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ORIGINAL ARTICLE

Metabolic Profile Elucidation of *Ventilago calyculata* Aqueous Extract Attenuating Sequelae of Aspirin Retarded Wound Healing

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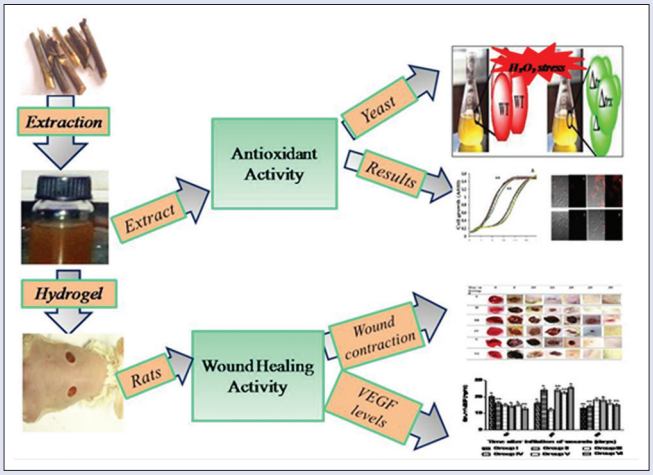
ABSTRACT

Background: Impaired wound healing due to aspirin is a common cause of delay in healing. *Ventilago calyculata* Tul. (*Rhamnaceae*) is used extensively in the Indian traditional medicines for skin problems. **Objective:** The objective of this work was to test the potential of formulations prepared from the extracts of *V. calyculata* against aspirin-retarded wound healing in rats and investigate the probable mechanism of action. **Materials and Methods:** The effect of topical administration of fractionates of *V. calyculata* against aspirin-delayed wound healing was assessed in Wistar albino rats. The chemo-profiling (liquid chromatography-mass spectroscopy [LC-MS]) of the extract with the most potent wound healing activity was carried out. Further, the mechanism of the most potent *Ventilago calyculata* aqueous extract (VCA) was determined by viability and plasma membrane integrity assays in H_2O_2-challenged wild type *Saccharomyces cerevisiae* BY4743) and knock-out strain (Δ*trx2*) strains. **Results:** The results of our investigations showed that in excision wound model; all formulations had statistically significant ($P < 0.01$) wound healing activity compared to the negative control. However, aqueous extract treatment (HF_3) exhibited maximum activity, and the chemo-profiling of VCA by LCMS suggested that the potent activity may be due to individual and/or synergistic effect of the identified pharmacologically active phytoconstituents. The study on yeast indicates that VCA was able to act intracellularly also as it was able to overcome the growth inhibitory effect of the H_2O_2 significantly ($P < 0.01$). **Conclusion:** We propose that treatment with HF_3 (VCA) is a therapeutically beneficial method of decelerating wound retardation caused by aspirin intake in patients on long-term aspirin therapy.

Key words: Aspirin, chemoprofiling, delayed wound healing, *Saccharomyces cerevisiae*, vascular endothelial growth factor, *Ventilago calyculata*

SUMMARY

- *Ventilago calyculata* is used extensively in the Indian traditional systems of medicine for thousands of years to treat ulcers, wounds, skin diseases, boils, etc. The various extracts from VCA were tested for the first time against aspirin-retarded wound healing in rats. The aqueous extract of VCA exhibited the strongest anti-oxidant and wound healing activities. The potent healing effect exhibited by HF_3 (or VCA) may be due to its ability to stimulate vascular endothelial growth factor expression; ability to scavenge reactive oxygen species molecules *in vitro* and *in vivo*. This must be attributed due to the individual and/or synergistic effect of the identified pharmacologically active phytoconstituents such as phenolic compounds, flavonoids, terpenoids, glycosides, hydroxy acids, vitamins, sugars, and medium-chain triglycerides in the bioactive extract by liquid chromatography-mass spectroscopy study. VCA could possibly be made use clinically for healing wounds in patients on long-term aspirin therapy.



Abbreviations used: VEGF: Vascular endothelial growth factor; YPD: Yeast extract peptone dextrose; ELISA: Enzyme-linked immunosorbent assay; FCR: Folin–Ciocalteu reagent; HRP: Horseradish peroxidase; LC-ESI-MS: Liquid chromatography-electrospray ionization-mass spectroscopy.

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INTRODUCTION

Wound healing is a product of the integrated response to an injury to tissue that leads to phases that require inflammation, the proliferation of fresh cells, maturation, and remodeling. Disruption into any of these stages could alter the repair process.[1]

Wound healing problems pose a serious challenge and are probable to intensify since they are linked to conditions such as diabetes, hypertension, obesity, and drug-induced delay. which may retard wounds, cause organ failure, and can even be life-threatening.

