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Comparative Cytotoxicity of *Glycyrrhiza glabra* Roots from Different Geographical Origins Against Immortal Human Keratinocyte (HaCaT), Lung Adenocarcinoma (A549) and Liver Carcinoma (HepG2) Cells

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Glycyrrhiza glabra L. (Fabaceae), commonly known as ‘liquorice’, is a well known medicinal plant. Roots of this plant have long been used as a sweetening and flavouring agent in food and pharmaceutical products, and also as a traditional remedy for cough, upper and lower respiratory ailments, kidney stones, hepatitis C, skin disorder, cardiovascular diseases, diabetes, gastrointestinal ulcers and stomach ache. Previous pharmacological and clinical studies have revealed its antitussive, anti-inflammatory, antiviral, antimicrobial, antioxidant, immunomodulatory, hepato- and cardio-protective properties. While glycyrrhizin, a sweet-tasting triterpene saponin, is the principal bioactive compound, several bioactive flavonoids and isoflavonoids are also present in the roots of this plant. In the present study, the cytotoxicity of the methanol extracts of nine samples of the roots of *G. glabra*, collected from various geographical origins, was assessed against immortal human keratinocyte (HaCaT), lung adenocarcinoma (A549) and liver carcinoma (HepG2) cell lines using the *in vitro* 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) cell toxicity/viability assay. Considerable variations in levels of cytotoxicity were observed among various samples of *G. glabra*.

Keywords: *Glycyrrhiza glabra*; liquorice; Fabaceae; cytotoxicity; cancer; MTT

INTRODUCTION

Glycyrrhiza glabra (common name: liquorice; family: Fabaceae) is a commercially important medicinal herb, which is well known for its established nutritional and therapeutic values (Zadeh et al., 2013). It is now widely cultivated in several parts of the world, including Afghanistan, China, Dagestan, Iran, Italy, Pakistan, Syria, Turkey and Uzbekistan (Hiroaki and Hiroshi, 2009; Russo et al., 2014). Owing to its sweet taste, liquorice is used as a sweetening and flavouring agent in food, tobacco, confectionery and pharmaceutical products.

Traditionally, liquorice has been used for the treatment of cough, upper and lower respiratory ailments, kidney stones, hepatitis C, skin disorder, cardiovascular diseases, diabetes, gastrointestinal ulcers and stomach ache (Marjan and Hossein, 2008). It is also an important ingredient in medicinal oils for epilepsy, paralysis, rheumatism and haemorrhagic diseases. The applications of liquorice in the treatment of diarrhoea, fevers, fever with delirium and anuria have also been well documented (Marjan and Hossein, 2008; Vispute and Khopade, 2011; Zadeh et al., 2013). The extract is used in auto-immune conditions, and has therapeutic benefit in immunodeficiency in humans. It is also used as a tonic, particularly, for the spleen and the stomach (Vispute and Khopade, 2011).

Previous pharmacological and clinical studies revealed that liquorice root extracts possess various useful pharmacological properties, e.g., antitussive, anti-inflammatory, antiviral, antimicrobial, antioxidant, chemopreventive, immunomodulatory, hepatoprotective and cardioprotective actions (Kalaiarasi et al.,

2009; Asha et al., 2012; Rajandeeep et al., 2013; Astafeva and Sukhenko, 2014; Dirican and Turkez, 2014). To date, more than 400 secondary metabolites have been isolated from various species of the genus *Glycyrrhiza*, and about 300 of them belong to the class of flavonoids (Marjan and Hossein, 2008). Among these constituents, glycyrrhizin, a sweet-tasting triterpene saponin, is the primary bioactive compound accounting for up to 2% of the dry material weight depending on species and growing regions, with other flavonoids such as flavanones, flavanols, chalcones, isoflavans, isoflavenes, flavones, flavonols, isoflavones, isoflavanones, and arylcoumarins (Marianna et al., 1995; Zhang and Ye, 2009) also being present in the plant.

