COMMUNAUTÉ FRANÇAISE DE BELGIQUE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Anticancer Effects of Soybean Bioactive Components and Anti-inflammatory Activities of the Soybean Peptide Lunasin

Yingying Zhu

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Co-promoteur: Guixing Ren

Promoteur: Nadia Everaert

2018

Résumé

ZHU Yingying. (2018). Effets anticancéreux des composants bioactifs du soja et les activités anti-inflammatoires de la peptide de soja Lunasine. (Thèse de doctorat en anglais). Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège, 106p., 7 table, 21 fig.

Il a été démontré que les produits à base de soja présentent des avantages pour la santé. Dans cette étude, nous avons étudié l'effet anticancéreux de 12 composants bioactifs provenant du soja dans des cellules de cancer du sein humain in vitro. Les résultats ont montré que la génistéine, la daidzéine, la glyciteine, la génistine et le dainzine présentaient une activité antiproliférative plus forte contre les cellules de MCF-7 avec une concentration médiane efficace (EC_{50}) de 66.98 ± 4.87 μM, 130.14 ± 2.10 μM, μM et 179.21 ± 6.37 μM, respectivement. Il y avait un effet synergique du traitement combiné de génistine plus daidzine dans des cellules MCF-7 avec un indice de combinaison d'inhibition à 50% (CI_{50}) de 0.89 ± 0.12. La génistéine, la glycitéine, la génistine et le β-sitostérol ont démontré une activité anti-proliférative plus forte contre les cellules MDA-MB-231 avec des valeurs de CE_{50} de 93.75 ± 5.15 μM, 142.67 ± 5.88 μM, 127.82 ± 4.70 μM et 196.28 ± 4.45 μM. L'effet synergique a été observé dans le mélange de génistéine plus génistine, génistéine plus β-sitostérol ou β-sitostérol plus génistine avec des valeurs de CI_{50} de 0.56 ± 0.13, 0.54 ± 0.20 et 0.45 ± 0.12, respectivement. Ces composants bioactifs sont capables d'inhiber l'invasion et la migration des cellules cancéreuses du sein et les traitements combinés ont amélioré l'effet d'inhibiteur. La régulation de la voie PI3K / Akt / mTOR semble être le principal mécanisme impliqué dans l'activité anticancéreuse.

Parmi tous les composants testés du soja, la lunasine a attiré notre intérêt car il s'agit d'un nouveau peptide et de nombreux problèmes concernant le lunasin restent à étudier. En ce qui concerne la lunasine, nous étions préoccupés par son activité anti-inflammatoire et par les voies de signalisation impliquées, ainsi que par sa production par la méthode du génie génétique. Dans cette étude, nous avons isolé la lunasine naturelle (N-lunasine) du soja et nous avons exprimé la lunasine recombinante (R-lunasine) de *Pichia (P.) pastoris*. Le niveau d'expression de la R-lunasine a atteint 240 mg / L de bouillon sans cellules dans les conditions optimales (pH initial 7.0, la concentration finale en méthanol est de 1.0% et l'induction pendant 72 h à 26 °C). L'activité anti-inflammatoire de la N-lunasine et de la R-lunasine a été accédée et comparée. Les résultats ont montré qu'il existait un effet comparable de la N-lunasine et de la R-lunasine sur l'inhibition de la libération d'oxyde nitrique (NO), du facteur de nécrose tumourale-α (TNF-α) et de l'interleukine-6 (IL-6) dans les lipopolysaccharides (LPS), des macrophages

RAW264.7 stimulés de manière de la dose-dépendante. En outre, l'analyse du réseau de signalisation intracellulaire a mis en évidence des niveaux régulés baissés d'Akt phosphorylée, du cible mécanistique de la rapamycine (mTOR) et de la p70 s6 kinase (p70s6k) et un niveau régulé élevé de la glycogène synthase kinase-3β (GSK-3β) après le traitement de R- lunasin. Ces résultats suggèrent que la lunasine exerçait une activité anti-inflammatoire dans les cellules d'RAW264.7 stimulées par les LPS en inhibant partiellement l'activation de la voie de signalisation d'Akt / mTOR / p70s6k.

Le soja est largement cultivé du nord de la Chine au sud de la Chine. Nous nous sommes également intéressés à la variation de la teneur en lunasine dans les graines de soja. Dans cette étude, nous avons déterminé la teneur en lunasine de 413 échantillons de soja prélevés dans les quatre grandes régions de Chine récoltées en 2014 et 2015 pour révéler la répartition régionale de la teneur en lunasine de soja en Chine et les effets des facteurs climatiques. Les résultats ont montré que le cultivar Changmidou 30 récolté dans la province de Jilin en 2015 présentait la plus forte teneur en lunasine (3.25 mg / g de graines sèches). Les données de 2014 et 2015 ont montré que la teneur en lunasine du soja collecté dans le nord de la Chine était significativement plus élevée ($p < 0.05$) que celle du sud de la Chine. Il y avait une corrélation positive ($p < 0.01$) entre la teneur en lunasine et les heures d'ensoleillement (HS), ainsi qu'une plage de température diurne (DTR), mais une corrélation négative ($p < 0.01$) entre la teneur en lunasine et les précipitations (RF). De plus, l'analyse combinée des données de 2014 et 2015 a montré que le DTR était le facteur dominant qui affectait la teneur en lunasine avec un coefficient de la voie directe de 0.301.

Les résultats de notre étude sont prévues d'améliorer notre connaissance sur les composants bioactifs du soja et de leurs bioactivités.

Mots-clés: Soja; Composants bioactifs; Anticancéreux; La lunasine; Anti-inflammatoire; *P. Pastoris*; Facteurs climatiques

Abstract

ZHU Yingying. (2018). Anticancer Effects of Soybean Bioactive Components and Anti-inflammatory Activities of the Soybean Peptide Lunasin (PhD Dissertation in English). Gembloux, Belgium, Gembloux Agro-Bio Tech, Liège University, 106 pages., 7 tables, 21 figures.

Soybean products have been demonstrated to have health-promoting benefits. In this study, we investigated the anticancer effect of 12 bioactive components from soybean on human breast cancer cells *in vitro*. Results showed that genistein, daidzein, glycitein, genistin and dainzin showed stronger anti-proliferative activity against MCF-7 cells with median effective concentration (EC_{50}) values of 66.98±4.87 μM, 130.14±2.10 μM, 190.67±5.65 μM, 72.82±2.66 μM and 179.21±6.37 μM, respectively. There was a synergistic effect of combination treatment of genistin plus daidzin in MCF-7 cells with combination index at inhibition of 50% (CI_{50}) of 0.89±0.12. Genistein, glycitein, genistin and β-sitosterol were demonstrated to have a stronger anti-proliferative activity against MDA-MB-231 cells with EC_{50} values of 93.75±5.15 μM, 142.67±5.88 μM, 127.82±4.70 μM and 196.28±4.45 μM. The synergistic effect was observed in the mixture of genistein plus genistin, genistein plus β-sitosterol or β-sitosterol plus genistin with CI_{50} values of 0.56±0.13, 0.54±0.20 and 0.45±0.12, respectively. These bioactive components were able to inhibit invasion and migration in breast cancer cells and the combination treatments enhanced the inhibitory effect. Regulation of PI3K/Akt/mTOR pathway seems to be the main mechanism involved in the anticancer activity.

Among all the tested components from soybean, lunasin attracted our interests because that it is a novel peptide and many problems about lunasin remained to be studied. About lunasin, we were concerned about its anti-inflammatory activity and involved signalling pathways, as well as its production using genetic engineering method. In this study, we isolated natural lunasin (N-lunasin) from soybean and expressed recombinant lunasin (R-lunasin) from *Pichia (P.) pastoris*. The expression level of R-lunasin reached 240 mg/L cell-free broth at the optimum condition (initial pH 7.0, 1.0% final methanol concentration and induction for 72 h at 26 ℃). The anti-inflammatory activity of N-lunasin and R-lunasin was accessed and compared. Results showed that there was a comparable effect of N-lunsin and R-lunasin on inhibition of release of nitric oxide (NO), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages in a dose-dependent manner. In addition, intracellular signalling array analysis demonstrated down-regulated levels of phosphorylated Akt, mechanistic target of rapamycin (mTOR) and p70 s6 kinase (p70s6k) and an up-regulated level of glycogen synthase kinase-3β (GSK-3β) after R-lunasin treatment. These results suggest that lunasin exerted anti-inflammatory activity in LPS-stimulated RAW264.7 cells partly via inhibiting the activation of Akt/mTOR/p70s6k signalling pathway.

Soybean is widely cultivated from north China to south China. We were also interested in the variation in lunasin content in soybean seeds. In this study, we determined the lunasin content in a total of 413 soybean samples that were collected from four major regions in China and harvested in 2014 and 2015 to reveal the regional distribution of soybean lunasin content in China and the effects of climate factors. The results showed that the cultivar Changmidou 30 collected from Jilin province and harvested in 2015 had the highest lunasin content (3.25 mg/g dry seeds). The data from both 2014 and 2015 showed that the lunasin content in soybean collected from north China was significantly higher ($p<0.05$) than that from south China. There was a positive correlation ($p<0.01$) between lunasin content and hours of sunshine (HS) as well as diurnal temperature range (DTR); however, there was a negative correlation ($p<0.01$) between the lunasin content and rainfall (RF). In addition, the combined analysis of data from 2014 and 2015 demonstrated that DTR was the dominant factor that affects the lunasin content with a direct path-coefficient of 0.301.

These results generated from our study are anticipated to improve our knowledge of bioactive components in soybean as well as their bioactivities.

Keywords: Soybean; Bioactive Components; Anticancer; Lunasin; Anti-inflammatory; *P. Pastoris*; Climate Factors

Acknowledgements

First of all, I would like to express my deepest gratitude to my supervisors, Prof. Nadia Everaert and Prof. Guixing Ren (CAAS). Thank for their continuous support of my Ph.D study and related researches, and for their patience and motivation. Their guidance helped me in realization of this work successfully.

I would also like to express my gratitude to my thesis committee and jury members. Thanks for taking their valuable time to read, evaluate and give pertinent suggestions to my research and thesis. Thanks for all your valuable time and genuine help.

I would also like to thank Prof. Tiandu Han, Dr. Wenwen Song, Prof. Baoqing Dun, Dr. Zhi Wang, Dr. Yang Yao and Dr. Xiushi Yang. Thank Prof. Tianfu Han and Dr. Wenwen Song for providing soybean samples collected from China. Thank Prof. Baoqing Dun and Dr. Zhi Wang for providing equipment for the expression of recombinant lunasin. With Dr. Yang Yao, I learned many important aspects of conducting a research project successfully to its end, and thank her for the long and instructive discussions that we had over the years about and around my PhD project. Thank Dr. Xiushi Yang for his reasonable management of the lab in China.

I am also very grateful to the staff of the Institute of Crop Science, Chinese Academy of Agricultural Sciences (IAS, CAAS, Beijing) for all the help given. Thank all the members from Precision Livestock and Nutrition Unit of Gembloux Agro-Bio tech. Thank you all for welcoming me, for your support, for your kindness and your friendship.

My most affectionate thanks go to my family. My parents generously provided a financial support for my daily life in Belgium and took careful of my little baby when I was not in China. My husb and always encouraged me to overcome difficulties and gave me the energy to go forward. I most appreciate that my baby came to my life. She gives me infinite happiness.

Finally, I would like to thank sincerely all the people who helped me to carry it out successfully

ZHU Yingying

13/07/2018 in Gembloux, Belgium

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BBI: Bowman-Birk protease inhibitor
ROS: reactive oxygen species
NO: nitric oxide
TNF-α: tumour necrosis factor-α
IL-6: interleukin-6
***E. coli*:** *Escherichia coli*
***P. pastoris*:** *Pichia pastoris*
ELISA: enzyme-linked immunosorbent assay
CI_{50}: combination index at inhibition of 50%
EC_{50}: median effective concentration
GFP: green fluorescent protein
ER: estrogen receptor
HEPES: 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulphonic acid
HBSS: Hank's balancedsalt solution
FBS: fetal bovine serum
PBS: phosphate-buffered saline
α-MEM: α-minimum essential medium
DMSO: dimethyl sulfoxide
ECM: extracellular matrix
AMPK α: 5'AMP-activated protein kinase α
mTOR: mammalian target of rapamycin
LPS: lipopolysaccharide
p70s6k: p70 s6 kinase
GSK-3β: glycogen synthase kinase-3β
N-lunasin: natural lunasin
R-lunasin: recombinant lunasin analog
NESR: Northeast Spring Planting Sub-region
NWSR: Northwest Spring Planting Sub-region
HHHR: Huang-Huai-Hai Valleys Summer Planting Region
SMCR: and the South Multiple Cropping region
AT15: accumulated temperature ≥15 °C
MDT: mean daily temperature
RF: rainfall
HS: hours of sunshine
DTR: diurnal temperature range

GPR1-R8: growth period from R1 to R8
CV: coefficient of variation
iNOS: inducible nitric oxide synthase

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General Introduction

1. General context

1.1 Anticancer effect of soybean food

The incidence and mortality of cancer are generally increasing throughout the world, which have been a major public health problem. Notably, the occurrence of cancer in different populations varies significantly depending on genetic tendencies, living environment and lifestyle (Torre et al., 2015). It has been reported that the incidence and mortality of cancer are slightly lower in the East than in the West. People living in China, Japan, and Korea are 4-10 times less likely to be diagnosed with cancer than people in the United States (Goldstein and Parkin, 2006). In a previous study, cancer incidence among Korean-American immigrant populations in the United States and native Koreans in South Korea was observed. The risks of prostate, colon, and rectal cancers for men were higher in Korean-American immigrants than in their native counterparts. In addition, the risks of breast, lung, colon, rectal, and endometrial cancers for women also increased (Lee et al., 2007). This study provided important evidence for the hypothesis that living environment and lifestyle are key factors in determining cancer frequency (Foumier et al., 1998). It has been well known that the most obvious difference in lifestyle between the East and the West is the dietary pattern. The eastern diet contains many more plant foods, particularly in protein sources, than the western diet. People in Asia rely on legume, whereas people in Europe and America rely on animal protein.

Legumes have been cultivated for thousands of years and play an important role in the daily diet of many regions throughout the world. In Asia, soybean (**Fig. 1**) is the most common legume and has been widely used as both food and medicine for many centuries (Messina, 1995). The average intake of soy protein in Asia varies from 10 g/day in China to 30-50 g/day in Japan and Taiwan. In contrast, Americans eat no more than 1-3 g/day (Park et al., 2005; Yamaya et al., 2007). Increasing evidence has indicated that the consumption of soybean food is associated with a reduced risk of chronic diseases, such as type 2 diabetes, obesity (Ali et al., 2004), heart disease (Messina and Lane, 2007) and cancer (Messina, 1995). The anticancer effect of soy-based food has received increasing attention and has been the subject of numerous studies. In the past several decades, several meta-analyses of the relationship between soybean food consumption and various types of cancer, including breast, stomach, prostate, lung, ovarian and gastric, have been conducted (He and Chen, 2013; Ji et al., 2001; Yan and Spitznagel, 2009; Yang et al., 2011; Zeng et al., 2015). As reported by Foumier et al. (1998) and Kerwin et al. (2004), the main components responsible for the anticancer effect of soybean are isoflavones, saponins and peptides. In addition, lectins, phytosterols and polysaccharides were also reported to contribute to the anticancer effect of soybean.

Figure 1. Soybean plants, soybean seeds and soybean products (toufu).

1.1.1 Isoflavones

Isoflavones are a group of naturally occurring heterocyclic phenols. Soybean contains three types and four chemical forms of isoflavones: daidzein, genistein, glycitein, daidzin, genistin, glycitin, 6″-*O*-acetyldaidzin, 6″-*O*-acetylgenistin, 6″-*O*-acetylglycitin, 6″-*O*-malonyldaidzin, 6″-*O*-malonylgenistin and 6″-*O*-malonylglycitin (Wang and Murphy, 1994). Genistein and daidzein, occurring as their β-glucosides genistin and daidzin, account for approximately 90% of the total soybean isoflavone content and are the principal isoflavones in soybean (Foumier et al., 1998). Soybean isoflavones have been demonstrated to possess possible anticancer activity. Li et al. (2005) reported that soybean genistein can inactivate NF-kappa B, inhibit proliferation and increase apoptosis in prostate, breast, lung and pancreatic cancer cells. Daidzein was found to protection against mammary carcinogenesis in female Sprague-Dawley rats (Constantinou et al., 2005). When the concentration of added glycitein was greater than 30 mg/mL, cell growth and DNA synthesis were inhibited in a dose-dependent manner in SKBR-3 human breast cancer cells (Bo et al., 2015).

After oral consumption, isoflavone daidzein can be further metabolized to equol by intestinal microflora before final absorption. The changes in structure are shown in **Fig. 2** (De et al., 2012). Equol has been demonstrated to have an inhibitory effect on the proliferation and invasion of cancer cells. In addition, it was shown to induce cancer cell cycle arrest and apoptosis (Samayoa et al., 2013; Zheng et al., 2012). However, it has been reported that only 30-50% of humans have the gut microflora necessary to convert daidzein to equol (De et al., 2012).

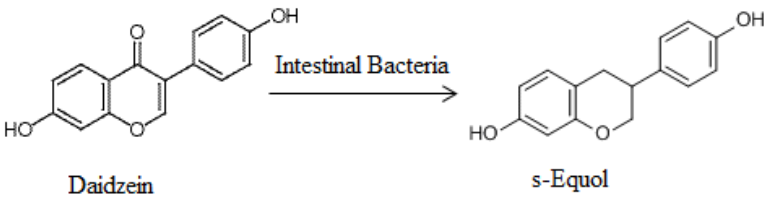


Figure 2. Structure of daidzein and equol

1.1.2 Saponins

Saponins are glycosides of triterpenoid or steroid aglycones with a varying number of sugar side chains (Negi et al., 2011), and their chemical characteristics are described as hydrophobic, polar, and acidic. Saponins are widely distributed in various plants. Soybean is recognized as an important source of dietary saponins. It has been reported that soyasaponins could not be destroyed during processing or cooking. Soyasaponins have been detected in baked soybean, soymilk, tofu and fermented soybean. Increasing evidence suggests that soyasaponins contribute greatly to the anticancer effect of soybean food. Kim et al. found that crude soyasaponin extract (Kim et al., 2004) inhibited the proliferation of HT-29 human colon cancer cells in dose- and time-dependent manners *in vitro*. An in *vivo* study also demonstrated the anticancer effect of soyasaponins on colon cancer in rats (Guo et al., 2013). Presently, the proposed mechanisms of the anticancer effect of soyasaponins are summarized as follows: high bioavailability in animals (Popovich and Kitts, 2006); selective toxicity towards cancer cells; immune modulation; regulation of cell proliferation (Foumier et al., 1998). However, research on the effect of soyasaponin on other cancer cells has been limited.

1.1.3 Bioactive peptide

Increasing attention has been paid to plant peptides due to their bioactivity and ability to promote health and prevent disease. Epidemiological studies have shown that plant peptides have the potential to prevent different stages of cancer, including initiation, promotion and progression (Ortizmartinez et al., 2014). Soybean is one of the important resources of anticancer peptides. The Bowman-Birk protease inhibitor (BBI) is an 8 kDa polypeptide consisting of 71 amino acids. There are two protease inhibitor sites, one for trypsin and one for chymotrypsin (Clair, 1991). The anticancer effect of BBI has been reported decades ago. Clair et al. (1990) reported that BBI, introduced as 0.5% or less of the diet, has the ability to suppress carcinogenesis in mice with no observed adverse effects on health. Kennedy et al. (1996) found that BBI can protect A549 human cancer cells from radiation- and cis-platinum-induced cytotoxicity. In addition, a novel anticancer peptide, lunasin, was isolated from soybean seeds in 1987 by a Japanese research team. Lunasin is a 43 amino acid soy peptide and has been reported to be able to prevent various types of cancer, such as breast cancer (Hsieh et al., 2010), skin cancer (Badole and Mahamuni, 2013), colon cancer (Dia and Mejia, 2010) and lung cancer (Mcconnell et al., 2015). The associated mechanisms of the anticancer effect of soybean peptides include the induction of apoptosis, blockage of intermediate tumour generation and regulation of the immune system (Ortizmartinez et al., 2014).

1.1.4 Others

Lectins are described as sugar-binding proteins or glycoproteins of non-immune origin excluded from sugar-binding antibodies and enzymes (Song et al., 2008). Tande et al. (2016) found that lectins from soybean seeds showed anticancer effects against the colorectal type of the HCT-116cancer cell line and were proved

to be a new link for developing anticancer drugs specific to colorectal adenomas. Soybean is the main resource of dietary phytosterols. Epidemiological and experimental studies have suggested a protective role of phytosterols in the development of cancer (Awad et al., 2000). Bakar et al. (2010) reported the chemo-preventive potential of β-sitosterol in colon cancer, and Awad et al. (2000) found inhibitory effect of β-sitosterol on the growth of breast cancer cells. In addition to these simple constituents, soybean polysaccharides, a class of soluble polysaccharides derived from soybean cotyledons, have been demonstrated to add value in biological applications due to their anticancer effect (Jia et al., 2015). Ko et al. (2013) suggested that soybean-soluble polysaccharides can induce apoptosis in HCT-116 human colon cancer cells via reactive oxygen species generation.

Among all types of cancers, breast cancer is the most commonly diagnosed cancer for women. Data from the American Cancer Society show that breast cancer is expected to account for 29% of all cancer diagnoses in women (Miller et al., 2016). Various anticancer constituents in soybean can prevent and treat breast cancer through dietary modification. It has been reported that total isoflavones account for 1-3 mg/g, BBI accounts for 14.15-186.27 mg/g, lunasin accounts for 1-13.3 mg/g, phytosterol accounts for 202-694 μg/g, and lectin accounts for 2.81-6.52 mg/g in soybean seeds (Gu et al., 2010; Gonzalez et al., 2004; Shiyakumar et al., 2015; Wang et al., 1994; Yamaya et al., 2007). It is well known that the anticancer effects of most of these bioactive components are dose-dependent. However, their low concentrations limit their clinical application. It has been reported that chemotherapeutic combination approaches can reach a greater effect than a single active compound at equal concentrations (Yang and Liu, 2009); this is called the synergistic effect. Synergistic anticancer effects have been observed with different compounds or bioactive components derived from natural plants, such as arctigenin, quercetin (Wang et al., 2015), phytochemical-enriched apple extracts and quercetin 3-β-D-glucoside (Yang and Liu, 2009). However, there is limited knowledge regarding the synergistic effect of the bioactive components in soybean on the inhibition of proliferation of breast cancer cells.

1.2 Lunasin properties

Among all the anticarcinogens in soybean, the bioactive peptide lunasin has attracted increasing attention. Lunasin is a novel therapeutic peptide and has recently been proposed to have beneficial effects on human health. It was initially isolated from soybean and contains 43 amino acids (Lumen, 2010). Studies have shown that lunasin has a variety of physiological functions. The cancer-preventive property of lunasin was recently demonstrated by de Lumen (Lumen, 2005). It has been reported that lunasin can suppress carcinogenesis caused by chemical carcinogens and oncogenes in *in vitro* models and a mouse model for skin cancer (Galvez et al., 2001). Lunasin was also demonstrated to have the ability to scavenge DPPH radicals and ABTS+ radicals (Ren et al., 2017). Galvez et al. (Galvez et al., 2012) found that the consumption of lunasin can reduce the risk of cardiovascular disease. In addition, in addition to anticancer activity, the anti-inflammatory activity of lunasin in RAW 264.7 macrophages has been the focus of

most studies (Hernández-Ledesma et al., 2009). It was demonstrated that the lunasin peptide exists not only in soybean but also in other cereals, such as barley, wheat, quinoa, oats, and solanum nigrum (Ortimartinez et al., 2014). However, the content of lunasin is low in natural raw materials, and the separation and purification of lunasin from natural raw materials is costly. Currently, with the development of molecular biology, researchers have begun to produce lunasin using genetic engineering methods

1.2.1 Discovery and structure of lunasin

The screening of protease inhibitors from soybean seeds was conducted in 1987 at Niigata University School of Medicine in Japan. During this work, a promising anticancer peptide was isolated and identified (Liu et al., 2014). In 1996, researchers at the University of California-Berkeley named this novel peptide lunasin. The word 'lunasin' was chosen from the Tagalog word '1unas', which means 'cure' (http://lunasin.com/). The 2S albumin, encoded by Gm2S-1 in soybean cotyledons, is composed of large subunits, small subunits, connective peptides and signal peptides, and the small subunit is lunasin (Galvez and Lumen, 1999). Although the lunasin gene was cloned from soybean, most recent experiments have used synthetic lunasin, which is composed of 43 amino acid residues, and its sequence is SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDDD with a molecular weight of approximately 5.5 kDa (**Fig. 3**). Synthetic lunasin contains 9 D-residues at the carboxyl terminus, an Arg-Gly-Asp cell adhesion motif and a structurally conserved helix region (KHIMEKIQ). The poly-D chain has a histone-binding property that allows lunasin to act as a potent inhibitor of positively charged H3 and H4 histone acetylation. The RGD motif has a cell-line-specific adhesion function and is responsible for the attachment of lunasin to the extracellular matrix. The KHIMEKIQ region can be recognized by histone H3/H4 to ensure that lunasin can enter the nucleus (Hernández-Ledesma et al., 2009). However, recently, researchers found that the structure of natural lunasin from soybean is not very different from the generally reported structure of lunasin. Dia et al. (2009) isolated and purified lunasin from soybean and found three peptide fractions with positive immunoreactivity against lunasin mouse monoclonal antibody (against lunasin epitope-EKHIMEKIQGRGDDDDD) corresponding to 5, 8 and 14 kDa. LC/MS-MS analysis showed that both the 5 kDa and 14 kDa peptides contained the lunasin epitope RGDDDDDDDDD, while the 8 kDa peptide showed high homology with 2 S soy albumin, which is a lunasin precursor. Seber et al. (2012) also characterized lunasin from soybean using mass spectrometry, and the results showed that the purified soybean lunasin (>99% purity) is 44 amino acids in length. Additionally, there is an C-terminal asparagine residue.

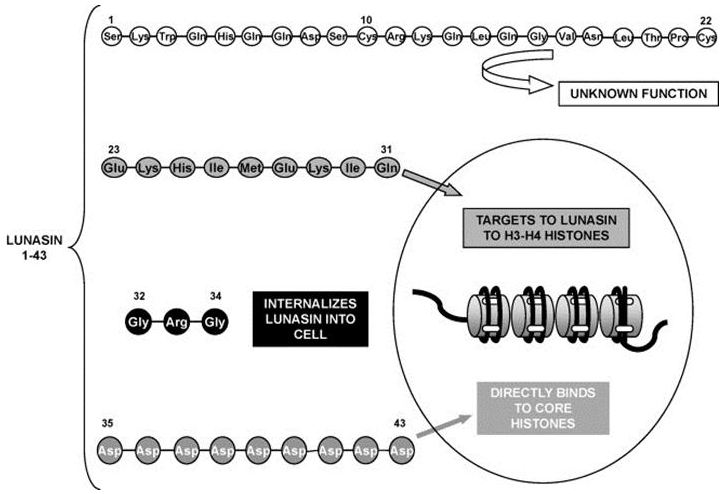


Figure 3. Amino acid sequence of lunasin peptide and demonstrated activity of the different fragments of its sequence.

1.2.2 Bioactivity of lunasin

Studies on the bioactivity of lunasin did not begin until Lumen et al. observed in 1996 that the peptide arrested mitosis in cancer cells by binding to the cell's chromatin and breaking the cell apart (Lumen, 2010).

Anticancer activity. Histone acetylation and deacetylation are often involved in the cell cycle. When histone undergoes acetylation or low acetylation, the centromeric complex in chromatin cannot form correctly, which cannot be captured by microtubules, leading to the suspension of mitosis; this affects gene transcription and expression and causes the occurrence of tumour. If lunasin is applied to cancer cells, cancer may be alleviated or even cured. To test this hypothesis, similar studies have been conducted. Studies have shown that lunasin can block the formation of skin papilloma in mice but does not affect the growth, proliferation, and morphology of normal cells (Jeong et al., 2007). Lunasin can inhibit the transformation of normal cells into cancer cells from chemical and viral oncogenes, ras and ElA, in mammals (Galvez et al., 2001). Dia and Mejia (2010) found that lunasin can activate the mitochondrial pathway and induce the expression of nuclear cluster proteins, thus promoting human breast cancer cells into apoptosis. In addition, lunasin can inhibit the growth of rat cancer cells such as breast cancer, lung cancer, and colon cancer. The researchers made the following conclusions about the anticancer activity of lunasin: (1) Lunasin can enter the cells through the RGD sequence; (2) lunasin then co-localizes with deacetylated chromatin; (3) lunasin binds preferentially to deacetylated histone H4; (4) lunasin inhibits the acetylation of histone H3, H4; (5) lunasin alleviates and blocks the formation of cancer cells. In conclusion, lunasin plays an important role in changing the chromosome and inhibiting the growth and proliferation of tumour cells caused by either chemical carcinogens or viral oncogenes.

Antioxidant activity. Oxidative stress is one of the most critical factors implicated in many disorders of the gastrointestinal tract, such as inflammatory bowel diseases and colon cancer (García-Nebot and Hernández-Ledesma, 2014). A study by Hernández-Ledesma et al. (2009) demonstrated that lunasin can inhibit linoleic acid oxidation and act as an ABTS radical scavenger. Furthermore, it can reduce the production of reactive oxygen species (ROS) by LPS-induced macrophages. In addition, they found that lunasin remained partially intact during incubation with cells, preventing the oxidative damage induced by both chemical agents (García-Nebot and Hernández-Ledesma, 2014). Ren et al. (2017) observed weak DPPH-radical-scavenging activity but strong oxygen-radical-scavenging activity of lunasin.

Lipid-lowering activity. It has been reported that lunasin can combine to the nucleosome of liver cells, prevent chromosome acetylation, block the transcription of HMG CoA reductase, and reduce cholesterol synthesis. Moreover, lunasin can activate the transcription of the LDL receptor and its activator SP1, promote the uptake of LDL in the blood and lower LDL levels (Jeong et al., 2007; Moon et al, 2009). Alfredo showed that the serum LDL-C level of obese pigs (LDL receptor defect type) can significantly decrease with the daily intake of 250 mg or 500 mg lunasin extracts (the content of lunasin is above 20%) (Galvez et al., 2013). Research on the mechanism of lipid-lowering activity of lunasin will provide important new ideas for the prevention and treatment of obesity.

