

INVESTIGATION OF ACTIVITES RELATED TO WOUND HEALING OF *SECAMONE AFZELII*

A.Y. Mensah^{1*}, P.J. Houghton², C. Agyare³, G Komlaga¹, M.L.K Mensah¹,
T.C. Fleischer¹ and K. Sarpong¹

¹Department of Pharmacognosy, Faculty of Pharmacy & Pharmaceutical Sciences,
Kwame Nkrumah University of Science and Technology

²Pharmacognosy Research Laboratories, Department of Pharmacy, King's College London,
150 Stamford Street, London SE1 9NN, UK

³Department of Pharmaceutics, Faculty of Pharmacy & Pharmaceutical Sciences,
College of Health Sciences
Kwame Nkrumah University of Science and Technology

ABSTRACT

Secamone afzelii has been used traditionally for wound healing. To confirm the folkloric uses of the plant, the methanol extract of the plant was tested for antimicrobial and antioxidant activities, since antimicrobial agents and antioxidants facilitate wound healing. The extract showed some level of antimicrobial and antioxidant activities. The selective antimicrobial and significant antioxidant activities suggest that the folkloric use of the plant for wound healing may be based on the antimicrobial and antioxidant roles.

Key words: *Secamone afzelii*, ringworm fungi, bacteria, wound healing

INTRODUCTION

Secamone afzelii Rhoem. (Asclepiadaceae) is a climbing or scrambling shrub mostly found in the secondary forest in Ghana (Irvine, 1961; Nielson, 1965). Aqueous extracts of the plant is used as wash for wounds and some concentrated extract is applied to the wounds and is reputed to aid its healing (Burkill, 1985; Abbiw *et al.*, 2000). We have previously shown that the plant has antioxidant activity and inhibited the peroxidation of bovine brain liposomes. The compound contributing to the antioxidant activity was identified to be alpha-tocopherol (Vitamin E) (Mensah *et al.*, 2004a).

The process of wound healing involves a number of overlapping stages which can be monitored in vitro using a number of models (Cherry *et al.*, 1994; Bodeker and Hughes, 1996; Tran *et al.*, 1997). Some assays used to test for wound healing activities of an extract include antioxidant and antimicrobial assays (Tran *et al.*, 1997; Mensah *et al.*, 2006).

A search of literature shows no evidence of the scientific justification of the traditional uses of *Secamone afzelii* as a wound healing agent; we therefore decided to try and confirm the folkloric uses of the plant as a wound healing agent.

MATERIALS AND METHODS

Plant material and preparation of extract

The aerial parts of *Secamone afzelii* were col-

lected in May from the campus of KNUST, Kumasi, Ghana and were authenticated by Dr. Fleischer of the Department of Pharmacognosy. Voucher specimen of the *Secamone afzelii* labelled SAS2 is deposited at the Faculty of Pharmacy, Pharmaceutical Sciences, KNUST in Kumasi, Ghana. The plant material was dried in the sunlight and powdered. 250gm of the powdered material was extracted with MeOH in a Soxhlet apparatus for twelve hours to obtain 1.6g of dark green viscous extract of the *Secamone afzelii*.

Part of the dried powdered material was subjected to preliminary phytochemical testing for the major chemical groups. The test showed the presence of tannins in the plant, absence of alkaloids, anthraquinones, cardiac glycosides and saponins (Harborne, 1991).

Test organisms and preparation of test inocula

Strains of actively growing cultures of bacteria, yeast and moulds were used as test organisms in the antimicrobial assay. The following microorganisms were used: *Candida albicans* (ATCC 10231); *Saccharomyces cerevisiae* (NCTC 080178); *Microsporum gypseum* (NCPF261); *Trichophyton interdigitale* (NCPF 654); *Trichophyton tonsurans* (NCPF 656); *Escherichia coli* (ATCC 25922); *Bacillus subtilis* (ATCC 6633); *Staphylococcus aureus* (ATCC 2593) and *Pseudomonas aeruginosa* (NCTC 5055).

The bacteria were maintained on nutrient agar at 37°C and the yeast and moulds on Sabouraud's dextrose agar maintained at 30°C and 26°C respectively. The cultured microorganisms were harvested by adding sterile phosphate buffered saline (PBS) to the agar and gently scraping the surface of the agar to release the organism, which were then transferred to sterile test tubes. The moulds were filtered through sterile glass wool to remove the mycelium but allowing the spores to pass through. Freshly prepared bacteria, yeast and spore suspensions were prepared for each assay. The inoculated agars were then incu-

bated at the appropriate temperatures (37°C for the bacteria, and 30°C and 26°C for the yeast and moulds respectively). The bacteria and yeasts were incubated for 48 hours and the moulds for ten days and were observed daily for growth.

