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ATR-FTIR ANALYSIS OF TOPICAL CREAMS FORMULATED WITH CHROMOLAENA ODORATA ETHANOLIC EXTRACT AND HONEY: A WOUND HEALING STUDY

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ABSTRACT

This study was carried out to evaluate wound healing effect of topical creams formulated with Chromolaena odorata ethanolic extract and honey. The topical creams were formulated as follows: cream A (95% base + 5% C. odorata ethanolic extract), cream B (90% cream base + 5% C.odorata ethanolic extract + 5% honey) and cream C (95% cream base + 5% honey). The experimental animals were segregated into five groups (each group consisting of five rats) as follows: group 1 (treated with cream A), group 2 (treated with cream B), group 3 (treated with cream C), group 4 (treated with positive control - Aloe vera cream) and group 5 (treated with negative control - cream base). Epithelization period less than 14 days was observed in group 1 and group 4 while the other animal groups showed the epithelization period more than 14 days. Cream A demonstrated the highest wound healing rate, followed by cream C and cream B. Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) analysis demonstrated that there were five new absorption peaks [wavenumbers: 1159.2 cm^{-1} (amines), 1259.4 cm^{-1} (amines), 1456.7 cm^{-1} (alkanes), 1508.8 cm^{-1} (aromatics) and 2921.9 cm^{-1} (carboxylic acids)] present in spectrum of cream B as compared with those of other formulated topical creams and cream base. We proposed that aromatic diamines were produced during formulation of cream B and caused the delay of wound healing process in the experimental animals.

KEY WORDS

Topical cream; wound healing; Chromolaena odorata; honey

INTRODUCTION

Wound is a type of tissue damage associated with disruption of its anatomic continuity [1]. It could be caused by physical, chemical, thermal, microbial or immunological insult to the tissue. Restoration of wounded tissue basically consists of integrated cellular and biochemical processes which lead to re-establishment of physical, structural and functional integrities of the wounded tissue. In particular, the wound healing process which begins just after surface lesions and or harmful exposures, involves various body responses such as coagulation, inflammation, formation of granulation tissue and tissue remodeling [2]. Currently, there has been a great interest in exploring natural resources for wound healing potential.

Chromolaena odorata, a perennial belonging to the plant family Asteraceae, is widely distributed in Southern Asia and Western Africa. It is a weed of 13 crops in 23 countries and has been described as the world's worst weed [3]. In addition to its antibacterial activity [4], *C. odorata* leaf extract has also been reported to exhibit promising wound healing effect.

According to [5], *C. odorata* ethanolic extract demonstrated protective effect on fibroblasts and keratinocytes against oxidative stress, which was due to its antioxidant content. By using liquid chromatography coupled with UV spectroscopy and mass spectrometry, the antioxidant contents of *C. odorata* ethanolic extract were found to be phenolic acids (protocatechuic, p-hydroxybenzoic, p-coumaric, ferulic and vanillic acids) and lipophilic flavonoid aglycones (flavanones, flavonols, flavones and chalcones). They concluded that a mixture of powerful antioxidant compounds contained in *C. odorata* ethanolic extract might be one of potential mechanism contributing to enhanced wound healing. Another *in vitro* study on wound healing activity of *C. odorata* ethanolic extract was conducted by [6]. It was observed that for fibroblasts, toxicity of hydrogen peroxide or hypoxanthine xanthine oxidase on cells was dose-dependent. At 400 and 800 microg/ml, *C. odorata* ethanolic extract showed maximum and consistent protective cellular effect on oxidant toxicity at low or high doses of oxidants while at 50 microg/ml concentration, it also had significant and slightly protective effects on fibroblasts against hydrogen peroxide and hypoxanthine-xanthine oxidase induced damage. Meanwhile, for keratinocytes, a dose-dependent relationship of oxidant toxicity was only seen with hydrogen peroxide but the protective action of *C. odorata* ethanolic extract correlated with oxidant dosage. *C. odorata* ethanolic extract at 400 and 800 microg/ml showed dose-dependent effects with both low and high concentration of oxidants whilst at 50 microg/ml, it had no effect on keratinocytes. In that study, it was suggested that protection of cells against destruction by inflammatory mediators may be one of the ways in which *C. odorata* ethanolic extract contributes to wound healing. Taken together, *C. odorata* ethanolic extract possesses a high wound healing potential. However, its synergistic wound healing effect in combination with honey which is well known to have wound healing activity remains uncertain. Therefore, this study was carried out to evaluate wound healing effect of topical creams formulated with *C. odorata* ethanolic extract and honey using excision wound model and attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR).

