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An investigation of the antimicrobial and anti-inflammatory activities of crocodile oil

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ABSTRACT

Ethnopharmacological relevance: Crocodile oil has been used by traditional practitioners world-wide to treat microbial infections and inflammatory conditions. However, the scientific rationale behind its use is not completely understood. This study provides an updated fatty acid profile and novel scientific evidence of the antimicrobial and anti-inflammatory properties of crocodile oil, obtained from the Nile crocodile (*Crocodylus niloticus*), justifying its use by traditional healers.

Materials and methods: The fatty acid content of the oil was determined by gas chromatography and the major fatty acids were identified. A microplate method was used to assess activity of the oil against *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Candida albicans*. The anti-inflammatory activity of the oil was assessed by oral administration and topical application, utilising a mouse model of acute croton oil-induced contact dermatitis.

Results: Sixteen fatty acids were identified with oleic, palmitic and linoleic acid being the major components of the oil. The optimal activity of the oil against the bacteria and fungus was obtained with 15% and 6% (w/v) oil respectively. No significant selectivity was observed against the bacterial species, but *Candida albicans* was more susceptible. The anti-inflammatory assays showed optimal activity at 3 h after the oral administration of oil ($60.8 \pm 5.5\%$) and at 12 h after topical application ($57.5 \pm 5.9\%$). This suggested a short duration of action when the oil was orally administered, and a longer duration of action when it was topically applied.

Conclusions: Subsequent studies may be directed towards the investigation of the mechanisms of action of the antimicrobial and anti-inflammatory activities of crocodile oil and its fatty acids.

1. Introduction

It has been observed by scientists that crocodiles live in environments that constantly expose them to various pathogens and microbes (Merchant et al., 2005; Merchant et al., 2006a; Merchant et al., 2006b). During fights, the limbs of crocodiles are sometimes torn and they are left with gaping wounds or even limbless. However, despite the harsh environment that they live in, they appear to heal rapidly and almost always infection-free (Merchant, Britton, 2006; Merchant et al., 2006c; Siroski et al., 2009). Research unveiled how powerful the crocodile's immune system is, as opposed to the human immune system (Shaharabany et al., 1999; Merchant et al., 2005; Preecharram et al., 2008). It is able to effectively destroy resistant bacteria, as well as viruses including the human immunodeficiency virus (HIV) (Merchant et al., 2005; Merchant, Britton, 2006; Merchant et al., 2006a; Siroski et al., 2009).

Crocodile oil has been used for centuries by traditional practitioners and has been documented to be very effective in the treatment of ailments ranging from skin conditions to cancer (Shim-Prydon & Camacho-Barreto, 2007). In Mexico, crocodile oil is used for illnesses such as asthma, emphysema, influenza and for a constant phlegmatic cough (Shim-Prydon & Camacho-Barreto, 2007). In Madagascar, the oil is prescribed to assist in the healing of burns, skin ulcers and cancer (Shim-Prydon & Camacho-Barreto, 2007). In Africa, crocodile oil is used for ailments such as skin rashes and to promote wound healing, and according to testimonial evidence, it showed extraordinary healing efficacy with an almost immediate observed effect

Abbreviations: ANOVA, analysis of variance; ATCC, American Type Culture Collection; DMSO, dimethyl sulphoxide; HIV-1, Human Immunodeficiency Virus-1; IL, interleukin; IC_{50}, concentration of crocodile serum that reduced viral load by 50%; MUFA, monounsaturated fatty acid; NSAID, non-steroidal anti-inflammatory drug; *p*-INT, *p*-iodonitrotetrazolium chloride violet; PUFA, polyunsaturated fatty acid; TNF, tumour necrosis factor

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(Shim-Prydon & Camacho-Barreto, 2007). There is also evidence of crocodile oil being used traditionally in South Africa. Many people in South Africa consult traditional healers and they play an important role in expanding the healthcare system in rural areas (Lindsey et al., 1999; De Villiers & Ledwaba, 2003; Griffiths & Cheetham, 1982; Ellis, 1996; Elgorashi et al., 2003). The fat is mixed with the ground bark of *Cryptocarya latifolia* and used by the Zulu people to treat chest ailments (Lall & Meyer, 1999). In Zululand, crocodile fat is used as protection against illnesses and lightning by the Tsonga people (Kelly, 2006).

