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Wound healing activity of the ethanolic extract and agnuside isolated from *Vitex negundo* L. Leaves

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Abstract
The wound healing effects of ethanolic extracts of leaves of *Vitex negundo* L. (Fam.Verbenaceae) as well as of the major iridoid, agnuside against the incision and excision wounds on Wistar albino rats were examined and elaborated in this study. The ethanolic extract and 25% agnuside which was enriched from the extract were used in this study. These experiments were designed in four groups of Wistar albino rats consisting of group I (control), group II (treated with *Vitex negundo* ethanolic extract), group III (treated with 25% agnuside) and group IV (treated with standard drug, Soframycin) for 0-15 days. This is the first report of the effects of 25% agnuside on wound healing in *in-vivo* experiments. Quick and effective incision and excision wound healing was observed in group III animals treated with 25% agnuside than in any other groups. The results were comparable to the treatment with soframycin. It is therefore, concluded that the active principle, agnuside in the traditional wound healing treatment using *Vitex negundo*.

Keywords: Agnuside, *Vitex negundo*, Iridoid, Wound healing activity

Introduction
Vitex negundo Linn., a small tree or large aromatic shrub commonly known as '*nirgundi*' in Ayurveda, is found throughout India from sea level to an altitude of 1500 m. This is a commonly used plant in traditional medicine, both non-codified and codified. The Ayurvedic Pharmacopoeia of India illustrates its anti-inflammatory and hepatoprotective potential [1]. The plant holds great secure as a commonly available medicinal plant (Fig.1) and it is indeed no overwhelm that the plant is referred to in the Indian traditional circles as *'sarvaroganivarini'* – the solution for all diseases [2]. *V.negundo* is a component of various commercially existing herbal formulations and had shown beneficial effects in a range of ailments [2, 3]. It is reported to possess various pharmacological activities, together with antioxidant [4], analgesic [5], anti-genotoxic [6], anti-inflammatory [7], anti-convulsant and central nervous system (CNS) depressant activity [8]. A wellknown neighborhood statement of the Bhangalis in the Western Himalayan locale of India which deciphers as –"A man can't kick the bucket of malady in a territory where *Vitex negundo, Adhatoda vasica and Acorus calamus* are found". [9]
The plant has a pungent, bitter; acrid taste. This plant has been used for various medicinal purposes in the ayurvedic and unani systems of medicine. Leaves are aromatic tonic and vermifuge. Decoction of nirgudi is given along with pepper in catarrhal fewer. Juice of leaves is said to have property of removing worms from ulcers. Oil is applied to sinuses [10]. Leaves are useful in dispersing swelling and useful as an antiallergic [11, 12]. Dried leaves are smoked for headache. All the parts are utilized, yet the leaves and the roots are critical as medications. Petroleum ether extract of *Vitex negundo* leaves has shown significant analgesic activity and anticonvulsant activity against strychnine and leptazole [13-15]. Dried leaves powder of *Vitex negundo* showed antiarthritic activity in rats [16]. *V. negundo* is reported to contain terpenoids [17], flavonoids [18, 19], iridoids [20], and lignans [21, 22].

Fig 1: *Vitex negundo* leaves and inflorescence

The most important therapeutic property of this plant is its use in traditional medicine for wound healing. The wound healing activity has been reported, the aqueous and ethanolic extract of the leaves were used [23], the aqueous extracts of the leaves only used [24] and The methanolic extracts of the leaves to see their effect on healing of artificially made wounds in white Wistar rats [25].

None of these investigators have analysed the chemical composition of these extracts and have tried the effect of any particular chemical constituent of these extracts. Hence,in the present study the effect of not only ethanolic extracts of the leaves but also the effect of agnuside, an important constituent of the extract are reported so as to highlight whether extracts are preferable or pure chemical is preferred for wound healing activity. 25% agnuside enriched from extract was evaluated for wound healing activity for the first time from this plant.

Materials and methods

Plant materials

The plant with leaves was collected from Kallakurichi, Villupuram district, Tamil Nadu, India during June 2013. The voucher specimen (Ref.RRCBI-AP4666) was deposited at National Ayurveda Dietetics Research Institute (NADRI), Bangalore -560 011, India. The taxonomic identification was done by Dr. B.N.Sridhar, Assistant Director, (NADRI), Bangalore -560 011. The fresh leaves were washed with tap water, rinsed with distilled water, air-dried for an hour, washed and rinsed once again thoroughly in distilled water and shade-dried in open air. The dried leaves were then pulverized and the coarse powder obtained was sieved in 20 mesh sieve.

Extraction

1 Kg of the powdered leaf is extracted with 100% ethanol at Soxhlet apparatus at 60° - 65°C for 3h. The extraction was repeated three more times. The extracts of leaves were filtered through polypropylene cloth concentrated and dried in vacuum; the yield was recorded. The extract obtained was used for phytochemical analysis and for studying wound healing activities.

Isolation of Agnuside

Agnuside is an Iridoid glycoside which is an ester of acubin and parahydroxybenzoic acid. Although the leaves of *Vitex* contains agnuside and Negundoside.Present interest was on agnuside only (Fig.2). The details of the chemicals obtained from this plant is given below

Agnuside

Name of the Compound	: Agnuside
Molecular formula	: $C_{22}H_{26}O_{11}$
Molecular weight	: 466.439 g/mol

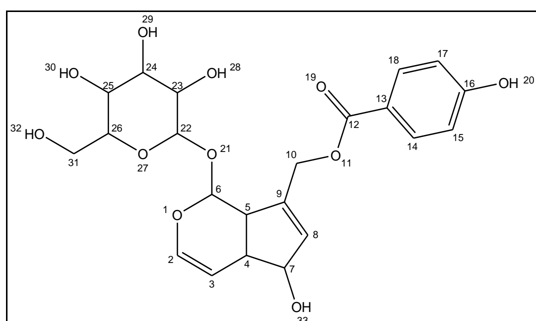

Fig 2: Structure of Agnuside

1 kg of leaves is extracted with four volumes of 100% Ethanol at reflux temperature (60°- 65°C) for three times and filtered through a polypropelene cloth. The filtrate was collected and all the ethanol extract was pooled and concentrated in vaccuo. Yield of extract is 12%. The ethanolic extract is dissolved in 30% w/v of ethanol. Six volumes of acetone were added and precipitated and allowed to settle. The mother liquor was collected and the solvent was stripped off in vacuum at 60°C. The concentrated extract was redissolved in 30% ethanol then the extract was partitioned with 2 volumes of chloroform X three times. The aqueous ethanol phase was collected and concentrated under vaccuo.