Glycyrrhizin, also known as glycyrrhizic acid, which is the major bioactive principle in *G. glabra*, has been shown to have numerous pharmacological effects including inhibition of viral replication on numerous RNA and DNA viruses, such as hepatitis A and C, herpes simplex, herpes zoster, HIV, varicella zoster and CMV (Hirabayashi et al., 1991; Lakshmi and Geetha, 2013; Li et al., 2014). Glycyrrhizin inhibits hepatic metabolism of aldosterone (Lakshmi and Geetha, 2011), and possesses mineralocorticoid and glucocorticoid activity (Zadeh et al., 2013). Several other secondary metabolites from *G. glabra* display hydrocortisone-like anti-inflammatory activity (Li et al., 2015). The anti-inflammatory activity is probably owing to inhibition of phospholipase A2, which is implicated to various inflammatory processes (Okimasu et al., 1983). Glycyrrhizic acid inhibits several factors of inflammatory process, e.g., cyclooxygenase activity, prostaglandin formation and to some extent, platelet aggregation (Akamatsu et al., 1991).

In our previous work, quantification of glycyrrhizin in the methanol extracts of nine samples of *G. glabra* from different geographical origins was carried out by the

semi-preparative reversed-phase HPLC-PDA method (Basar et al., 2014). Concentration levels of glycyrrhizin were between 0.177 to 0.688 % w/w of dry material in those samples. We now report on the comparative cytotoxicity studies of the methanol extracts of nine samples of *G. glabra* against three cell lines, immortal human keratinocyte (HaCaT), lung adenocarcinoma (A549) and liver carcinoma (HepG2), using the *in-vitro* 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) cell toxicity assay.

MATERIALS AND METHODS

Reagents and chemicals. Unless otherwise stated all chemicals were purchased from Sigma-Aldrich (Dorset, UK). Solvents were purchased from Fischer Scientific (Loughborough, UK). All cell culture reagents were purchased from Biosera (Nauaille, France).

Plant materials. Commercial or experimental samples of roots of *Glycyrrhiza glabra* L. were collected from different geographical origins, identified appropriately by the taxonomists in the source countries and stored under dry and cool condition (15°C) (Table 1). Further macroscopic and chromatographic (HPLC) identification of all samples was carried out by Prof S Sarker at the Medicinal Chemistry and Natural Products Lab in Liverpool John Moores University.

Extraction and preparation of plant samples. Ground roots (15 g each) were Soxhlet-extracted, sequentially, with *n*-hexane and methanol (MeOH), 400 mL each. Ten cycles were allowed for each extraction, and the temperature of the heating mantle for all extractions was kept constant at 60°C. The extracts were filtered and evaporated to dryness in a rotary evaporator at a temperature not exceeding 45°C.

Cell lines and cell cultures. Immortal human keratinocyte (HaCaT), lung adenocarcinoma (A549) and human liver carcinoma (HepG2) cells lines were treated with the different root extracts to determine their cytotoxic potential. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10%

foetal bovine serum (FBS) and antibiotics. All cells were cultured at 37°C in 95% air and 5% CO₂. For the MTT assay, cells were seeded into 96 well plates at density 1.2 x 10⁴ cells/well in a working volume of 200 µL/well and allowed to grow for 24 h before each experiment was commenced.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) cell toxicity/viability assay. The procedure was based on the method as described by Mosmann (1983). Briefly, a stock solution of each extract was prepared in 100% dimethylsulphoxide (DMSO) at the concentration of 1 mg/mL. Prior to use, the stock solution was further diluted in the medium to final working concentrations. The final concentration of DMSO was less than 0.01% in each well. Cells were treated with the extracts at various concentrations, e.g., 15.625 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL or 250 µg/mL for 24 h. A set of untreated control cells was included in each experiment, with preliminary experiments having shown the absence of toxicity at a concentration up to 0.1% DMSO (data not shown). Following incubation with the extracts for 24 h, 20 µL of MTT solution (5 mg/mL) was added to each well. After 3 h of incubation at 37°C, the medium was discarded and replaced with 100 µL of DMSO. The OD₅₇₀ was determined with a microplate reader (CLARIO Star Microplate reader, BMG Labtech, UK) and each experiment was repeated three times with five replicates in each experiment. The OD₅₇₀ obtained for the controls cells (no extract) was arbitrarily set at 100% and the OD₅₇₀ value for cells treated with extracts were expressed as a percentage of this control. The IC₅₀ values were calculated using the software GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

