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ANALYSIS OF PHYTOCHEMICAL CONSTITUENTS AND PHARMACOLOGICAL PROPERTIES OF *ABRUS PRECATORIUS L*

ABHILASHA SHOURIE[1] AND KUNTAL KALRA*[2]

*[*1, 2]Department of Biotechnology,Faculty of Engineering and Technology ,Manav Rachna International University , Faridabad ,Haryana ,India*

ABSTRACT

Abrus precatorius is known to possess antiseptic and anti-inflammatory activity, which makes it useful in treatment of wounds. In the present study, different concentrations of ethanolic extracts of *A. precatorius* stem were investigated for evaluation of wound healing activity in rats. Wistar albino rats have been grouped into three sets- first set was uninfected with wounds, second set was infected with *Staphylococcus Aureus* and third set was infected with *Candida albicans*. Different concentrations of ethanolic extracts of *A.precatorius* (60, 90, 120mg\g) were applied topically in the form of ointment to wounds inflicted on rats and healing was assessed by the rate of wound contraction and epithelialization period. On 15th day, the extract treated rats exhibited 71 % reduction in uninfected wound area as compared to control which exhibited 59 % contraction. In case of wound infected with S.aureus, the extract treated rats exhibited 72 % reduction in wound area as compared to control which exhibited 51 % contraction approximately. In antimicrobial testing, ethanolic extracts of *A.precatorius* were screened against clinical wound isolates of *Staphylococcus aureus* , *Pseudomonas aeruginosa* and *Candida albicans*. All extracts were found to be active against the pathogens, while the ethanolic extract exhibited highest inhibition and document the beneficial effects for acceleration of wound healing activity in rats. Phytochemical tests showed the presence of tannins, triterpenes, glycosides, alkaloids, antraquinones and carbohydrates in crude extracts of *A.precatorius.*

***KEY WORDS:** Abrus precatorius , anti-microbial, epithelization, ,infected wound , healing*

KUNTAL KALRA
Department of Biotechnology,Faculty of Engineering and Technology , Manav Rachna International University, Faridabad ,Haryana ,India

INTRODUCTION

'Abrus precatorius' locally known as Rosary pea or Ratti, is indigenous to India and is commonly found in other tropical and sub-tropical regions. It is a beautiful deciduous, climbing plant, which belongs to the family of Fabaceae. Its seeds have remarkably uniform weight of 1/10th of a gram, therefore were used by goldsmiths as standard weights for weighing gold and silver in previous time. It was first recognized and mentioned as a homoeopathic medicine by Dr.William Boericke in his Homoeopathic Materia Medica under the heading of Jequirity [1]. The plant is used in some traditional medicine to treat scratches, sores and wounds caused by dogs, cats and mice, and are also used with other ingredients to treat leucoderma, tetanus and rabies. They are ground with lime and applied on acne sores, boils and abscesses. The seeds are considered abortifacient[2], aphrodisiac and antimicrobial [3]. Dry seeds of *A. precatorius* are also used to cure worm infection.

Traditional use of this plant for its antimicrobial and anti-suppurative properties led us to investigate its wound healing potential. Healing of wounds occurs through a series of processes which involves phagocytosis of invading bacteria or fungi and debris in inflammatory phase; cell divison, angiogenesis granulation tissue formation and epithelialization in proliferative phase; followed by remodeling and realignment of collagen in remodeling phase [4, 5] . Wound surface colonization by polymicrobial communities is a critical factor in causing delay in wound healing. Topical antimicrobial therapy has long been known to be one of the most important methods of wound care [6, 7], aiming towards prevention of wound contamination and microbial colonization causing delay in wound healing. Current methods used to treat chronic wounds include debridement, antibiotics, tissue grafts and proteolytic enzymes, which possess major drawbacks and unwanted side effects. The routine or systemic use of topical antibiotics is not justified for colonised or infected wounds. Moreover, resistance to antibiotics has become a serious problem in recent years particularly with the rise of epidemic strains of microbes. The overuse of broad-spectrum antibiotics serves to exacerbate the situation and calls for alternative therapeutic remedies such as herbal medicines. In view of this, present study has been carried out in order to establish the antimicrobial and wound healing potential of *A. precatorius*. Methanolic extracts of seeds of *A. precatorius* are reported to have potent antimicrobial activity [8]. Similarly, effectiveness of the seed extracts and fractions of A. *precatorius* in controlling the infection was shown *in vivo* [9] by several researchers.

