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Perspective paper

In vitro tests and ethnopharmacological investigations: Wound healing as an example

P.J. Houghton [a,*], P.J. Hylands [b], A.Y. Mensah [b], A. Hensel [c], A.M. Deters [c]

[a] *Pharmacognosy Research Laboratories, Department of Pharmacy, King's College London, 150 Stamford Street, London SE1 9NH, UK*
[b] *Department of Pharmacognosy, Faculty of Pharmacy, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana*
[c] *Institute for Pharmaceutical Biology and Phytochemistry, University of Muenster, Hittorfstr. 56, Münster, Germany*

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Abstract

In vitro tests are now widely employed in ethnopharmacological research because of ethical reasons and their usefulness in bioactive-guided fractionation and determination of active compounds. For many disease conditions, a variety of in vitro tests can now be employed as the biochemical mechanisms underlying disease and healing processes are understood. Approaches to the in vitro investigations of wound healing processes are exemplified by studies on extracts of *Buddleja* species and three Ghanaian species *Spathodea campanulata*, *Commelina diffusa* and *Secamone afzelii*. Most studies have been carried out on *Buddleja officinalis* or *Buddleja globosa*. The extracts have been shown to have anti-inflammatory and antioxidant properties due to flavonoids, triterpenoids, diterpenoids and caffeic acid derivatives. There appears to a slight effect on proliferation of fibroblasts at lower concentrations, but this was not significant, and higher concentrations appeared to be cytotoxic. Novel findings are the ability of *Buddleja globosa* leaf extracts to induce differentiation in keratinocytes and to alter the profile of proteins produced by cultured fibroblasts. Extracts also had some effect on lattice contraction. The three Ghanaian species examined show a mixture of antimicrobial and antioxidant activities. The evolution over recent years of tests for wound healing, from in vivo tests to cell-based systems and chemical reactions and on to investigations into effects on secondary messengers and protein expression, is described.

Keywords: *Buddleja*; Wound-healing; Bioassays; Anti-inflammatory; Cell proliferation; Antioxidant; Proteomics; Keratinocytes; Flavonoids; Phenylethanoids

1. Introduction

Over the last 20 years ethnopharmacological studies have increasingly included in vitro bioassays as a replacement for experiments using tissues or whole animals. This is partly due to the ethical and commercial problems of using animals but also the unsuitability of such practices for bioassay-guided fractionation of the compounds responsible for any activity observed (Houghton, 2000). These procedures may be based on cultured cells, enzyme studies or receptor-ligand binding experiments and nowadays also gene-expression-arrays are extensively used, not only in ethnopharmacological research, but also by the pharmaceutical industry. Both constituencies use the techniques for screening large numbers of natural extracts and fractions, but industrial concerns are often highly automated to enable high throughput screening and tend to more often screen synthetic compounds and mixtures.

In spite of the widespread use of in vitro assays, it is important to point out the deficiencies of such an approach. It is very unusual for one in vitro assay alone to represent a disease state and the use of a battery of relevant tests is preferred, since most disease states are complex and several mechanisms are involved, all of which may offer targets for compounds which effect amelioration of the condition. However, even with a variety of relevant tests, it is generally acknowledged that in vitro tests are too reductionist to extrapolate their results to provide evidence for clinical efficacy, and that eventually animal testing and clinical trials have to be performed. Fractionation, which is often carried out when in vitro tests are employed, often results in loss of activity, or less increase per fraction than might be expected.

* Corresponding author. Fax: +44 20 7848 4775.
E-mail address: peter.houghton@kcl.ac.uk (P.J. Houghton).

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This may be due to decomposition of the active products or to breakup of synergistic relationships.

In spite of these negative aspects, in vitro bioassays have resulted in the discovery of some novel therapeutic agents and are continually revealing compounds from traditional medicines, which help explain their usage. They may be valuable also in providing evidence of the modes of action of materials, which have shown clinical or in vivo activity. This paper seeks to exemplify some of the issues concerning in vitro tests for wound healing by reviewing existing data, and providing novel findings, concerning the leaves and flowers of the genus *Buddleja* (Loganiaceae) and also illustrates other in vitro tests used to investigate three Ghanaian species.

