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An Ethnopharmacological Study of Medicinal Plants in New South Wales

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Abstract: The Australian Aboriginal people have used plants as medicine and food for thousands of years, however, this traditional knowledge is documented only to a limited extent, and is in danger of being lost. The Indigenous Bioresources Research Group (IBRG) aims to help Australian Aboriginal communities to preserve their customary medicinal knowledge, and to provide information that can be used for their cultural or educational purposes, as well as for scientific advancement. This work is undertaken in close collaboration with Australian Aboriginal communities in New South Wales. The project is multidisciplinary, combining an ethnobotanical and an ethnopharmacological approach, which includes biological and chemical investigations, as well as developing best practices for protecting traditional knowledge.

This paper describes the general strategy of the project as well as methods used in the ethnopharmacological study. Ethnobotanical databases are set up for each participating community. Plant material is collected, extracted, and active compounds are isolated using a bioassay-guided fractionation approach. All extracts and compounds are tested for biological activity in antimicrobial assays (disc diffusion, resazurin, fluorescein

diacetate), neurological assays or anti-inflammatory assays, depending on their traditional use.

Keywords: Natural products, Aboriginal medicine, ethnobotany, ethnopharmacology, bioassay-guided fractionation.

Introduction

Australian Aboriginal people have used plants for food and medicinal purposes for thousands of years and have acquired a vast knowledge of Australia's unique flora [1]. This traditional knowledge, however, is documented only to a limited extent, and is in danger of being lost. This is largely due to social changes within the communities, such as dislocation and westernisation, and the death of the elders with this knowledge [2]. This trend in loss of traditional knowledge is being seen worldwide in most indigenous societies. The World Health Organization (WHO) acknowledges the value of traditional medicine and the preservation and protection of this knowledge is one of their objectives [3]. In recent decades, considerable ethnopharmacological research has been done worldwide, especially in North and South America, Asia and Africa [4]. In Australia some ethnopharmacological work has been done in the Northern Territory and Western Australia [1,5]. Only limited ethnopharmacological studies have been undertaken in New South Wales and this has been done predominantly without Aboriginal community involvement [6]. We have established a research group to preserve and expand this medicinal plant knowledge of Aboriginal communities. The Indigenous Bioresources Research Group (IBRG) is based at Macquarie University, Sydney, Australia and brings together an interdisciplinary team with experience in ethnobotany, natural products, medicinal chemistry, microbiology and law, and most importantly works in collaboration with Aboriginal communities through the Land Councils. The IBRG aims to document first hand traditional and contemporary knowledge; to provide information to the communities that can be used for their cultural or educational purposes; to identify biologically active compounds using bioassay-guided fractionation; and to develop best practices for collaborative research with Aboriginal communities. The IBRG is currently involved in studies in coastal and inland New South Wales and the Kimberley, and will be extending the study to include other areas of Australia.

The IBRG project consists of three parts – the development of best practices in protecting Aboriginal traditional knowledge; an ethnobotanical study; and an ethnopharmacological study, which includes biological and chemical investigations. This approach conforms to the ethical guidelines proposed by the National Health and Medical Research Council for working with Aboriginal people [7], and represents best practice in relation to benefit sharing with Indigenous owners of traditional knowledge, as documented in the 1993 International Convention on Biodiversity [8], to which Australia is a signatory. All stages of the project are done with full cooperation of the Aboriginal communities and have been approved by the Macquarie University Ethics Review Committee (Human

Research, approval number HE27FEB2004-R02750) and the Macquarie University Biosafety Committee (approval number 01/01/LAB). This paper describes the strategies and general methodology of the project, with a particular focus on the ethnopharmacological study.

Discussion

Development of best practices

The Participatory Action Research methods published by UNESCO [9] have been adopted for developing cooperative research relationships with Aboriginal communities. The Participatory Action Research strategy consists of the following steps: 1) meet with community members who constitute appropriate authorising persons to discuss the project; 2) obtain authorisation to proceed with the project; 3) meet with community members to design research activities and to identify interested participants; 4) implement the study plan with participants; 5) summarise data with input from participants, both field workers and others; 6) discuss the expansion of the project and seek community advice as to how best to provide in kind support strategies to the community as recompense for their efforts; 7) discuss the most appropriate way of storing and transmitting the information to the greater community; and 8) clarify with community members how ongoing research will proceed, what further field work may be necessary and how they might be involved [9].

The IBRG project abides by the above principles and the following conditions: research will only take place with the consent of the appropriate local Aboriginal authorising body and community representatives; all publications resulting from this research will be jointly prepared and any commercial products will also be jointly developed with the Aboriginal community; the data obtained in the study will be confidential unless desired otherwise by the community. Collaboration with the Centre for Environmental Law and Warawara Indigenous Studies, of Macquarie University, ensures the protection of the indigenous knowledge and adherence to the above conditions, as well as provides further advice on the development of best practices.

