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Commiphora Myrrha (Nees) Engl. Resin Extracts Induce Phase-I Cytochrome P450 2C8, 2C9, 2C19, and 3A4 Isoenzyme Expressions in Human Hepatocellular Carcinoma (HepG2) Cells

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Abstract

Background: *Commiphora myrrha* (Nees) Engl. (*C. myrrha*) resin is one of the oldest Middle Eastern herbal medicine used for treatment and prevention of numerous diseases. This resin is prepared in different methods and widely consumed among Saudi Arabian patients. Despite its popularity, no studies have been done on potential modulation effects of *C. myrrha* resin extracts on human cytochrome P450 (CYP) drug-metabolizing enzyme expression.

Methods: The *C. myrrha* extracts were prepared by two different methods of sonication and boiling resembling the most popular traditional preparations of maceration and decoction, respectively. Both extracts were fingerprinted using high-performance liquid chromatography equipped with ultra-violet detector (HPLC-UVD). The viability of HepG2 cells treated with aqueous extracts was determined using Promega CellTiter-Glo® assay in order to select the efficient and non-toxic resin extract concentrations for phase-I metabolic isoenzyme expression analysis. The isoenzyme gene and protein expression levels of CYP 2C8, 2C9, 2C19, and 3A4 were measured using reverse transcription-quantitative polymerase chain reaction and Western blot technologies, respectively.

Results: The HPLC-UVD fingerprinting revealed different chromatograms for *C. myrrha* extracts indicating possible differences in their modulation effects on CYP expression. Both aqueous extracts were toxic to HepG2 cells when tested at concentrations exceeding 150 µg/ml of dry crude extract. The CYP 2C8, 2C9, and 2C19 mRNA expression levels increased up to 4.0-fold after cell treatment with concentrations ranging from 1 to 30 µg/ml *C. myrrha* extracts, as compared with the untreated cells. However, the modulation of CYP3A4 mRNA expression levels was only significant at 30 µg/ml of crude extract exceeding the 2.0-fold cutoff. The up-regulation of CYP mRNA expression levels induced by *C. myrrha* extracts was confirmed at the CYP protein expression levels as well.

Conclusions: The *C. myrrha* aqueous extracts modulate CYP 2C8, 2C9, 2C19, and 3A4 gene expression at clinically-relevant concentrations regardless of preparation methods. Further *in vitro* and *in vivo* experiments are required for the establishment of herb-drug interaction profile for these traditional medicinal resin extracts.

Introduction

The Middle Eastern medicine of "Myrrah" is a botanical resin obtained from scratching the tree bark of *Commiphora myrrha* (Nees) Engl. (*C. myrrha*) (Synonym, *Commiphora molmol* (Engl.) Engl. ex Tschirch) and is worldwide known as "Mir'rah" (in Arabic, □□□□□), "Hira'bol" (in Hindi, □□□□□□□) in the Indian subcontinent, and "Mò'yào" (in simplified Chinese, □□) in China. This ancient natural medicine from the *Burseraceae* family is considered as an Ayurveda, Chinese, and Greco-Arab (Unani) traditional medicine and is commonly known nowadays as the "True Myrrha" or "Molmol Myrrha". The resin of *C. myrrha* is used for the treatment of arthritis, hyperlipidemia, obesity, pain, fractures, cancer, wounds, and bacterial/parasitic infections [1]. Traditionally, *C. myrrha* herbal medicine is prepared using different

extraction methods depending on desired clinical treatment. The resin is either boiled, macerated, prepared as an ointment, or used directly in raw resin form [2]. The highly desired therapeutic properties of *C. myrrha* resin might be due to its rich composition of tannins, flavonoids, alkaloids, glycosides, steroids, saponins, and terpenoids [3].

Numerous survey studies confirm that resin of *C. myrrha* is one of the most popular traditional medicines used by individuals in Saudi Arabia. In 2003, a survey reported that approximately 35 percent of 1408 healthy volunteers were consuming *C. myrrha* resins within the year [4]. Another study of 100 healthy volunteers has found that 59 percent at least used *C. myrrha* resins mainly as an antiseptic treatment [5]. Among Saudi Arabian patients, a study published that 45 percent among 51 diabetic patients use *C. myrrha* resins [6]. In another survey, approximately 7 percent of asthmatic out-patients used *C. myrrha* resins [7]. Furthermore, Jazieh et al. [8] reported that 90 percent of hospitalized cancer patients used complementary medicine including 10 percent consuming *C. myrrha* resins. Despite its popular use among patients, no clinical studies assessed the safety of consuming *C. myrrha* along with drugs primarily metabolized by hepatic cytochrome P450 (CYP) enzymes.

Cytochrome P450 enzymes are a group of phase-I metabolic detoxifying enzymes located mainly in the liver and enterocytes. More than 50 percent of overall elimination of pharmaceutical drugs are mediated by CYP enzymes [9]. The consumption of phytochemicals can interfere with the drug-metabolizing activity of such hepatic enzymes. Such interferences are referred to as food-drug and herb-drug interactions which can pose serious health consequences. *In vitro* cell models are used to predict such interactions using primary enterocytes or hepatocytes [10]. However, HepG2 cells are preferred from primary hepatocytes as the later has many disadvantages including special cell culture requirements and limited lifespan to name a few [11]. Common cells used for CYP-related studies include liver cancer cell lines such as HepG2, Hep3B, SK-Heb-1, Huh-7, and HepaRG [12, 13]. Non-hepatic cancerous cells, including intestinal Caco-2 cells, have been used but is known to have much lower basal CYP expression levels [14].

If consumed concomitantly, certain traditional medicines can modulate by up-regulating or down-regulating *in vivo* CYP gene expression levels resulting in less efficient drug therapy or over-dose drug toxicity [15]. This can be predicted *in vitro* by exposing the hepatoma cells to different concentrations of the traditional medicine crude extract. The effect can be measured at the transcriptional level using quantitative PCR (q-PCR) technique, and at the translational level using Western blot or mass spectrometry technologies. The rapid advances in molecular techniques have allowed the accurate and sensitive measurements of mRNA and protein levels in hepatoma cells. Generally, the hepatoma cells are known to have lower basal CYP mRNA and protein levels in comparison to the gold *in vitro* model option of freshly-harvested primary human hepatocytes [13].

In this present study, we determined the comparative modulatory effects of boiled and sonicated aqueous extracts of *C. myrrha* resins on CYP 2C8, 2C9, 2C19, and 3A4 isoenzyme expression at both mRNA and protein levels.

Materials And Methods

Source of *C. myrrha* resin

The resin was purchased from Boswellness (Colchester, VT) with product number 10018 and lot number CM000105. The herb was certified to be organic and free of pesticides, preservatives, and alterations as the resin was wildcrafted by experts in the Federal Republic of Somalia. The resins were stored in a dark and cool cabinet until processed. The remaining resin sample is kept in our department for future reference.

Source of HepG2 cells

Human Hepatocellular Carcinoma (HepG2) cells were kindly gifted by Dr. Ahmed Al-Qahtani, (King Faisal Specialist Hospital and Research Center; Riyadh, Saudi Arabia), originally purchased from the American Type Culture Collection (ATCC, Manassas, VA). The HepG2 cell line is derived from a fifteen-year-old Caucasian adolescent male patient diagnosed with hepatocellular carcinoma.

Chemicals and reagents

Rifampicin (USP grade, ≥97 percent) was obtained from UFC Biotechnology Fine Chemicals (Amherst, NY). Dulbecco's Modified Eagle Medium (DMEM) plus GlutaMax-1 (4.5g/l D-Glucose, 25 mM HEPES, Pyruvate), fetal bovine serum (FBS), TrypLE™ Express, and Dulbecco's phosphate-buffered saline (PBS) were provided from Gibco® (Waltham, MA). NP40 cell lysis buffer was purchased Thermo Fisher Invitrogen (Carlsbad, CA). Methanol (chromatography grade, ≥99 percent) was obtained from Honeywell Riedel-de Haen (Seelze, Germany). Purified carbon dioxide (CO_2) gas was provided by Saudi Industrial Gas (Dammam, Saudi Arabia). Ultrapure water was produced using a Millipore (Billerica, MA) system with a resistivity reading of 18.2 MΩ·cm at 25°C.

Preparation of *C. myrrha* aqueous extracts

The extracts were prepared as 100 percent water by sonication and 100 percent water by boiling. Prior extraction, resin of *C. myrrha* was fine powered using an electric grinder. For the sonicated extraction, approximately 500 mg of the fine powder were mixed with 10 ml of ultra-pure water and sonicated for 30 min at high-power mode using a Sonics (Newton, CT) Vibra-Cell Ultrasonic Liquid Processor Model GEX-130 probe-sonicator. For the boiling water, approximately 500 mg of resin were mixed with 10 ml of ultra-pure water and boiled until half of the volume was evaporated. The extracts were filtered using a Sartorius stedim biotech (Göttingen, Germany) quantitive ashless paper filter and dried in an incubator set at 50˚C for 3 to 4 days. The remaining dried pellets were weighted and reconstituted with 1 ml of ultra-

pure water by vortex until completely dissolved. The reconstituted extracts were stored at cool temperature in dark until used.

Fingerprinting of *C. myrrha* aqueous extracts by HPLC-UVD

The chromatograms of *C. myrrha* aqueous extracts were performed using an Agilent (Santa Clara, CA) 1260 infinity high-performance liquid chromatography (HPLC) equipped with an ultra-violet detector (UVD). The major components of *C. myrrha* were separated using a Phenomenex (Torrance, CA) C18 Kinetex column (250 × 4.6 mm, 5 µm). The multi-wave UVD was set at wavelengths ranging from 225 to 375 nm at 25 nm segments without reference. The gradient programming of methanol and ultra-pure water was as follows: separation-phase (5 to 100 percent methanol, 0 to 35 min), washing-phase (100 percent methanol, 35 to 40 min), and equilibrium-phase (100 to 5 percent methanol, 40 to 45 min). The injection volume was 10 µl at ambient temperature and the total run time was 45 min.