It is not only the oil that is traditionally used in remedies. In China, the blood, oil, bile and gall bladder of crocodiles are used for conditions such as bronchitis, coughing, allergy, skin problems, high blood pressure and cancer (Shim-Prydon & Camacho-Barreto, 2007). Potent antimicrobial activity has been documented for the serum obtained from the American alligator *(Alligator mississippiensis)* against enveloped viruses in cell-based assays (Merchant et al., 2005). The serum showed activity against HIV-1 (IC_{50}=0.9%), West Nile virus (IC_{50}=4.3%) and the Herpes simplex type 1 virus (IC_{50}=3.4%). Serum from the Siamese crocodile *(Crocodylus siamensis)* also showed antibacterial activity against a variety of microorganisms, including *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Vibrio cholerae* (Preecharram et al., 2008).

The fatty acid profile of crocodile oil indicates that it consists mainly of fatty acids that were individually shown to possess antimicrobial and anti-inflammatory properties (Kabara et al., 1972; Maroon & Bost, 2006). Fatty acids play a vital role in the biosynthesis of hormone-like substances, such as prostaglandins which regulate many body functions, including heart rate, blood pressure, blood clotting, fertility and conception (Smith & De Witt, 1995; Lipsky, 1999).

The scientific evaluation of traditional medicines, including crocodile oil, is important and the aim of this study was to provide a current fatty acid profile of the Nile crocodile and to determine the antimicrobial and anti-inflammatory activities associated with the oil.

2. Materials and methods

2.1. Chemicals

Chloroform, croton oil, dimethyl sulphoxide (DMSO), fatty acid methyl ester standards, indomethacin, *p*-iodonitrotetrazolium chloride violet (*p*-INT), linoleic acid, methanol, Mueller–Hinton broth, oleic acid, palmitic acid, pentadecanoic acid (internal standard), Sabouraud dextrose broth, sodium chloride and sodium sulphate (anhydrous) were obtained from Sigma (Darmstadt, Germany).

The fatty acid analysis was based on a study by Franco and co-workers who investigated the fatty acid profile of the Celta pig breed (Franco et al., 2006). The standards consisted of methyl esters of the following fatty acids:

- Std 1: C12 (undecanoic acid);
- Std 2: C13 (tridecanoic acid), C14 (myristic acid), C15 (pentadecanoic acid), C16 (palmitic acid), C17 (heptadecanoic acid);
- Std 3: C16:1 (palmitoleic acid), C18:1 (*cis*-9-oleic acid), C20:1 (*cis*-11-eicosanoic acid), C22:1 (erucic acid);
- Std 4: C18 (stearic acid), C18:1 (oleic acid), C18:1 (*trans*-9-elaidic acid), C18:2 (linoleic acid), C18:3 (linolelaidic acid), C20 (arachidic acid);
- Std 5: C20:1 (*cis*-11-eicosenoic acid), C20:2 (*cis*-11,14-eicosadienoic acid), C20:4 (arachidonic acid), C20:5 (*cis*-11,8,14,17-ecosapentaenoic acid);
- Std 6: C24 (lignoceric acid).

2.2. Analysis of fatty acids

2.2.1. Sample preparation

Samples for this study were obtained from the tail fat of slaughtered male and female *Crocodylus niloticus* (Nile crocodile) collected from a crocodile farm in the Msunduzi Municipality, Pietermaritzburg, South Africa. The crude fat samples were transported to the laboratory under refrigeration and were immediately stored at −80 °C until analysed.

2.2.2. Extraction

The fat of the samples was extracted according to the procedure described by Folch and co-workers, but with slight modifications (Folch et al., 1957). Briefly, a crude sample (25 g) was homogenised with chloroform:methanol (2:1) (75 ml) and the mixture was stirred for 45 min on a magnetic stirrer. The mixture was then filtered through fat-free Whatman no. 1 filter paper (Whatman, Maidstone, UK). The filtrate was transferred into a separating funnel, and distilled water saturated with NaCl (35 ml) was added. The aqueous layer was discarded and anhydrous sodium sulphate (5 g) was added to the organic phase as a drying agent. The organic phase was filtered twice through the filter paper into a round bottom flask. The solvent was then removed using a Heidolph rotary evaporator (Heidolph, Darmstadt, Germany) set at 50 °C.