MATERIALS AND METHODS

Plant Material and Extract preparation

The stem and bark of *A. precatorius* (Fabaceae) were collected from the local flora in Palwal district, Haryana in the month of September. The plant material was air dried completely and ground into powder using electric blender. The fine powder (200 gm) was macerated in 500 ml of 80% ethanol (Himedia ,India) for 48 hrs in water bath at 40^{o}C. The mixture was filtered using a fine cloth and followed by filter paper (Whattman No.1) and concentrated in-vacuo to afford 21.40g (10.70% w/w) of dry extract. The extract was subjected to preliminary phytochemical tests.

Percentage yield = (Weight of Extract / Weight of ground plant material) * 100

Animals

Wistar albino Rats (150-220g) were obtained from the animal house, Scientific Biotech Pvt. Ltd. The animals were housed in galvanized wire cages and acclimatized to animal room conditions and were maintained on commercial pellet diet and water *ad libitum*. All the rats were periodically weighed before and during the experiment. All procedures with animals were conducted strictly in accordance with the guidelines approved by the University's Animal Ethical Committee. During the experiments, maximum care was taken to minimize animal suffering and, in addition, the number of rats used was kept at minimum.

Test Mico-organisms

Wounds may be grouped according to the cause, the environment in which they occur, their extent, and whether they are clean or contaminated. Bacteria, fungi, and viruses can cause wound infections. The micro-organisms used for this experiment include clinical isolates of potential wound pathogens *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. Treatment sample ointment was applied after forty-eight hours of wound infection.

Antimicrobial Studies

The strains of *S. aureus* and *P. aeruginosa* were grown to exponential phase in Mueller-Hinton broth by incubating at 37^{o} C for 24 hrs and fungal strain *C. albicans* was cultured on Sabouraud dextrose agar (SDA) at 35^{o}C for 48 hrs with agitation (50rpm). The microbial suspensions were then inoculated in the respective media supplemented with serial dilutions of extracts ranging from 10µg/ml to 100µg/ml for determination of antimicrobial activity.

The inoculum density of *S. aureus* and *P. aeruginosa* was approximately 1X10^{8} CFU/ml each (McFarland 0.5 density giving absorbance of 0.1). Similarly, the inoculum density of the fungal strain *C. albicans* was approximately 2.5X10^{3} CFU /ml [10, 11]. After 24 hrs incubation, the growth was monitored both visually and spectrophotometrically (at 450 nm for bacteria and 405 nm for fungi). The effective inhibitory concentration was expressed as the lowest concentration of extract that significantly inhibited macroscopic growth at the end of 24 hrs incubation[12].

Determination of Post Antibiotic Effect (PAE)

Concentrations of plant extract to be applied for testing wound healing activity and antibiotic representing 10 x MIC, were added to the broth containing each test species at 1 x l0^{8} cfu/ml. After incubation for 1 hr at 37°C in a shaking water bath, the antibiotic content was reduced to an inactive level by diluting the cultures to 1 x 10^{-3} in pre-warmed fresh medium. The absorbance was taken for every hour at 450 nm and PAE was determined by bacterial viable count. The control culture was also subjected to 1 x 10^{-3} dilution and growth rate was determined under identical conditions without antibiotic or plant extract exposure[12].

For assessment of fungicidal activity, C. albicans in the log growth phase were used to prepare suspensions of 10^{4} CFU/ml in Sabouraud dextrose broth. The antifungal drug fluconazole at concentrations of 2 × MFC and ethanolic extracts of *A. precatorius* were added to the suspensions. Aliquots were removed at 60 min after starting the experiment and plated onto Sabouraud dextrose agar. Absorbance was measured at 405 nm for 28 hrs. Turbidimetric growth curves were obtained depending on the changes in the OD of fungal growth for each drug concentration and the drug-free growth control.

