ejpmr, 2017,4(2), 473-480

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

HISTOPATHOLOGY ACTIVE BIPHENYL PYRROLE COMPOUND ISOLATED FROM MARINE *BREVIBACTERIUM* SPECIES

V. Srilekha[a,d], B. Prasanna*[b] A. Sridhar Rao[c], P. ShivaKrishna[a], V. Sheshasrinivas[d], M. A. Singaracharya*a

[a]Department of Microbiology, Kakatiya University, Warangal, Telangana, India.
[b]Department of Chemistry, Chaitanya Postgraduate College(Autonomous), Hanamkonda, Warangal, Telangana, India.
[c]Department of Chemistry, Mahatma Gandhi University, Nalgonda, Telangana, India.
[d]Department of Biotechnology, SNIST, Ghatkesar, JNTU Hyderabad, Telangana, India.

***Corresponding Author: M. A. Singaracharya**

Department of Microbiology, Kakatiya University, Warangal, Telangana, India.

Article Received on 21/11/2016 **Article Revised on 11/12/2016** **Article Accepted on 31/12/2016**

ABSTRACT

A Biphenyl pyrrole **(1)** was identified in the marine *Brevibacterium sp* which was extracted from crude pigment and purified through RP-HPLC. Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that the bacterium was closely related to the type strains of Brevibacterium species (99%). The bacterial strains which showed significant antimicrobial activity were identified to genus level by PCR amplification of the 16S rRNA gene, BLAST analysis, and comparison with sequences in the GenBank nucleotide database and the species level was identified as the nearest phylogenetic neighbor with sequences >99% sequence similarity. The Wound healing activity against Wistar Albino rats was also studied by using Safromycin ointment as standard drug. The complete structure of compound **(1)** was confirmed through IR, NMR and Mass spectral data.

KEYWORDS: Brevibacterium sp, Marine bacteria, Biphenyl pyrrole, Wound healing, Wistar Albino.

INTRODUCTION

The search for new antimicrobial agents is necessary due to the increase in drug resistance in many common bacterial pathogens. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from microorganisms, many based on their use in traditional medicine. Microorganisms have been the source of many valuable compounds in medicine, industry and agriculture. Most of these compounds are derived from microorganisms surviving in terrestrial habitats. Since the terrestrial resources have been greatly explored, the academic and industry researchers are striving to get lead molecules from the microorganisms existing in the oceans.

The world's oceans, which cover more than 70% of the surface of the earth, contain an exceptional biological diversity, accounting for more than 95% of the whole biosphere[1] **(Bull *et al.*, 2000)**. In recent years marine microorganisms have become important in the study of novel microbial products exhibiting antimicrobial, antiviral, antitumor as well as anticoagulant and cardio active properties **(Marderosian,1969*;* Austin, 1989; Molinski,1993*)*** [2-4].The diversity of marine organisms and the complex living circumstances surrounding these microbes have resulted in the production of novel and unique secondary metabolites with much stronger bioactivities compared with those of terrestrial organisms **(Carte, 1996**; **Rinehart, 2000; Schwartsmann *et al.*, 2001)**[5-7]. Competition among microbes for space and nutrients in marine environment is a powerful selection pressure that endows marine microorganisms to produce many natural products of medical and industrial value **(Armstrong et *al.* 2001)**[8]. Many marine heterotrophic bacteria are known to produce antibacterial substances **(Burgess et *al.*, 1991)**[9]. During the past two decades, research on marine bacteria has highlighted the tremendous potential of these microorganisms as a source of new bioactive metabolites **(Anand *et al.*, 2006**; **Uzair *et al.*, 2008)**[10-11].