MATERIALS AND METHODS

Plant extract

Leaves of *C. odorata* and pure honey were collected from Kuala Rompin, town in the Rompin district of the State of Pahang, Malaysia. The leaves were washed, dried and ground into powder form. One hundred grams of the powder was then soaked in one liter of 100% ethanol. The solvent was then filtered using a Whatman No. 1 paper and removed using a rotary evaporator.

Topical creams

Topical creams were formulated using a combination of fermented rice as a cream base, *C. odorata* ethanolic extract and pure honey as follows: cream A (95% base + 5% *C. odorata* ethanolic extract), cream B (90% cream base + 5% *C.odorata* ethanolic extract + 5% honey), cream C (95% cream base + 5% honey).

Phytochemical screening

Chemical test for saponins, tannins and flavonoids were performed on the formulated topical creams using standard protocols.

Experimental animals and wound healing activity

A total of 25 male Sprague Dawley rats of approximately the same age, weighing about 150-250 g were used for the study. They were

fed with standard diet and water *ad libitum*. They were housed in polypropylene cages and maintained under standard conditions (12/12 hr light/dark cycle; 25^0C-30^0C). They were segregated into five groups (each group consisting of five rats) as follows: group 1 (treated with cream A), group 2 (treated with cream B), group 3 (treated with cream C), group 4 (treated with positive control - Aloe vera cream) and group 5 (treated with negative control - cream base). The experimental animals were subjected to ether anesthesia and their dorsal skin was shaved. Excision wound (four cm length) was created on the dorsal back of experimental animals using a forceps, surgical blade and pointed scissor. The formulated creams were then applied topically on the wound site on daily basis. Within 14 days of wound healing period, wound length and epithelization period were monitored. The procedures used in this animal study were in accordance with the ethical standard from the UiTM Research Committee on the Ethical Use in Research (UiTM Care).

Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR)

The samples (cream base and formulated topical creams) were placed in direct contact with horizontal attenuated total reflectance (ZnSe crystal) at controlled ambient temperature. The infra red spectra were recorded in the frequency range of 4000 cm^{-1} to 400 cm^{-1}. Comparison of infra red spectra was made between the cream base and formulated creams.

Statistical analysis

Wound length (cm) was expressed as mean ± standard error measurement. The significant difference ($p<0.05$) between the animal groups was determined using one-way ANOVA.

RESULTS AND DISCUSSIONS

Solvent extraction has been a common method to extract phytochemical constituents from medicinal plants. In this study, ethanol solvent was used because it is known to be effective in extracting the important bioactive compounds that exhibit wound healing potential. The leaves of *C. odorata* were extracted using 100 % ethanol solvent in four different duration of soaking times namely 24, 48, 72 and 96 hours. **Table 1** shows effect of soaking time on *C. odorata* plant extract yield. It was found that 72 hour soaking time produced the highest percentage (13.12±0.29%) of *C. odorata* plant extract yield whilst 24 hour soaking time produced the lowest percentage (11.11±0.09%) of *C. odorata* plant extract yield. It seems that the soaking time did not apparently affect the percentage of *C. odorata* plant extract yield.

Phytochemical constituents play key role in determining biological activities of medicinal plants. In this study, topical creams were prepared by formulating cream base (fermented rice) with *C. odorata* ethanolic extract and pure honey. With respect to wound healing activity of *C. odorata* ethanolic extract, the phytochemical screening was conducted on the formulated topical creams in order to determine the presence of saponins, tannins and flavonoids compounds. **Table 2** shows the presence (indicated by +) or absence (indicated by -) of those chemical compounds in base (fermented rice) and formulated topical creams. It was observed that saponin compounds were absent in cream base and cream A, and present in cream B and C. Meanwhile, tannin and flavonoid compounds were present in all test samples except cream base. This result was slightly different from a previous study [7] reporting the presence of saponins in the ethanolic extract of *C. odorata*, which could be attributed to

different geographical locations where the plants were collected. The leaves of *C. odorata* in that study were collected from the University of the Philippines Diliman, Quezon City, Philippines.