Preparation of herbal ointment

Sample ointment containing the ethanolic extract of *A. precatorius* was prepared in a ceramic mortar and pestle using soft paraffin ointment base [13]. Three batches of the ointment containing 60, 90 and 120 mg/g of the extract were prepared for the studies. Blank paraffin was used as a negative control, Neomycin ointment (containing Neomycin

sulphate 0.5%w/w) as a positive control treatment against bacterial wound infection and Clotrimazole ointment (1%w/w) obtained from Rexcin Pharmaceutical Pvt. Ltd., India as a positive control treatment against fungal wound infection. The ointments were stored in the refrigerator until they were used.

Wound Healing Activity

Excision Wound Model[14] was used to study three categories of dermal wounds: Uninfected Wound, Wound infected by *S. aureus*, and Wound infected by *C. albicans*. The rats were weakly anesthetized under chloroform prior and during creation of wounds. Rats were depilated on dorsal trunk, sterilized with ethanol and inflicted with a full thickness circular excision wound of 314 mm^2 area and 2 mm depth using forceps, scalpel and pointed scissors. The animals were allowed to achieve complete homeostasis and full recovery from anesthesia.

The rats were randomly divided into groups comprising three categories: the first category was left uninfected, while the second and the third categories were applied with activated cultures of *S.aureus and C. albicans* respectively [13]. Each of the categories was further divided into five sub-groups and treated as follows: Group 1 (negative control) was treated with blank soft paraffin base (CDH, India), Group 2 (Positive control) was treated with 0.5% Neomycin ointment (Rexcin Pharmaceutical Pvt. Ltd.). Group 3, 4, and 5 were treated with 60, 90, and 120 mg/g of extract of *A. precatorius*.

The wound area was measured with a translucent paper and estimated on a $1mm^2$ graph sheet at every 3 days interval until completed wound closure was recorded. Wound contraction was calculated as a percentage of the original wound size. Period of complete epithelialization was also recorded.

Phytochemical Screening Methods

Saponins

Extract (300 mg) was boiled with 5 ml water for 2 mins; the mixture was cooled and mixed vigorously and left for 3 mins. The formation of frothing indicates the presence of saponins [15].

Tannins

1 ml of extract (300 mg ml^{-1}) was added to 2 ml of sodium chloride (2%), filtered and mixed with 5 ml 1% (w/v) gelatin solution. Precipitation indicates the presence of tannins[15].

Triterpenes

Extract (300 mg) was mixed with 5 ml chloroform and warmed at 80°C for 30 mins, few drops of concentrated sulfuric acid was added and mixed well. The appearance of red color indicates the presence of triterpenes [16].

Alkaloids

Extract of each plant sample (0.5g) was separately stirred with 1% hydrochloric acid (HCl) on a steam bath. The solution obtained was filtered and 1ml of the filtrate was treated with two drops of Mayer's reagent. The two solutions were mixed and made up to 100ml with distilled water. Turbidity of the extract filtrate on addition of Mayer's reagent was regarded as the evidence for the presence of alkaloids in the extract [15].

Flavonoids

The presence of flavonoids was determined by using 1% aluminum chloride solution in methanol, concentrated HCl, magnesium turnins and potassium hydroxide solution[15].