Antimicrobial Assay Procedure

The agar dilution method was used for the assay (Vanden Berghe and Vlietinck 1991). Stock solutions of the extract were prepared by dissolving 4mg of each extract in 50µL of DMSO and sonicating to ensure thorough mixing. Sterile water was then added to make the volume up to 1mL. Serial dilution of the extract in molten double strength nutrient and Sabouraud's dextrose agar were made to obtain following concentrations: 125, 250, 500, 750, 1000 µg/mL in normal strength which were then used in assay as previously described by Mensah *et al.* (2000). 200 µL of each dilution was poured into well in a 96 well plate. The microorganisms suspensions were standardised using the total and viable count methods (Mensah *et al.*, 2000). A fresh standardised solution of the bacteria suspensions was prepared and 100µL added to the nutrient agar in the 96 well plate such that 100µL of the bacteria suspension contains 10⁶ CFU of bacteria. A similar procedure was followed for the yeasts and moulds in the Sabouraud's dextrose agar such that 100 µL of the mould suspension contains 10⁵ CFU of organisms. The bacteria and yeasts were incubated for 48 hours and the moulds for 10 days and examined daily. The minimum inhibitory concentration (MIC) is the concentration of the extract where there is no visible growth of the microorganisms. Chloramphenicol and Clotrimazole were used as positive control for the bacteria and fungi respectively whilst the culture media used for the assay were used as negative control to ensure that the microorganisms were viable in the absence of the extract and positive control. The assay was repeated three times.

In vitro test for protection against damage to MRC-5 by oxygen free radicals

Confluent MRC-5 cell line (Sigma, UK) was used for the experiment. Cells from the 15 to 21 passage were used for the assay. The cells were trypsinized, centrifuged and resuspended in (EMEM)/10% foetal calf serum (FCS) containing 2mM Glutamine and 1% Non essential amino acids (NEAA) (Sigma). The cells were diluted in the same mixture to give a standardized suspension of 1×10^4 cells/mL. The cells were seeded at a density of 4×10^3 cells per well in a 96 well plate and incubated for 24 hours. The protection against damage to MRC-5 by oxygen free radicals assay procedure was based on that developed by Murrell *et al.* (1990). To determine a suitable concentration of H_2O_2 which would induce a recoverable damage on MRC-5 cells of concentration 4×10^3 /well, a modification of the method developed by Murrell *et al.* (1990) was employed and a dose of 1×10^{-4} M H_2O_2 was selected for use in the experiments. The antioxidant assay used was that described by Yamasaki *et al.* (1994) and is outlined below. MRC-5 cells were seeded at 4×10^3 cells/well in 96 well plates and grown until almost confluent. The culture media was washed and replaced with serial dilutions of each extract in Hanks' Balanced Salt Solution (HBSS). The assay protocol employed involved the addition of the extract followed by the H_2O_2 to the cells. Catalase (250 IU/mL), a scavenger of H_2O_2 , was used as the positive antioxidant control and normal control consisted of the cells alone (exposed only to the HBSS). The cells were treated with three doses of the extract (1 μ g/mL, 5 μ g/mL, and 10 μ g/mL) and the Neutral Red assay was used to assess the protection offered by the doses (Zhang *et al.*, 1990). The cells were also visually examined to check for any visible sign of damage and also for microbial contamination since this contamination can produce false strong optical density signals. Visual examination of the cells will show signs of contamination if any and cell damage such as disruption of monolayers, vacuolization, and changes in shape from the extended strands to

rounded shape and also detachment from the well. Each experiment was repeated seven times for statistical purposes. The Neutral Red assay is described below. The culture medium was discarded from the cells; the cells washed with phosphate buffered saline (PBS), 100 μ L freshly prepared Neutral Red solution (1.2 mL Neutral Red solution (Sigma) in 78.8 mL Hank's Balanced Salt Solution (Sigma) added and the cells were incubated at 37 °C for 3 hours. The Neutral Red was then removed and the cells washed rapidly with 1% HCHO/1% $CaCl_2$. The Neutral Red in the lysosomes was eluted with 100 μ L of 1% acetic acid/50% ethanol and the plate placed in the incubator for 30 min, shaken on an orbital shaker and the optical density measured at 540 nm using a microtiter plate reader. Four separate experiments were conducted and, in all cases, seven replicates were performed and the results obtained are expressed as mean \pm standard error of the mean of the absorbance. The data from the experiment were analyzed by one-way analysis of variance and Dunnett's test using Microsoft Excel package. Differences at the 95% level were considered to be significant.

