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Utilization of the ability to induce activation of the nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) to assess potential cancer chemopreventive activity of liquorice samples

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ABSTRACT:

Introduction – Nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) is a transcription factor that regulates expression of many detoxification enzymes. Nrf2-antioxidant responsive element (Nrf2-ARE) signalling pathway can be a target for cancer chemoprevention. *Glycyrrhiza glabra*, common name, 'liquorice', is used as a sweetening and flavouring agent, and traditionally, to treat various ailments, and implicated to chemoprevention. However, its chemopreventive property has not yet been scientifically substantiated.

Objective – To assess the ability of liquorice root samples to induce Nrf2 activation correlating to their potential chemopreventive property.

Methods – The ability of nine methanolic extracts of liquorice root samples, collected from various geographical origins, to induce Nrf2 activation was determined by the luciferase reporter assay using the ARE-reporter cell line, AREc32. The antioxidant properties were determined by the 2,2-diphenyl-1-picryhydrazyl (DPPH) and the ferric-reducing antioxidant power (FRAP) assays.

Results – All extracts exhibited free-radical-scavenging property (RC_{50} = 136.39-635.66 μg/mL). The reducing capacity of ferrous ion was 214.46-465.59 μM Fe(II)/g. Nrf2 activation indicated that all extracts induced expression of ARE-driven luciferase activity with a maximum induction of 2.3 fold relative to control. These activities varied for samples from one geographical location to another.

Conclusions – The present findings add to the existing knowledge of cancer chemoprevention by plant-derived extracts or purified phytochemicals, particularly the potential use of liquorice for this purpose.

Keywords: Cancer chemoprevention; nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2); 2,2-diphenyl-1-picryhydrazyl (DPPH); ferric-reducing antioxidant power (FRAP); *Glycyrrhiza glabra;* Fabaceae

Introduction

Nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) is a transcription factor that regulates expression of many detoxification or antioxidant enzymes. Nrf2-antioxidant responsive element (Nrf2-ARE) signalling pathway is considered as a potential target for cancer chemoprevention, because activation of this pathway leads to the expression of a battery of cytoprotective genes that may hold the key to suppressing, delaying or reversing the progression of cancers (Kwak and Kensler, 2010). Thus, assessment of the ability of any natural products to induce cellular protein based antioxidant defence systems through the activation of Nrf2 transcription factor as determined by the luciferase assay using the antioxidant response element (ARE) reporter cell line, AREc32, can reveal potential cancer chemopreventive property of those natural products. This strategy was adopted in the present work to assess cancer chemopreventive potential of liquorice (*Glycyrrhiza glabra*) samples collected from various geographical origins.

Glycyrrhiza glabra L. (family: Fabaceae), commonly known as 'liquorice' and widely cultivated in several parts of the world, including Afghanistan, China, Dagestan, Iran, Italy, Pakistan, Syria, Turkey and Uzbekistan, is a commercially valuable medicinal herb that is well known for its nutritional and medicinal properties for centuries (Hiroki and Hiroshi, 2009; Montoro *et al.*, 2011; Zadeh *et al.*, 2013; Russo *et al.*, 2014). Because of its sweet taste, liquorice is used as an important sweetening and flavouring agent in food, tobacco and confectionery products. Traditionally, it has been used for the treatment of various human ailments, *e.g.*, cough, upper and lower respiratory complications, 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 benefits of liquorice in the treatment of diarrhoea, fevers, fever with delirium and anuria have also been well established (Marjan and Hossein, 2008; Vispute and Khopade, 2011; Zadeh *et al.*, 2013). Extracts have been found to be useful in treating auto-immune conditions, and possess therapeutic benefit in immunodeficiency conditions. It is also used as a tonic, particularly, for the spleen and the stomach, and implicated to chemoprevention (Vispute and Khopade, 2011). However, its chemopreventive property has not yet been substantiated by any mechanistic scientific evidence.