Determination of endotoxin level in *C. myrrha* aqueous extracts

The levels of endotoxins in the *C. myrrha* aqueous extracts were determined using the Thermo Scientific™ Pierce™ Chromogenic Endotoxin Quant kit (catalog number, A39553) as per the manufacturer's instructions.

Culture and treatment of HepG2 cells

The HepG2 cells were cultured in complete DMEM Glutamax medium containing 10 percent (*v/v*) heat-inactivated FBS, and 1 percent (*v/v*) of penicillin G (100 IU/ml) and of streptomycin (100 mg/ml) and were incubated at 37˚C in 5 percent CO_2 and 95 percent relative humidity. The media was changed 2 to 3 days and sub-cultured when the cell population density reached 70 to 80 percent confluency. Cells were seeded at an appropriate density according to each experimental design. For the cell viability experiment, concentrations ranging from 0.05 to 2000 µg dry extract weight per ml were used for each boiled and sonicated extract. For gene expression assessment, the HepG2 cells were treated with three different concentrations of 1, 15, and 30 µg/ml for each boiled and sonicated aqueous *C. myrrha* extracts. Different rifampicin concentrations ranging between 0.001 and 50 µM inducing CYP isoenzyme gene expression were used as positive controls.

Cytotoxicity of *C. myrrha* extracts on HepG2 cells

The percentage of cell viability was determined using Promega CellTiter-Glo® assay (Madison, WI) according to the manufacturer's instructions. Briefly, the HepG2 cells were seeded at a density of 4×10^3

cells per well. The untreated and treated cells were incubated for 48 h under cell culture conditions previously described. After incubation, the plate was left at room temperature for 30 min and 100 µl of Cell Titer-Glo® reagent were added for each well and mixed on a shaker for 2min. The plate was left at room temperature for 10 min to settle and the luminescence of each well was read by the Perkin Elmer (Waltham, MA) EnVision Multilabel Reader.

Extraction of mRNA from HepG2 cells

The untreated and treated HepG2 cells were washed with PBS and harvested using TrypLE™ Express solution. The cells were transferred to a 1.5 ml tube and centrifuged at 15,000 rpm for 5 min. The cell pellet was stored at -20°C. The whole RNA was extracted from the treated and untreated cell pellets using the Qiagen (Hilden, Germany) RNeasy Plus Mini kit (catalog number, 74106) as per the manufacturer's instructions.

Extraction of proteins from HepG2 cells

The HepG2 cells were washed with PBS and 80 µl of NP40 lysis buffer were added to the cell pellet and incubated on ice for 30 min with repeated vortexing at 10 min intervals. The cell lysate was transferred to 1.5 ml tube and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant containing the whole cell lysate rich in proteins was transferred to fresh 1.5 tube and kept at -20°C. The amount of proteins was measured using the Molecular Probes® Life Technologies (Eugene, OR) Qubit® Protein Assay kit (catalog number, Q33211) as per the manufacturer's instructions.

RT-qPCR for CYP Gene Expression assessment

Complementary DNAs (cDNAs) at 50.0 ng per well were first produced from the whole RNA extracts *via* reverse transcription using Thermo Fisher Scientific Applied Biosystems™ High Capacity cDNA Reverse Transcription kit. The quantitative real-time PCR was performed using a Thermo Fisher Scientific SYBR Green PCR Master Mix kit and performed on Applied Biosystems 7900 real-time PCR system. For each analysis, a negative control was prepared using all reagents except the cDNA template. The housekeeping gene (β-actin) was used as an internal control and cytochrome enzymes primers (Table 1) were at 10.0 pmol/µl concentration.

Western blot for CYP protein expression analysis

Protein samples (120 µg per well) were separated on 11 percent SDS–polyacrylamide gels and electro-blotted onto a Thermo Fisher polyvinylidene difluoride (PVDF) transfer membrane 0.45 mm (catalog number, 88518). Primary antibodies (Invitrogen) against mouse monoclonal anti-CYP3A4 antibody

(catalog number, MA5-17064), rabbit polyclonal anti-CYP2C19 antibody (catalog number, PA5-13669), mouse monoclonal anti-CYP2C9 antibody (catalog number, MA5-25748) were used as probes to stain the PVDF membranes. Secondary LI-COR (Lincolin, NE) IRDye® goat anti mouse (catalog number, 926-32210) and goat anti- rabbit antibodies (catalog number, 926-32211) were used for detection and measurement of targeted CYP expression. In all gels, a LI-COR Chameleon Due Pre-Stained Protein Ladder (catalog number, 928-60000) was included. The housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control and was detected by Abcam (Cambridge, UK) mouse monoclonal anti-GAPDH antibody (catalog number, ab9485).

Data Analysis

The cytotoxicity values were estimated based on the plotting of common log (log_{10}) values of dry extract weight of *C. myrrha* versus percentage inhibition of untreated control using the log-inhibition variable-slop (four-parameter) model in Prism GraphPad (San Diego, CA) software version 5.04. For q-PCR, the gene expression values were normalized to β-actin and considered to be positive for induction or suppression if exceeding the two-fold relative to untreated control (*i.e.* 0.5 or 2.0-fold cutoff) (USFDA, 2020). The results are expressed as mean ± standard deviation from a minimum of three independent cell culture experiments. For Western blot analysis, the protein expression level detected in treated cells was related to the basal protein expression level detected in untreated cells using ImageJ software (http://rsbweb.nih.gov/ij/index.html).

Results

Fingerprinting boiled and sonicated aqueous extracts of C. myrrha resins and their cytotoxic effects on HepG2 cells

In this study, we investigated whether the boiled and sonicated aqueous *C. myrrha* resin extracts could modulate the gene expression of CYP isoenzymes in HepG2 cells. We firstly performed the fingerprinting of the two extracts and noticed different chromatograms, indicating possible differences in their potential modulation effects on CYP isoenzyme gene expression (Fig. 1). Prior cell treatment, we determined the bacterial endotoxin levels in both *C. myrrha* aqueous extracts. Low endotoxin levels were detected in both boiled and sonicated extracts and corresponding to 1.47 and 2.09 Endotoxin Unit (EU)/ml, respectively. To determine the *C. myrrha* aqueous extracts concentrations resulting in HepG2 cytotoxicity, we assessed the viability of HepG2 cells at different concentrations of *C. myrrha* aqueous extracts. Compared with the high viability of untreated cells, cell viability decreased by 20 percent (IC_{20}) after HepG2 cell exposure to concentrations determined at 183.8 and 632.0 µg/ml for sonicated and boiled aqueous extracts, respectively (Fig. 2). Half-maximum inhibitory concentrations (IC_{50}) on HepG2 cell viability were determined at 467.9 and 1066.0 µg/ml of sonicated and boiled extracts, respectively (Fig. 2). Based on cytotoxic concentrations of both *C. myrrha* aqueous extracts, further biological investigation was

performed by treating HepG2 cells with non-toxic concentrations of each extract, tested at 1, 15, and 30 µg/ml.

Modulatory effects of boiled and sonicated aqueous extracts of C. myrrha resins on CYP gene and protein expression

Figure 3a displays the effects of *C. myrrha* resin aqueous extracts on CYP 2C8 gene expression in HepG2 cells. Both sonicated and boiled extracts increased mean gene expression ranging from 2.3 to 3.5-fold change. The boiled concentrations at 1 and 15 µg/ml shows mean values just below 3.0-fold and lowered at concentration 30 µg/ml to 2.3-fold. On the other hand, the sonicated extracts had similar mean values of 2.5-fold and spiked to 3.5-fold at 30 µg/ml. The positive inducer, rifampicin, increased the gene expression of CYP 2C8 up to 4.0-fold change. Figure 3b displays the most representative Western blot results which show increased CYP 2C8 protein expression in HepG2 cells treated with 1 and 15 µg/ml of boiled aqueous extracts and in HepG2 cells treated with 30 µg/ml of sonicated aqueous extracts.

Figure 4a displays the effect of *C. myrrha* aqueous extracts on CYP 2C9 gene expression levels in HepG2 cells. Both selected boiled and sonicated aqueous extracts stimulated the expression of CYP 2C9 gene at levels exceeding 2.0-fold in comparison to the basal expression level monitored in untreated cells. At the lowest concentration of boiled-derived aqueous extract (1 µg/ml), CYP 2C9 gene expression level trended to increase by 3.0-fold followed by a descending decrease at higher concentrations. Concerning sonicated aqueous extracts, only one concentration at 30 µg/ml stimulated CYP 2C9 gene expression with more than 2.0-fold increase. Figure 4b shows that extracts upregulated CYP 2C9 protein expression, which confirmed their stimulatory effect at the gene expression level observed in Fig. 4a. In comparison with the basal expression level detected in the untreated cells, an induction of CYP 2C9 protein expression level was observed in all treatments and especially in cells treated with boiled extracted tested at 1 and 15 µg/ml and sonicated extract tested at 30 µg/ml.

Figure 5a displays the effect of *C. myrrha* aqueous extracts on CYP 2C19 gene expression levels in HepG2 cells. Both boiled and sonicated extracts induced CYP 2C19 gene expression at levels exceeding 2.0-fold in comparison to untreated cells. The CYP 2C19 gene expression modulated by the boiled extract at 15 µg/ml reached 4.4-fold which is the highest in this study for cell treatment using *C. myrrha* extract. Figure 5b displays the effects by extracts on CYP 2C19 protein expression which agreed with the observed effects on gene expression level presented in Fig. 5a. In comparison with the basal protein expression level detected in untreated cells, the CYP 2C19 protein expression was induced in all treatments especially boiling extracts of 15 and 30 µg/ml and sonicated 30 µg/ml concentrations.