Glycosides

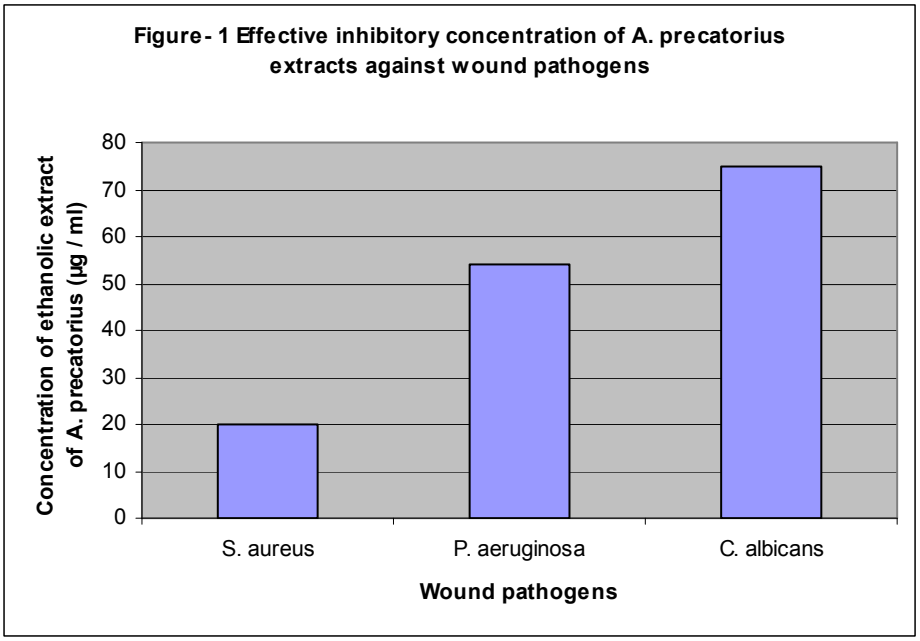
Coarsely powered plant material (1g) was boiled with 1.0 ml of Sulfuric acid in a test tube. It was filtered while hot and then cooled. Then equivolume chloroform was added. The chloroform layer of the mixture was separated and to it 10 ml of ammonia was added. The presence of reddish brown precipitate in the filtrate was taken as positive for glycosides[17].

Statistical Analysis of Data:

Results were expressed as mean ± Standard Error of mean. The statistical difference between the groups in terms of the mean of Wound Closure Rate measured in wound area (mm^2) was calculated using Student's *t*-test.

RESULTS

Ethanolic extracts of A. *precatorius* were screened for their antimicrobial activity against the laboratory strains of three micro-organisms *S. aureus, P. aeruginosa and C. albicans* in comparision to standard antibiotic and antifungal agents. All the three micro-organisms which are potential wound pathogens were found to be susceptible to the ethanolic extracts of A. *precatorius.* Antimicrobial effect was visualized by the lack of visual turbidity and effective inhibitory concentration was determined as the concentration at which there was a sharp decline in the absorbance value. As shown in Figure – 1, the ethanolic extracts of A. *precatorius* were found to be inhibitory for all the three wound pathogens, exhibiting most effective inhibition against *S. aureus* at concentration as less as 20µg/ml. The other two micro-organisms *P. aeruginosa* and *C. albicans* were also inhibited at concentrations was 54 µg/ml and 75µg/ml respectively.



Post-antibiotic effect (PAE) was observed in relation to PAE of standard antibiotics as well as untreated control. The growth curves of all the three microbes *S. aureus, P. aeruginosa and C. albicans* showed the extension of lag phase in dose related manner when serial dilutions of extracts were added to the broth depicting their inhibitory effect on microbial growth. Extract concentration 120mg/g was found to be the most effective in all cases as compared to the untreated controls and was also quite comparable to the standard antibiotic ampicillin and antifungal fluconazole agents. (Figure 2, 3 and 4)

Figure-2 Post-antibiotic effect of antimicrobial agents on growth curve of S. aureus