Anti-inflammatory activity. Chronic inflammation has been found to underlie our most destructive chronic diseases, such as cancer, cardiovascular diseases, type 2 diabetes, and Alzheimer's disease. Studies have shown that there are some common signal molecules, such as proteins, in the formation of inflammation and tumours related to invasion, migration, and metabolism in cells, cytokines and their corresponding receptors. Therefore, in recent years, the anti-inflammatory activity of lunasin has attracted more attention than its other activities. Lunasin has been demonstrated to be able to inhibit the release of nitric oxide (NO) and pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), in LPS-stimulated RAW264.7 cells (Hernándezledesma and Lumen, 2009; Ren et al., 2017). In addition, Cam et al. (2013) evaluated the anti-inflammatory activity of lunasin in human THP-1 macrophages and found that the internalization of lunasin into macrophages is amplified in inflammatory conditions and is primarily mediated by endocytic mechanisms. Hsieh et al. (2017) investigated the effect of lunasin on obesity-related inflammation and claimed that lunasin acts as a potential anti-inflammatory agent not only in macrophages but also in adipocytes.

Dia et al. (2009) reported that 100 μM lunasin decreased the expression of iNOS and COX-2 in LPS-stimulated RAW264.7 cells, which suggested that lunasin might contribute to the suppression of inflammation via inhibition of the COX-2/PGE 2 and iNOS/NO pathways. Mejia and Dia (2009) found that lunasin and lunasin-like peptides inhibited inflammation in LPS-induced RAW 264.7 macrophages by suppressing the NF-κB pathway.

1.2.3 Bioavailability of lunasin

A key question about the bioavailability of lunasin is whether lunasin survives digestion in the gastrointestinal tract when ingested orally. A human trial conducted by Dia et al. (2009) showed that a 5 kDa peptide similar to synthetic lunasin was present in the plasma samples of people who consumed soy protein while the peptide was absent in the baseline plasma samples from the same individuals, which suggested that lunasin is bioavailable in humans. Park et al. (2007) investigated the stabilities of lunasin and BBI during *in vitro* digestion by simulated intestinal fluid and simulated gastric fluid and found that the purified lunasin from soybean and synthetic lunasin were easily digested after 2 min of *in vitro* digestion; in contrast, lunasin from soy protein containing BBI was relatively stable. These results showed that the purified lunasin from soybean and synthetic lunasin are easily digested after 2 min of *in vitro* digestion. In contrast, lunasin from soy protein containing BBI is relatively stable after digestion in simulated intestinal fluid and simulated gastric fluid.

1.2.4 Production of lunasin

At present, the production of lunasin mainly includes 1) chemical synthesis, 2) isolation and purification from natural substances, and 3) biosynthesis.

Chemically synthesized lunasin is the most widely used material in present experiments for evaluating the bioactivity of lunasin. Chemical synthesis, based on the amino acid sequence of lunasin, is a process of repeated addition of amino acids. The order of synthesis is generally from the C-terminal to the N-terminal (Wang, 1991; Zhang et al., 2015). However, the chemical synthesis method is more suitable for short-chain amino acid sequences (fewer than 35 amino acids) (Kyle et al., 2012). Forty-three amino acid sequences of lunasin make the scaling production costly and problematic.

The method of isolation of lunasin from natural substances has been well developed. Jeong et al. (2003, 2007) pre-processed soybean and barley with extraction, centrifugation, and dialysis and then obtained lunasin through an anion-exchange column and immunoaffinity chromatography. Cavazos et al. (2012) pre-processed commercial defatted soybean powder with resuspension and centrifugation. After passing the DEAE column, ultrafiltration and desalination were conducted, and lunasin with a purity of more than 90% was obtained. Seber et al. (2012) utilized the sequential application of anion-exchange chromatography, ultrafiltration, and reversed-phase chromatography to develop a large-scale method to generate highly purified lunasin from defatted soy flour. As a result, this method generates lunasin preparations of >99% purity with a yield of 442 mg/kg defatted soy flour. Although we can obtain natural lunasin, the extraction and purification processes are complicated and toilsome.

Biosynthesis, based on an organism's expression system, can be used to express the target protein. With the development of molecular biology and genetic engineering, researchers pay more attention to DNA recombinant technology, which recombines the target gene with an appropriate vector and then transfers the recombinant into the genome of receptor cells for genetically modified organisms (Luo et al., 2006). Existing exogenous protein expression systems include the coli expression system, the yeast cell expression system, animals, and the plant

expression system. Exogenous protein expression systems are an effective way to transfer lunasin into the plant genome to express the target protein through the plant eukaryotic expression system. Davis et al. (2012) established a transient expression system based on the tobacco mosaic virus vector for large-scale production of modified forms of recombinant lunasin (lunasin peptides as C-terminal fusions to green fluorescent protein (GFP)) that includes a linker sequence containing a thrombin cleavage site in tobacco. This system can produce GFP-lunasin at levels >100 mg/kg fresh weight tissue. Ren et al. transferred lunasin into the rice genome for the first time and found that lunasin can be successfully expressed in rice and behaved as an antioxidant and anti-inflammatory agent *in vitro* (unpublished data). *Escherichia (E.) coli* is the first microorganism to be used in genetic engineering with a short growth cycle and rapid reproduction and has played an important role in biotechnology. Liu et al. (2010) used the *E. coli* expression system to express the lunasin peptide, which can produce 4.73 mg recombinant lunasin in every litre of Luria-Bertani culture medium. When the *E. coli* expression system was used, the expressed fusion protein had a similar activity as soybean lunasin (Ge, 2010). However, there were some deficiencies in expressing lunasin through the prokaryotic expression system. Exogenous proteins expressed in prokaryotic cells sometimes cannot properly modify and fold, which seriously affects the activity of the expressed protein and leads to undissolved inclusions (Nuc et al., 2005). *Pichia* yeast is a unicellular eukaryote; compared with the *E. coli* expression system, the yeast cell expression system exhibits high expression levels, which are 10~100 times higher than those of other bacteria. In addition, *pichia* yeast is relatively safe in genetic engineering, does not contain a specific virus and produces no toxin. In addition, it exhibits a favourable growth situation and stable expression level in simple medium, which is easy to control. Moreover, the target protein can be modified after translation in eukaryotic expression systems, such as signal peptide shearing, disulfide bond formation, and protein glycosylation. In 1987, Gregg et al. (1995) used *pichia* yeast as the expression vector to express HBsAg for the first time. Since then, with the improvement in the expression system, *pichia* has been applied to the multiple expression of exogenous proteins. Hundreds of exogenous proteins have been expressed in *pichia* at home and abroad (Annadana et al., 2003; Kawi et al., 2003; Wang et al., 2003). Zhu et al. used *Pichia (P.) pastoris* GS115 to express a native lunasin gene and obtained the purity recombinant lunasin through ultrafiltration and ion-exchange chromatography (unpublished data). However, the yield was very low. In addition to optimizing the yield, it is also important to evaluate the bioactivity of recombinant lunasin.

1.3 Lunasin content in natural plants

Lunasin was initially found in soybean, and its content is affected by both genetic and environmental factors, such as cultivar, planting temperature, soil moisture and light condition (Wang et al., 2008). Gonza et al. (2004) determined the lunasin concentration in 144 diverse soybean accessions from the U.S. Department of Agriculture Soybean Germplasm Collection using an enzyme-linked immunosorbent assay (ELISA). Differences that exceeded 100% were observed

among accessions of similar maturity that were grown in the same environment, indicating that genetic differences in soybeans exist for lunasin. Park et al. (2005) investigated the effect of light and dark conditions on lunasin content in soybean during the stages of seed development and sprouting. Lunasin appeared at 6 weeks after flowering, and its content increased gradually as the seed matured. During sprouting under light and dark conditions, lunasin content started to decrease 2 days after soaking and disappeared at 7 days. In China, researchers found that lunasin contents varied significantly in 6 different soybean cultivars (Gao et al., 2018). Most of these results were based on controlled experiments in green-houses or with limited in sampling, and a comprehensive and in-depth analysis of variation in lunasin content in soybean seeds by extensively sampling over broad ecological regions is required.

In addition to soybean seeds, researchers have identified that lunasin also exists in other natural plants. Ren et al. (2017) detected lunasin in quinoa for the first time, and its content was 1.01-4.89 μ/g dry seeds. Nakurte et al. (2013) found that the lunasin content in oats was 0.197 mg/g seeds. Jeong et al. (2002) identified lunasin in barley, Solanum nigrum (2007a) and wheat (2007b). The concentrations of lunasin in natural plants are listed in **Table1**.

Table 1. Lunasin content in natural plants.

Plant	References	Contents of lunasin	
		mg lunasin/g protein	mg lunasin/g seed
Soybean	Gonza et al., 2004; Gao et al., 2018	4.4-70.5	0.5-8.1
Quinoa	Ren et al., 2017	--	$1.01\text{-}4.89*10^{-3}$
Oats	Nakurte et al., 2013	--	0.195
Barley	Jeong et al., 2002	5.9-8.7	0.01-0.02
Solanum nigrumL.	Jeong et al., 2007a	36.4	--
Solanum lyratum Thunb.	Jeong et al., 2007b	22.3	--
Amaranth	Silvas ánchez et al., 2008	9.5-12.1	--
Wheat	Dineli et al., 2014	--	0.2-0.3

2. Objective and outline of the thesis

2.1 Objective and hypothesis

The main objectives of this thesis were to improve our knowledge of 1) the anticancer effect of bioactive components from soybean, 2) the anti-inflammatory activity and biosynthesis of lunasin, and 3) the natural variation in lunasin concentration in soybean seeds cultivated in China. To achieve these objectives, we first evaluated the anticancer effect of 12 bioactive components from soybean on human breast cancer cells by single or two-way combination treatments *in vitro*. Second, lunasin was selected, and the anti-inflammatory activity of natural lunasin

from soybean and recombinant lunasin from *P. pastoris* was conducted. Finally, we deterimined the lunasin content in 413 soybean samples collected from four major regions in China.

As such, for the first part of our research, we compared the anti-proliferative effect of isoflavones, equol, soyasaponin, soy β-sitosterol, lectin, BBI and lunasin in MDA-MB-231 and MCF-7 human breast cancer cells *in vitro*; additionally, we also aimed to determine their anti-proliferative effect by two-way combination treatments. We assumed that the two-way combination treatments would enhance the anticancer activity of these components and that a synergistic effect would be generated.

The second part of our research focuse on one bioactive component in soybean, i.e., soybean 'lunasin'. Natural lunasin was isolated and purified from defatted soybean flour. Recombinant lunasin was obtained by genetic engineering using *P. pastoris* as an expression system. We aimed to evaluate and compare the anti-inflammatory activity of soybean lunasin and recombinant lunasin. We assumed that the genetic engineering method is a suitable way to produce large amounts of lunasin with equal bioactivity as that of natural lunasin, which may then contribute to an *in vivo* study of lunasin in the future.

The third part of this PhD dicsusses the variations in lunasin content in a total of 413 soybean samples collected from four major regions in China harvested in 2014 and 2015. We assumed that high lunasin content soybean can be cultivated if we know more about how the geography factor and climate factors influence the lunasin content in soybean seeds.

2.2 Outline of the thesis

This thesis is based on three scientific papers that have been published or are under review. It is structured as follows:

Chapter 1 (current chapter) introduces the subject of the thesis, gives an overview of the context and describes the overall structure of the PhD thesis (**Fig. 4**).

Chapter 2 investigates the anti-proliferative activity of 12 bioactive components from soybean by single or combination treatment in MCF-7 and MDA-MB-231 human breast cancer cells. Then, a wound healing assay and a transwell assay were used to evaluate the inhibitory activity (invasion and migration) of the cancer cells. At last, the involved signalling pathways were explored by examination of the protein expression levels of 18 intracellular signalling molecules.
Reference: Zhu, Y., Yao, Y., Shi, Z., Everaert, N., & Ren, G. (2018). Synergistic effect of bioactive anticarcinogens from soybean on anti-proliferative activity in mda-mb-231 and mcf-7 human breast cancer cells in vitro. Molecules, 23(7).

Chapter 3 investigates the anti-inflammatory activity of natural lunasin from soybean and recombinant lunasin from *P. pastoris* in LPS-stimulated RAW264.7 cells.
Reference: Zhu, Y., Everaert, N., Yao, Y., Shi, Z., & Ren, G. (2018). Tandem repeated expression of lunasin gene in pichia pastoris and its anti-inflammatory activity inÂ vitro. Journal of Bioscience & Bioengineering.

Chapter 4 quantifies the variations in lunasin content in a total of 413 soybean samples collected from four major regions in China harvested in 2014 and 2015. *Reference: Zhu,Y., Song, W., Everaert, N, Shi, Z., & Ren, G. Revealing regional distribution of soybean lunasin content in China and the effects of climate factors by extensively sampling. (Under revision)*

Chapter 5 discusses the main results of the thesis and perspectives. Finally, the conclusion of our research is given.

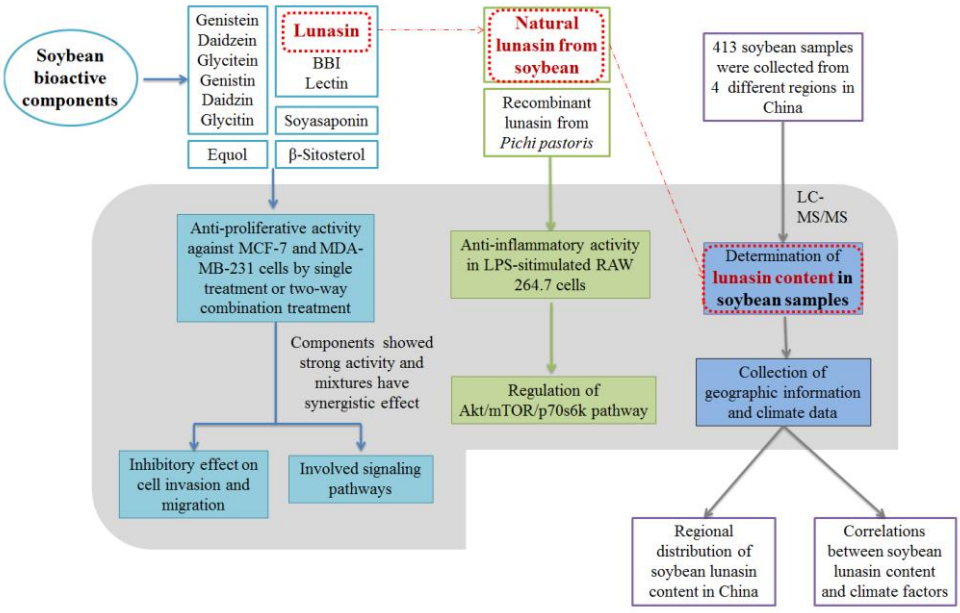


Figure 4. The experimental design of this thesis.

3. References

Ali A. A., Velasquez M. T., Hansen C. T., Mohamed A. I., and Bhathena S. J. 2004. Effects of soybean isoflavones, probiotics, and their interactions on lipid metabolism and endocrine system in an animal model of obesity and diabetes. Journal of Nutritional Biochemistry, 15(10), 583-590.

Annadana S., Schipper B. and Beekwilder J., 2003. Cloning functional expression in Pichia pastoris and purification of potato cystatin and multicystatin. J. Biosci. Bioeng., 95, 118-123.

Awad A. B., Downie A. C., and Fink, C. S. 2000. Inhibition of growth and stimulation of apoptosis by beta-sitosterol treatment of mda-mb-231 human breast

cancer cells in culture. International Journal of Molecular Medicine, 5(5), 541-545.

Baskar A. A., Ignacimuthu S., Paulraj G. M., and Numair, K. S. A. 2010. Chemopreventive potential of β-sitosterol in experimental colon cancer model - an in vitro, and in vivo, study. Bmc Complementary & Alternative Medicine, 10(1), 24.

Bo Z., Su J. P., Yang B., Jie L., and Liu Y. H. 2015. Inhibitory effects of o-methylated isoflavone glycitein on human breast cancer skbr-3 cells. International Journal of Clinical & Experimental Pathology, 8(7), 7809.

Badole S. L., and Mahamuni S. P. 2013. Soybean: Key Role in Skin Cancer. Bioactive Dietary Factors and Plant Extracts in Dermatology. Humana Press.

Cam A., Sivaguru M., and Gonzalez d. M. E. 2013. Endocytic mechanism of internalization of dietary peptide lunasin into macrophages in inflammatory condition associated with cardiovascular disease. Plos One, 8(9), e72115.

Cavazos A., Morales E. & Dia V.P., 2012. Analysis of lunasin in commercial and pilot plant produced soybean products and an improved method of lunasin purification. J. Food Sci., 77, C539-45.

Clair W. H. S. 1991. Suppression of 3-methylcholanthrene-induced celluar tranformation by timed administration of the bowman-birk protease inhibitor. Carcinogenesis, 12(5), 935.

Clair W. H., Billings P. C., Carew J. A., Keller-Mcgandy C., Newberne P., and Kennedy A. R. 1990. Suppression of dimethylhydrazine-induced carcinogenesis in mice by dietary addition of the bowman-birk protease inhibitor. Cancer Research, 50(3), 580-6.

Constantinou A. I., White B. E., Tonetti D., Yang Y., Liang W., and Li W., 2005. The soy isoflavone daidzein improves the capacity of tamoxifen to prevent mammary tumours. European Journal of Cancer, 41(4), 647-654.

Davis K. R., Seber L. E., Mcconnell E. J., and Boles K. 2012. Abstract 3850: production of recombinant lunasin peptides with enhanced anticancer activity using transient expression in tobacco. Cancer Research, 72(8 Supplement), 3850-3850.

De l. P. C., Otero-Franqui E., Martinez-Montemayor M., and Dharmawardhane, S. 2012. The soy isoflavone equol may increase cancer malignancy via up-regulation of eukaryotic protein synthesis initiation factor eif4g. Journal of Biological Chemistry, 287(50), 41640-41650.

Dinelli G., Bregola V., Bosi S., Fiori J., Gotti R., and Simonetti E. 2014. Lunasin in wheat: a chemical and molecular study on its presence or absence. Food Chemistry, 151(4), 520-525.

Dia V. P., Wang W., Oh V. L., Lumen B. O. D., and Mejia E. G. D. 2009. Isolation, purification and characterisation of lunasin from defatted soybean flour and in vitro evaluation of its anti-inflammatory activity. Food Chemistry, 114(1), 108-115.

Dia V. P., and Mejia, E. G. 2010. Lunasin promotes apoptosis in human colon cancer cells by mitochondrial pathway activation and induction of nuclear clusterin expression. Cancer Letters, 295(1), 44-53.

Fournier D. B., Jr E. J., and Gordon, G. B. 1998. Soy, its components, and cancer prevention: a review of the in vitro, animal, and human data. Cancer Epidemiol Biomarkers Prev, 7(11), 1055-1065.

Fenwick D. E., and Oakenfull D. 1983. Saponin content of food products and prepared foods. Journal of the Science of Food & Agriculture, 34(2), 186-191.

Gao M. D., Meng X. B., Xing C. C., Zhang Y., Deng H. T., and Wang F. Z. 2018. Determination of polypeptide lunasin content in six different cultivars of soybean using nanolc-orbitrap technique. Modern Food Science & Technology.

Galvez A. F. and de Lumen B. O., 1999. A soybean cDNA encoding a chromatin-binding peptide inhibits mitosis of mammalian cells. Nat. Biotechnol., 17, 495-500.

Galvez A. F., Chen N., Macasieb J., and Ben O. 2001. Chemopreventive property of a soybean peptide (lunasin) that binds to deacetylated histones and inhibits acetylation. Cancer research, 61(20), 7473-7478

Galvez A. F. 2012. identification of lunasin as the active component in soy protein responsible for reducing LDL cholesterol and risk of cardiovascular disease.

Galvez A. F., Matel H., Ivey J.. Lunasin-Enriched Soy Extract (LunaRich X™), in Combination with the Dietary Supplement Reliv Now, Reduces Free Fatty Acid by Increasing Plasma Leptin and Adiponectin Levels in LDL-Receptor Mutant Pigs. 2013, PRWEB [Internet]

Garcá-Nebot M. J., Recio I., and Hernández-Ledesma B. 2014. Antioxidant activity and protective effects of peptide lunasin against oxidative stress in intestinal caco-2 cells. Food & Chemical Toxicology, 65(1), 155-161.

Ge W. L., 2010. Functional study of soybean bioactive peptide-prokaryotic expression, purification, and activity identification of Lunasin. Huazhong University of Science and Technology.

Gregg D. and Saddler J. N., 1995. Bioconversion of lignocellulosic residue to ethanol: process flowsheet development. Biomass and Bioenergy., 9, 287-302.

Goldstein B. Y., and Parkin D. M. 2006. Cancer Burden and Trends,and Prevention Strategy in the Eastern Asia Region.

Guo Y. W., Chen Y. H., Chiu W. C., Liao H., and Lin S. H. 2013. Soy saponins meditate the progression of colon cancer in rats by inhibiting the activity of β-glucuronidase and the number of aberrant crypt foci but not cyclooxygenase-2 activity. Isrn Oncology, 2, 645817.

Gu C., Pan H., Sun Z., and Qin G. 2010. Effect of soybean variety on anti-nutritional factors content, and growth performance and nutrients metabolism in rat. International journal of molecular sciences, 11(3), 1048-1056

Gonzalez de Mejia, E., Vásconez, M., de Lumen, B. O., and Nelson, R. 2004. Lunasin concentration in different soybean genotypes, commercial soy protein, and isoflavone products. Journal of agricultural and food chemistry, 52(19), 5882-5887.

Gonza M. E., Vasconez M., Lumen B. O. and Nelson R., 2004. Lunasin Concentration in Different Soybean Genotypes, Commercial Soy Protein, and Isoflavone Products. J. Agric. Food Chem., 52, 5882-5887.

He F. J., and Chen J. Q. 2013. Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: differences between chinese women and women in western countries and possible mechanisms. Food Science & Human Wellness, 2(3-4), 146-161.

Hsieh C. C., Hernándezledesma B., and Lumen B. O. D. 2010. Lunasin, a novel seed peptide, sensitizes human breast cancer mda-mb-231 cells to aspirin-arrested cell cycle and induced apoptosis. Chemico-Biological Interactions, 186(2), 127-134.

Hsieh, C. C., Chou M. J., and Wang C. H. 2017. Lunasin attenuates obesity-related inflammation in raw264.7 cells and 3t3-l1 adipocytes by inhibiting inflammatory cytokine production. Plos One, 12(2), e0171969.

Hernández-Ledesma B., Hsieh C. C., and Ben, O. 2009. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. Biochemical and Biophysical Research Communications, 390(3), 803-808.

Ji B. T., Chow W. H., Yang G., Jin F., Gao Y. T., and Jr F. J. 2001. Correspondence re: a. h. wu et al. a meta-analysis of soyfoods and risk of stomach cancer: the problem of potential confounders. cancer epidemiol. biomark. prev. 9: 1051-1058, 2000. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 10(5), 570.

Jia X., Chen M., Wan J. B., Su H., and He C. 2015. Review on the extraction, characterization and application of soybean polysaccharide. Rsc Advances, 5(90), 73525-73534.

Jeong H. J., Lam Y. I. and de Lumen B. O., 2002. Barley Lunasin Suppresses ras-Induced Colony Formation and Inhibits Core Histone Acetylation in Mammalian Cells. J. Agric. Food Chem, 50, 5903-5908.

Jeong H. J., Park J. H. & Lam Y., 2003. Characterization of Lunasin Isolated from Soybean. J. Agric. Food Chem., 51, 7901-7906.

Jeong J. B., Jeong H. J. & Park J. H., 2007. Cancer-Preventive Peptide Lunasin from Solanum nigrum L. Inhibits Acetylation of Core Histones H3 and H4 and Phosphorylation of Retinoblastoma Protein. J. Agric. Food Chem., 55, 10707-13.

Jeong H. J., Jeong J. B. & Kim D. S., 2007. The Cancer Preventive Peptide Lunasin from Wheat Inhibits Core Histone Acetylation. Cancer Lett., 255, 42-48.

Kawai R. et al., 2003. Production and characterization of recombinant Phanerochaete chrysosporium beta-glucosidase in the methylotrophic yeast Pichia pastoris. Biosci. Biotechnol. Biochem., 67, 1-7.

Kyle S., James K. A., and McPherson, M. J. 2012. Recombinant production of the therapeutic peptide lunasin. Microb Cell Fact, 11, 28.

Kennedy C. W., Donahue J. J., and Wan X. S. 1996. Effects of the bowman‐birk protease inhibitor on survival of fibroblasts and cancer cells exposed to radiation and ‐ platinum. Nutrition & Cancer, 26(2), 209-17.

Kerwin S. M. 2004. Soy saponins and the anticancer effects of soybeans and soy-based foods. Curr Med Chem Anticancer Agents., 4(3), 263-272.

Kim H. Y., Yu R., Kim J. S., Kim Y. K., and Sung M. K. 2004. Antiproliferative crude soy saponin extract modulates the expression of iκbα, protein kinase c, and cyclooxygenase-2 in human colon cancer cells. Cancer Letters, 210(1), 1-6.

Ko Y. J., Jeong J. W., Choi Y. H., and Ryu, C. H. 2013. Soy soluble polysaccharide induces apoptosis in hct‐116 human colon cancer cells via reactive oxygen species generation. Molecular Medicine Reports, 8(6), 1767-1772.

Lee J., Demissie K., Lu S. E. and Rhoads G. G. 2007. Cancer incidence among korean-american immigrants in the united states and native koreans in south korea. Cancer Control, 14(1), 78-85.

Li Y., Ahmed F., Ali S., Philip P. A., Kucuk O., and Sarkar F. H. 2005. Inactivation of nuclear factor kappab by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. Cancer Research, 65(15), 6934-6942.

Liu C.F. and Pan T. M., 2010. Recombinant expression of bioactive peptide lunasin in Escherichia coli. Appl. Microbiol. Biotechnol., 88, 177-86.

Liu J., Jia S. H., Kirberger M., and Chen N. 2014. Lunasin as a promising health-beneficial peptide. European Review for Medical & Pharmacological Sciences, 18(14), 2070-5.

Luo J. F. and Li Z. Y., 2006. Principles and techniques of biological engineering, Beijing. Science Press, pp, 103-120.

Lumen D. B. O. (2005). Lunasin: A Cancer‐Preventive Soy Peptide. Nutrition reviews, 63(1), 16-21.

Lumen D. B. O. (2010). Lunasin: a cancer-preventive soy peptide. Nutrition Reviews, 63(1), 16-21.

Mcconnell E. J., Devapatla B., Yaddanapudi K., and Davis K. R. 2015. The soybean-derived peptide lunasin inhibits non-small cell lung cancer cell proliferation by suppressing phosphorylation of the retinoblastoma protein. Oncotarget, 6(7), 4649-62.

Messina M. 1995. Modern applications for an ancient bean: soybeans and the prevention and treatment of chronic disease. Journal of Nutrition, 125(3 Suppl),

567S.

Messina M., and Lane B. 2007. Soy protein, soybean isoflavones and coronary heart disease risk: where do we stand?. Future Lipidol, 2(1), 55-74.

Mejia E. G., and Dia V. P. 2009. Lunasin and lunasin-like peptides inhibit inflammation through suppression of nf-kappab pathway in the macrophage. Peptides, 30(12), 2388-2398.

Miller K. D., Siegel R. L., Lin C. C., Mariotto A. B., Kramer J. L., Rowland J. H., and Jemal, A. 2016. Cancer treatment and survivorship statistics, 2016. CA: a cancer journal for clinicians, 66(4), 271-289.

Moon C., Han J. R. and Park H. J., 2009. Synthetic RGDS peptide attenuates lipopolysaccharide-induced pulmonary inflammation by inhibiting integrin signaled MAP kinase pathways. Respir. Res., 10, 18.

Nakurte I., Kirhnere I., Namniece J., and Saleniece K., 2013. Detection of the lunasin peptide in oats (Avena sativa, L). J. Cereal Sci, 57, 319-324.

Negi J. S., Pramod S., Nee P. G. J., and Rawat M. S. M. 2011. High-performance liquid chromatography analysis of plant saponins: an update 2005-2010. Pharmacognosy Reviews, 5(10), 155-158.

Nuc P. and Nuc K., 2005. Recombinant protein production in Escherichia coli, Postepy biochem., 52, 448-456.

Ortizmartinez M., Winkler R., and Garc álara S. 2014. Preventive and therapeutic potential of peptides from cereals against cancer. Journal of Proteomics, 111, 165-183.

Park J. H., Jeong H. J., and de Lumen B. O. 2005. Contents and bioactivities of lunasin, bowman− birk inhibitor, and isoflavones in soybean seed. Journal of agricultural and food chemistry, 53(20), 7686-7690

Park J. H., Jeong H. J., and Lumen B. O. 2007. In vitro digestibility of the cancer-preventive soy peptides lunasin and bbi. J Agric Food Chem, 55(26), 10703-10706.]

Popovich D. G., and Kitts D. D. 2006. Anticancer Activity of Ginseng and Soy Saponins. Nutrition and Cancer Prevention.

Ren G., Zhu Y., Shi Z., and Li J. 2017. Detection of lunasin in quinoa (Chenopodium quinoa Willd.) and the in vitro evaluation of its antioxidant and anti - inflammatory activities. Journal of the Science of Food and Agriculture, 97(12), 4110-4116

Samayoa C., Kotha A., Krishnegowda N. K., Vadlamudi R. K., and Tekmal R. R. 2013. Abstract 1312: the estrogen receptor β agonists, liquiritigenin and s-equol, inhibit breast cancer cell proliferation through the activation of tumour-suppressor and other pathways. Cancer Research, 73(8 Supplement), 1312-1312.

Seber L. E., Barnett B. W., Mcconnell E. J., Hume S. D., Cai J., and Boles K., et al. 2012. Scalable purification and characterization of the anticancer lunasin

peptide from soybean. Plos One, 7(4), e35409.

