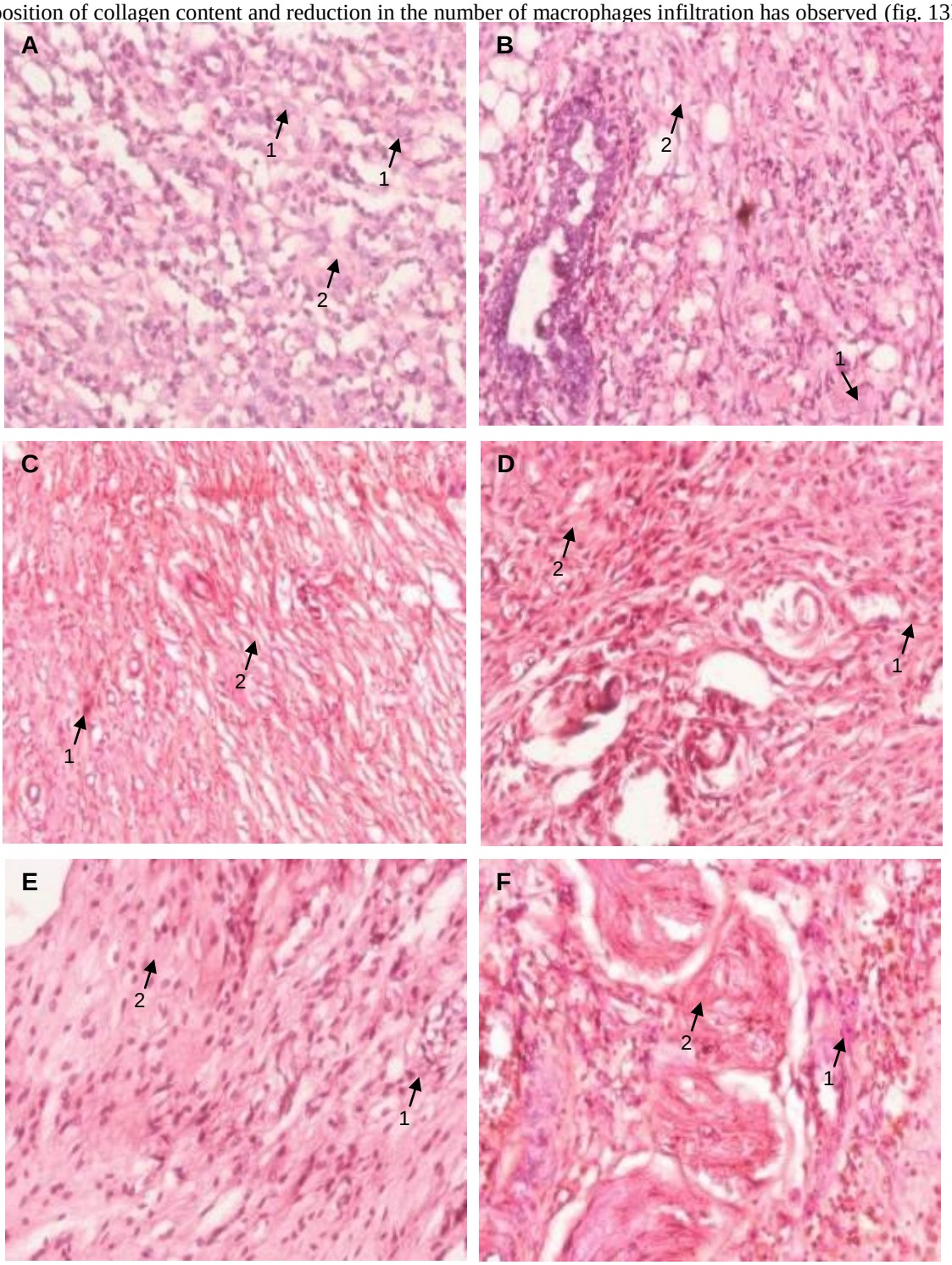


Figure 13: Histology of granulation tissue of RAT wound model showing wound healing effect of extracts and isolated compounds of *C. bonducella*. A) Control, B) Leaves chloroform extract treated, C) Leaves ethanol

extract treated, D)Stembark chloroform extract treated, E) Stem bark ethanol extract treated, F) LC3 treated. (1. Macrophages; 2, Collagen fibers).

High deposition of collagen and significant reduction of macrophages infiltration has been noticed in histology of wound section of the animals treated with SC2: (Methyl(4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate) which supports the significant wound healing promoting activity (fig. 13G). Similarly, the animals treated with povidone iodine also showed increased collagenation and decreased accumulation of macrophages at the site of injury (fig. 13H).

