Original article

Novel plant inducers of PXR-dependent cytochrome P450 3A4 expression in HepG2 cells

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ABSTRACT

The cytochrome P450 3A4 (CYP3A4) is the most abundant CYP450 enzyme involved in the metabolism of endogenous products and xenobiotics, including prescription drugs and herbal products. Modulation of hepatic CYP3A4 gene expression via nuclear receptors, like pregnane X receptor (PXR), is a major cause of adverse effects like drug-unresponsiveness and toxicity. In the present study, ethanol extracts of 58 medicinal plants, belonging to 27 families, were evaluated for potential activities in CYP3A4 induction in HepG2 cells by reporter gene assay. For PXR-mediated CYP3A4 induction, a 50 μg/ml concentration was used for all non-cytotoxic plant extracts. Rifampicin (10 μM) and DMSO (0.1%) were used as standard inducer and untreated (negative) control, respectively. The comparative fold-induction of CYP3A4 by the plant extracts in relation to the untreated control was determined. As a result, Dodonaea angustifolia (2.62 fold; \( P < 0.0001 \)) was found to be the most promising inducer of CYP3A4, followed by Euphorbia tirucalli (1.95 fold; \( P = 0.0004 \)), Alternanthera pungens (1.74 fold, \( P = 0.0035 \)), and Ficus palmata (1.65 fold; \( P = 0.0097 \)). Further phytochemical characterizations of the active plants are therefore, warranted.

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1. Introduction

Cytochrome P450 (CYP) is a superfamily of drug-metabolizing enzymes that is involved in the metabolism of endogenous compounds, xenobiotics and pharmaceuticals (Anzenbacher and Anzenbacherová, 2001). The major CYP involved in the hepatic metabolism of most of the drugs include CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 (Anzenbacher and Anzenbacherová, 2001; Al-Dosari and Parvez, 2016). Since CYP is involved in phase-1 metabolism of >70% of prescription drugs, modulation of its expressions is a major cause of adverse drug-drug interactions, including decreased drug efficacy (Tyagi et al., 2010; Zhang et al., 2010). Expression of CYP3A4 is markedly induced both in vivo and in cultured human hepatocytes in vitro in response to a variety of xenobiotics (e.g., dexamethasone and rifampicin) as well as medicinal herbs like, St. John’s wort (Hypericum perforatum) (Kolars et al., 1992; Schuetz and Guzelian, 1984; Zhou and Lai, 2008; Harmsen et al., 2008; Quatrochi and Guzelian, 2001; Moore et al., 2000).

Further, gene expression of CYP is regulated by a set of nuclear receptors in response to a wide spectrum of xenobiotics (Pelkonen et al., 1998; Quatrochi and Guzelian, 2001; Al-Dosari and Parvez, 2016). Of these, the pregnane X receptor (PXR), also known as xenobiotic or pregnane-activated receptor regulates CYP3A4 gene induction (Matic et al., 2007; Hustert et al., 2001). The most common clinical implication for the PXR activation is the drug-drug interactions, mediated by the upregulation of CYP3A4. Notably, PXR itself is activated by rifampicin and other xenobiotics (Lehmann et al., 1998) as well as plant secondary metabolites like, hyperforin from H. perforatum (Zhou and Lai, 2008, Moore et al., 2000). In addition to this, PXR and its target genes also play an important role in maintaining normal physiological function and homeostasis. For example, artemisinin (Artemisia annua), piperine (Piper nigrum) and notoginsenoside (Panax notoginseng), the known activators of PXR, have been shown to prevent severity of colonic inflammatory bowel disease by inducing CYP3A4 expression (Hu et al., 2015, 2014; Zhang et al., 2015).

Hepatic PXR has broad substrate specificity and thus may be activated by a large number of chemically-diverse secondary metabolites found in dietary supplements and therapeutic herbs. Since such natural products are often orally consumed, the high concentration of their phytoconstituents in gut and liver may
potentially affect the CYP activity. In this report, we therefore, intended to screen the novel PXR-dependent CYP3A4 activation potential of 58 medicinal plants of 27 families using cultured hepatocytes and reporter gene assay.

2. Experimental methods

2.1. Plant materials and extraction

The studied plant extracts included pre-identified, non-cytotoxic medicinal plants (Table 1) with their traditionally known or published therapeutic values (Arbab et al., 2017). The dried plant parts were ground to a coarse powder using mortar-pestle and extracted with 80% ethanol (Merck, Germany) for three days with periodic shaking and filtered using Whatman No. 1 paper (Sigma, Germany). After removal of the solvent under reduced pressure using rotary evaporator (4°C) and complete drying, their yield percentage were calculated. Stock of each extract (100 mg/ml) was prepared by dissolving in dimethyl sulfoxide (DMSO, Sigma, USA), and stored at -20°C.