TLC analysis of aqueous layer confirm the presence of agnuside (Solvent system: ethyl acetate: methanol: water: glacial acetic acid (80:12:6:2). Concentrated aqueous ethanol layer was subjected to column chromatography using Silica Gel (60-120 mesh) prepacked with Chloroform. This was eluted with chloroform and followed by 0 to 12% methanol to collect the fractions. 10% methanol fraction gave single spot in TLC system. The collected fractions were concentrated and crystallized by mixture of chloroform and methanol got off white amorphous powder. Pure compound weight is recorded. The isolated compounds were characterised by using various spectral instruments like LCMS, NMR (^{1}H and ^{13}C NMR).

Procedure

^{1}H NMR (300 MHz) and ^{13}C NMR (100 MHz): TMS as int. standard. Silica gel 60 (70-270 mesh, Merck) was used for CC and silica gel $G_{60}F_{254}$ (Merck) plates for TLC. Substances were detected by UV light or by spraying H_2SO_4-EtOH (10:90). HPLC LaChrom, DAD detector (Merck-Hitashi).

HPTLC finger print analysis

HPTLC chromatography is an advanced technique which is used to detect the phytochemicals present in two altered wavelengths (254 nm and 366 nm). CAMAG HPTLC System, equipped with a Linomat V with CAMAG 100µL syringe sample applicator, a model Camag twin trough glass chamber (20cm × 10cm) and a thin layer chromatography (TLC) scanner and Camag TLC scanner 3 software Win Cats 4.3.1 version were used in the study. 10 µl of ethanolic extract and 25% agnuside were applied to tracks in TLC aluminium sheets (10 cm × 10 cm) of silica gel G $_{60}$ F 254. The plates were developed with up to 90 mm after chamber saturation conditions. After air drying the solvent, the plates were taken a photograph and recorded.

HPTLC Fingerprinting profile

HPTLC fingerprinting profile is attempted using different mobile phases for the separation of ethanolic extracts along with the isolated compound, Agnuside. Screening was done using three different solvent systems: ethyl acetate: methanol: water: glacial acetic acid (80:12:6:2), another mobile phase as hexane: ethyl acetate: methanol (7:3:1) and $CHCl_3$: MeOH: (85:15). However, the suitable mobile phase is ethyl acetate: methanol: water: glacial acetic acid (80:12:6:2), the application volume was 10 µL each.

HPLC Analysis

100 mg of the ethanol extract was dissolved in HPLC methanol and diluted to 100 mL to obtain a concentration of 2.5 mg/mL. The prepared ethanol extract solution was passed through a 0.45 µm filter to acquire a clear solution. 25mg of standard agnuside was prepared in the same manner as like sample solution to obtain concentrations 2.5µg/mL of

agnuside. The ethanol extract and the standard solution were subjected to HPLC separately. A gradient HPLC (Shimadzu HPLC Class VP series) with two LC- 10 AT VP pumps (Shimadzu), UV visible detector SPD-10A, an SCL-10A VP system controller (Shimadzu), a reverse phase Luna® 5 µm C18 (2) YMC ODS column (250 mm X 4.0 mm) was used. The mobile phase constitutes mixture of water and methanol (90:10) ratio (Mobile Phase A) and mixture of water and methanol (50:50) ratio (Mobile Phase B) which were filtered through a 0.2 µm membrane filters before use and pumped from the solvent reservoir at a flow rate of 1.2 ml/min. About 20 µL of the respective sample was injected by using a Rheodyne syringe (Model 7202, Hamilton) and the HPLC peaks were observed at UV detector at 258 nm. Further analysis was carried out to compare the HPLC chromatogram of *Vitex negundo* ethanol extract against standard compound agnuside.

In –Vivo studies on wound healing

Selection of experimental rats

Wistar albino rats weighing 250-280 g of both sexes were used for the study. The animals were kept in standard conditions. The animals were fed pellets (HLL, Kolkatta) the room temperature was maintained 22°±2°C with food and water *adlibitum*. The animals were transferred to the lab at least 2h before start of the experiments. The procedures for conducting the wound healing activities in rats were permitted by Institutional Animal Ethics Committee. (IAEC) (Regn. No: SAC/IAEC/BC/2015/Ph.D-002 dated 14.07.2015).

Wound Incision in a rat

Incision in a rodent Incision wound was made under light ether anesthesia; two paraventral incisions of 6 cm were made through the whole thickness of skin on either side of vertebral section with the assistance of a sharp cutting edge. After the incision was made the separated skin was kept together and sutured with nylon string by 0.5cm. Careful string (No.000) and curved needle of No.11 were utilized for suturing. The nonstop string of both injury edges was fixed for good selection of wounds. Tensile strength represents the support of wound healing. (After creating the wounds, all the animals in the groups were kept independently for 0-3 days) [26].

Wound excision in a rat

An excision wound was made on the dorsal side of rats. The dorsal side of rats was shaved with an extremely sharp edge razor blade. Excision wound of size 2.5cm^2 areas of skin in long, 0.2cm^2 in deep inside was created by utilising surgical scissors. Haemostasis was achieved by smearing the wound with cotton swab absorbed in ordinary saline. The wound was left undressed to the open condition. (After creating the wounds, all the animals in the groups were kept independently for 0-3 days) [26].

Preparation of the ointment base

The ointment was prepared using plant extracts with Simple Ointment Base (Petroleum Ointment). For each batch, 100 g of blank petroleum jelly B.P was weighed into a beaker and then melted using thermostatic water bath. The required quantities of the extracts and the compounds were weighed and added to the molten ointment base and then homogenized well. 5g of the prepared ointment was topically applied on the surface of the wounds of the experimental animals.