Pigmentation is widespread among bacteria and pigments found in marine heterotrophic bacteria consist of carotenoid, flexirubin, xanthomonadine, and prodigiosin **(Kim *et al.*, 2007)**[12]. Antimicrobial activities of pigments from microbial sources have been reported by many workers. Pigments like prodigiosin (red), violacein (violet), and pyocyanin (blue green) are known to have active compounds exhibiting activities such as antimicrobial activity, antiviral, antitumor, antiprotozoa, antioxidant and anticancer. **(Ferreira *et al.*, 2004; Matz *et al.*, 2004; Deorukhkar *et al.*2007; Kim *et al.*, 2010)**[13-16]. Pigments are colorants which have been widely used in food, cloth, painting, cosmetics, pharmaceuticals and plastics **(Yuan Lu *et al*, 2009)**[17].

There is growing interest in microbial pigments due to their natural character, medicinal properties and nutritive value; production being independent of season, geographical conditions, controllable and predictable yield and safety to use **(Francis, 2000; Johnson and Schroeder, 1996.)**[18-19]. In view of the growing importance of pigmented compounds, the present investigation aims at screening of marine heterotrophic bacteria for their pigments from ***Vishakhapatnam coastal*** waters in India. The compound 3,4-diphenylpyrrole **1** was isolated, purified and confirmed by spectroscopic data. The compound was assessed for wound healing activity and exhibited potential activity.

MATERIAL AND METHODS

General experimental procedures

^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) and 2D-NMR spectra were recorded using the residual solvent signal as internal standard on a Varian 400 MHz spectrometer. IR spectra were measured on a Bruker Tensor 27 FTIR spectrometer. HRESIMS were obtained on an Agilent Series 1100 SL mass spectrometer. TLC was carried out on aluminum-backed plates precoated with silica gel F254 (20 × 20 cm, 200 µm, 60 Å, Merck). Visualization was accomplished by spraying with *p*-anisaldehyde [0.5 mL in glacial acetic acid (50 mL) and sulfuric acid (97%, 1 mL)] spray reagent followed by heating. Flash silica gel (60-120 µm, 60 Å, SiliCycle), SiliaBond C18 silica gel (40-63 µm, 60 Å, 17% carbon loading, SiliCycle) and Sephadex LH-20 (25-100 µm, lipophilic, Sigma-Aldrich) were used for column chromatography.

2,3-Diphenyl-1H-pyrrole (1): mp 125-126^{o}C; IR (ATR) ν =3331, 1504, 1492, 1101, 893, 772, 757, 740, 687, 669, 605, 580, 551 cm-1 ; ^{1}H NMR (400 MHz, DMSO-d6); δ7.42-7.38 (m, 4H, Ph), 7.36-7.23 (m, 4H, Ph), 7.23-7.18 (m, 1H, Ph), 7.05-7.12 (m, 1H, Ph), 6.78 (t, J=2.6 Hz, 1H, H5), 6.15 (t, J=2.6 Hz, 1H, H4); 13C NMR (100.6MHz, DMSO-d6) δ=136.1 (C100), 133.3 (C10), 127.3 (2C), 127.2 (2C), 126.9 (2C), 126.3 (Cq), 126.3 (2C), 125.2, 124.3 (C4′ , C4″), 121.0 (Cq), 118.6 (C5), 110.0 (C4); EI-MS (m/z) 219 (100) [M], 129 (22), 117 (68), 103 (51), 90 (36), 83 (18), 77 (30), 55 (43), 40 (28); HR-MS calcd for [C16H13N] 219.1048, found 219.1078.

RESULTS AND DISCUSSION

The IR spectrum of compound **1** displayed intense characteristic absorption band at 3331, 1544, 1472, 1100, 873, 792, 767, 744, 687, 649, 615, 560 cm^{-1} presence of an aromatic moiety with pyrrole ring. The molecular formula of $C_{16}H_{13}N$ was deduced from the HRMS [HR-MS calcd for [$C_{16}H_{13}N$] 219.1048, found 219.1078]. The ^{1}H NMR (DMSO-d_6) resonances δ 7.42-7.38 (m, 4H, Ph), 7.36-7.23 (m, 4H, Ph), 7.23-7.18 (m, 1H, Ph), 7.05-7.12 (m, 1H, Ph) and its corresponding ^{13}C (DMSO-d_6) resonates are found in experimental procedure, respectively. The HMBC & COSY correlation of compound **1** clearly indicating the isolated compound **1** is related to biphenyl pyrrole, all the possible correlations shown in **Figure 1**. The compound (1) was isolated first time from the marine *Brevibacterium* specie and synthetically known[20]. Based on above data and with available literature, the compound **1** is confirmed as Biphenyl pyrrole.