2.2. Cell culture and reagents

Human hepatoblastoma cell line, HepG2 (López-Terrada et al., 2009) was maintained in T75 culture flask (Corning, USA) in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Inc., MA, USA).

| No. | Specimen no. | Plants name          | Family               | Plant part used |
|-----|--------------|----------------------|----------------------|-----------------|
| 1   | 16011        | Achyranthe aspera    | Amaranthaceae        | Shoots          |
| 2   | 16391        | Alternanthera pungens | Amaranthaceae        | Shoots          |
| 3   | 16189        | Amaranthus alba      | Amaranthaceae        | Shoots          |
| 4   | 16196        | Avera javanica       | Avera javanica       | Shoots          |
| 5   | 16198        | Flaveria trineriva   | Asteraceae           | Shoots          |
| 6   | 16083        | Puslicaria crispia   | Puslicaria crispia   | Shoots          |
| 7   | 16075        | Pepergaria tomentosa  | Asclepiadaceae       | Shoots          |
| 8   | 161318       | Eruca sativa         | Brassicaceae         | Leaves, Stems   |
| 9   | 15841        | Capparis decidua     | Capparaceae          | Stems           |
| 10  | 16195        | Artiplex suberecta   | Chenopodiaceae       | Shoots          |
| 11  | 15496        | Combretum molle      | Combretaceae         | Bark            |
| 12  | 798          | Guiera senegalensis  | Combretaceae         | Leaves          |
| 13  | 16075        | Ipomoea cairica      | Convolvulaceae       | Shoots          |
| 14  | 16179        | Juniperus phonicia   | Cupressaceae         | Leaves, Stems   |
| 15  | 16194        | Juniperus procerca   | Cupressaceae         | Leaves, Stems   |
| 16  | 15830        | Cleome droserifolia  | Cruciferae           | Shoots          |
| 17  | 16275        | Coccinia grandis     | Crassulaceae         | Leaves, Stems   |
| 18  | 16393        | Coreloocarpus epigeus| Crassulaceae         | Leaves          |
| 19  | 16395        | Momordica balsamina  | Fabaceae             | Leaves          |
| 20  | 16181        | Chenopodium ambrosioides| Aarial parts       |
| 21  | 16197        | Chenopodium glauca   | Fabaceae             | leaves, Stems   |
| 22  | 16172        | Euphorbia tirucalli  | Euphorbiaceae        | Stems           |
| 23  | 16084        | Euphorbia hirta      | Euphorbiaceae        | Shoots          |
| 24  | 15189        | Jatropha curcas      | Euphorbiaceae        | Shoots          |
| 25  | 14005        | Ricinus communis     | Euphorbiaceae        | Shoots          |
| 26  | 16281        | Acacia mellifera     | Fabaceae             | Leaves          |
| 27  | 16221        | Acacia hamulosa      | Fabaceae             | Leaves, Stems   |
| 28  | 16187        | Acacia asi            | Fabaceae             | Leaves          |
| 29  | 16385        | Acacia ehrenbergiana | Fabaceae             | Stems           |
| 30  | 16390        | Acacia laeta         | Fabaceae             | Stems           |
| 31  | 16389        | Acacia oerfota       | Fabaceae             | Stems           |
| 32  | 15007        | Acacia salicina      | Fabaceae             | Leaves          |
| 33  | 14977        | Acacia tortilis      | Fabaceae             | Stems           |
| 34  | 16182        | Albizia procera      | Fabaceae             | Leaves          |
| 35  | 16035        | Delonix elata        | Fabaceae             | Leaves          |
| 36  | 16183        | Delonix regia        | Fabaceae             | Leaves          |
| 37  | 16392        | Indigofera tinctoria | Fabaceae             | Stems           |
| 38  | 16390        | Indigofera tinctoria | Fabaceae             | Stems           |
| 39  | 160322       | Senna obtusifolia    | Fabaceae             | Fruits          |
| 40  | 155009       | Senna occidentalis   | Fabaceae             | Fruits          |
| 41  | 16245        | Senna alexandrina    | Fabaceae             | Leaves          |
| 42  | 16301        | Fumaria parviflora   | Fumariaceae          | Leaves, Stems   |
| 43  | 16043        | Marrubium vulgare    | Fumariaceae          | Sheets          |
| 44  | 15716        | Cassytha filiformis  | Lauraceae            | Stems           |
| 45  | 16082        | Abutilon figuranum   | Malvaceae            | Leaves          |
| 46  | 16080        | Ficus benghalensis   | Moraceae             | Leaves, Stems   |
| 47  | 15448        | Ficus palmata        | Moraceae             | Leaves          |
| 48  | 16085        | Psidium guajava      | Myrtaceae            | Leaves          |
| 49  | 16184        | Boerhavia diffusa    | Myrtaceae            | Leaves          |
| 50  | 16177        | Bougainvillea spectabilis| Sprengeriaceae     |
| 51  | 16185        | Argemone ochroleuca  | Papaveraceae         | Shoots          |
| 52  | 16186        | Rumex dentatus       | Papaveraceae         | Shoots          |
| 53  | 16173        | Citrus maxima        | Rutaceae             | Leaves          |
| 54  | 15787        | Dodonea angustifolia | Rutaceae             | Leaves          |
| 55  | 15604        | Datura noxia         | Rutaceae             | Leaves          |
| 56  | 16386        | Solanum surrattense  | Solanaceae           | Leaves          |
| 57  | 12788        | Chlodendrum inermi   | Verbenaceae          | Leaves, Stems   |
| 58  | 560          | Balanites aegyptiaca | Zygophyllaceae       | Bark            |
USA), supplemented with 10% heat-inactivated bovine serum (Gibco, MA, USA), 1× penicillin-streptomycin (Gibco, MA, USA) and 1× sodium pyruvate (GE Healthcare Life Sci., UT, USA) in an incubator at 37°C with 5% CO2 supply. Dimethyl sulphoxide (DMSO; Sigma, Germany) was used as standard PXR-mediated CYP3A4 inducer or positive control.