RESULTS AND DISCUSSION

Antimicrobial Assay

The results of the antimicrobial assay are presented in Table 1. The bacteria and fungi used in this assay were chosen primarily on the basis of their association with infected wounds and also being opportunistic pathogens of humans (Hollinworth, 1997; Jones *et al.*, 2000). Two Gram positive bacteria (*S. aureus* and *B. subtilis*) and two Gram negative organisms were selected (*E. coli* and *P. aeruginosa*). The moulds were selected because they are ringworm fungi responsible for disfiguring the nails and other parts of the body and the yeasts are opportunistic pathogens (Mensah *et al.* 2004b). The results of the present investigation indicate the existence of a weak level of antimicrobial activity in the crude extract of the *Secamone afzelii* plant. The extract showed weak antibacterial activity against the *S. aureus* and the *E. coli*, however, it

Table 1: Results of the antimicrobial assay

Fungi	<i>Secamone afzelii</i> (500µg/ml)	Clotrimazole (100µg/ml)
<i>C. albicans</i>	+	+++
<i>S. cerevisiae</i>	+	+++
<i>M. gypseum</i>	-	+++
<i>E. floccosum</i>	-	+++
<i>T. interdigitale</i>	-	+++
<i>T. tonsurans</i>	-	+++
Bacteria	<i>Secamone afzelii</i> (1000µg/ml)	Chloramphenicol (100µg/ml)
<i>B. subtilis</i>	-	+++
<i>S. aureus</i>	++	+++
<i>E. coli</i>	+	+++
<i>P. aeruginosa</i>	-	++

Key += activity

- = No activity

had no activity against the *B. Subtilis* and *P. aeruginosa*. The extract showed antifungal activity against the yeast but no activity against the dermatophytes. This may partly be explained by the different cell wall compositions of the dermatophytes and yeast. The cell walls of some

yeasts such as Saccharomycetaceae and Cryptococceae consist mainly of a mannan-glucan complex whilst those of the dermatophytes have cell walls comprising of chitin and glucan (Webster, 1980). Further phytochemical studies are required to isolate and characterise the compounds responsible for the reported activity.

In vitro test for protection against damage to MRC-5 by oxygen free radicals

The methanolic extract of *Secamone afzelii* at concentrations of 1, 5 and 10µg/mL gave statistically significant protection of the MRC-5 cells against hydrogen peroxide-induced damage (Fig. 1). Exposure to H₂O₂ alone reduced the absorbance to 20.6% of the control (i.e. cells exposed only to the HBSS). The absorbance obtained for the cells treated with 1µg/mL and 5µg/mL of *S. afzelii* extract was 84% of the control (i.e. cells exposed only to the HBSS), the absorbance readings for the 10µg/mL was 93% of the control whilst catalase the positive control gave an absorbance reading of 84% of the control showing that it significantly protected the cell from hydrogen peroxide induced damage compared to those exposed only to the H₂O₂. There was significant difference between the results obtained for the cells exposed to H₂O₂ alone and those treated with the extract (p<0.05) implying that

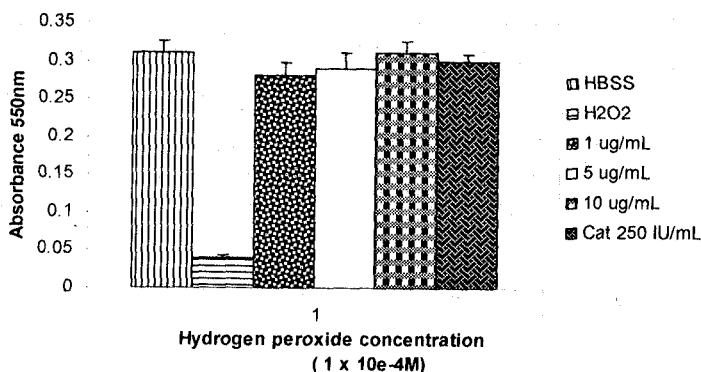


Fig. 1: Neutral Red assay of the protective effect of *Secamone afzelii* on MRC-5 (P20) against hydrogen peroxide induced oxidant injury

the extract protected the cells against the oxidant damage induced by the H_2O_2 . The result also shows the $10\mu\text{g/mL}$ dose to be the most effective protective dose. The $1\mu\text{g/ml}$ and $5\mu\text{g/ml}$ doses also offered some level of protection to the cells but were less than that offered by the $10\mu\text{g/ml}$.