Table 1: Soaking time and percentage of plant extract yield

Soaking Time (Hours)	Percentage of plant extract yield (%)				
	1	2	3	Average	S.E.M
24	13.11	13.22	13.00	11.11	0.09
48	11.89	12.83	12.41	12.38	0.38
72	13.44	13.18	12.74	13.12	0.29
96	12.82	12.32	13.27	12.95	0.39

Table 2: Phytochemical screening of formulated creams

Creams	Bioactive components	Saponins	Tannins	Flavonoids
Cream base	-	-	-	-
Cream A	*C.odorata* ethanolic extract	-	+	+
Cream B	*C.odorata* ethanolic extract + honey	+	+	+
Cream C	honey	+	+	+

Table 3 Effect of topical application of formulated creams on epithelization period

Animal groups	Creams	Epithelization period (days)
Group 1	Cream A	< 14
Group 2	Cream B	> 14
Group 3	Cream C	> 14
Group 4	+ve Control	< 14
Group 5	-ve Control	> 14

Epithelization period is a common method used in the study of wound healing activity of the medicinal plants. In brief, epithelization means growth of epithelium at the centre of wound site after injury. In this study, the epithelization period was determined within 14 days following topical application of the formulated creams on dorsal back of experimental animals. **Table 3** shows the epithelization period recorded for all animal groups. The epithelization period less than 14 days was observed in group 1 (Cream A – cream base + *C. odorata* ethanolic extract) and group 4 (positive control – Aloe vera cream) while the other animal groups showed the epithelization period more than 14 days. It was suggested that the wound healing activity of cream A was comparable with that of positive control. In addition to the epithelization period, wound length also has been one of the common parameters to evaluate wound healing activity of medicinal plant. It is normally measured on daily basis after wound excision has been made on dorsal back of animals. In this study, the wound length was measured using analytical scale within 14 days following topical application of the formulated creams. **Table 4** and **Figure 1** show length and healing of wound of different animal groups. To evaluate the wound healing effect of the formulated topical creams clearly, day 2, 8 and 14 post surgical wound were

highlighted. It was observed that, the wound length apparently reduced from day 2 onwards in all animal groups except group 5. At day 8, it was also found that the wound healing activity of cream A (1.36±0.15 cm) was comparable with that of positive control (1.32 ± 0.43 cm). Meanwhile at day 14, all animal groups exhibited the wound length less than one cm except group 5 (2.20±0.45 cm). Taken together, cream A demonstrated the highest wound healing rate, followed by cream C and cream B. Because the cream B was formulated with *C. odorata* ethanolic extract and pure honey, it was possible that the formulation suppressed its wound healing effect. *In vivo* wound healing effect of *C. odorata* leaf extract and pure honey has also been investigated by [8]. In that study, *C. odorata* aqueous extract and pure honey were topically applied on the posterior neck area of Sprague Dawley rats. In particular, group 1, 2, 3, and 4 were treated with normal saline (negative control), honey, a combination of honey and *C. odorata* aqueous extract, and solcoceryl jelly (positive control) respectively. It was found that the wounds treated with solcoceryl jelly and honey in combination with *C. odorata* aqueous extract healed significantly earlier than honey alone which indicated that the synergistic effect resulting from the combination was comparable with the positive control. However, our finding was not in agreement with [8] which might be due to different plant extraction method.

Table 4 Effect of topical application of formulated creams on wound length (cm). Values are expressed as mean ± standard error measurement with n=5.

Animal groups	Creams	Wound length (cm)						
		Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Group 1	Cream A	3.06±0.17	2.44±0.09	1.94±0.24	1.36±0.15	1.00±0.23	0.48±0.20	0.10±0.14
Group 2	Cream B	3.06±0.23	2.62±0.22	2.36±0.38	2.18±0.38	1.92±0.50	1.30±0.41	0.92±0.37
Group 3	Cream C	3.12±0.19	2.94±0.05	2.40±0.26	2.22±0.26	1.74±0.27	1.04±0.29	0.36±0.18
Group 4	+ve Control	3.12±0.41	2.68±0.32	2.12±0.40	1.32±0.43	0.68±0.26	0.12±0.16	0.00±0.00
Group 5	-ve Control	3.54±0.11	3.22±0.11	2.92±0.23	2.76±0.34	2.62±0.37	2.40±0.44	2.20±0.45