Figure 6a displays the effect of *C. myrrha* aqueous extracts on the CYP 3A4 expression levels monitored in HepG2 cells. Unlike the previous isoenzymes, only sonicated 30 µg/ml exceeded the 2.0-fold expression in comparison to untreated cells. The boiled extracts of 1 and 15 µg/ml were slightly below

the cutoff value set at two-fold. Figure 6b displays the effects of both extracts on CYP 3A4 protein expression levels, which roughly aligned with the variations of gene expression levels shown in Fig. 6a. In comparison with the basal expression level detected in untreated cells, the CYP 3A4 protein expression level was induced in all treatments used, and especially by both sonicated and boiled extracts tested at 30.0 µg/ml.

Discussion

We demonstrated in this study the induction of important CYP isoenzymes by *C. myrrha* resins. We chose these CYP isoenzymes as they present the main isoenzymes that are involved in the metabolism of more than 60 percent of CYP-mediated clinical pharmaceutical drugs including those with narrow therapeutic indices such as S-warfarin, S-mephenytoin, cyclosporine, sirolimus, and tacrolimus [16]. We performed the extractions using sonicated and boiled water methods resembling the local traditional preparation methods of maceration and decoction, respectively. Prior to cell treatment, we determined the levels of endotoxin contamination and the cytotoxicity of *C. myrrha* on HepG2 cells to select the concentrations for cell treatment and subsequent CYP gene and protein expression studies. It should be noted that we chose a 2-day treatment with replenishment of treatment after 24 hours to mimic the real-life therapy situation as this traditional medicine is typically consumed for several days [17].

The levels of endotoxins in aqueous extracts is measured prior use in experiments as endotoxins (20 EU/kg body weight) are shown to inhibit CYP isoenzymes levels in volunteers; thus, interfere with CYP expression levels [18]. However, the acceptable level of endotoxins in mammalian cell culture is not firmly established and varies among cell types and laboratories. In one study, Epstein et al. [19] exposed several common cell lines with several endotoxin concentrations up to 100 EU/ml and found minimal effects on cell proliferation, thus establishing < 5 EU/ml as a conservative acceptable level for endotoxin contamination in cell culture. Based on this conservative level, the *C. myrrha* extracts in this study are considered acceptable for cell culture considering that these extracts were further diluted twice, in preparation of extract concentration serial dilutions and final cell treatment dilution reaching an estimated maximum endotoxin contamination at 0.003 EU/ml at final cell treatment concentration for both *C. myrrh* aqueous extracts. Based on this final maximum endotoxin concentration, we found it unnecessary to include an endotoxin removal step prior HepG2 cell culture for the two aqueous extracts.

The cytotoxicity of *C. myrrha* extracts on HepG2 cells was performed to determine the range of concentrations to be used for drug-metabolizing CYP isoenzyme expression analysis. The concentrations of *C. myrrha* selected for HepG2 cell treatment are much below the measured IC_{20} by approximately 2.0 or 4.0-fold for sonicated and boiled *C. myrrha* aqueous extracts, respectively. The difference in their cytotoxicity reflects the HPLC-UVD-based fingerprinting, suggesting that the heat-sensitive chemicals could increase the cytotoxic potency of *C. myrrha* sonicated aqueous extract on HepG2 cells by approximately a factor of 2.0-fold in comparison to the boiled extract. Based on the relatively low

cytotoxic effects of *C. myrrha* resin extracts on HepG2 cell viability, we chose the range of treatment concentrations from 1 to 50 µg/ml, which is considered clinically relevant taken note that the typical dosage is 3–5 g per day for several days [17]. It is worth mentioning that we have also determined the IC_{50} value for a methanolic extract of *C. myrrha* resin on HepG2 cells at 29.9 ± 5.7 µg/ml which is much potent than the sonicated *C. myrrha* aqueous extract despite showing similar fingerprinting chromatograms at 275 nm absorbance (unpublished data). This supports the approach that research on safety use of natural health products should be performed using extraction methods resembling common traditional preparations prepared by consumers.

Based on the United States Food and Drug Administration Industry Guidelines for Drug Interaction Studies, rifampicin tested at 10 µM should be performed in CYP 2C and 3A-related induction studies and used as a positive control for CYP expression stimulation [20, 21]. In this study, we have tested increasing concentrations of rifampicin to demonstrate dose-dependency and to compare its induction effects with established CYP expression induction values reported in the industry guidelines. The induction of CYP gene expression in HepG2 cells by rifampicin in this study is comparable to those previously published in the guidelines. The typical induction of CYP gene expression for CYP 2C8, 2C9, 2C19, and 3A4 isoenzymes at 10 µM treatment concentration are approximately 2.0–4.0, 4.0, 20.0, and 4.0-fold, respectively [20, 21]. We used lower rifampicin concentrations and observed a similar trend with CYP 2C19 showing the highest induction expression reaching 13.6-fold in one experiment. Despite the low induction of CYP 2C9 expression observed at the transcriptional level, rifampicin clearly upregulated CYP 2C9 protein expression in HepG2 cells, when tested at all concentrations and especially at 0.001 and 0.01 µM concentrations, suggesting a positive regulatory system prompting CYP 2C9 expression at the translational level.

Previous studies involving *C. myrrha* resins and 2C/3A isoenzyme expression and activity have been reported although very limited. For CYP 3A, Al-Janoobi et al. [22] measured the bioavailability of cyclosporin A, a substrate for CYP 3A4, before and after an eight-day-treatment with *C. myrrha* resin (60 mg/kg) in rats. They found the bioavailability has significantly decreased by 45 percent, which suggests induction of *in vivo* CYP 3A isoenzyme activity, which agrees with our results despite the known differences between rat and human equivalent CYP isoenzymes [23, 24]. For CYP 2C9, Al Faraj [25] reported a clinical case for a patient that consumed *C. myrrha* resin along with warfarin and noticed significant decrease in anti-platelet activity which suggest induced CYP 2C9 expression as one possible reason. This study agrees with the aforementioned clinical report as the lowest concentration of *C. myrrha* resin extract (*i.e.* 1.0 µg/ml) is shown to strongly induce CYP 2C9 expression at both transcriptional and protein levels (Fig. 4) in this study. Despite our best effort, no previous studies involving the remaining CYP isoenzymes (*i.e.* CYP 2C8 and 2C19) and *C. myrrha* resins were found.

Despite the evidence of induction at both transcriptional and translational levels, the *in vivo* situation can be completely different. Several pharmacokinetic factors including gastric digestion of extract, gut absorption of phytochemicals, and first-pass effect in humans are not accounted in this present study. The role of *in vivo* chemical interactions among *C. myrrha* phytochemicals should also be investigated.

Worth mentioning is the natural variation of phytochemicals in *C. myrrha* resin which might be different based on harvest location, processing method, and storage condition. Thus, the modulation effect might be stronger from the lot used in this study. Nevertheless, this study cautions patients and health care providers for concomitant consumption of *C. myrrha* resin and drugs mainly metabolized by CYP 2C8, 2C9, 2C19, and 3A4 isoenzymes.

Conclusion

This is the first study to report the modulation effects of *C. myrrha* resin extracts on major phase-I metabolic isoenzyme (*i.e.* CYP 2C8, 2C9, 2C19, and 3A4) expressions at transcriptional and translational levels using a human liver carcinoma cell line. The results suggest highly-possible herb-drug interaction occurrences regardless of preparation method and could be used to predict clinical drug adverse-effects especially for CYP 2C isoenzyme drug substrates. Further *in vitro* and human studies are needed to investigate the effects of *C. myrrha* resin on phase-I and II isoenzymes to complete the safety profile for this popular traditional medicine.

Abbreviations

p>cDNA, complementary DNA; CO_2, carbon dioxide; CYP, cytochrome P450; DMEM, Dulbecco's Modified Eagle Medium; EU, endotoxin unit; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC-UVD, high-performance liquid chromatography and ultra-violet detector; mRNA, messenger ribonucleic acid; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PBS, phosphate-buffered saline

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request

Competing interests

The authors declare that there are no conflicts of interest.

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Authors' contributions

ZA conceived and conducted the study. GA, AN, AA, HA, MA, EH, and NA carried out the experiments, collected the data, analyzed the data and reviewed the manuscript. ZA and SMN interpreted the data and wrote the manuscript.

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Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