Experimental design in Incision and Excision wounding

Six rats were used as controls and the wounds were treated with crude extract and pure allopathy drug.

Group I: Normal incision/ excision wounded animals without any treatment (Control)

Group II: Normal Incision/excision wounded animals treated with *Vitex negundo* leaf ethanol extract for 15 days.

Group III: Normal Incision/excision wounded animals treated with 25% Agnuside for 15 days.

Group IV: Normal incision/excision wounded animals treated with standard drug soframycin ointment for 15 days.

Analysis of wound contraction

The level of wound decrease was dictated by the decrease in wound size. The injury estimate was estimated routinely by following injury site with follow paper and estimated graphically. After incision (0 to 15 days), the staying wound was estimated by utilizing vernier caliper. The surface region of the injury was estimated while leading the analysis. The decrease of wound size was checked from the underlying and last zone of the injury. The standard deviations and mean deviations were given in cm.

$$\text{The wound reduction \%} = \frac{\text{Area of the initial wound - area of the actual wound}}{\text{Area of the initial wound}} \times 100$$

The rats were sacrificed after 15 days of study, after taking the bleed samples.

Biochemical estimations

The following biochemical measurements/estimations were made on the experimental rats and control rats: (i) Tensile Strength of skin [27] (ii) Analysis of granuloma weight [28] (iii) Analysis of platelet derived growth factor, (iv) Analysis of TNF alphas, (v) Analysis of hydroxyproline [29] (vi) Analysis of hexosamine in granulated tissue [30], (vii) Analysis of enzymes in experimental animals, (viii) Analysis of the serum Ascorbic acid levels in experimental animals, (ix) Analysis of nucleic acid content by diphenylamine reaction [31], (x) Analysis of RNA content [32].

Statistical analysis

All values are mentioned as the mean ±SD. Animal group comparison was statistically made under the null hypothesis in one-way analysis of variance (ANOVA) test followed by multiple range tests employing SPSS 11.0 for windows. $P < 0.05$ was considered statistically significant by suing T test.

Results

Characterization of the compound

The structure of the isolated compound (Fig. 2) obtained by column chromatography was off white amorphous powder, weight is 16 gm (1.6%, 72% recovery), with melting point 148.9°C; the characterization of this compound was carried out using spectroscopic techniques. The molecular weight of the compound was obtained by using Liquid Chromatography-Mass Spectrometry (LC-MS). The mass spectrum showed parent molecular ion m/z at 465.06 $(M-H)^-$ APCI, Negative ion mode (Fig.3) which confirms the assignment of molecular formula of $C_{22}H_{26}O_{11}$.

Recorded ^1H NMR and ^{13}C NMR spectrum (Fig 4), values are given in Table 1

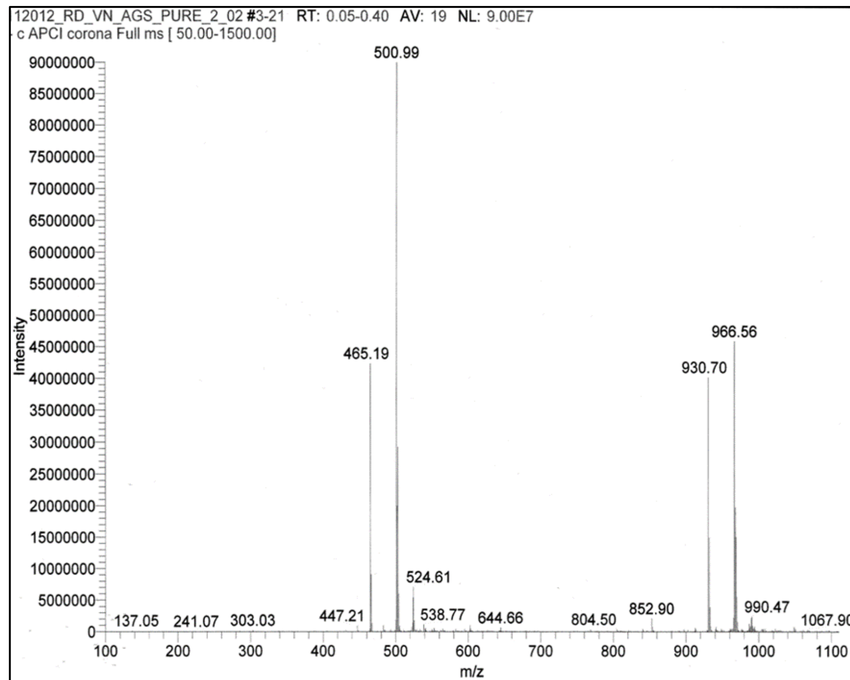

Fig 3: Mass fragmentation pattern of Agnuside

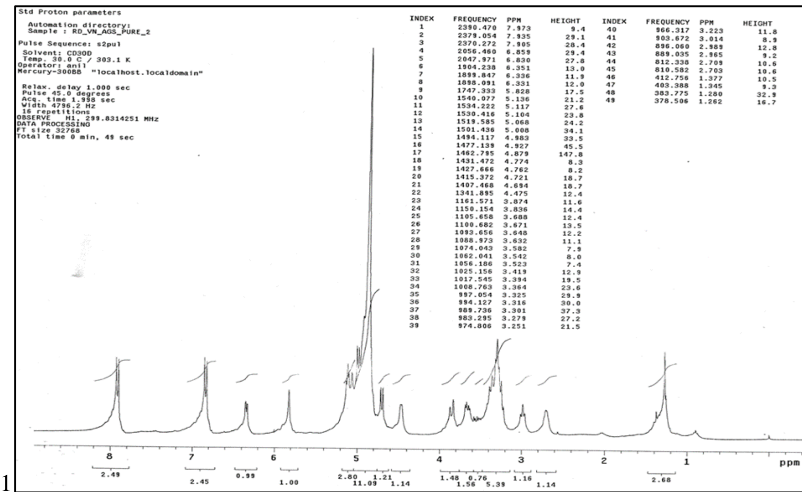

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Fig 4(a): Expansion representation of ^{1}HNMR spectra of agnuside

