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WOUND HEALING ACTIVE CONSTITUENT FROM *Cryptolepis buchanani*

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INTRODUCTION

Non-healing wounds are a challenging problem to the patient as well as to the healthcare professionals and the health care system. It is recorded that they significantly impair the quality of life for millions of peoples in the USA (Jorge de la Torre 2007). This is true not only to the USA but to the rest of the world as well. It requires intensive treatment which imparts an enormous burden on the society in terms of lost productivity and healthcare especially in the developing countries such as ours, as the total health care system depends heavily on foreign exchange.

Wound healing, or wound repair, is a complex natural process of the body, which involves regeneration of dermal and epidermal tissue. During the wound healing process, a set of overlapping events take place in a predictable fashion to repair the damage (Stadelmann, *et al.* 1998).

Wound infection is likely the most common reason for poor wound healing. Common aerobic pathogens responsible for such infections are *Staphylococcus aureus*, *Enterococci* species, Coagulase-negative *Staphylococci, Pseudomonas aeruginosa, Enterobacter* species, *Proteus mirabilis, Klebsiella .pneumoniae,* and *Candida* species (Jorge de la Torre 2007).

There are many reputed wound healing medicinal oils that are currently in use in the Indigenous and Ayurvedhic systems of medicine, some of which are known to be in existence for more than 2000 years. Therefore a systematic study of these medicinal preparations to identify the active constituents and/or active fractions responsible for wound healing was undertaken. 'Pinda oil' is one of the reputed medicinal oils which is widely used for wound healing (Jayasinghe D. M. 1976) and it is prepared by boiling dried aerial parts of *Rubia cordifolia*, *Cryptolepis buchanani* and *Glycyrrhiza glabra* in sesame oil and water until all the water is evaporated and adding plant ingredient. Herein we report the isolation and identification of a plant metabolite with positive Wound Healing Activity (WHA) from *Cryptolepis buchanani*.

METHODOLOGY

Extraction of *C. buchanani* and fractionation

The extraction was taken by soaking dried and coarsely ground plant materials (300 g / batch) in water (3 L) at room temperature for one day and then boiling for 20 hours. The aqueous extract was filtered and partitioned sequentially with hexane, dichloromethane, and ethyl acetate and each organic layer was evaporated to dryness under reduced pressure at 40 °C. The residual plant material was boiled again with 1.5 L of water for 18 hours and the water extract was sequentially partitioned with hexane, dichloromethane and ethyl acetate. A total of 900 g of plant material was subjected to the above extraction procedure.

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The hexane extract (850.0 mg) of *C. buchanani was* chromatographed on a column of silica gel (35-70 mesh) eluted with mixtures of hexane, ethyl acetate and methanol. Purified compounds were identified by using NMR data.

Anti Bacterial Assay

The extracts and/or fractions were dissolved in methanol and used to determine antibiotic activity against (*Bacillus* sp. *E. coli* and *Klebsiella* sp.) using Kirby-Bauer Disc Diffusion method (Pommerville and Alcamo 2004). Filter paper discs containing Amoxicillin 25 µg (positive control) test samples (500 µg of extracts dissolved in 10µL of methanol) and discs with 10µL of methanol (negative control) were made and dried for 24 hours prior to the experiment. The test was carried out at ~ 35 °C. Results are shown in Table 1.

Wound healing Assay

The wound healing assay was carried out (Yarrow *et al)*on a monolayer of Madin-Darby Canine Kidney cell line. Cells were grown in Dulbecco's Modification of Eagles Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) with Penicillin / Streptomycin (5000 Units) and Fluconazole (150mg). Cells were distributed at a density of 3.0 X 10^4 cells/well in 200µl on 12 well tissue culture plates (SARSTEDT) with clear bottoms. Cells were incubated at 37 °C with 5% CO_2 overnight in a humidified incubator. Scratch wounds were introduced after 24 hours by using a micro pipette tip. Extracted and purified test samples were dissolved in carrier medium, dimethyl sulfoxide (DMSO) and 10µl of this solution were added to the cell cultures where the scratch wounds were made. 10µl of DMSO was used in the control experiment. Cultures were observed and the size of the scratch wounds was measured at different time intervals. Results are shown in Table 2 and Figure 1.

RESULTS AND DISCUSSION

C. buchanani (900 g) yielded combined hexane extract (1.0509 g), CH_2Cl_2 extract (0.8072 g) and EtOAc extract (0.9768 g). An antibacterial disc sensitivity test was carried out with the above crude extracts, on *Bacillus* sp., *E. coli,* and *Klebsiella* sp. and the hexane extract showed antibacterial activity against *Bacillus sp. E. coli* and *Klebsiella* sp while the other two extracts did not show significant antibacterial activity (See Table 1).