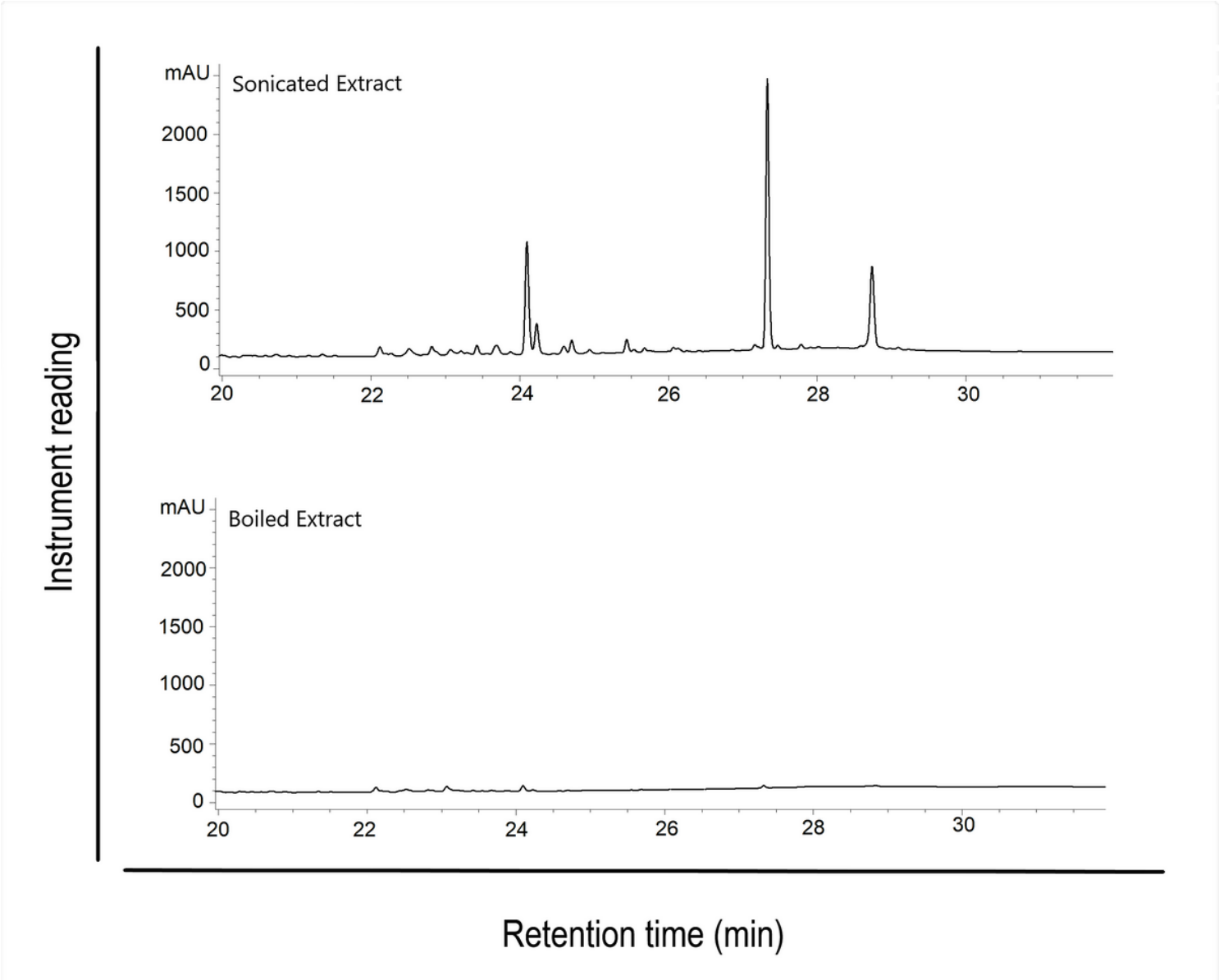
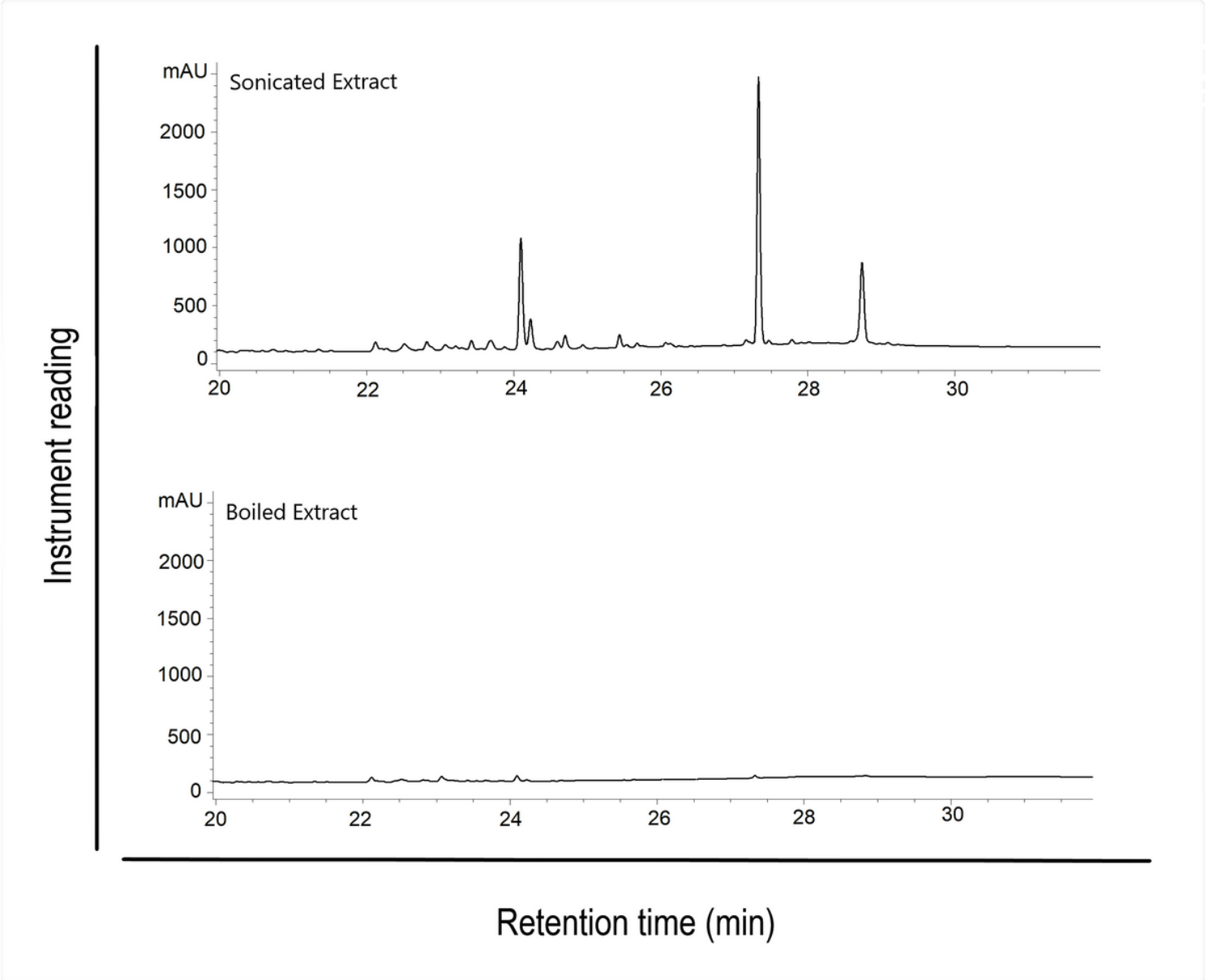


Figure 1

Comparative fingerprinting chromatograms for boiled and sonicated aqueous extracts of C. myrrha resins using HPLC with UVD detector set at 275 nm without reference.



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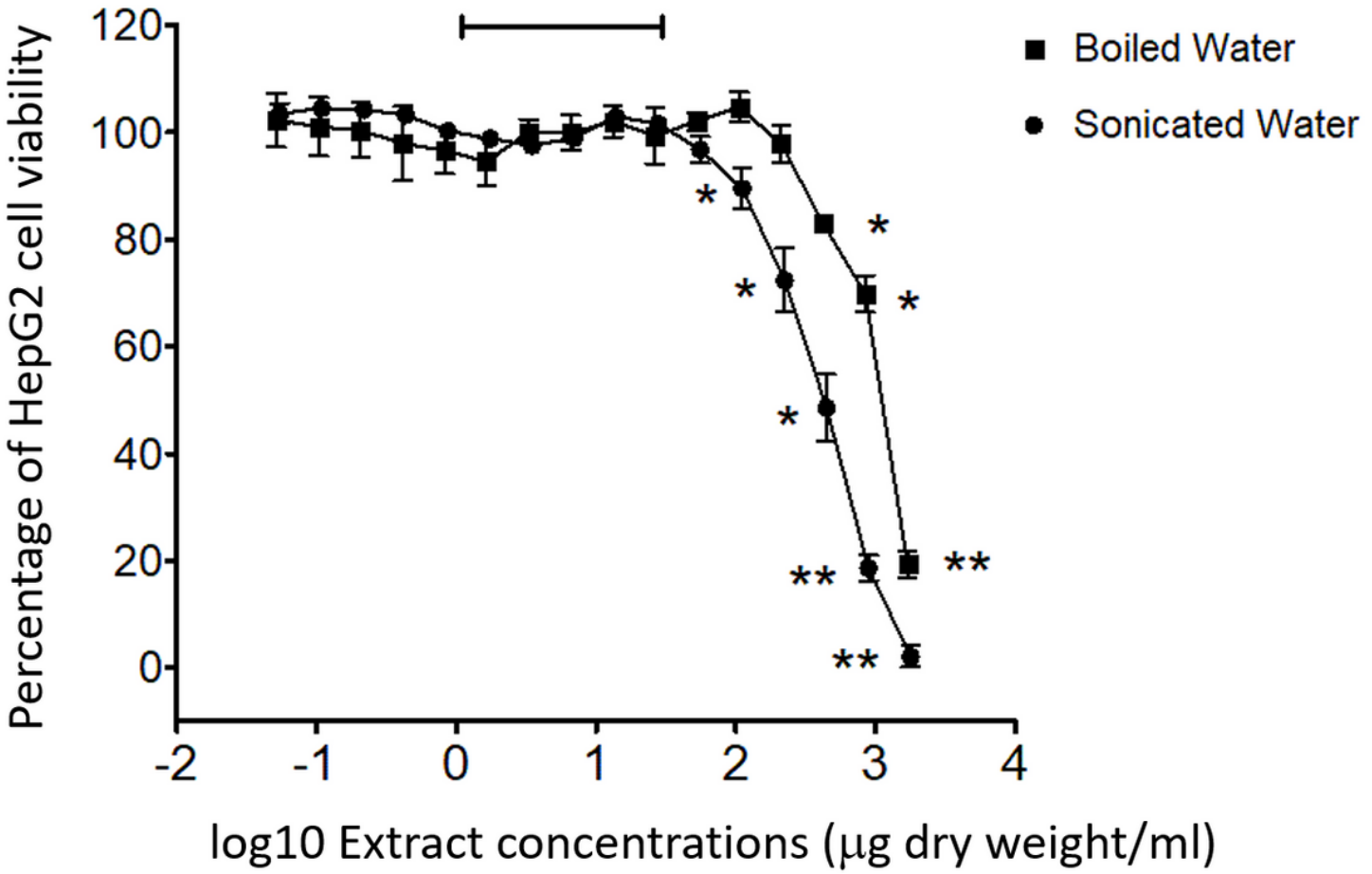
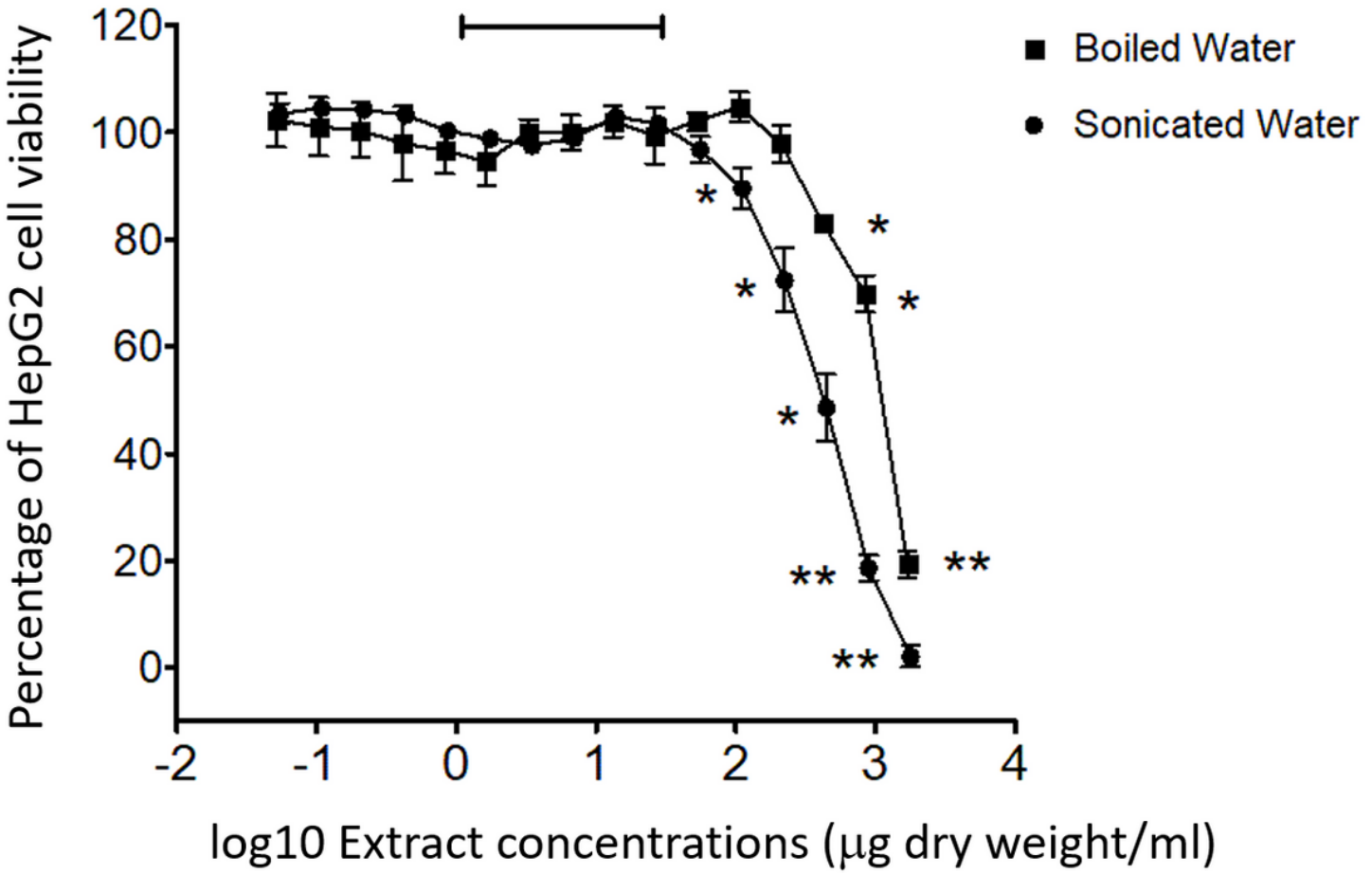


