



A Study of the Antimicrobial and Wound Healing Activities of the Ethanolic Leaf Extract of *Anchomanes difformis* (Blume) Engl. Pallidus

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: The aim of this current research is to ascertain the antimicrobial and wound healing properties of the leaves of *Anchomanes difformis* (Araceae).

Methods: Powdered leaves were extracted by cold maceration with 96.4% ethanol. Phytochemical analysis was carried on both powdered leaves and the extract to determine the presence of some secondary plant metabolites. Agar well diffusion method was used to determine the zone of inhibition on some microorganisms that infect wounds and the MIC of the extract was determined. Excision wound model was used for the wound healing analysis in Wister albino rats.

Results: Tannins, saponins, alkaloids, flavonoids, and phenols were detected in both the powdered leaves and the extract. Glycoside was detected in the powdered leaves however, not detected in the extract. The *A. difformis* extract demonstrated antimicrobial activity with MICs of >20mg/ml, 10mg/ml, 1.25mg/mL, 0.2679mg/mL for *Klebsiella pneumonia*, *Staph. aureus*, *E. coli*, *P. aeruginosa* respectively. Percentage wound healing contractions of 83.51%, 90.79%, 94.66% and 98.65% were recorded for 2.5%, 5%, 10%, and 15% concentrations of *A. difformis* extracts respectively.

Conclusion: From the result above, *A. difformis* has shown to have antimicrobial and wound healing properties. The study provides justification for the plant's traditional usage to treat infections and wounds.

Keywords: Wounds; medicinal plants; antimicrobial agents; MIC; agar well diffusion.

1. INTRODUCTION

A wound is a disruption of the cellular and anatomical continuity of a tissue (Cooper et al, 2001). The disruption may be produced by physical, chemical, thermal, microbial or immunological insult to the tissue. Current estimate indicates that 1.51 to 2.21 per 1000 persons worldwide suffer from chronic wounds [1]. Chronic wounds constantly produce inflammatory mediators that induce pain and swelling at the wound site. Chronic wound may lead to multiple organ failure or septicaemia which may lead to death of patient [2].

Chronic wounds represent a significant burden to patients and healthcare professionals affecting 5.7 million patients costing an estimated \$20 billion annually. To effectively manage these problems, one must understand the normal healing process and engineer a healthy physical and biochemical environment [3].

Traditional medicine has a long history of use and constitutes an important aspect of the culture and life of Africans [4]. They are widely used in the treatment of infections and diseases [5]. Africa is endowed with vast and diverse vegetation; making medicinal plants very accessible [6]. The goal of treating a wound is to hasten the healing process or to minimize the undesirable consequences like infections and inflammation [7]. Medicinal plants are enriched with bioactives which act synergistically to

stimulate wound healing. These bioactives are able to accelerate the proliferation and differentiation, prevent microbial contamination and enhance re-epithelization of the broken skin tissues. Medicinal plants are also able to prevent oxidative stress caused by reactive oxygen species that may have implication on wound healing. Plants documented to have wound healing properties include *Aloe vera*, *Vinca rosea*, *Rosemarinus officinalis*, *Camellia sinensis*, *Carrica papaya* and *Moringa oleifera* etc. *Curcuma domestica*, *Daucus carota*, *Emblca officinalis*, *Glycyrrhiza glabra*, *Mangifera indica*, *Allium sativum*, *Momordica charantia* just to mention a few [6,8,9].

Anchomanes difformis (Blume) Engl. Pallidus, commonly known as forest Anchomanes is a plant of the family Araceae. *Anchomanes difformis* is distributed widely in wetlands and terrestrial areas of west tropical Africa including Nigeria, Ghana, Ivory Coast, Sierra Leone, Senegal and Togo [10]. According to a study by [6] *Anchomanes difformis*, possesses antimicrobial, anti-inflammatory and antioxidant properties. The leaf has three [3] main divisions with each leaflet measuring 10cm long by 8 cm wide, an olive-green petiole and dull darkish-brown spot at the base of the short and rigid prickles. It has green stout prickly stem. The tuber is harvested from the wild as an emergency food in times of need. It is a multi-purpose plant and each of the part has a medicinal property. Folklorically, the roots are used to treat cough,