Extract	Dose (µg/disc)	Diameter of Clear Zone (mm)		
		Bacillus sp.	*E. coli*	*Klebsiella* sp
Hexane	500	10.50	12.50	10.50
CH_2Cl_2	500	7.0	7.0	No clear zone
EtOAc	500	No clear zone		
Amoxicillin (+)	25	10.5	22.50	No clear zone

Table 1: Results of the antibacterial assay of hexane, CH_2Cl_2 and EtOAc extracts of *C. buchanani*

Prompted with these positive results, the fractionation of the hexane extract (850 mg) was carried out using column chromatography, 60 fractions collected and pooled according to their TLC patterns (F-1 to F-14) and subjected to antibacterial assay. None of the fractions showed significant antibacterial activity, suggesting either the activity has been distributed among the fractions or lost due to the adsorption to the column material.

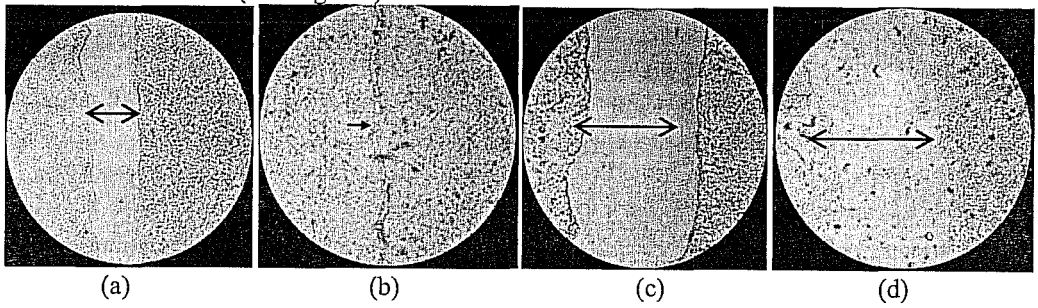
TLC examinations have shown the column fraction F-1 to be a single compound while fraction F-3 consisted one major compound with slight impurities, which gave pure compound (RK/21/33/01) on further purification on preparative TLC on silica gel (eluant: 4% EtOAc in hexane). The remaining fractions, F-2 and F-4 to F-14 were found to be mixtures of compounds.

Both these compounds were subjected to wound healing assay as described in the methodology. Different concentrations of RK/21/33/01 were added into the wells with scratched wounds on Madin-Darby Canine Kidney monolayer of cells. The healing of the wounds was observed at different time intervals for 24 hours (Table 2) and photographed and Figure 1).

Samples	Concentration of the sample (%)	12 hours	24 hours
RK/21/33/01	50	Partially healed	Healed
RK/21/33/01	40	Partially healed	Healed
RK/21/33/01	30	Partially healed	Healed
RK/21/33/01	20	Partially healed	Healed
RK/21/33/01	10	Partially healed	Healed
RK/21/33/01	5	Not healed	Not Healed
RK/21/33/01	2.5	Not healed	Not Healed
Control(DMEM)		Not healed	Not Healed
10% GM		Partially healed	Healed
20% GM		Partially healed	Healed

Table 2: Results of the wound healing assay of RK/21/33/01

It was observed that RK/21/33/01was capable of affecting the wound healing activity at a concentration of 10% (See Figure1).



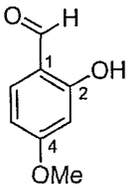
(a) (b) (c) (d)

Figure -1 Images of scratched wounds of monolayer of Madin-Darby Canine Kidney cells
(a) Initial stage (b) after 24 hours with the test sample at 10%
(c) Initial stage (d) after 24 hours of the control

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of RK/21/33/01 shows 6 signals while ^{13}C NMR showed 8 carbons. ^{1}H NMR showed the presence of CHO (δ 11.5, s), OH (9.7, s) and OMe (δ 3.8, s) and 1,2,4-trisubstituted benzene ring (See Table 3). Analysis of HSQC and HMBC correlations revealed the compound to be 2-hydroxy-4-methoxybenzaldehyde (**1**).

#	δ_H (multiplicity)	δ_c	gHMBC
1		115.0	C-6, C-5, C-3
2		164.5	C-6, C-3
3	6.4 (d, J = 2.4 Hz)	100.6	C-2, C-5
4		108.4	OMe, C-6, C-3
5	6,5(dd, J =2.4 and 8.8 Hz)	135.2	C-3
6	7.4 (d, J = 8.8 Hz)	166.8	C-2
CHO	11.5 (s)	194.4	C-6
2'-OH	9.7 (s)		
4'-OMe	3.8 (s)	55.7	C-4

Table 3: NMR data ($CDCl_3$; 400MHz) of 2-hydroxy-4-methoxybenzaldehyde (RK/21/33/01)



(1)

The compound RK/21/31/01 isolated from *Cryptolepis buchanani* was found to be a long chain hydrocarbon by spectral analysis.

CONCLUSIONS/RECOMMENDATIONS

2-Hydroxy-4-methoxybenzaldehyde isolated from *C. buchanani was* found to be enhancing the cell migration ability on wound healing assay. The development of the bioassay methodology is underway to study such activity quantitatively.

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