2.2.3. Identification and quantification

The procedure described by Shehata and co-workers was essentially followed but with slight modifications (Shehata et al., 1970). Identification and quantification of the fatty acids were carried out by gas chromatography (Varian CP-3800, Walnut Creek, CA, USA). The instrument was fitted with a model 1177 split/splitless injector, a flame ionisation detector and a CP-8400 auto-sampler using a Star chromatography workstation version 6.00. Peak areas were used for quantitation. Identification of fatty acids was achieved by comparison of retention times with the retention times of authentic standard fatty acid methyl esters.

Fatty acid separation was carried out on a PONA column (TR-50.2 PONA, Teknokroma, Barcelona, Spain) with a length of 50 m, an internal diameter of 0.25 mm and a stationary phase film thickness of 0.25 μm. The temperature programme used was as described in Table 1. The gases used were air (350 ml/min), hydrogen (355 ml/min), helium (carrier gas) (0.5 ml/min).

2.3. Antimicrobial susceptibility assays

2.3.1. Microbial strains

The following microorganisms were used (American Type Culture Collection—ATCC, Manassas, VA, USA):

- *Staphylococcus aureus* (ATCC strain 12600);
- *Klebsiella pneumoniae* (ATCC strain 13883);
- *Candida albicans* (ATCC strain 10231).

Staphylococcus aureus and *Klebsiella pneumoniae* were cultured in Mueller–Hinton broth (optical density of 0.6–0.8 at 490 nm) and *Candida albicans* (optical density of 0.3–0.5 at 630 nm) in

Table 1
Gas chromatography conditions.

Temperature ramps	°C/min	Temperature (°C)	Duration (min)
Initial	5	50	1
Ramp 1		248	40
Ramp 2		248	6

Sabouraud dextrose broth. Broths were prepared according to the manufacturer's protocols.

2.3.2. Susceptibility tests

Tests were conducted in clear, sterile 96-well microtitre plates (Corning Life Sciences, Acton, MA, USA). The assay was based on the microplate method by Eloff, but with modifications (Eloff, 1998). Briefly, the following steps were followed and the plate tapped after each step where compounds were added:

- a 100 μl/well of appropriate broth was added;
- this was followed by a 100 μl/well of the appropriate microbial culture;
- and finally the appropriate volumes of water (94, 79, 76, 73, 70, 64, or 49 μl), crocodile oil (0, 15, 18, 21, 24, 30, or 45 μl) followed by 6 μl DMSO in that order. The water, crocodile oil and DMSO constitute a total constant volume of 100 μl. The DMSO was added last to ensure that it never exceeded the 2% v/v limit. Pilot studies were conducted on the microbial species, and it was determined that this DMSO concentration did not adversely affect microbial growth. Therefore, with a total final volume of 300 μl/well (including the broth and culture) and a dilution factor of 3 ×, the final concentration of DMSO/well was constant 2% v/v and the final concentrations of the crocodile oil/well were 5% (50 μl/ml), 6% (60 μl/ml), 7% (70 μl/ml), 8% (80 μl/ml), 10% (100 μl/ml) and 15% (150 μl/ml);
- the plate was incubated at 37 °C for 18 (*Staphylococcus aureus* and *Klebsiella pneumoniae*) or 24 h *(Candida albicans)*;
- for *Staphylococcus aureus* and *Klebsiella pneumoniae*, 40 μl/well of *p*-INT (400 μg/ml in water) was added and plates were incubated (37 °C; 15 min);
- microbial growth was quantified by colourimetry (490 nm) for bacteria and optical density (690 nm) for *Candida albicans* in a microplate reader (BioTek ELx800, Winooski, VT, USA).

The positive controls were neomycin (*Staphylococcus aureus* and *Klebsiella pneumoniae*) and amphotericin B *(Candida albicans)* and the final concentrations in the wells were 100 μM.