diabetes, dysentery and throat infections [11]. The stem and rhizome are used to ease child birth, constipation, hernia, kidney pain, abdominal pain and treat diabetes, gonorrhoea, asthma, epilepsy. More importantly, the leaves are used as galactagogue, antitussive, purgative, for wounds and minor cuts [12]. Indigenous using *Anchomanes difformis* apply directly into their wound as a poultice. Herbal products intended to be used as wound healing products must be scientifically validated [13]. In view of this, this research work investigated antimicrobial properties and the wound healing activities of the leaves of *Anchomanes difformis*. Also, the plant material and extracts were screened for phytochemicals. The aim is to scientifically validate the use of *A. difformis* for treating wounds and infections.

2. MATERIALS AND METHODS

2.1 Materials

Chemicals Used: Ethanol 98%v/v (GPR, BDH, Poole, UK.), chloroform 99.9%v/v (AR, Marek, UK.), normal saline infusion 0.9%w/v (Kabi Pot. Ltd. Pune, India.), Fehling's solution A and B (GPR, BDH, Poole, UK), Sulphuric acid 98.5%v/v (GPR, BDH, Poole, UK), Hydrochloric acid 36%v/v (GPR, BDH, Poole, UK), Sodium hydroxide 96%v/v (GPR, BDH, Poole, UK), Dragendorff's reagent 50%v/v (AR, Marek, UK), Ammonia solution 30%v/v (AR, Marek, UK) and Iron (II) chloride 97%v/v (GPR, BDH, Poole, UK).

2.1.1 Instruments/reference drugs: Amoxicillin (Bristol Laboratories, UK) (for antimicrobial assay) and Drez ointment (Stedman, India) (for the wound healing test).

Instruments and equipment/Glass ware: Rotary evaporation-R 10 (Buchi, Germany), Hot air oven (Sanyo, OMT Oven, Gallenkamp, UK), Gallenkamp Plus II Cooled Incubator (Gallenkamp, UK), Thermostatically controlled water bath (R76 New Brunswick, Edison N.J, USA Electronic weighing balance (Ohaus corporation, Pine Brook, N.J, UK), No. 5 Cork borer (Gerber Instruments, AG, Holland), Portable autoclave (Basildon, Ltd., UK), 500ml Separating funnel (GMBH, Wertheim, Germany), Beakers 50ml, 250ml, 500ml and 1L (GMBH, Wertheim, Germany), Test tubes (GMBH,

Wertheim, Germany), 1ml, 10ml dropping pipette (GMBH, Wertheim, Germany), 250ml, 500ml conical flask (GMBH, Wertheim, Germany) and Petri dish (GMBH, Wertheim, Germany).

2.1.2 Test organisms: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 26923), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC-BAA 1705).

2.2 Plant Collection and Preparation

The leaves together with the stems of *Anchomanes difformis* (Blume) were collected from Adansi North (Asokwa junction) in Ashanti Region. The collected specimen was authenticated by Miss Miriam Tagoe, the head of the School of Pharmacy Herbarium, and voucher number (CUC/ANK/008) was deposited in the Department's Herbarium. All dried plant materials were milled to coarse powders with mechanical milling machine and stored in airtight amber glass containers and well covered.

The fresh leaves were washed, dried under shade in the laboratory, and milled into powder. The obtained powder was stored in a dry container until ready for use.

2.3 Plant Extraction

The powdered leaves (430.56g) was extracted using 96.4% ethanol solvent by cold maceration. The ethanol extract was concentrated using rotary evaporator and a deep green mass of weight 41.89g was obtained. The ethanolic *Anchomanes difformis* extract was coded as ADC.

2.4 Phytochemical Screening

Phytochemical components of *Anchomanes difformis* (Blume) leaf extract was identified using conventional techniques outlined by [14].

2.5 Antimicrobial Studies

The leaf extract of *Anchomanes difformis* (Blume) was investigated for the antimicrobial activity against *Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* using agar well diffusion method and the zone of inhibition was measured [15].