Silvas ánchez C., Ap D. L. R., Le óngalv án M. F., de Lumen B. O., De L. A., and de Mej á E. G. 2008. Bioactive peptides in amaranth (amaranthus hypochondriacus) seed. Journal of Agricultural & Food Chemistry, 56(4), 1233.

Song L., Zhang M., and Liang L. I. 2008. Screening of catalyst for hydrogenation of soy lecithin. Food Science, 29(12), 350-352.

Shivakumar M., Verma K., Talukdar A., Srivastava N., Lal S. K., Sapra R. L., and Singh K. P. 2015. Genetic variability and effect of heat treatment on trypsin inhibitor content in soybeanGlycine max (L.) Merrill.. Legume Res, 38(1), 60-5.

Torre L. A., Bray F., Siegel R. L., Ferlay J., Lortet‐Tieulent J., and Jemal A. 2015. Global cancer statistics, 2012. CA: a cancer journal for clinicians, 65(2), 87-108.

Tande A. T. 2016. Screening of soy lectin: as new era cancer healthing agent. IJPSR, 7(5): 2147-2151.

Wang D. X., 1991. Advances in the research on the protection and cleavage method in the synthesis of solid phase peptide. Chemistry, 02, 1-7.

Wang H., and Murphy P. A. 1994. Isoflavone content in commercial soybean foods. J.agric.food Chem, 42(8), 1666-1673.

Wang H. J., and Murphy P. A. 1994. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. Journal of Agricultural and Food Chemistry, 42(8), 1674-1677.

Wang W., Sun Y. H. & Wang Y. P., 2003. Expression of grass carp growth hormone in the yeast Pichia pastoris. Acta genetica Sinica., 30, 301-306.

Wang W., Dia V. P., Vasconez M., de Mejia E. G. & Nelson R. L., 2008. Analysis of soybean protein-derived peptides and the effect of cultivar, environmental conditions, and processing on lunasin concentration in soybean and soy products. J. AOAC. Int, 91, 936-946.

Wang P., Phan T., Gordon D., Chung S., Henning S. M., and Vadgama J. V. 2015. Arctigenin in combination with quercetin synergistically enhances the anti-proliferative effect in prostate cancer cells. Molecular Nutrition & Food Research, 59(2), 250-261.

Yamaya A., Endo Y., Fujimoto K., and Kitamura K. 2007. Effects of genetic variability and planting location on the phytosterol content and composition in soybean seeds. Food chemistry, 102(4), 1071-107.

Yan L., and Spitznagel E. L. 2009. Soy consumption and prostate cancer risk in men: a revisit of a meta-analysis. American Journal of Clinical Nutrition, 89(4), 1155-63.

Yang W. S., Va P., Wong M. Y., Zhang H. L., and Xiang Y. B. 2011. Soy intake is associated with lower lung cancer risk: results from a meta-analysis of epidemiologic studies. American Journal of Clinical Nutrition, 94(6), 1575-83.

Yang J., and Liu R. H. 2009. Synergistic effect of apple extracts and quercetin 3-β-d-glucoside combination on antiproliferative activity in mcf-7 human breast cancer cells in vitro. Journal of Agricultural & Food Chemistry, 57(18), 8581.

Zhang K. P., Su S. L. & Liu Y. L., 2015. Research Progress on Function and Preparation of Bioactive Peptides. Aem Roducts Rocessing, 12, 61-64.

Zeng Y., Bai J., Deng L. C., Xie Y. P., Zhao F., and Huang Y. 2015. Association of the glutathione s-transferase t1 null genotype with risk of gastric cancer: a meta-analysis in asian populations. Asian Pac J Cancer Prev, 17(3), 1141-1148.

Zheng W., Zhang Y., Ma D., Shi Y., Liu C., and Wang P. 2012. (±)equol inhibits invasion in prostate cancer du145 cells possibly via down-regulation of matrix metalloproteinase-9, matrix metalloproteinase-2 and urokinase-type plasminogen activator by antioxidant activity. Journal of Clinical Biochemistry & Nutrition, 51(1), 61-67.

2

Synergistic Anticancer Effect of Bioactive Components from Soybean in MDA-MB-231 and MCF-7 Human Breast Cancer Cells *in Vitro*

In this chapter, six soy isoflavones (genistein, daidzein, glycitein, genistin, daidzin, glycitin), a soy isoflavones natural estrogenic metabolite (equol), soyasaponin, soy β-sitosterol, lunasin, BBI and lectin were collected. Firstly, the anti-proliferative activity of these bioactive components from soybean in MDA-MB-231 and MCF-7 human breast cancer cells was evaluated by single treatment in vitro. Then, components showing stronger anti-proliferative activity were taken forward to investigate whether there is a synergistic effect by two-way combination treatment.

This chapter is adapted from the published article:

Zhu Y., Yao Y., Shi Z., Everaert N., Ren G. 2018. Synergistic effect of bioactive anticarcinogens from soybean on anti-proliferative activity in MDA-MB-231 and MCF-7 human breast cancer cells *in vitro*. Molecules. 23(7): 1557.

1. Abstract

Consumption of soybean products has been implicated in the prevention of breast cancer. This study provides insights into the anti-proliferative activity of 12 anticarcinogens from soybean (lunasin, BBI, lectin, genistein, daidzein, glycitein, genistin, daidzin, glycitin, equol, soyasaponin and β-sitosterol) by single or two-way combination treatment against MCF-7 and MDA-MB-231 human breast cancer cells. Results showed that genistein, daidzein, glycitein, genistin and dainzin showed stronger anti-proliferative activity against MCF-7 cells with the median effective concentration (EC_{50}) values of 66.98±4.87 μM, 130.14±2.10 μM, 190.67±5.65 μM, 72.82±2.66 μM and 179.21±6.37 μM, respectively. There is a synergistic effect of combination treatment of genistin plus daidzin in MCF-7 cells with combination index at inhibition of 50% (CI_{50}) of 0.89±0.12. Genistein, glycitein, genistin and β-sitosterol were demonstrated to have a stronger anti-proliferative activity against MDA-MB-231 cells with EC_{50} values of 93.75±5.15 μM, 142.67±5.88 μM, 127.82±4.70 μM and 196.28±4.45 μM. The synergistic effect was observed in the mixture of genistein plus genistin, genistein plus β-sitosterol or β-sitosterol plus genistin with CI_{50} values of 0.56±0.13, 0.54±0.20 and 0.45±0.12, respectively. These bioactive anticarcinogens were able to inhibit invasion and migration of breast cancer cells and the combination treatments enhanced the inhibitory effect. Regulation of PI3K/Akt/mTOR pathway seems to be the main mechanisms involved in the anticancer activity.

Keywords: Soybean; Synergistic Effect; MDA-MB-231; MCF-7; Anti-proliferation

2. Introduction

Epidemiological evidence suggests that an increased consumption of soybean food is associated with a decreased cancer risk (Messina et al., 1994). Soybean food has been recommended in cancer prevention for a number of anticarcinogens exist in it. Soybean isoflavones, a family of nonnutritive compounds, were demonstrated to be associated with modulation of a variety of biological processes in carcinogenesis (Clubbs and Bomser, 2007). Equol, derived from isoflavones by the action of gut microflora, was reported to inhibit cancer cell growth and invasion with higher bioavailability (Magee et al., 2006). Phytosterols are plant sterols chemically similar to cholesterol but exclusively in plants, particularly legumes. Awad et al. (2003) found that it could induce apoptosis in breast cancer cells. In addition, a previous study indicated that soybean saponins could suppress the growth of colon tumour cells *in vitro* (Kim et al., 2004). Except these phytochemicals, soybean also provides a source of bioactive proteins and polypeptides which are now being intensively studied as cancer chemopreventive agents, such as trypsin inhibitor, lectin and a recently discovered peptide lunasin (Gladysheva et al., 2001; Hsieh et al., 2018; Prashanta et al., 2014).

Breast cancer is the most frequently diagnosed cancer and one of the major causes of mortality in woman (Siegel et al., 2016). The conventional treatment methods of breast cancer would bring irreversible side effects to patients. Although a lot of work on prevention and intensive treatment of breast cancer has been done, effective strategies with minor side effects are still lacking (Yang and Liu, 2009). Breast cancer is a heterogeneous disease, classified into different subtypes according to the gene expression profile or immunohisto chemical markers such as estrogen receptor (ER) (Leung et al., 2016). MCF-7 and MDA-MB-231 human cancer cell lines are ER+ and ER- cells, which are well-established *in vitro* model for evaluation of estrogen responsive or estrogen independent antineoplastic drugs (Kaushik et al., 2016). Recently, increasing evidences suggested that the abundant anticarcinogens in soybean have obvious ability to prevent and treat breast cancer. However, it now remains to identify the most bioactive anticarcinogens from soybean on different subtypes of breast cancer cells.

The low concentration and low bioavailability of active components in natural plants limited their clinical application (Henning et al., 2011). It is reported that chemotherapeutic combination approaches could reach a greater effect than with one active compounds single at equal concentrations (Yang and Liu, 2009)，which is called a synergistic effect. Synergistic anticancer effect has been observed with different compounds or bioactive components derived from natural plants such as arctigenin and quercetin (Wang et al., 2015), and phytochemicals enriched apple extracts and quercetin 3-β-D-glucoside (Yang and Liu, 2009). However, limited

knowledge is available regarding the synergistic effect of anticarcinogens in soybean on inhibition of proliferation of breast cancer cells.

In this study, the anti-proliferative activity of 12 reported anticarcinogens individually from soybean in MDA-MB-231 and MCF-7 human breast cancer cells was firstly evaluated by single treatment. Then, anticarcinogens showing stronger activity were taken forward to investigate whether there is a synergistic effect on human breast cancer cell proliferation by two-way combination treatment.

3. Materials and methods

3.1 Chemicals

Genistein, daidzein, glycitein, genistin, daidzin, glycitin, equol, soyasaponin, β-sitosterol, BBI, lectin and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lunasin was synthesized by the American Peptide Company (CA, Sunnyvale, USA). 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulphonic acid (HEPES), Hank's balancedsalt solution (HBSS), fetal bovine serum (FBS), penicillin-streptomycin, phosphate-buffered saline (PBS) and α-minimum essential medium (α-MEM) were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Methylene blue was purchased from BBL (Cockeysville, MD, USA). All reagents are of analytical grade.

3.2 Preparation of treatment solution

Genistein, daidzein, glycitein, genistin, daidzin, glycitin, equol, soyasaponin and β-sitosterol screened were prepared in 100 % dimethyl sulfoxide (DMSO) at 10000 μM. BBI, lunasin and lectin were prepared in growth media at 5000 μM. These solutions were stored at -80 ℃ and used as stock solution. Working solutions of all treatments were made through supplement with growth media until the final concentrations of 0-200 μM.

3.3 Cell culture

The MDA-MB-231 and MCF-7 human breast cancer cell lines were obtained from American Tissue Type Culture Collection (ATCC, Bethesda, MD). MDA-MB-231 cells were maintained in α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin and 1 mM sodium pyruvate. MCF-7 cells were maintained in α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate and 10 μg/mL insulin. All cells were grown in a humidified atmosphere at 37 ℃, with 5% CO_2 in air.

3.4 Cell proliferation assay

The cell proliferation was determined using the methylene blue (98% HBSS,

0.67% glutaraldehyde, 0.6% methylene blue) assay as previously reported by Felice et al. (Felice et al., 2009). MDA-MB-231 and MCF-7 human breast cancer cells were respectively seeded into a 96-well plate at a density of 2.5×10^5 cells/well. After 16 h for MDA-MB-231 cells or 8 h for MCF-7 cells, the growth media containing various concentrations (0-200 μM) of different treatment solutions were added to MDA-MB-231 cells. After 72 h of incubation, cell proliferation was determined by measuring the absorbance at 570 nm using a microplate reader (Bio-Rad, MA, USA). An inhibition curve of cell proliferation was achieved for each treatment solution and the EC_{50} was determined.

3.5 Combination study

The samples that showed stronger anti-proliferative effects (EC_{50} value <200 μM) towards human breast cancer cells were selected for the combination study. According to the EC_{50} value of each treatment solution, two-way combination treatments were conducted. In detail, MCF-7 and MDA-MB-231 cells were respectively seeded into a 96-well plate at a density of 2.5×10^5 cells/well. For MCF-7 cells, the growth media was changed to media containing a series of concentrations at different ratios of EC_{50} ($0.125\times EC_{50}$, $0.25\times EC_{50}$, $0.50\times EC_{50}$, $0.75\times EC_{50}$, $1.00\times EC_{50}$, and $1.25\times EC_{50}$) value of two selected samples, which include genistein plus daidzein, enistein plus daidzein, genistein plus glycitein, genistein plus genistin, genistein plus daidzin, daidzein plus glycitein, daidzeinplus genistin, daidzein plus daidzin, glycitein plus genistin, glycitein plus daidzin or genistin plus daidzin, after 16 h incubation. For MDA-MB-231 cells, the growth media was changed to media containing a series of concentrations at different ratios of EC_{50} ($0.125\times EC_{50}$, $0.25\times EC_{50}$, $0.50\times EC_{50}$, $0.75\times EC_{50}$, $1.00\times EC_{50}$, and $1.25\times EC_{50}$) value of genistein plus glycitein, genistein plus genistin, genistein plus β-sitosterol, glycitein plus genistin, glycitein plus β-sitosterol, β-sitosterol plus genistin, after 8 h incubation. After 72 h of incubation, cell proliferation was determined and the new EC_{50} value for each sample in combination study was calculated. Finally, a CI was calculated for each combination treatment at inhibition of 50% according to the method of Yang et al (Yang and Liu, 2009). The CI_{50} value of a combination treatment less than 1 indicates a synergistic effect.

3.6 Cytotoxicity assay

Cell cytotoxicity of bioactive anticarcinogens by single or two-way combination treatment, at the concentration of their EC_{50} values, was assessed using the methylene blue assay (Cuenda and Rousseau, 2007; Yang and Liu, 2009) with some modification. MDA-MB-231 and MCF-7 human breast cancer cells were respectively seeded into a 96-well plate at density of 5×10^5 cells/well and incubated for 24 h. For MCF-7 cells, cells were then treated with genistein at 66.98

μM, daidzein at 130.14 μM, glycitein at 190.67 μM, genistin at 72.82 μM, daidzin at 179.21 μM and genistin plus daidzin at 26.21 μM plus 64.50 μM, or control for 24 h, respectively. For MDA-MB-231 cells, cells were treated with genistein at 93.75 μM, glycitein at 142.67 μM, genistin at 127.82 μM, β-sitosterol at 196.28 μM, genistein plus genistin at 33.61 μM plus 52.21 μM, genistein plus β-sitosterol at 24.08 μM plus 50.42 μM and β-sitosterol plus genistin at 37.71 μM plus 24.55 μM, or control for 24 h, respectively. The viability of cells was expressed as % of control.

3.7 Wound healing assay

Samples which showed stronger anti-proliferative effects by single and combination treatments having a synergistic effect were taken forwards to further investigate their capacity to inhibit breast cancer cells invasion and migration.

Firstly, a wound-healing assay was conducted as reported by Kozlova et al. (2016). Briefly, MCF-7 and MDA-MB-231 cells were respectively seeded into 6-well plate at 5×10^5/well and grown in growth media until 90-100% confluency. After starvation in serum-free media overnight, a cell-free area (wound) was constructed using 200 μL pipette tip in each well and washed gently with ice-cold PBS for three times. Then serum-free media containing different samples were added to cells, or control. The concentration of each sample used was the same with that described in cytotoxicity assay. Healing of the wound was observed after 24 h by a light microscopy (CX-2, Olympus) and analyzed using Image J software (NIH, USA).

3.8 Transwell chamber assay

A transwell chamber assay was conducted using an extracellular matrix (ECM) invasion assay kit (ECMatrix Cell Invasion Assay (Millipore, Billerica, MA) according to the manufacturer's instruction. Briefly, MCF-7 and MDA-MB-231 cells were respectively suspended at 5×10^5 cells in 300 μL of serum-free media and plated on the top of an ECM-coated membrane insert. The assay was terminated after 48 h with different treatment solutions or control. The concentration of each sample used was the same with that described in the cytotoxicity assay. Then the cells on the upper side of the insert were wiped off with a cotton swab and the cells on the lower side were stained with the solution included in the invasion assay. The number of invasive cells was determined as described earlier for the matrigel invasion assays in co-culture (Tapia-Pizarro et al., 2013).

3.9 Intracellular signalling array

MCF-7 and MDA-MB-231 cells were respectively seeded into a 6-well plate at a density of 2.5×10^5 cells/well and treated with the different treatment solutions, or

control, as described in the anti-proliferative activity assay. The concentration of each sample used was the same with that described in the cytotoxicity assay. Whole cell lysates were collected using ice-cold lysis RIPA buffer (Fluorescent Readout, Cell Signalling Technology, Danvers, MA, USA) supplemented with 1% PMSF and stored at -20 ℃ until analysis. The PathScan Intracellular Signalling array kit (Fluorescent Readout, Cell Signalling Technology, Danvers, MA, USA) was used according to the manufacturer's instructions, to simultaneously detect 18 phosphorylated or cleaved signalling molecules. The images were captured and quantified using a LiCor Odyssey imaging system (Li-Cor Biosciences, Lincoln Nebraska). A transwell chamber assay was conduct.

3.10 Western blot assay

The whole cell lysates collected in the last section were used to the western-blot assay performed according to a previously described method (Zhu et al., 2016). Total cellular protein was solubilized in ice-cold lysis RIPA buffer. Anti-PI3K p85 HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. GAPDH protein was used as control. Target proteins were detected and quantified using SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA).

3.11 Data analysis

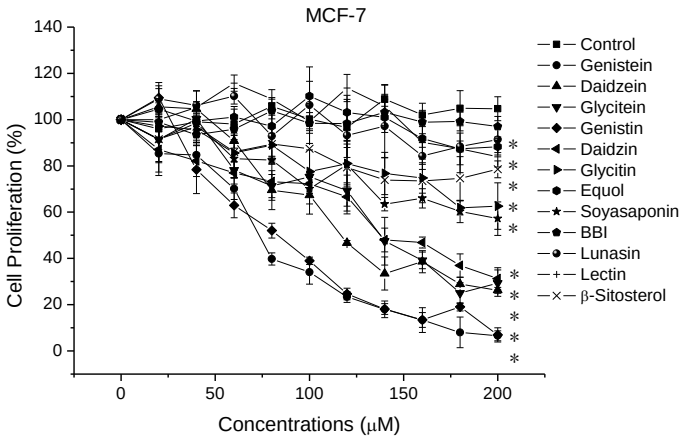
Data in this study were analyzed using one way ANOVA and Tukey's test performed by SPSS (Statistics for Social Science) version 17.0 (IBM, New York，USA). All the assays were conducted in triplicate (n=3). Significant differences were reported at $p<0.05$.

4. Results and discussion

4.1 Anti-proliferative effects of 12 treatment solutions on human breast cancer cells by single treatment and combination treatment

To realize the aims of this study, 12 reported anticarcinogens from soybean were collected and screened for their anti-proliferative activity against the MCF-7 and MDA-MB-231 human breast cancer cells. As shown in **Fig. 5a and 5b**, within the concentrations determined (0-200 μM), BBI and lunasin have no significant anti-proliferative effect on MCF-7 cells. Genistein, daidzein, glycitein, genistin, daidzin, glycitin, equol, soyasaponin, β-sitosteroland lectin inhibited the MCF-7 cell proliferation in a dose-dependent manner. Among these 10 bioactive anticarcinogens, genistein, daidzein, glycitein, genistin and daidzin were demonstrated to have stronger anti-proliferative activity, with the EC_{50} values (**Fig.**

6a) of 66.98±4.87 μM, 130.14±2.10 μM, 190.67±5.65 μM, 72.82±2.66 μM and 179.21±6.37 μM, respectively. MCF-7 human breast cancer cells are estrogen-responsive breast cancer cells. The anti-proliferative activity of soybean isoflavones has been previously and widely demonstrated in estrogen-responsive breast cancer cells *in vitro* (Szliszka and Krol, 2011). Recently, considerable interest has focused on the triple negative breast cancer which is an aggressive subtype of breast cancer and more frequent in younger and pre-menopausal women (Dent et al., 2007). MDA-MB-231 human breast cancer cells are the estrogen-independent, triple negative breast cancer cells. Results of the present study showed that single treatments with daidzin, BBI, equol, lectin and lunasin at concentrations less or equal than 200 μM showed no significant inhibition effects on MDA-MB-231 cell proliferation. However, genistein, glycitein, genistin and β-sitosterol exhibited stronger anti-proliferative effects against MDA-MB-231 cells in a dose-dependent manner, with the EC_{50} values (**Figure 6b**) of 93.75±5.15 μM, 142.67±5.88 μM, 127.82±4.70 μM and 196.28±4.45 μM, respectively. These results suggested that MCF-7 cells were more sensitive to anticarcinogens from soybean than MDA-MB-231 cells.



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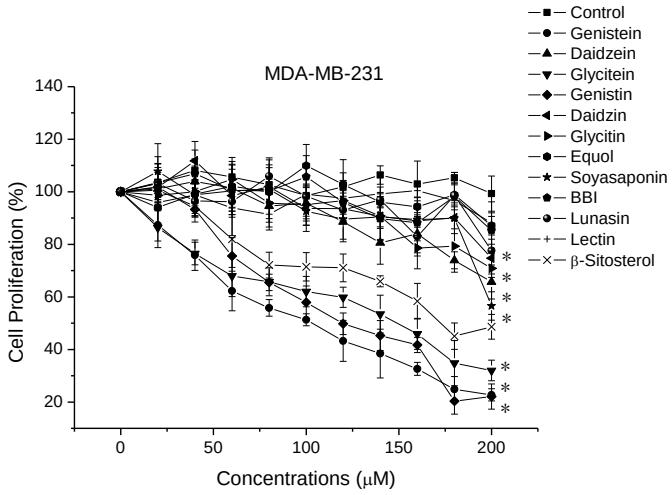
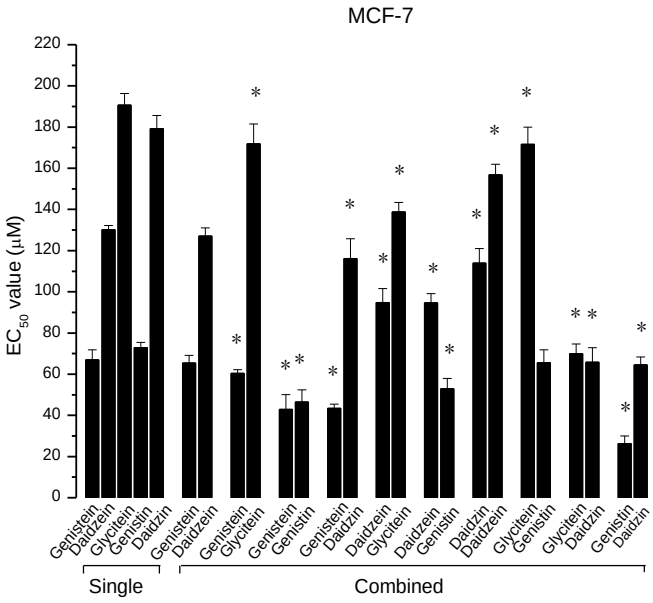


b

Figure 5. Percent inhibition of MCF-7 (a) and MDA-MB-231 (b) human cancer cells proliferation by 12 anticarcinogens from soybean. Cells were respectively treated with different concentrations (0-200 μM) of 12 samples. Data are presented as mean ± SD. *indicates a significant difference compared to the control in cell proliferation assay at concentration of 200 μM ($p<0.05$).



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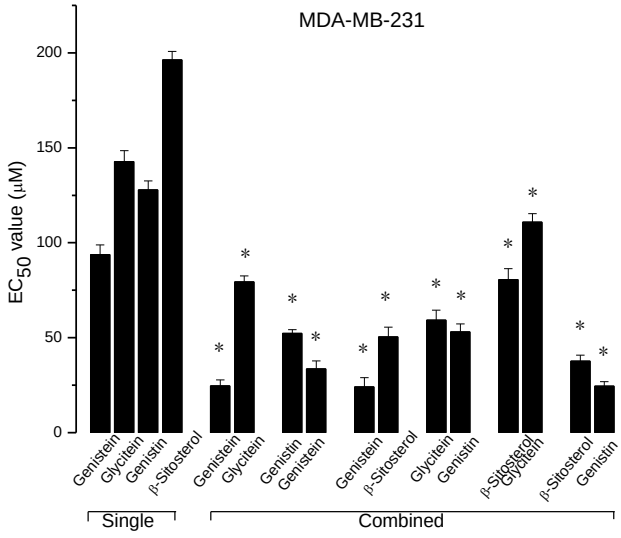


b

Figure 6. EC_{50} values of bioactive anticarcinogens by single or two-way combination treatment in MCF-7 (a) and MDA-MB-231 (b) human cancer cells. Data are presented as

mean ± SD. *Indicates a significant difference compared to the EC_{50} value of single treatment ($p<0.05$).

Due to obvious anti-proliferative activity of compounds from natural plants, the dietary modification is thought to be an alternative strategy to prevent and reduce the risk of breast cancer (Yang and Liu, 2009). However, the effective doses of these compounds can barely be achieved by oral consumption. Nowadays, the synergistic effect generated from drug combination has attracted great attention due to the advantage of increased anti-cancer effect, less drug dose, reduced side effects. To further investigate whether there is a synergistic effect of anticarcinogens from soybean, samples showing stronger anti-proliferative activity by single treatment were selected and two-way combination treatments were conducted respectively in MCF-7 and MDA-MB-231 cells. Ten combination treatments including genistein plus daidzein, genistein plus glycitein, genistein plus genistin, genistein plus daidzin, daidzein plus glycitein, daidzein plus genistin, daidzein plus daidzin, glycitein plus genistin, glycitein plus daidzin and genistin plus daidzin were performed on MCF-7 cells (**Fig. 6a**). The CI values were calculated for all the ten combination treatment at 50% inhibition of MCF-7 proliferation. Results (**Table 2**) showed that only the CI_{50} value of the combination of genistin plus daidzin was less than 1 (0.89±0.12), indicating that there was a synergistic effect by the combined treatment of genistin plus daidzin in MCF-7 cells. The EC_{50} value of genistin and daidzin were reduced to 26.21±3.72 μM and 64.50±3.88 μM, respectively, due to the synergistic effect. Six combination treatments including genistein plus glycitein, genistein plus genistin, genistein plus β-sitosterol, glycitein plus genistin, glycitein plus β-sitosterol and β-sitosterol plus genistin were conductedtoward MDA-MB-231 cells and results are presented in **Fig. 6b**. The CI_{50} values of two-way combination treatments of genistein plus genistin, genistein plus β-sitosterol and β-sitosterol plus genistin for MDA-MB-231 cell proliferation were 0.56±0.13, 0.54±0.20 and 0.45±0.12, respectively (**Table 2**). These results suggested that there were significant synergistic effects of these three combination treatments. The EC_{50} values of genistein, genistin and β-sitosterol in these two-way combination treatments were significantly lower than in single treatments. The EC_{50} values of β-sitosterol and genistin toward MDA-MB-231 reduced to 37.71 μM and 24.55 μM, less than 50 μM. It is reported that soybean isoflavone concentrations in prostatic fluid can reach up to 50 μM in persons with a long-term soybean-rich dietary habits (Hedlund et al., 2005). The synergistic effects of these combination treatments increase the possible application of natural anticarcinogens in humans.

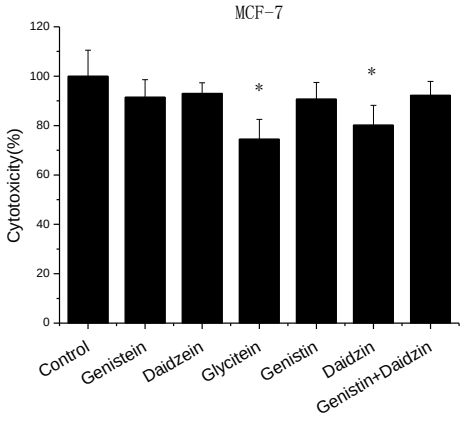
Table 2 CI_{50} values[a] of bioactive anticarcinogens by single or two-way combination treatment in MCF-7 and MDA-MB-231 human cancer cells.

	Combined treatments	CI value at inhibition of 50%
MCF-7	Genistein+Daidzein	2.02±0.30
	Genistein+Glycitein	1.86±0.22
	Genistein+Genistin	1.53±0.33
	Genistein+Daidzin	1.52±0.18
	Daidzein+Glycitein	1.68±0.44
	Daidzein+Genistin	1.69±0.31
	Daidzein+Daidzin	1.89±0.23
	Glycitein+Genistin	1.92±0.58
	Glycitein+Daidzin	1.01±0.10
	Genistin+Daidzin	0.89±0.12
MBA-MD-231	Genistein+Glycitein	1.30±0.08
	Genistein+Genistin	0.56±0.13
	Genistein+β-Sitosterol	0.54±0.20
	Glycitein+Genistin	1.05±0.10
	Glycitein+β-Sitosterol	1.29±0.07
	β-Sitosterol+Genistin	0.45±0.12

[a]The combination index at inhibition of 50%.

4.2 Cytotocicity of bioactive anticarcinogens by single or two-way combination treatment

At the concentration of the EC_{50} values, the cytotoxicity of genistein, daidzein, glycitein, genistin, daidzin and genistin plus daidzin toward MCF-7 cells, and the cytotoxicity of genistein, glycitein, genistin, β-sitosterol, genistein plus genistin, genistein plus β-sitosterol and β-sitosterol plus genistin toward MDA-MB-231 cells are shown in **Fig. 7a and 7b**. No cytotoxicity was observed in genistein, daidzein, genistin and genistin plus daidzin toward MCF-7 cells. Also, there was no cytotoxicity in genistein, genistin, genistein plus genistin, genistein plus β-sitosterol and β-sitosterol plus genistin toward MDA-MB-231 cells.



