FORMULATION STRATEGY FOR THE PRE-CLINICAL AND EFFICACY EVALUATION OF HERBAL PRODUCTS WITH MEDICINAL PROPERTIES.

Tembe-Fokunang EA\(^1,3\), Horan I\(^2\), Fokunang CN\(^1,2\), Tomkins PT\(^2\)

\(^1\)Faculty of Medicine and Biomedical Sciences, University of Yaoundé 1, Cameroon.
\(^2\)Toxicology Unit Bioserv Ltd; Athlone Institute of Technology, Athlone, Republic of Ireland.
\(^3\)Pfizer Research and Development, Clinical Pharmacology, Sandwich, United Kingdom.

Corresponding author: Dr Estella Tembe-Fokunang, Faculty of Medicine and Biomedical Sciences, University of Yaoundé 1, Cameroon; Email: estellafokunang@yahoo.co.uk.

ABSTRACT

Introduction

Natural products constitute a major source of therapeutics, accounting for about 40% of all new US drug approvals in the decade to 2010 and more than 60,000 plant species are exploited for their medicinal properties. A specific category of these compounds are herbal medicines which are assuming a greater clinical and commercial importance in line with a growing interest in traditional and alternative medicines in many developed countries. Despite the fact that herbal medicines now represent more than US 56 billion in sales per year in Europe, with the exception of a guideline on the quality of herbal preparations, there are no uniform regulatory criteria.

Objective of Study: This study aims at formulating a hierarchical strategy for evaluating complex mixtures of ethanolic extracts for dermal application by using an initial analytical screen followed by an in vitro toxicology and in vitro modeling of possible activity.

Materials and Methods: A combination of UV/VIS, FTIR, GC-MS, polarimetry, and amino acid (AA) techniques applied to ultrafiltrates and Sephadex column fractions was used for the identification of major active species in the complex. The use of MTT, SRB and NR cytotoxicity assays with organotypic cells was also applied to permits a rapid evaluation of gross toxicity which when applied in conjunction with separation methods allowed the activity to be associated with components of known molecular weight. Determination of mutagenic and antiseptic potential of the extract was assessed by virtue of Ames assay and disinfectant assays respectively. Finally, a highest order potential toxicity and possible mechanisms of efficacy action was evaluated using specific 3D culture models based on primary cells.

Results: The protein and carbohydrate content of the herbal extract and the final product showed a significant variation. The herbal extract showed a protein content of 25.17±1.3mg/ml while the final product had a significant reduced protein content of 0.044±0.0mg/ml. For sugar content Herbal extract had a reducing sugar of 235±35.0 mg/ml as compared to 15.33± 2.55mg/ml. Protein purification procedures identified two different proteins the molecular weights of which were estimated as follows: The herbal extract using ultrafiltration showed a molecular weight of 38914±1,266 kDa. Although UV/VIS analysis is not diagnostic for a particular compound a number of absorptive peaks were evident in the 200-220nm region which is indicative of flavones (phenolic structures). Acid hydrolysis indicated the presence of polysaccharides/hydrolysable glycosides, also the precipitation of phlobaphenes were indicative of the presence of tannins. Ames assay: results showed no mutagenic potential of the extract with and without metabolic activation, these results also achieved significance at a 95% confidence level. However it was observed that at high concentrations a bactericidal effect was induced. A disinfectant assay using \(\textit{E. coli}\), \(\textit{S. aureus}\), \(\textit{S. typhimurium}\) and \(\textit{C. sporogenes}\) proved the herbal extract to contain antibiotic constituents, although its cell killing properties were influenced by final ethanol concentration.

Key words: pre-clinical, efficacy evaluation, herbal products, medicinal properties

RESUME

INTRODUCTION

Les produits naturels constituent les sources majeurs des thérapeutiques, contribuant a environ 40% de toutes les nouveaux médicaments brevetées aux états unis dans les derniers décennies jusqu’à 2010 et plus de 60,000 espèces des plantes sont exploites pour leurs propriétés médicinales. Une de catégorie spécifique de cette composes sont les
INTRODUCTION

Plants and plant products are present in 14 of the 15 therapeutic categories of pharmaceutical preparations which are currently recommended to medicinal practitioners in the developed countries. (Phillipson and Anderson 1989). An examination of the list of drugs derived from natural sources, included in any pharmacopoeia, reveals that the majority are derived from Spermatophyta- the dominant seed bearing plants although fungi and algae also contain biologically active substances. Medicinal compounds such as digoxin, morphine, codeine, atropine, quinine, ephedrine and colchicine are botanically derived.