In the context of medicinal agent, synergistic effect is an increased intensity of healing or protective effect due to a combination of two or more bioactive substances. *C. odorata* ethanolic extract was mixed with pure honey of known wound healing agent in order to investigate the possible synergistic wound healing effect of the formulated topical cream. However, based on **Table 4** and **Figure 1**, the cream B (cream base + *C.odorata* ethanolic extract + honey) was found to exhibit wound healing effect less than cream A and C. Therefore, to understand this, ATR-FTIR analysis was carried out to determine chemical composition of all the formulated topical creams. **Figure 2** displays ATR-FTIR spectra of the formulated topical creams and cream base. It was demonstrated that there were five new absorption peaks present in spectrum of cream B as compared with those of other formulated topical creams and cream base. The

wavenumber of those new absorption peaks were as follows: 1159.2 cm^{-1} (amines), 1259.4 cm^{-1} (amines), 1456.7 cm^{-1} (alkanes), 1508.8 cm^{-1} (aromatics) and 2921.9 cm^{-1} (carboxylic acids). This indicated that aromatic diamines were possibly formed when the cream base formulated with *C. odorata* ethanolic extract and pure honey. In 2005, Shin et al. [9] studied suppressive effect of novel aromatic diamines on nuclear factor-kappa B (NF-KB), an inflammatory factor. It was noted that the aromatic diamine compound (N1-benzyl-4-methylbenzene-1, 2-diamine, BMD) suppressed activation of NF-KB which in turn reduced the inflammation. It was also shown that the BMD compound had therapeutic potential in nitric oxide (NO)-associated inflammatory diseases. Furthermore, dermal absorption of the aromatic diamines has been investigated by [10]. They found that the aromatic diamines (2, 5-toluylenediamine, 2, 5-TDA) which is a contaminant in hair dye formulation, was rapidly absorbed dermally based on GCMS method. After a distribution phase of 12 hours, 2, 5-TDA was excreted with a half-time of 8 hours. Excretion was 90% complete within 24 hours after application. The doses of 2, 5-TDA excreted within 48 hours were 700 µg for application of a brown-reddish hair dye cream and 1.5 mg for the application of a brown-black hair dye cream. In relation to these, the aromatic diamines observed in cream B might play a role in the wound healing effect. Considering the fact that the inflammation is one of the important stages in acceleration of wound healing [2], it was proposed that the aromatic diamines partially suppressed the inflammation which in turn delayed the wound healing process in the experimental animals topically treated with cream B. However, it remains poorly understood how the aromatic diamines could be produced during formulation of cream B.

Figure 1 Photographs of rats showing various phases of wound healing.
A: cream A (cream base + *C.odorata* ethanolic extract); B: cream B (cream base + *C.odorata* ethanolic extract + honey); C: cream C (cream base + honey); D: positive Control (Aloe Vera cream).