Ethnobotanical study

Ethnobotany is the study of plants used by indigenous societies for food, medicine, building materials, economic application or ceremony [10]. An ethnobotanical study is carried out by gathering data of traditionally used plants by interviewing people with specific knowledge. In the Australian context, this usually includes Aboriginal women, since they often possess the most detailed knowledge [11]. For this study plants are being collected from the field under guidance of Aboriginal advisers. The plants are then vouchered and identified, and the ethnobotanical information is documented in databases, separately for each participating community. Information stored in the ethnobotanical databases includes the botanical and Aboriginal names of the plants, what parts are used and at which stages of growth, collection data (date, time and place), the use and preparation in published literature (if any), and most importantly, the way of preparation and application of the remedy by the

communities. Since these databases reflect the traditional knowledge of the communities, they are password protected with access restricted, based on the communities' wishes.

Ethnopharmacological study

Ethnopharmacology involves the observation, description, and experimental investigation of indigenous medicines and their biological activities as an approach to drug discovery [12]. In our study, plants used by the Aboriginal communities that show significant medicinal potential are being investigated for their biologically active compounds, using bioassay-guided fractionation. Indigenous societies in general tend to focus on remedies for dermatological ailments, inflammation, gastrointestinal ailments and diseases of the nervous system [13]. The IBRG places particular attention on plants used traditionally to treat bacterial or fungal infections, conditions derived from neurological disturbances (*e.g.* pain, anxiety, depression, memory or sleep loss), and inflammation. These conditions affect both the Aboriginal communities and the population worldwide, and the development of new treatments has a high research priority. Antimicrobial resistance is a significant global health concern in treating infectious diseases. It seriously reduces the therapeutic value of the available antimicrobial agents and makes the search for agents with novel antimicrobial activity of major importance. Current trends suggest that some diseases caused by resistant strains will have no effective therapy within the next ten years, if no new drugs are developed [14]. According to the WHO, 25% percent of people worldwide will develop one or more mental or behavioural disorders at some stage in their lives [14]. Among these disorders are depression, schizophrenia and ageing diseases, such as Alzheimer's disease, which are expected to increase over the next 20 years with the ageing of populations [15]. Alzheimer's disease is already the most common neurological disease of adulthood [16]. Inflammation is an essential component of host defence, however, seriously debilitating diseases can occur when this process is inappropriately activated. This includes osteoarthritis and rheumatoid arthritis, which affect 15% of the total population in Australia [17].

Extraction and isolation

In this study, a bioassay-guided fractionation approach is used to obtain pure, active compounds through a set of purification methods [18]. Several conditions are used to extract the plant material. One of the most important methods is preparation of the plant material analogous to the method of preparation by the communities, to validate its use. This includes preparation of water-based extractions such as decoctions or infusions. Other extraction procedures include the use of ethanol or a series of solvents of increasing polarity (hexane, dichloromethane, ethyl acetate, butanol and water) against which the plant material is directly partitioned [18]. The crude water and ethanol extracts are cleaned up by partitioning them against such solvents. Specific compounds are also targeted, such as alkaloids or tannins, since these usually have biological activity [18]. After the initial extraction, the compounds present in the crude extracts are separated by general chromatography techniques such as reversed-phase and normal-phase liquid chromatography, and preparative TLC. Identification of these

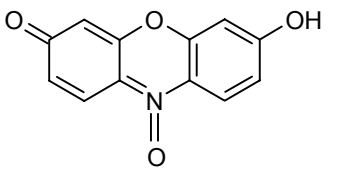
compounds is done using spectroscopic methods, including nuclear magnetic resonance spectroscopy and mass spectrometry.

Antimicrobial assays

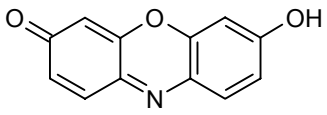
Three different assays are used to determine the antimicrobial activity of either the extracts, fractions or pure compounds and where possible compared to commercially available antimicrobial agents, such as chloramphenicol, ampicillin, tetracycline and gentamicin. The distinction between microbicidal and microbiostatic activity of the extracts is also made in some of these assays by subculturing onto agar plates, followed by a colony count after incubation [19].

In the disc diffusion assay, small sterile paper discs are impregnated with the extracts or pure compounds. These are then placed on agar plates that have been inoculated with microbes, and the plates are incubated overnight. The extracts or test compounds diffuse into the agar, giving rise to a gradually changing gradient of concentration in the agar around the disc [20]. If the compound has any activity against the microbes, a clear inhibition zone can be seen around the disc after incubation. The size of this zone, while depending on various factors (hydrophobicity, molecular size, diffusability, *etc*), is in general proportional to its antimicrobial activity and can be measured easily.