Changes in the morphology were also observed in the appearances of some of the cells. The cells exposed only to the HBSS showed the characteristic elongated strands of normal MRC-5 cells stained red by the Neutral Red dye. The cells treated only with the hydrogen peroxide appeared slightly rounded, less red coloured and some were detached from the wells which showed that they were damaged. The cells treated with the $1\mu\text{g/mL}$, $5\mu\text{g/mL}$ and $10\mu\text{g/mL}$ of the *Secamone afzelii* extract also had elongated red coloured strands like normal cells although the cells treated with the $10\mu\text{g/mL}$ appeared more confluent than those treated with the $1\mu\text{g/ml}$ and $5\mu\text{g/mL}$ (Fig not shown). This suggests that the $1\mu\text{g/mL}$, $5\mu\text{g/mL}$ and $10\mu\text{g/mL}$ concentration of the *Secamone afzelii* extract gave some protection to the cells against oxidant damage induced by the hydrogen peroxide.

The antioxidant experiments involving the cell line were monitored by Neutral Red assay method and also by visual examination under an inverted microscope. The Neutral Red release assay has proven useful for comparing the cytotoxic effect of various harmful agents on human cell lines (Korting, et al., 1995). The uptake of Neutral Red dye is proportional to the number of viable cells and particularly the number of lysosomes. The dye can be extracted from the lysosomes for quantitative measurement of cell viability and cytotoxicity (Zhang, 1990). Damage to cell surface or lysosomal membranes by a variety of substance results in decreased dye uptake. In the experiment the MRC-5 were exposed to H_2O_2 in the culture medium to mimic an environment of oxygen radicals *in vivo*. The experiment has shown that H_2O_2 induced significant damage of the cells as evidenced by the reduction in the Neutral Red absorbance reading

of cells exposed to the H_2O_2 only, (20%). The cells treated with the extract, significantly protected the cells from the oxidant injury.

It has been shown by previous documented reports that the results obtained in this type of antioxidant assay involving cell lines is not due to direct interaction of the extract and hydrogen peroxide. However, the extract may have altered the cell membrane thus limiting the damage induced by the hydrogen peroxide (Tran et al. 1997; Mensah et al., 2001). Further investigation is necessary to identify the other compound(s) present in the extract responsible for the protective activity.

CONCLUSION

The antioxidant activity of methanolic extract of *Secamone afzelii* confirmed that there is some scientific justification for the traditional use of the plant as a wound healing agent in Ghana. This is partly explained by the antioxidant activity of the extract as shown in this study. The antimicrobial activity is selective and unlikely to be very effective against infection of wounds at the concentration used. Further studies will be needed to identify the compounds responsible for this activity. In addition other tests for properties such as fibroblast stimulation or mechanisms associated with anti-inflammatory action would be of interest to provide a wider spectrum of activities that relate to wound-healing properties.

ACKNOWLEDGEMENT

Abraham Yeboah Mensah is grateful to the Wellcome Trust Fund for providing the financial support for the research.

REFERENCES

- Abbiw, D. K. (1990). Useful Plants of Ghana: West African Uses of Wild and Cultivated Plants. Kew: Intermediate Technology Publications and the Kew Botanic Gardens, , pp 135-210