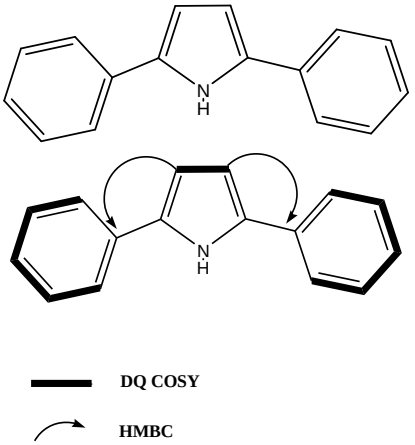


Figure 1: Structure and its possible correlations of compound (1)

Isolation of marine bacteria

Seawater samples were collected in the intertidal zone of Visakhapatnam coastal region, India. The water samples were collected in clean, sanitized and autoclaved bottles and brought to the laboratory. About 8 µl of sample water was spread over the entire surface of Zobell marine agar 2216 **(Himedia, India)** plates consisting of g l: peptone 5.0, yeast extract 1.0, ferric citrate 0.1, sodium chloride 19.45, magnesium chloride 8.8, sodium sulphate 3.24, calcium chloride 1.8, potassium chloride 0.55, sodium bicarbonate 0.16, potassium bromide 0.08, strontium chloride 0.034, boric acid 0.022, sodium silicate 0.004, sodium fluoride 0.0024, ammonium nitrate 0.0016, disodium phosphate 0.008, agar 15.0. After incubation at 25°C for 48 h, all colonies were screened and those with different morphology were isolated.

Selection of potential strain

The potential strain was selected based on the colour of the pigment. In the first phase, all chromogenic cultures that showed bright pigmentation were short listed. During the second phase, the bacteria that produced intense yellow pigmentation on Zobell agar medium was selected for further studies. The criteria employed for selection of potential strain included production of intense colour.

Screening of isolate for antimicrobial activity

Preparation of crude extract

The marine bacteria were cultured in 300 ml Marine Broth (peptone 5 g, yeast extract 1 g, dissolved in 1 L seawater, pH 7.2-7.6) in 500 ml Erlenmeyer flasks for the production of cells. Flasks were incubated on a rotator shaker at 220 rpm for 3 days at 25 °C. Marine bacterial cells were separated from the spent broth by

centrifugation (at 4000 *g* for 15 min at 4 °C) and washed twice with sterile water. The yellow pigment was extracted from the cells with methanol at 4 °C until the pellet becomes white. The crude extract was tested for antimicrobial activity and methanol (5%) was also used as a negative control.

Molecular Identification

The bacterial strains which showed significant antimicrobial activity were identified to Genus level by PCR amplification of the 16S rRNA gene, BLAST analysis, and comparison with sequences in the GenBank nucleotide database. Specifically, the 16S rRNA gene from strains was amplified using universal primers 27f (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') and PCR conditions were as described previously **(Acinas et al. 1999)**[21]. PCR products were purified and sequenced by the Dingan Bio-company (Shanghai, China). The sequences were compared with known sequences in the GenBank nucleotide database and the species level was identified as the nearest phylogenetic neighbour with sequences >99% sequence similarity **(Hentschel *et al*., 2001)**[22].

Extraction of the pigment

Extraction of the pigment was done following the method given by **Asker** and **Ohta** (1999)[23] with modifications.1 ml aliquots of cultures were centrifuged at 10000 rpm for 10 min at 4^{o}C. The harvested cells were re-suspended in distilled water for cell Lysis to occur. The pigment was then extracted with methanol by repeated centrifugation until the cell debris turned colorless.