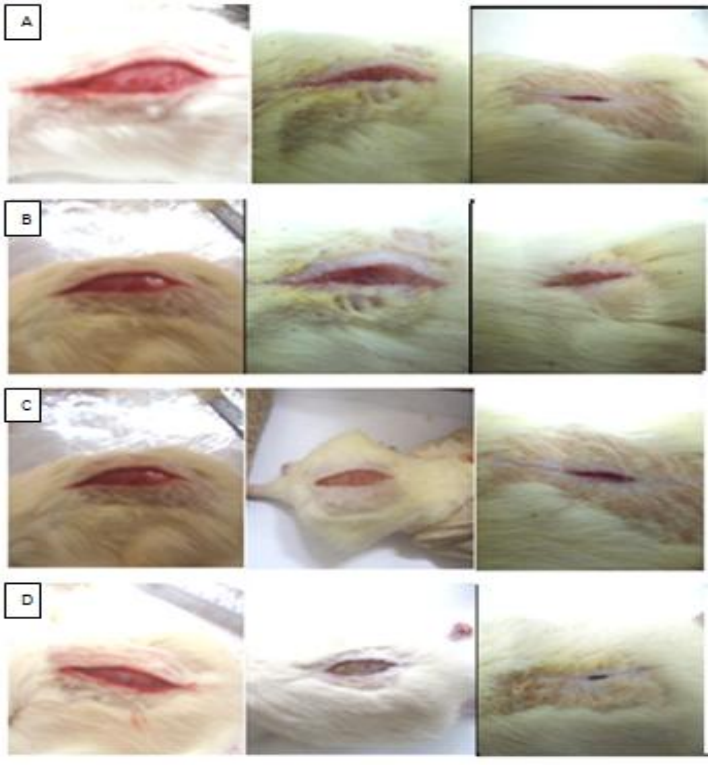
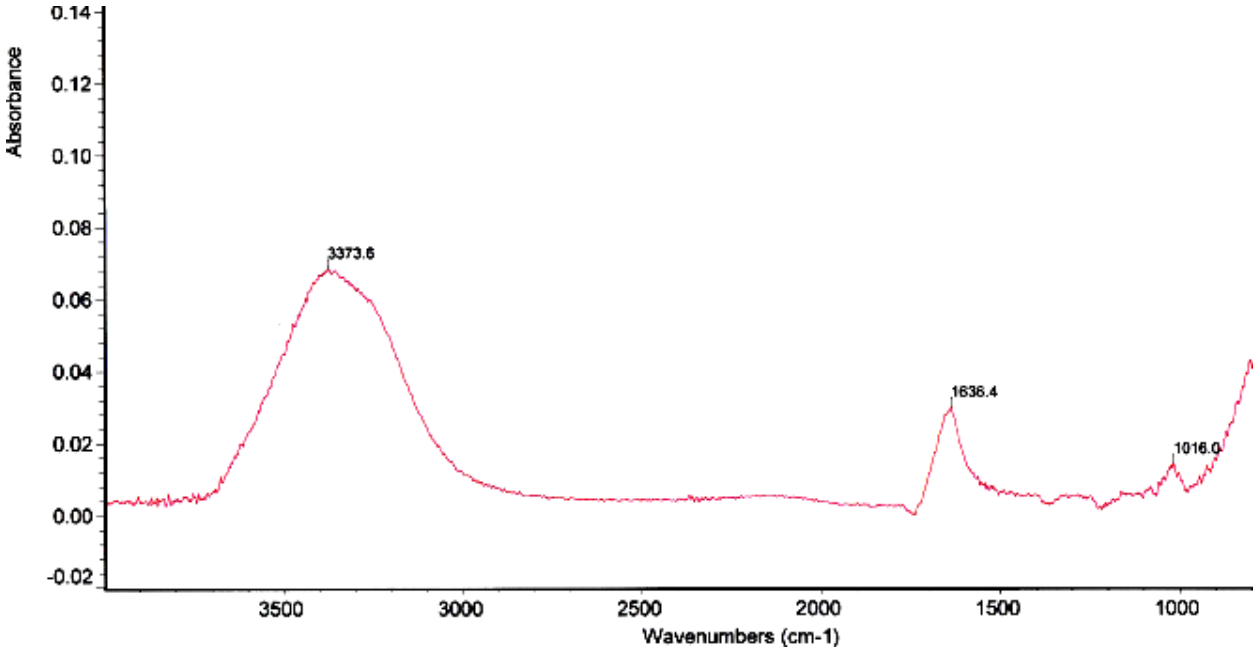