Statistical analysis. All experiments were carried out in triplicate. Data were expressed as means \pm standard error of the mean (SEM). The graph was plotted using non-linear regression with the use of GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Soxhlet extraction of nine different root samples of *G. glabra*, successively, with *n*-hexane and MeOH, yielded extracts of various amounts (Basar et al., 2014). *n*-Hexane was used mainly to get rid of any fatty materials or alkanes from the roots, and to get fat-free clean extracts from the extraction using MeOH.

The MeOH extracts of nine different root samples (Table 1) exhibited different levels of cytotoxicity against immortal human keratinocyte (HaCaT), lung adenocarcinoma (A549) and liver carcinoma (HepG2) cell lines in the *in vitro* 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) cell toxicity/viability assay (Figure 1). Generally, in terms of cytotoxicity, the HepG2 cells were the least susceptible to the test extracts (eight out of nine extracts) (Figure 1), while the HaCaT cells were the most susceptible to the extracts at the concentration of 250 µg/mL. Among the samples, P01 (obtained from Calabria, Italy) was the least cytotoxic against all cell lines, i.e., demonstrated the highest cell viability following exposure for 24 h (Figure 1), whereas another sample collected from the same geographical origin (P03) showed cytotoxicity against A549 and HaCaT cell lines ($IC_{50} = 189.1$ and 241.9 µg/mL, respectively). Also, P04 and P05, collected from Dagestan and Uzbekistan, respectively, did not show any considerable cytotoxicity (<50% cell death) at the highest test concentration (250 µg/mL). The IC_{50} values of the extracts that showed a cytotoxicity of >50% cell death are shown in Table 2. P14 (obtained from Afghanistan) was the most active extract and showed considerable cytotoxicity against all three cell lines, particularly against HaCaT cell line ($IC_{50} = 158.8$ µg/mL) (Table 2). However, a

second sample from Afghanistan, P12, was only cytotoxic to A549 cell line ($IC_{50} = 205.6 \mu\text{g/mL}$) (Table 2). Although three of the samples (P01, P03 and P22) were obtained from young plants, their cytotoxicity profiles were quite variable (Table 2). Interestingly, two of them (P01 and P03) were from the same geographical origin, Calabria, Italy (Table 1). In a recent study, considerable variations in the amounts of glycyrrhizin, which is one of the major compounds found in *G. glabra*, in P01 and P03, have been reported (Basar et al., 2014). Although both samples came from Calabria and thus, major regional variation is unlikely, the two samples were collected in different years. The observed differences in cytotoxicity may reflect more general variation in chemical profiles between the two samples which were collected in different years. This may explain the variation observed with the cytotoxicity profiles of the samples from Italy. Basar et al. (2014) also showed that samples P04 (from Dagestan) and P05 (from Uzbekistan) had the highest level of glycyrrhizin, but in the current study none of these samples were particularly cytotoxic (Figure 1; Table 2). This indicates that glycyrrhizin may not positively contribute to the cytotoxicity of *G. glabra* extract.

Current finding suggested that the age of *G. glabra* or its geographical origin might not be major determinants for its cytotoxicity, but differences in climatic conditions within a geographical area might contribute to the variations. However, several published reports suggested that the age of a plant might be a determinant factor for its chemical composition, and thus its bioactivity (Centre and Wright, 1991; Santos et al., 2012).