***In vivo* Wound healing activity**

Experiment animals

Wistar albino rats weighing about 8-10 weeks (150–250 g), were used for the study (Mahaveer Agencies, Hyderabad). The rats were fed with standard rodent pellet diet and tap water *ad libitum*. They were housed in polypropylene cages maintained under standard laboratory conditions of temperature (25 ± 3 °C), light dark cycles of (12 hour light-dark cycle) and relative humidity of (35–60%). The animals were left for ten days to the laboratory environment for acclimatization before commencement of experiment. The experimental rats used and the protocols followed in this study were reviewed and approved by the Institutional Animal Ethical Committee (44/SPIPS/IAEC/13) before the initiation of the experiment.

Preparation of Ointment

For assessment of wound healing activity by excision wound model the extracts were formulated in the form of ointment. The ointment was prepared by fusion method. For preparation of simple ointment the ingredients used includes wool fat, hard paraffin, cetostearyl alcohol, white soft paraffin which were mixed and heated gently according to increasing order of their melting point and mixed gently with stirring followed by cooling and then packed in a wide mouth container. In same manner 10% ointment of yellow pigment was prepared and packed in a wide mouth container.

***In vivo* wound healing activity Excision wound model**

The cutaneous excision model was used to assess the wound healing activity of the yellow pigment extract. The wound was inflicted at dorsal side on the rats as described in the literature **(Mori *et al*., 2002).**[24] Before inflicting the excision wounds, the experimental rats were anesthetized by intraperitoneal administration of ketamine (70 mg/kg body weight), and the fur on the dorsal side of the animals was shaved using an aseptic surgical blade and disinfected with 40% of ethanol. A circular excision wound, of 300 mm^2 and 0.2 cm depth, was inflicted with a surgical blade on the dorsal surface at the thoracolumbar region of each of the experimental rats, under sterile conditions. To each experimental animal, 10% formulation of yellow pigment extract ointment was applied topically twice a day on the wound until they are completely healed. The progressive changes in wound were monitored planimetrically by tracing the wound margin on graph paper every alternate day. Epithelialisation time was noted as a number of days after wound required for the scar to fall off leaving no raw wound behind. From the healed wound, a specimen sample of tissue is isolated from each group of rats for histopathological examination**.**

Dosage used in wound healing studies

The effect of yellow pigment extract ointment on wound healing activity was studied in male Wistar rats. A total of 18 rats weighing 150–250 g were randomly selected and divided into three groups consisting of six rats in each group. Rats in each of the different experimental groups topically received an application of test and standard ointment in the following manner.

- Group I: Received no treatment and served as control
- Group II: Received application of standard drug ointment (2% Soframycin ointment)
- Group III: Received application of 10% yellow pigment extract ointment served as test group.

Measurement of wound index

The wound indices were measured after every 2 days of wound formation following a random scoring system.[38] The healing property was evaluated as percentage of wound contraction, measuring the length and size of the wound with digital callipers following the Walker and Mason formula.[39] Significance in wound healing of the test groups was derived by comparing the healed wound area, on the respective days, with the healed wound area of the control group. The rate of wound contraction was calculated using the given formula:

%Wound contraction=

$$\frac{\text{Initial area wound-nth day area of wound}}{\text{Initial area of wound}}$$

Skin irritation studies
This study was performed to observe any skin irritation for the animal model. Three sites were selected on the dorsal side of the rat. One side serve as control and other two sides was applied with standard ointment and extracted 10% yellow pigment ointment and observed the results for skin irritation.