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Aspirin is a nonsteroidal anti-inflammatory drug, which is most commonly used for handling pain, arthritis, ischemic heart diseases, cerebrovascular diseases and the prevention of colon cancer, etc.[2] It is found to delay the wound healing process in living tissues by impeding the inflammatory response. The anti-inflammatory action of aspirin is based on the inhibition of nuclear factor-κB activation (targets kinase pathways that activate genes translating pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, inducible enzymes, such as COX-2), mediating the release of adenosine (specifically inhibit IKK-β activity to reduce ATP binding).[3-5] It is found to delay the wound healing process in living tissues by impeding the inflammatory response.

According to the ancient literature, *Ventilago calyculata* Tul. (*Rhamnaceae*) syn. *Ventilago denticulata*[6,7] is used extensively in the Indian (both Ayurveda and Siddha), Chinese, American, and African traditional medicines for skin diseases, itch, contusions, sprains, wound healing, inflammation, pain, etc.

It was hypothesized that formulations prepared from the extracts of *V. calyculata* may be able to accelerate wound healing suppressed due to aspirin administration in rats.

MATERIALS AND METHODS

Materials

Disprin® tablet was used to retard wound healing in rats. SSDee^{Ultra} (Silver sulfadiazine/chlorhexidine gluconate/*Aloe vera*) cream was used as the standard wound-healing drug. Ray Bio® Rat vascular endothelial growth factor (VEGF)-A enzyme-linked immunosorbent assay (ELISA) Kit (Ray Biotech, Norcross, GA, USA) was used for determining VEGF level in rats. Rest of the reagents and solvents used in the present work were procured from Sigma Chemical Co., (St. Louis, MO, USA).

Identification, preparation, and extraction of the plant materials

The stem bark of *V. calyculata* (VC) was collected in October from a hilly area of Bhopal. The samples were identified by Dr. Zia-Ul-Hasan, HOD, Department of Botany, Saifia College, Bhopal (Voucher specimen no.:415/Saifia/Botany/15).

The plant material was washed and dried in shade, powdered, sieved through (#60 mesh), and stored in air-tight containers. The dried powdered materials (500 g) were defatted with petroleum ether to remove the greasy material, and the extract was subjected to Thin layer chromatography (TLC) studies ensuring for the presence of terpenoids. As no terpenoids were found to be present in the filtrate, and hence, it was extracted successively with ethyl acetate (EA) and methanol in a Soxhlet extractor. Further, aqueous extract of the material was obtained by macerating in distilled water chloroform: water (95:5) for 72 h at room temperature with occasional shaking. The percentage yield of stem bark of VC extracts, namely ethyl acetate (VCEA), methanolic (VCM), and aqueous extracts were found to be 6.08%, 9.24%, and 11.33% w/w, respectively.

Experimental protocol for evaluating the wound repair potential of the formulations against the nonsteroidal drug (aspirin)

Animals

Inbred Wistar albino rats (150–250 g) of both sexes were chosen for assessing pharmacological activity. The animals were housed in well-ventilated and hygienic cages during the experimental period, under 12:12 h day and night schedules with a temperature between 18°C and 20°C, maintained by air conditioner. They received commercial pellet feed during the study. The animal care and pharmacological studies were conducted in the Division of Pharmacology of our institution and were in compliance with the requirements of the Institutional Animal Ethical Committee (CPCSEA protocol no.PH/IAEC/VNS/2K13/16).

Preparation and evaluation of the carbopol gel formulations for animal study

Carbopol gel was prepared by adding 1% w/v polymer Carbopol 934 (polyacrylic acid) to distilled water (60 mL), kept overnight and added methyl/propyl paraben-0.02% and sodium meta bisulphite-0.2%. The mixture was neutralized by adding triethanolamine dropwise and added (2.5%) of respective *V. calyculata* extracts, i.e., VCEA, VCM and aqueous (VCA).[8] Similarly, the control was prepared without adding any extract. All the formulations were tested for homogeneity, pH, spreadability, viscosity, etc.[8]

Skin irritation potential of HF_1, HF_2, and HF_3 was conducted in-house as per the Organization for Economic Co-operation and Development Guideline number 404 (2000b)[9] on rats weighing approximately 150–200 g by Patch skin irritation test. A total of 0.5 g each of the test formulation (containing 2.5% of the respective extracts in the formulation) was applied, and the animals were observed every day for the next 3 days. The nearby areas of untreated skin from each animal acted as controls and were checked for erythema and edema.

Wound repair activity of the formulations in excision wound model against aspirin retardation

Disprin® (aspirin) was fed to rats by oral route (150 mg/kg body weight of rats) for 10 days, starting 1 day before wound induction in excision model.[10] The formulations were topically applied once daily for a period of 30 days or until the complete repair of the wound, whichever was early.