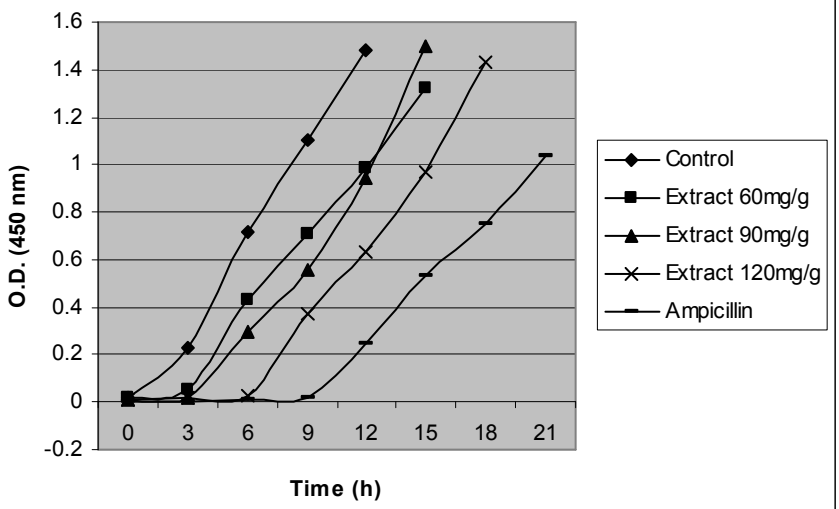
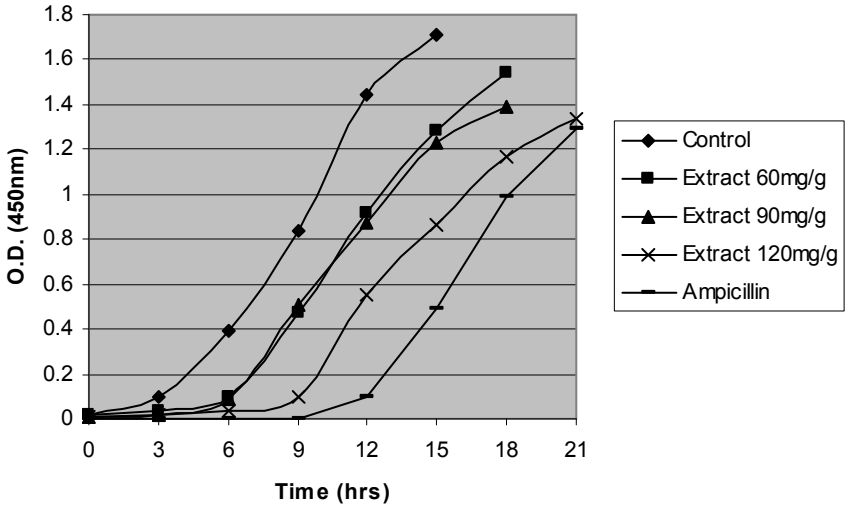
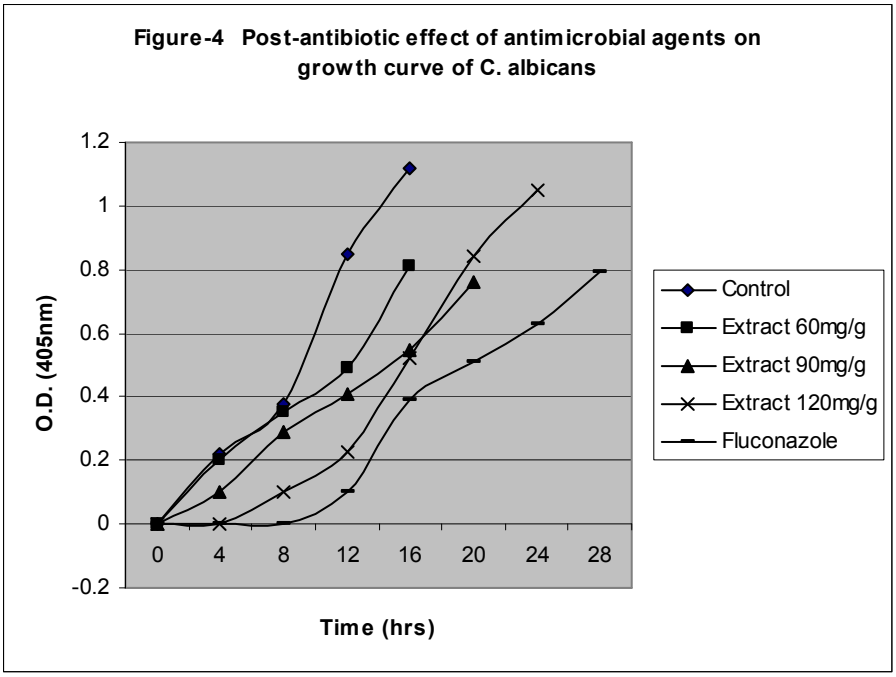