Histopathology studies
The histological changes, i.e. epithelialization, granulation tissue formation, and cell migration, were observed during the process of wound healing in individual experimental rats that were treated with yellow pigment extract. On the 18th day of post wounding, all the animals were sacrificed, and the granulation tissue formed on and around the excision wounds of the untreated and treated rats was carefully dissected with a sterile surgical knife and carefully collected without any folding, and weighed. Later, the sample tissues were fixed in 10% buffered formalin solution (pH 7.4) and stored. After the usual processing of the tissue in dehydrated alcohol, these tissues were cleared in xylene and were embedded in paraffin wax (melting point 55 °C).Skin samples from wound healing sites were taken for histopathological studies. These tissues were stained with haematoxylin-eosin stain and viewed under microscope **(Hassan *et al.*, 2011)**[25] for histological examination. The sections were then observed under a light microscope (Olympus BX51) for qualitative assessment of the degree of necrosis, epithelialization, collagen formation and fibroblast proliferation in the wound tissues. Congestion, edema, PNL, mononuclear cells, fibroblasts and vascularization were also qualitatively evaluated for treated and untreated rats. From the healed wound, a specimen sample of tissue was collected from each rat for histopathological examination (**Taranalli *et al.*, 2004; Anderson, 1980).**[26, 27]

Statistical analysis The values were calculated as mean ± S.E.M. The significance of the difference of the mean value with respect to control group was analyzed by one way ANOVA followed by Dunnet's t- test using statistica 8.0. Statistically significant at a level of $P<0.05$ or above was considered to be significant.

The present study was carried out to investigate *in vivo* wound healing properties of yellow pigment extract of marine pigmented bacterium. Wounds may be defined as loss or breaking of cellular and anatomic or functional continuity of living tissues **(Patial *et al.*, 2001).**[28] Wound healing is an intricate and continual cascade of events, with various cellular and biochemical processes, ultimately resulting in the reconstruction and regeneration of damaged tissue (**Phillips *et al.*, 1991).**[29] Excision wound model was used to determine the wound healing activity of yellow pigment extracts. In this method, the wound of 300 mm^2 was induced and topically applied with yellow pigment extract ointment two times a day. The ointment of yellow pigment extract, reference standard was applied to wound twice daily, until recovery to respective group of animals. The observation of percentage wound closer was made on 3rd, 6^{th}, 9th, 12^{th}, 15^{th}, 18th and 21^{st} post wounding days(Figure.2&3). Wound contraction was measured in each 2 days interval, until complete wound healing and expressed in percentage of healed wound area **(Sadaf *et al.*, 2006).**[30]

In the present study the methanol extract of pigment from *Brevibacterium* sp. was able to reduce the time required for wound healing. The results were obtained from the faster contraction of the wound treated with pigment extract in comparison with untreated group **(Figure 2&3).**Wound contraction was expressed as percentage reduction of original wound size **(Mukherjee *et al.*, 2000)**[31]. Healing was determined by reduction in the size of wound area and found to be effective in the functional recovery of the healing of wounds and also in histopathological alterations. In general, the wound healing process is characterized by dynamic, interactive events involving soluble mediators, blood cells, extracellular matrix, and parenchymal cells that result in the permanent restoration of anatomic and functional integrity **(Singer and Clark, 1999).**[32] Histological evaluation was carried out for the treated and untreated samples. The methanol extract of pigment from *Brevibacterium* sp. was able to reduce the time required for wound healing. Ten percent yellow pigment extract as ointment showed better wound healing activity when compared to control. Further observation revealed that the wound contracting ability of the 10% (w/w) yellow pigment extract ointment treated groups showed better wound healing from the sixth day onwards. The administration of the yellow pigment extract accelerated the progression of wound healing by 6^{th} day. All treated groups were compared with the control groups. From the results, it was observed that pigment extract ointment showed better and fast wound healing activity when compared to untreated control **(Fig 2)**. Moreover, the animal behavior was normal during the entire experimental period, and the rats did not show any signs of compound-related toxicity or other related skin-sensitive side effects. The compound showed promising wound healing activity, without any abnormal behavior patterns, such as fatigue, stress, aggressiveness, weight loss, change in movement, or signs of infection among the rats during the course of the study. The percentage of wound contraction was tabulated in **Table-1.**

The percentage of wound contraction in standard and test is 100 percentage and in control group the percentage of wound contraction was comparatively less because the wound was left open so it has taken more time to get healed completely. Wound healing is a process by which a damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound. There was a reduction in wound area from day three onwards in treated mice and also on later days the closure rate was much faster than

when compared with control mice. Table shows the effect of pigment extracts on excision wound model in mice.