Figure 2

The viability of HepG2 cells at different concentrations of boiled and sonicated C. myrrha resin extracts. Each point represents the mean and standard deviation based on three individual experiments. The above bar indicates the range of C. myrrha concentrations used in downstream HepG2 cell treatments. (*) and (**) signify a statistically significant different ($p < 0.05$ and $p < 0.01$) in comparison with the control, 100%.



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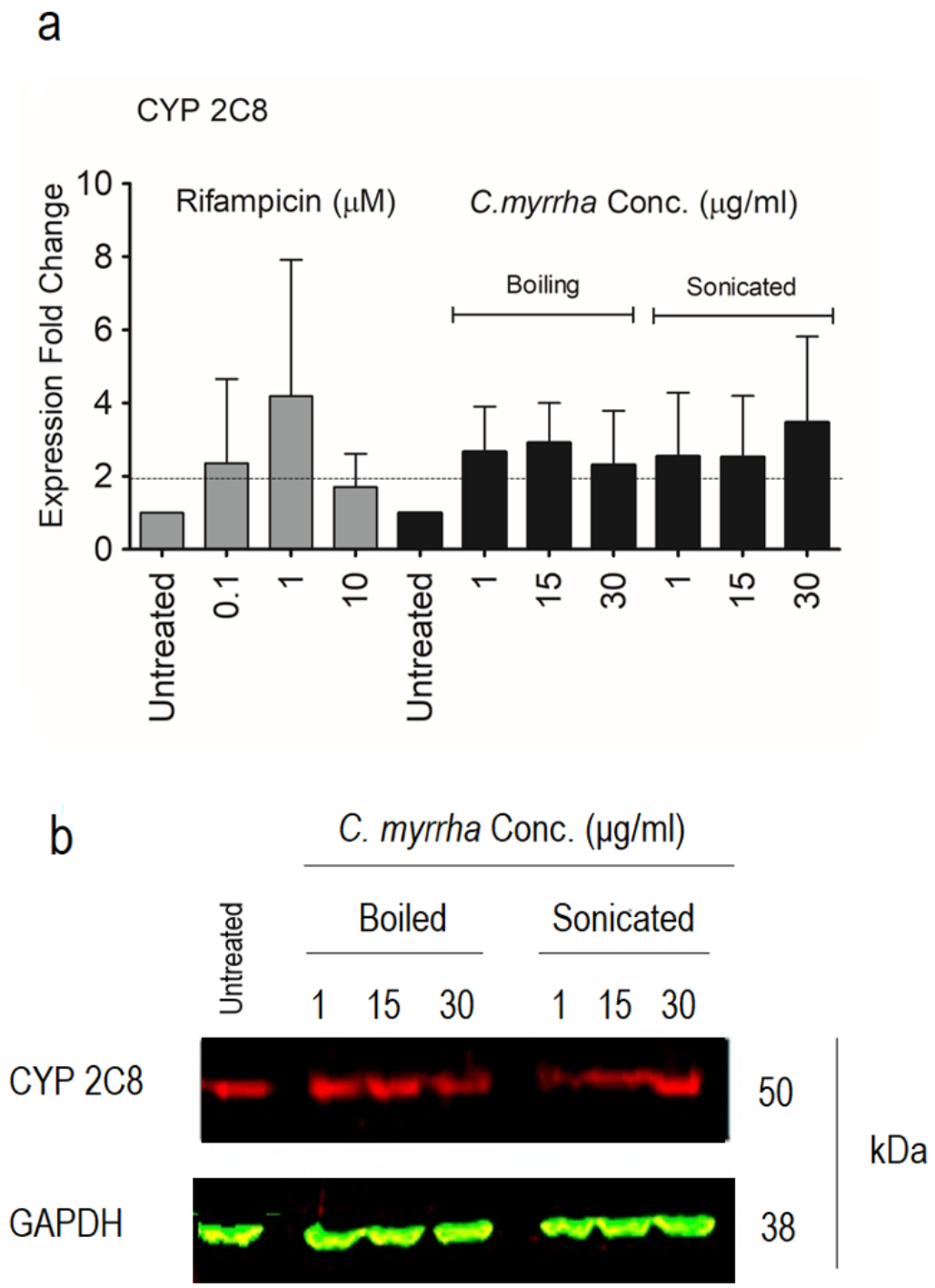
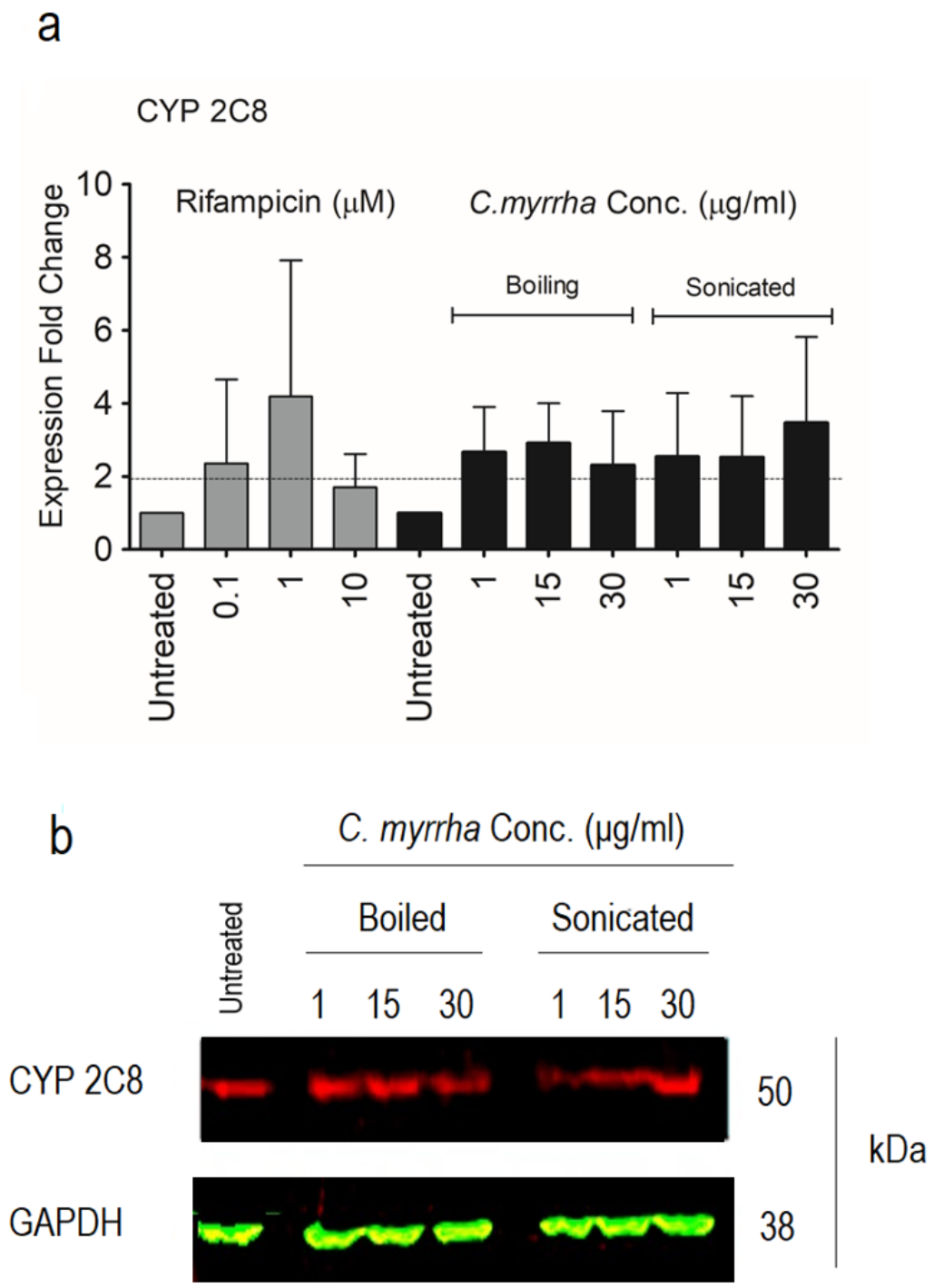