Previous studies suggested that liquorice root extracts possess various useful pharmacological properties, including, anti-inflammatory, antimicrobial, antioxidant, antitussive, antiviral, cardioprotective, hepatoprotective and immunomodulatory actions (Kalaiarasi *et al.*, 2009; Asha *et al.*, 2012; Rajandeep *et al.*, 2013; Astafeva and Sukhenko, 2014; Dirican and Turkez, 2014). To date, more than 400 compounds have been isolated from various *Glycyrrhiza* species, and *ca.* 300 of these compounds are flavonoids (Marjan and Hossein, 2008). Among the compounds found in the genus *Glycyrrhiza* L, glycyrrhizin (also known as glycyrrhizic acid), a sweet-tasting triterpene saponin is the main active compound accounting for up to 2% of the dry material weight depending on species and growing regions, with other flavonoids such as arylcoumarins, chalcones, flavanones, flavanonols, flavones, flavonols, isoflavones, isoflavans, isoflavenes and isoflavanones (Marianna *et al.*, 1995; Zhang and Ye, 2009) also being present in the plant.

Glycyrrhizin has been shown to possess several pharmacological properties 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, 2011; Li *et al.*, 2014). It 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* showed hydrocortisone-like anti-inflammatory activity (Li *et al.*, 2014), which was probably owing to inhibition of phospholipase A2 generally associated with various inflammatory processes (Okimasu *et al.*, 1983). Glycyrrhizin also 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 extract. We now report on the comparative antioxidant capacity of these extracts as assessed by the 2,2-diphenyl-1-picryhydrazyl (DPPH) and the ferric reducing antioxidant power (FRAP) assays, and potential cancer chemopreventive property of the extracts through induction of cellular protein based antioxidant defence systems *via* the activation of the nuclear factor (erythroid-

derived 2)-like factor 2 (Nrf2) transcription factor as determined using the antioxidant response element (ARE) reporter cell line, AREc32 (Wang *et al.*, 2006).

Experimental

Reagents and chemicals

All chemicals were purchased from Sigma-Aldrich (Dorset, UK), unless otherwise stated. Solvents were purchased form Fischer Scientific (Loughborough, UK). All cell culture reagents were purchased from Biosera (Nauaille, France). Luciferase reporter assay system was purchased from Promega (Southampton, UK).

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 conditions (15^{o}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, UK.

Extraction and preparation of plant samples

Ground dried roots (15 g each) were Soxhlet-extracted, sequentially, with *n*-hexane and methanol (MeOH), 400 mL each (Basar *et al.*, 2014). Ten cycles were allowed for each extraction, and the temperature of the heating mantle for all extractions was kept constant at 60^{o}C. The extracts were filtered and evaporated to dryness in a rotary evaporator at a temperature not exceeding 45^{o}C.

DPPH radical scavenging capacity

The capacity of samples to scavenge DPPH$^{\bullet}$ was assessed as previously reported (Takao *et al.*, 1994) with suitable modifications (Kumarasamy *et al.*, 2007; Chima *et al.*, 2014). DPPH (4 mg) was dissolved in MeOH (50 mL) to obtain a concentration of 80 μg/mL. The MeOH extracts were reconstituted in MeOH to obtain the test concentration of 10 mg/mL. Dilutions were made to obtain concentrations of 1.0, 0.1, 0.01, 0.001, 0.0001 and 0.00001 mg/mL. Diluted solutions (1.00 mL each) were mixed with DPPH (1.00 mL) and allowed to

stand in a dark chamber for 30 min for any reaction to take place. The DPPH•-scavenging effect was evaluated by spectrophotometric at 517 nm against a blank. The values are reported as mean ±SD of three determinations.

The percentage scavenging effect was calculated as:

Scavenging rate, $RC_{50} = [(A_1 - A_2) / A_0] \times 100\%$

Where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample, A_2 is the absorbance of sample without DPPH radical.

The scavenging ability of the samples was expressed as RC_{50} value, which is the effective concentration at which 50% of DPPH radicals were scavenged. The RC_{50} values were calculated from the relationship curve of scavenging activities (%) versus concentrations of respective sample. The experiment was performed in triplicate, and the average absorption was noted for each concentration. The decrease in absorption induced by the test compounds was compared with the the positive controls, ascorbic acid (1 mg/mL in MeOH). The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated as follows (Scherer and Godoy, 2009).