Plate 1. Concentration of ADC using rotary evaporator

2.5.1 Sterilization of materials

All glassware, test tubes and petri dishes were washed with detergents and rinse in distilled water properly. These items were air dried and then sterilized in autoclave at 121 degrees Celsius for 15 minutes. Nutrient agar was prepared according to [16] and sterilized in autoclave at 121 degrees celsius for 15 minutes. Cork borer, glass rods and forceps were sterilized by dipping in 70% ethanol which was then flamed in Bunsen flame. The inoculating loop was also sterilized by heating to redness using naked flame before and after each use as described in [17].

2.5.2 Zones of inhibition assay

The antibacterial activity of the leaf extracts were carried out using agar well diffusion and a modified method described in [17]. Organisms known to affect wounds such as Gram positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*) were selected. The microorganisms were seeded on Muller–Hinton plates and then incubated in a bacteriological oven at $37\pm 1^{\circ}\text{C}$ for 24 hours. Bacterial suspension (inoculum) was diluted with sterile physiological solution to 0.5 McFarland barium sulfate standard (1×10^8 CFU/mL). Concentration above the Minimum inhibitory concentrations of the plant according to [18,19] to were selected and serially diluted to produce of 2.5, 5.0, 10.0 and 15.0 mg/ml in 200 μL with the aid of a micropipette into the plates. The zones of growth inhibition were measured after 24 hours incubation at 37°C . The antimicrobial activities of the extracts were compared with the activity of a standard antibiotic, amoxicillin (1 mg/mL). Experiments were carried out in triplicates,

and the antibacterial activity was expressed as the mean of the inhibition diameters (mm) produced. Amoxicillin was used as standard. Zones of inhibition was examined, measured (mm) and recorded.

2.5.3 Minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) of the extracts and the standard drug were evaluated using a modified solid media technique described in [20]. The minimum inhibitory concentration was calculated from the zones of inhibition by a graph method. The zones of inhibition were plotted against the log concentration on a graph sheet. The line of best fit was extended to intercept with the x axis (log concentration). The MIC was calculated as the antilog of x. This was repeated for all the test organisms.

2.6 Wound Healing Assay

2.6.1 Preparation of animals

Wistar albino rats (males and females) of weight between 150-170g were obtained from Noguchi Memorial Institute for Medical Research (NMIMR), Ghana. These rats were kept in stainless steel cages at the animal house, Department of Pharmacology, Central University, under ambient temperature (25°C), light, and relative humidity (55 to 60%). The rats were maintained on standard pellet diet and water daily for one week prior to the experiments for acclimatization at room temperature.

2.6.2 Excision wound healing model

The wound healing analysis was carried out by methods described by [21]. Excision wound

model was used to evaluate the rate of wound contraction and epithelization. Thirty-five Wistar rats of both sexes with body weight 120-170 g were used for this study. The rats were fed with standard feed and clean water. The rats were divided into six groups of five rats as indicated on Table 1. The rats were shaved and anesthetized with ketamine (30mg/kg, ip). Approximately 2cm wound area of was marked on the back of the rats using marker filled with ammonium oxalate violet paint. The marked shaved skin zone was then cut carefully in its thickness using razor blade and cleaned with 70% ethanol. The created wounds were allowed to assume it normal size as the tissues stretches and the initial wound size was taken and recorded using digital caliper. Wound treatment with formulated creams and Drez ointment began 24 hours post-injury and lasted for 14 days. The rate of wound contraction was calculated as given in the formula below:

$$\% \text{ wound contraction} = \frac{\text{Healed area}}{\text{Original wound area}} \times 100$$

(Healed area = original wound area – present wound area) [22,23].