The animals were categorized into six groups each group consisting of six animals where $n = 6$ in excision wound model (as two wounds inflicted on each animal by punch biopsy). In the animals of Group I (normal group-only hydrogel base was applied topically, administered saline only-without aspirin); Group II (standard group was fed aspirin [150 mg/kg/body weight] orally for 10 days and SSDee^{Ultra} cream was applied topically to wounds); Group III (negative control group was fed aspirin [150 mg/kg/body weight] orally for 10 days and hydrogel base was applied topically to wounds); Group IV–VI (treatment groups were fed aspirin orally for 10 days [150 mg/kg/body weight] and HF_1, HF_2, and HF_3 were applied respectively). Excision wounds were created on the 2nd day of aspirin dosing.

Full-thickness excision wounds were inflicted on the dorsal side of rats under deep sedation with 90 mg/kg ketamine (Neon Laboratories Ltd.) and 50 mg/kg xylazine (Indian Immunologicals Ltd.) intraperitoneally. The dorsal hair was shaved and wiped with ethanol and anesthetized with lidocaine gel. Two 6-mm diameter full-thickness circular open wounds were inflicted on the skin.[11] This model was used to screen for the most potent extract against aspirin-induced wound delay in rats by assessing the following parameters:

Measurement of % wound contraction

The percentage of wound contracture was computed using the following formula:

$$\%\text{Wound contracture} = \frac{\text{Wound size on the initial day} - \text{Wound size on a specific day}}{\text{Wound size on the initial day}} \times 100$$

Determination of period of epithelialization

The period of epithelialization was determined by counting the number of days necessary for the falling of eschar (dead tissue) from the skin, leaving no raw wound behind.[12]

Biochemical analyses (vascular endothelial growth factor level) of biopsy tissue

VEGF is one of the most important pro-angiogenic molecules in the skin. Serum VEGF levels were detected by sandwich ELISA (double-antibody sandwich ELISA method) by collecting blood samples (retro-orbital puncture) from rats of each group at different time intervals (10, 20, and 30 days) postsurgery. The standard or rat serum (test samples) were analyzed following manufacturer's instructions-Ray Bio®Rat VEGF-A ELISA Kit using a microplate reader (BioTEK, USA) at λ_{max} 450 nm. All the samples were taken in triplicate and absorbance were then estimated by a standard curve of VEGF.

Chemo profiling of VCA as analyzed by liquid chromatography-electrospray ionization-mass spectrometry

Fractionate was found to possess the maximum antioxidant and wound healing potential, i.e., VCA was subjected to liquid chromatography-electrospray ionization coupled with mass spectrometry (LC-ESI-MS). The purified samples were analyzed qualitatively by a high-performance LC/ESI-MS system in positive ion and negative modes using an Agilent 1260 Binary LC system and separated on Agilent Zorbax Eclipse Plus C18column (2.1 mm × 50 mm 1.8 µM).

The gradient of solvents: water (95%) with 0.1% formic acid (solvent A) and acetonitrile (5%) LC-MS grade, J. T. Baker (solvent B) was used as the mobile phase. Total time of analysis was 30 min, with a stable flow rate at 0.3 mL/min. Injection volume for extracts was 1 µL. ESI-quadrupole-time-of-flight-MS analysis was performed according to the following parameters of the ion Source: dual spray jet stream ESI, (+)-positive and negative ion modes, gas flow rate: 8 L/min., nebulizer pressure: 30 psig, gas temp.: 325°C; mass range 60–1600 m/z, with ACQ Method: CAMS-Metabolite_ms-N, VCap (V): 3500 with MS scan rate of 2 Hz, skimmer: 45 V, fragmentor: 150 V. The data acquisition was carried out on Mass Hunter workstation software v.B.05.01, (Agilent Technologies, Inc., Santa Clara, CA, USA) and METLIN database.

Antioxidant analysis of the most potent extract (VCA) in *Saccharomyces cerevisiae*-wild and mutant strains (*in vivo* method)

Yeast strains, growth media, and culture conditions

The wild-type (WT) parental *Saccharomyces cerevisiae* strain BY4743 (MATa/MATa*his3Δ1/his3Δ1leu2Δ0/leu2Δ0met15Δ0*/MET15LYS2 */lys2Δ0g/ura3Δ0*) and its isogenic mutants-Δ*cta1* (encoding catalase A), Δ*ctt1* (encoding cytosolic catalase T), Δ*grx1* (encoding glutaredoxin 1), Δ*grx2* (encoding glutaredoxin 2), Δ*rad9* (encoding DNA damage-dependent checkpoint protein), Δ*sod1* (encoding cytoplasmic superoxide dismutases), Δ*sod2* (encoding mitochondrial superoxide dismutases), and Δ*trx2* (encoding cytoplasmic thioredoxins isoenzyme) were used in the study (All the strains were acquired from Open Biosystems [Thermo Scientific]). For each study, colonies from freshly streaked yeast extract peptone dextrose (YPD) plates were used. Stock culture of WT BY4743/knockout strains were maintained in standard liquid YPD medium containing 1% yeast extract, 2% peptone and 2% dextrose (Difco Laboratories, Detroit, MI, USA) on an orbital shaker at 180 rpm or on a YPD plate, the medium was solidified with 2% agar at a temperature of 23°C. Liquid YPD media were inoculated with one colony per 5 ml.

