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A metabolomic approach to identify anti-hepatocarcinogenic compounds from plants used traditionally in the treatment of liver diseases

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ABSTRACT

Liver cancer is a major health burden in Southeast Asia, and most patients turn towards the use of medicinal plants to alleviate their symptoms. The aim of this work was to apply to Southeast Asian plants traditionally used to treat liver disorders, a successive ranking strategy based on a comprehensive review of the literature and metabolomic data in order to relate ethnopharmacological relevance to chemical entities of interest. We analyzed 45 publications resulting in a list of 378 plant species, and our point system based on the frequency of citation in the literature allowed the selection of 10 top ranked species for further collection and extraction. Extracts of these plants were tested for their *in vitro* anti-proliferative activities on HepG2 cells. Ethanolic extracts of *Andrographis paniculata*, *Oroxylum indicum*, *Orthosiphon aristatus* and *Willughbeia edulis* showed the highest anti-proliferative effects (IC_{50} = 195.9, 64.1, 71.3 and 66.7 μg/ml, respectively). A metabolomic ranking model was performed to annotate compounds responsible for the anti-proliferative properties of *A. paniculata* (andrographolactone and dehydroandrographolide), *O. indicum* (baicalein, chrysin, oroxylin A and scutellarein), *O. aristatus* (5-desmethylsinensetin) and *W. edulis* (parabaroside C and procyanidin). Overall, our dereplicative approach combined with a bibliographic scoring system allowed us to rapidly decipher the molecular basis of traditionally used medicinal plants.

1. Introduction

Liver cancer represents the second leading cause of cancer-related death worldwide and is therefore a major public health problem [1], with hepatocellular carcinoma (HCC) being responsible for 80% of all liver cancers. The geographical distribution of HCC is heterogeneous with a high prevalence in developing regions of Eastern and Southeastern Asia [2]. Curative treatments for HCC are mainly based on surgical resection, liver transplantation and local ablation. Although these therapies can improve prognosis, they are indicated mainly for patients diagnosed at an early stage [3]. However, in developing countries, most patients are diagnosed at a late stage, and thus only receive palliative treatments [4,5]. Moreover, the high cost of treatment does not allow poorer people to have access to good quality healthcare. Therefore, recent studies have highlighted that liver cancer patients from low-income countries often turn towards the use of traditional, complementary and alternative medicine (TCAM), and in particular medicinal plants [6,7].

Hepatocarcinogenesis is a complex process involving a series of individual steps related to proliferation, cell cycle regulation, differentiation, apoptosis, angiogenesis, invasion and metastasis [8,9]. Natural agents can exert their therapeutic effects at one or more of the specific stages of this multistep process. As an example, milk thistle (*Silybum marianum* (L.) Gaertn.) has been used for the treatment of liver disorders since the ancient Greeks, and is by far the most studied hepatoprotective plant species [10]. The active agent in milk thistle is known to be a flavanolignan/flavonoid mixture, called silymarin, which can be isolated from the seeds and fruits of the plant. Its anticarcinogenic effect has been attributed to various actions: anti-inflammation, cell cycle regulation, induction of apoptosis, growth inhibition, inhibition of angiogenesis, and inhibition of invasion and metastasis [11,12]. Despite their known limitations, *in vitro* anti-proliferative assays on human hepatoma (HepG2) cells are widely used as a starting point for the evaluation of anti-hepatocarcinogenic effects [13–15]. Bio-assay guided fractionation can be coupled to a dereplication approach and multivariate data analysis in order to rank and identify compounds *in mixture* responsible for the anti-proliferative effects of plant extracts, and provide a better understanding of the

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mechanisms of action of medicinal plants [16,17]. Recently, Chervin et al. described a new method for the dereplication of natural products in complex mixture, which they employed for the identification of redox active compounds from *Viola* extracts [18].

Most of the research into medicinal plants used for the treatment of chronic liver diseases is based on the study of pharmacopeias of major traditional medicines (*i.e.* Traditional Chinese Medicine (TCM), Ayurvedic medicine (India), Kampo medicine (Japan) or Western herbal medicine) [19]. However, in Southeast Asia, information on medicinal plants used to treat liver diseases is scattered and, to the best of our knowledge, only one relevant publication exists, which focuses on medicinal plants used by Khmer traditional healers in the Phnom Penh area (Cambodia) for liver pathologies [20]. In this study, the authors documented the use of 83 plant species, and discovered that most of them have been shown to possess liver-related pharmacological properties (*i.e.* hepatoprotective, anti-microbial and anti-inflammatory activities), thus justifying their use with varying degrees of certainty. Still, for many of these plant species, further in-depth investigation is required in order to determine their mechanism of action and recommend their use.

In Southeast Asia, many patients suffering from liver cancer consult traditional healers, and rely on medicinal plants for treatment. Therefore, we used a bibliographic scoring system to select the 10 most used TCAM plant species in Southeast Asia, and evaluated their anti-proliferative activity on HepG2 cells. Then, after identifying the most anti-proliferative extracts, we used a dereplication approach to putatively identify active compounds.