2.7 Preparation of Aqueous Creams

Aqueous creams used for the wound healing experiment were prepared according to the method described in the British Pharmacopoeia (2013). Preservatives were excluded from the cream to prevent its interference with the wound healing activity of the extracts. Aqueous cream (100 g) was prepared by mixing 30 g of emulsifying ointment in 70 mL of sterile distilled water maintained in a water bath maintained at temperature 60 °C. The mixture was then stirred until it was melted and allowed to cool. Different concentrations of *Anchomanes difformis* extract (i.e., 2.5 % w/w; 5.0 % w/w; 10 % w/w; 15.0% w/w) were incorporated into the aqueous cream and kept in a labelled ointment container. The physical stability of the creams was monitored for phase separation, colour, odour, and texture.

Table 1. Grouping of rats for wound healing treatment

Groups	Description
	14 days treatment
I	2.5% <i>A. difformis</i> extract
II	5% <i>A. difformis</i> extract
III	10% <i>A. difformis</i> extract
IV	15% <i>A. difformis</i> extract
V	Drez ointment (PC)
VI	Untreated (0.9% sodium chloride) NC

PC= Positive Control, NC= Negative Control



Plate 2. Picture showing the excision wound process



Plate 3. Picture showing ADC aqueous creams

3. RESULTS

In the phytochemical screening tannins, saponins, flavonoids, alkaloids and phenols were detected in both the powdered plant material and plant extract. Glycosides were detected in the powdered plant material and but not detected in the plant extract as seen in Table 2. Antimicrobial assay was carried out on the extract as a preliminary test to determine the concentrations that would be used in the preparation of the ointment and also to confirm the antimicrobial

effect of *Anchomanes difformis*. Sensitivity test: agar cup plate method used. The zones of inhibition were measured and recorded as shown in Table 3. MIC of the extracts, which measure the minimum concentration of the extracts to cause inhibition in a microorganism were also evaluated as seen on Table 4. The excision wound model was employed. Both treated and untreated groups were analyzed for a period of 14 days. Figs. 1, 2 and 3 have the results from the wound healing assay.

3.1 Phytochemical Analysis

Table 2. Results of phytochemical analysis of the dried leaves powder and the extract of *A. difformis*

Phytochemical test	<i>Anchomanes Difformis</i> dried leaves powder	<i>Anchomanes Difformis</i> yield extract
Tannins	+	+
Saponins	+	+
Flavonoids	+	+
Phenols	+	+
Alkaloids (Wagner test)	+	+
Glycoside	+	-

Key: Detected = (+) and Not detected = (-)

Table 3. Zone of inhibition of the extracts and reference drug

Microorganism	Concentration/ Zone of inhibition (mm) ±STD				
	Amoxicillin (mg/mL)	20mg/ml	10mg/ml	5mg/ml	2.5mg/ml
<i>P. aeruginosa</i>	14 ±06	11 ±45	10 ±04	7 ±43	6 ±03
<i>Staph. aureus</i>	18 ±08	6 ±76	-	-	-
<i>E. coli</i>	5 ±03	8 ±23	5 ±09	4 ±12	2 ±09
<i>Klebsiella pneumonia</i>	5 ±11	-	-	-	-

STD, Standard deviation

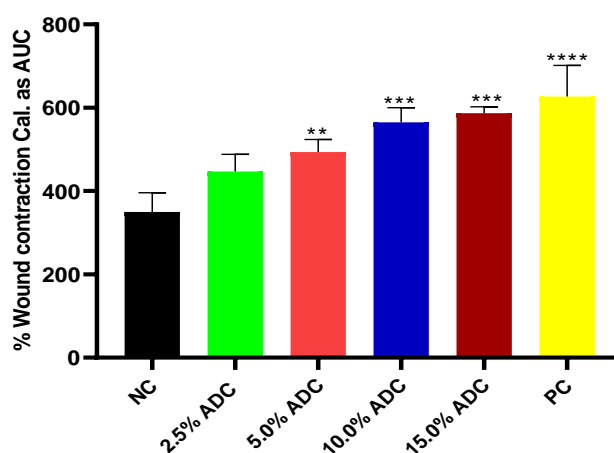


Fig. 1. This represents the wound healing response (AUC) using Wister albino rat. The data is expressed as mean±/SED (n=5), **p<0.05 (i.e. p=0.0065) as compared with the 0.9% sterile sodium chloride solution (negative control). PC (positive control); Drez ointment. One way ANOVA was used for this analysis followed by Dunnet's multiple comparison