2.3. Plasmid DNA preparations

The nuclear receptor expression vector pCDG-hPXR and CYP3A4 firefly-luciferase reporter construct pGL3-CYP3A4-XREM were kind gifts from Dr. Ron Evans (The Salk Institute for Biological Studies, La Jolla, USA) and Dr. Richard Kim (Department of Physiology and Pharmacology, University of Western Ontario, London, Canada), respectively. The renilla-luciferase expression plasmid (pRL-TK; Promega, USA) served as internal control. All plasmid DNA were transformed into DH5α XL competent cells (Invitrogen, Canada), respectively. The renilla-luciferase expression plasmid DNA stocks were prepared (Qiagen Plasmid Maxi-prep Kit, Germany), quantified (Nanodrop 3300) and stored at −20°C.

2.4. Transient transfection

HepG2 cells were seeded in 24-well culture plates (Corning, USA) and incubated overnight to reach up to 60–70% confluency. Next day, cells were co-transfected with pGL3-CYP3A4-XREM (CYP, 400 ng), pCDG-hPXR (PXR, 400 ng) and pRL-TK (200 ng), using transfection reagent FuGENE6 (Promega, USA) per well. A mock transfection control (negative, without plasmid) was also included. For a 24-well plate (200 μl media/well), the amount of FuGENE6 to DNA per well (3:1) was optimized as per the FuGENE6 manual. After 24 h, the medium was removed and 200 μl/well of fresh medium containing DMSO (0.1%) or rifampicin (10 μM) or plant extracts (50 μg/ml) was added. The treated cells were further incubated for 24 h at 37°C. The cells were transfected in triplicate for all samples, including controls.

2.5. Luciferase reporter gene assay

After 24 h of treatment (48 h post-transfection), the reporter activities of firefly-luciferase and renilla-luciferase were measured with the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer’s manual. Briefly, reagents were brought to room temperature (RT), and reconstituted. Meanwhile, media were discarded and cells were carefully washed with 1× PBS (200 μl/well). The passive lysis buffer (1× PLB; 60 μl/well) was added and cells were allowed to lyse for 15 min at RT by gentle rocking (Heidolph DuoMax 1030, Heidolph instruments, GmBH, Germany). Total cell lysates were properly mixed and carefully harvested into pre-labeled 1.5 ml Eppendorf tubes. Lysates were quickly cleared at 10000 rpm for 30 sec (Eppendorf 5415D, USA) and placed in ice bath. The assay was instantly performed in round bottom high-clarity polypropylene tubes (5 ml; Falcon, USA) with 100 μl Luciferase Assay Reagent II or Stop & Glo Reagent and 10 μl lystate, using an illuminometer (Berthhold Lumat LB9507, Berthhold Technologies, USA). The assay was performed in triplicate for all samples and repeated. The firefly-luciferase signal was normalized to renilla-luciferase signal for each sample. To calculate fold-inductions, the ratios of all tested extracts were compared with normalized signals of the control. To determine the potency of the active extracts, concentrations <50 μg/ml were also tested. All tests were performed in triplicate and repeated twice. Data was analyzed and represented as bar graph (Excel 2010; Microsoft, OK, USA).