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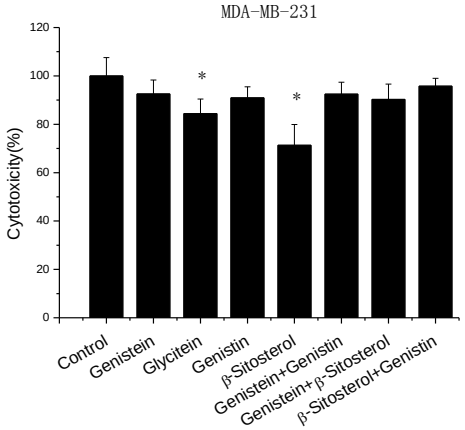


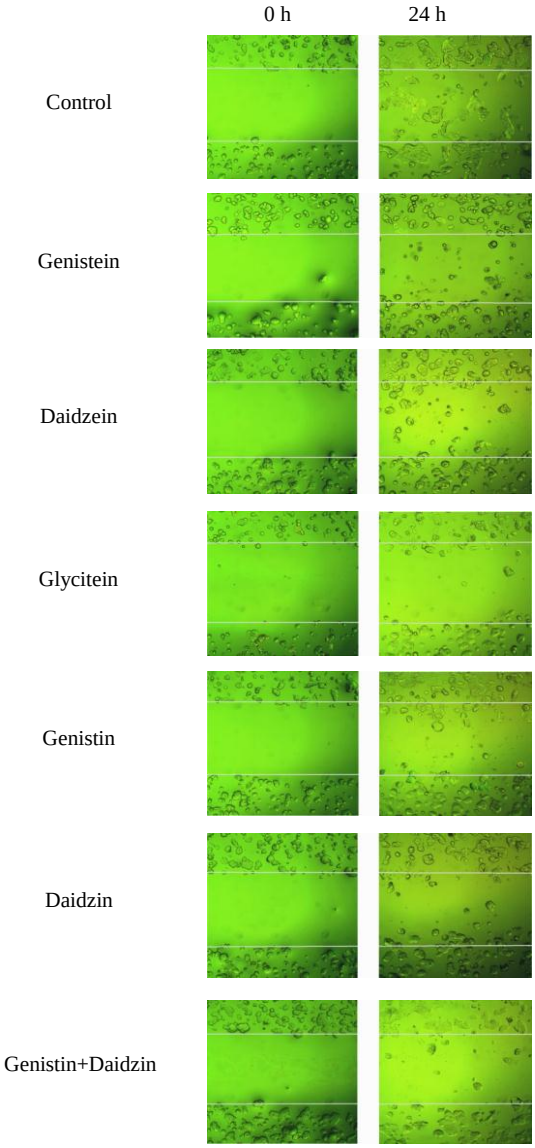
b

Figure 7. Cytotoxicity of bioactive anticarcinogens by alone or two-way combination treatment in MCF-7 (a) and MDA-MB-231 (b) human cancer cells. Data are presented as mean ± SD. *Indicates a significant difference compared to the control ($p<0.05$).

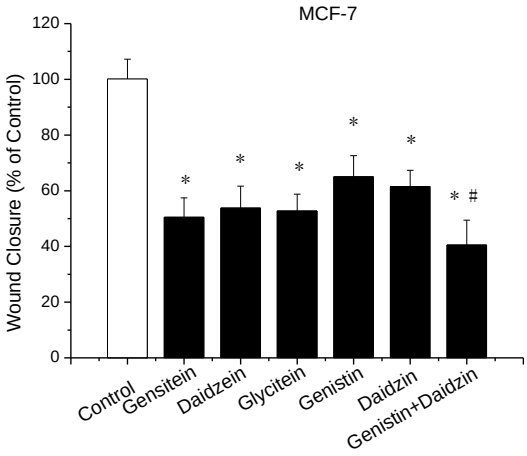
4.3 Inhibition of cell invasion and migration

The invasive and migratory ability is the important characteristic of metastasis of tumour cells (Chun and Kim, 2013). To further evaluate the pharmacological activity of these anticarcinogens against cancer metastasis, wound-healing assay and transwell chamber assay were performed to evaluate the inhibition of cell invasion and migration. As shown in **Fig. 8a and 8b**, treatment of MCF-7 cells

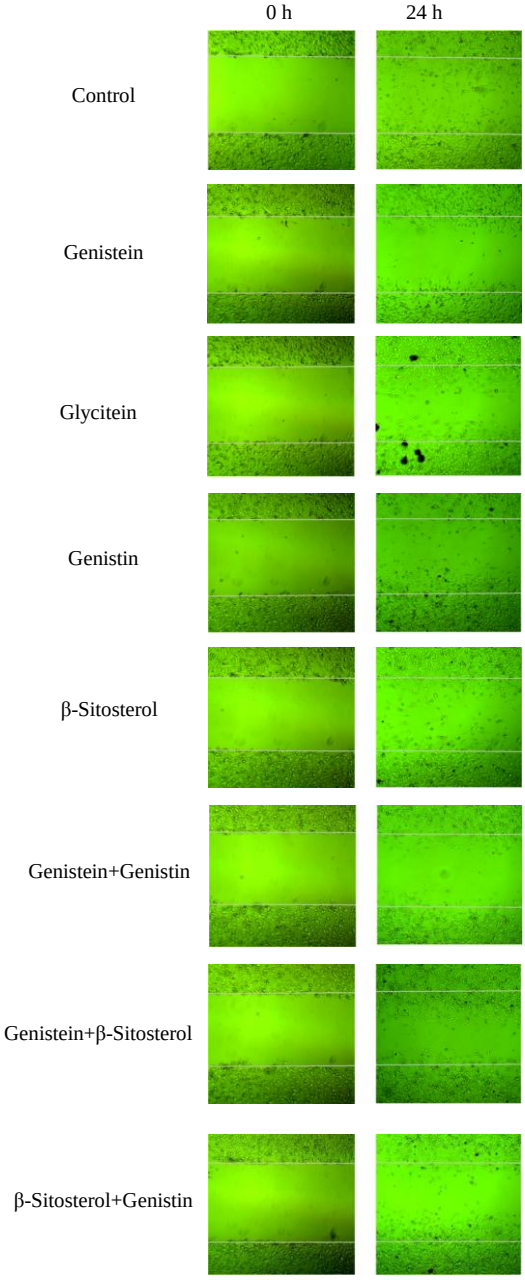
with genistein, daidzein, glycitein, genistin or daidzin single led to decreased wound closure compared to control cells by 49.49%, 46.22%, 47.24%, 34.98% and 38.49%, respectively, suggesting that these samples reduced the motility of MCF-7 cells. The mixture of genistin plus daidzin inhibited the wound closure by 59.48%, significantly more ($p<0.05$) than treatment with genistin or daidzin single. Genistein, glycitein, genistin and β-sitosterol were able to inhibit the wound closure in MDA-MB-231 (**Fig. 8c and 8d**). They decreased wound closure compared to control cells by 57.27%, 24.93%, 5.19% and 15.67%. Genistein plus genistin, genistein plus β-sitosterol, and β-sitosterol plus genistin decreased wound closure by 55.56%, 27.49% and 45.51%. However, among these combination treatments, only the mixture of β-sitosterol and genistin showed significantly stronger ($p<0.05$) inhibitory effect than single treatment in MDA-MB-231 cells.


0 h
24 h
Control
Genistein
Daidzein
Glycitein
Genistin
Daidzin
Genistin+Daidzin

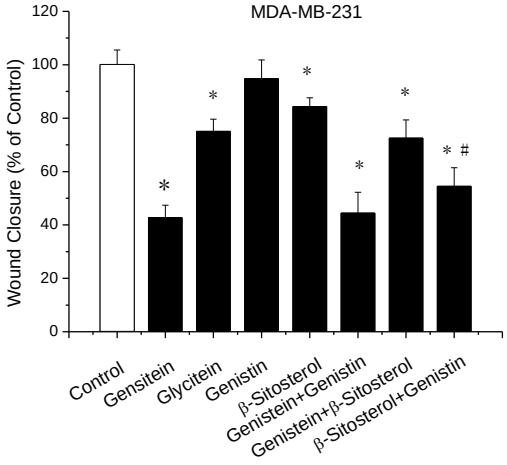
a


MCF-7
Wound Closure (% of Control)
120
100
80
60
40
20
0
*
*
*
*
*
* #
Control
Gensitein
Daidzein
Glycitein
Genistin
Daidzin
Genistin+Daidzin

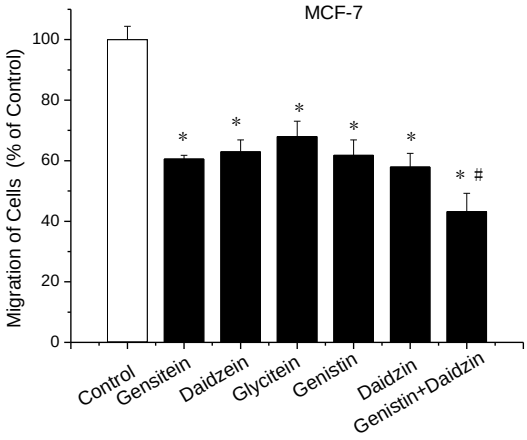
b


0 h
24 h
Control
Genistein
Glycitein
Genistin
β-Sitosterol
Genistein+Genistin
Genistein+β-Sitosterol
β-Sitosterol+Genistin

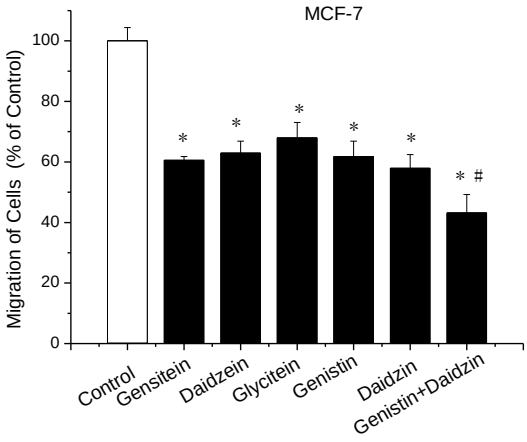
C


MDA-MB-231
Wound Closure (% of Control)
120
100
80
60
40
20
0
Control
Gensitein
Glycitein
Genistin
β-Sitosterol
Genistein+Genistin
Genistein+β-Sitosterol
β-Sitosterol+Genistin

d


MCF-7
Migration of Cells (% of Control)
100
80
60
40
20
0
Control
Gensitein
Daidzein
Glycitein
Genistin
Daidzin
Genistin+Daidzin

e



f

Figure 8. Inhibition of cell invasion and migration measured by wound healing assay and transwell chamber assay. For wound-healing assay, wounds were made when cells were 90-100% confluent. After overnight, cells were treated with samples, or control. The closure of wounds in MCF-7 cells and MDA-MB-231 cells were imaged (a, c) and quantitatively measured (b, d) at 0 h and 24 h. For transwell chamber assay, MCF-7 (e) and MDA-MB-231 (f) Cells were treated with samples, or control for 48 h. Cells suspended in serum-free medium were seeded on the upper membrane of transwell chamber and incubated for 48 h. Complete growth medium was added on the bottom. Cells on the lower membrane of chambers were counted. Data are presented as mean ± SD.* indicates a significant difference compared to the control ($p<0.05$). #indicates a significant difference compared to treatment with samples by single ($p<0.05$).

Results of the transwell assay showed that gensitein, daidzein, glycitein, genistin and daidzin have a positive effect on MCF-7 cell migration (**Fig. 8e**). The migration of MCF-7 cells was respectively inhibited by 65.43%, 57.04%, 42.04%, 38.19% and 62.03% with gensitein, daidzein, glycitein, genistin and daidzin treatment compared to control. The combination treatment of genistin plus daidzin significantly enhanced ($p<0.05$) the inhibition compared to genistin and daidzin alone. The inhibitory effects of genistein, glycitein, genistin and β-sitosterol on MDA-MB-231cell migration were also examined (**Fig. 8f**). Similar to results of the wound-healing assay, genistein, glycitein, genistin and β-sitosterol led to a significant decrease ($p<0.05$) in MDA-MB-231 cell migration, however, genistin had no significant effect by single treatment. Three combination treatments also showed significant inhibitory effects on MDA-MB-231 cell migration, however, only combination of genistin plus β-sitosterol enhanced the effects compared to genistin and β-sitosterol alone.

Overall, all the data indicate that the bioactive anticarcinogens, except genistin, from soybean effectively decreased the invasive and migratory ability of MCF-7 cells and MDA-MB-231 cells. The combination treatment could enhance the inhibitory effect. The inhibitory effect on the invasion and migration of cancer cells is not due to the cytotoxicity.

4.3 Modulations of protein expression and signalling pathways

The anti-proliferative effect and inhibitory effect on cell invasion and migration of anticarcinogens was associated with changes in multiple signalling pathways (Wang et al., 2015). In order to explore the involved signalling pathways of single treatments and combination treatments in MCF-7 and MD-MB-231 cells, the protein expression levels of 18 intracellular signalling molecules (**Fig. 9a**) were examined.

In MCF-7 cells (**Fig. 9b and 9c**), results showed that genistein significantly ($p<0.05$) decreased phosphorylation of stat3, Akt, and PRAS40; Daidzein significantly ($p<0.05$) decreased phosphorylation of stat3 and PRAS40; Glycitein significantly ($p<0.05$) decreased phosphorylation of stat3, and increased phosphorylation of 5'AMP-activated protein kinase (AMPK) α; Daidzin significantly ($p<0.05$) increased phosphorylation of AMPKα. By combination treatment of genistin plus daidzin, phosphorylation of stat3, mammalian target of rapamycin (mTOR) and PRAS40 were significantly ($p<0.05$) decreased, and phosphorylation of AMPKα was significantly ($p<0.05$) increased. In MDA-MB-231 cells, genisitein significantly ($p<0.05$) down-regulated phosphorylation of Akt, mTOR, p38, PRAS40 and GSK-3β, and up-regulated phosphorylation of BAD and p53; Glycitein significantly ($p<0.05$) down-regulated phosphorylation of Akt, mTOR, p38, PRAS40 and GSK-3β, and up-regulated phosphorylation of AMPKα; Genistin significantly ($p<0.05$) down-regulated phosphorylation of Akt, mTOR and GSK-3β, and up-regulated phosphorylation of BAD and p53; β-Sitosterol significantly down-regulated phosphorylation of Akt, BAD, p53, p38 PRAS40 and GSK-3β, and up-regulated AMPKα. Three combination treatments showed similar pattern with significantly ($p<0.05$) up-regulation of phosphorylated AMPKα, BAD and p53, but not significantly ($p>0.05$) down-regulation of phosphorylated Akt and mTOR. These results suggested that these anticarcinogens exerted anticancer effect in MCF-7 and MDA-MB-231 cells via different signalling pathways.

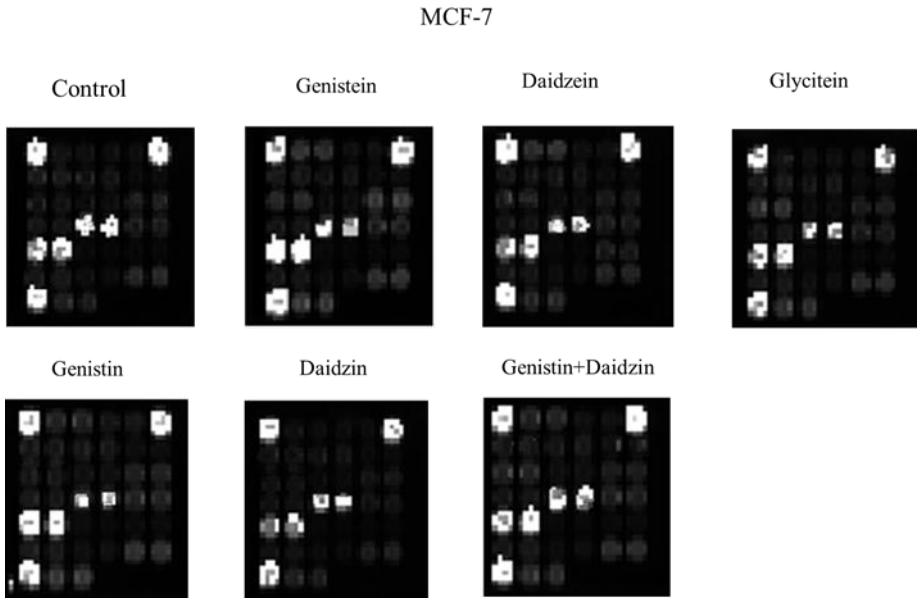
From these results, almost all treatments up-regulated phosphorylation of AMPKα in varied degrees. AMPK is a serine/threonine protein kinase which responsible for cellular energy homeostasis (Chiang et al., 2010). Published studies indicate that AMPK activation strongly suppresses cell proliferation in non-malignant cells as well as in tumour cells. These actions of AMPK appear to

be mediated through multiple mechanisms including regulation of the cell cycle and inhibition of protein synthesis, de novo fatty acid synthesis, specifically the generation of mevalonate as well as other products downstream of mevalonate in the cholesterol synthesis pathway. It is reported that AMPK can regulate cell proliferation, cell growth and autophagy. It has been proposed to be a potential target for cancer chemotherapy (Høyer-Hansen and Jäättelä, 2007).

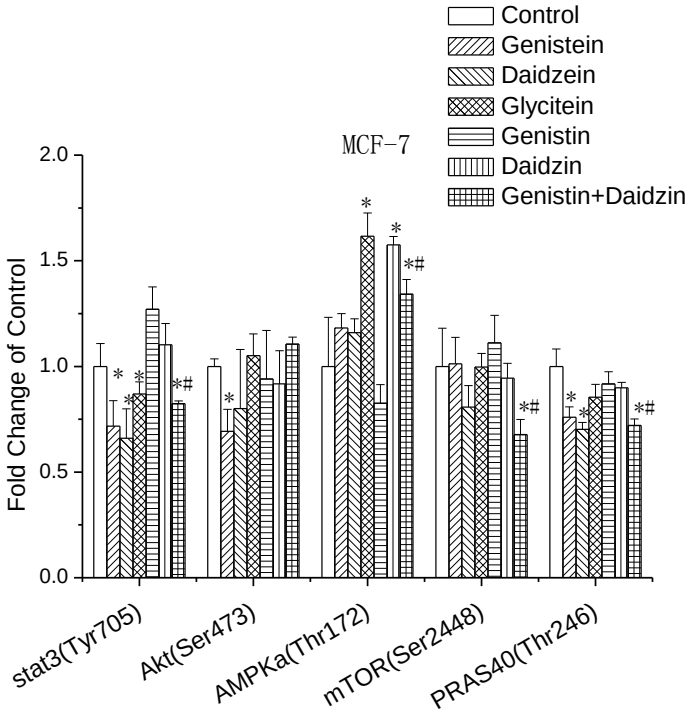
Intracellular Signaling

	Target	Phosphorylation Site	Modification
1	Positive Control	N/A	N/A
2	Negative Control	N/A	N/A
3	ERK1/2	Thr202/Tyr204	Phosphorylation
4	Stat1	Tyr701	Phosphorylation
5	Stat3	Tyr705	Phosphorylation
6	Akt	Thr308	Phosphorylation
7	Akt	Ser473	Phosphorylation
8	AMPKα	Thr172	Phosphorylation
9	S6 Ribosomal Protein	Ser235/236	Phosphorylation
10	mTOR	Ser2448	Phosphorylation
11	HSP27	Ser78	Phosphorylation
12	Bad	Ser112	Phosphorylation
13	p70 S6 Kinase	Thr389	Phosphorylation
14	PRAS40	Thr246	Phosphorylation
15	p53	Ser15	Phosphorylation
16	p38	Thr180/Tyr182	Phosphorylation
17	SAPK/JNK	Thr183/Tyr185	Phosphorylation
18	PARP	Asp214	Cleavage
19	Caspase-3	Asp175	Cleavage
20	GSK-3β	Ser9	Phosphorylation

a

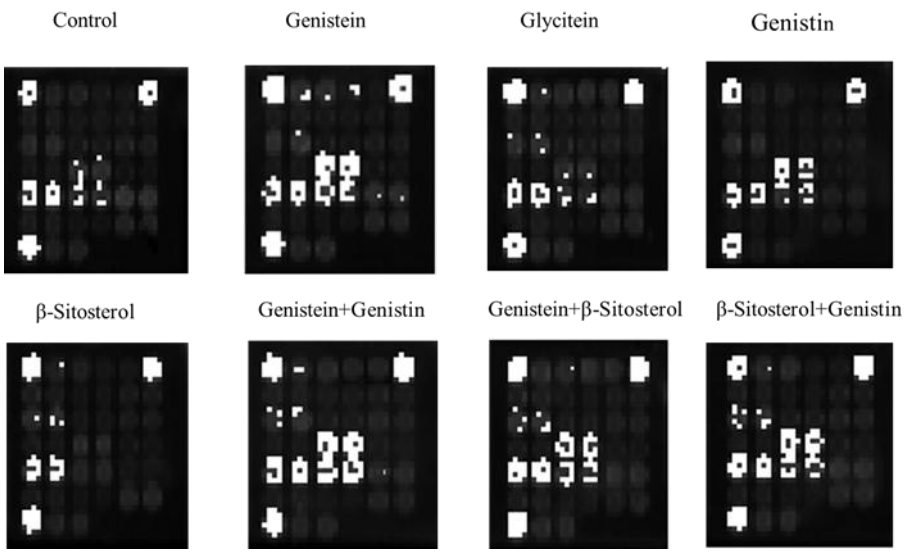

MCF-7
Control
Genistein
Daidzein
Glycitein
Genistin
Daidzin
Genistin+Daidzin

b

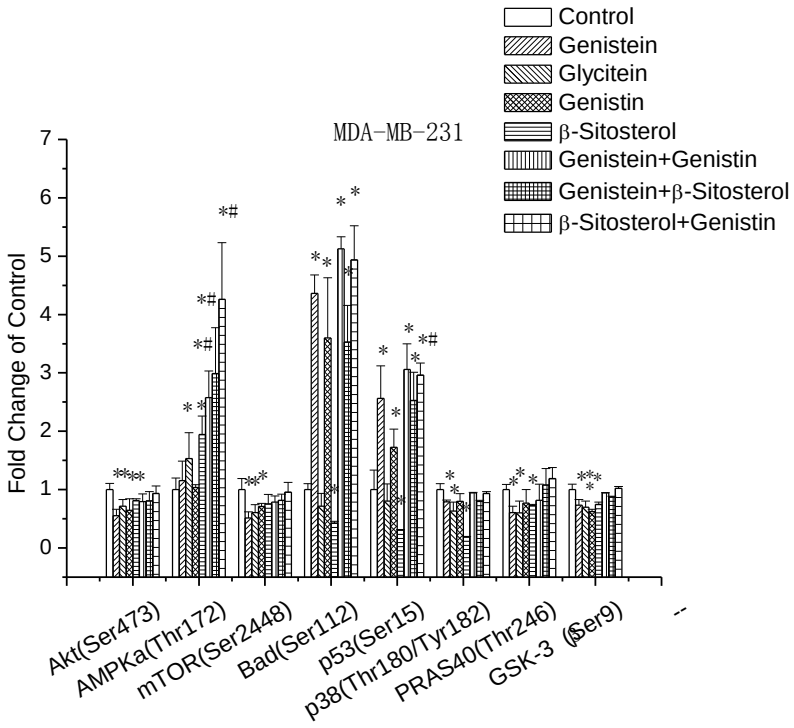

Control
Genistein
Daidzein
Glycitein
Genistin
Daidzin
Genistin+Daidzin
MCF-7
Fold Change of Control
2.0
1.5
1.0
0.5
0.0
stat3(Tyr705)
Akt(Ser473)
AMPKa(Thr172)
mTOR(Ser2448)
PRAS40(Thr246)

c

MDA-MB-231


Control
Genistein
Glycitein
Genistin
β-Sitosterol
Genistein+Genistin
Genistein+β-Sitosterol
β-Sitosterol+Genistin

d

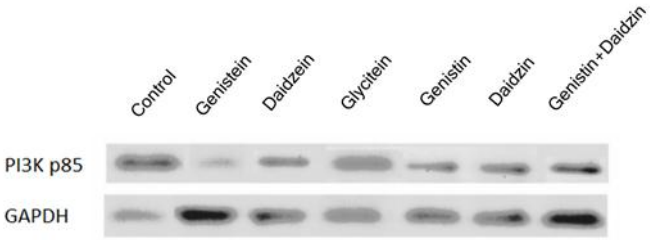


e

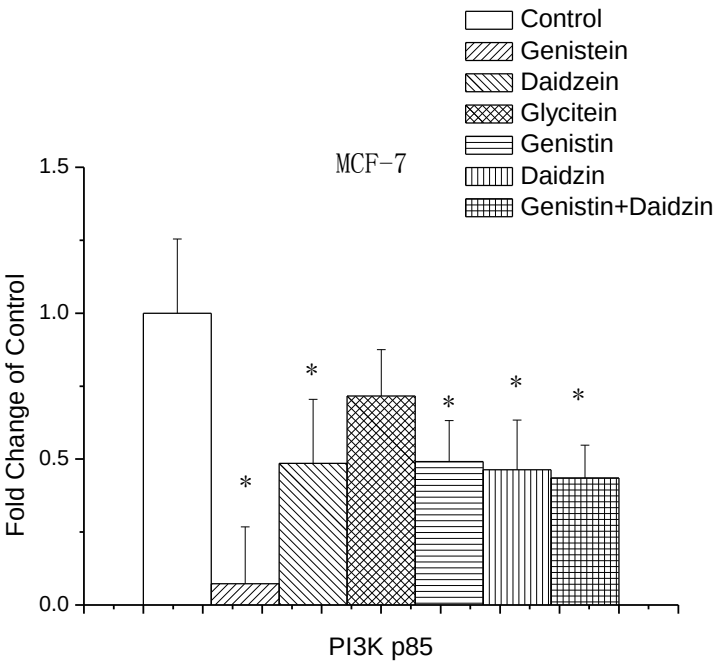
Figure 9. Modulations of intracellular signalling pathways. Cells were treated with samples, or control for 72 h. Total protein was extracted for the analysis. A slide-based antibody array was used for simultaneous detection of 18 signalling molecules (a) when phosphorylated or cleaved using a PathScan Intracellular Signalling Array kit. Image (b) and quantitative data (c) for MCF-7 cells as well as image (d) and quantitative (e) data for MDA-MB-23 cells were obtained using a LiCor Odyssey imaging system. Data are presented as mean ± SD. * indicates a significant difference compared to the control ($p<0.05$). #indicates a significant difference compared to treatment with samples by single ($p<0.05$).

It reported that PI3K/Akt/mTOR signalling pathway activation is heavily implicated in cancer pathogenesis (Lin et al., 2017). Inhibited phosphorylation of Akt and mTOR in this study suggested that inactivation of PI3K/Akt/mTOR pathway seems to be the main mechanism involved in anticancer activity of bioactive anticarcinogens from soybean both in MCF-7 and MDA-MB-231 and cells. To further confirm this hypothesis, the protein expression level of PI3K

p85 was determined using western-blot assay (**Fig. 10a, 10b, 10c and 10d**). As shown, reduction in the expression level of PI3K p85 was observed in these tested samples, which supported our hypothesis. In addition, change in the phosphorylation of molecular proteins including stat3 (a key target in cancer cell growth), Bad (a pro-apoptoic protein from Akt pathway), p53 (a a pro-poptotic protein well known tumour suppressor), p38 (a member of MAPK), PRAS40 (a protein which can inhibit Akt pathway) and GSK-3β (a key regulator in tumour development) was observed in this study (Cuenda and Rousseau, 2007; Hollstein et al., 1991; Luo et al., 2009; Madhunapantula et al., 2007).



a



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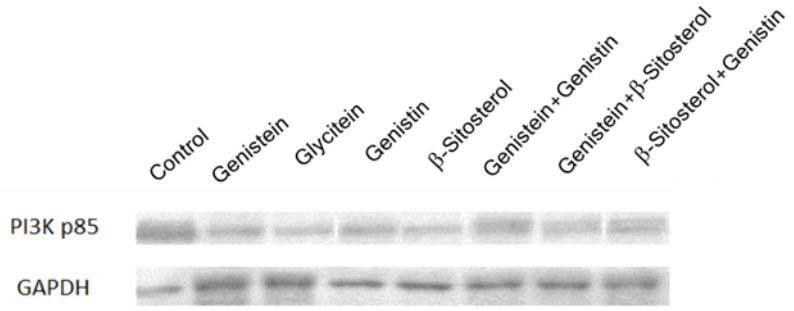
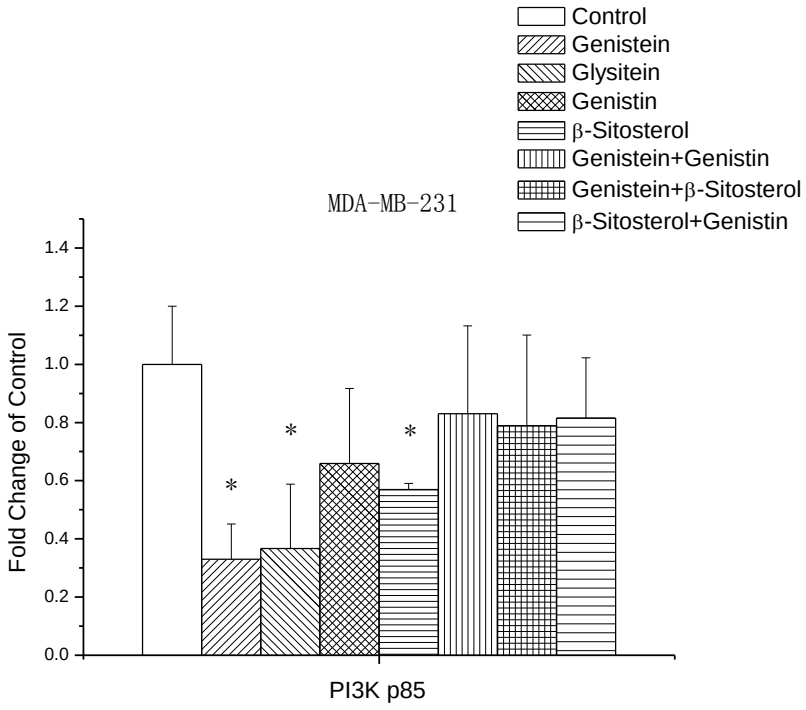


c



d

Figure 10. The protein expression level of PI3K p85. Cells were treated with samples, or control for 72 h. Total protein was extracted for the analysis using western blot. The signal

of target protein in MCF-7 cells (a, b) and MDA-MB-231 cells (c, d) was detected and quantified using SuperSignal ELISA Pico Chemiluminescent Substrate. Data are presented as mean ± SD. * indicates a significant difference compared to the control ($p<0.05$). #indicates a significant difference compared to treatment with samples by single ($p<0.05$).