Table 4. MIC result obtained for microorganism (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staph. aureus*, and *Klebsiella pneumonia*)

Organism	MIC (mg/ml) of ADC	MIC of Amoxicillin (mg/ml)
<i>P. aeruginosa</i>	0.2679	< 0.5
<i>E. coli</i>	1.25	< 0.5
<i>Staph. aureus</i>	> 10	< 0.5
<i>Klebsiella pneumonia</i> .	>20	< 0.5

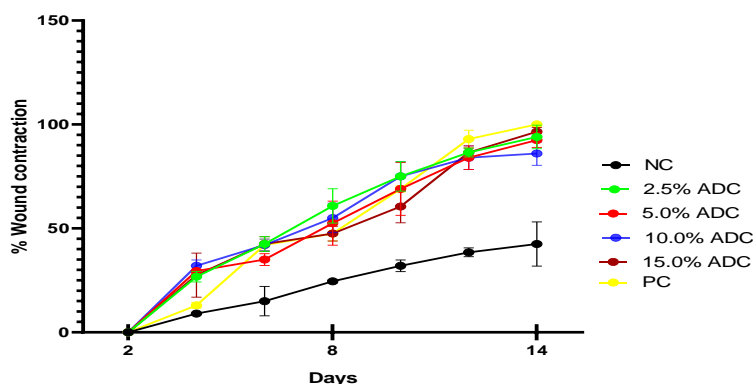


Fig. 2. Represents the time course for the healing and percentage wound contraction. The data is expressed as mean \pm SED (n=5), **p<0.05 (i.e. p=0.0065) as compared with the 0.9% sterile sodium chloride solution (negative control). One way ANOVA was used for this analysis followed by Dunnet's multiple comparison

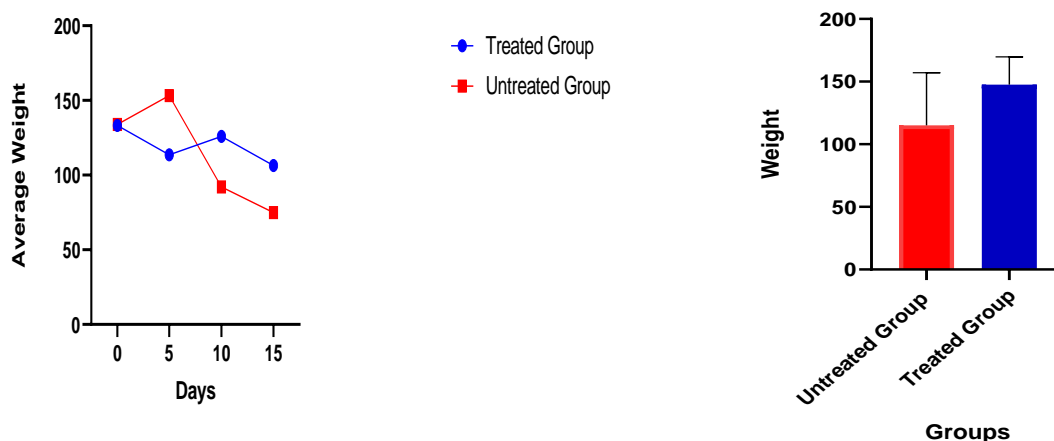


Fig. 3. (A) Average weight of rats over 15-day study period and (B) average weight treated and untreated groups on day 15

4. DISCUSSION

Wound healing is a natural phenomenon which arise from wound contraction [6,24,25]. Usually wound contraction begins with proliferation phase which covers angiogenesis, collagen deposition, fibroblast proliferation, granulation, tissue formation, epithelialization, and wound contraction. As the wound contract, it leads to the restoration of the lost and damaged cellular structures and epidermal tissue layers. Plants in

general have played an important role in wound healing and in management of diverse clinical conditions. It has been revealed that approximately 80% of Africans use therapeutic plants to manage their infections and conditions; malaria, diabetes, chronic wounds and skin ulcers due to their perceived affordability and accessibility. Plants have been known to contain important metabolites that is responsible for their pharmacological activity they exhibit.