2.6. Statistical analysis

All experiments were performed in triplicate and data were presented as the mean ± standard error, and were analyzed by One Way ANOVA using GraphPad Prism 7.04. The statistical differences between the control (CYP + PXR only) and treatment groups were carried out using Dunnett’s Test (P value <0.05).

3. Results

3.1. PXR-dependent CYP3A4 induction by plant extracts

For PXR-dependent CYP3A4 induction, a 50 μg/ml concentration was used for all non-cytotoxic plants ethanol extracts. Of the 58 plants extracts screened, four showed induction of
PXR-mediated CYP3A4 expression. The comparative fold-induction of CYP3A4 by the plant extracts in relation to the untreated (DMSO) control was determined (Fig. 1). The induction activities of the tested plants were in the order, Dodonaea angustifolia (2.62 fold, \( P < 0.0001 \)), Euphorbia tirucalli (1.95 fold, \( P = 0.0004 \)), Alternanthera pungens (1.74 fold, \( P = 0.0035 \)), Ficus palmata (1.65 fold, \( P = 0.0097 \)); and the rifampicin mediated fold of induction was 1.42 (\( P = 0.1209 \)).

4. Discussion

The PXR-mediated CYP3A4 expression is markedly induced in cultured human hepatocytes in response to a variety of xenobiotics and drugs, including some bioactive plant products. In this study, we have therefore, screened ethanol extracts of 58 medicinal plants using HepG2 cell culture and dual-luciferase assay for their PXR-mediated CYP3A4 activation potential. All the non-toxic extracts were tested at the safe concentration 50 \( \mu \)g/ml as compared to a similar study where extracts at 100 \( \mu \)g/ml doses were used (Mooiman et al., 2013).

Dodonaea angustifolia (sand olive) occurs naturally in Arabia and southern Africa, including Australia and New Zealand. An important traditional medicine of Africa, its leaves decoction is used for fever, cough, earache, neuralgia and rheumatism (Duke, 1983). In this study, we have demonstrated the novel PXR-mediated CYP3A4 activation potential of Dodonaea angustifolia (Kunth) is a ruderal plant of roadsides, path verges and waste places. Though a native of South America, its leaves decoction is used as hypoglycemic, anti-tumour, anti-ulcer, anti-inflammatory, antidiabetic, lipid lowering and antifungal remedy, including nephro-hepatoprotective effect (Joshi et al., 2014). In this report, we have shown a novel PXR-mediated CYP3A4 inducing activity of Dodonaea angustifolia in HepG2 cells.

Alternanthera pungens (Kunth) is a ruderal plant of roadsides, path verges and waste places. Though a native of South America, it is also reported from other tropical countries including India (Jalakar and Daheya, 2017). Compared to its other species, Alternanthera pungens is poorly studied. In a very recent study, its crude extract is shown to have a wide spectrum antibacterial activity as well as good antioxidant potential (Jakhar and Dahiya, 2017). Here, we have demonstrated the novel PXR-mediated CYP3A4 activation potential of Alternanthera pungens in HepG2 cells.

Ficus palmata (Fegra or Wild Himalayan Fig) occurs in North West India, Afghanistan, Iran, Arabia and Africa (Joshi et al., 2014). It is used as hypoglycemic, anti-tumour, anti-ulcer, anti-diabetic, lipid lowering and antifungal remedy, including nephro-hepatoprotective effect (Joshi et al., 2014). In this report, we have shown a novel PXR-mediated CYP3A4 inducing activity of Ficus palmata in HepG2 cells.

5. Conclusion

Our screening of ethanol extracts of 58 medicinal plants using HepG2 cells and reporter gene assay, has demonstrated the novel PXR-mediated CYP3A4 gene induction potential of four plants. Of these, Dodonaea angustifolia was found to be the most promising CYP3A4 activator, followed by Euphorbia tirucalli, Alternanthera pungens, and Ficus palmata. Further phytochemical characterizations, including isolation of active principles are therefore, warranted.