Figure-3 Post-antibiotic effect of antimicrobial agents on growth curve of P. aeruginosa





In the assessment of wound healing potential of *A. precatorius*, it was found that incorporation of extract (60, 90 and 120 mg/g) into the applied ointment enhanced the rate of wound closure considerably and reduced the period of epithelialization (Figure-5) in dose related manner. In case of uninfected wound category (Table-I), most pronounced effect was seen on application of 120 mg/g extract, exhibiting 97.90% wound closure on 27th post wounding day and period of epithelialization was 28.60 days. Similar observations were recorded for wounds infected with *S. aureus* and *C.albicans* (Table-II & III); exhibiting rapid wound closure 94, 90% and 97.10% on 27th post wounding day respectively and the period of epithelialization was 27.20 and 27.40 days respectively. However, the extract was found to be comparatively less effective in healing of wounds infected with *C. albicans* as compared to the other two models i.e. uninfected wound and that infected with *S. aureus*. The results correspond to the antimicrobial testing of the extract that shows its lesser efficacy towards inhibition of growth of *C. albicans*.

Figure-5
Epithelialization of wound

Table- I

Effect of A. precatorius extract applied as ointment on rate of wound closure of uninfected excision wounds in rats

Treatment Group	Area under Epithelization (mm^2)					Epithelization Period (Days)
	Day 3	Day 9	Day 15	Day 21	Day 27	
Untreated Control	36.42±13.34 (11.64%)	97.50±14.57 (31.05%)	185.26±9.01 (59.00%)	244.45±8.39 (77.85%)	290.92±4.86 (92.65%)	29.4±0.56
Extract 1 (60 mg/g)	36.42±13.34* (11.64%)	127.64±13.69* (40.65%)	204.26±16.09* (65.05%)	244.45±8.39* (77.85%)	298.61±3.97* (95.10%)	27.4±0.56*
Extract 2 (90 mg/g)	48.04±16.33* (15.36%)	168.62±11.92* (53.70%)	207.59±5.58* (66.11%)	252.77±12.05* (80.50%)	300.03±3.24* (95.55%)	29.2±0.46*
Extract 3 (120 mg/g)	76.15±15.45* (24.25%)	189.19±11.04* (60.25%)	222.00±14.75* (70.70%)	273.18±5.41* (87.00%)	307.41±3.97* (97.90%)	28.6±0.56*
Neomycin (0.5%w/w)	102.68±14.57* (32.70%)	193.11±11.04* (61.50%)	235.19±11.41* (74.90%)	281.66±5.74* (89.70%)	308.19±4.25* (98.15%)	27.6±0.56*

Table- II

Effect of A. precatorius extract applied as ointment on rate of wound closure of excision wounds infected by S. aureus

Treatment Group	Area under Epithelization (mm^2)					Epithelization Period (Days)
	Day 3	Day 9	Day 15	Day 21	Day 27	
Untreated Control	36.42±13.34 (11.60%)	81.64±12.62 (26.00%)	155.27±18.98 (49.45%)	238.48±6.85 (75.95%)	288.88±8.57 (92.00%)	30.00±0.73
Extract 1 (60 mg/g)	42.23±16.33* (13.45%)	122.77±13.69* (39.10%)	168.62±11.92* (53.70%)	247.43±6.85* (78.80%)	288.88±8.57* (92.00%)	28.80±0.86*
Extract 2 (90 mg/g)	48.04±16.33* (15.30%)	168.62±11.92* (53.70%)	211.79±10.15* (67.45%)	255.75±7.51* (81.45%)	293.75±8.07* (93.55%)	28.60±0.92*
Extract 3 (120 mg/g)	47.73±26.05* (15.20%)	180.71±20.96* (57.55%)	225.61±9.27* (71.85%)	275.22±7.99* (87.65%)	297.99±8.39* (94.90%)	27.20±0.86*
Neomycin (0.5%w/w)	59.35±20.52* (18.90%)	185.26±9.01* (59.00%)	234.87±16.12* (74.80%)	279.62±5.74* (89.05%)	307.41±3.97* (97.90%)	26.60±0.92*