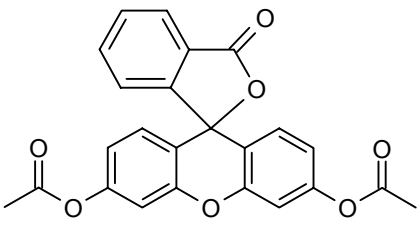
The resazurin and the fluorescein diacetate (FDA) assays are used in microtitre plate format, and are based on the incubation of microbes in wells with the extracts or test compounds. After an overnight incubation, the reagent (either resazurin or FDA) is added and a colour change is visible after an additional 2-3 hour incubation. In the case of the resazurin assay, the blue dye resazurin **(1)** is reduced to the pink-coloured resorufin **(2)** in the medium by cell activity (growing microbes) [21,22]. This assay depends on an easily recognised colour change from blue (indicating inhibition of the microbes) to mauve or pink (no inhibition) and can be determined visually or measured spectrophotometrically [22]. An example of this colour change can be seen in Figure 1A.



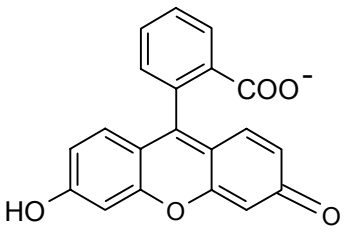
resazurin **(1)**



resorufin **(2)**



fluorescein diacetate **(3)**



fluorescein **(4)**

The FDA assay is based on the conversion of colourless fluorescein diacetate **(3)** to the yellow-green fluorescent compound fluorescein **(4)** by non-specific esterases present in growing microbes [23,24]. A low level of fluorescence indicates the inhibition of microbial growth, and hence the presence of a compound with antimicrobial activity. A high level of fluorescence indicates no inhibition of microbial growth. An example of the assay can be seen in Figure 1B.

Figure 1: Resazurin (A) and FDA (B) assay examples of the activity of compounds on the growth of microbes

A: wells 1 – 3 remained blue after adding resazurin, indicating that the microbes are non-viable and an antimicrobial test compound is present.; wells 4 – 6 turned pink, indicating that the test compound had no inhibitory effect on the microbial growth.

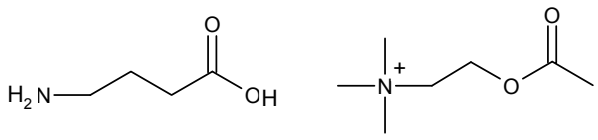
B: wells 1 - 3 show low levels of fluorescence after adding FDA, indicating that the microbes are non-viable and an antimicrobial test compound is present; wells 4 – 6 have high levels of fluorescence, indicating that the test compound had no inhibitory effect on the microbial growth.

A B

The three antimicrobial assays are employed because of their complementarity. For example, coloured compounds could interfere with the absorbance and fluorescence assays, and hydrophobic compounds and large molecules are known to give poor results in the disc diffusion assay. In addition, the resazurin assay is not suitable for *P. aeruginosa* [22], and the FDA assay is not well suited for *E. coli* [25].

Neurological assays

Plants that have been used to treat pain, anxiety, depression, memory loss or other neurological disturbances will be tested for their neurological activity. This will be done using *Xenopus* oocytes (immature eggs of the South African clawed frog *Xenopus laevis*) that can express functional neurotransmitter receptors such as gamma-aminobutyric acid (GABA) **(5)** or acetyl choline **(6)** on their surface [26,27]. These classes of neurotransmitter receptors are ligand-gated ion channels and are easily expressed in the membranes of the frog eggs by injecting mRNA or cDNA of the receptors into the cells. The activity of the extracts or pure compounds can be measured with a two electrode voltage clamp (TEVC) recorder, in which the compounds are added to the frog eggs and the electric current over the cell is determined. The effect of the compounds can either be inhibitory or stimulatory and can be measured in the absence or presence of the neurotransmitter. For this study the assay will be done on an automated system called the Roboocyte [27].