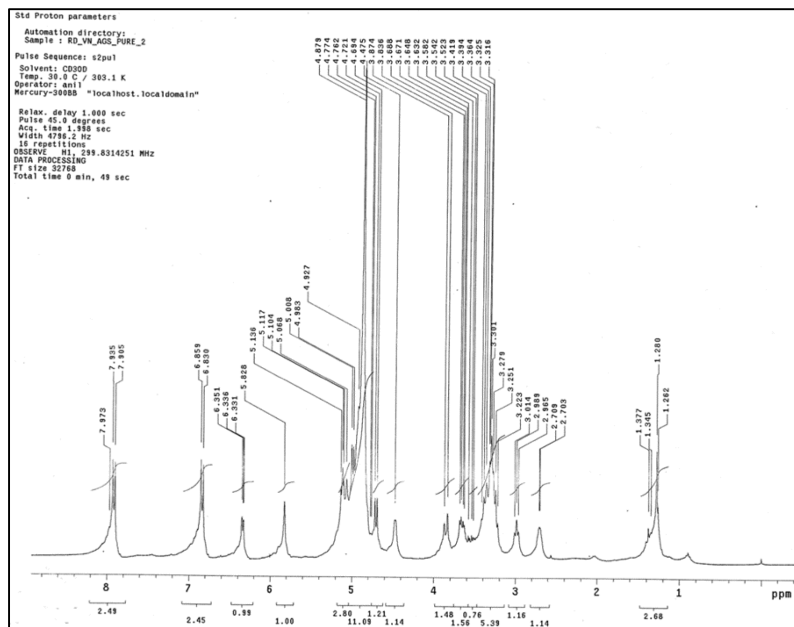

Fig 4(b): Expansion representation of ^{1}HNMR spectra of agnuside

^{1}HNMR (CD_3OD,300 MHz); δ 3.0 – 4.0 (m, 4H), δ 4.4(s,1H), δ6.35(s,1H), δ5.82(s,1H), δ4.8 – δ5.1 (m,1H), δ4.69(m,6OH), δ6.85(d,J=8.7Hz,1H), δ7.93(d,J=9Hz,1H).

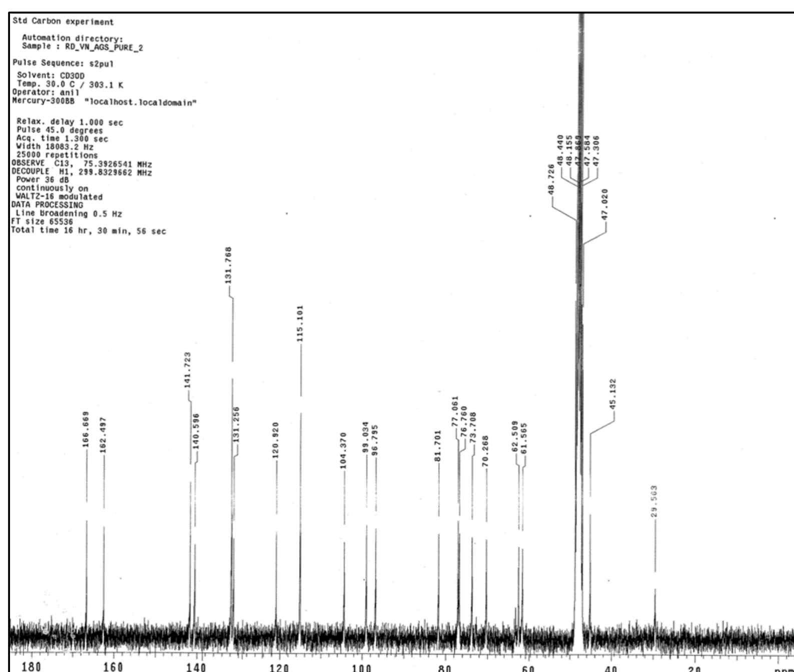

Fig 5: ^{13}C NMR spectra of the isolated compound (agnuside)

Table 1: ^{13}C NMR (CD_3OD) data of agnuside

Carbon atom No	Chemical shift (ppm)	Carbon atom No	Chemical Shift (ppm)	Carbon atom No	Chemical Shift (ppm)
1	96.795	1’	99.034	1’’	120.920
3	140.596	2’	73.708	2’’,6’’	131.256
4	104.370	3’	77.061	3’’, 5’’	115.101
5	45.132	4’	70.268	4’’	162.497
6	81.701	5’	76.760	C=O	166.669
7	131.768	6’	61.565		
8	141.723				
9	48.720				
10	62.909				

Based on the spectroscopic data (Fig 4(a & b), 5 & Table 1), the structure of the compound was derived as Agnuside and ^{1}H,^{13} C NMR values are well matches with the reported data. [33]

The Reference standard agnuside was eluted at RT 17.0 min, and our samples also eluted more or less the same RT at 17.20 min (Fig 6). The Agnuside content in dried *vitex negundo* extract is 2.2% of agnuside on raw material basis (ORB).

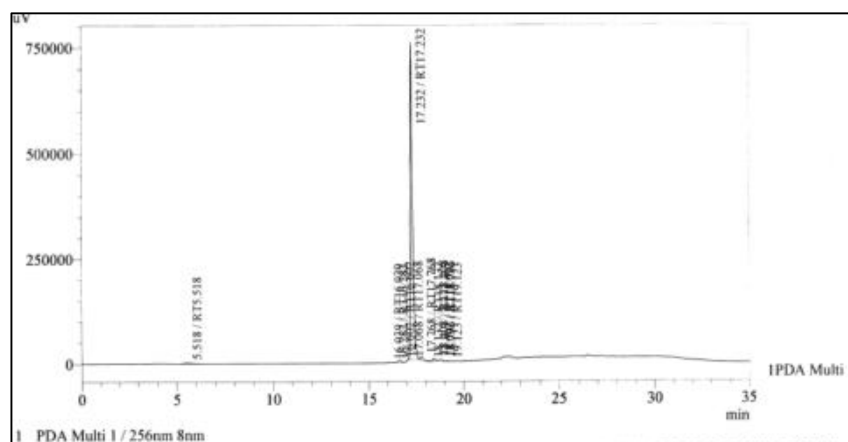

Fig 6: HPLC chromatogram of the isolated compound (agnuside)

HPTLC Fingerprinting profile

From our results, it was clear that the calibration curve was linear; the correlation coefficient value of 0.99 indicated the good linearity between the area and the concentration (results not shown). The obtained Rf values from the chromatogram matched well with

From our results, it was clear that the calibration curve was linear; The correlation coefficient value of 0.99 indicated the good linearity between the area and the concentration (results not shown). The obtained Rf values from the chromatogram matched well with The obtained R_f values from the chromatogram matched well with reference standard agnuside in the peak display at 10 uL applied volume. From figure 7, it is further observed that, R_f 0.27 confirmed the presence of agnuside. HPTLC fingerprinting at two different wavelengths and after derivatisation with anisaldehyde sulphuric acid reagent as shown in the representation fig. 7 a,b and c.