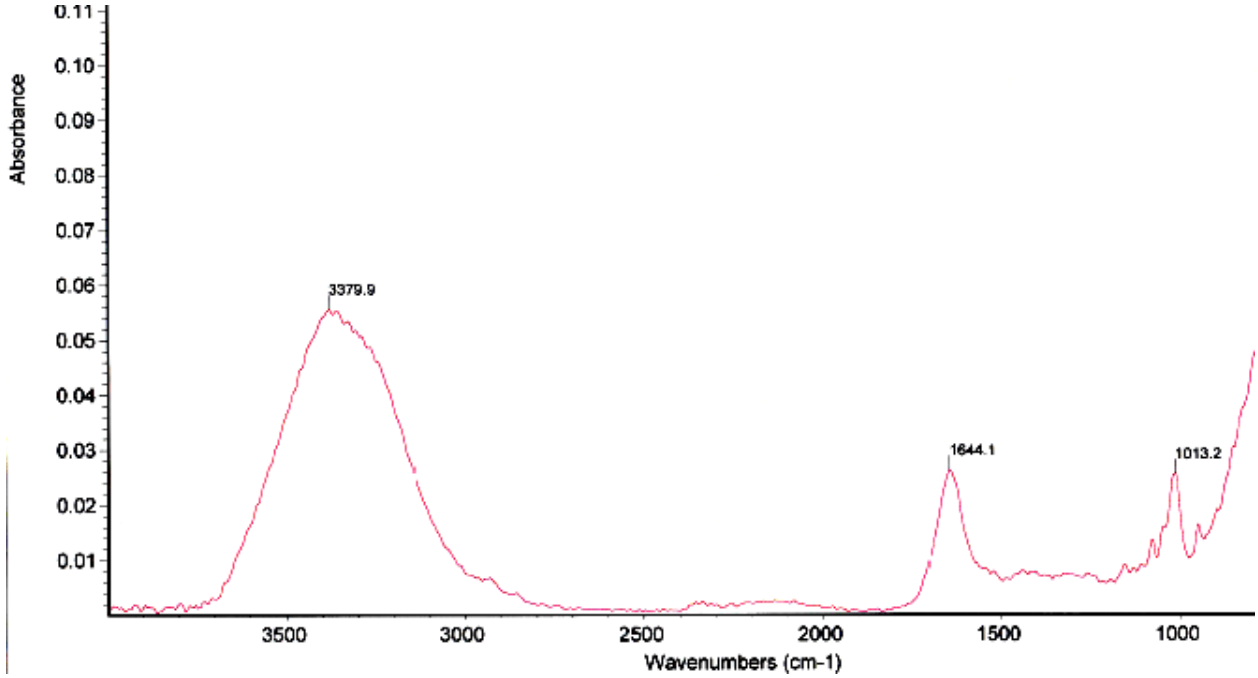