AAI = [final concentration of DPPH (μg/mL) / RC_{50} (μg/mL)] x 100

Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was carried out according to the procedure previously reported by Benzie and Strain (1996). The FRAP reagent was prepared by mixing acetate buffer (25 mL, 300 mmol/L, pH 3.6), 10 mmol/L TPTZ solution (2.5 mL) in 40 mmol/L HCl and 20 mmol/L $FeCl_3$ solution (2.5 mL) in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh and warmed to 37°C in a water bath prior to use. 200 μL of the samples (1 mg/mL) was added to the FRAP reagent (1.8 mL). The reaction mixture was incubated in a water bath for 30 min at 37°C. Then, the absorbance of the samples was measured at 593 nm. The difference between absorbance of sample and the absorbance of blank was calculated and used to calculate the FRAP value. FRAP value was expressed in terms of μmol ferrous ion equivalent per gram of sample dry weight using ferrous sulphate standard curve ($y = 1.5596x + 0.1502$, $R^2 = 0.9891$). All measurements were calculated from the value obtained from triplicate assays.

Cell lines and cell culture

The stable human mammary ARE-reporter cell line (AREc32) was utilized to investigate the ability of extracts to activate the Nrf2 transcription factor. All cell-lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), penicillin-streptomycin antibiotics suspension and geneticin (G418; 0.8 mg/mL). All cells were cultured at 37°C in 95% air and 5% CO_2. The cells were seeded into 96 well plates at density 1.2 x 10^4 cells/well in a working volume of 200 μL/well and allowed to grow for 24 h before each experiment commenced. The ability of the extract to induce Nrf2 activity was determined by the luciferase reporter assay.

Luciferase reporter assay

AREc32 cells were treated with 100 μg/mL of the different root extracts of *G. glabra* for 24 h. Cells were then washed with phosphate buffered saline (PBS) and luciferase reporter lysis buffer (Promega, USA) was added to each well followed by a freeze-thaw cycle to achieve complete cell lysis. The cell lysate was then aspirated and dispensed into opaque 96-well plates. Luciferase reporter substrate was then added to each well and immediately the enzymatic activity was measured using a plate reader (ClarioStar). Levels of luciferase activity expressed by AREc32 cells following treatment with extracts was compared to the basal level of luciferase activity in controls (no treatment) and presented as a fold increase (relative to controls). Each experiment was repeated at least three times with five replicates in each repeat.

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means ± standard deviation (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

Antioxidant activities

Two different experimental approaches were employed for the determination of antioxidant activities. The DPPH assay is based on the capacity of biological reagents to scavenge the DPPH radical and is widely used in natural antioxidant studies because of its simplicity and sensitivity, whilst the FRAP assay has been extensively used to evaluate total antioxidant potential of plant extracts, and it assesses the ability of any test samples to reduce the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to ferrous tripyridyltriazine (Fe(II)-TPTZ) at a low pH (Benzie and Strain, 1996). In this study, methanol extracts of nine different samples of the roots of *G. glabra* collected from different geographical regions (Table 1) (Basar *et al.*, 2014) were tested to compare their antioxidant activity.

In the DPPH assay (Chima *et al.*, 2014), sample P25 (collected from Uzbekistan) had the highest capability to scavenge DPPH free-radical with a RC_{50} value of 136.39 μg/mL (Table 2). Samples collected from the same cultivation region (Afghanistan) displayed significant differences in the DPPH radical scavenging activity as shown by the samples P12 (RC_{50} 336.70 μg/mL) and P14 (IC_{50} = 712.46 μg/mL). The Antioxidant Activity Index (AAI) of all extracts was below 0.5, suggesting low antioxidant activity (Scherer and Godoy, 2009). Although the DPPH radical-scavenging abilities of the extracts were significantly lower than those of ascorbic acid, it was evident that the extracts did show some proton-donating ability and could serve as free-radical inhibitors or scavengers, acting possibly as primary antioxidants.

The ferric reducing antioxidant power (in the FRAP assay) of the extracts (1 mg/mL) was in the range of 214.46-465.59 μmol Fe (II)/g (Table 3). The standard curve was generated in the range of 100 to 1000 μM of ferrous sulphate and the results were expressed as μmol ferrous ion equivalent per gram of sample dry weight (y = 1.5596x + 0.1502, r2 = 0.9891). All samples showed approximately lower ferric reducing capacity compared to the standard reference ascorbic acid (889.63 ± 2.2 μmol Fe (II)/g) (Table 3). Sample P12 (Afghanistan) exhibited higher capacity in reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) than to scavenging free-radicals with FRAP value 465.59 ± 3.2 μmol Fe (II)/g), whereas a commercial sample, P04 (Dagestan) had the lowest FRAP value 214.46 ± 1.1 μmol Fe (II)/g). In general, samples collected from Afghanistan (P12), Uzbekistan (P25) and