Figure 3

(a) The modulation effects of boiled and sonicated aqueous extracts of C. myrrha resins on gene expression of CYP 2C8 isoenzyme. Three independent cell treatments were performed for each C. myrrha resin extract concentration. Rifampicin tested at three different concentrations (0.1, 1, and 10 μM) was used as a positive control. Dotted line represents the up-regulation cutoff level of 2.0-fold. (b)

Representative Western blot showing the effects of different C. myrrha boiled and sonicated extract concentrations (1, 15, and 30 µg/ml) on the protein expression of CYP 2C8 isoenzyme in HepG2 cells.



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(a) The modulation effects of boiled and sonicated aqueous extracts of C. myrrha resins on gene expression of CYP 2C8 isoenzyme. Three independent cell treatments were performed for each C. myrrha resin extract concentration. Rifampicin tested at three different concentrations (0.1, 1, and 10 µM) was

used as a positive control. Dotted line represents the up-regulation cutoff level of 2.0-fold. (b) Representative Western blot showing the effects of different C. myrrha boiled and sonicated extract concentrations (1, 15, and 30 µg/ml) on the protein expression of CYP 2C8 isoenzyme in HepG2 cells.

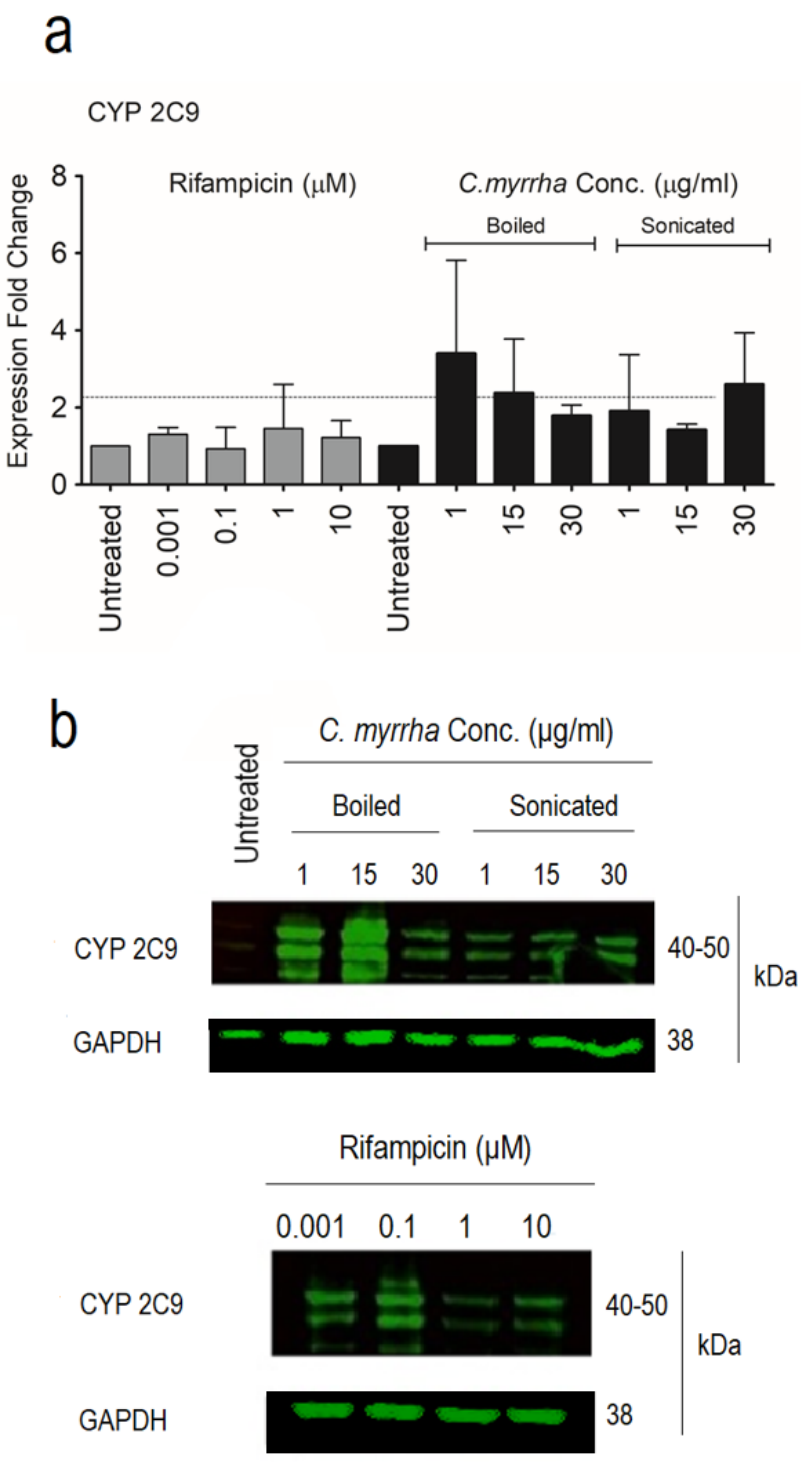

Figure 4

(a) The modulation effects of boiled and sonicated aqueous extracts of C. myrrha resins on gene expression of CYP 2C9 isoenzyme. Three independent cell treatments were performed for each C. myrrha

resin extract concentration. Dotted line represents the up-regulation cutoff level of 2.0-fold. (b) Representative Western blot showing the effects of different C. myrrha boiled and sonicated extract concentrations (1, 15, and 30 µg/ml) on the protein expression of CYP 2C9 isoenzyme in HepG2 cells. Rifampicin tested at four different concentrations (0.001, 0.01, 1, and 10 µM) was used as a positive control.