- Bodeker, G. and Hughes, M. A. (1996). Wound healing, traditional treatments and research policy. In: H.D.V. Prendergast, N.L, Etkin, D.R. Harris and P.J. Houghton (Eds.), *Plants for Food and Medicine*. London: Royal Botanic Gardens Kew, pp. 345-359.
- Burkhill, H.M. (1985). *The Useful Plants of West Tropical Africa*. London: Royal Botanic Gardens Kew, pp. 431-432.
- Cherry, G.W., Hughes, M.A., Kingsnorth, A.N. and Arnold, F.W. (1994). Wound healing. In: P.J. Morris and R.A. Malt (Eds.), *Oxford Textbook of Surgery*, Oxford: I. Oxford University Press, pp. 3-23.
- Harborne, J.B. (1991). *Phytochemical Analysis: A Guide to Modern Techniques of Plant Analysis 2nd ed.* London. Chapman and Hall pp 5-80.
- Hollinworth, H. (1997). "The management of infected wounds". *Professional Nurse Study 12, (Suppl.): 8-11*.
- Irvine, F.R. (1961). *Woody Plants of Ghana*. Oxford: Oxford University Press. :p. 653.
- Jones, N.P., Arnason, J.T., Abou-zaid, M., Akpagana, K., Sanchez-Vindas, P. and Smith, M.L., (2000). "Antifungal activity of extracts from medicinal plants used by first nation's peoples of Eastern Canada". *Journal of Ethnopharmacology 73: 191-198*.
- Korting, H.C., Hulsebus, E., Kerscher, M, Greber, R. and Schaferkorting, M. (1995). "Discrimination of the toxic potential of chemically differing topical glucocorticoids using a Neutral Red release assay with human keratinocytes and fibroblasts". *British Journal of Dermatology 133: 54-59*.
- Laupattarakasem, P., Houghton, P.J., Hoult, J.R.S. and Itharat, A. (2003). "An evaluation of the activity related to inflammation of four plants used in Thailand to treat arthritis". *Journal of Ethnopharmacology 85: 207-215*.
- Mensah, A.Y., Houghton, P.J., Bloomfield, S.F., Vanden Berghe, D.A. and Vlietnick, A.J. (2000). "Known and novel terpenes from *Buddleja globosa* displaying selective anti-fungal activity against dermatophytes". *Journal of Natural Products 63: 1210-1213*.
- Mensah, A.Y., Sampson, J., Houghton, P.J., Hylands, P.J., Westbrook, J., Dunn, M., Hughes, M.A. and Cherry, G.W. (2001). "Effects of *Buddleja globosa* leaf and its constituents relevant to wound healing". *Journal of Ethnopharmacology 77: 219-226*.
- Mensah, A.Y., Houghton, P.J., Akyirem, G.N., Fleischer, T.C., Mensah, M.L., Sarpong, K. and Adosraku, R. (2004a). "Evaluation of the antioxidant and free radical scavenging properties of *Secamone afzelii* Rhoem". *Phytotherapy Research 18:1031-1032*.
- Mensah, A.Y., Houghton, P.J. and Woode, E. (2004b). "Fungi, Friends or Foes?" *The Ghana Pharmaceutical Journal 28: 22-36*.
- Mensah, A.Y., Houghton, P.J., Akyirem, G.N., Fleischer, T.C., Mensah, M.L., Sarpong, K. and Adosraku, R. (2006). "In Vitro evaluation of effects two Ghanaian plants relevant to wound healing". *Phytotherapy Research 20:941-944*.
- Morrison, M. and Kindlen, S. (1997). The physiology of wound healing. In: M. Morrison, C. Moffat, and J. Bridel-Nixon, (Eds.), *Nursing Management of Chronic Wounds*. Barcelona: Mosby. pp. 1-51.
- Murrell, G.A.C., Francis, M.J.O. and Bromley, L. (1990). "Modulation of fibroblast proliferation by oxygen free radical". *Biochemical Journal 265: 659-665*.
- Nielson, S. M. (1965). *Introduction to the Flowering Plants of West Africa*; London: University of London Press. 202-204.
- Tran, V.H., Hughes, M.A. and Cherry, G.W. (1997). "In vitro studies on the antioxidant

- and growth stimulatory activities of a polyphenolic extract from *Cudrania cochinchinensis* in the treatment of wounds in Vietnam". *Wound Repair and Regeneration* 5: 159-167.
- Vanden Berghe, D.A. and Vlietnick, A.J. (1991). Screening methods for antibacterial and antiviral agents from higher plants. In: Hostettmann (Ed.), *Methods in Plant Biochemistry* 6. Assays for Bioactivity. London: Academic Press. pp. 47-69.
- Webster, J. (1980). *Introduction to Fungi* 2nd ed. London: Cambridge University Press. pp. 57-97.
- Yamasaki, T., Li, L. and Lau, B.H.S. (1994). "Garlic compounds protect vascular endothelial cells from hydrogen peroxide-induced oxidant injury". *Phytotherapy Research* 8: 408-412.
- Zhang, S., Lipsky, M.M., Trump, B.F. and Hsu, I. (1990). "Neutral Red (NR) assays for cell viability and enobiotics induced cytotoxicity in primary cultures and rat hepatocytes". *Cell Biology and Toxicology* 6: 219-234.