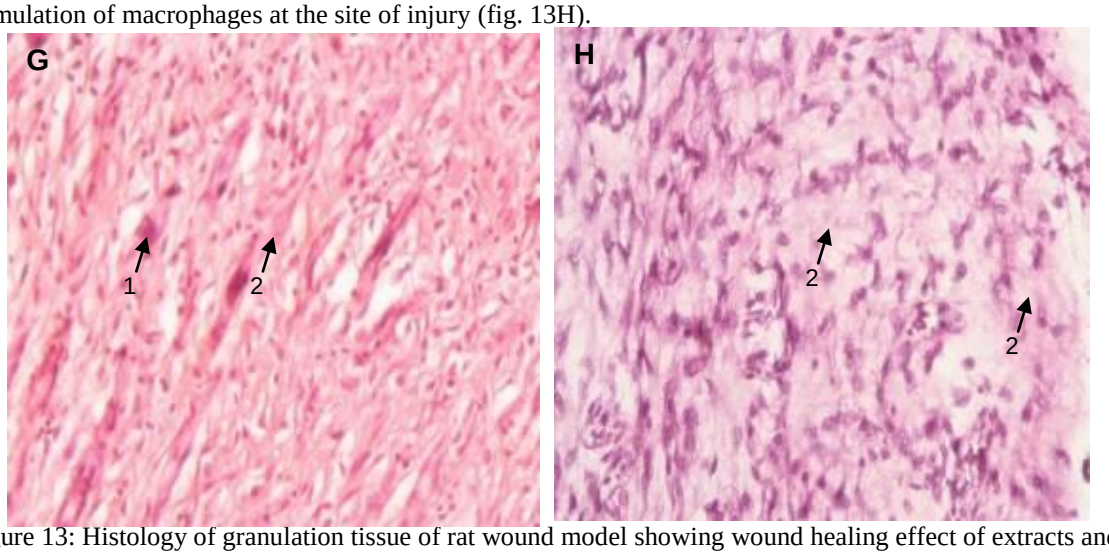


Figure 13: Histology of granulation tissue of rat wound model showing wound healing effect of extracts and isolated compounds of *C. bonducella*. G) SC2 treated, H)povidone iodine treated, (1. Macrophages; 2, Collagen fibers).

Discussion:

In the present investigation, it has been observed that the *C. bonducella* contains an amazing diversity of secondary metabolites like flavonoids, phenolic acids, alkaloids, saponins, tannins and steroids. Many investigators reported the terpenoids from the seeds of *C. bonducella* namely, Caesalpin ($C_{24}H_{32}O_8$) (1-ketone 6,7-diacetylcassane) M.W.-448.512, a-caesalpin ($C_{20}H_{28}O_6$) (1-ketone 5, 6, 7, 14-tetrahydroxy voucapanone) M. W. -364.438 and a-caesalpin ($C_{34}H_{56}O_7$) (o-tetradecanoylvoucapanediterpenoid) M. W.-576.812 were the bitter diterpenoids isolated from the seeds of *C. crista*(Blagoveshehenski&Aleksandrova, 1981). However, only the phytoconstituents of the leaves and the stem bark has not been explored in detail. Hence, in the present investigation has been under taken to screen the therapeutically active constituents of *C. bonducella.* Preliminary tests for qualitative phytochemical found that the leaves chloroform extract has shown positive results for the presence of terpenoids, sterols, flavonoids, saponins, glycosides and carbohydrates. From this extract The LC3: β - Sitosterol ($C_{29}H_{48}O$) were isolated. Based on the rules of IUPAC nomenclature the stem bark chloroform extract found SC2: Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxy phenyl}pent-4-enoate and were not yet reported by the earlier investigations.

Wound healing is a complex series of reactions and interactions among cells and mediators. In the present investigation, three different models viz., excision, incision and dead space wound were used to assess the effect of various extracts and the isolated phytoconstituents. The povidone-iodine was used as a standard reference to assess the healing potency of the crude drug and the constituents against the control (George Broughton & Attinger, 2006). Ghosh *et al*., (2012) also used povidone iodine as the standard drug to evaluate the wound healing effect of *Pedilanthustithymaloide*.In excision wound model, the significant wound healing activity was observed in the animals treated with stem bark chloroform extract. Significant decrease in the period of epithelialization and increase in wound contraction rate were observed in these group animals. In povidone-iodine treated animals, the epithelialization was completed on (14.5 ± 0.29 days) post wounding day. While in stem bark chloroform extract treated animals, the epithelialization was completed on 14.25±0.48 days. The phytoconstituents LC3 and SC2 showed epithelialization on 17.5±0.87 and 15.25±0.25 days respectively. In case of control animals, the rate of wound was extended up to 21.75 ± 0.25 days. In the incision wound model, the animals treated with stem bark chloroform extract significant increase in the breaking strength on 10^{th} post wounding day. Hydroxyproline, hexosamine and uronic acid estimation revealed that the stem bark chloroform extract and leaves ethanol extract significantly increased. These results are agreed with the report (Lingeraju et al., 2012). Compounds isolated from the stem bark chloroform extract *viz.*, SC2: methyl(4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate treated wounds have shown highest hydroxyproline (3.98 ± 0.07 mg/100 mg dry tissue respectively), hexosamine (0.51 ± 0.02 µg/100 mg dry tissue respectively) and uronic acid content (0.2 ± 0.01µg/100 mg dry tissue respectively) than other isolated compounds wound healing activity. The significant wound healing activity was observed in the animals treated

with the stem bark chloroform extract and its constituent SC2: (Methyl (4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl} pent-4-enoate).

Considerable decrease in the period of epithelialization, increase in the wound contraction rate, increase in the tensile strength and restoration in the levels hydroxyproline, hexosamine and uronic acid content of the wound tissue were observed in these groups of animals. Whereas, moderate and least wound healing activity was observed in the animals treated with the leaves ethanol extract. Histological study of the granulation tissue provides further evidence on the wound healing efficacy of the extracts and the constituents. The section of the granulation tissue of the untreated animals showed monocytes and fibroblasts. Incomplete healing was evidenced with lesser epithelialization, fibrosis and collagen formation. The sections of the granulation tissues of the animals treated with stem bark chloroform extract showed complete epithelialization, fibrosis and collagen formation. Whereas, high deposition of collagen and significant reduction of macrophages infiltration has been noticed in histology of wound section treated with SC2: (Methyl (4*E*)-5-{2-[(1*E*)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate).

Conclusion

The traditional claim indicated that the leaves and stem bark of *C. bonducella* have potential source of drug for wound healing. This investigation supported the traditional claim that stem bark chloroform extract and its isolated constituent Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate showed significant wound healing property. Further clinical trials are essential to confirm the therapeutic efficacy of these phytoconstituents.

Conflict of Interest:

The authors declare that there is no conflict of interests regarding the publication of this paper.

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