Table- III

Effect of A. precatorius extract applied as ointment on rate of wound closure of excision wounds infected by C. albicans

Treatment Group	Area under Epithelization (mm^2)					Epithelization Period (Days)
	Day 3	Day 9	Day 15	Day 21	Day 27	
Untreated Control	30.62±0.00 (9.75%)	76.15±15.45 (24.25)	159.83±15.96 (50.90)	246.80±17.02 (78.60)	281.34±8.75 (89.60)	28.00±1.03
Extract 1 (60 mg/g)	36.42±13.34* (11.60)	117.28±27.16* (37.35)	181.02±14.83* (57.65)	247.43±6.85* (78.80)	290.92±4.86* (92.65)	27.60±0.56*
Extract 2 (90 mg/g)	36.42±13.34* (11.60)	155.27±18.98* (49.45)	215.09±14.94* (68.50)	250.10±10.27* (79.65)	292.65±3.97* (93.20)	28.00±0.00*
Extract 3 (120 mg/g)	53.85±13.34* (17.15)	185.26±9.01* (59.00)	231.89±13.59* (73.85)	270.51±10.19* (86.15)	304.89±7.22* (97.10)	27.40±0.56*
Neomycin (0.5%w/w)	92.32±11.90* (29.40)	189.19±11.04* (60.25)	238.17±13.40* (75.85)	285.43±6.85* (90.90)	311.02±2.64* (99.05)	27.20±0.46*

- ***Significant at P< 0.05***
- ***percentage wound closure in parenthesis***

The phytochemical analysis of the A. precatorius extract by qualitative study showed the presence of phytochemicals saponins, tannins, triterpenes, alkaloids, flavonoids and glycosides (Table-IV).

Table- IV
Phytochemical constituents of crude extracts of A. precatorius

S.No	Phytoconstituents	Aqueous Extract	Methanolic Extract	Ethanolic Extract
1.	Saponins	+++	-	+
2.	Tannins	++	+	++
3.	Triterpenes	-	-	+++
4.	Alkaloids	-	+	+
5.	Flavonoids	+	-	+++
6.	Glycosides	+++	+++	+++

DISCUSSION

Medicinal plants have long been the inevitable source of topical preparations[12]. *A. precatorius* is mentioned for its therapeutic properties in our traditional system of medicine 'Ayurveda'. In this study, we demonstrated the potential of *A. precatorius* in accelerating the wound healing process using excision wound model. The ethanolic extract applied in the form of ointment showed significant/ considerable reduction in epithelization period and wound contraction time, which can be due to several effects, direct or indirect [14, 18]. Direct effect can be the stimulation of release of factors contributing in wound healing, enhancement of localised cell proliferation in the wound area and rapid collagen deposition [14]. Indirect effects can be the anti-inflammatory property of extract or the antimicrobial activity particularly against potential wound microbes like *S. aureus*, *P.aeruginosa* and *C. albicans*. The eradication of colonizing organisms and inhibition of microbial contamination creates a suitable environment for the healing process [19, 20]. The antibacterial and antifungal activities of *A. precatorius* extract might contribute remarkably to faster wound healing rate as evident by the experimentally treated groups. These properties can be attributed to the phytochemicals identified in *A. precatorius* extract such as alkaloids, flavonoids, triterpenes etc. all of which are known to have some therapeutic properties like anti-microbial, anti-inflammatory or pro-wound healing activity, which makes the plant medicinally valuable. Flavonoids and triterpenes are potential antioxidants[20] and probably exhibit radical scavenging and iron chelating properties. Scavanging of reactive oxygen species, super oxide and hydroxyl radicals by these phytochemicals [21] decreases the risk of oxidative damage to the tissues contributing to rapid and efficient wound healing.

The present study reveals that ethanolic extracts of *A. precatorius* has remarkable antibacterial, anti-fungal and wound healing properties and is a promising candidate to be used for preparation of a herbal formulation for treatment of wounds, sores and boils as claimed in folk lores and traditional information.

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