The leaves of a number of species of *Buddleja* are reported to be applied topically as a poultice or lotion for healing of wounds (Houghton, 1984). Studies on wound-healing properties have focused on the South American species *Buddleja globosa* Hope which is endemic to Chile and Argentina, the leaves being used by the indigenous Mapuche for the treatment of ulcers and wounds (Houghton and Manby, 1985). Studies have also been carried out on the Far Eastern *Buddleja officinalis* L. whose flowers are used to treat sore and damaged eyes, a condition which is similar to skin wounds (Houghton, 1984). The fairly detailed knowledge of the constituents present in *Buddleja* enabled speculation that the presence of saponins, flavonoids and other phenolics could contribute to wound healing because of their detergent ability to remove grease, dirt and bacteria from tissue and act as antimicrobials (Houghton and Mensah, 1997). However, this speculation had no experimental support at the time because of the comparative lack of in vitro tests related to wound healing.

Interviews with traditional Ashanti healers in Ghana revealed a variety of plants used to treat wounds. Three species were selected on the basis of little previous chemical or biological work having been carried out and these were the stembark of *Spathodea campanulata* P. Beauv. (Bignoniaceae), the leafy shoots of *Secamone afzelii* Rhoem. (Asclepiadaceae) and the herb of *Commelina diffusa* Burn. (Commelinaceae). All three plants are used as poultices of lotions made by steeping the material in hot water or local alcoholic spirits (Abbiw, 1990).

2. The wound healing process and relevant biological tests

In vivo models of wound healing generally use small rodents such as guinea pigs or rats. The backs are shaved and wounds induced by scarification or burning. Placebo and test solutions are applied to the wound and the times taken for the wounds to reach recognized stages of the wound healing process for test substance and controls are noted (Saha et al., 1997). Another approach is to measure the surface area and tensile strength of the wound (Rashed et al., 2003). However, in many parts of the world, the use of animals in experiments is being severely curtailed for financial and ethical reasons so in vitro models relevant to wound healing have been developed.

The process of wound healing involves a variety of processes such as inflammation, cell proliferation and contraction of the collagen lattice formed (Bodeker and Hughes, 1998). In addition, the healing process may be hampered by the presence of oxygen free radicals or microbial infection. Over the last 15 years, the in vitro tests developed have exploited all of these processes as targets. The different phases of the wound healing process overlap and ideally a plant-based remedy should affect at least two different processes before it can be said to have some scientific support for its traditional use. The effects of pharmacological agents which modulate many of these processes, such as fibroblast proliferation or reduction of oxidative stress, can be assessed by in vitro experiments. A summary of these tests is shown in Table 1.

2.1. Inflammation and oxidative damage

When wounding occurs, it is accompanied within quite a short time by pain, and reddening and edema of the surrounding tissue. These are all classical symptoms of inflammation and are caused by the release of the eicosanoids, prostaglandins and leukotrienes, and of reactive oxygen species (ROS). Thus, inhibition of eicosanoid synthesis and antioxidant activity are both properties of extracts and their constituents which can be tested in vitro.

Various in vitro techniques have been developed to assess the inhibition by compounds on the synthetic cascade that eventually leads to the formation of prostaglandins and leukotrienes. Inhibition of NFκB synthesis has also recently been employed extensively in the study of anti-inflammatory activity (Bremner et al., 2004), but it has not been applied as yet to any extent specifically in the study of wound-healing plants. A more common approach has been to investigate the enzymes 5-lipoxgenase and cycloxygenase which convert arachidonic acid to leukotrienes and prostaglandins respectively. ^{14}C-labelled arachidonic acid is fed to isolated rat leukocytes, stimulated with the calcium ionophore A23187 to produce eicosanoids, and the amounts of thromboxane B_2 and leukotriene B_4 produced after specified times were measured by radioimmunoassay. The amounts produced by cells treated with extract or compounds are compared over a range of doses and significant reductions in the amount of one or both the two products formed indicate that anti-inflammatory properties might be shown by the test material. Although most ethnopharmacological experiments of this type have been connected with arthritis as an inflammatory disease, compounds extracted from the leaves of *Buddleja asiatica* and flowers of *Buddleja officinalis* were tested in this way (Liao et al., 1999). Both species are used in traditional Chinese medicine for wound healing. The methanol extracts showed significant eicosanoid synthesis inhibition at 50 μg/ml and several types of compounds were