In this study, eight H_2O_2-stress-responsive knock-out yeast strains (Δ*cta1*, Δ*ctt1*, Δ*grx1*, Δ*grx2*, Δ*rad9*, Δ*sod1*, Δ*sod2,* and Δ*trx2*) were selected and screened for their tolerance toward H_2O_2 (*Saccharomyces* Genome Database). The oxidant hydrogen peroxide was analyzed for determining the growth-arrest concentration (1 mM, 2 mM, and 4 mM) and its duration (15 min, 30 min, and 45 min) of incubation. The optimum stress conditions were found out to be 4 mM and 60 min in wild and mutant yeast strains.

Viability assay of wild-type (BY4743) and Δtrx2 in the presence of VCA/ascorbic acid in liquid medium (growth curve assay)

During the viability assay, a single colony of WT and Δ*trx2* was inoculated into YPD media, kept overnight. Different concentrations of VCA were prepared and sterilized. Ascorbic acid (10 mM) served as a positive control for the study. 4 mM H_2O_2 was added to all the wells except the control and incubated for 24 h in Eon microplate reader (BioTEK, USA). Yeast growth (OD_{600}) was measured every 30 min for 24 h. The assay was performed in a minimum of three replicates to check variability and error and cell densities, and the average reading was plotted.[13]

Plasma membrane integrity (propidium iodide staining) in wild-type (BY4743) and its mutant strain (Δtrx2) after treatment with VCA

Yeast cells WT and Δ*trx2* were cultured as described for viability assay above and diluted to OD_{600} 0.5. All the groups, except the control group, were exposed to H_2O_2 (4 mM). The test groups were treated with either VCA (1.6 mg/ml) or ascorbic acid (10 mM). After incubation (30°C) in the dark, live cells were harvested (12,000 rpm/30 s) and suspended in phosphate-buffered saline (PBS) buffer (200 µl). Propidium iodide (PI) was added to the cell suspension to a final concentration of 1 µg/ml. After incubation with PI for 5 min in the dark, yeast cells were harvested and resuspended in PBS. The PI uptake by yeast cells was visualized using fluorescence light microscope (Zeiss Apotome, Germany) at ×100 (ex535 nm/em617 nm). The processing of images was performed using Zeiss ZEN software (Version 2012) (Carl Zeiss Microscopy GmbH, Jena, Germany).[14]

Statistical analysis

All the results were expressed as mean ± standard deviation. The data for anti-oxidant studies and wound healing activity were analyzed using one-way ANOVA, followed by Dunnett's multiple comparison tests. The analyses were performed using GraphPad Prism version 5.00 (Graph Pad, San Diego, CA), $P < 0.01$ were considered as statistically significant.

RESULTS

Animal study for wound healing potential against aspirin (Disprin®) in Wistar rats

Evaluation of all the topical herbal hydrogels (HF_1, HF_2, and HF_3) revealed that they were homogeneous formulations with pH value in the close range of neutral pH (6.4–7.4). The spreadability and viscosity were also found to be suitable for an ideal hydrogel preparation. The dermal irritation studies showed no noticeable irritation (erythema and edema) up to 72 h following the application of medicated hydrogels.

In this aspirin-delayed excision model, the rate of wound contraction was found to be 100% in Group I (normal group-hydrogel base + saline) on the 20th day, Group II (positive control-SSDee^{Ultra} cream + Disprin®) was 100% on the 25th day which was significantly ($P < 0.01$) different from Group III (negative control-Disprin®) that received aspirin only, was found to be only 40.19% and 59.80% on 20th and 25th day, respectively. In addition to that Group III failed to achieve complete wound healing even till the 30th day. Wound healing in Groups IV–VI (treatment groups) was found to be delayed initially, but after discontinuing aspirin, the rate of healing fastened and wound contraction was 100% between 25 and 30 days [Table 1].

The period of re-epithelialization (calculated from the initial day) was found to be significantly reduced in Group I and in Group II and in various treatment Groups IV–VI ($P < 0.01$), [Table 1] relative to Group III which was found to be maximum ($P < 0.01$).

VEGF levels in the serum of rats of the various groups were assessed on days 10, 20, and 30 [Figure 1]. The aqueous extract (VCA) showed the highest wound healing potential against aspirin retardation.

Chemo profiling of VCA as analyzed by liquid chromatography-electrospray ionization-mass spectrometry

The LC-ESI-MS of aqueous fraction of *V. calyculata* (VCA) identified the presence of several pharmacologically important primary, secondary and intermediate metabolites [Table 2]. The analysis successfully confirmed therapeutically important polar metabolites belonging to the class phenols and their derivatives, flavonoids, glycosides, organic acids, carbohydrates, short-chain amino acids, nonprotein amino acids, fatty acids, etc.