To the best of our knowledge, there is no previous report on the cytotoxicity of any methanol extract of the roots of *G. glabra* against immortal human keratinocyte (HaCaT), lung adenocarcinoma (A549) and liver carcinoma (HepG2) cell lines. Current

study, for the first time, has demonstrated certain level of cytotoxicity of the extracts of various samples of liquorice roots of different geographical origins, albeit not so strong, against three cell lines as mentioned above. However, Schmidt et al. (2013) evaluated the cytotoxicity of herbal extracts, *G. glabra* being one of those, used for treatment of prostatic disease on head and neck carcinoma cell lines and non-malignant primary mucosal cells, and Yo et al. (2009) showed *G. glabra* and its component licochalcone A (Figure 2), a chalcone, to induce autophagy in addition to apoptosis in human LNCaP prostate cancer cells.

In a recent study, isoliquiritigenin (Figure 2), another chalcone from *G. glabra*, has been shown to inhibit human breast cancer metastasis (Zheng et al., 2014). The inhibition was due to prevention of anoikis resistance, migration and invasion through down-regulation of cyclooxygenase (COX)-2 and cytochrome P450 (CYP) 4A signalling. Formononetin (Figure 2), an isoflavone also found in this plant, could inhibit cell proliferation and cell cycle progression, as well as induction of apoptosis in various cancer cell lines (Zhou et al., 2014). The effect of this compound on the migration and invasion of breast cancer cells MDA-MB-231 and 4T1 *in vitro* and *in vivo* was demonstrated by Zhou et al (2014). Another isoflavonoid, glabridin (Figure 2), from the roots of *G. glabra* has recently been shown to possess considerable effect on the metastasis of tumour cells, particularly, on the migration and invasion of human hepatocellular carcinoma (HHC) cells, and to effectively suppress the tumour formation in the hepatoma xenograft model *in vivo* (Hsieh et al., 2014). The apoptotic mechanism of 18 β -glycyrrhetic acid (or simply glycyrrhetic acid) (Figure 2), a triterpene acid from this plant, in non-small cell lung cancer cells (NSCLCCs) has been established (Sharma et al., 2012). This compound could considerably suppress the viability of the

non-small lung cancer cells, e.g., NCI-H460 and A549. It has also been shown that this compound could induce apoptosis via inhibition of protein kinase C α/β II and activate c-Jun *N*-terminal kinase (JNK) in NCI-H460 (Song et al., 2014). Glycyrrhetic acid also preferentially inhibits proliferation of human breast cancer cells, MCF-7, apparently through apoptosis (as evident from phosphatidyl serine externalization and DNA fragmentation), but does not affect immortalised normal mammary epithelial cell line (MCF-10A) (Sharma et al., 2012).

