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Research Paper

HPLC-based activity profiling for antiplasmodial compounds in the traditional Indonesian medicinal plant *Carica papaya* L

Tasqiah Julianti [a,c], Maria De Mieri [a], Stefanie Zimmermann [a,b], Samad N. Ebrahimi [a,d], Marcel Kaiser [b], Markus Neuburger [e], Melanie Raith [a], Reto Brun [b], Matthias Hamburger [a,*]

[a] Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, Basel 4056, Switzerland
[b] Swiss Tropical and Public Health Institute, Basel 4051, Switzerland
[c] Faculty of Pharmacy, Pancasila University, Jakarta 12640, Indonesia
[d] Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, G. C., Evin, Tehran, Iran
[e] Inorganic Chemistry, Department of Chemistry, University of Basel, Basel 4056, Switzerland

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ABSTRACT

Ethnopharmacological relevance: Leaf decoctions of *Carica papaya* have been traditionally used in some parts of Indonesia to treat and prevent malaria. Leaf extracts and fraction have been previously shown to possess antiplasmodial activity *in vitro* and *in vivo*.

Materials and methods: Antiplasmodial activity of extracts was confirmed and the active fractions in the extract were identified by HPLC-based activity profiling, a gradient HPLC fractionation of a single injection of the extract, followed by offline bioassay of the obtained microfractions. For preparative isolation of compounds, an alkaloidal fraction was obtained *via* adsorption on cationic ion exchange resin. Active compounds were purified by HPLC–MS and MPLC–ELSD. Structures were established by HR-ESI-MS and NMR spectroscopy. For compounds **5** and **7** absolute configuration was confirmed by comparison of experimental and calculated electronic circular dichroism (ECD) spectroscopy data, and by X-ray crystallography. Compounds were tested for bioactivity *in vitro* against four parasites (*Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum*), and in the *Plasmodium berghei* mouse model.

Results: Profiling indicated flavonoids and alkaloids in the active time windows. A total of nine compounds were isolated. Four were known flavonols – manghaslin, clitorin, rutin, and nicotiflorin. Five compounds isolated from the alkaloidal fraction were piperidine alkaloids. Compounds **5** and **6** were inactive carpamic acid and methyl carpamate, while three alkaloids **7**–**9** showed high antiplasmodial activity and low cytotoxicity. When tested in the *Plasmodium berghei* mouse model, carpaine (**7**) did not increase the survival time of animals.

Conclusions: The antiplasmodial activity of papaya leaves could be linked to alkaloids. Among these, carpaine was highly active and selective *in vitro*. The high *in vitro* activity could not be substantiated with the *in vivo* murine model. Further investigations are needed to clarify the divergence between our negative *in vivo* results for carpaine, and previous reports of *in vivo* activity with papaya leaf extracts.

1. Introduction

Based on the number of cases reported, malaria is considered as a controlled disease in Indonesia (Feachem et al., 2010). However, malaria is still a major health concern in the densely forested parts of eastern Indonesia. Current primary treatment for malaria is Artemisinin Combination Therapies (ATCs), but artemisinin-resistant *Plasmodium falciparum* strains have been reported (Miller et al., 2013). Thus, a continued effort for discovery of novel antimalarial compounds is needed.

Traditional remedies to prevent and treat malaria remain in use in Indonesia. Among these, papaya leaf decoctions are widely used in Papua and Maluku islands. Other than malaria therapy, papaya leaves are empirically used in Indonesia to enhance breast milk production, for deworming and boosting appetite, and for reducing fever (Rehena, 2009; Sastroamidjojo, 2001; Tjahjadi, 1990). Use of papaya leaves as an antimalarial remedy has also been reported from India, and from some Latin American and African

* Corresponding author. Tel.: +41 61 267 14 25; fax: +41 61 267 14 74.
E-mail address: matthias.hamburger@unibas.ch (M. Hamburger).