2.4. Anti-inflammatory assays

Ethical approval (003/09/Animal) from the University of KwaZulu-Natal Animal Ethics Sub-committee was obtained prior to the investigation of croton oil-induced oedema in a mouse model. Guidelines by the University of KwaZulu-Natal Animal Ethics Subcommittee and Biomedical Resource Unit for the maintenance and treatment of laboratory animals were followed.

Young adult male Balb/c mice weighing 29–32 g were obtained from the Biomedical Resource Unit of the University of KwaZulu-Natal. The mice were maintained under controlled laboratory conditions with free access to food and water and with a normal day/night cycle.

For the topical anti-inflammatory assays, the study design of Lopez et al., 1999 was essentially followed, but with modifications (Lopez et al., 1999). For the oral anti-inflammatory assays, the study designs of Ghannadi and co-workers (Ghannadi et al., 2005) and that of Hong and co-workers (Hong et al., 2001) were implemented, but with modifications. Equal volumes of croton oil (25 μl) and acetone as vehicle (25 μl) were mixed and applied (50 μl total volume; 1 h) onto the inner surface of the right auricle of each mouse to induce oedema. The right auricle of each mouse was treated and the left auricle was used as the negative control and was left untreated. For the topical assays, crocodile oil (50 or 70 μl; 3, 6 or 12 h treatment) was applied onto the right auricle. The non-steroidal anti-inflammatory drug indomethacin (2 mg/100 μl acetone; 6 h treatment) was included as positive control. For the oral assays, crocodile oil (200 μl; 3, 6, or 12 h treatment) was administered through an oral gavage (Pharmed, Durban, South Africa). Indomethacin (2 mg/100 μl in 10% v/v DMSO) was used as positive control. Acetone as a vehicle has not been documented to have an anti-inflammatory effect by itself (Fretland et al., 1990). Mice were euthanised in a CO_2 chamber after treatment for 3, 6 and 12 h. From each mouse, left and right auricle biopsy specimens were obtained with a 6 mm biopsy punch and immediately weighed on an analytical balance.

2.5. Data analysis

Data are reported as the mean ± standard error of the mean of at least four to five independent experiments with duplicate measurements. For the antimicrobial assays, microbial growth was quantified as a percentage of the control without any test compound. For the anti-inflammatory assays, oedema was quantified by calculating the difference in weights of the right and left auricle biopsy specimens and expressed as a percentage of the croton oil control. Statistical comparisons were made by one-way ANOVA followed by Bonferroni's post-test for multiple comparisons, or by Student's two-tailed paired *t*-test to determine *P* values. A value of $P < 0.05$ was considered significant.

3. Results and discussion

This study investigated the fatty acid profile as well as the antimicrobial and anti-inflammatory activities of the Nile crocodile.

3.1. Identification and quantification of fatty acids

The crocodile oil extracted from the fat of *Crocodylus niloticus* was analysed using gas chromatography to determine the fatty acids. The crude oil was methylated to produce polar, non-reactive and volatile derivatives (methyl esters) of fatty acids, allowing rapid analysis by gas chromatography (Morrison, Smith, 1964; Hallmann et al., 2008). The fatty acid profile (as methyl esters) is depicted in Table 2.

From Table 2 it is evident that 16 fatty acids were detected. In comparison, Gunstone and Russell (1954) and Hoffman et al. (2000) detected 13 fatty acids each. Lignoceric acid has not been identified previously. The arachidate and erucate contents are

Table 2
The fatty acid profile of methylated oil obtained from the Nile crocodile, *Crocodylus niloticus*. Data are the mean ± standard error of the mean of triplicate measurements from 6 independent experiments.

Fatty acid	% Methylated content
Undedecanoate	0.139 ± 0.092
Tridecanoate	0.017 ± 0.006
Myristate	1.156 ± 0.167
Pentadecanoate	0.250 ± 0.000
Palmitoleate	3.138 ± 0.231
Palmitate	15.436 ± 1.038
Heptadecanoate	0.476 ± 0.054
Linoleate	4.031 ± 0.776
Linolelaidic	0.161 ± 0.093
Oleate	19.593 ± 1.764
Elaidate	0.205 ± 0.086
Stearate	1.359 ± 0.738
Eicosenoate	0.051 ± 0.024
Arachidate	0.002 ± 0.002
Erucate	< 0.001
Lignoceric acid	1.339 ± 0.675

considered insignificant (< 0.1%). Eight long-chain saturated fatty acids in the range C12–C24 were identified, as well as four mono-unsaturated fatty acids (MUFAs) and four poly-unsaturated fatty acids (PUFAs). The three fatty acids with the highest content were oleic (~20%), palmitic (~15%) and linoleic acid (~4%).