Anti-oxidant analysis of VCA in *Saccharomyces cerevisiae*-wild and mutant strains

Viability assay of wild-type (BY4743) and Δtrx2 in the presence of VCA/ascorbic acid in a liquid medium

In the growth curve assay, the anti-oxidant activity was assessed by the capacity of yeast cells in liquid culture. There was a significant reduction ($P < 0.01$) in the yeast cells (WT) population in H_2O_2 treated culture compared to the normal control, after 24 h incubation. The H_2O_2-induced growth arrest was restored by VCA in a concentration-dependent manner in both the WT strain. These results indicate that VCA has potent *in vivo* antioxidant activity [Figure 2a and b].

Our results of plasma membrane integrity assay indicate that the incubation with H_2O_2-triggered oxidative stress in cells disrupted the plasma membranes and allowed PI penetration over 40.42% ± 1.40% for WT cells and 68.4% ± 2.9% for Δ*trx2*, yeast cells. The ascorbic acid-treated group resulted in approximately 5 times reduction in PI stained WT and Δ*trx2 cells* in comparison to the negative control. At the same time, the adaptive treatment with VCA resulted in a reduction

Table 1: Effect of topical application of reference formulation and hydrogels formulated from various extracts of *Ventilago calyculata* on wound area (mm^2) ± standard deviation (percentage wound contraction) and epithelization period against aspirin-induced delay in excision wound model in rats

Postwounding days	Group I (%)	Group II (%)	Group III (%)	Group IV (%)	Group V (%)	Group VI (%)
Oral administration of Disprin® (aspirin) initiated						
0th day	6.42±0.13	6.72±0.20	6.75±0.33	6.64±0.18**	6.54±0.29**	6.65±0.28**
5th day	5.37±0.41** (16.39)	6.9±0.05** (7.84)	6.5±0.37 (3.46)	6.25±0.02* (5.9)	6.06±0.08** (7.34)	6.01±0.40** (5.83)
10th day	3.96±0.05** (38.38)	6.3±0.37** (10.83)	6.4±0.12 (5.03)	5.94±0.11** (10.49)	5.56±0.31** (14.98)	5.83±0.41** (12.32)
Administration of Disprin® (aspirin) discontinued and application of prepared herbal formulations/marketed preparation started						
15th day	1.57±0.39** (75.46)	4.2±0.29** (13.33)	6.07±0.35 (9.97)	5.37±0.16** (19.02)	4.9±0.05** (25.07)	5.51±0.30** (17.18)
20th day	0.0±0** (100)	2.6±0.21** (79.97)	4.03±0.11 (40.19)	3.36±0.30** (49.27)	2.30±0.02** (64.73)	2.47±0.18** (62.82)
25th day	0.0±0** (100)	0.0±0** (100)	2.71±0.37 (59.80)	0.80±0.20** (87.90)	0.48±0.16* (92.61)	0.67±0.24** (89.98)
30th day	0.0±0** (100)	0.0±0** (100)	1.19±0.20 (82.27)	0.0±0** (100)	0.0±0** (100)	0.0±0** (100)
Epithelization period	14.67±1.9**	17.66±1.6*	27.6±0.9*	22.67±3.3**	18.33±0.4**	20.67±1.8**

Values are presented as mean±SD; n=6. *P<0.05; **P<0.01 (comparison of I, II, IV, V, VI with III) where. Group I: Normal group (hydrogel base + saline); Group II: Standard group. (SSDee^{Ultra+} + Disprin®); Group III: Negative control group (Disprin® only); Group IV-VI: treatment groups administered with HF_1+ Disprin®/HF_2+ Disprin®/HF_3+ Disprin® respectively. SD: Standard deviation