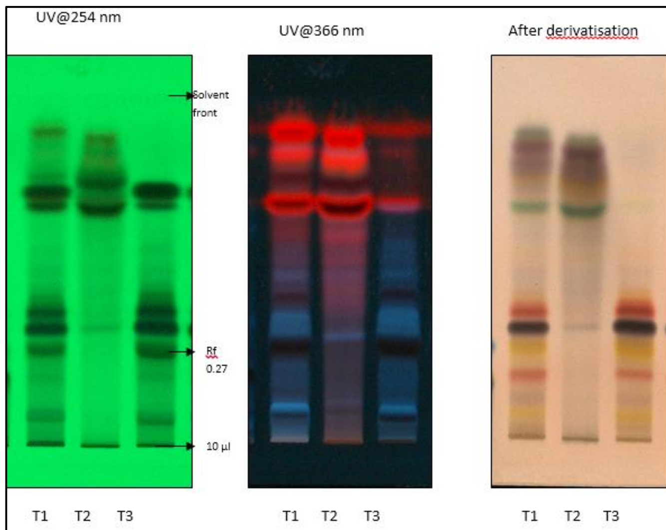

Fig 7 (a, b & c): Picture of HPTLC plates at UV @254 nm, UV 366 nm and after derivatisation with Anisaldehyde sulphuric acid reagent

HPTLC Track Legends:

T1: *Vitex negundo* ethanol extract; T2: Chloroform fraction; T3: 25% agnuside extract

Biological activity

Acute Toxicity study

The ethanolic extract was studied for acute toxicity. It was found to be safe and there was no mortality upto the dose of 2000mg/kg body weight in rats. So, the drug was safe for the study. The drug is practically nontoxic, as its LD_{50} dose recorded was 7.5 g/kg/wt. [34]

Wound healing activity

Our studies showed an improved rate of wound contraction and reduction in healing time in animals treated with ointment containing *V.negundo* leaf extracts in both incision (Table 2) and excision wounds (Table 3). 25%Agnuside (Group III) was found to be the most effective and quickest when compared to the ethanolic extract (Group II) tried in both types of wounds. The wound healing rate was significantly greater than that of the control and almost nearer to that of the standard drug, soframycin. (Table 2 & 3)

Table 2: Rate of Wound Contraction in experimental animals – Incision wound

Groups	Wound Contraction (cm)		Wound Contraction (%)
	0th Day	15th Day	
Group I	2.07±0.03	1.64±0.01	20.48
Group II	2.15±0.02	0.94±0.01 a	56.12
Group III	2.05±0.02	0.35±0.01 a	82.93
Group IV	2.14±0.01	0.27±0.01 a	87.21

Values are Mean ± SE, n = 6; a $p < 0.05$ - Statistically significant when compared to Group I.

Table 3: Rate of Wound Contraction in experimental animals – Excision wound

Groups	Wound Contraction (cm)		Wound Contraction %
	0th Day	15th Day	
Group I	2.05±0.04	1.57±0.07	23.58
Group II	2.15±0.03	0.80±0.03a	62.79
Group III	2.07±0.03	0.42±0.02 a	79.84
Group IV	2.20±0.05	0.35±0.04 a	84.09

Values are Mean±SE, n = 6;a $p<0.05$ - Statistically significant when compared to Group I.

This result of ours (Table 2 & 3) indicates that the active component of this plant agnuside is responsible for wound healing effect.

Wound healing is a complex process and involves quite a lot of physiological and structural changes beginning with wound closure, repair and replacing of injured tissues with new tissue formation. This complexity can be better understood by studying tensile strength [35], indirectly indicates concentration and stabilisation of collagen fibres [36] which forms the muscle tissue during wound healing. Results of studies made on tensile strength in incision wounds are summarised in Table 4. Here 25% concentration of agnuside promoted wound healing with greater tensile strength of skin than other treatments and was fairly nearer to soframycin treatment. ANOVA test clearly indicates this significance of the values obtained and presented in Table 4. This result indicates that the agnuside of this plant are probably involved in promoting tensile strength, epithelialisation and granuloma weight.

Table 4: Tensile strength of skin, Epithelialisation and Granuloma weight in experimental animals – Incision wound

Groups	Tensile strength of skin (g)	Epithelialization (Days)	Granuloma weight (mg/100g)
Group I	213.50±1.15	14.17±0.31	36.33±0.42
Group II	326.00±1.93 a	7.83±0.40 a	63.17±0.40 a
Group III	404.33±1.89 a	5.67±0.33 a	76.33±0.56 a
Group IV	396.83±1.17 a	6.83±0.48 a	83.33±0.61 a

Values are Mean±SE, n = 6;a $p< 0.05$ - Statistically significant when compared to Group I

Animals treated with ethanol extracts showed faster epithelialization of wound (16 days) than the wistar albino rats treated with aqueous extract (17 days). The period of epithelialization was 14 days with respect to standard drug used. [37]

Higher granuloma weights are associated with 25% agnuside treatment and soframycin. This results conclude that these two are higher to other treatments in wound healing and the results obtained are statistically significant ($p<0.05$) (Table 4). An increase in breaking strength which may be due to the increase in collagen concentration and stabilization of the fibres [38]. The collagen molecules synthesized are set down at the wound site and turn into cross-linked to form fibre. Wound strength is acquired from both remodelling of collagen and the formation of stable intra- and intermolecular cross links. 25% agnuside showed greater breaking strength which may be due to increased collagen synthesis.