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countries (Asasea et al., 2010; Kovendan et al., 2012; Stangelanda et al., 2011; Valadeau et al., 2009). The traditional use as an antimalarial remedy was substantiated by *in vitro* studies (Lina, 1996; Rehena, 2009), and by testing of an alkaloid containing polar leaf extract in a mouse model (Sulistyowati, 2000).

Papaya (*Carica papaya* L., Caricaceae) grows widely in tropical and subtropical regions around the world (Garrett, 1995). The trees are mainly cultivated for their fruits, but the leaves, seeds, and latex are traditionally known to benefit health. Papaya leaves contain flavonoids and other phenolic compounds, saponins, cardiac glycosides, anthraquinones, and alkaloids (Afzan et al., 2012; Canini et al., 2007; Sherwani et al., 2013). Alkaloids reported include carpaine (Greshoff, 1890), pseudocarpaine (Govindachari et al., 1954), and dehydrocarpaine I and II (Tang, 1979).

Considering the easy accessibility of plant material, the traditional use as an antiplasmodial, and the reported *in vivo* activity, we embarked on an activity-driven characterization of active principles in papaya leaves. For an efficient localization of active constituents in the extract we employed HPLC-based activity profiling (Potterat and Hamburger, 2013), using a protocol established for the discovery of antiprotozoal compounds in complex matrices (Adams et al., 2009). Analytical HPLC connected to PDA and MS detectors, and parallel micro-fractionation of the column effluent for off-line bioassay link biological and structural information with chromatographic peaks in the chromatogram.

We here report on the identification of *in vitro* antiplasmodial compounds in papaya leaves, and on the outcomes from a testing of the main alkaloid, carpaine, in the murine *Plasmodium berghei* model.

2. Materials and methods

2.1. Materials

Analytical grade solvents for extraction and HPLC grade solvents were from Scharlau (Barcelona, Spain). Ammonium hydroxide (26%) was from Riedel-de Häen (Seelze, Germany). Formic acid (98–100%) was from Sigma-Aldrich (Buchs, Switzerland). DMSO was from Reuss Chemie (Tägerig, Switzerland). HPLC grade water was obtained by an EASYpure II (Barnstead, Dubuque, USA) water purification system. Cationic exchange resin Lewatit® MonoPlus SP 112 was from Lanxess (Cologne, Germany). Reference drugs for bioactivity tests were melarsoprol (Arsobal®, Sanofi-Aventis, Switzerland), benznidazole (Sigma-Aldrich), miltefosine (VWR), chloroquine (Sigma-Aldrich), artesunate (Mepha, Switzerland), and podophyllotoxin (Sigma-Aldrich).

Parasites for *in vitro* activity tests were *Trypanosoma brucei rhodesiense*, STIB 900 strain, trypomastigote stage; *Trypanosoma cruzi*, Tulahuen C4 strain, amastigote stage; *Leishmania donovani*, MHOM-ET-67/L82 strain, amastigote stage; *Plasmodium falciparum*, K1 strain (chloroquine- and pyrimethamine-resistant), erythrocytic stage. Cytotoxicity was determined with rat skeletal myoblast cells (L6). The *in vivo* efficacy study was carried out in the *Plasmodium berghei* mouse model. Adult female NMRI mice were purchased from RCC Janvier.

2.2. General experimental procedures

Extract screening and HPLC-based activity profiling including mass spectral data analysis were carried out as previously described by Adams et al. (2009). A HPLC SunFire RP-18 column (3.5 μm, 3 × 150 mm i.d., Waters; Wexvord, Ireland) was used. Minute-based microfractionation and offline data collection for HPLC based activity profiling were carried out with a series 1100 HPLC system consisting of a degasser, a binary high pressure mixing pump, a column oven and a PDA detector with 250 μL loop (all from Agilent; Waldbronn, Germany) connected to an Esquire 3000 Plus ion trap mass spectrometer with an electrospray interface (Bruker Daltonics; Bremen, Germany). MS spectra were recorded in positive and negative mode in the range of *m*/*z* 200–1500. Hystar 3.0 software (Bruker Daltonics; Bremen, Germany) was used for controlling the LC–MS system.