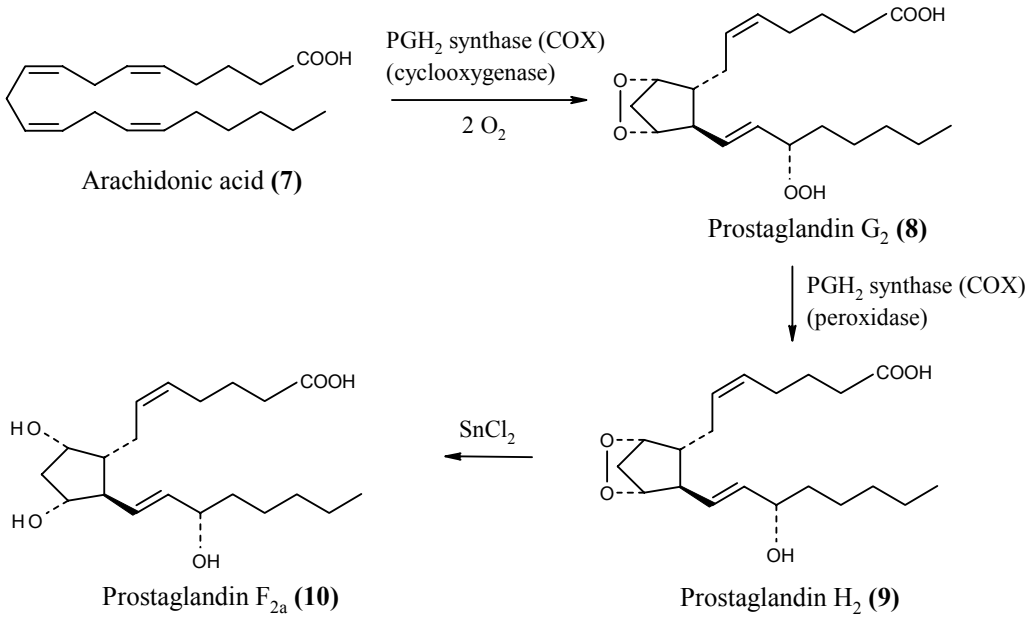
gamma-aminobutyric acid **(5)** acetyl choline **(6)**

Anti-inflammatory assays

Lipoxygenase (LO) and cyclooxygenase (COX) enzyme assays will be used to test extracts of plants that have been employed to treat pain and inflammation. COX is the rate limiting enzyme involved in the synthesis of prostaglandins, which are local hormones responsible for the pathology of inflammation and fever. They are also involved in blood clotting, ovulation, wound healing, kidney function and blood vessel tone [28,29]. Three isoforms of COX have been described, COX-1, COX-2 and COX-3 [30]. All have a cyclooxygenase and a peroxidase function and convert arachidonic acid **(7)** to prostaglandin G_2 **(8)** and further to prostaglandin H_2 **(9)**, as can be seen in Figure 2. LO catalyses the initial step in the biosynthesis of leukotrienes, which are potent mediators of inflammation. Three lipoxygenases exist in mammals, the most biologically important is arachidonate-5-lipoxygenase, which catalyses the formation of the parent leukotriene (leukotriene A_4) [31].

COX-2 selective inhibitors have been of considerable interest as anti-inflammatory agents because of their lower gastro-duodenal and renal side effects compared to COX-1 inhibitors [29,32], however, the safety of COX-2 inhibitors is now being questioned [33]. Our study therefore involves screening for COX-1, COX-2 and LO inhibition.

Figure 2: COX enzyme assay – conversion of arachidonic acid to prostaglandin $F_{2\alpha}$ (adapted from [28])



The COX and LO assays are done as per manufacturers instructions [34,35]. For example, for the COX assay, COX-1 and COX-2 are tested separately by incubating arachidonic acid with the enzymes and the extracts, followed by a microtitre plate based enzyme immunoassay (EIA) [34]. The product of the enzymatic reaction (prostaglandin H_2) can be converted chemically to the more stable prostaglandin $F_{2\alpha}$ **(10)** by adding $SnCl_2$ (see Figure 2). Prostaglandin $F_{2\alpha}$ binds to the wells of the microtitre plate by a broadly specific antibody. This is in competition with the binding of PGE_2 coupled with acetylcholine esterase. Addition of acetylthiocholine leads to the formation of thiocholine by acetylcholine esterase. Thiocholine then reacts with added Ellman's reagent (5,5'-dithio-*bis*[2-nitrobenzoic acid], or DTNB) to form the yellow thionitrobenzoic acid anion (TNB^{2-}) [34]. The absorbance of the solution can be measured and high absorbance values are indicative of an inhibitory effect of the test compounds on COX.

Conclusions

The Indigenous Bioresources Research Group (IBRG) is a unique multidisciplinary collaboration between scientists with expertise in life sciences, law and indigenous studies, and has established research partnerships with Australian Aboriginal communities. Guidelines developed by UNSECO have been adapted to ensure best practice in developing cooperative research relationships. The IBRG has documented traditional and contemporary medicinal plant knowledge of NSW Aboriginal communities and conducted subsequent ethnopharmacological studies. Bioactive compounds are being identified following bioassay-guided fractionation using antimicrobial, neurological and anti-inflammatory assays. Possible spin offs of this project include the discovery of new medicinal agents and a better understanding of Australia's biodiversity.

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