Ultimately, the goal in surveying plants for biologically active compounds should be to isolate the one or more constituents responsible for a particular activity, although the constituents may act additively or synergistically. Knowledge of the phytochemicals present can help explain or predict a variety of events relating to the efficacy and toxicity of herbal preparations (Farnsworth, 1990). At present there are large national differences in the regulatory status of herbal remedies. In the U.S and U.K, such products can circumvent the regulatory premarket drug evaluation which is obligatory for conventional medicines; they do so by posing as dietary supplements (Desmet, 1989; Desmet 1995; Desmet & Rivier, 1989;; Tyler, 2005). Also medicinal products which are currently recommended to practitioners as dietary supplements (Desmet, 1989; Desmet 1995; Desmet & Rivier, 1989;) are considered food in the U.S, and herbal products which are currently recommended to practitioners as dietary supplements (Desmet, 1989) are considered food in the U.S, and medicinal products which are currently recommended to practitioners as dietary supplements (Desmet, 1989) are considered food in the U.S, and medicinal products which are currently recommended to practitioners as dietary supplements (Desmet, 1989) are considered food in the U.S, and medicinal products which are currently recommended to practitioners as dietary supplements (Desmet, 1989) are considered food in the U.S. In the U.S and U.K. such products can circumvent the regulatory premarket drug evaluation which is obligatory for conventional medicines; they do so by posing as dietary supplements (Desmet, 1989; Desmet 1995; Desmet & Rivier, 1989; Tyler, 2005). Also finished herbal medicinal products fall within the scope of Council Directive 65/65/EEC and thus they have access to the European decentralization procedure (Benzi, 1998). When a herbal remedy has adequate product quality and a wide safety margin, there is no need for regulatory authorities to require pharmacokinetic-pharmacodynamic evaluation (PPE) data. Examples would include *Achillea millefolium* (yarrow) and *Taxacarum officinale* (dandelion). However when herbal remedies are required for more serious disorders, harmonization is required between medical authorities of European member states (Desmet and Jacobus 1997).
In this study we are attempting to define a strategy for the evaluation of efficacy and toxicological properties of an ethanolic extract of mixed herbs. The intended target condition for this preparation is psoriasis and eczema. Psoriasis is characterized by increased epidermal proliferation (hyperkeratosis) resulting in accumulation in the stratum corneum (Krueger et al., 1984). Psoriasis may be an autoimmune disorder implying potential target molecules such as arachidonic acid, colony-stimulating factors, interferon, T-cells, B-cells, macrophages and the complement system (Bentner et al. 1979). Cyclic nucleotides are believed to play a key role (Hamet, 1983), so phosphodiesterase inhibition essays could serve as a screening and bioassay tool for the detection and isolation of biologically active principles from the herbal concentrate (Petkov et al., 1983, Nikaido et al., 1983). It has been reported by Lien et al that polysaccharides derived from various Chinese medicinal herbs e.g. *Angelica acutiloba* regulate components of the immune system (Lien et al., 1996).

Eczematous dermatitis is an epidermal eruption that may be acute, chronic, localized or generalized. Intercellular edema causes spongiosis which induces vesiculation. Eczema may also represent an allergic Type 4 reaction with increased levels of IgE, activated memory T-lymphocytes, Langerhans cells and RF DI+ antigen presenting cells at the site of eruption (Rajka, 1975, Maurer and Stingl, 1995; Bieber et al., 1992). Down-regulating the local T-cell mediated reaction is known to exert an immune-suppressive and anti-inflammatory effect (Saski et al 1989; Chang et al., 1987).

**MATERIALS AND METHODS**

Detection of biologically active natural products plays a strategic role in the phytochemical investigation of crude plant extracts. In order to perform an efficient screening of the extract both chemical analysis and biological essays were performed. Chemical analysis: involved the analysis of the herbal extract by UV/VIS, SDS-PAGE, colourimetric determination (Verpoorte, 1980; Porter, 2001; Gupta and Seshadri, 2002) and FTIR. The concentration of protein and reducing sugars were determined by standard essays (Guthrie, 1974; Brandon and Tooze, 1991). Protein purification was achieved by elution through a Sephadex G-100 column followed by ultrafiltration.

Cell culture and induction of cytotoxicity: NRK cells and XB-2 cells were routinely grown and exposure to the extract was evaluated by MTT, NR and SRB (Mosmann, 1983; Borenford and Peurer, 1985) assays with and without an microsomal fraction. Ames Assay: The standard plate incorporation test methods were used according to Ames procedure methods (Ames et al 1975) and Maron and Ames (Maron and Ames 1983), using six different strains of *Salmonella typhimurium* with and without a metabolic activator S9. Disinfectant Test: Oxoidculti-loops were dissolved in TSB and RCM and plated following exposure to 1 ml of extract.