Considering the cytotoxicity observed in the current study, and on the basis of the previously published reports on antitumour/anticancer properties of *G. glabra* and its various constituents as outlined above, it is reasonable to state that *G. glabra* may have certain potential as an herbal anticancer remedy. However, the cytotoxic/anticancer potential of *G. glabra* can be considerably variable depending on the collection sources and time.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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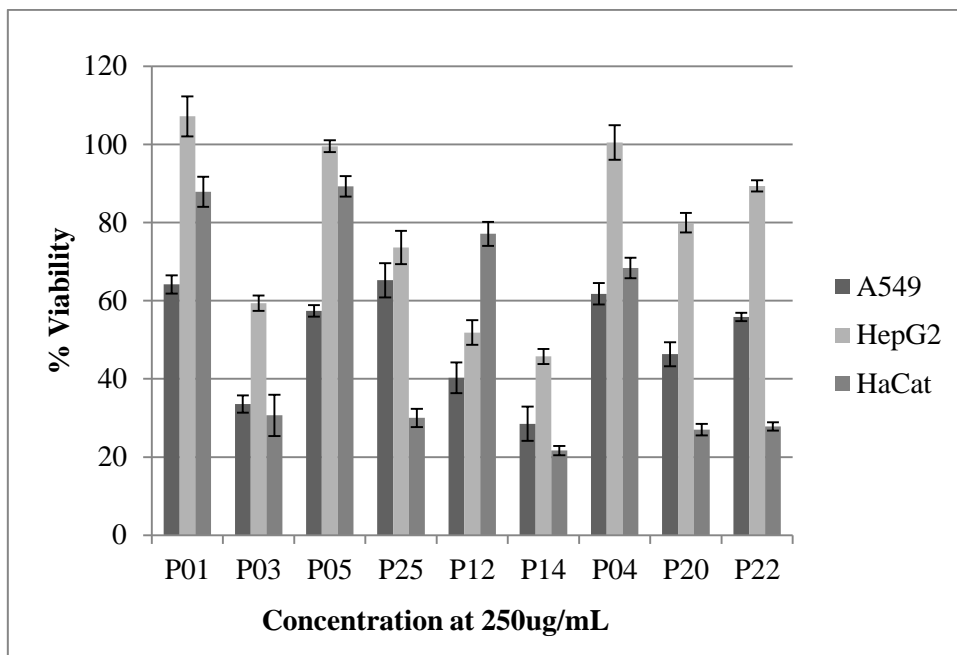


Figure 1. Differences in the level of cytotoxicity/viability of the methanol extracts of nine root samples of *G. glabra* (250 µg/mL) against A549, HepG2 and HaCaT cells