5. Conclusion

In conclusion, the present study provides insights into the anti-proliferative activity of 12 anticarcinogens from soybean against MCF-7 and MDA-MB-231 human breast cancer cell lines by single or two-way combination treatment. Genistein, daidzein, gycitein, genistin and daidzin were demonstrated to have stronger anti-proliferative activity against MCF-7cells with EC_{50} values less than 200 μM. There is a synergistic effect of the combination treatment of genistin plus daidzinin MCF-7 cells. Gensitein, glycitein, genistin and β-sitosterol showed stronger anti-proliferative activity (EC_{50} values <200 μM) against MDA-MB-231 cells and a synergistic effect was observed in the combination of genistein plus genistin, genistein plus β-sitosterol and β-sitosterol plus genistin. In addition, these bioactive anticarcinogens were also able to inhibit invasion and migration of breast cancer cells and combination treatment enhanced the inhibitory effect. Regulation of PI3K/Akt/mTOR pathway seems to be the main mechanisms involved in the anticancer activity. Our results may partly contribute to the understanding of anticancer effect of dietary components in soybean. However, further studies are needed in clinical trials.

6. Reference

Awad A.B., Roy R., and Fink C.S. 2003. β-sitosterol, a plant sterol, induces apoptosis and activates key caspases in MDA-MB-231 human breast cancer cells. Oncol. Rep. 10(2): 497-500.

Clubbs E.A., and Bomser J.A. 2007. Glycitein activates extracellular signal-regulated kinase via vascular endothelial growth factor receptor signalling in nontumourigenic (RWPE-1) prostate epithelial cells. J. Nutr. Biochem. 18(8): 525-532.

Chun J., and Kim Y.S. 2013.Platycodin D inhibits migration, invasion, and growth of MDA-MB-231 human breast cancer cells via suppression of EGFR-mediated Akt and MAPK pathways. Chem-Bioll Interact. 205(3): 212-221.

Chiang P.C., Lin S.C., Pan,S.L., Kuo C.H., Tsai I. L., Kuo M.T., and Guh, J.H. 2010. Antroquinonol displays anticancer potential against human hepatocellular carcinoma cells: a crucial role of AMPK and mTOR pathways. Biochem. Pharmacol. 79(2): 162-171.

Cuenda A., and Rousseau S. 2007. p38 MAP-kinases pathway regulation,

function and role in human diseases. Bba-Mol. Cell Res. 1773(8): 1358-1375.

Dent R., Trudeau M., Pritchard K.I., Hanna W.M., Kahn H.K., Sawka C.A., and Narod S.A. 2007. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin. Cancer Res. 13(15): 4429-4434.

Felice D.L., Sun J., and Liu R.H. 2009. A modified methylene blue assay for accurate cell counting. J. Funct. Foods 1: 109-118.

Gladysheva I.P., Moroz N.A., Karmakova T.A. Nemtsova E.R., Yakubovskaya R.I., and Larionova N.I. 2001. Immunoconjugates of soybean Bowman-Birk protease inhibitor as targeted antitumour polymeric agents. J. Drug Target 9(5): 303-316.

Hsieh C.C., Martínez-Villaluenga C., de Lumen B.O., and Hernández-Ledesma B. 2018. Updating the research on the chemopreventive and therapeutic role of peptide lunasin. J. Sci. Food Agr. 98: 2070-2079.

Henning S.M., Wang P., and Heber D. 2011. Chemopreventive effects of tea in prostate cancer: green tea versus black tea. Mol. Nutr. Food Res. 55(6): 905-920.

Hedlund T.E., Maroni P.D., Ferucci P.G., Dayton R., Barnes S., Jones K., and Gray K.J. 2005. Long-term dietary habits affect soy isoflavone metabolism and accumulation in prostatic fluid in caucasian men. J. Nutr. 135(6): 1400-1406.

Høyer-Hansen M., and Jäättelä M. 2007. AMP-activated protein kinase: a universal regulator of autophagy? Autophagy 3(4): 381-383.

Hodge D.R., Hurt E.M., and Farrar W.L. 2005. The role of il-6 and stat3 in inflammation and cancer. Eur. J. Cancer Care 41(16): 2502-2512.

Hollstein M., Sidransky D., Vogelstein B., and Harris C.C. 1991. p53 mutations in human cancers. Science 253: 49-53.

Kim H.Y., Yu R., Kim J.S., Kim Y.K., and Sung M.K. 2004. Antiproliferative crude soy saponin extract modulates the expression of IκBα, protein kinase C, and cyclooxygenase-2 in human colon cancer cells. Cancer Letts. 210(1): 1-6.

Kaushik S., Shyam H., Sharma R., and Balapure A.K. 2016. Genistein synergizes centchroman action in human breast cancer cells. Indian J. Pharmacol. 48(6): 637.

Kozlova N., Samoylenko A., Drobot L., and Kietzmann T. 2016. Urokinase is a negative modulator of Egf‐dependent proliferation and motility in the two breast cancer cell lines MCF-7 and MDA-MB-231. Molecular Carcinogen 55(2): 170-181.

Leung H.W., Wong L.S., Yue G.G.L., Tsang J.Y.S., Lin Z.X., Tse G.M.K., and Lau C.B.S. 2016. Differential proliferative responses of estrogenic Chinese herbal medicines in breast cancer cells of different molecular subtypes. Planta. Medica. 82(S 01): P808.

Lin W., Chen S., and Liu X. 2017. Cisplatin regulates cell autophagy in endometrial cancer cells via the pi3k/akt/mtor signalling pathway. Oncol. Letter

13(5): 3567-3571.

Luo J. 2009. The role of glycogen synthase kinase 3β (gsk3β) in tumourigenesis and cancer chemotherapy. Cancer Letter 273(2): 194-200.

Messina M.J., Persky V., Setchell K.D., and Barnes S. 1994. Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data. Nutr. Cancer 21(2): 113-131.

Magee P.J., Raschke M., Steiner C., Duffin J.G., Pool-Zobel B.L., Jokela T., and Rowland I.R. 2006. Equol: a comparison of the effects of the racemic compound with that of the purified S-enantiomer on the growth, invasion, and DNA integrity of breast and prostate cells in vitro. Nutr. Cancer 54(2): 232-242.

Madhunapantula S.V., Sharma A., and Robertson G.P. 2007. Pras40 deregulates apoptosis in malignant melanoma. Cancer Res. 67(8): 3626.

Prashanta Kumar P., Subhadip M., Birendra B., Chandra Sekhar B., Sandeep D., and Durgesh Nandini D. 2014. Antitumour effect of soybean lectin mediated through reactive oxygen species-dependent pathway. Life Sci. 111(1-2): 27-35.

Siegel R.L., Miller K.D., and Jemal A. 2016. Cancer statistics. CA-cancer J. Clin. 66(1): 7-30.

Szliszka E., and Krol W. 2011. Soy isoflavones augment the effect of trail-mediated apoptotic death in prostate cancer cells. Oncol. Rep. 26(3): 533-541.

Tapia-Pizarro A., Argandoña F., Palomino W.A., and Devoto L. 2013. Human chorionic gonadotropin (hCG) modulation of TIMP1 secretion by human endometrial stromal cells facilitates extravillous trophoblast invasion *in vitro*. Human Reproduction 28(8): 2215-2227.

Wang P., Phan T., Gordon D., Chung S., Henning S. M., and Vadgama J. V. 2015. Arctigenin in combination with quercetin synergistically enhances the antiproliferative effect in prostate cancer cells. Mol. Nutr. Food Res. 59(2): 250-261.

Yang J., and Liu R. H. 2009. Synergistic effect of apple extracts and quercetin 3-β-D-glucoside combination on antiproliferative activity in MCF-7 human breast cancer cells *in vitro*. J. Agr. Food Chem. 57(18): 8581-8586.

Zhu Y., Yao Y., Gao Y., Hu Y., Shi Z., and Ren G. 2016. Suppressive Effects of Barley β−Glucans with Different Molecular Weight on 3T3−L1 Adipocyte Differentiation. J. Food Sci. 81(3): H786-H793.

3

Anti-inflammatory Activity of Natural Lunasin from Soybean and Recombinant Lunasin from *Pichia Pastoris* in LPS-stimulated RAW264.7 Cells

In chapter 2, we accessed the anticancer effect of 12 bioactive components from soybean. Although lunasin is not the most effective one, it succefully attracted our interests due to that it is a novel peptide and many problems about lunasin remained to be studied. In this study, natural lunasin was isolated and purified from defatted soybean and recombinant lunasin was obtained from P. pastoris. The anti-inflammatory activity of natural lunasin and recombinant lunasin was evaluated and compared in LPS-stimulated RAW264.7 cells. And the effect of lunasin on mTOR signalling pathways was investigated.

This chapter is mainly based upon the following publised article with the addition of unpublished data.

Zhu, Y., Nadia, E., Yao, Y., Shi, Z., & Ren, G. (2018). Tandem repeated expression of lunasin gene in pichia pastoris and its anti-inflammatory activity in vitro. Journal of Bioscience & Bioengineering.

1. Abstract

Lunasin is a novel promising health-beneficial peptide derived from soybean and has been demonstrated to have various bioactivities. In this study, we isolated natural lunasin (N-lunasin) from soybean and express recombinant lunasin (R-lunasin) from *P. pastoris*. The anti-inflammatory activity of N-lunasin and R-lunasin was compared. Results showed that there was a comparable effect of N-lunsin and R-lunasin on inhibition of release of NO, TNF-α and IL-6 in LPS-stimulated RAW264.7 macrophages in a dose-dependent manner. In addition, intracellular signalling array analysis demonstrated down-regulated levels of phosphorylated Akt, mTOR and p70 s6 kinase (p70s6k) and an up-regulated level of glycogen synthase kinase-3β (GSK-3β) after R-lunasin treatment. These results suggest that lunasin exerted anti-inflammatory activities in LPS-stimulated RAW264.7 cells partly via inhibiting the activation of Akt/mTOR/p70s6k signalling pathway.

Keywords: lunasin, soybean, *P. pastoris*, anti-inflammatory activity, Akt/mTOR/p70s6k pathway

2. Introduction

Soybean (*Glycine max*), an ancient food crop in East Asia, is generally consumed as a major and excellence source of dietary protein (Lule et al., 2015). Soy product consumption has been suggested to reduce the risk of various disorders such as different types of cancers (Ye et al., 2009) and cardiovascular disease (Rebholz et al., 2013). These effects are partially attributed to bioactive peptides derived from soybean protein. Among them, increasing interests have been focused on lunasin which is a unique 43-amino acid peptide sequence encoded within the soybean Gm2s-1 gene. Both *in vitro* and animal studies have demonstrated that lunasin peptide has anti-cancer, anti-inflammatory, antioxidant and cholesterol-lowing effects (Hernándezledesma et al., 2009; Hisieh et al., 2010). Chronic Inflammation has been found to underlie our most destructive chronic diseases such as cancer, cardiovascular diseases, type 2 diabetes, and Alzheimer's disease. Studies have shown that there were some common signal molecules in the formation of inflammation and tumour, such as the proteins related with the invasion, migration, and metabolism in cells, cytokines and its corresponding receptors. Therefore, in recent years, the anti-inflammatory activity of lunasin has attracted more attention than its other activities. Lunasin has been demonstrated to be able to inhibit the release of NO and pro-inflammatory cytokines including TNF-α and IL-6 in LPS-stimulated RAW264.7 cells (Hernándezledesma et al., 2009; Ren et al., 2017) by suppressing COX-2/PGE 2, iNOS/NO (Dia et al., 2009) and NF-κB pathways (Mejia and Dia, 2009). Recent studies have indicated that mechanistic target of rapamycin (mTOR) signalling also can be activated by LPS stimulation (Dos et al., 2007; Kawai et al., 2010). mTOR is the catalytic subunit of two multiprotein complexes, mTORC1 and mTORC2 (Hsu et al., 2011). It is believed that Akt-induced activation of NF-κB needs the participation of the mTORC1-associated protein raptor (Lee et al., 2016). However, whether lunasin inhibit mTOR signalling pathway has not been clarified.

To obtain lunasin, different methods including extraction of lunasin from natural plants, synthesis of lunasin through chemical method and expression of recombinant lunasin through genetic engineering have been developed (Kyle et al., 2009). Among these ways, genetic engineering is reported to be a practical and economical method to express target proteins. The yeast *Pichia pastoris* (*P. pastoris*) expression system is reported to have several advantages i. e. high production yield, genetically stable expression strain, the potential to secrete recombinant proteins freely into the medium, and inexpensive culture conditions for production of foreign proteins (Hou et al., 2007). In our previous study, recombinant lunasin was successfully produced from recombinant *P. pastoris* GS115 strain (unpublished data). However, the recombinant lunasin has a low

expression level (12.13 mmg/mL cell free broth) and was difficult to purify. Therefore, it is necessary to put forward a new expression strategy. In addition, the bioactivity of recombinant lunasin remained to be evaluated.

In this study, we isolated and purified natural lunasin (N-lunasin) from defatted soybean flour. At the same time, a formation lunasin gene was expressed in *P. pastoris*, resulting in the production of a dimer formation protein, and then the recombinant lunasin analog (R-lunasin) was obtained by enterokinase digestion. The anti-inflammatory activity of N-lunasin and R-lunasin was evaluated in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. The effect on mTOR signalling pathway was investigated.

3. Materials and methods

3.1 Isolation and purification of N-lunasin from defatted soybean flour

Defatted soybean flour was commercially available. The isolation and purification of N-lunasin was performed using methods reported by Seber et al. (2012) with some modification. Briefly, 100 g of quinoa flour was dissolved in PBS buffer (0.1 M, pH 7.4, 1:15, w/v) by shaking for 48 h at 4 ℃ and the supernatant was collected after two runs of high speed centrifugation (12000 ×g, 30 min). The low molecular weight fraction (>1 kDa, < 10 kDa) was obtained from the supernatant through ultrafiltration at 4 ℃ (Millipore ultrafiltration tube, cut offs of 1 kDa and 10 kDa; Millipore Co., USA). This lunasin enriched faction was further purified using Q-Sepharose FF chromatography. As reported, a 0.35 M NaCl elution fraction (lunasin-enriched fraction) was collected and analysed by UPLC-ESI-MS. For further purification, DEAE ion-exchange chromatography was used. Lunasin was eluted using a step gradient (0-1 M NaCl) at a flow rate of 4 mL/min, and the column fractions were analysed by western-blot assay with anti-lunasin epitope-EKHIMEKIQGRGDDDDD. The lunasin-enriched fractions were collected and desalted.

3.2 Expression and purification of R-lunasin in P. pastoris

A gene fragment encoding 4 repeat lunasin analogs with optimized codon for *P. pastoris* was designed as follows:
TCCAAATGGCAGCATCAGCAGGACAGTTGCAGAAAGCAGCTGCAAGGT
GTTAATCTTACACCTTGCGAAAAGCACATCATGGAAAAGATACAAGGC
AGAGGCGACGACGACGATGATGATGATGACGACCTGGTGCCGCGCGGC
AGCTCAAAGTGGCAACATCAGCAGGATTCCTGTAGGAAACAGTTACAA
GGTGTCAACCTTACTCCCTGCGAAAAACACATTATGGAAAAAATACAA
GGAAGAGGTGACGACGACGACGATGACGACGACGACCTGGTGCCGCGC

GGCAGCAGTAAGTGGCAACATCAACAAGACTCTTGCAGAAAACAGCTA
CAAGGAGTCAATTTAACCCCCTGCGAAAAACATATCATGGAAAAAATA
CAGGGAAGAGGTGATGATGATGACGACGATGATGACGATCTGGTGCCG
CGCGGCAGCAGTAAATGGCAACATCAGCAAGATAGTTGCAGAAAGCAA
TTGCAGGGCGTGAATCTAACGCCATGCGAGAAGCACATCATGGAGAAG
ATTCAAGGCAGGGGAGACGACGATGATGATGACGACGACGATCTGGTG
CCGCGCGGCAGCAGCAGCGGCCACCATCACCATCACCATTAA. The first 4 underlined nucleotides indicate the location of enterokinase digestion. The last underlined nucleotide indicate a tag of six histidine residues (6×His) at its C end. The construction of recombinant strain was conducted by Sangon Biotech Co. Ltd (Shanghai, China) using GS115 strain. The clones grown on 4 mg/mL G418 YPD plates were selected as recombinant expression strain. Small-scale expression was performed to test the production of the recombinant protein. An SDS-page and an western-blot assay was carried out as previously described (Zhu et al., 2016). Mouse monoclonal antibody against His-tag was used as the primary antibody. Recombinant protein was purified using a Ni-NTA column and freeze-dried. Then more small-scale expression was performed to optimize the expression conditions. The factors including initial pH value (5.0-8.0), final methanol concentration (0.25-2.0 %), incubation time (24-96 h) and incubation temperature (26-30 ℃) were tested. The expression level was determined by densitometric scan using GelDoc system (Bio-Rad Laboratories, Italy). The purified recombinant protein was used as standard (results were performed in the annex).

Large scale expression was performed under the optimum condition and recombinant protein was purified. Then enterokinase digestion was conducted by adding 1 unit of enzyme per μg of protein at 37 ℃ for 2 h. The digestion solution was further separated through ultrafiltration (Millipore ultrafiltration tube, cut offs of 3 kDa and 10 kDa; Millipore Co., USA). Concentrated solution between 3-10 kDa was collected and freezed-dried to obtain R-lunasin.

3.3. Cell culture

The mouse macrophage cell line RAW264.7 was purchased from the Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 medium supplemented with 1% penicillin/streptomycin/glutamine (100×) and 10% FBS at 37 ℃ in 5% CO_2/95% air.

3.4. Cell cytotoxicity assay

The cytotoxicity of N-lunasin and R-lunasin towards RAW264.7 cells was evaluated using the MTT assay as reported by Zhu et al. (2016) with some modifications. RAW264.7 cells were seeded at a density of 1×10^4 cells per well in

a 96-well plate, allowed to adhere overnight, and incubated for 24 h in the absence (as control group) or presence of different concentrations (0.03-0.5 mg/mL) of N-lunasin or R-lunasin. At the end of the treatment, 20 μL of MTT solution (1 mg/mL) was added to each well and the plates were incubated at 37 ℃ for 4 h in a dark environment. Then the supernatant was removed and 200 μL of DMSO was added to each well to solubilize formazan crystals. The absorbance at 570 nm was read using a microplate reader (Bio-Rad, MA, USA). The results were expressed as percentage of the control.

3.5. Anti-inflammatory activity assay

The anti-inflammatory activity was determined according to a previously described method reported by Dia et al. (2009). RAW264.7 cells were seeded into 96-well plates at a density of 2×10^5 cells per well. After 80-90% confluency, cells were treated with or without (as positive control) different concentrations (0.03-0.5 mg/mL) of N-lunasin or R-lunasin for 24 h in presence of LPS (1 μg/mL). Cells cultured in RPMI-1640 medium without any treatment were used as negative control. The supernatants were collected from each well and stored at -20 ℃ until analysis.

The accumulated NO in the medium was measured using griess reagent (Sigma-Aldrich, St. Louis, MO, USA). TNF-α and IL-6 levels were measured using commercial ELISA assay kits (BD Bioscience, San Jose, CA, USA).

3.6. Intracellular signal pathways assay

RAW264.7 cells were seeded into a 6-well plate at a density of 2×10^5 cells per well and treated with or without R-lunasin (0.5 mg/mL) as described above. After 24 h treatment, whole cell lysates were collected using ice-cold lysis RIPA buffer (Fluorescent Readout, Cell Signalling Technology, Danvers, MA, USA) supplemented with 1% PMSF and stored at -20 ℃ until analysis. The PathScan Intracellular Signalling array kit (Fluorescent Readout, Cell Signalling Technology, Danvers, MA, USA) was used according to the manufacturer's instructions, to simultaneously detect 18 phosphorylated or cleaved signalling molecules. The images were captured by a LiCor Odyssey imaging system (Li-Cor Biosciences, Lincoln Nebraska) and quantified data were obtained using a LiCor Odyssey imaging system (Li-Cor Biosciences, Lincoln Nebraska).

3.7. Statistical analysis

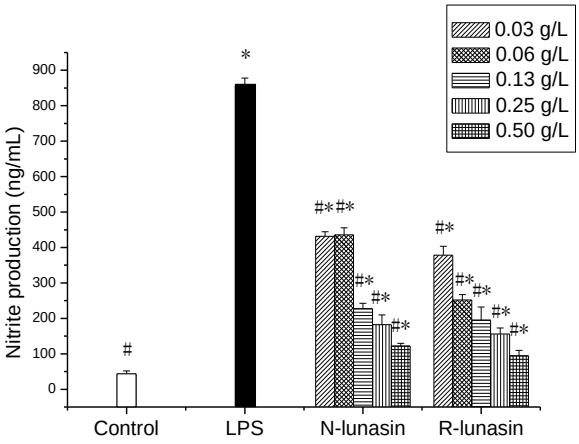
Data in this study were analyzed using one way ANOVA and Tukey's test performed by SPSS (Statistics for Social Science) version 17.0 (IBM, New York , USA). All the experiments were conducted in triplicate (n=3). Significant

differences were reported at $p<0.05$.

4. Results and discussion

4.1. Anti-inflammatory activity

The yeast *P. pastoris* expression system (GS115 strain) was demonstrated to be an effective tool for production of R-lunasin, and the highest expression level at the optimal condition was 240 mg/L cell-free broth (**shown in the annex**). In this study, both the purified N-lunasin and R-lunasin were successfully obtained. We next compared the anti-inflammatory activity of N-lunasin and R-lunasin. Inflammation is involved in many chronic diseases such as cardiovascular disease and cancer (Moutsopoulos et al., 2010). LPS is a major component of the outer membrane of Gram negative bacteria and can promote the production of pro-inflammatory cytokines in phagocytic cells (Hernández-ledesma et al., 2009). Both chemical synthetic lunasin and natural lunasin from various plants have been reported to exert anti-inflammatory activity on LPS-stimulated RAW264.7 macrophages cells (Hernández-ledesma et al., 2009; Dia et al., 2009). Results of the present study showed that the production of NO, TNF-α and IL-6 were significantly increased ($p<0.05$) in LPS-stimulated RAW264.7 cells (**Fig. 11a, 11b and 11c**). Treatment with N-lunasin and R-lunasin inhibited this increase in a dose-dependent manner. These results suggested that both N-lunasin and R-lunasin has anti-inflammatory activity on macrophage cells. As shown, R-lunasin was more effective in decrease of the NO and TNF-α levels, while N-lunasin was more effective in decrease of IL-6 level.



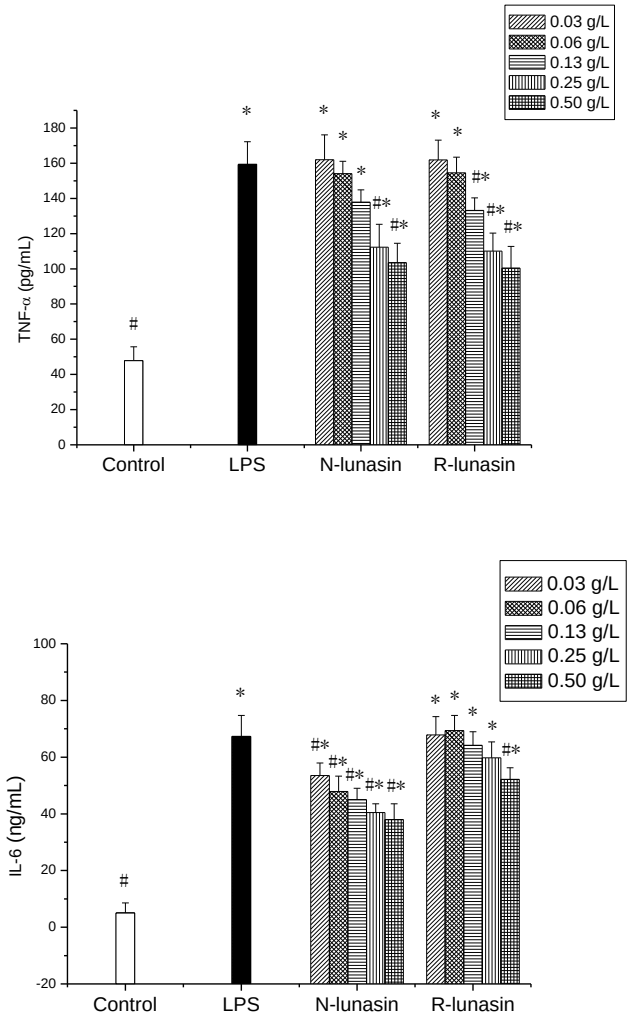


Figure 11. Anti-inflammatory activity of N-lunasin and R-lunasin. The inhibitory effects of lunasin analog (0.03-0.50 g/L) on release of NO (a), TNF-α (b) and IL-6 (c) in LPS-stimulated RAW264.7 cells. Data were shown as mean ± SD, n=3. *p<0.05 compared to the control group. #p<0.05 compared to the LPS stimulated group.

4.2. Cell cytotoxiicity

In the cell cytotoxicity assay (**Fig. 12**), no significant difference in cell viability was observed at the tested concentrations (0.03-0.50 mg/mL), which indicated that

both N-lunasin and R-lunasin showed no cytotoxicity towards RAW264.7 cells at these concentrations. Consistent with our results, both Hsieh et al. (2017) and Mejia et al. (2009) reported that lunasin has no effects on the cell viability of RAW264.7 cells.

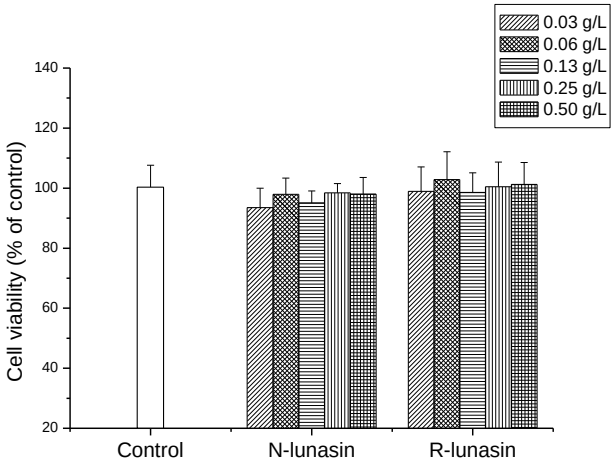


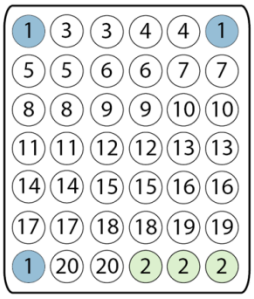
Figure 12. Cell cytotoxicityivity of N-lunasin and R-lunasin. MTT assay results (0.03-0.50 mg/mL) towards RAW264.7. Data were shown as mean ± SD, n=3. *p<0.05 compared to the control group. #p<0.05 compared to the LPS stimulated group.

4.3. Modulations of signalling pathway

LPS activates a broad range of signalling pathways in macrophages (Mendes et al., 2009). A previous study have demonstrated the capability of lunasin to inhibit inflammation by inhibiting the LPS-stimulated transactivation of NF-κB pathway and NF-kB is the major targets of lunasin to exert its anti-inflammatory action (Mejia and Dia, 2009). Recent studies have indicated that mechanistic target of rapamycin (mTOR) signalling also can be activated by LPS stimulation (Dos et al., 2007; Kawai et al., 2010). mTOR is the catalytic subunit of two multiprotein complexes, mTORC1 and mTORC2 (Hsu et al., 2011). It is believed that Akt-induced activation of NF-κB needs the participation of the mTORC1-associated protein Raptor (Lee et al., 2016). However, whether lunasin inhibit mTOR signalling pathway has not been clarified. In this study, R-lunasin showed a almost comparable anti-inflammatory activity with N-lunasin. So we just used R-lunasin to clarify its effect on mTOR signalling pathway. A slide-based antibody array was used for simultaneous detection of 18 important and well-characterized signalling molecules. These molecules were listed in **Fig. 13a**. Results showed that LPS significantly increased (p<0.05) phosphorylation levels of Akt at Ser 473 residues

accompanied by mTOR and p70s6k activation (**Fig. 13b and 13c**), which is consistent with Wang et al. (2014). Akt is an upstream kinase regulating mTOR activity and p70s6k is downstream to mTOR. R-lunasin treatment strongly inhibited the phosphorylation of Akt, mTOR and p70s6k at the concentration of 0.5 g/L, which suggested that lunasin could suppress LPS-induced activation of Akt/mTOR signalling. Wang et al. (2014) and Weinstein et al. (2000) found that elevated mTOR signalling might be responsible for the increased TNF-α and NO production in macrophages in response to LPS stimulation. This suggested that lunasin may inhibit TNF-α and NO production via mTOR pathway.

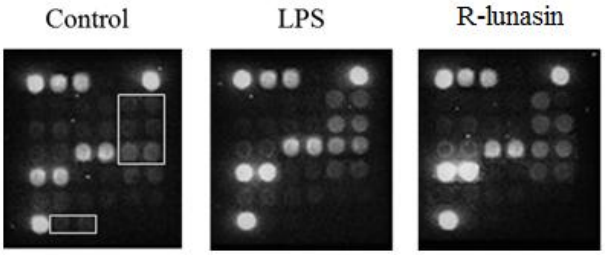
We determined that the inhibition of GSK-3β expression in LPS-stimulated cells was up-regulated by R-lunasin treatment **(Fig. 13b and 13c)**. Previous study proved that the phosphorylation of GSK-3β at Ser9 decreases the expression of IL-6 and inducible nitric oxide synthase (iNOS) in LPS-stimulated cells (Beurel and Jope, 2009). Consequently, it was concluded that the down-regulation of IL-6 and NO was also dependent on GSK-3β inhibition.