**RESULTS:**

**TABLE 1. Evaluation of Protein and Carbohydrate Concentrations.**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>COMPONENT</th>
<th>CONC. (Mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herbal extract (protein)</td>
<td>25.17±1.3</td>
<td></td>
</tr>
<tr>
<td>Final Product (protein)</td>
<td>0.044±0.0</td>
<td></td>
</tr>
<tr>
<td>Herbal extract (reducing sugars)</td>
<td>235±35.0</td>
<td></td>
</tr>
<tr>
<td>Final product reducing sugars</td>
<td>15.33± 2.55</td>
<td></td>
</tr>
</tbody>
</table>

The protein and carbohydrate content of the herbal extract and the final product showed a significant variation. The herbal extract showed a protein content of 25.17±1.3mg/ml while the final product had a significant reduced protein content of 0.044±0.0mg/ml. For sugar content, the herbal extract had a reducing sugar of 235±35.0 mg/ml as compared to 15.33± 2.55mg/ml.

Protein purification procedures identified two different proteins the molecular weights of which were estimated as follows as shown in table 2 below:

**TABLE 2. Protein Molecular Weight Estimation.**

<table>
<thead>
<tr>
<th>Ultrafiltration</th>
<th>Mol. Weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retenate (Centricon 30)</td>
<td>&gt;30,000</td>
</tr>
<tr>
<td>Filtrate (Centricon 30)</td>
<td>&lt;30,000</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>m.w (kDa)</td>
</tr>
<tr>
<td>Herbal extract</td>
<td>38914±1,266</td>
</tr>
<tr>
<td>TCA precipitate</td>
<td>43651-79960±2961</td>
</tr>
<tr>
<td>Supernatant</td>
<td>27542-55085±5372</td>
</tr>
</tbody>
</table>

The herbal extract using ultrafiltration showed a molecular weight of 38914±1,266 kDa. Although
UV/VIS analysis is not diagnostic for a particular compound as a number of absorptive peaks were evident in the 200-220nm region which is indicative of flavones (phenolic structures). Acid hydrolysis indicated the presence of polysaccharides/hydrolysable glycosides, also the precipitation of phlobaphenes were indicative of the presence of tannins.

FTIR analysis coupled to software library matching elucidated the following components using the ethyl acetate, hexane, ethanol and water solvent. With the ethyl acetate, the FTIR detected high levels of tannic acid, nutmeg oils, tolu balsams. While for the pure water solvent citric acid and d-glucitol was detected. Inositol, sucrose, mono-oleate and oxalic acid was detected in the ethanol extract as shown in Table 3.

**TABLE 3. Fourier Tungsten Infra Red (FTIR) analysis in various solvents**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Detected compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Acetate</td>
<td>Tannic acid, Nutmeg oils, Tolu balsams</td>
</tr>
<tr>
<td>Hexane</td>
<td>Grapefruit oils, waxy substances. Mannitol</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Inositol, Sucrose mono-oleate, oxalic acid</td>
</tr>
<tr>
<td>Water</td>
<td>Citric acid, d-glucitol</td>
</tr>
</tbody>
</table>

For the evaluation of the LD₅₀ from the cytotoxic assays the results obtained are shown in Table 4 below.

**TABLE 4. LD₅₀ values from cytotoxicity assays**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cell line</th>
<th>Protein conc. (mg/ml)</th>
<th>S9 mix (16.6%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>(NRK cells)</td>
<td>5.1±0.1</td>
<td>7.6±0.25</td>
</tr>
<tr>
<td></td>
<td>(XB-2 cells)</td>
<td>6.4±0.2</td>
<td>4.85±0.45</td>
</tr>
<tr>
<td>SRB</td>
<td>(NRK cells)</td>
<td>7.75±0.25</td>
<td>4.8±0.58</td>
</tr>
<tr>
<td></td>
<td>(XB-2 cells)</td>
<td>5.7±1.2</td>
<td>8.0±0</td>
</tr>
<tr>
<td>MTT</td>
<td>(NRK cells)</td>
<td>7.1±0.45</td>
<td>7.1±0</td>
</tr>
<tr>
<td></td>
<td>(XB-2 cells)</td>
<td>5.25±0.25</td>
<td>7.05±0</td>
</tr>
</tbody>
</table>

The estimation of LD₅₀ from the Neutral red (NR), MTT and SRB assay showed that the protein concentration was high 7.75±0.25 (mg/ml) in SRB using NRK cell lines, followed by 7.1±0.45mg/ml for MTT assay using NRK cells.