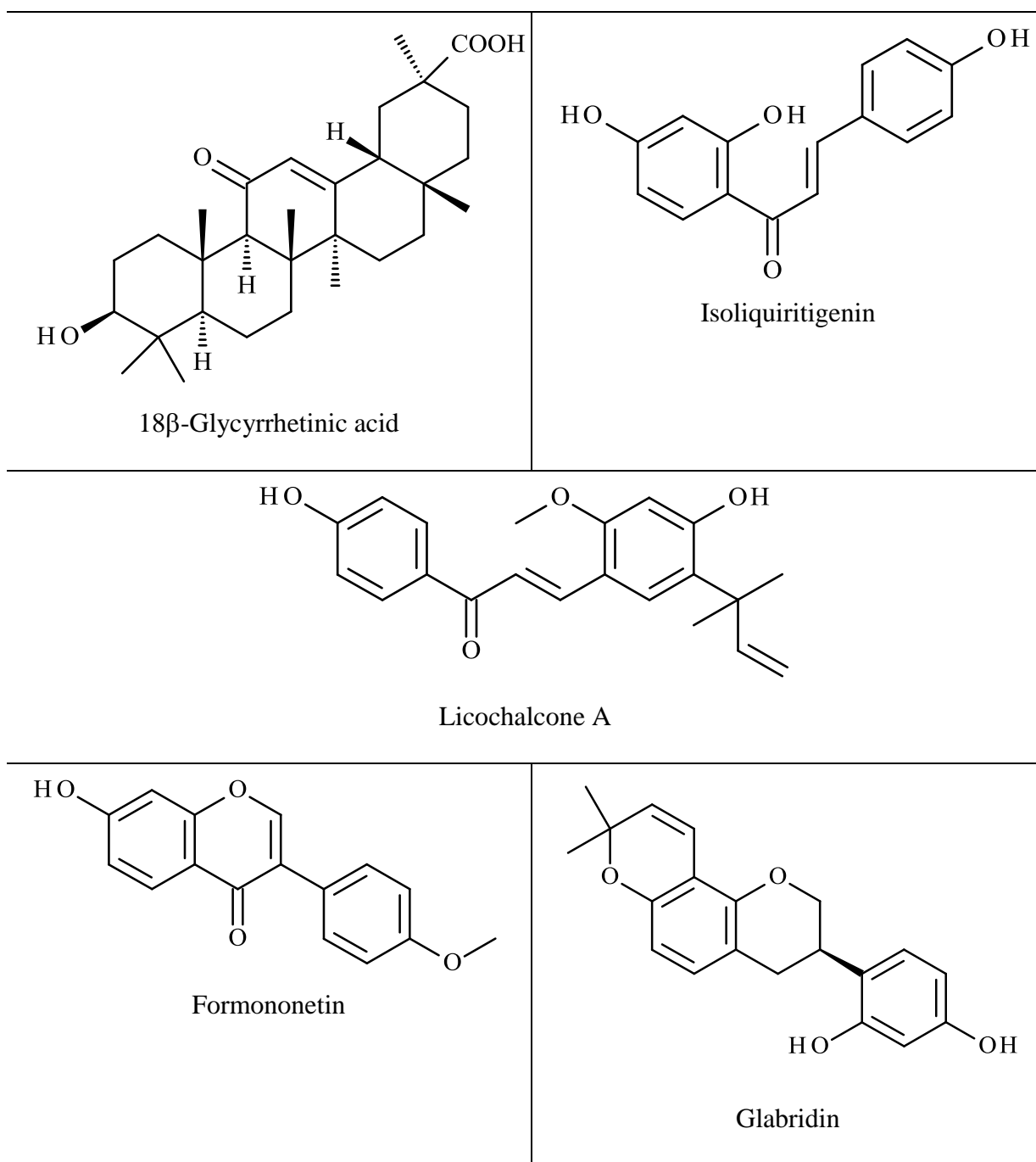


Figure 2. Examples of some potential anticancer compounds from *G. glabra*

Table 1. *Glycyrrhiza glabra* L. roots collected from different geographical origins

Sample codes	Geographical origins	Supplier details	Procurement time
P01	Consenza, Calabria, Italy	Experimental sample from young plant, provided by Romano Radice di Liquirizia (Liquorice exporters), Cosenza, Calabria, Italy (www.radicediliquirizia.com)	November 2009
P03			July 2010
P04	Dagestan	Commercial sample provided by Kamil Aliev, Mitrada, Mahachkala, Dagestan, Russia http://mitrada.en.ec21.com/	July 2010
P05	Uzbekistan	Experimental sample provided by Dr Habibjon Kushiev, Gulistan State University, Uzbekistan and Dr Akmal Karimov, IWMI Tashkent, Uzbekistan	July 2010
P12	Afghanistan	Commercial sample (unpeeled cut pieces) provided by Alfarid Corp., Karachi, Pakistan (www.alfarid.org)	August 2010
P14			August 2010
P20	Damascus area, Syria	Commercial chopped root sample from Philippe Robert Bittar, Liquorice exporters, Damascus (www.bonetwork.com/bflicorice)	September 2010
P22	Anatolia, Turkey	Experimental sample from young plant grown in Selçuk University experimental garden, Konya, Turkey. Provided by Dr Yuksel Kan.	September 2010
P25	Uzbekistan	Experimental sample provided by Dr Habibjon Kushiev, Gulistan State University, Uzbekistan and Dr Akmal Karimov, IWMI Tashkent, Uzbekistan	September 2011

Table 2. IC₅₀ values of extracts of *G. glabra* against three cell lines

Extract codes	IC ₅₀ values (µg/mL)		
	A549	HepG2	HaCaT
P01	NA	NA	NA
P03	189.1	NA	241.9
P04	NA	NA	NA
P05	NA	NA	NA
P12	205.6	NA	NA
P14	191.3	248.5	158.8
P20	238.9	NA	193.8
P22	NA	NA	196.6
P25	NA	NA	221.7

NA = < 50% cell death at the highest test concentration (250 µg/mL); IC₅₀ value could not be calculated