Figure 2: ATR-FTIR spectra of cream base and formulated topical creams. Arrows indicate the new absorption peaks [wavenumbers: 1159.2 cm^{-1} (amines), 1259.4 cm^{-1} (amines), 1456.7 cm^{-1} (alkanes), 1508.8 cm^{-1} (aromatics) and 2921.9 cm^{-1} (carboxylic acids)] in comparison of all spectra.

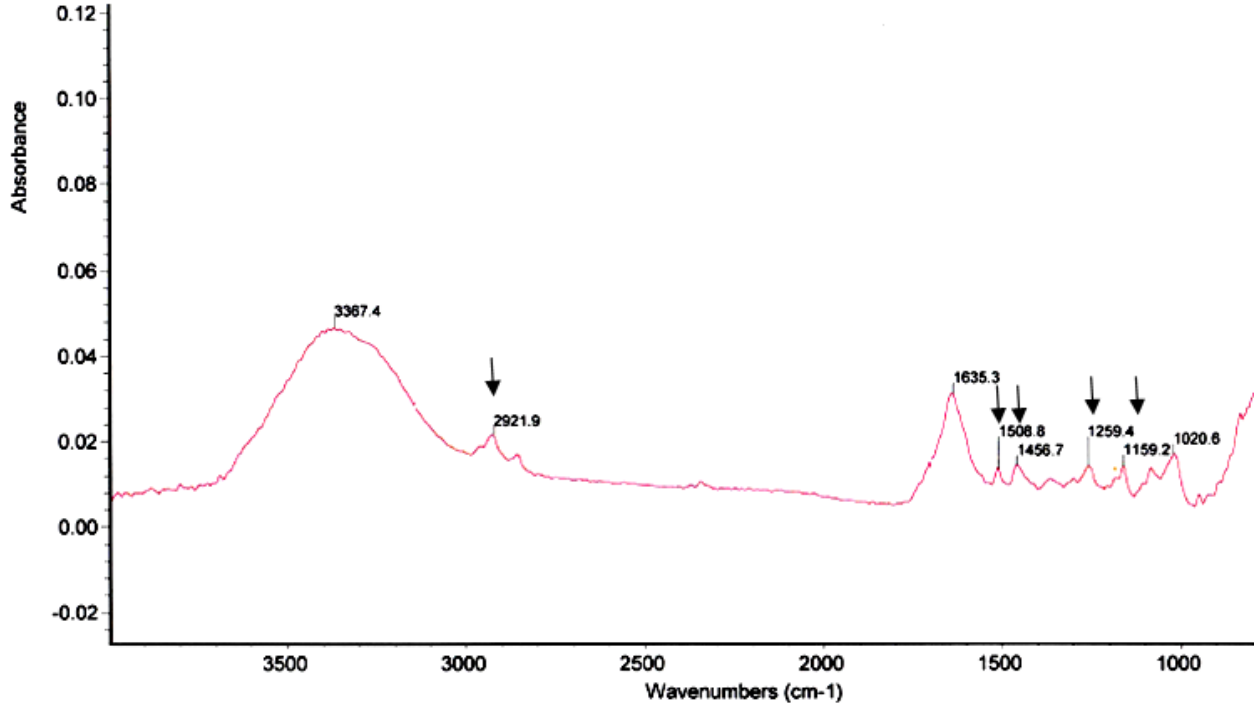
Base



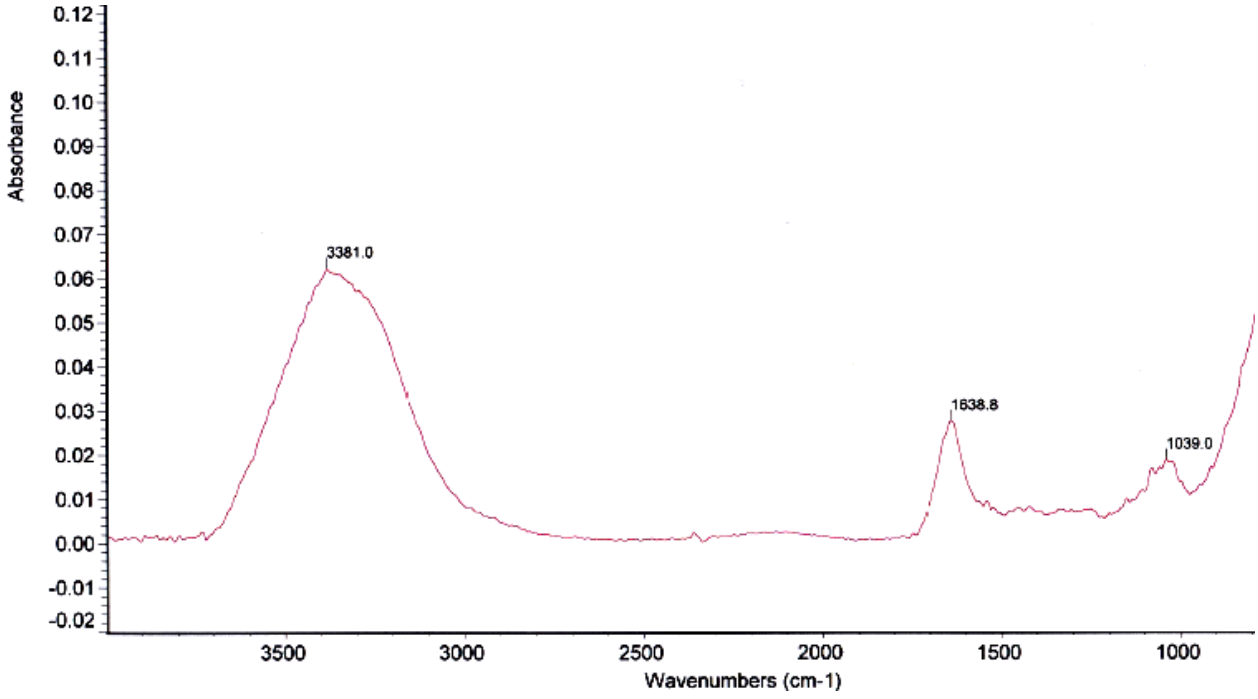
Cream A (95% base + 5% *C. odorata* ethanolic extract)



Cream B (90% cream base + 5% *C.odorata* ethanolic extract + 5% honey)



Cream C (95% cream base + 5% honey)



CONCLUSION

We have demonstrated that cream A (cream base + *C. odorata* ethanolic extract) exhibited the highest wound healing rate, followed by cream C (cream base + honey) and cream B (cream base + *C. odorata* ethanolic extract + honey). The ATR-FTIR spectrum of cream B was found to contain five new absorption peaks

which correspond to amines, alkanes, aromatics and carboxylic acids, when compared with spectra of other formulated topical creams and cream base. It was likely that the aromatic diamines were produced during formulation of cream B and caused the delay of wound healing process in the experimental animals.

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