These results are expressed as mean and standard error of the mean based on protein concentration of the herbal extract. A statistical comparison between plates treated with and without S9 mix indicated that there was no significant difference between both sets of data at a 3% significance level. The ID₅₀ value for the final product was 7.2±0.42mg/ml protein.

Ames assay: results showed no mutagenic potential of the extract with and without metabolic activation, these results also achieved significance at a 95% confidence level. However it was observed that at high concentrations a bactericidal effect was induced. A disinfectant assay using *E. coli*, *S. aureus*, *S. typhimurium* and *C. sporogenes* proved the herbal extract to contain antibiotic constituents, although its cell killing properties were influenced by final ethanol concentration.

**DISCUSSION.**

A preliminary solvent extraction procedure indicated that most of the components of the herbal concentrate were present within the neutral fraction. FTIR analysis indicated that a number of essential and volatile oils were present. Such compounds are frequently associated with gums and resins and are generally mixtures of hydrocarbons and oxygenated compounds derived from hydrocarbons. Many oils are terpenoid in origin and some contain aromatic derivatives, which are often associated in odour fractions such as the phenylpropnods. Some monoterpenes occur in plants in the glycosidic form (Skopp and Horster, 2006). It is unlikely that tolu balsams are present but it may be inferred that monohydric aromatic alcohols were present to some extent. Mannitol which is a hexahydric aliphatic alcohol is isomeric with glucitol (mol. Wt=182.18) a group of plant alcohols related to the alicyclic sugar alcohols are the carboxylic inositols (mol.wt.=180.16) (Plouvier, 1993).

Therapeutically, the phytochemicals present may have the following properties with regard to treating skin disease volatile/essential oils (demulcent and antibiotic); mucilagens/waxes (emollient); polysaccharides (absorbant) and tannins (astringent). The antiseptic nature of the extract was evaluated on a range of micro-organisms. The most common phenolic compounds are the polymeric astringent tannins whose protein binding properties are well documented. Characteristic of some orders of the cotyledenous plants are the “hydrolysable tannins” based on gallic acid residues linked, often as esterified.
chains, to glucose or another polyhydric alcohol. More widespread are the “condensed tannins”, oligomers of the flavonoid catechin linked by interflavon bonds (Pierpont 1996). A simple technique for the precipitation of flavonoids using lead acetate gave a positive result. Polyphenolic compounds are known to contain free radical quenching antioxidants (Aruoma et al., 1992), and such an activity has been implicated in the treatment of atopic eczema using Chinese herbs (Kirby and Schmidt, 2007).

The protein concentration of the herbal mixture was estimated to be 26mg/ml. However this may be attributed to Folin’s reagent used in the procedure which is documented to react with phenolics which were also present (Harbone, 1987). Ultrafiltration results indicate that there were two different protein species present, the molecular weights of which were in the range 27,000-38,000kDa and 55,000-80,000kDa. However it has been concluded that neither protein induces a deleterious effect to cells in culture. The results obtained from three different assays show toxicity to be induced at an ID50 value of 5-8mg/ml protein, using two organotypic cell lines. Mutagenicity screening of the extract and final product indicates no mutagenic potential with or without metabolic activation. The modulating effects of naturally occurring phytochemicals that protect against mutagenesis and carcinogenesis have been reported (Bertran et al., 1987). Limonene a terpenoid substance inhibits chemically induced lung tumours in mice (Wattenberg, 2001) and tannic acid inhibits benzo(a) pyrene metabolites is S. typhimurium and in Chinese hamster V-79 cells (Huang and Wood, 1983). A report issued regarding the screening of plant extracts for genetic toxicology states that unless knowledge is available concerning the constituents of a herbal mixture, data will not be accepted relating to mutagenic or antimutagenic assessment (Shelby, 1997). The next phase of the study will involve the analysis of other properties of the extract including antioxidant potential and their relationship to purified sub-fractions.

CONCLUSION.

This study was aimed at evaluating the formulation strategy for preclinical and efficacy evaluation of herbal products with medical properties. Therapeutically, the phytochemicals present may have the following properties with regard to treating skin disease – volatile/essential oils (demulcent and antibiotic); mucilagens/waxes (emollient); polysaccharides (absorbant) and tannins (astringent). The medical product evaluated with the herbal extract showed that neither protein induces a deleterious effect to cells in culture. Mutagenicity screening of the extract and final product indicates no mutagenic potential with or without metabolic activation. Herbal products continue to have and to serve as a high potential source of new chemical entities for developing new drugs in the drug discovery and development pipelines. There is therefore a need for a concerted effort towards research and development of potential lead compound from the rich cocktail of medicinal plants in Cameroon and the humid tropical dense forest of Central Africa.

REFERENCES.