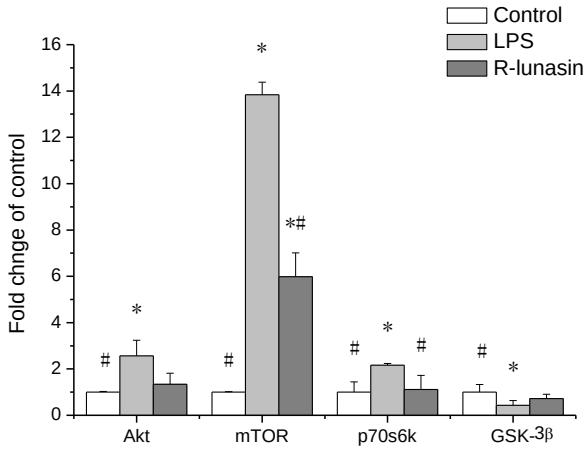
Intracellular Signaling

	Target	Phosphorylation Site	Modification
1	Positive Control	N/A	N/A
2	Negative Control	N/A	N/A
3	ERK1/2	Thr202/Tyr204	Phosphorylation
4	Stat1	Tyr701	Phosphorylation
5	Stat3	Tyr705	Phosphorylation
6	Akt	Thr308	Phosphorylation
7	Akt	Ser473	Phosphorylation
8	AMPKα	Thr172	Phosphorylation
9	S6 Ribosomal Protein	Ser235/236	Phosphorylation
10	mTOR	Ser2448	Phosphorylation
11	HSP27	Ser78	Phosphorylation
12	Bad	Ser112	Phosphorylation
13	p70 S6 Kinase	Thr389	Phosphorylation
14	PRAS40	Thr246	Phosphorylation
15	p53	Ser15	Phosphorylation
16	p38	Thr180/Tyr182	Phosphorylation
17	SAPK/JNK	Thr183/Tyr185	Phosphorylation
18	PARP	Asp214	Cleavage
19	Caspase-3	Asp175	Cleavage
20	GSK-3β	Ser9	Phosphorylation

a



b



c

Figure 13 Modulations of intracellular signalling pathways. A slide-based antibody array was used for simultaneous detection of 18 signalling molecules (a) when phosphorylated or cleaved using a PathScan Intracellular Signalling Array kit. Image (b) and quantitative data (c) was obtained using a LiCor Odyssey imaging system. Data are presented as mean ± SD, n=3. *p<0.05 compared to the control group. #p<0.05 compared to the LPS stimulated group.

5. Conclusion

In the present study, we isolated natural lunasin from soybean and expressed recombinant lunasin from *P. pastoris*. Then we demonstrated anti-inflammatory activity of N-lunasin and R-lunasin in LPS-stimulated RAW264.7 cells. R-lunasin exerted anti-inflammatory effect via inhibiting the activation of Akt/mTOR/p70s6k

signalling pathway. In addition, the expression level at the optimal condition (initial pH 7.0, 1.0% final methanol concentration and induction for 72 h at 26 ℃) was 240 mg/L cell-free broth.

6. Reference

Beurel E., and Jope R.S. 2009. Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain, J. Neuroinflamm., 9, 23.

Dos Santos S., Delattre A. I., De Longueville F., Bult H., and Raes M. 2007. Gene expression profiling of LPS-stimulated murine macrophages and role of the NF-κB and PI3K/mTOR signalling pathways, Ann. Ny. Acad. Sci., 1096, 70-77.

Dia V. P., Wang W., Oh V. L., Lumen B. O. D., and Mejia E. G. D. 2009. Isolation, purification and characterisation of lunasin from defatted soybean flour and in vitro evaluation of its anti-inflammatory activity. Food Chemistry, 114(1), 108-115.

Gorgenes J. F., Van Zyl W. H., Knoetze J. H., and Hahn-Hagerdal B. 2005. Amino acid supplementation improves heterologous protein production by Saccharomyces cerevisiae in defined medium, Appl. Microbiol. Biotechnol., 67, 684–691.

Hernández ledesma B., Hsieh C. C., and de Lumen B. O. 2009. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW264.7 macrophages, Biochem. Biophys. Res. Commun., 390(3), 803-808.

Hou J., Yan R., Yang L., Zhiqiang W. U., Wang C., Ding D., Li N., Ma C., and Li M. 2007. High-level expression of fusion protein containing 10 tandem repeated glp-1 analogs in yeast and its biological activity in a diabetic rat model, Biosci. Biotechnol. Biochem., 71(6), 1462-1469.

Hsieh C. C., Chou M. J., and Wang C. H. 2017. Lunasin attenuates obesity-related inflammation in raw264.7 cells and 3t3-l1 adipocytes by inhibiting inflammatory cytokine production, Plos One, 12(2), e0171969.

Hsieh C. C., Hernández ledesma B., and Lumen B. O. D. 2010. Soybean peptide lunasin suppresses in vitro and in vivo 7,12-dimethylbenz[a]anthracene-induced tumourigenesis. J. Food Sci., 75(9), H311-H316.

Hsu P. P., Kang S. A., Rameseder J., Zhang Y., Ottina K. A., Lim D., Peterson T. R., Choi Y., Gray N. S., Yaffe M. B., Marto J. A., and Marto J. A. 2011. The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signalling, Science, 332(6035), 1317-1322.

Kawai T., and Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, Nat. Immunol., 11(5), 373-384.

Kyle S., Aggeli A., Ingham E., and McPherson M. J. 2009. Production of self-assembling biomaterials for tissue engineering. Trends Biotechnol, 27, 423-433

Lee d. H., and Lee C. S. 2016. Flavonoid myricetin inhibits tnf-α-stimulated production of inflammatory mediators by suppressing the akt, mtor and nf-κb pathways in human keratinocytes, Eur. J. Pharmacol., 784, 164-172.

Lule V. K., Garg S., Pophaly S. D., and Tomar S. K. 2015. Potential health benefits of lunasin: a multifaceted soy-derived bioactive peptide, J. Food Sci., 80(3), R485-R494.

Liu C.F. and Pan T. M., 2010. Recombinant expression of bioactive peptide lunasin in Escherichia coli. Appl. Microbiol. Biotechnol., 88, 177-86.

Mejia E. G., and Dia V. P. 2009. Lunasin and lunasin-like peptides inhibit inflammation through suppression of nf-kappab pathway in the macrophage. Peptides, 30(12), 2388-2398.

Mendes S. D. S., Candi A., Vansteenbrugge M., Pignon M. R., Bult H., Boudjeltia K. Z., Munaut C., and Raes M. 2009. Microarray analyses of the effects of NF-κB or PI3K pathway inhibitors on the LPS-induced gene expression profile in RAW264. 7 cells: synergistic effects of rapamycin on LPS-induced MMP9-overexpression, Cell. Signal., 21(7), 1109-1122.

Moutsopoulos N. M., and Madianos P. N. 2010. Low-Grade Inflammation in Chronic Infectious Diseases, Ann. NY. Acad. Sci., 1088(1), 251-264.

Rebholz C. M., Reynolds K., Wofford M. R., Chen J., Kelly T. N., and Mei H., Whelton P. K., and He J. 2013. Effect of soybean protein on novel cardiovascular disease risk factors: a randomized controlled trial, Eur. J. Clin. Nutr, 67(1), 58-63.

Ren G., Zhu Y., Shi Z., and Li J. 2017. Detection of lunasin in quinoa (chenopodium quinoa willd.) and the in vitro evaluation of its antioxidant and anti-inflammatory activities. J Sci Food Agric, 97(12), 4110-4116.

Seber L. E., Barnett B. W., Mcconnell E. J., Hume S. D., Cai J., and Boles K., et al. 2012. Scalable purification and characterization of the anticancer lunasin peptide from soybean. Plos One, 7(4), e35409.

Wang Y., Liu Y., Zhang X. Y., Xu L. H., Ouyang D. Y., Liu K. P., Pan H., He J., and He X. H. 2014. Ginsenoside Rg1 regulates innate immune responses in macrophages through differentially modulating the NF-κB and PI3K/Akt/mTOR pathways, Int. Immunopharmac., 23(1), 77-84.

Weinstein S. L., Finn A. J., Davé S. H., Meng F., Lowell C. A., Sanghera J. S., and DeFranco A. L. 2000. Phosphatidylinositol 3-kinase and mtor mediate lipopolysaccharide-stimulated nitric oxide production in macrophages via interferon-beta, J. Leukocyte Biol., 67(3), 405-414.

Ye W. H., Soo Y. K., Sun H. J., Youn N. K., and Chung M. N. 2009. Soy food consumption and risk of prostate cancer: a meta-analysis of observational studies, Nutr. Cancer, 61(5), 598-606.

Zhu Y., Yao Y., Gao Y., Hu Y., Shi Z., and Ren G. 2016. Suppressive Effects of

Barley β-Glucans with Different Molecular Weight on 3T3-L1 Adipocyte Differentiation, J. Food Sci, 81, H786-H793.

7. Annex

Fig. 14 showed the construction of recombinant vector conducted by Sangon Biotech Co. (Shanghai, China). Transformants that resist a high concentration (4 mg/mL) of G418 (**Fig. 15**), was selected to verify genomic integration of the expression cassette by colony PCR. PCR results were shown in **Fig. 16**, a fragment about 2.2 K and a fragment about 0.5 K was amplified from the negative control respectively suggesting the presence of the complete AOX1 gene and a partial AOX1 gene. However, a fragment about 2.2 K and a fragment about 1.1 K was amplified from transformants of L1-L7. These results demonstrated that the desingnd lunasin gene fragment was successfully integrated into the *P. pastoris* GS115 genome.The production of recombinant protein was determined by SDS-PAGE and western-blot assay (**Fig. 17**). A new ~45 kDa protein secreted from positive transformants, compared to the negative strain. According to the designed gene fragment, theoretically, the recombinant protein is a ~23 kDa protein. These results suggested that a process of dimerization may occur during expression of recombinant protein. Then western-blot assay further supported it. It is reported that optimization and tight control of induction conditions are necessary to increase the recombinant protein expression level (Gorgenes et al., 2005). Therefore, after confirmation of production of remcobinant protein, the induction conditions were optimized to improve the expression level. As shown in **Fig. 18**, the optimal expression condition was as follows: initial pH 7.0, 1.0% final methanol concentration and induction for 72 h at 26 ℃. The highest expression level of the recombinant protein at the optimal condition was 240 mg/L cell-free broth. Liu et al. (Liu et al., 2010) had used the *E. coli* expression system to express the lunasin peptide, which can produce 4.73 mg recombinant lunasin in every liter of Luria-Bertani culture medium. Our results showed that the yeast system is more suitable than *E. coli* expression system. Compared with extraction of lunasin from soybean, it is easier to purify. Finally, R-lunasin was obtained from recombinant protein through enterokinase digestion and ultrafiltration. The results of the SDS-PAGE revealed that the molecular weight of R-lunasin was about 4.6 kDa (**Fig. 19**).

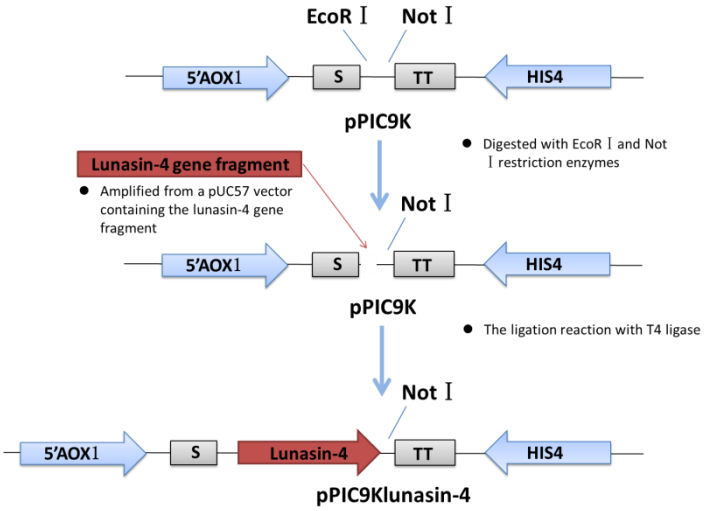


Figure 14 The schematic diagram showing the designed lunasin gene expression within pPIC9K vector.

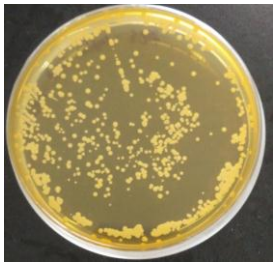


Figure 15 G418 selection (4 mg/mL) of P. pastoris transformants.

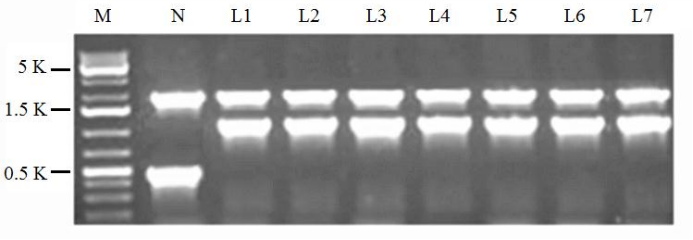


Figure 16 Verification of transformants by PCR analysis with 5'-and 3'-AOX1 primers. M, protein maker; N, negative control (GS115 strain transformed with pPIC9K); L1-L7, seven positive transformants.

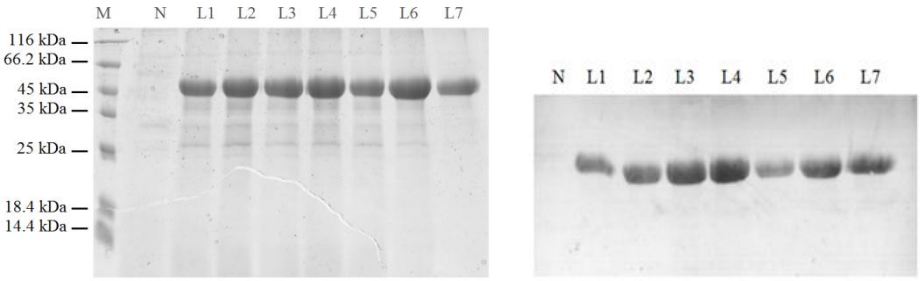


Figure 17 Production of recombinant protein. M, protein maker; N, negative control (GS115 strain transformed with pPIC9K); L1-L7, seven positive transformants.

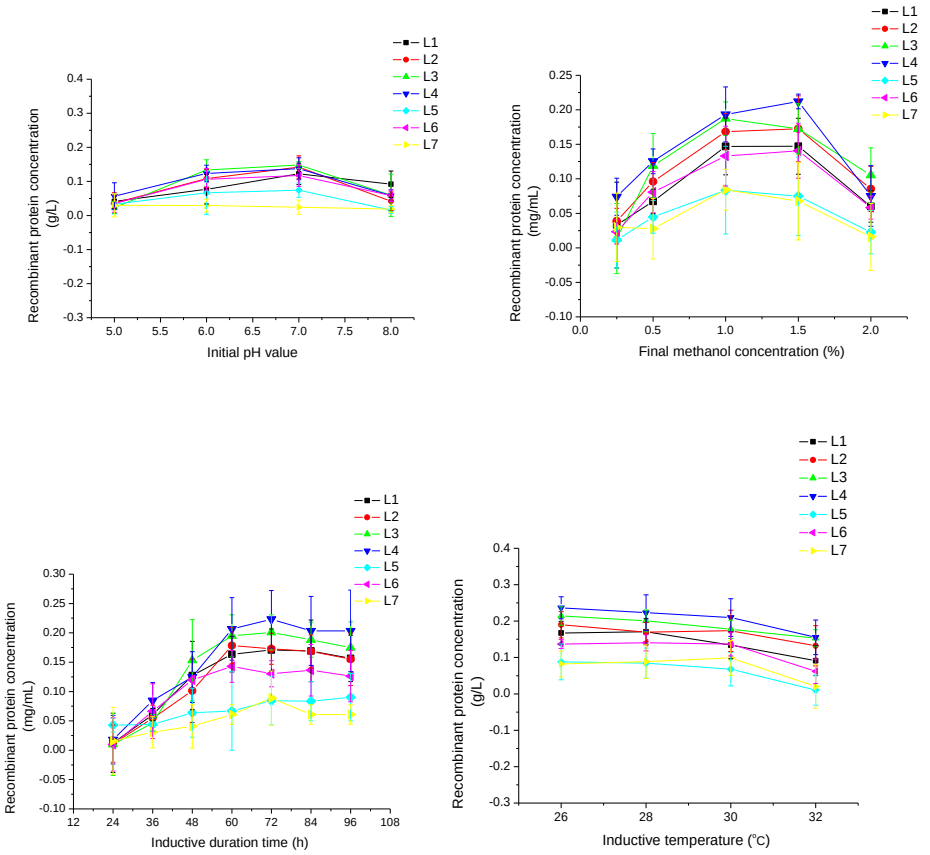


Figure 18 Optimization of expression conditions. L1-L7, seven positive transformants. Quantitative data was obtained by densitometric scan using GelDoc system. Data are presented as mean ± SD, n=3.

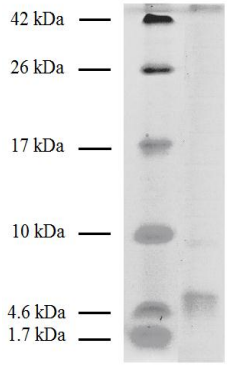


Figure 19 SDS-PAGE analysis of R-lunasin.

4

Revealing Regional Distribution of Soybean Lunasin Content in China and the Effects of Climate Factors by Extensively Sampling

In chapter 3, to obtain natural lunasin, the method of isolation from soybean was used. As previously reported, the highest yield of lunasin isolated from soybean is 442 mg/kg defatted soy flour. We hypothesized that if there is a high lunasin content soybean cultivar, the yield could be improved. Therefore, in this chapter, we collected 413 soybean samples from 4 different regions in China and gathered the climate data of these regions. The lunasin content was determined. We aimed to screen the high lunasin content soybean cultivar from these samples, reveal the regional distribution of soybean lunasin content in China, and analyze the effects of climate factors on lunasin content.

This chapter is adapted from a submitted paper.

Zhu, Y., Song, W., Everaert, N., Shi, Z., Han, T.,& Ren, G. Revealing Regional Distribution of Soybean Lunasin Content in China and the Effects of Climate Factors by Extensively Sampling. Journal of the Science of Food and Agriculture (under revision)

1. Abstract

Lunasin is a novel therapeutic peptide that was initially isolated from soybean. Soybean is widely cultivated from north China to south China. In this study, we quantified the variations in lunasin content in a total of 413 soybean samples that were collected from four major regions in China and harvested in 2014 and 2015 to reveal regional the distribution of soybean lunasin content in China and the effects of climate factors. The results showed that the cultivar Changmidou 30 collected from Jilin province and harvested in 2015 had the highest lunasin content (3.25 mg/g dry seeds). The data from both 2014 and 2015 showed that the lunasin content in soybean collected from north China was significantly higher ($p<0.05$) than that from south China. There was a positive correlation ($p<0.01$) between lunasin content and hours of sunshine (HS) as well as diurnal temperature range (DTR); however, there was a negative correlation ($p<0.01$) between lunasin content and rainfall (RF). In addition, combined analysis of data from 2014 and 2015 demonstrated that DTR was the dominant factor that affected lunasin content with a direct path-coefficient of 0.301. These results could contribute to guide the cultivation of soybean with high lunasin content.

Keywords: Soybean, lunasin content, regional distribution, LC/MS-MS, climate factors

2.Introduction

Soybean (*Glycine max*) is an ancient and important industrial crop in Asia. Currently, the potential health benefits of soybean food have attracted much attention. Lunasin, a unique peptide composed of 43 amino acids with a molecular mass of 5.5 kDa, was first isolated from soybean at the Niigata University School of Medicine in Japan in 1987 (Odani et al., 1987). It contains an Arg-Gly-Asp cell adhesion motif and a conserved chromatin-binding region for binding to histone H3/H4 (Singh et al., 2012). It contains 9 Asps at its C-terminus end, which might be involved in anti-m itotic functions (Hernández et al., 2011). Lunasin has been widely demonstrated to have various bioactivities, such as anticancer, anti-inflammatory, anti-oxidant and cholesterol-lowering activities (Dia et al., 2009; Gavez et al., 2012; Hernández et al., 2009; Lam et al., 2003). The bioactivity of lunasin depends on its concentration in materials (Wang et al., 2008). Therefore, soybean accession possessing a high lunasin content will be an ideal functional food. It has been reported that the lunasin content in soybean seeds is influenced by both genetic and environmental factors, such as cultivar, planting temperature and light conditions (Wang et al., 2008). However, most of these results were based on controlled experiments in green-houses or with limited sampling (Gonzalez et al., 2004; Park et al., 2005;). A comprehensive and indepth analysis of lunasin content in soybean seeds by extensively sampling over broad ecological regions is required to reveal the regional distribution of lunasin content and the effect of different eco-physiological factors of lunasin content on soybean seeds.

In China, soybean is widely cultivated from north China to south China, and the yields were 12.15, 11.78 million tons and 6.79, 6.80, 651 million hectares in 2014 and 2015, respectively (http://zdscxx.moa.gov.cn:8080/misportal/public/dataChannelRedStyle.jsp). According to the variations in environmental conditions, the soybean cultivation area was divided into four major regions, which include the Northeast Spring Planting Sub-region (NESR), the Northwest Spring Planting Sub-region (NWSR), the Huang-Huai-Hai Valleys Summer Planting Region (HHHR) and the South Multiple Cropping region (SMCR) (Yang et al., 2012). Such planting situation of soybean enables the investigation of the regional distribution of lunasin content in soybean seeds and the relationship between soybean lunasin content and climate factors on a national scale. In this study, a total of 204 and 209 soybean samples were collected from four major cultivation regions in 2014 and 2015, respectively. The lunasin contents in these soybean samples were analysed using an UPLC/MS-MS system.

3. Materials and Methods

3.1 Plant Materials

A total of 238 and 236 soybean samples were collected from 2014 and 2015, respectively. These samples represented four major soybean cultivation regions of China: the NESR, NWSR, HHHR and SMCR (**Fig. 20 and Table 3**). The number of samples, the maturity groups and the geographical conditions in each region are shown in **Table 3**.

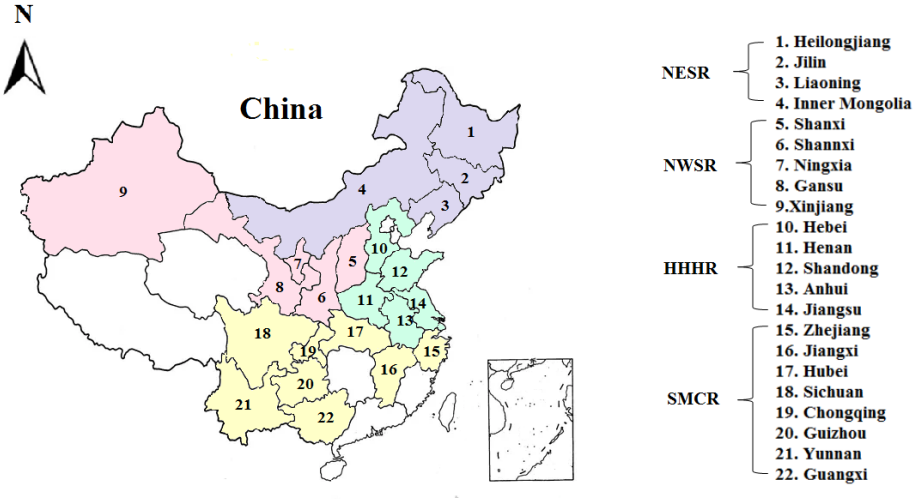


Figure 20 Four major regions where we collected soybean samples in China.

Table 3. Geographic characterization, varieties information and climate conditions of four major regions of China.

	NESR[#]	NWSR[#]	HHHR[#]	SMCR[#]
Province	Heilongjiang, Jilin, Liaoning, Inner Mongolia	Shaanxi, Gansu, Ningxia, Xinjiang, Shanxi	Hebei, Shandong, Henan, Anhui, Jiangsu	Zhejiang, Jiangxi, Hubei Sichuan, Chongqing, Guizhou, Yunnan Guangxi

Latitude & longitude		38.72-53.53 ℃N 116.88-134.76 ℃E	31.75-49.04 ℃N 73.74-114.40 ℃E		32.61-42.58 ℃N 110.60-122.65 ℃E	18.17-34.32 ℃N 97.37-123.50 ℃E		
Sowing ecotype		Spring sown	Spring sown	Summer sown	Summer sown	Spring sown	Summer sown	Autumn sown
GP_{R1-R8}*		July 1-Sept 30	July 1-Sept 30	July 1-Sept 30	July 15-Sept 30	June 1-July 31	Aug 1-sept 30	Aug 1-Oct 31
Sampled varieties	2014	94	25		45		40	
	2015	117	17		42		33	
AT_{15}Φ (℃)	2014	1548.81	1693.53		2143.38		2077.49	
	2015	1777.35	3461.09		2265.04		2078.78	
MDTΦ (℃)	2014	19.07	19.83		23.64		24.06	
	2015	21.15	27.60		24.65		23.16	
RFΦ (mm)	2014	291.64	310.87		386.59		476.05	
	2015	269.58	151.8		295.72		531.92	
HSΦ (h)	2014	641.02	644.58		467.44		567.54	
	2015	651.62	706.49		560.11		386.48	
DTRΦ (℃)	2014	11.68	11.03		8.42		7.27	
	2015	11.30	12.47		8.84		7.65	

*GPR1−R8, growth period from R1 to R8.

NESR, Northeast Spring Planting Subregion; NWSR, Northwest Spring Planting Subregion; HHHR, Huang-Huai-Hai Valleys Summer Planting Region; SMCR, South Multiple Cropping Region.

ΦAT15 accumulated temperature ≥15 °C; MDT, mean daily temperature; RF, rainfall; HS, hours of sunshine; DTR, diurnal temperature range.

3.2 Chemicals

The lunasin standard was synthesized by the American Peptide Company (CA, Sunnyvale, USA). The protease inhibitor cocktail was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were of analytical or chromatography grade.

3.3 Sample preparation

The whole soybean seeds of each sample were dried and ground using a mill (FOSS Tecator, Hoganas, Sweden) and passed through a 60-mesh sieve. The

isolation of lunasin from defatted soybean flour was performed using the method reported by Nakurte et al. (2013) with some modification. Briefly, one gram of the defatted sample was extracted with 15 mL PBS buffer (0.1 M, pH 7.4) supplemented with a fresh protease inhibitor cocktail at a concentration of 1% v/v for 48 h at 4 ℃. After centrifugation (13000×g, 20 min, 4 ℃), the supernatant was collected in a new tube and then re-centrifuged (13000×g, 20 min, 4 ℃). Macromolecular impurity components in the supernatant were removed using a Millipore ultrafiltration tube (cut off of 10 kDa; Millipore Co., USA). Then the low molecular weight fraction was collected and passed through 0.22-μm Millipore filters and stored at 4 ℃ until analysis.

3.4 UPLC/MS-MS analysis

The contents of lunasin in the extract samples from soybean were detected by UPLC/MS-MS. The lunasin standard was used as a reference compound. It was dissolved in ultrapure water by a series of gradient of dilutions (0-1000 pg/mL). The UPLC was coupled to an XEVO TQ-S mass spectrometer (Waters; Etten-Leur, The Netherlands) that contained an electrospray ionization source (ESI). The MS instrument was operated in the ion electrospray ionization mode with multiple reaction monitoring (MRM). The lunasin ion at m/z 1257.31 corresponding to the $[M + 4H]^{4+}$ multicharged form can be detected through the MRM. Chromatographic analysis was carried out using an ACQUITY UPLC peptide CSH C18 column (1.7 μm, 2.1mm×150mm) whose mobile phase was a mixture of 0.1% trifluoroacetic acid in water (A) and methanol (B). The flow rate was 0.2 mL/min and was conducted in the following steps: 0 min 95% A, 5 min 5% A, 5.1 min 95% A, and 6 min 95% A, until the initial condition was reached. Quantitative analysis of lunasin was calculated by measuring the peak area. The calibration curve was available within a certain concentration range. The results of lunasin content are expressed as milligram of lunasin per gram of dry flour (mg/g).

3.5 Climate data

The climate data were obtained from the China Meteorological Data Sharing Service System (http://cdc.nmic.cn/home.do). The dates of the first flowering and full maturity for the 474 samples were provided by sample providers, and the missing data refer to the study of Song et al. (2016). The details, which include accumulated temperature ≥15 ℃ (AT15), mean daily temperature (MDT), rainfall (RF), hours of sunshine (HS) and diurnal temperature range (DTR), are shown in Table 1.

3.6 Statistics

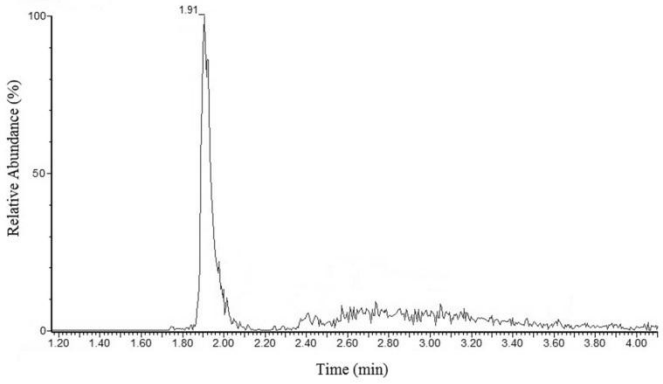
All data are reported as the mean ± standard deviation (SD) of experiments run in

triplicate. Lunsain content is expressed as dry weight of defatted flour. The data were subjected to analysis of variance (ANOVA) using one-way ANOVA procedure in SPSS (Version 17.0, IBM Corporation, NY, USA). Comparisons between means were made using Scheffe Multiple Comparison at a 0.05 probability level when the homogeneity of variance test indicated equal variance and model significance. Pearson's correlation coefficients between lunasin content and climate factors were also investigated using SPSS 17.0 software. Then the climate factors were separated into direct and indirect effects via path-coefficient analysis according to methods reported by Williams et al. (1990).