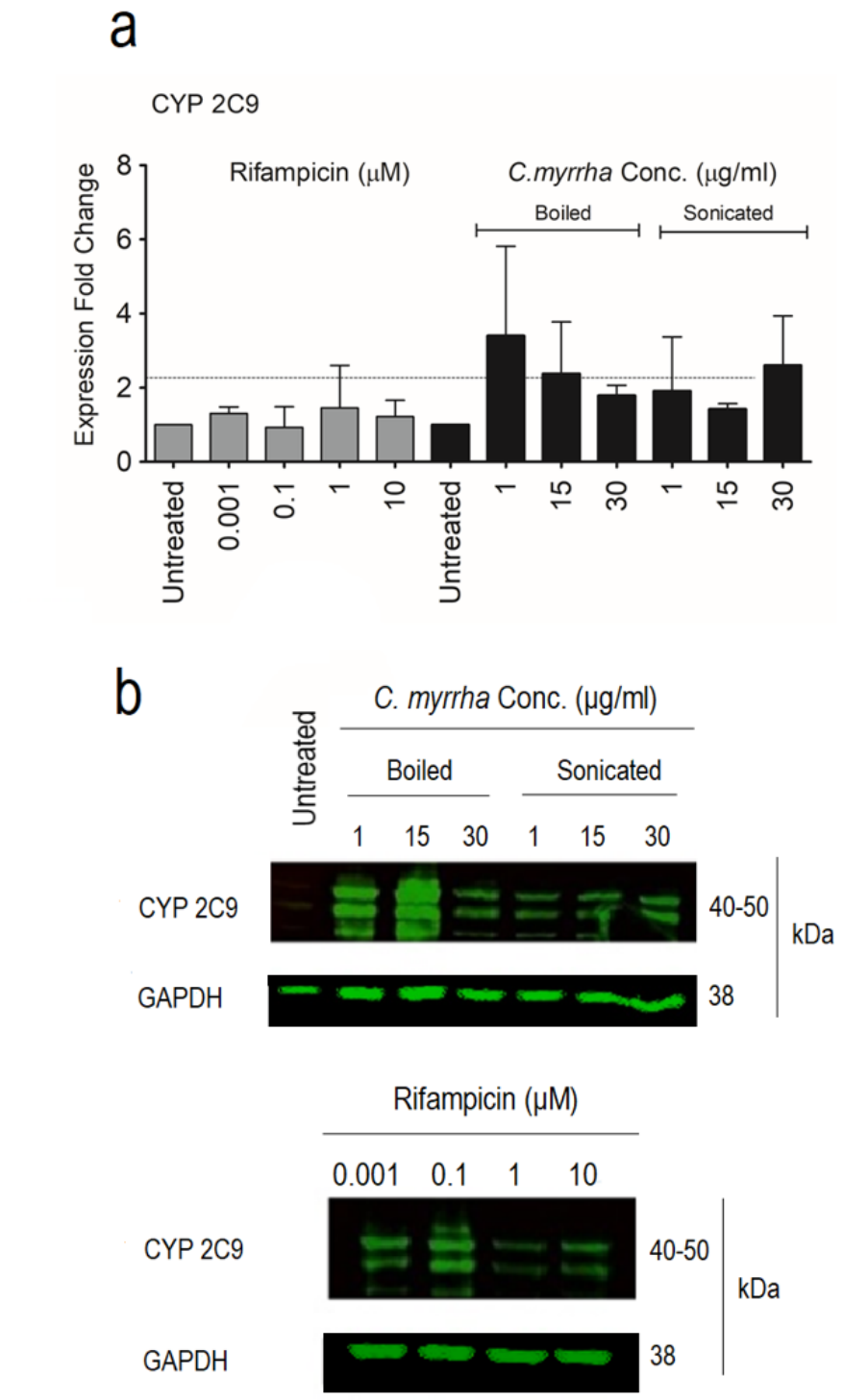

Figure 4

(a) The modulation effects of boiled and sonicated aqueous extracts of C. myrrha resins on gene expression of CYP 2C9 isoenzyme. Three independent cell treatments were performed for each C. myrrha resin extract concentration. Dotted line represents the up-regulation cutoff level of 2.0-fold. (b) Representative Western blot showing the effects of different C. myrrha boiled and sonicated extract concentrations (1, 15, and 30 µg/ml) on the protein expression of CYP 2C9 isoenzyme in HepG2 cells. Rifampicin tested at four different concentrations (0.001, 0.01, 1, and 10 µM) was used as a positive control.

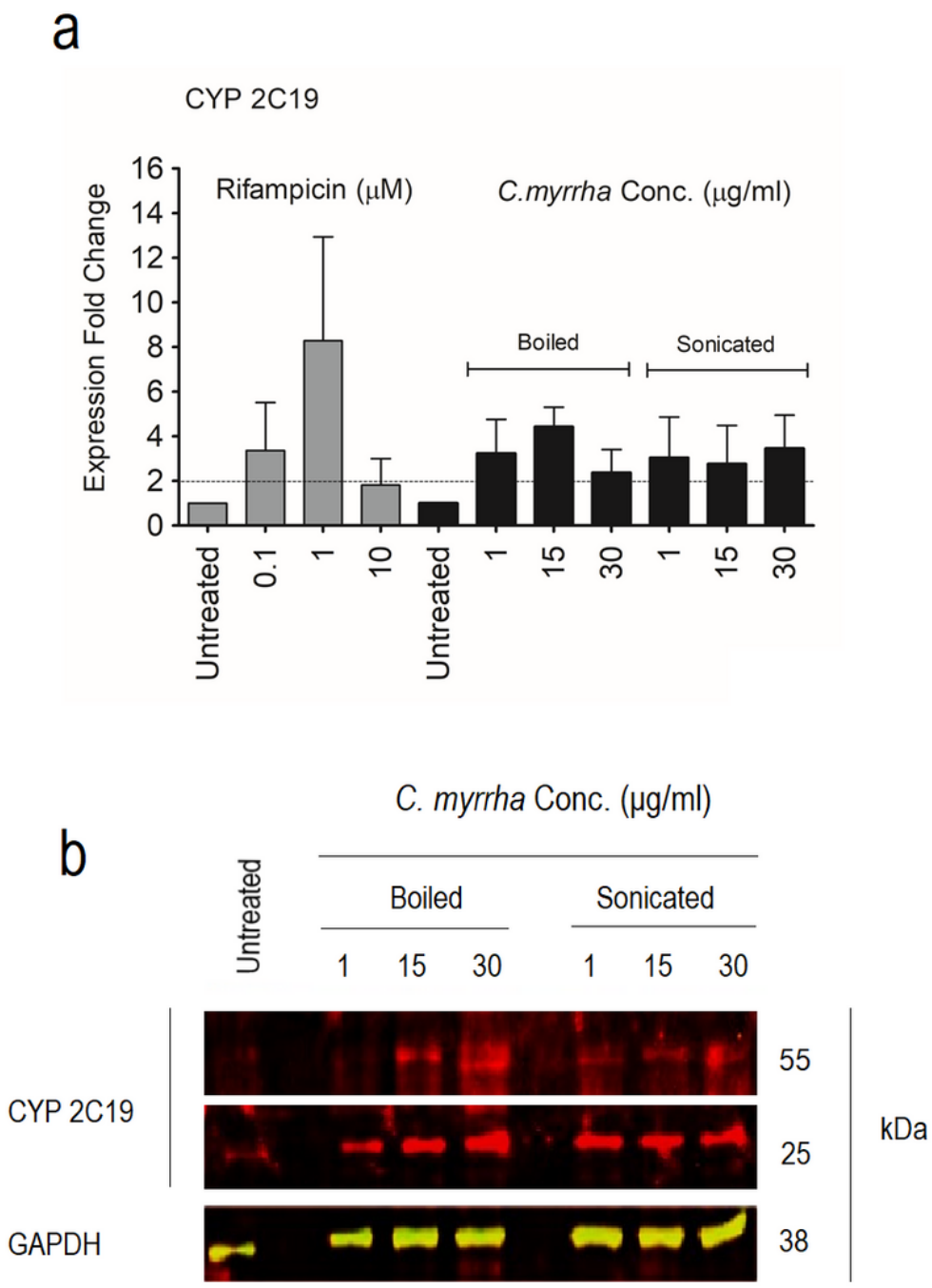

Figure 5

(a) The modulation effects of boiled and sonicated aqueous extracts of C. myrrha resins on gene expression of CYP 2C19 isoenzyme. Three independent cell treatments were performed for each C. myrrha resin extract concentration. Rifampicin tested at three different concentrations (0.1, 1, and 10 µM) was used as a positive control. Dotted line represents the up-regulation cutoff level of 2.0-fold. (b) Representative Western blot showing the effects of different C. myrrha boiled and sonicated resin extract concentrations (1, 15, and 30 µg/ml) on the protein expression of CYP 2C19 isoenzyme in HepG2 cells.

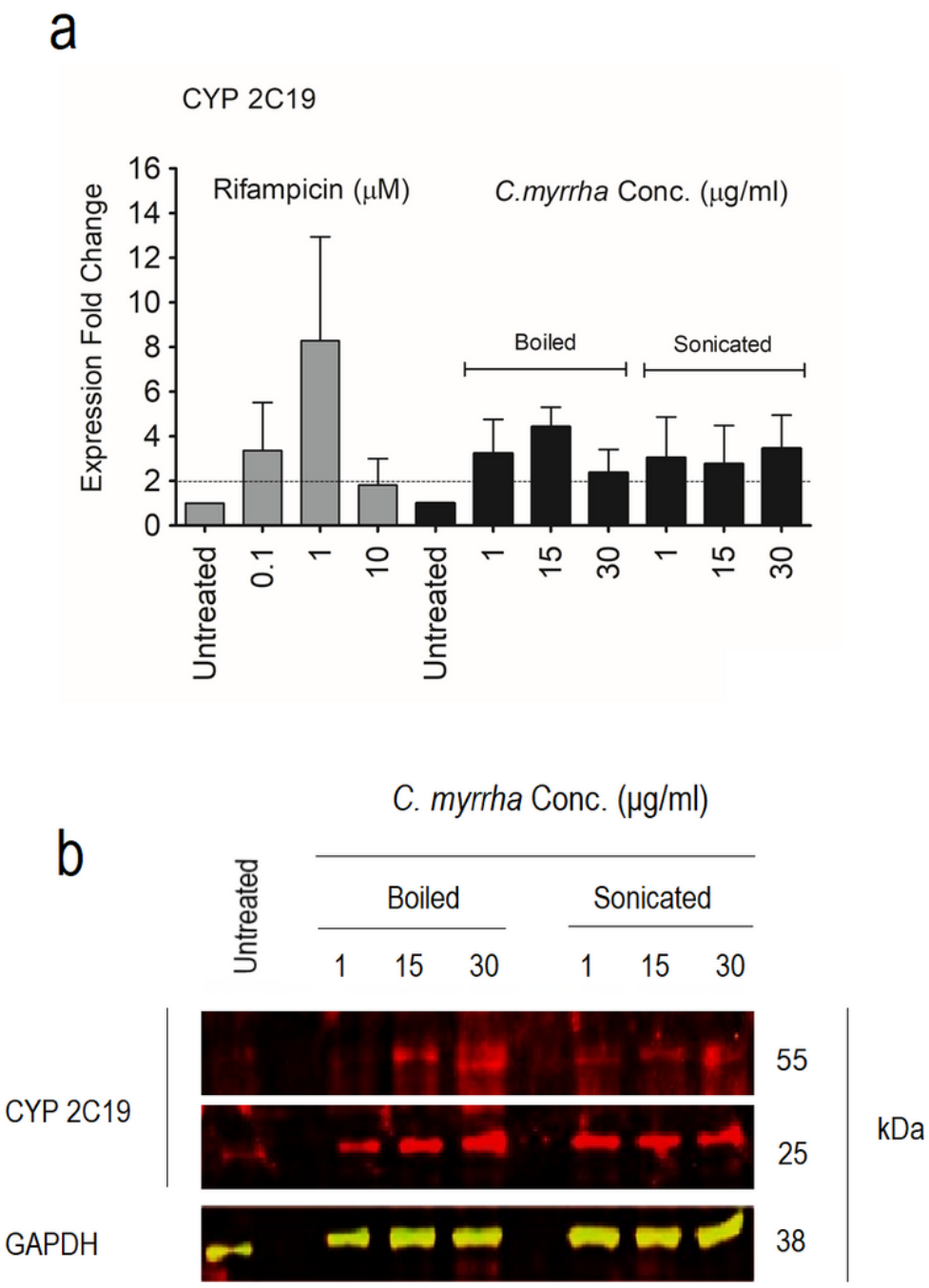

Figure 5

(a) The modulation effects of boiled and sonicated aqueous extracts of C. myrrha resins on gene expression of CYP 2C19 isoenzyme. Three independent cell treatments were performed for each C. myrrha resin extract concentration. Rifampicin tested at three different concentrations (0.1, 1, and 10 µM) was used as a positive control. Dotted line represents the up-regulation cutoff level of 2.0-fold. (b) Representative Western blot showing the effects of different C. myrrha boiled and sonicated resin extract concentrations (1, 15, and 30 µg/ml) on the protein expression of CYP 2C19 isoenzyme in HepG2 cells.