Syria (P20) demonstrated significant radical-scavenging activity in the DPPH and the FRAP assays. Previous phytochemical studies reported the presence of phenolic compounds (*e.g.*, flavonoids), which are likely to be responsible for the antioxidant activity of *G. glabra* (Li *et al.*, 2000; Kinoshita *et al.*, 2005; Li *et al.*, 2005; Sara-Franceschelli *et al.*, 2011; Fu *et al.*, 2013; Dong et al., 2014). The chemical structure and substitution pattern of hydroxyl groups of flavonoids dictate their antioxidant activity (Bors *et al.*, 1990). In addition, quantitative differences of phenolic compounds in the extracts derived from various sources of *G. glabra* (Montoro *et al.*, 2011), secondary metabolite profiles are subject to considerable variability not only according to geographic area, but also in relation to stage of plant maturity, genotype, environmental conditions, harvesting, processing and also diversity between populations (Douglas *et al.*, 2004; Duffy *et al.*, 2009; Zhang *et al.*, 2011; Yu *et al.*, 2015). The changes in the composition of the plant material affect its therapeutic value as well as the pharmacological activity.

Assessment of induction of activation of Nrf2activity, and the luciferase assay

Nrf2 is a bZIP protein encoded by the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) gene, which contains conserved JUN and FOS regions that form the activator protein-1 (AP-1) transcription factor for rendering various cellular processes linked to cell differentiation, proliferation and apoptosis (Ameyar *et al.*, 2003; Lee and Johnson, 2004). Extensive research into the process of carcinogenesis has revealed the Nrf2/ARE signalling pathway as a potential target for cancer chemoprevention (Copple *et al.*, 2008; Petri *et al.*, 2012; Kou *et al.*, 2013; Yang et al., 2015). Activation of this pathway leads to the expression of a battery of cytoprotective genes that may hold the key to suppressing, delaying or reversing the progression of neoplastic diseases. Transcriptional activation of protective genes is mediated by a cis-acting element called the antioxidant responsive element (ARE), where the transcription factor Nrf2 (NF-E2-related factor 2) binds to. Activation of this pathway protects cells from oxidative stress-induced cell death. It is already known that many phytochemicals can act as Nrf2 inducers (Kou et al., 2013) and prominent examples are epigallocatechin gallate from green tea, resveratrol from grapes and sulforaphane produced by cruciferous vegetables. To complete the investigation into the antioxidant and chemopreventive potential of the extracts of *G. glabra*, the AREc32 cell line was utilized to identify extracts capable of activating the transcription factor Nrf2, which has been reported

to initiate the expression of up to 200 genes, many of which are involved in cellular defense against oxidative or toxic insult including heme-oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferases (GSTs) (Wang *et al.*, 2006; Copple *et al.*, 2008).

Prior to carrying out the luciferase assay, cellular toxicity assays (MTT) were conducted on the AREc32 cells using the extracts at a concentration of 100 μg/mL, and no toxicity was detected in any extract (results not shown). All the extracts induced Nrf2 activity with P20 producing the highest induction of 2.3 fold, followed by P12 (1.8 fold induction) (Figure 1). The ability of these two extracts to induce Nrf2 activity was consistent with the result of the antioxidant capacity of P20 and P12 in the FRAP assay, in which they showed the highest antioxidant activity (Table 3); in fact, the FRAP and Nrf2 activities were found to correlate quite well. Results from the present study have shown that the chemopreventive potential of *G. glabra* is mediated by the activation of Nrf2-dependent antioxidant defense mechanisms, similar to a recent finding where the anti-inflammatory potential of another medicinal plant, *Antrodia salmonea*, was found to be mediated by the activation of Nrf2-dependent antioxidant defense mechanisms (Yang et al., 2015). The fact that Nrf2 activation has been shown previously to be induced by plant phenolic compounds, e.g., flavonoids and stibenes (Bhullar and Rupasinghe, 2015), and that *G. glabra* is known to biosynthesise various flavonoids, it is reasonable to assume that the ability of *G. glabra* root extracts to activate Nrf2 is owing to the presence of various flavonoids.