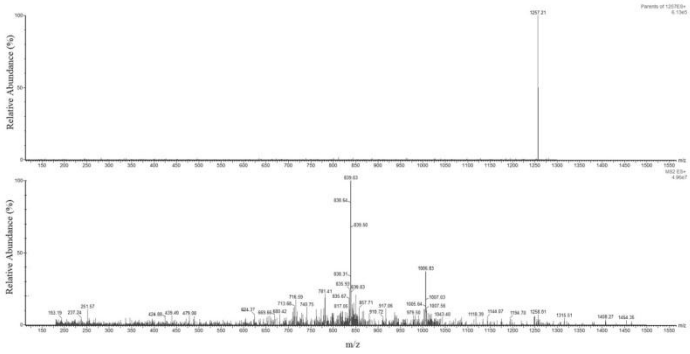
4. Results and discussion

4.1 Regional distribution of soybean lunasin content in China

As shown in **Fig 21a**, the MRM chromatographic retention time for the lunasin standard was 1.91 min within a total run time of 6 min. The ESI mass spectrum from m/z 0 to m/z 1550 of the lunasin standard is presented in **Fig. 21b**. It exhibita a multicharged profile, with fragments of m/z 839.03, m/z 1006.83 and m/z 1257.21 corresponding to the $[M+6H]^{6+}$, $[M+5H]^{5+}$ and $[M+4H]^{4+}$, respectively, which was consistent with a previous study (Dinelli et al., 2014). The MRM chromatogram of the lunasin extract from the soybean samples exhibits a complicated signal due to the complex matrix in the extract, but a peak at a retention time of 1.71 min can be found (**Fig. 21c**). The lunasin content was measured based on this peak.



a



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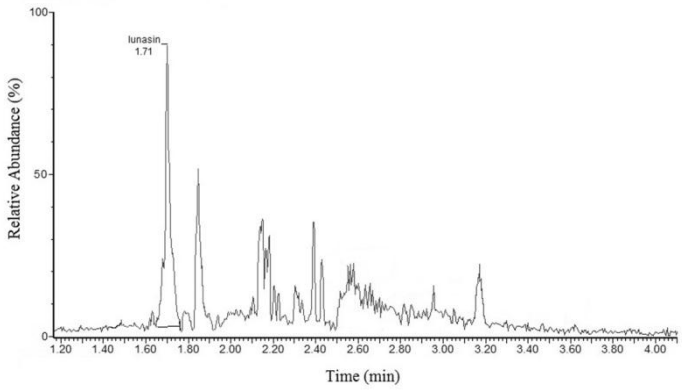

c

Figure 21. UPLC/MS-MS analysis. (a) MRM chromatogram of lunasin standard. (b) Mass spectrum acquired on peak at 1.91 min in the MRM chromatogram of lunasin standard. (c) A typical MRM chromatogram of extract sample from soybean.

The variation in lunasin content of soybean samples from the four regions is shown in **Table 2** and **Table 7**. Lunasin content in 204 samples harvested in 2014 ranged from 0.53±0.09 mg/g to 3.24±0.19 mg/g with a coefficient of variation (CV) value of 38.11 and a mean content of 1.72±0.66 mg/g. The lunasin content in 209 samples harvested in 2015 ranged from 0.71±0.14 mg/g to 3.25±0.10 mg/g with a CV value of 34.10 and a mean content of 1.83±0.62 mg/g. These results are consistent with the lunasin content (1-13.3 mg lunasin per g defatted soybean flour) reported by Gonzalez et al., (2004) who determined the lunasin content in 144 selected diverse soybean accessions from the U.S. using an enzyme-linked immunosorbent assay (ELISA). Gao et al. (2018) quantified the lunasin content of 6 soybean cultivars planted in China using the NanoLC-Orbitrap technique. Their results showed that the lunasin content ranged from 2.03 mg lunasin per g defatted soybean flour to 4.66 mg lunasin per g defatted soybean flour, which was also consistent with our results. Among all the samples, cultivar Changmidou 30, which

was planted in Jilin, possessed the highest lunasin content. This result indicated that the soybean cultivar Changmidou 30 collected from Jilin province has the potential to be an ideal source for the isolation of natural lunasin peptide.

Lunasin content varied significantly among smples from NESR, NWSR, HHHR and SMCR. Both the data from 2014 and the data from 2015 showed that the mean lunasin content in soybean seeds from NESR and NWSR were significantly higher ($p<0.05$) than those from HHHR and SMCR, which showed an increasing trend from south China to north China. This is the first evidence regarding the regional distribution of soybean lunasin content in China. Though our results are from different soybean cultivars and genetic factors cannot be ignored, the extensive and high-density sampling as well as the similar tend observed from two years make the results tremendously credible. In addition, a substantial variation in lunasin content in soybean cultivars planted in the same region was observed. As shown in **Table 4**, the CV values for lunasin content in NESR, NWSR, HHHR and SMCR were 32.21, 31.29, 37.02 and 37.81 in 2014, respectively, and 31.44, 21.52, 30.07 and 28.48 in 2015, respectively. However, these results could not provide fully credible evidence to support the previous opinion that soybean genotype has a significant effect on lunasin content due to the differences in climate factors among the planting locations (Hernandez et al., 2009). Therefore, we next evaluated the effects of climate factors on lunasin content.

Table 4. Lunasin content of different soybean cultivars grown in four regions of China*

		NESR#	NWSR#	HHHR#	SMCR#	total
2014	Range	0.63-3.24	0.71-2.88	0.53-2.51	0.58-2.42	0.53-3.24
	Mean	2.03[a]	1.82[a]	1.45[b]	1.33[b]	1.72
	SD	0.65	0.57	0.54	0.50	0.65
	CV, %	32.21	31.29	37.02	37.81	38.11
2015	Range	0.71-3.25	0.79-2.40	0.74-2.65	0.77-1.88	0.71-3.25
	Mean	2.04[a]	1.96[a]	1.58[b]	1.31[b]	1.83
	SD	0.64	0.42	0.48	0.37	0.62

	CV, %	31.44	21.52	30.07	28.48	34.10

*Values in the same raw with different letters are significantly different, $p<0.05$.

NESR, Northeast Spring Planting Subregion; NWSR, Northwest Spring Planting Subregion; HHHR, Huang-Huai-Hai Valleys Summer Planting Region; SMCR, South Multiple Cropping Region.

4.2 Correlations between soybean lunasin content and climate factors

Wang et al. (2008) reported that soil moisture conditions and growing temperature significantly affect lunasin content in soybean seeds, which suggests that climate factors may influence lunasin content. To evaluate the correlation between lunasin content and climate factors, we extracted detailed climate information of the planting locations for each sample (results were shown in the annex in **Table 7**). As shown in **Table 5**, the correlations were almost consistent in 2014 and 2015. Park et al. (2005) reported that light and dark conditions have no influence on lunasin content in soybean. Therefore, the influence of photoperiod was not considered in this study. However, our results showed that, on a national scale, soybean lunasin content was positively correlated ($p<0.01$) with HS and DTR. As shown, we found that lunasin content was negatively correlated ($p<0.01$) with RF. High rainfall will increase the soil moisture and will delay the maturity of soybean seeds (Lozovaya et al., 2005). It has been reported that lunasin content positively correlates with seed maturation (Hernandez et al., 2013). These results may explain why high rainfall reduces lunasin content. Minor differences in correlation between AT15 and lunasin content as well as between MDT and lunasin content observed in 2014 and 2015. In 2014, there were negative ($p<0.05$) correlations between lunasin content and AT_{15} as well as between lunasin content and MDT with low R values of -0.168 and -0.177, respectively. In 2015, no significant correlation was observed between lunasin content and either of these two factors. In a previous study, high (23/33 ℃ with mean a temperature of 28 ℃) or low (13/23 ℃ with a mean temperature of 18 ℃) temperatures led to lower lunasin than moderate temperature (18/28 ℃ with a mean temperature of 23 ℃), and no significant linear correlation between lunasin content and environmental temperature was found (Vasconez et al., 2004).

Table 5. Correlation between lunasin content and climate factors[a]

		$AT_{15}{}^{\Phi}$ (℃)	MDT^{Φ} (℃)	RF^{Φ} (nm)	HS^{Φ} (h)	DTR^{Φ} (℃)
2014	R value	-0.168*	-0.177*	-0.375**	0.322**	0.454**

	P value	0.016	0.011	0.001	0.001	0.001
2015	R value	-0.077	-0.057	-0.244**	0.631**	0.320**
	P value	0.265	0.412	0.001	0.001	0.001

[a]Correlation between lunasin content and climate factors.

* $p < 0.05$; **, $p < 0.01$. Values without asterisks are not significant at $p < 0.05$.

ΦAT15 accumulated temperature ≥15 ℃; MDT, mean daily temperature; RF, rainfall; HS, hours of sunshine; DTR, diurnal temperature range.

4.3 Path-Coefficient analysis of climate factors on soybean quality

Path-coefficient analysis was used to examine and assess the possible causal linkage between climate factors and relative effects on soybean quality traits (**Table 6**). In this study, the combined data from 2014 and 2015 were used to performe the path-coefficient analysis. The results showed that the direct path-coefficient of DTR was the greatest (0.301), which suggested that DTR had a dominant and direct effect on soybean lunasin content.

Table 6. Path coefficient of soybean quality traits and climate factors

	AT_{15}^{Φ} (℃)	MDT^{Φ} (℃)	RF^{Φ} (mm)	HS^{Φ} (h)	DTR^{Φ} (℃)
Direct coefficient	0.299	-0.309	-0.109	0.262	0.301
Indirect coefficient	-0.23856	0.119131	-0.3906	0.228941	0.099166

ΦAT_{15} accumulated temperature ≥15 ℃; MDT, mean daily temperature; RF, rainfall; HS, hours of sunshine; DTR, diurnal temperature range.

5. Conclusion

In conclusion, the highest lunasin content was observed in cultivar Changmidou 30 collected from Jilin province and harvested in 2015. The regional and climate factors indeed affected the lunasin content in the soybean seeds. The content

showed an increasing trend from south China to north China. There was a positive correlation between lunasin content and HS as well as DTR; however, a negative correlation between lunasin content and RF was found. DTR was demonstrated as the dominant factors that affect lunasin content. This result can help to select soybean samples that can be used as an ideal source for the isolation of natural lunasin and can guide the cultivation of soybeans with high lunasin content.

6. Reference

Dia V. P., Wang W., Oh V. L., De Lumen B. O., and De Mejia E. G. 2009. Isolation, purification and characterisation of lunasin from defatted soybean flour and in vitro evaluation of its anti-inflammatory activity. Food Chemistry, 114(1), 108-115.

Dinelli G., Bregola V., Bosi S., Fiori J., Gotti R., Simonetti E., and Malaguti M. 2014. Lunasin in wheat: A chemical and molecular study on its presence or absence. Food chemistry, 151, 520-525.

Galvez A. F. 2012. identification of lunasin as the active component in soy protein responsible for reducing LDL cholesterol and risk of cardiovascular disease.

Gao M. D., Meng X. B., Xing C. C., Zhang Y., Deng H. T., and Wang F.Z. 2018. Determination of polypeptide lunasin content in six different cultivars of soybean using nanolc-orbitrap technique. Modern Food Science & Technology (in Chinese).

Gonzalez de Mejia E., Vásconez M., de Lumen B. O., and Nelson R. 2004. Lunasin concentration in different soybean genotypes, commercial soy protein, and isoflavone products. Journal of agricultural and food chemistry, 52(19), 5882-5887.

Hernandez-Ledesma B., C Hsieh C., and O de Lumen B. 2013. Chemopreventive properties of Peptide Lunasin: a review. Protein and peptide letters, 20(4), 424-432.

Hernández-Ledesma B., Hsieh C. C., and Ben O. 2009. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. Biochemical and Biophysical Research Communications, 390(3), 803-808.

Hernandez-Ledesma B., Hsieh C. C., and Ben O. 2009. Lunasin, a novel seed peptide for cancer prevention. Peptides, 30(2), 426-430.

Hernández-Ledesma B., Hsieh C. C., and de Lumen B. O. 2011. Relationship between lunasin's sequence and its inhibitory activity of histones H3 and H4 acetylation. Molecular nutrition & food research, 55(7), 989-998.

Lam Y., Galvez A., and de Lumen B. O. 2003. Lunasin™ suppresses E1A-mediated transformation of mammalian cells but does not inhibit growth of immortalized and established cancer cell lines. Nutrition and cancer, 47(1), 88-94.

Lozovaya V. V., Lygin A. V., Ulanov A. V., Nelson R. L., Dayd é J., and Widholm J. M. 2005. Effect of temperature and soil moisture status during seed development on soybean seed isoflavone concentration and composition. Crop science, 45(5), 1934-1940.

Nakurte I., Kirhnere I., Namniece J., Saleniece K., Krigere L., Mekss P., and Muceniece R. 2013. Detection of the lunasin peptide in oats (Avena sativa L). Journal of cereal science, 57(3), 319-324.

Odani S., Koide T., and Ono T. 1987. Amino acid sequence of a soybean (Glycine max) seed polypeptide having a poly (L-aspartic acid) structure. Journal of Biological Chemistry, 262(22), 10502-10505.

Park J. H., Jeong H. J., and de Lumen B. O. 2005. Contents and bioactivities of lunasin, bowman− birk inhibitor, and isoflavones in soybean seed. Journal of agricultural and food chemistry, 53(20), 7686-7690.

Singh P., and Bisetty K. 2012. A molecular dynamics study of lunasin. South African Journal of Chemistry, 65(1), 115-124.

Song W., Yang R., Wu T., Wu C., Sun S., Zhang S., and Han T. 2016. Analyzing the effects of climate factors on soybean protein, oil contents, and composition by extensive and high-density sampling in China. Journal of agricultural and food chemistry, 64(20), 4121-4130.

Vasconez-Costa M. 2004. Effect of genotype, environment and processing on the level of lectin and lunasin in soybean. Urbana-Champaign: Master Thesis, University of Illinois.

Wang W., Dia V. P., Vasconez M., de Mejia E. G., and Nelson R. L. 2008. Analysis of soybean protein-derived peptides and the effect of cultivar, environmental conditions, and processing on lunasin concentration in soybean and soy products. Journal of AOAC International, 91(4), 936-946.

Williams W. A., Demment M. W., and Jones M. B. 1990. A concise table for path analysis statistics. Agronomy journal, 82(5), 1022-1024.

Yang R. P., Song W. W., Sun S., Wu C. X., Wang H. J., and Han T. F. 2012. Comparison of soybean yield and yield-related traits of agri-technology demonstration counties in different regions of China. Soybean Sci, 31, 557-567.

7. Annex

Table 7. Varieties information, lunasin content and climate conditions for each sample in 2014

Sample No.	Cultivar	Location	Lunasin content[a]	RF[b]	HS[b]	AT_{15}[b]	MDT[b]	DTR[b]

			(mg/g)	(mm)	(h)	(℃)	(℃)	(℃)
14001	Fudou 9	Anhui	0.55±0.01	546.00	376.70	2166.10	23.79	7.69
14002	Lingbi 5	Anhui	0.61±0.02	341.80	529.10	2099.60	23.06	8.78
14003	Fudou 9	Anhui	0.73±0.02	473.20	295.40	2300.60	25.27	6.40
14004	Fudou 9	Anhui	0.79±0.01	549.90	417.40	2257.00	24.79	6.99
14005	Wandou 15	Anhui	0.8±0.07	546.00	376.70	2166.10	23.79	7.69
14006	Zhonghuang 13	Anhui	0.89±0.04	546.00	376.70	2166.10	23.79	7.69
14007	Fudou 9	Anhui	1±0.11	546.00	376.70	2166.10	23.79	7.69
14008	Zhonghuang 13	Anhui	1.1±0.08	549.90	417.40	2257.00	24.79	6.99
14009	Fudou 9	Anhui	1.74±0.06	546.00	376.70	2166.10	23.79	7.69
14010	Zhonghuang 13	Anhui	1.87±0.02	540.30	459.90	2205.90	24.23	7.40
14011	Zhuike 998	Anhui	1.95±0	540.30	459.90	2205.90	24.23	7.40
14012	Zhonghuang 13	Anhui	2.11±0.03	540.30	459.90	2205.90	24.23	7.40
14013	Wandou 15	Anhui	2.37±0.13	540.30	459.90	2205.90	24.23	7.40
14014	Nandou 12	Chongqing	1.34±0.09	731.70	344.20	2157.00	23.61	7.12
14015	Zhonghuang 30	Gansu	1.08±0	418.00	627.10	1476.60	18.32	9.81
14016	Jidou 17	Gansu	1.6±0.01	418.00	627.10	1476.60	18.32	9.81
14017	Zhonghuang 30	Gansu	1.76±0.03	65.40	812.40	1613.10	19.08	13.82
14018	Jindou 23	Gansu	1.96±0.04	418.00	627.10	1476.60	18.32	9.81

14019	Zhonghuang 39	Gansu	2.05±0.08	188.40	745.70	1687.90	19.70	12.44
14020	Jidou 17	Gansu	2.24±0.06	138.80	749.10	1655.30	19.24	12.11
14021	Tieshudadou	Gansu	2.43±0.01	25.50	853.70	1561.30	18.67	14.29
14022	Guixia 3	Guangxi	0.88±0.09	335.80	571.40	2419.80	26.31	8.11
14023	Landrace	Guangxi	1.01±0.14	504.10	471.50	2367.10	25.74	8.22
14024	Guixia 3	Guangxi	1.02±0.02	504.10	471.50	2367.10	25.74	8.22
14025	Landrace	Guangxi	1.11±0.01	504.10	471.50	2367.10	25.74	8.22
14026	Mifeng	Guangxi	1.21±0.07	504.10	471.50	2367.10	25.74	8.22
14027	Guixia 1	Guangxi	2.35±0.13	504.10	471.50	2367.10	25.74	8.22
14028	Maozao	Guizhou	0.64±0.06	564.10	311.70	1813.60	19.92	6.76
14029	Qingpidou	Guizhou	0.89±0.1	512.10	414.00	2159.10	23.72	7.27
14030	Landrace	Guizhou	1.04±0	626.80	399.10	1906.30	20.94	5.71
14031	Landrace	Guizhou	1.17±0.15	809.10	355.00	1987.00	21.81	5.89
14032	Landrace	Guizhou	1.21±0.04	797.90	332.30	2304.30	25.31	6.93
14033	Qiandou	Guizhou	1.26±0.04	797.90	332.30	2304.30	25.31	6.93
14034	Landrace	Guizhou	1.37±0	626.80	399.10	1906.30	20.94	5.71
14035	Xifeng	Guizhou	1.47±0.01	506.00	358.00	1939.30	21.29	5.98
14036	Weixianhuangdou	Hebei	0.69±0.03	197.00	605.50	2157.10	23.83	9.42
14037	Shidou 1	Hebei	1.06±0.02	164.20	664.70	1947.30	21.65	9.24

14038	Gandou 5	Hebei	1.09±0.07	179.80	333.40	2224.30	24.57	8.88
14039	Cangdou 10	Hebei	1.15±0.06	179.80	333.40	2224.30	24.57	8.88
14040	Yangyuanhuangdou	Hebei	1.16±0.07	197.00	605.50	2157.10	23.83	9.42
14041	Jihuang 13	Hebei	1.49±0.09	179.80	333.40	2224.30	24.57	8.88
14042	Shidou 1	Hebei	1.57±0.04	179.80	333.40	2224.30	24.57	8.88
14043	Kefeng 6	Hebei	1.75±0.02	204.20	706.50	1711.70	19.78	11.63
14044	Zhonghuang 13	Hebei	1.86±0.05	197.00	605.50	2157.10	23.83	9.42
14045	Cangdou 6	Hebei	2±0.01	197.00	605.50	2157.10	23.83	9.42
14046	Cangdou 10	Hebei	2.08±0.11	204.20	706.50	1711.70	19.78	11.63
14047	Shidou 8	Hebei	2.4±0.01	233.40	550.10	2088.90	23.07	11.20
14048	Hefeng 57	Heilongjang	2.46±0.04	199.80	752.50	1297.50	16.74	15.47
14049	Keshan 1	Heilongjiang	0.63±0.02	362.00	535.00	1357.00	17.72	12.63
14050	KenLandraceg 2	Heilongjiang	0.66±0	362.00	535.00	1357.00	17.72	12.63
14051	Keshan 1	Heilongjiang	0.69±0	362.00	535.00	1357.00	17.72	12.63
14052	QiLandraceg 1	Heilongjiang	0.75±0.06	362.00	535.00	1357.00	17.72	12.63
14053	Keshan 1	Heilongjiang	0.96±0.01	362.00	535.00	1357.00	17.72	12.63

14054	Hefeng 56	Heilongjiang	0.97±0.06	362.00	535.00	1357.00	17.72	12.63
14055	Keshan 1	Heilongjiang	1.12±0.01	371.50	608.80	1525.70	19.23	10.97
14056	Heihe 38	Heilongjiang	1.17±0.05	268.10	595.60	1414.20	17.89	12.99
14057	Heihe 43	Heilongjiang	1.17±0.07	299.10	685.10	1634.60	19.92	10.41
14058	Heihe 38	Heilongjiang	1.24±0.07	486.70	607.70	1474.50	18.70	10.76
14059	Huajiang 2	Heilongjiang	1.25±0.03	390.30	598.60	1487.80	18.63	10.89
14060	Heihe 43	Heilongjiang	1.26±0.04	390.30	598.60	1487.80	18.63	10.89
14061	Huajiang 2	Heilongjiang	1.26±0.05	390.30	598.60	1487.80	18.63	10.89
14062	SuiLandraceg 26	Heilongjiang	1.31±0.06	199.80	752.50	1297.50	16.74	15.47
14063	Heihe 38	heilongjiang	1.38±0.03	371.50	608.80	1525.70	19.23	10.97
14064	Kenfeng 16	Heilongjiang	1.4±0.01	362.00	535.00	1357.00	17.72	12.63
14065	Hefeng 50	Heilongjiang	1.42±0.06	362.00	535.00	1357.00	17.72	12.63
14066	HeiLandraceg 37	Heilongjia	1.43±0.15	327.50	440.60	1378.00	17.51	12.64

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14067	KengLandraceg 31	Heilongjia ng	1.51±0.05	199.80	752.50	1297.50	16.74	15.47
14068	Beidou 14	Heilongjia ng	1.55±0	327.50	440.60	1378.00	17.51	12.64
14069	Heihe 43	Heilongjia ng	1.57±0.1	327.50	440.60	1378.00	17.51	12.64
14070	HeiLandraceg 51	Heilongjia ng	1.57±0.17	327.50	440.60	1378.00	17.51	12.64
14071	DongLandraceg 53	Heilongjia ng	1.61±0	261.00	693.50	1461.20	18.46	10.22
14072	Kenfeng 16	Heilongjia ng	1.66±0.11	327.50	440.60	1378.00	17.51	12.64
14073	Heihe 43	Heilongjia ng	1.68±0.11	555.10	657.30	1436.30	18.25	11.44
14074	Heihe 38	Heilongjia ng	1.7±0.07	486.70	607.70	1474.50	18.70	10.76
14075	Hefeng 56	Heilongjia ng	1.76±0.06	362.00	535.00	1357.00	17.72	12.63
14076	Huajiang 4	Heilongjia ng	1.89±0.01	390.30	598.60	1487.80	18.63	10.89
14077	Keshan 1	Heilongjia ng	1.98±0.14	263.80	728.50	1693.70	20.10	11.28
14078	Beidou 20	Heilongjia ng	1.99±0.08	390.30	598.60	1487.80	18.63	10.89

14079	Heihe 43	Heilongjiang	2.01±0.07	362.00	535.00	1357.00	17.72	12.63
14080	Heihe 43	Heilongjiang	2.07±0	555.10	657.30	1436.30	18.25	11.44
14081	Kenjiandou 27	Heilongjiang	2.09±0.09	327.50	440.60	1378.00	17.51	12.64
14082	Kangxian 9	Heilongjiang	2.13±0.13	327.50	440.60	1378.00	17.51	12.64
14083	Keshan 1	Heilongjiang	2.15±0.05	234.30	687.50	1658.50	19.85	11.43
14084	Longda 1	Heilongjiang	2.2±0.02	261.00	693.50	1461.20	18.46	10.22
14085	Heihe 51	Heilongjiang	2.21±0.11	390.30	598.60	1487.80	18.63	10.89
14086	Heihe 43	Heilongjiang	2.23±0.09	390.30	598.60	1487.80	18.63	10.89
14087	SuiLandraceg 22	Heilongjiang	2.3±0.14	199.80	752.50	1297.50	16.74	15.47
14088	Hefeng 50	Heilongjiang	2.32±0.13	362.00	535.00	1357.00	17.72	12.63
14089	Heihe 43	Heilongjiang	2.34±0.02	555.10	657.30	1436.30	18.25	11.44
14090	SuiLandraceg 26	Heilongjiang	2.4±0.11	256.00	564.40	985.30	14.24	17.71
14091	Kenjiandou 27	Heilongjia	2.4±0.16	199.80	752.50	1297.50	16.74	15.47

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14092	Beidou 43	Heilongjiang	2.45±0.14	390.30	598.60	1487.80	18.63	10.89
14093	Beidou 28	Heilongjiang	2.48±0.02	338.50	784.40	1532.60	18.84	9.52
14094	Kenfeng 22	Heilongjiang	2.54±0.12	256.00	564.40	985.30	14.24	17.71
14095	Heihe 52	Heilongjiang	2.55±0.13	362.00	535.00	1357.00	17.72	12.63
14096	Heihe 43	Heilongjiang	2.6±0.14	390.30	598.60	1487.80	18.63	10.89
14097	Beidou 20	Heilongjiang	2.61±0.11	338.50	784.40	1532.60	18.84	9.52
14098	Kangxian 8	Heilongjiang	2.68±0.01	477.50	696.50	1356.80	17.63	11.98
14099	DongLandraceg48	Heilongjiang	2.78±0.1	555.10	657.30	1436.30	18.25	11.44
14100	Beijiang 9-1	Heilongjiang	2.82±0.06	263.80	728.50	1693.70	20.10	11.28
14101	Beidou 40	Heilongjiang	2.85±0.02	259.20	600.40	1541.00	19.22	10.20
14102	Heihe 45	Heilongjiang	2.88±0.2	209.80	633.40	1427.30	18.14	12.31
14103	Heihe 51	Heilongjiang	2.89±0.05	259.20	600.40	1541.00	19.22	10.20

14104	Heihe 34	Heilongjiang	2.91±0.12	354.90	544.00	4802.70	53.97	24.61
14105	Heihe 43	Heilongjiang	2.91±0.16	371.50	608.80	1525.70	19.23	10.97
14106	Landracegken 658	Helongjiang	1.46±0.05	327.50	440.60	1378.00	17.51	12.64
14107	Keshan 1	Helongjiang	1.61±0.08	327.50	440.60	1378.00	17.51	12.64
14108	Beidou 40	Helongjiang	1.91±0.19	327.50	440.60	1378.00	17.51	12.64
14109	Keshan 1	Helongjiang	1.94±0.13	327.50	440.60	1378.00	17.51	12.64
14110	Kenfeng 16	Helongjiang	2.01±0	327.50	440.60	1378.00	17.51	12.64
14111	Kenfeng 17	Helongjiang	2.1±0.06	327.50	440.60	1378.00	17.51	12.64
14112	Beidou 14	Helongjiang	2.98±0.08	327.50	440.60	1378.00	17.51	12.64
14113	Zheng 196	Henan	1.04±0.15	311.60	485.40	2185.20	24.00	8.08
14114	Zhudou 12	Henan	1.16±0.06	378.40	439.90	2177.50	23.91	8.06
14115	Zhudou 02-19	Henan	1.18±0	378.40	439.90	2177.50	23.91	8.06
14116	Zheng 196	Henan	1.34±0.01	513.00	368.70	2086.90	23.08	8.98
14117	Zhudou 02-19	Henan	1.71±0.06	378.40	439.90	2177.50	23.91	8.06

14118	Zhudou 02-19	Henan	1.75±0.03	378.40	439.90	2177.50	23.91	8.06
14119	Zhonghuang 13	Henan	1.8±0.09	412.80	422.40	2194.90	24.11	8.11
14120	Zheng 9805	Henan	1.82±0.03	513.00	368.70	2086.90	23.08	8.98
14121	Ludou 1	Henan	1.82±0.08	293.90	435.10	2092.30	23.14	9.67
14122	Shangdou 6	Henan	2.02±0.06	254.20	442.50	2173.00	23.87	8.32
14123	Zhonghuang 13	Hubei	0.6±0.06	198.20	253.70	1574.20	26.24	6.63
14124	Zhongdou 8	Hubei	0.72±0.02	108.70	342.60	1571.20	26.19	6.81
14125	Zhongdou 39	Hubei	0.83±0.01	108.70	342.60	1571.20	26.19	6.81
14126	Hedou 13	Hubei	0.95±0.1	347.60	269.20	1422.60	23.71	6.53
14127	Endou 31	Hubei	1.55±0.03	198.20	253.70	1574.20	26.24	6.63
14128	Edou 8	Hubei	1.66±0.08	347.60	269.20	1422.60	23.71	6.53
14129	Hefeng 25	Inner Mongolia	0.86±0.05	247.60	649.30	1437.10	18.06	11.82
14130	Landrace	Inner Mongolia	2.01±0.01	113.30	863.30	1574.00	19.14	12.61
14131	Qingchadou	Inner Mongolia	2.14±0.07	109.00	688.40	1818.10	21.02	11.89
14132	Landrace	Inner Mongolia	2.34±0.08	97.70	890.10	1480.40	18.56	13.09
14133	Fudou 1	Inner Mongolia	2.41±0.1	112.00	844.60	1639.10	19.55	12.95
14134	Beidou 5	Inner	2.42±0.07	486.70	607.70	1474.50	18.70	10.76