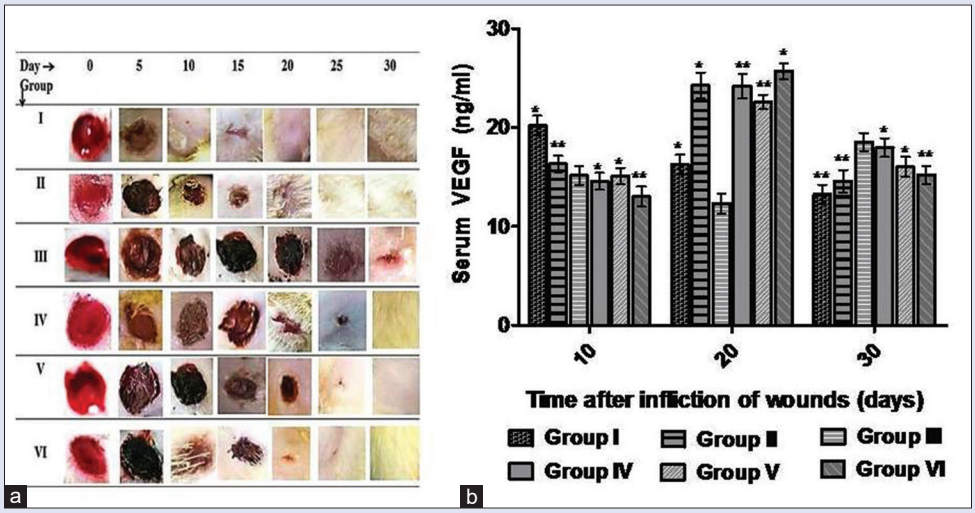

Figure 1: (a) Morphological representation of wound contraction area after 6 mm punch biopsy on different days of control, standard and hydrogel of successive extracts of *Ventilago calyculata* treated against aspirin-induced wound delay in wild-type rats. (b) Vascular endothelial growth factor levels in serum of rats after 10, 20 and 30 days of infliction of excision wounds (ns: nonsignificant, *P < 0.05, **P < 0.01 when compared to negative control). Group I: Normal group (hydrogel base + saline); Group II: Standard group (SSDee^{Ultra+} + Disprin®); Group III: Negative control group (Disprin® only); Group IV–VI: Treatment groups

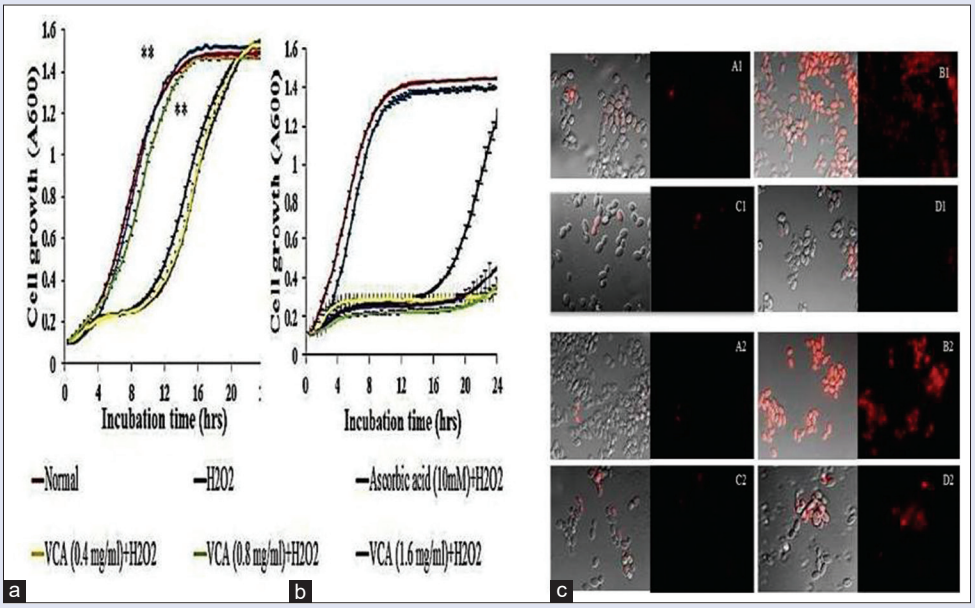

Figure 2: (a) Growth curve for wild-type yeast strains treated with VCA (0.4 mg/ml, 0.8 mg/ml, 1.6 mg/ml). (b) Growth curve for *Δtrx2* yeast strains treated by VCA (0.4 mg/ml, 0.8 mg/ml, 1.6 mg/ml) (**$P < 0.01$). (c) Plasma membrane integrity determination in yeast wild-type (A1-D1) and Δ*trx2* (A2-D2) cells stained with propidium iodide, A1 and A2: Normal cells; B1 and B2: Cells stressed with H_2O_2; C1 and C2: Cells pretreated with ascorbic acid and stressed with H_2O_2; D1 and D2: Cells pretreated with VCA and stressed with H_2O_2

Table 2: Qualitative analysis of pharmacologically important secondary metabolites found in *Ventilago calyculata* aqueous fraction of stem bark (liquid chromatography-mass spectrometry)

Name of metabolite	Empirical formula	mass to charge ratio (m/z)	Fragment ions (m/z)	Difference (ppm)	Detection mode/RT (min)	Category	Pharmacological activity
Atrolactic acid	$C_9H_{10}O_3$	149.059 $[M+H]^+$	149.059 150.062	3.59	13.23	Phenolics	Skin diseases[15]
Duartin (−)	$C_{18}H_{20}O_6$	337.103 $[M+H]^+$	337.103 338.107 339.110 340.109	2.56	13.23	Flavonoid (isoflavan)	Antioxidant, trypanosomicidal[16]
Isorhamnetin	$C_{16}H_{12}O_7$	315.053 $[M-H]^-$	315.053 316.056 317.059	−6.2	8.59	o-methylated flavonol	Antioxidant, wound healing[17]
Dihydrokaempferol	$C_{15}H_{12}O_6$	269.046 $[M-H]^-$	269.046 270.050 271.052	−3.8	9.79	Flavanonol	Antioxidant, wound healing[17]
Morin	$C_{15}H_{10}O_7$	329.032 $[M-H]^-$	329.032 330.035 331.035	−6.81	9.44	Flavanonol	Anti-inflammatory activity[18]
Apionic acid	$C_5H_{10}O_6$	165.040 $[M-H]^-$	165.040 166.045 167.045	−0.42	0.49	Hydroxy acids	Skin ailments[19]
Sedoheptulose	$C_7H_{14}O_7$	209.06 $[M-H]^-$	209.066 210.072 211.074	−1.38	0.49	Sugar	Wound healing[20]
Thiamine monophosphate	$C_{12}H_{18}ClN_4O_4PS$	379.041 $[M-H]^-$	379.041 380.044 381.039 382.042 383.040	−4.4	0.50	Vitamin	Skin ulcers, diabetic wounds[21]

Contd...