Excision wound showed faster healing compared with control group. The faster wound contraction may be due to stimulation of interleukin-8, an inflammatory α-chemokine which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes, and may increase the gap junctional intracellular communication in fibroblasts, and induces a more rapid maturation of granulation tissue [39]

A few biochemical parameters were contemplated in the animals subjected to wound healing in order to understand the biochemical changes connected with wounding and wound healing the results of these studies are presented in Tables 5 to 11 under both incision wounds and excision wounds.

The level of Platelet derived growth factor (PDGF) is an essential factor in managing cell development and division. Since wound healing involves cell development, cell division

and cell separation, PDGF expect incredible significance in wound healing. [40].
The results of PDGF assay in different treatment are given in table 4 under incision/excision wound. It is evident from this table that the level of PDGF in control wounds is the minimum among values observed in ethanolic extract while the values in all other treatments are almost the same as that of soframycin.

Table 5: Platelet derived growth factor and TNF - Alpha in experimental animals

Groups	Platelet derived growth factor (pg/ml)		TNF - Alpha (pg/ml)	
	Incision	Excision	Incision	Excision
Group I	6.25±0.38	9.74±0.44	191.79±1.52	220.33±1.52
Group II	27.50±0.56 [a]	30.44±0.50 [a]	106.29±0.60 [a]	135.83±0.60 [a]
Group III	23.33±0.49 [a]	26.49±0.33 [a]	84.12±1.23 [a]	114.33±1.23 [a]
Group IV	29.83±0.60 [a]	33.43±0.76 [a]	104.10±0.88 [a]	135.33±0.88 [a]

Values are Mean±SE, n = 6;[a] $p < 0.05$ - Statistically significant when compared to Group I

The inflammatory phase in wound healing is suspicious to be a preparatory process for the structure of new tissue. A monocyte-derived cytokine, tumor necrosis factor-alpha (TNF-alpha), is a highly conserved molecule that is known to play a major role in the pathogenesis. TNF-alpha may have either a valuable or detrimental role in wound healing. [41]
The results of TNF - alpha assay made by us in different treatment are given in table 5. It is obvious from this table that the level of TNF - alpha in control wounds is more when compared to values observed in other treatments (Table 5). It is also very significant to note that TNF alpha level is the least in 25% agnuside treated rats; probably here wound healing proceeds without much inflammation of wounded area. This is also a very important in the wound healing process.
The hydroxyproline content in rat treated with ointment containing 25% agnuside was noteworthy (see Table 6) and was more or less comparable to the soframycin treatment. Since hydroxyproline binds to protein to develop a hydroxyproline-rich proteins under stress conditions, the wound healing most likely involves these proteins and promotes faster healing as well as to help the animal to bear this stress. This is also supported by the high level of hydroxyproline experimental in soframycin treated rats.

Table 6: Levels of Hydroxyproline and Hexosamine in granulated tissue of experimental animals – Incision/ Excision wounds

Groups	Hydroxyproline (mg/ g tissue)		Hexosamine (mg/ g tissue)	
	Incision	Excision	Incision	Excision
Group I	31.56±1.78	30.67±1.82	3.19±0.32	3.51±0.31
Group II	55.56±0.44 [a]	58.00±2.20 [a]	7.14±0.44 [a]	7.38±0.44 [a]
Group III	73.56±1.26 [a]	75.33±3.61 [a]	10.92±0.41 [a]	11.16±0.41 [a]
Group IV	82.67±0.69 [a]	88.00±2.04 [a]	12.46±0.17 [a]	12.70±0.17 [a]

Values are Mean±SE, n = 6; [a] $p < 0.05$ - Statistically significant when compared to Group I.

Hexosamine HXA content was significantly increased in treated group compared to the control group. ($p < 0.05$). The results with 25% agnuside treatment were comparable with Soframycin drug (Table 6).
Lipid peroxidation (LPO) is the process in which free radicals "steal" electrons from the lipids in cell membranes, causing cell damage. The end products of lipid peroxidation are reactive aldehydes, such as malondialdehyde (MDA), the major bioactive marker of lipid peroxidation. It is due to its numerous biological activities resembling activities of reactive oxygen species. (Table 7)

Table 7: Levels of non-antioxidant enzymes in granulated tissue of experimental animals –Incision / Excision wounds

Groups	LPO (nmol MDA/g tissue)		GSH (µg/ g tissue)	
	Incision	Excision	Incision	Excision
Group I	117.96±1.42	119.25±1.41	1.4±0.08	3.1±0.09
Group II	63.08±1.24 [a]	63.61±1.31 [a]	2.6±0.23 [a]	4.3±0.21 [a]
Group III	41.48±0.70 [a]	42.31±0.57 [a]	4.2±0.20 [a]	5.9±0.20 [a]
Group IV	43.38±3.35 [a]	42.78±1.83 [a]	5.5±0.54 [a]	6.8±0.40 [a]

Values are Mean±SE, n = 6;[a] $p < 0.05$ - Statistically significant when compared to Group I

Glutathione (GSH) enzyme in granulated tissue is used in metabolic and biochemical reactions such as DNA synthesis and repair, protein synthesis, prostaglandin synthesis, amino acid transport, and enzyme activation. Thus, every system in the body can be affected by the state of the glutathione system, especially the immune system, the nervous system, the gastrointestinal system, and the lungs. GSH has a vital function in wound healing through the control gated channels in cell membranes. Glutathione (GSH) participates in leukotriene synthesis and is a cofactor for the enzyme glutathione peroxidase. [42]
Superoxide dismutase (SOD) has powerful anti-inflammatory activity. For example, SOD is a highly effective experimental treatment of chronic inflammation in colitis. Treatment with SOD decreases reactive oxygen species generation and oxidative stress and thus, inhibits endothelial activation. The higher levels of SOD in 25% Agnuside and soframycin treated wounds support the above statement. (Table 8)

Table 8: Levels of antioxidant enzymes in granulated tissue of experimental animals– Incision/Excision wounds