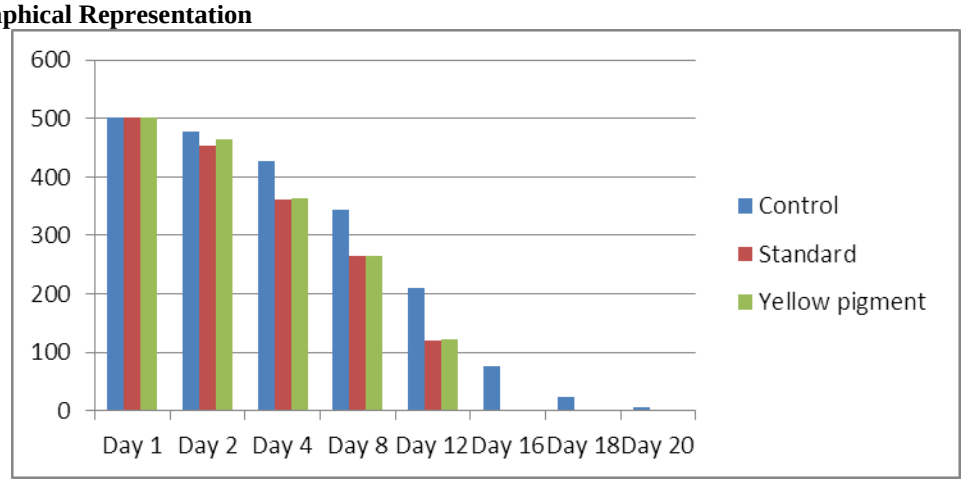
The wound closure time was lesser, as well as the percentage of wound contraction was more with the 10 % yellow pigment extract ointment treated group. The results of the present study indicated that the methanol extract of yellow pigment was able to reduce the time required for wound healing.

Histopathological studies of the wound tissues of the rats that received yellow pigment extract ointment showed morphological changes, such as higher collagen content, granulation tissue formation, and increased migration of macrophages and fibroblasts cells, which aid in granulation tissue formation and wound tissue repair. Antimicrobial property of yellow pigment extract massively reduced the bacterial population, thereby indirectly reducing the inflammatory cells on the wound site. Complete wound epithelialization with increased collagen formation was observed on the 16th day of wound healing in the experimental rats that received yellow pigment extract. The visual observation of wound confirmed that growth of the granulation tissue initiated from the base of the wound and proceeded to fill the entire wounds following treatment with both the yellow pigment extract and soframycin. The histopathological slides obtained from the animals during wound healing process also showed clear cut differences in between control and treatment. The granulation tissue formed was pink-red in color, moist, and shiny. The percentage of wound contraction and reepithelialization after topically administered yellow pigment extract was promising when compared with the control group studied. Thus the study clearly demonstrated that the yellow pigment extract ointment accelerated the process of wound healing compared with the untreated animals. Furthermore, all the three (ointment base, 10% yellow pigment extract ointment and 2% soframycin) was shown negative results for skin irritation studies. From the results obtained in the present study, it is possible to conclude that the ointment of the extract of yellow pigmented bacteria has promising wound healing activity. Present study confirms the potent wound healing activity of methanol extract of yellow pigment of marine *Brevibacterium* sp.