2. Materials and methods

2.1. Ethnopharmacological selection of plants

A comprehensive review of the scientific literature was undertaken from May to July 2016, specifically focusing on Southeast Asian ethnobotanical documents. Books, PhD or master's theses, and reports available in Lao, Cambodian and French libraries were reviewed. Scientific articles published in international journals were screened by consulting electronic databases (Google Scholar, PubMed and Web of Science), using these specific keywords: “traditional medicine” and “medicinal plants”, combined with terms related to liver disease: “liver”, “jaundice”, “icterus” and “hepatitis”; and geographical names: “Southeast Asia”, “Cambodia”, “Laos”, “Vietnam”, “Thailand” and “China”.

References in French, English, Cambodian and Lao were consulted, and translated if necessary. Botanical names were validated by referring to the Plant List database (http://www.theplantlist.org/). Overall, 45 references were thoroughly investigated.

As described in Elkington et al., a point system was employed to rank the species recorded among the 45 references [21]. Each plant species was assigned one point: (i) for each citation in the overall literature, (ii) for each Southeast Asian country where the plant is cited, (iii) for each people interviewed who cited the plant in the references, if available. The weighted score was obtained by multiplying the three values. All the plants recorded were entered in an Excel spreadsheet (Microsoft 2013) (Supplementary material S1). Altogether 378 species were listed. From this list, we selected species with: (i) the highest score, (ii) no previous pharmacological evaluation of their crude extract on HepG2 cells, and (iii) a high frequency and abundance in the prospected area.

2.2. Plant materials

Plants were collected in July 2016 in Champasak province, Lao People's Democratic Republic (PDR) with the assistance of traditional healers working in the Traditional Medicine Department, Champasak Regional Hospital. Authorization to collect the plants was obtained from the relevant authorities. Ethical approval was obtained in February 2016 from the National Ethics Committee for Health Research (NECHR) under the supervision of the Ministry of Health of Lao PDR (010NIOPH/NECHR).

Voucher specimens were deposited at the herbarium of the Department of Biology, Faculty of Sciences, National University of Laos (NUoL) in Vientiane. Botanical identification was initially realized by F. Chassagne, and confirmed by specialists.

2.3. Plant extraction and fractionation procedures

All selected plant materials were shade-dried at room temperature for seven days and pulverized into a fine powder. Then, the air-dried and powdered plants (15 g) were exhaustively macerated with 80% ethanol (v/v, 2 × 150 ml) at room temperature. The ethanolic solutions were filtered, then concentrated under reduced pressure at 40 °C to obtain the crude extracts. In parallel, aqueous extracts were obtained as follows: 15 g of dried powdered plants were stirred in 150 ml of deionized water, in a water bath at 90 °C for 30 min, then kept at room temperature for 2 h. The solutions were filtered, stored at −20 °C for one day, and freeze-dried for three days.

The ethanolic and aqueous dry extracts of each bioactive plants were fractionated using solid phase extraction (SPE) (1 g Sep-Pak® C18 cartridge, Waters, USA). The SPE cartridges were primarily activated with 10 ml of methanol 100% (v/v), then equilibrated with 10 ml of methanol 5% (v/v). Plant extract (100 mg) was dissolved in 1 ml of methanol 5% (ethanolic extracts) or 1 ml of water (aqueous extracts). Then, the dissolved plant extract was loaded on the column and eluted successively by passing six aqueous methanolic solutions (15 ml each) of increasing organic solvent concentration, starting with 5% MeOH/95% water (Fr1), then 20% MeOH/80% water (Fr2), 40% MeOH/60% water (Fr3), 60% MeOH/40% water (Fr4), 80% MeOH/20% water (Fr5) and finally 100% MeOH (Fr6). The six fractions collected for each sample were then concentrated under reduce pressure

For each fraction and extract, stock solutions at a concentration of 10 mg/ml in 100% dimethyl sulfoxide (DMSO) (Fisher Chemicals, UK) (v/v) were prepared for anti-proliferative assays using the HepG2 model; and at a concentration of 2 mg/ml in 100% methanol (v/v) for ultra-high-performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) analysis.

2.4. Cell culture and anti-proliferative assay

HepG2 cell lines were purchased from the American Type Culture Collection (ATCC, USA). They were cultured at 37 °C, 5% CO_2, in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco®, Life Technologies, USA), supplemented with 10% fetal bovine serum, and penicillin/streptomycin (100 U/ml). For the anti-proliferative assays, HepG2 cells were cultured in the same conditions but without antibiotics. The medium was renewed twice a week.

HepG2 cells under exponential growth were seeded in 96-well plates at a density of 10,000 cells per well. After overnight growth, cells were treated with various concentrations of extracts/fractions for 72 h. The concentrations tested ranged from 1.95 to 250 µg/ml. Then, the cells were washed with phosphate-buffered saline (Gibco®, Life Technologies, USA), and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, USA) at a concentration of 0.5 mg/ml for 4 h. Next, DMSO (100 µl/well) was added into each well, and the plates were read at 570 nm using a microplate reader (EON, BioTek, USA). The cell growth and inhibition rate were calculated and IC_{50} values were determined using Graph Pad Prism version 6 software (Graph Pad, USA). For each set of experiments, a positive control (Triton X-100 1%) was used to induce 100% cell death. All experiments were performed in triplicate.