Groups	SOD (U/mg protein)		CAT (U/mg protein)		GPX (U/mg protein)	
	Incision	Excision	Incision	Excision	Incision	Excision
Group I	3.81±0.25	4.51±0.35	3.80±0.81	6.00±0.64	2.04±0.19	2.44±0.06
Group II	6.15±0.51 [a]	6.32±0.55 [a]	8.52±0.78 [a]	10.20±0.53 [a]	4.33±0.16 [a]	4.95±0.05 [a]
Group III	10.39±0.44 [a]	10.83±0.43 [a]	11.70±0.43 [a]	13.20±0.46 [a]	7.84±0.10 [a]	8.34±0.14 [a]
Group IV	11.10±0.92 [a]	11.73±0.91 [a]	11.95±0.45 [a]	13.58±0.45 [a]	7.74±0.11 [a]	8.20±0.02 [a]

Values are Mean±SE, n = 6;[a] $p < 0.05$ - Statistically significant when compared to Group I

Chloramphenicol acetyl transferase (CAT) is a bacterial enzyme that detoxifies the antibiotic chloramphenicol and is responsible for chloramphenicol resistance in bacteria. This enzyme covalently attaches an acetyl group from acetyl-CoA to chloramphenicol, which prevents chloramphenicol from binding to ribosomes A histidine residue, located in the C-terminal section of the enzyme, plays a central role in its catalytic mechanism.Higher levels of this enzyme activity is observed in 25% agnuside when compared to the control and soframycin treated animals indicating the high anti-oxidant activities in these treatments.

Glutathione peroxidise (GPX) is an antioxidant enzyme. GPX functions in the scavenging and inactivating of hydrogen and lipid peroxides, thereby protecting the body against oxidative stress. High levels of this enzyme in group III and group IV animals indicate its importance as a strong antioxidant. (Table 8)

Along with RNA and proteins, DNA forms one of the three major macromolecules that are essential for all known forms of life.

Table 9: Levels of nucleic acid content in granulated tissue of experimental animals - Incision / Excision wounds

Groups	DNA (mg/g of tissue)		RNA (mg/g of tissue)	
	Incision	Excision	Incision	Excision
Group I	4.54±0.33	5.42±0.47	7.4±0.4	7.9±0.5
Group II	9.84±0.89 [a]	11.38±0.98 [a]	15.0±0.5 [a]	16.6±1.0 [a]
Group III	15.80±1.36 [a]	16.79±1.45 [a]	17.8±0.5 [a]	24.5±1.5 [a]
Group IV	15.78±0.85 [a]	17.33±1.50 [a]	19.7±0.3 [a]	25.3±1.5 [a]

Values are Mean±SE, n = 6;[a] $p< 0.05$-Statistically significant when compared to Group I

25% agnuside and soframycin show a significantly higher DNA content compared to the control and hence it can be assumed that there is a greater amount of DNA synthesis needed for new cells and for repairing DNA of existing cells. (Table 9)

From table 10, one can conclude that levels of serum ascorbic acid in experimental animals shows significant improvement.

Table 10: Levels of Serum Ascorbic Acid in experimental animals - Incision/Excision wounds

Groups	Ascorbic Acid (mg/dl)	
	Incision	Excision
Group I	1.01±0.04	1.36±0.0458
Group II	2.34±0.10 [a]	2.73±0.0915 [a]
Group III	2.96±0.12 [a]	3.41±0.1144 [a]
Group IV	3.17±0.10 [a]	3.55±0.1190 [a]

Values are Mean±SE, n = 6;[a] $p< 0.05$ - Statistically significant when compared to Group I

Before sacrificing the animals, Red blood cells count, White blood cells count and Haemoglobin were checked. The values are listed in Table11. Finally the blood analysis report indicates that, 25% concentration agnuside gave significant results when compared to the control.

Table 11: Hematological profiles in experimental animals – Excision wound

Groups	RBC (10^6 Cells/mm^3)	WBC (10^3 Cells/mm^3)	Hb (%)
Group I	2.85±0.033	9.92±0.0822	7.30±0.07
Group II	3.73±0.046[a]	5.36±0.0444 [a]	9.48±0.09 [a]
Group III	5.30±0.058 [a]	4.01±0.0191 [a]	12.57±0.06 [a]
Group IV	5.54±0.067 [a]	3.59±0.0653 [a]	13.59±0.02 [a]

Values are Mean±SE, n = 6; [a] $p< 0.05$ - Statistically significant when compared to Group I.

Group I

Initial (0 Day) **Final (15 day)**

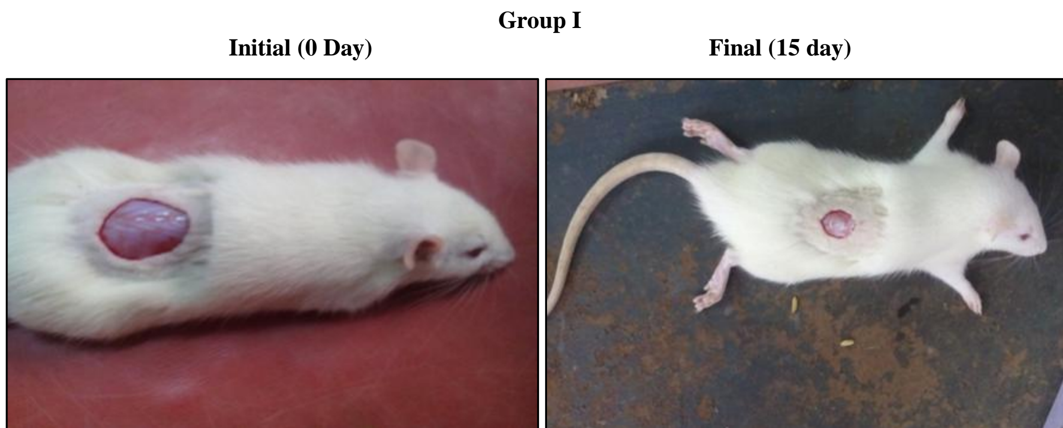

Fig 10: Wistor albino treated without drug (Control)