Table-1: Wound healing studies of methanolic extract of yellow pigment showing percentage reduction of wound size in rats (% contraction)

Post wound healing days	Control	Standard	Yellow pigment
Day 1	501	501.3333333	501
Day 2	477.666667	454	464
Day 4	426	360.3333333	363.6666667
Day 8	342.666667	264.6666667	264.6666667
Day 12	211	121	122.6666667
Day 16	77	0	0
Day 18	24	0	0
Day 20	6.66666667	0	0

Figure 1: Graphical Representation



Control - Control group
Standard - Soframycin ointment
Yellow pigment - Yellow pigment extract ointment

Figure.2. Photographical representation of wound contraction in albino rats treated with yellow pigment extract during 20 days of treatment.

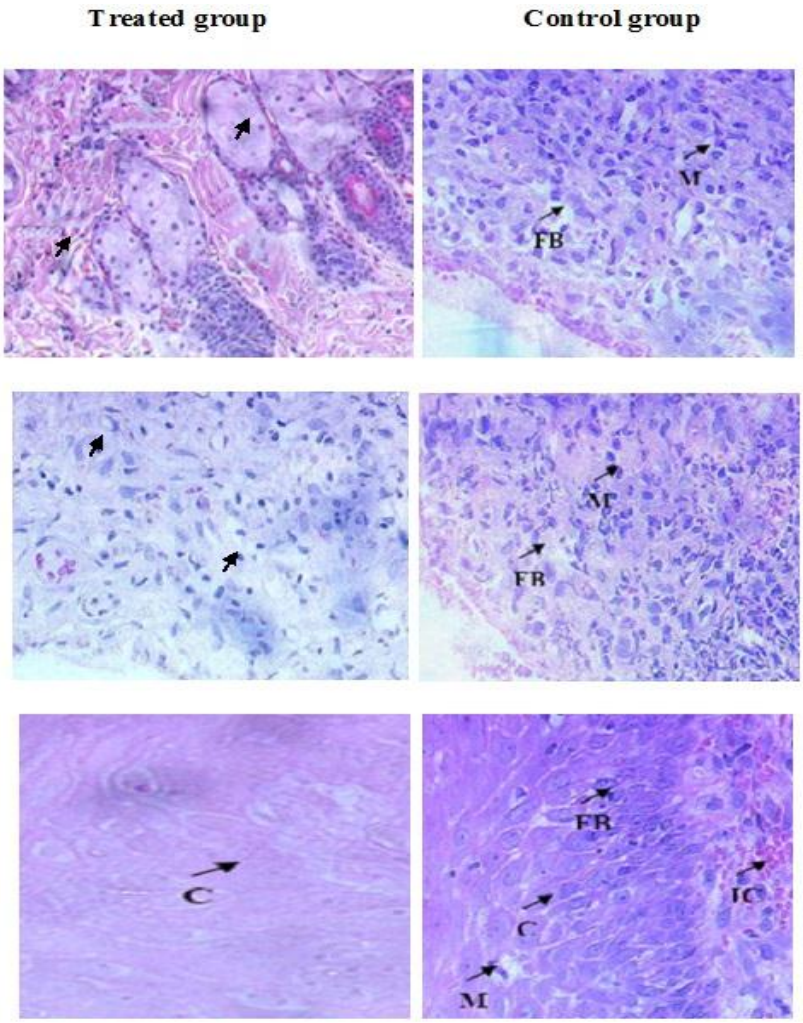


Figure-3: Histopathological slides of wound tissues showing wound healing process in excision wound model treated with yellow pigment extracts.

ACKNOWLEDGEMENTS

The authors are thankful to the **Dr. Ch. V. Purushotham Reddy**, Chairman, Chaitanya Colleges, Hanamkonda, Warangal for providing facilities and financial support.