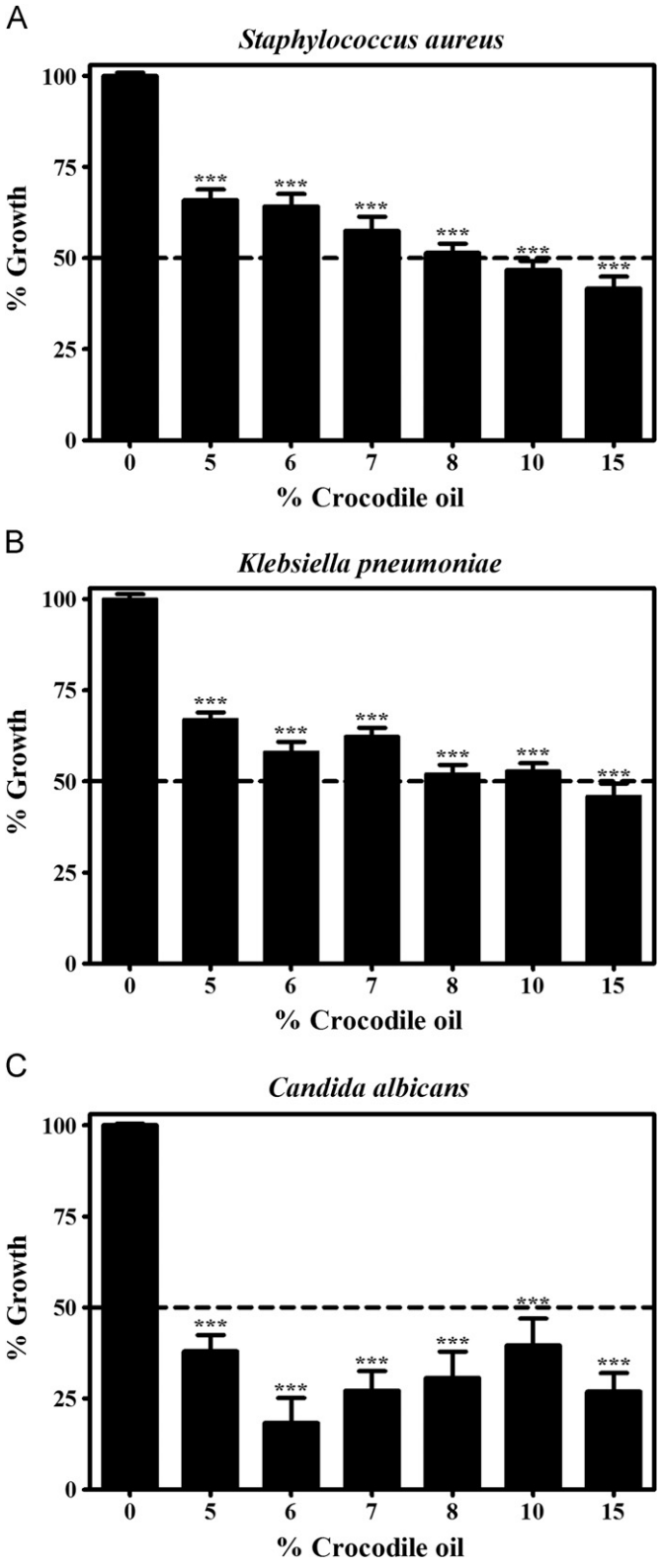

Fig. 1. Microbial susceptibility to oil obtained from the Nile crocodile, *Crocodylus niloticus*. The percentage growth of *Staphylococcus aureus* (A), *Klebsiella pneumoniae* (B) and *Candida albicans* (C) was measured against increasing concentrations oil (% v/v). Data are the mean ± standard error of the mean of duplicate measurements from 7–9 independent experiments. Statistical comparisons of activity were by one-way ANOVA followed by Bonferroni's post-test; ***, $P < 0.001$ versus no oil control.