Group II

Initial (0 Day) **Final (15 day)**

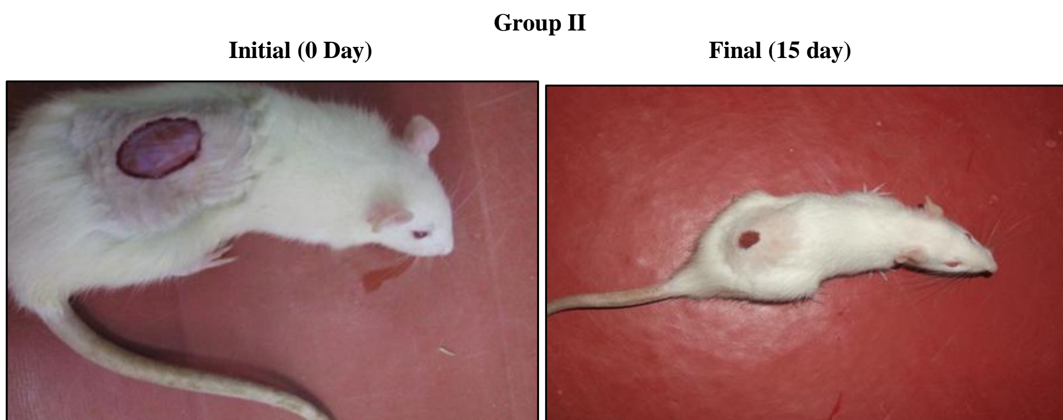

Fig 11: Wounded animals treated with ethanolic extract

Group III

INITIAL (0 Day) **FINAL (15 day)**

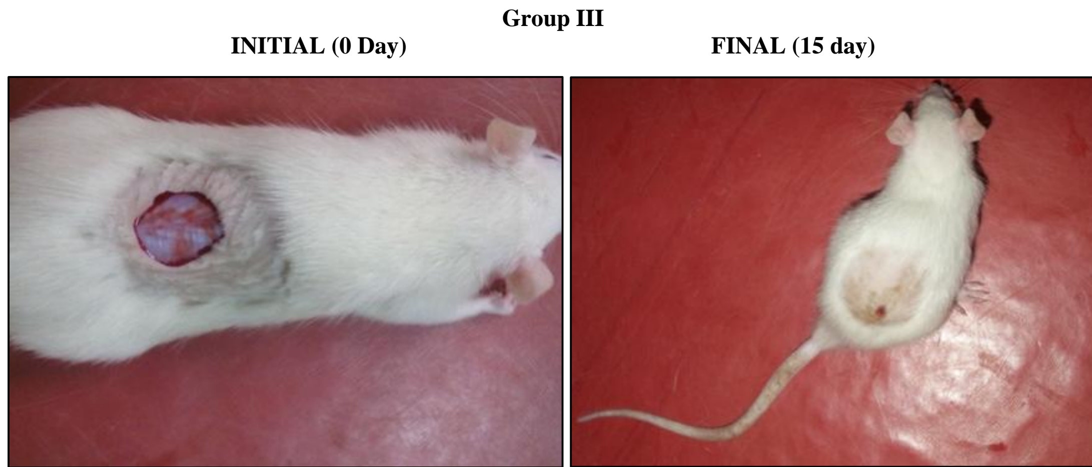

Fig 12: Wounded animals treated with 25% agnuside

Group IV

INITIAL (0 Day) **FINAL (15 day)**

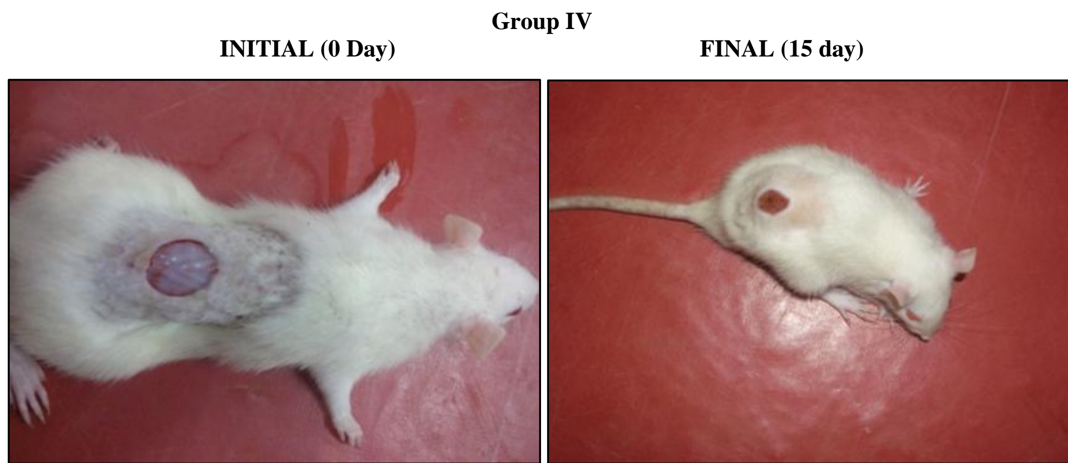

Fig 13: Wounded rats treated with Soframycin

Fig.10 -13: wound healing potential of *Vitex negundo* extracts and its agnusides on Wistor albino rats

Conclusion

Vitex negundo leaves are traditionally used in alternated medical systems of India like Siddha, Ayurveda and Unani. Although the leaves have been claimed to have several therapeutic potentials, they are most important in wound healing and anti-inflammatory activities. This investigation was conducted to understand the mechanism of its wound healing activity. Experiments were carried out on artificially wound Wistar rats which were divided into four groups. Group I are control (Fig.10), Group II are wounded animals treated with ethanolic extracts (Fig.11), Group III are wounded animals treated with 25% agnusides (Fig.12) which are one of the active constituents of the leaves and Group 4 are wounded rats treated with Soframycin (Fig.13). It was found that, 25% agnusides promoted wound healing almost similar to agnusides. All biochemical and physiological parameters measured during wound healing process were similar in agnuside treated rats to those of soframycin treated rats. Hence, It is concluded that, the active principle of *Vitex negundo* leaves involved in wound healing is agnuside.

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Conflict of Interest

The authors do not have any conflict of interest.

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