REFERENCES

1. Bull AT, Ward AC, Good fellow, M, Microbiol, Mol. Biol. Rev, 2000; 64: 573-606.
2. Marderosian AD, Pharm. Sci. 1969; 58: 1-30.
3. Austin B, J, Appl, Bacteriol, 1989; 67: 461-470.
4. Molinski TF, Nat, Prod, 1993; 56: 1-8.
5. Carte BK, Bioscience, 1996; 46: 271-286.
6. Rinehart K L, Medical Research Reviews, 2000; 20: 1-27.
7. Schwartsmann G, Rocha AB, Berlinck RGS, Jimeno J, Lancet Oncology, 2001; 2: 221-225.
8. Armstrong E, Yan L, Boyd KG, Wright PC, Burgess JG, Hydrobiologia, 2001; 461: 37- 40.
9. Burgess JG, Miyashita H, Sudo H, and Matsunaga T, FEMS Microbiol, Lett, 1991; 4: 301-306.
10. Anand TP, Bhat AW, Shouche YS, Roy U, Siddharth J, and Sharma SP, Microbiol, Res, 2006; 161: 252-62.
11. Uzair B, Ahmed N, Ahmad VU, Mohammad FV, Edwards DH, Microbiol. Lett, 2008; 279: 243-240.
12. Kim D, Lee J S, Park YK, Kim JF, Jeong H, Oh, TK, Kim BS, and Lee, CH, Journal of Applied Microbiology, 2007; 102: 937-944.
13. Ferreira CV, Bos C L, Versteeg HH, Justo GZ, Duran N, Peppelenbosch MP, The American Society of Hematology, 2004; 104: 1459-1464.
14. Matz C, Deines P, Boenigk J, Arndt H, Erberl L, Kjelleberg S, and Jurgens K, Applied and Environmental Microbiology, 2004; 70: 1593-1599.
15. Deorukhkar AA, Chander R, Ghosh SB, and Sainis KB, Research in Microbiology, 2007; 158: 399-404.
16. Kim JS, Harikrishnan R, Kim MC, Balasundaram C, and Heo MS, Fish and Shellfish Immunology, 2010; 29: 104-110.
17. Yuan Lu, Liyan Wang, Yuan Xue, Chong Zhiang, Xin-Hui Xing, Kai Lou, Bioche, Engi. Journal, 2009; 43: 135–141.
18. Francis FJ, Cereal Food World, 2000; 45: 198–203.
19. Johnson EA, and Schroeder W,. Advances in Biochemical Engineering and Biotechnol, 1996; 53: 119-178.

20. Ines Bergner, Christine Wiebe, Nino Meyer, and Till Opatz, J. Org. Chem.2009; 74: 8243–8253.
21. Acinas SG, Anton J, and Rodriguez-Valera F, Applied and Environmental Microbiology, 1999; 65: 514–522.
22. Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C, Hacker J. FEMS Microbiol. Ecol, 2001; 35: 305–312.
23. Asker D and Ohta Y, J. Biosci. Bioeng, 1999; 88: 617-621.
24. Mori R, Kondo T, Ohshima T, Ishida Y, Mukaida N, FASEB J, 2002; 16(9): 963–974.
25. Hassan KA, Deogratius O, Nyafuono JF, Francis O, Engeu OP, Afr. J. Pharm. Pharacol. 2011; 5: 132-136.
26. Taranalli AD, Tipare SV, Kumar S, Torgal SS, Ind. J. Pharm. Sci. 2004; 66: 444-6.
27. Anderson JE. Muirs Text Book of Pathology. 11 th ed. ELBS; 1980; 77-85.
28. Patial MB, Jalalpure SS, Ashraf A, Indian Drugs, 2001; 38(6): 288-293.
29. Phillips GD, Whitehead RA, Kinghton DR, Am, J, Anat, 1991; 192(3): 257-262.
30. Sadaf F, Saleem R, Ahmed M, Ahmad SI, Navaid-ul-Zafar, J, Ethnopharmacol, 2006; 107: 161–3.
31. Mukherjee PK, Verpoorte R, Suresh B, J, Ethnopharmacol, 2000; 70: 315-21.
32. Singer AJ, Clark RAF. The New England Journal of Medicine, 1999; 341: 738-746.