3.2. Microbial susceptibility

3.2.1. Crocodile oil

Antimicrobial activity of crocodile oil is depicted in Fig. 1. For *Staphylococcus aureus* and *Klebsiella pneumoniae*, crocodile oil inhibited the growth in a dose-dependent manner with 15% v/v exhibiting the highest activity (58.4 ± 3.3% and 54.0 ± 3.4% respectively). For *Candida albicans*, a multiple dose-response relationship was observed with the highest activities at 6% (v/v) (81.7 ± 6.9%) and 15% v/v (73.1 ± 5.1%). These results suggest that no specificity exists between the Gram-positive *Staphylococcus aureus* and Gram-negative *Klebsiella pneumoniae*, and that structural differences in the cell wall do not modulate the efficacy of crocodile oil. However, the activity against *Candida albicans* was more pronounced, suggesting fungal specificity. The multiple activities against *Candida albicans* suggest that at different concentrations, multiple binding sites with different affinities may be involved (a high affinity binding site at lower concentrations, and a low affinity binding site at higher concentrations).

3.3. Anti-inflammatory activity

3.3.1. Oral administration

Fig. 2 depicts the oral anti-inflammatory activity of crocodile oil. The maximum oral dose of crocodile oil administered to mice was determined as 200 μl. Optimal activity was reached after 3 h treatment (60.8 ± 5.5%) and activity decreased after 6 h (46.9 ± 2.8%), but decreased significantly after 12 h treatment (35.3 ± 6.8%). Interestingly, there was no significant difference in the activity of the NSAID indomethacin after 3 and 6 h, but activity increased from 6 h (30.7 ± 4.1) to 12 h (44.3 ± 3.1%) treatment. These results suggest that indomethacin exhibited a longer duration of action, while crocodile oil resulted in an acute, relatively short acting anti-inflammatory response.

In a study by Zhao and co-workers, the anti-inflammatory activity of linoleic acid was confirmed and it was suggested that

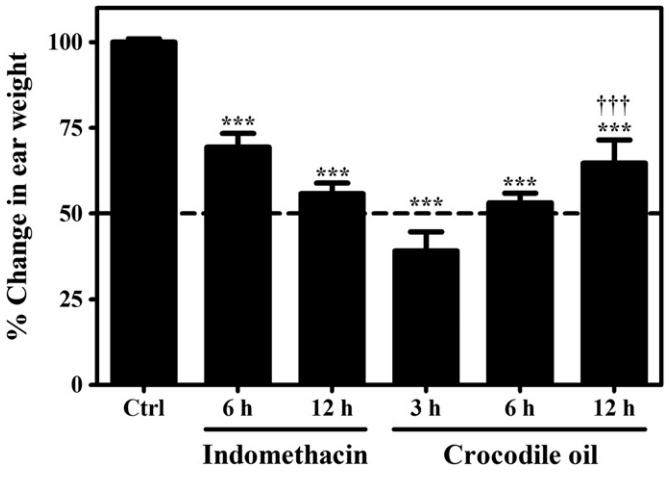

Fig. 2. The oral anti-inflammatory activity of oil obtained from the Nile crocodile, *Crocodylus niloticus*. Activity was measured in a mouse model of acute contact dermatitis. Indomethacin (2 mg/100 μl) and crocodile oil (200 μl) was orally administered and the mice treated for 3, 6 or 12 h. After treatment, the reduction in swelling of the auricular biopsies was determined and the change in weight was expressed as a percentage. Data is the mean ± standard error of the mean of duplicate measurements from at least 4 independent experiments. Statistical comparisons of activity were by one-way ANOVA followed by Bonferroni's post-test, or by Student's two-tailed, paired *t* test; ***, $P < 0.001$ versus no indomethacin and oil control; †††, $P < 0.001$ versus 3 h treatment with oil.

the mechanism of action was through the inhibition of IL-6, IL-1β and TNF-α (Zhao et al., 2005). This mechanism of action has advantages above the cyclooxygenase inhibitors in terms of side effects, such as gastric ulceration (Bjarnason & Rainsford, 2001; Gary & Green, 2001). According to the fatty acid profile of crocodile oil, linoleic acid contributed to 4% of the total fatty acid content. The activity was in agreement with anecdotal evidence of the successful treatment of many inflammatory diseases world-wide.