Semipreparative HPLC separations of flavonoids and alkaloid **9** were carried out on an 1100 series HPLC system consisting of a quaternary low-pressure mixing pump with a degasser module, a column oven, and a PDA detector with a 1000 μL loop (all Agilent; Waldbronn, Germany). A SunFire C18 column (5 μm, 10 × 150 mm; Waters) was used. The separation of flavonoids was monitored following UV-detection. For the alkaloid, the separation was monitored using an Esquire 3000 Plus ion trap mass spectrometer with an electrospray interface (Bruker Daltonics; Bremen, Germany) connected *via* a T-splitter (split ratio 3: 997). Data analysis and controlling of the MS were with Hystar 3.2 software (Bruker Daltonics; Bremen, Germany). Spectra were recorded in positive mode in the range of *m*/*z* 200–1000.

Semipreparative separations of alkaloids **5**–**8** were carried out with a PuriFlash 4100 system consisting of a mixing HPLC pump, a UV detector dual length DAD, a fraction collector, and a sample loading module (Interchim; Montluçon, France). For system controlling and process monitoring, Interchim Software 5.0 was used. Detection was done with a 2000ES ELSD (Alltech; Woodridge, Illinois, USA). The following ELSD settings were used: temperature 60 °C, gas flow 2.4 L/min, and gain of 4 or 8, with impactor on.

High resolution MS were recorded with an Agilent 1100 series HPLC linked to a microTOF-ESI-MS system (Bruker Daltonics). HyStar 3.0 software (Bruker Daltonics) was used for data acquisition and processing.

NMR spectra were recorded in CD_3OD and $CDCl_3$ on a Bruker Avance III spectrometer (Bruker; Fällanden, Switzerland) operating at 500.13 MHz for ^1H, and 125.77 MHz for ^{13}C. ^1H NMR and 2D (COSY, HSQC, and HMBC) spectra were measured with a 1 mm TXI probe at 18 °C. ^{13}C NMR spectra were recorded with a 5 mm BBO probe at 23 °C. Spectra were processed and analyzed by Bruker TopSpin 3.0 software.

ECD spectra of compounds were recorded, at 500 μg/mL in MeOH or MeCN, on an AVIV Model 62ADS CD spectrometer, and analyzed with the AVIV 60DS V4.1 software.

X-ray crystallography was performed with a Bruker Kappa Apex2 diffractometer at 123 K using graphite-monochromated Cu K_α-radiation. Structure solution used program SIR92 (Altomare et al., 1994) and SHELX 86 (Sheldrick, 1985). Structure refinement employed CRYSTALS (Betteridge et al., 2003). Data analysis and visualization utilized Mercury v.3.0 software.

2.3. Plant material and extraction

Extract screening and HPLC-based activity profiling were carried out with a leaf sample purchased from a Thai food store in Basel. Isolation of compounds was carried out with papaya leaves purchased from Dixa AG (St. Gallen, Switzerland), a supplier of pharmaceutical herbs. Vouchers have been deposited under identification nos. 910 and 647 at the Division of Pharmaceutical Biology, University of Basel, Switzerland. Authentication of the material was carried out by M. Hamburger.

In the initial screening, the extracts of methanol, ethyl acetate, and petroleum ether were prepared by pressurized liquid extraction using an ASE 200 extractor with solvent module (Dionex; Sunnyvale, CA, USA). Extraction was performed with 1 g of ground leaves in a 22-mL cartridge. Instrument setting for three extraction cycles were of temperature 100 °C, preheating time 1 min, heating time 5 min, static extraction 5 min, flush 100% solvent of cell volume, purge 120 s with nitrogen, and pressure 120 bar. The