The pharmacological prowess of a plant is a reflection of its bioactive constituents [26]. The phytochemical analysis performed on *A. difformis* showed the presence of major secondary plant metabolites such as alkaloids, tannins, saponins, flavonoids, and tannins both on the extract and the powdered samples. Additionally, glycoside was present in the powdered samples however, it was absent in the extract (Table 2). [27] indicated that concentration of ethanol have significant effect in plant extraction. The lower the ethanol concentration the higher and better the glucosidal yield in the extract. This phytochemical are responsible for various pharmacological properties exhibited by plant extract. Some plant metabolites such as flavonoids, tannins and saponins are known to promote wound healing. Flavonoids help in dwindling the lipid peroxidation which causes necrosis and reduces angiogenesis. Tannins, on the other hand have antimicrobial and antioxidant which is crucial in improving and promoting wound healing. Saponins are known to augment the immune status to facilitate healing. The wound healing contraction observed may be due to the ability of some phytochemicals in the creams that caused collagen expression, its subsequent maturity, angiogenesis which increased wound tensile strength during the proliferation phase of wound healing [28-31].

The susceptibility pattern of *A. difformis* was determined using the zone of inhibition (which is the clear zone around each well of the agents) for the various test organisms. There were linear relationship between the extract dose and the susceptibility pattern of the test organisms. The highest concentration recorded the highest zone of inhibition as seen Table 3. *A. difformis* was more sensitive in *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The *A. difformis* was found to have an inhibitory effect on *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia* with MICs of, 0.2679mg/ml, 1.25mg/ml, >10mg/ml and >20mg/ml respectively (Table 3). Saponins and alkaloids have been known to possess selective antibacterial properties. This antibacterial effect observed might be due to presence of saponins and alkaloid present in the plant as shown on Table 2 [32].

A. difformis extract exhibited a significant ($p < 0.05$) dose depended wound healing response, as the percentage wound contraction increased with increasing concentration of the

Anchomanes difformis extract which is illustrated in Fig. 2. The respective percentage wound contractions were 83.51%, 90.79%, 94.66%, and 98.65% for 2.5%, 5%, 10% and 15% concentrations of aqueous cream of *A. difformis* (Fig. 2). Wound healing generally has the following phases; Hemostasis, Inflammation, Proliferation and Remodeling. In the inflammation phase, proliferation of inflammatory mediators (neutrophils and monocyte etc.) are triggered [31,33]. Inflammation can be classified as mild, moderate and severe (excessive) based on the level of circulating cell types and quantity. Various wound healing studies have proposed that the inflammatory phase has an effect on the final healed wound. A moderate inflammation is vital for wound healing. However, an excessive inflammation normally results in scarring. *A. difformis* extract healed wounds had neither scars nor hypertrophic scar. It therefore imperative to state that the *A. difformis* extract caused no pain to the rats upon application of the formulations because there was no sign of restlessness and scratching of the wound site on extract application. The extract was able to enhance the feeding ability of the test rats and therefore causing an increase in their overall weight (Fig. 3). Despite the wound healing activity exhibited by wound closure, histological studies will be needed to determine inflammation rate and tissue development.

5. CONCLUSION

The highest concentration of the formulated cream exhibited a good healing activity close to the control drug. This research further reveals that *Anchomanes difformis* extract has antimicrobial activity against *P. aeruginosa*, *E. coli*, *Staph. aureus*, and *Klebsiella pneumonia*. *A. difformis* extract can therefore be formulated and used in wound management.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Rats were handled according to guidelines for care and use of laboratory animals laid down by the National Institute of Health (NIH, Department of Health and Human Services Publication No. 5, Revised 1985) [23]. All animal studies in this research were consented to by the Animal Ethical Committee at the School of Pharmacy, Central University, Accra, Ghana Committee with

the ethical clearance number SOP-AEC/CA08/18.

DATA AVAILABILITY

The data used to support the findings of this study are included in the article and also available from the corresponding author upon request.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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