Table 2: Contd...

Name of metabolite	Empirical formula	(m/z)	Fragment ions (m/z)	Difference (ppm)	Detection mode/RT (min)	Category	Pharmacological activity
Salicin	$C_{13}H_{18}O_7$	267.089 $[M-H]^-$	267.089 268.092 269.098	−6.1	5.72	Sesquiterpene lactone	Anti-inflammatory activity[22]
Arbutin	$C_{12}H_{16}O_7$	295.078 [M+H]	295.078 296.082 297.083	1.31	5.49	Sesquiterpene lactone	Antioxidant, anti-inflammatory activity[23]
Terephthalic acid	$C_8H_6O_4$	149.023 $[M+H]^+$	149.023 150.026	0.3	15.6	Aromatic dicarboxylic acid	Antioxidant[24]

RT: Retention time

in PI stained WT and Δ*trx2* strain approximately by 2.5 and 2 times, respectively, as compared to the negative control. The results indicate that VCA and ascorbic acid bring about adaptive changes in cells which could counteract against the reactive oxygen species (ROS) and can keep the cell membrane intact against H_2O_2-challenge. The cell membrane protective effect of VCA is comparable with that of the standard [Figure 2c].

DISCUSSION

The present study was undertaken to investigate the potential of *V. calyculata* (stem bark) against wound healing retardation caused by the nonsteroidal drug-aspirin.

It is evident from the results of excision wound model that the healing was delayed initially due to the administration of aspirin in all the groups (except the normal) till aspirin was fed orally (day 10).

Wound contraction leads to wound closure which is necessary for restoring the functional barrier. An incredible repair pattern was noted in the extract-treated groups after the 10th day resulting in complete wound closure between 20 and 25 days in comparison to the negative control which failed to heal even till day 30.

Furthermore, it was revealed that the Group VI applied with HF_3/VCA hydrogel showed high ROS scavenging potential (*in vitro*), maximum-wound contraction, a shortened period of epithelialization and increased levels of VEGF after discontinuing aspirin and thus supporting our hypothesis. Many studies have shown that VEGF is a critical regulator of angiogenesis in wound healing.[25] As suggested by the current study, it is likely that VEGF release may regulate the healing of the cutaneous wounds.

Owing to the incredible therapeutic potential of VCA, it was investigated further using yeast as the model to determine the probable mechanism of action.

Interestingly, the results of growth curve and staining with PI in yeast revealed that VCA protects WT yeast cells from H_2O_2-induced stress by reducing cell membrane damage but were unable to rescue mutant strain (Δ*trx2*) from stress. Although in our previous work under similar experimental conditions, the aqueous extract of *Sida cordifolia* (SCA) was found to be able to rescue both WT and Δ*trx2* yeast strains from stress and it was concluded that it might be due to stimulation of the enzyme thioredoxin-II to restore redox homeostasis, thus elucidating probable mechanism of SCA.[26]

In future, mechanistic studies assessing the role of individual H_2O_2-stress-responsive knock-out yeast strains (Δ*cta1*, Δ*ctt1*, Δ*grx1*, Δ*grx2*, Δ*rad9*, Δ*sod1*, and Δ*sod2*) in VCA could be undertaken to elucidate their involvement in wound healing.

The remarkable effect of VCA in promoting wound healing might be attributed to the individual and/or synergistic effect of the pharmacologically active phytoconstituents such as phenolic compounds, flavonoids, terpenoids, glycosides, hydroxyl acids, vitamins, sugars, and medium-chain triglycerides identified in the bioactive extract by LC-MS study [Table 2].