Herbal medicines, various nutraceuticals, dietary supplements, and phytochemicals are well known as effective cancer chemopreventive agents, which may retard, block or reverse carcinogenesis (Fazio and Ricciardielo, 2014). As standard chemotherapeutic regimes against cancer often render severe side-effects and complications in the post therapeutic management of the disease, cancer chemoprevention may therefore be the way forward to fight against cancer (Ullah *et al.*, 2014). The present findings certainly add to the existing knowledge of cancer chemoprevention by plant-derived extracts or purified phytochemicals, particularly the potential use of liquorice for this purpose.

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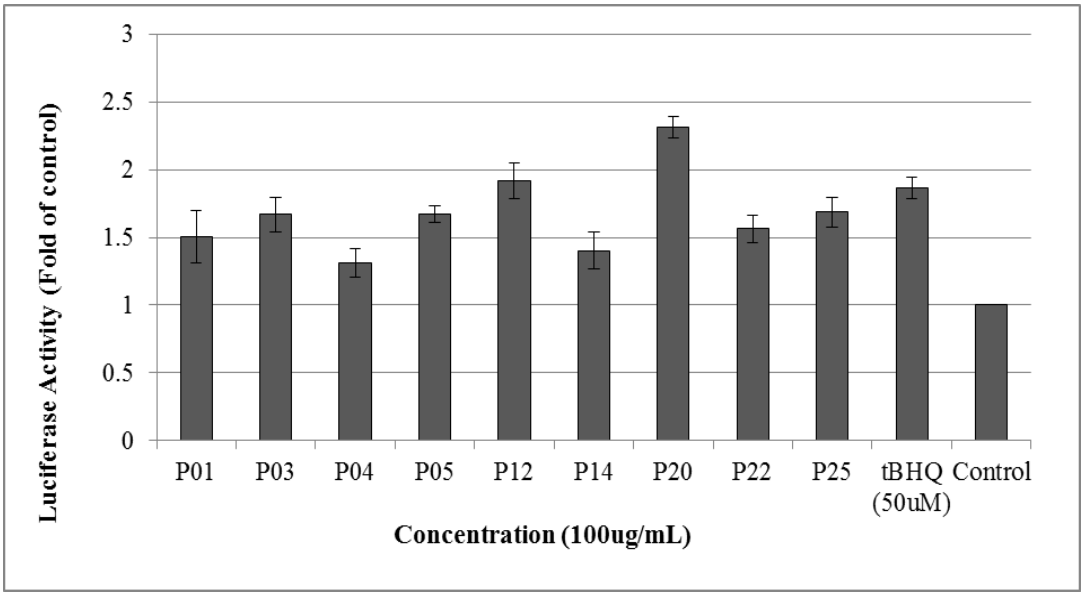

Figure 1. Induction of luciferase activity in AREc32 cells by the methanol extract of *G. glabra* root samples from different geographical locations

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

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

Table 2. Free-radical scavenging activities of the methanol extracts of *G. glabra* root samples and ascorbic acid determined by the DPPH assay

Sample	RC_{50} value μg/mL ± SD	Antioxidant Activity Index (AAI)
Ascorbic acid	14.70 ± 0.7	5.442
P01	635.66 ± 2.4	0.1259
P03	481.88 ± 3.6	0.1660
P05	566.08 ± 2.9	0.1413
P25	136.39 ± 0.9	0.5866
P12	336.70 ± 1.1	0.2376
P14	712.46 ±3.1	0.1123
P04	628.62 ± 6.3	0.1273
P20	411.22 ± 3.4	0.1945
P22	607.81 ± 3.2	0.1316

Table 3. The Ferric-Reducing Antioxidant Power (FRAP) activity for methanol extracts of *G. glabra* root samples and ascorbic acid

Sample (1 mg/mL)	FRAP μmol Fe (II)/g ± SD
Ascorbic acid	889.63 ± 2.2
P01	221.08 ± 1.3
P03	344.19 ± 2.5
P04	214.46 ± 1.1
P05	302.73 ± 1.0
P12	465.59 ± 3.2
P14	257.20 ± 4.3
P20	431.61 ± 2.4
P22	333.08 ± 1.3
P25	398.05 ± 1.4