3.3.2. Topical application

Fig. 3 depicts the topical anti-inflammatory activity of crocodile oil. Optimal activity was reached with a 50 µl dose and 12 h treatment (57.5 ± 5.9%). No significant differences were found in activity between the 50 and 70 µl doses at all times and the activity was more pronounced after 12 h treatment. These results suggest that the topical application of crocodile oil resulted in an acute, relatively long acting anti-inflammatory response. This differs from the oral administration route which exhibited a relatively short duration of action.

Oleic acid is present in crocodile oil at ~20% and it has been reported to increase skin permeability through the stratum corneum by increasing the fluidity of the lipid matrix (Santoyo & Ygartua, 2000). However, this process is associated with skin irritation (Tanojo et al., 1997). In comparison to the pure fatty acids which may cause skin irritation, the plant extract of *Botryococcus braunii* containing a combination of fatty acids (including oleic acid) accelerated the topical absorption of the NSAID flurbiprofen and it was suggested that a buffer effect is produced by other components in the plant extract that alleviates irritation caused by fatty acids (Fang et al., 2004). It is suggested that the reduction in skin irritation could be attributed to the combination fatty acids in the plant material. No reports on the topical use of crocodile oil for medicinal purposes indicated skin irritation.

In contrast to the results obtained with the oral administration of crocodile oil, there was a tendency that the topical application resulted in a sustained anti-inflammatory activity after 12 h treatment.

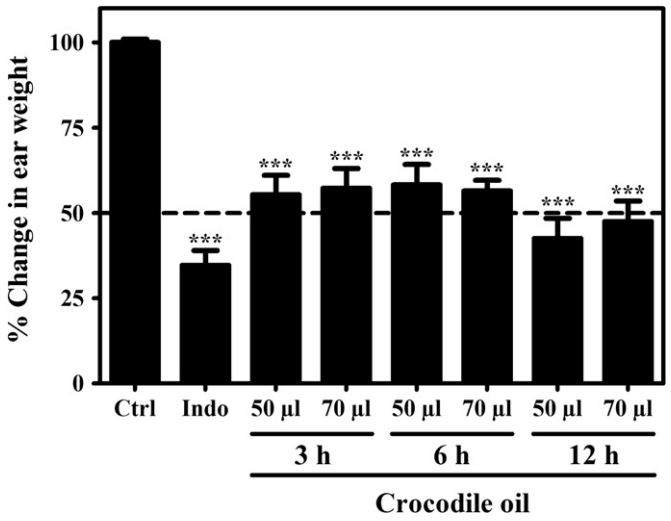

Fig. 3. The topical anti-inflammatory activity of oil obtained from the Nile crocodile, *Crocodylus niloticus*. Activity was measured in a mouse model of acute contact dermatitis. Indomethacin (2 mg/100 µl; 6 h) and crocodile oil (50 or 70 µl) was topically administered and the mice treated for 3, 6 or 12 h. After treatment, the reduction in swelling of the auricular biopsies was determined and the change in weight was expressed as a percentage. Data are the mean ± standard error of the mean of duplicate measurements from at least 4 independent experiments. Statistical comparisons of activity were by one-way ANOVA followed by Bonferroni's post-test; ***, $P < 0.001$ versus no indomethacin and oil control.

4. Conclusion

A new updated fatty acid profile was presented and compared to previous profiles. It was shown that crocodile oil exhibited antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* with no difference in susceptibility against the tested bacteria. It however exhibited fungal specificity against *Candida albicans*. Topical application of crocodile oil resulted in an acute, relatively long acting anti-inflammatory response which differs from the oral administration route where a relatively short duration of action was exhibited. This study provides novel scientific evidence of the antimicrobial and anti-inflammatory properties of crocodile oil, justifying its use by traditional healers. Subsequent studies may be directed towards the investigation of the mechanisms of action of the antimicrobial and anti-inflammatory activities of crocodile oil and its fatty acids.

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