CONCLUSION

Although traditional medicines offer a safe, inexpensive approach to the treatment of wounds, it has not received adequate attention. The current study justifies the wound healing related ethnopharmacological uses of *V. calyculata* which could possibly be made use clinically for healing wounds in patients on long-term aspirin therapy.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Nayak BS, Suresh R, Rao AV, Pillai GK, Davis EM, Ramkissoon V, *et al.* Evaluation of wound healing activity of *Vanda roxburghii* R.Br (Orchidacea): A preclinical study in a rat model. Int J Low Extrem Wounds 2005;4:200-4.
2. Schuna AA. Update on treatment of rheumatoid arthritis. J Am Pharm Assoc (Wash) 1998;38:728-35.
3. Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 1994;265:956-9.
4. Muscará MN, McKnight W, Asfaha S, Wallace JL. Wound collagen deposition in rats: Effects of an NO-NSAID and a selective COX-2 inhibitor. Br J Pharmacol 2000;129:681-6.
5. Schwenger P, Bellosta P, Vietor I, Basilico C, Skolnik EY, Vilcek J. Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-Jun N-terminal kinase/stress-activated protein kinase activation. Proc Natl Acad Sci U S A 1997;94:2869-73.
6. Kumar S, Balakrishnan N, Lakshmi PK, Toppo FA, Pawar RS. Pharmacognostical and phytochemical evaluation of *Ventilago calyculata* Tul. (Bark). Pharmacogn J 2015;7:271-5.
7. Parrotta K. Study on Healing Plants of Peninsular India. Wallingford, UK: CABI Publishing; 1978. p. 602-3.
8. Khan AW, Kotta S, Ansari SH, Sharma RK, Kumar A, Ali J. Formulation development,

optimization and evaluation of *Aloe vera* gel for wound healing. Pharmacogn Mag 2013;9:S6-10.

9. Organization for Economic Cooperation and Development. OECD Guideline for Testing of Chemicals, No. 404. Acute Dermal Toxicity-Fixed Dose Procedure. Paris, France: Organization for Economic Cooperation and Development Publishing; 2000b. p. 1-3.
10. Allen HL, Wase A, Bear WT. Indomethacin and aspirin: Effect of nonsteroidal anti-inflammatory agents on the rate of fracture repair in the rat. Acta Orthop Scand 1980;51:595-600.
11. Thakur R, Jain N, Pathak R, Sandhu SS. Practices in wound healing studies of plants. Evid Based Complement Alternat Med 2011;2011:438056.
12. Dwivedi D, Dwivedi M, Malviya S, Singh V. Evaluation of wound healing, anti-microbial and antioxidant potential of *Pongamia pinnata* in wistar rats. J Tradit Complement Med 2017;7:79-85.
13. Wu MJ, O'Doherty PJ, Fernandez HR, Lyons V, Rogers PJ, Dawes IW, *et al*. An antioxidant screening assay based on oxidant-induced growth arrest in *Saccharomyces cerevisiae*. FEMS Yeast Res 2011;11:379-87.
14. Liesche J, Marek M, Günther-Pomorski T. Cell wall staining with trypan blue enables quantitative analysis of morphological changes in yeast cells. Front Microbiol 2015;6:107.
15. Van Scott EJ, Ruey JY. Treatment of Skin Keratoses with α-Hydroxy Acids and Related Compounds. United States Patent US 4. 234,599; 18 November, 1980.
16. Promden W, Monthakantirat O, Umehara K, Noguchi H, De-Eknamkul W. Structure and antioxidant activity relationships of isoflavonoids from *Dalbergia parviflora*. Molecules 2014;19:2226-37.
17. Karodi R, Jadhav M, Rub R, Bafna A. Evaluation of the wound healing activity of a crude extract of *Rubia cordifolia* L. (Indian madder) in mice. Int J Appl Res Nat Prod 2009;2:12-8.
18. Fang SH, Hou YC, Chang WC, Hsiu SL, Chao PD, Chiang BL. Morin sulfates/glucuronides exert anti-inflammatory activity on activated macrophages and decreased the incidence of septic shock. Life Sci 2003;74:743-56.
19. Ruey JY, Van Scott EJ. Method of Treating Wrinkles using Mucic Acid or Mucolactone. United States Patent US: 5. 561,153. Tristrata Technology Inc.; 01 October, 1996.
20. Silvetti AN Sr., Silvetti AN Jr. Fructose Containing Wound Healing Preparation. United States patent US: 4. 889:844; 1989.
21. Caspe S. Accelerating Cellular Repair Composition for the Human Body and Method of Administering Same. United States Patent US: 4.308,257; 29 December, 1981.
22. Maroon JC, Bost JW, Maroon A. Natural anti-inflammatory agents for pain relief. Surg Neurol Int 2010;1:80.
23. Lee HJ, Kim KW. Anti-inflammatory effects of arbutin in lipopolysaccharide-stimulated BV2 microglial cells. Inflamm Res 2012;61:817-25.
24. Thakur RS, Jain MP, Hruban L, Santavý F. Terephthalic acid and its methyl esters from *Zizyphus* sativa. Planta Med 1975;28:172-3.
25. Johnson KE, Wilgus TA. Vascular endothelial growth factor and angiogenesis in the regulation of cutaneous wound repair. Adv Wound Care (New Rochelle) 2014;3:647-61.
26. Kumar S, Lakshmi PK, Sahi C, Pawar RS. Sida cordifolia accelerates wound healing process delayed by dexamethasone in rats: Effect on ROS and probable mechanism of action. J Ethnopharmacol 2019;235:279-92.