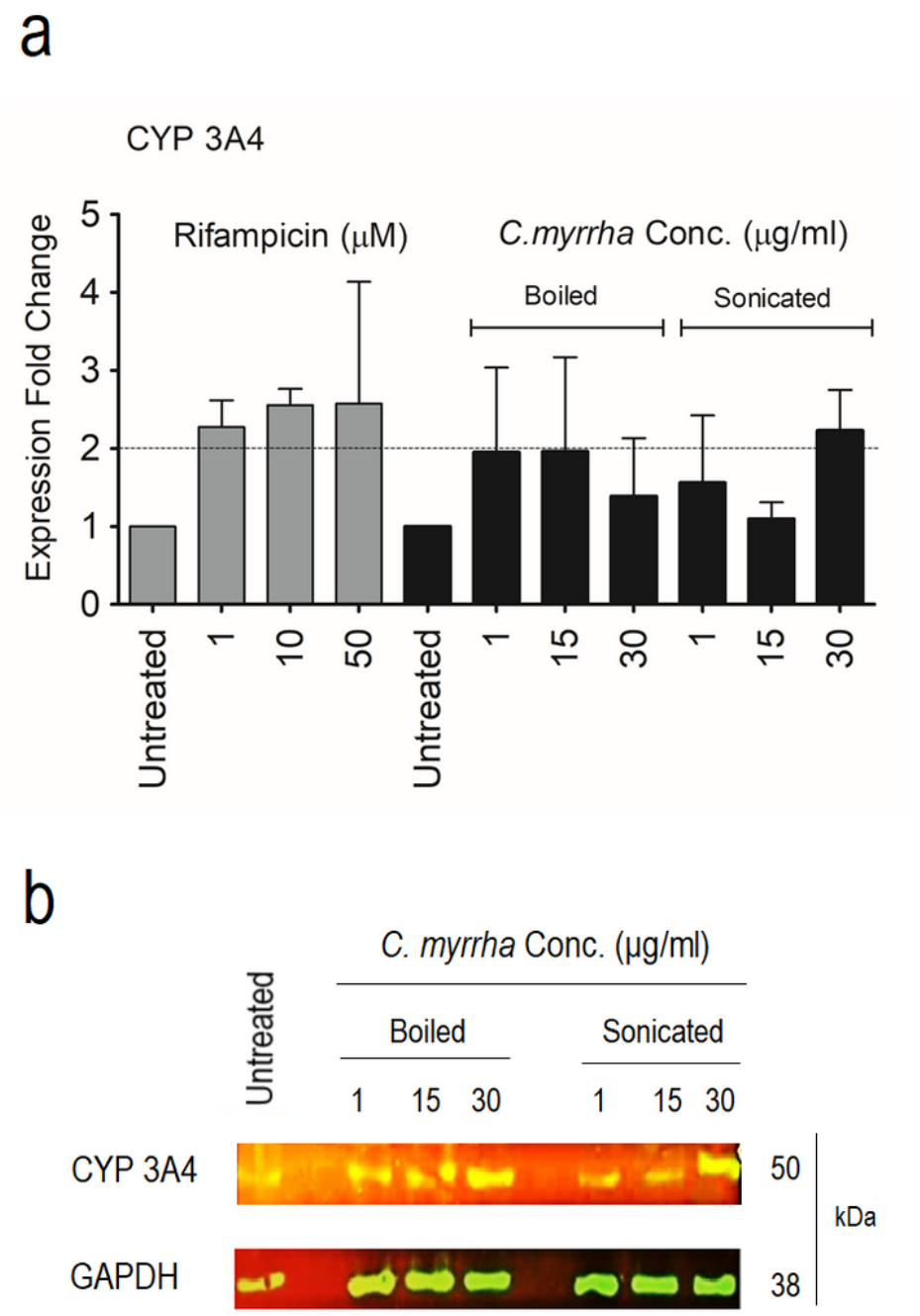

Figure 6

(a) The modulation effects of boiled and sonicated aqueous extracts of C. myrrha resins on gene expression of CYP 3A4 isoenzyme. Minimum of three independent cell treatments were performed for each C. myrrha resin extract concentration. Rifampicin tested at three different concentrations (1, 10, and 50 μM) was used as a positive control. Dotted line represents the up-regulation cutoff level of 2.0-fold. (b) Representative Western blot showing the effects of different C. myrrha boiled and sonicated resin extract concentrations (1, 15, and 30 μg/ml) on the protein expression of CYP 3A4 isoenzyme in HepG2 cells.

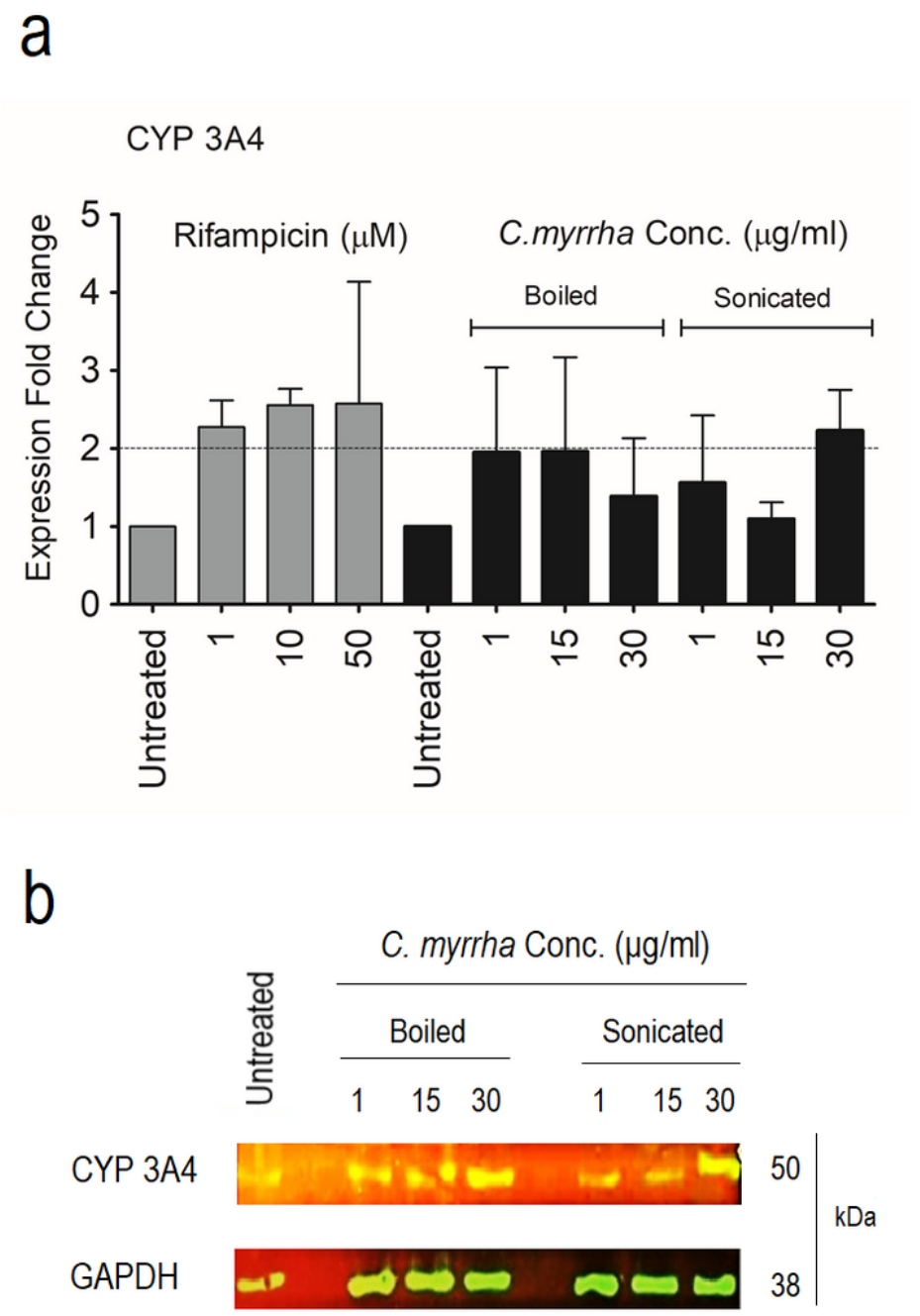

Figure 6

(a) The modulation effects of boiled and sonicated aqueous extracts of C. myrrha resins on gene expression of CYP 3A4 isoenzyme. Minimum of three independent cell treatments were performed for each C. myrrha resin extract concentration. Rifampicin tested at three different concentrations (1, 10, and 50 μM) was used as a positive control. Dotted line represents the up-regulation cutoff level of 2.0-fold. (b) Representative Western blot showing the effects of different C. myrrha boiled and sonicated resin extract concentrations (1, 15, and 30 μg/ml) on the protein expression of CYP 3A4 isoenzyme in HepG2 cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.xlsx
- Table1.xlsx