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14135	Xinfeng 1	Inner Mongolia	2.45±0.12	113.30	863.30	1574.00	19.14	12.61
14136	Hefeng 25	Inner Mongolia	2.65±0.07	113.30	863.30	1574.00	19.14	12.61
14137	HeiLandraceg 48	Inner Mongolia	2.69±0.08	113.30	863.30	1574.00	19.14	12.61
14138	Landrace	Inner Mongolia	2.74±0.18	113.30	863.30	1574.00	19.14	12.61
14139	Hefeng 25	Inner Mongolia	2.82±0.02	113.30	863.30	1574.00	19.14	12.61
14140	Hefeng 25	Inner Mongolia	2.85±0.2	113.30	863.30	1574.00	19.14	12.61
14141	Hefeng 25	Inner Mongolia	2.97±0.1	113.30	863.30	1574.00	19.14	12.61
14142	Landrace	Inner Mongolia	3.24±0.17	113.30	863.30	1574.00	19.14	12.61
14143	Huaidou 6	Jiangsu	0.53±0.09	634.40	341.10	2149.10	23.60	6.43
14144	Hedou 13	Jiangsu	1.01±0.01	435.40	537.00	2185.20	24.00	7.56
14145	Xudou 16	Jiangsu	1.09±0.04	840.60	446.20	2110.30	23.17	6.45
14146	Guandou 2	Jiangsu	1.16±0.05	702.00	414.10	2167.50	23.81	6.94
14147	Xudou 16	Jiangsu	1.71±0.08	438.50	401.00	2123.70	23.32	7.07
14148	Xudou 14	Jiangsu	1.94±0.06	392.10	422.40	2095.50	23.01	8.26

14149	Fudou 71	Jiangxi	1.77±0.05	219.60	644.20	2282.80	24.83	9.75
14150	NanLandraceg 99-10	Jiangxi	2.22±0.03	160.50	3794.30	2365.30	25.73	7.97
14151	Gandou 7	Jiangxi	2.33±0.03	160.50	3794.30	2365.30	25.73	7.97
14152	NanLandraceg 99-10	Jiangxi	2.42±0.08	207.40	625.80	2342.20	25.47	7.30
14153	Tiedou 43	Liaoning	1.19±0.02	219.10	725.70	2025.40	22.51	6.53
14154	Yushi 11	Liaoning	1.32±0.08	101.50	724.70	1848.90	21.04	12.93
14155	Liaodou 15	Liaoning	1.45±0.01	150.50	650.70	1818.90	20.90	11.96
14156	Jinyu 39	Liaoning	1.57±0.03	101.50	724.70	1848.90	21.04	12.93
14157	Liaodou 15	Liaoning	1.72±0.1	201.80	679.10	1908.10	21.45	12.96
14158	Tiefeng 31	Liaoning	1.94±0.07	607.60	730.90	1884.10	20.95	10.00
14159	Tiefeng 31	Liaoning	2.03±0.05	127.70	733.90	2041.50	22.64	10.37
14160	Kaiyu 12	Liaoning	2.12±0.04	219.10	725.70	2025.40	22.51	6.53
14161	Tiefeng 31	Liaoning	2.33±0.04	187.30	719.00	1973.90	21.90	9.84
14162	Liaodou 15	Liaoning	2.39±0.04	101.50	724.70	1848.90	21.04	12.93
14163	Tiefeng 29	Liaoning	2.5±0.12	169.60	825.90	1786.90	20.46	12.78
14164	Tiedou 37	Liaoning	2.69±0.04	133.40	625.90	1966.40	21.83	10.94
14165	Fudou 23	Liaoning	2.72±0.04	234.30	761.00	1655.00	19.56	13.64
14166	Liaodou 36	Liaoning	3.05±0.1	219.20	705.40	1817.00	20.84	10.41
14167	Dandou 13	Liaoning	3.24±0.19	217.40	754.80	1677.40	19.76	12.96

14168	Zhonghuang 30	Ningxia	1.55±0.04	68.90	770.40	1856.10	20.70	11.62
14169	Jidou 17	Shaanxi	0.71±0.04	429.30	536.80	1532.10	18.48	10.31
14170	Qindou 10	Shaanxi	1.01±0.02	459.30	652.40	1506.00	18.35	11.05
14171	Qindou 10	Shaanxi	1.1±0	459.30	652.40	1506.00	18.35	11.05
14172	Qindou 10	Shaanxi	1.17±0.06	448.80	491.90	2143.20	23.53	7.63
14173	Qindou 13	Shaanxi	1.2±0.08	404.10	520.90	2191.50	24.07	8.20
14174	Pingan 10	Shaanxi	1.48±0.04	309.50	569.10	1819.70	20.46	10.28
14175	Jindou 23	Shaanxi	1.49±0.08	429.30	536.80	1532.10	18.48	10.31
14176	Qingdou 13	Shaanxi	1.76±0.04	309.50	569.10	1819.70	20.46	10.28
14177	Zhonghuang 13	Shaanxi	1.78±0.11	459.30	652.40	1506.00	18.35	11.05
14178	Fuyu A	Shaanxi	1.98±0.1	429.30	536.80	1532.10	18.48	10.31
14179	Jidou 12	Shaanxi	2.31±0.02	429.30	536.80	1532.10	18.48	10.31
14180	Pingan 10	Shaanxi	2.47±0.03	286.20	459.50	1945.00	21.36	9.51
14181	Jihuang 34	Shandong	1.03±0.01	316.50	608.30	2134.50	23.44	9.25
14182	Hedou 19	Shandong	1.85±0.11	194.70	604.10	2134.60	23.76	9.62
14183	Jihuang 34	Shandong	1.86±0.01	316.50	608.30	2134.50	23.44	9.25
14184	Jihuang 34	Shandong	2.51±0.04	194.70	604.10	2134.60	23.76	9.62
14185	Fendou 78	Shanxi	2.16±0.08	248.00	652.70	1772.30	20.36	11.78
14186	Jindou 21	Shanxi	2.39±0.07	447.10	570.20	2140.00	23.51	9.37

14187	Fendou 95	Shanxi	2.58±0.16	367.70	555.90	1899.50	21.33	10.04
14188	Nandou 25	Sichuan	0.68±0.03	538.70	277.10	2045.40	22.39	6.79
14189	Nandou 12	Sichuan	0.97±0.07	527.80	224.70	2048.00	22.59	6.22
14190	Nanheidou 20	Sichuan	1.29±0.08	793.60	400.00	1715.00	20.18	8.36
14191	Heihe 38	Xinjiang	2.24±0.07	36.00	822.80	1409.80	18.54	15.29
14192	Hefeng 55	Xinjiang	2.88±0.05	78.80	985.40	1747.60	20.65	15.31
14193	Diandou 5	Yunnan	0.58±0.03	461.20	376.80	2200.50	24.19	7.24
14194	Diandou 5	Yunnan	0.85±0.01	471.80	459.70	1843.00	20.26	7.54
14195	Diandou 4	Yunnan	1.32±0.02	471.80	459.70	1843.00	20.26	7.54
14196	Diandou 4	Yunnan	1.33±0	461.20	376.80	2200.50	24.19	7.24
14197	Huangdou	Yunnan	1.46±0.06	700.10	384.50	2331.50	25.63	8.92
14198	Xiaobaidou	Yunnan	1.69±0.01	700.10	384.50	2331.50	25.63	8.92
14199	Daheidou	Yunnan	1.83±0.13	461.20	376.80	2200.50	24.19	7.24
14200	Xihuangdou	Yunnan	1.84±0.13	597.70	441.50	2092.40	23.00	8.50
14201	Tiegandou	Zhejiang	1.02±0.05	316.50	482.80	2303.70	25.05	7.66
14202	Shiyuehuang	Zhejiang	1.44±0.1	603.00	361.90	2223.80	24.18	6.37
14203	Bayuebai	Zhejiang	1.82±0.15	603.00	361.90	2223.80	24.18	6.37
14204	Gaoxiong 2	Zhejiang	2.08±0.09	448.90	568.60	2273.00	24.72	5.23

[a]lunasin content was expressed as milligram of lunasin per gram of dry flour

[b]AT_{15} accumulated temperature ≥15 °C; MDT, mean daily temperature; RF, rainfall; HS, hours of sunshine; DTR, diurnal temperature range.

5

General Discussion, Perspectives and Conclusion

1. General discussion

1.1 Synergistic anticancer effect of bioactive components from soybean

Synergistic anticancer effects. Accumulating evidence has suggested that the management of lifestyle habits, such as nutritional habits, is the best strategy for the prevention of chronic disorders. Chronic disorders, including cancer, diabetes, obesity, and cardiovascular and neurodegenerative diseases, are a leading cause of death worldwide. Interestingly, some dietary compounds have revealed desirable health-promoting benefits counteracting this public issue. It has been reported that the large geographic difference in the incidence and mortality of breast cancer between the United States and East Asia may be due to dietary variables (Chuang et al., 2006). Soybean is a main dietary source of phytochemicals, including isoflavones, saponins, sterols, phytates, phenolic acids, protease inhibitors, lectins, and bioactive peptides (Ahmad et al., 2014). Various bioactive components in soybean food have been hypothesized to be the main dietary factors that contribute to reducing breast cancer risk (Zhou et al., 1999). In this study, the anti-proliferative activity of 12 soybean components in MCF-7 and MDA-MB-231 cells was evaluated. We found that genistein, daidzein, glycitein, genistin, daidzin, glycitin, equol, soyasaponin, β-sitosterol and lectin inhibited MCF-7 cell proliferation, and genistein, daidzein, glycitein, genistin, glycitin, soyasaponin, and β-sitosterol inhibited MDA-MB-231 cell proliferation in a dose-dependent manner. From these results, we concluded that soybean isoflavones may be the major anticarcinogens in soybean, which is consistent with the hypothesis of Folkman et al. (1995). In our study, we found that although most of these soybean components inhibited the proliferation of breast cancer cells, the median effective concentration (EC_{50}) values of all these samples exceeded 65 μM. Xu et al. (1994) evaluated the bioavailability of soybean isoflavones for humans and found that the total plasma isoflavone concentration was increased to (4.4 +/- 2.5) nM at 6.5 h after receiving a single dose of 2.0 mg isoflavones/kg body wt in soybean milk as part of a liquid diet. Hedlund et al. (2005) reported that soybean isoflavone concentrations in prostatic fluid can reach up to 50 μM in persons with a long-term soybean-rich dietary habit. In a study by Hu et al., 8 healthy women ingested a single dose of concentrated soy extract containing 434 μM group B soyasaponins; however, after 5 days, only 36.3 +/- 10.2 M group B soyasaponin metabolites were detected in the faecal collection, which suggested that ingested soyasaponins have low absorbability and bioavailability in humans (Hu et al., 2004). Therefore, the effective single-treament dose obtained in this study is unlikely to occur *in vivo* only through the consumption of soybean. Currently, the synergistic effect generated from the combination of different drugs has attracted great attention due to the advantage of increased anticancer effects, lower drug dosages, and reduced side effects. Synergistic anticancer effects have been observed with different compounds or bioactive components derived from natural plants, such as arctigenin

and quercetin, and phytochemical enriched apple extracts and quercetin 3-β-D-glucoside (Wang et al., 2015; Yang et al., 2009). Therefore, the synergistic effect of two-way combination treatments of these bioactive components was investigated. We found that the mixture of genistin plus daidzin showed a synergistic effect towards MCF-7 cells with a CI_{50} value of 0.89. In addition, the mixture of genistein plus genistin, genistein plus β-sitosterol and β-sitosterol plus genistin showed synergistic effects towards MDA-MB-231 cells with combination index at inhibition of 50% (CI_{50}) values of 0.56, 0.54 and 0.45, respectively. Among all the combination treatments, due to the synergistic effects, the EC_{50} values of β-sitosterol and genistin towards MDA-MB-231 were respectively reduced to 37.71 μM and 24.55 μM, both less than 50 μM. Based upon the results of Hedlund et al. (2005), we hypothesized that less than 50 μM β-sitosterol and genistin less can also be reached in the breast of humans with long-term and high intake of soybean food. Thus, the decrease in EC_{50} value increases the possibility of application of natural anticarcinogens in humans. Invasive and migratory abilities are important characteristics tumour cells metastasis (Chun et al., 2013). We found that the tested samples were able to inhibit the invasion and migration of breast cancer cells. Combination treatments further enhanced the effects.

Signalling pathways. Anti-proliferative and inhibitory effects of anticarcinogens on cell invasion and migration were associated with changes in multiple signalling pathways (Wang et al., 2015). Among the 18 tested target proteins, AMPKα is an important molecular target of these bioactive anticarcinogens, which suggested that the regulation of the AMPKα signalling pathway is the main mechanism involved in the anti-proliferative activity of bioactive anticarcinogens from soybean in both MCF-7 and MDA-MB-231 cells. All the tested samples, expect genistin, significantly elevated the phosphorylation of AMPKα in both MCF-7 and MDA-231 cells. AMPK is a serine/threonine protein kinase that is responsible for cellular energy homeostasis (Chiang et al., 2010). It has been reported that AMPK can regulate cell proliferation, cell growth and autophagy and has been proposed to be a potential target for cancer chemotherapy (Høyer et al., 2007). AMPK activation strongly suppresses cell proliferation in non-malignant cells as well as in tumour cells (Motoshima et al., 2010). In addition, changes in the phosphorylation of molecular proteins, including stat1 (a key modulator of cell death), Bad (a pro-apoptic protein from Akt pathway), p53 (a well-known tumour suppressor), p38 (a member of MAPK), a mammalian target of rapamycin (mTOR) (a modulator of cell growth that can mediate the activation of Akt pathway) and Akt (a pro-apoptic protein), were observed in this several studies (Kim et al., 2007; Yuan et al., 2005; Hollstein et al., 1991; Cuenda et al., 2007; Guertin et al., 2007).

1.2 Anti-inflammatory activity of lunasin

Production of lunasin. Currently, large molecules in plants have attracted more attention than small molecules. Resistant soybean proteins, including BBI and 2S albumin storage proteins and polypeptides such as lunasin, have received particular

interest as bioactive components (Clemente & Olias, 2017). Lunasin is a novel bioactive peptide with higher potential against chronic disorders. Research on the bioactivity of lunasin began in this century, and many problems still remain to be studied. Therefore, in our following study, we focused on lunasin. Inflammation is involved in many chronic diseases, such as cardiovascular disease and cancer (Moutsopoulos et al., 2010). In previous studies, the anti-inflammatory activity of natural lunasin (N-lunasin) from soybean was demonstrated. (Hernández-ledesma et al., 2009; Dia et al., 2009). Recently, we attempted to produce lunasin through genetic engineering methods, and recombinant lunasin (R-lunasin) was successfully expressed from *P. pastoris* in the present study with a yield of 240 mg/L cell-free broth. The yield in our study is much higher than that in the study of Liu et al. (2010), who expressed recombinant lunasin from *E. coli* (4.73 mg per liter of Luria-Bertani culture medium).

Anti-inflammatory activity. Because the purified recombinant lunasin was obtained, the main objective of the present study was to compare its anti-inflammatory activity with that of natural lunasin. The results showed that R-lunasin and N-lunasin showed comparable inhibitory effects on the production of NO, TNF-α and IL-6 in LPS-stimulated RAW264.7 cells between N-lunasin and R-lunasin.

Signalling pathways. LPS activates a broad range of signalling pathways in macrophages (Mendes et al., 2009). The signalling pathways involved in the anti-inflammatory effects of lunasin are focused on NF-κB pathway (Mejia et al., 2009). Recent studies have indicated that mTOR signalling can also be activated by LPS stimulation (Dos et al., 2007; Kawai et al., 2010). However, whether lunasin inhibits the mTOR signalling pathway has not been clarified. In this respect, a slide-based antibody array was used for the simultaneous detection of 18 important and well-characterized signalling molecules. The results showed that R-lunasin treatment strongly inhibited the phosphorylation of Akt, mTOR and p70s6k elevated by LPS, which suggested that lunasin could suppress LPS-induced activation of Akt/mTOR signalling. Wang et al. (2014) and Weinstein et al. (2000) found that elevated mTOR signalling might be responsible for the increased TNF-α and NO production in macrophages in response to LPS stimulation. These results suggested that lunasin may inhibit TNF-α and NO production via the mTOR pathway. We determined that the inhibition of GSK-3β expression in LPS-stimulated cells was up-regulated by R-lunasin treatment. A previous study demonstrated that the phosphorylation of GSK-3β at Ser9 decreases the expression of IL-6 and inducible nitric oxide synthase (iNOS) in LPS-stimulated cells (Beurel et al., 2009). Consequently, it was concluded that the down-regulation of IL-6 and NO was also dependent on GSK-3β inhibition.

1.3 Lunasin content in 413 soybean samples collected from four major regions in China

Lunasin content. We are also interested in the presence as well as the

bioactivity of lunasin in soybean seeds. It has been reported that the lunasin content in soybean seeds is affected by both genetic and environmental factors, such as cultivar, planting temperature, soil moisture and light condition (Wang et al., 2008). However, most of these results were based on controlled experiments in green-houses or with limited sampling. In the present study, we collected 413 soybean samples from 4 regions in China. The results showed that the lunasin content ranged from 0.53 mg/g to 3.25 mg/g dry flour, which was consistent with the lunasin content (1-13.3 mg lunasin per g defatted soybean flour) reported by Gonzalez et al. (2004), who determined the lunasin content in 144 selected diverse soybean accessions from the U.S. using an ELISA assay. Gao et al. (2018) quantified the lunasin content of 6 soybean cultivars planted in China using the NanoLC-Orbitrap technique. Their results showed that the lunasin content ranged from 2.03 mg lunasin per g defatted soybean flour to 4.66 mg lunasin per g defatted soybean flour, which was also consistent with our results. These results further reflected a fact that natural plants contain low lunasin content. Gentic engineering method would be a better method to produce lunasin.

Influence factors. We found that lunasin content varied significantly in four major planting regions. It showed an increasing trend from south China to north China. This is the first evidence regarding the regional distribution of soybean lunasin content in China. Although our results are from different soybean cultivars and genetic factors cannot be ignored, the extensive and high-density sampling as well as the similar tend observed from two years make the results tremendously credible. The detected high lunasin content could meet the demand for an ideal material for the extraction of natural lunasin. Wang et al. (2008) reported that soil moisture conditions and growing temperature significantly affected lunasin content in soybean seeds, which suggested that climate factors may influence lunasin content. In the present study, a positive correlation between lunasin content and hours of sunshine (HS) as well as diurnal temperature range (DTR) and a negative correlation between lunasin content and rainfall (RF) were observed. High RF increased the soil moisture, which delayed the maturity of soybean seeds (Lozovaya et al., 2005). It has been reported that lunasin content positively correlates with seed maturation (Hernandez et al., 2013). These results may explain why high HF reduce lunasin content. The observed correlation between lunasin content and climate factors indicates that lunasin content in soybean can be increased through cultivation with different conditions.

2. Perspectives

2.1 A new strategy for future developments of natural health products was provided

In recent years, increasing research has focused on the prevention of disease through dietary modification with natural health, which are generally called functional foods or nutraceuticals. The main difference between natural health

produts and pharmaceuticals is that natural health products are 1) made from natural materials, 2) can be multi-targeted mixtures and 3) present at low concentrations (Shahidi, 2012). There are challenges for natural health product producers because such products should show clear bioactivity and visible effects (though much lower than those of pharmaceutical products) at low concentrations. Soybean food is well-known for its anticancer effect. Our results demonstrated synergistic anticancer effects of bioactive components in soybean *in vitro*, which clarified the effective constituents and reduced the effective dose. These results provide a strategy for future developments of natural health products. In the future, more work should be conducted on the combination of different groups of bioactive components to examine the possible synergistic effects. Furthermore, clinical trials are needed to confirm these effects *in vivo* in future studies.

2.2 Recombinant lunasin was provided as materials for future study on the bioavailability of lunasin in vivo.

The effect of the digestive process on the properties of orally consumed bioactive peptides is determined. The key question about the bioavailability of lunasin is whether lunasin survives digestion in the gastrointestinal tract when ingested orally. Previous *in vitro* studies confirmed the protective role of BBI on lunasin digestion and the intestinal absorption of this peptide and its digestion-derived fragment RKQLQGVN through epithelial paracellular diffusion. Interestingly, four regions comprising the entire 43 amino acid sequence of lunasin, SKWQHQQDSC, RKQLQGVN1810, LTPCEKHIME and KIQGRGDDDDDDDDD, showed particular resistance to the action of gastrointestinal enzymes, with SKWQHQQDSC being the main responsible fragment for the proven inhibitory activity of lunasin and its gastrointestinal digests in HT-29 colorectal cancer cells (Cruz-Huerta et al., 2015; Fernández-Tomé et al., 2018). In addition, the biological activity of prepared lunasin significantly decreased after its hydrolysis by pepsin-pancreatin, which is a simulated gastrointestinal environment (Price et al., 2017). One *in vivo* study of the bioavailability of lunasin was conducted by Dia et al. (2009) using soybean protein products rather than purified lunasin. They assessed the presence and concentration of lunasin in the blood of men fed soy protein products and estimated an average lunasin absorption of 4.5% (range of 2.2-7.8%) from the total lunasin ingested from 50 g of soy protein. Currently, studies on the metabolism and bioavailability of lunasin *in vivo* are severely limited by the high cost of purified lunasin. In the present study, a successful protocol for the expression of recombinant lunasin in *P. pastoris* with a high yield was used to provide purified materials for future *in vivo* studies on the metabolism and bioavailability of lunasin.

3. General conclusion

In this study, we first investigated the anticancer effect of 12 bioactive components from soybean on human breast cancer cells *in vitro*. Single treatment

of genistein showed the strongest anti-proliferative effect in both MCF-7 and MDA-MB-231 cells. In addition, synergistic anti-proliferative effects were observed in two-way combination treatments of genistin plus daidzin in MCF-7 cells and of genistein plus genistin, genistein plus β-sitosterol or β-sitosterol plus genistin in MDA-MB-231 cells. Among all the tested components, lunasin attracted our interest because it is a novel peptide and many problems about lunasin remain to be studied. Therefore, we isolated natural lunasin from soybean and successfully expressed recombinant lunasin from P. pastoris. Anti-inflammatory activity in LPS-stimulated RAW264.7 cells was demonstrated. Intracellular signalling array analysis revealed that lunasin inhibited the activation of the Akt/mTOR/p70s6k signalling pathway in LPS-stimulated RAW264.7 cells. We hypothesized that a high lunasin content soybean cultivar may help to improve the yield of lunasin extraction from soybean. Therefore, 413 soybean samples were collected and the lunasin content was determined. The highest lunasin content was observed in cultivar Changmidou 30 collected from Jilin province harvested in 2015. Regional and climate factors indeed affected lunasin content in the soybean seeds. The lunasin content showed an increasing trend from south China to north China. There was a positive correlation between lunasin content and HS as well as DTR; however, a negative correlation between lunasin content and RF was found. DTR was demonstrated as the dominant factor affecting lunasin content in soybean seeds. These results generated from our study were anticipated to improve our knowledge of bioactive components in soybean.

4. References

Ahmad, A., Hayat, I., Arif, S., Masud, T., Khalid, N., & Ahmed, A. (2014). Mechanisms involved in the therapeutic effects of soybean (Glycine max). International Journal of Food Properties,17, 1332–1354.

Beurel, E., and Jope, R.S. (2009). Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain, J. Neuroinflamm., 9, 23.

Chiang, P. C., Lin, S. C., Pan, S. L., Kuo, C. H., Tsai, I. L., Kuo, M. T., ... &Guh, J. H. (2010). Antroquinonol displays anticancer potential against human hepatocellular carcinoma cells: a crucial role of AMPK and mTOR pathways. Biochemical pharmacology, 7

Chuang SC, Chen W, Hashibe M, Li G, & Zhang ZF. (2006). Survival rates of invasive breast cancer among ethnic chinese women born in east asia and the united states. Asian Pac J Cancer Prev, 7(2), 221-226.

Chun, J., & Kim, Y. S. (2013). Platycodin d inhibits migration, invasion, and growth of mda-mb-231 human breast cancer cells via suppression of egfr-mediated akt and mapk pathways. Chemico-biological Interactions, 205(3), 212-221.

Clemente, A., & Olias, R. (2017). Beneficial effects of legumes in gut health. Current Opinion in Food Science, 14, 32-36.

Cuenda, A., & Rousseau, S. (2007). P38 map-kinases pathway regulation, function and role in human diseases. Biochimica Et Biophysica Acta, 1773(8), 1358-1375.

Dia, V. P, Wang, W, Oh, V. L, Lumen, B. O. de, & Mejia, E. G. de. (2009). Isolation, purification and characterisation of lunasin from defatted soybean flour and in vitro evaluation of its anti-inflammatory activity. Food Chemistry, 114(1), 108-115.

Dia, V. P., Torres, S., De Lumen, B. O., Jr, E. J., & De Mejia, E. G. (2009). Presence of lunasin in plasma of men after soy protein consumption. Journal of Agricultural & Food Chemistry, 57(4), 1260-6.

Dos Santos, S., Delattre, A. I., De Longueville, F., Bult, H., and Raes. M. (2007). Gene expression profiling of LPS-stimulated murine macrophages and role of the NF-κB and PI3K/mTOR signalling pathways, Ann. Ny. Acad. Sci., 1096, 70-77.

Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med. 1: 27–31.

Gao, MD., Meng, XB., Xing CC., Zhang, Y., Deng, HT., and Wang FZ. (2018). Determination of polypeptide lunasin content in six different cultivars of soybean using nanolc-orbitrap technique. Modern Food Science & Technology (in Chinese).

Gonzalez de Mejia, E., Vásconez, M., de Lumen, B. O., & Nelson, R. (2004). Lunasin concentration in different soybean genotypes, commercial soy protein, and isoflavone products. Journal of agricultural and food chemistry, 52(19), 5882-5887.

Gorgenes, J. F., Van Zyl, W. H., Knoetze, J. H., and Hahn-Hagerdal, B. (2005). Amino acid supplementation improves heterologous protein production by Saccharomyces cerevisiae in defined medium, Appl. Microbiol. Biotechnol., 67, 684–691.

Guertin, D. A., & Sabatini, D. M. (2007). Defining the role of mtor in cancer. Cancer Cell, 12(1), 9-22.

Hedlund, T. E., Maroni, P. D., Ferucci, P. G., Dayton, R., Barnes, S., Jones, K., ... & Gray, K. J. (2005). Long-term dietary habits affect soy isoflavone metabolism and accumulation in prostatic fluid in caucasian men. The Journal of nutrition, 135(6), 1400-1406.

Hernandez-Ledesma, B., C Hsieh, C., & O de Lumen, B. (2013). Chemopreventive properties of Peptide Lunasin: a review. Protein and peptide letters, 20(4), 424-432.

Hollstein, M., Sidransky, D., Vogelstein, B., & Harris, C. C. (1991). P53 mutations in human cancers. Science, 253(5015), 49-53.

Hu, J., Reddy, M. B., Hendrich, S., & Murphy, P. A. (2004). Soyasaponin i and sapongenol b have limited absorption by caco-2 intestinal cells and limited bioavailability in women. Journal of Nutrition, 134(8), 1867.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, Nat. Immunol., 11(5), 373-384.

Kim, H. S., & Lee, M. S. (2007). Stat1 as a key modulator of cell death. Cellular Signalling, 19(3), 454-465.]

Kyle, S., James, K. A., & McPherson, M. J. (2012). Recombinant production of the therapeutic peptide lunasin. Microbial cell factories, 11(1), 28.

Liu C.F. & Pan T. M., 2010. Recombinant expression of bioactive peptide lunasin in Escherichia coli. Appl. Microbiol. Biotechnol., 88, 177-86.

Lozovaya, V. V., Lygin, A. V., Ulanov, A. V., Nelson, R. L., Daydé, J., & Widholm, J. M. (2005). Effect of temperature and soil moisture status during seed development on soybean seed isoflavone concentration and composition. Crop science, 45(5), 1934-1940.

Moutsopoulos, N. M., and Madianos, P. N. (2010). Low-Grade Inflammation in Chronic Infectious Diseases, Ann. NY. Acad. Sci., 1088(1), 251-264.

Mejia, E. G. D., and Dia, V. P. (2009). Lunasin and lunasin-like peptides inhibit inflammation through suppression of nf-κb pathway in the macrophage, Peptides, 30(12), 2388-98.

Mendes, S. D. S., Candi, A., Vansteenbrugge, M., Pignon, M. R., Bult, H., Boudjeltia, K. Z., Munaut, C., and Raes, M. (2009). Microarray analyses of the effects of NF-κB or PI3K pathway inhibitors on the LPS-induced gene expression profile in RAW264. 7 cells: synergistic effects of rapamycin on LPS-induced MMP9-overexpression, Cell. Signal., 21(7), 1109-1122.

Motoshima, H., Goldstein, B. J., Igata, M., & Araki, E. (2010). Ampk and cell proliferation--ampk as a therapeutic target for atherosclerosis and cancer. Journal of Physiology, 574(1), 63-71.

Odani, S., Koide, T., & Ono, T. (1987). Amino acid sequence of a soybean (Glycine max) seed polypeptide having a poly (L-aspartic acid) structure. Journal of Biological Chemistry, 262(22), 10502-10505.

Shahidi, F. (2012). Nutraceuticals, functional foods and dietary supplements in health and disease. Journal of food and drug analysis, 20, 226-230.

Wang, P., Phan, T., Gordon, D., Chung, S., Henning, S. M., &Vadgama, J. V. (2015). Arctigenin in combination with quercetin synergistically enhances the anti-proliferative effect in prostate cancer cells. Molecular Nutrition & Food Research, 59(2), 250-261.

Wang, W., Dia, V. P., Vasconez, M., de Mejia, E. G., & Nelson, R. L. (2008).

Analysis of soybean protein-derived peptides and the effect of cultivar, environmental conditions, and processing on lunasin concentration in soybean and soy products. Journal of AOAC International, 91(4), 936-946.

Wang, Y., Liu, Y., Zhang, X. Y., Xu, L. H., Ouyang, D. Y., Liu, K. P., Pan, H., He, J., and He, X. H. (2014). Ginsenoside Rg1 regulates innate immune responses in macrophages through differentially modulating the NF-κB and PI3K/Akt/mTOR pathways, Int. Immunopharmac., 23(1), 77-84.

Weinstein, S. L., Finn, A. J., Davé, S. H., Meng, F., Lowell, C. A., Sanghera, J. S., and DeFranco, A. L. (2000). Phosphatidylinositol 3-kinase and mtor mediate lipopolysaccharide-stimulated nitric oxide production in macrophages via interferon-beta, J. Leukocyte Biol., 67(3), 405-414.

Xu, X., Wang, H. J., Murphy, P. A., Cook, L., & Hendrich, S. (1994). Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. Journal of Nutrition, 124(6), 825-32.

Yang, J., & Liu, R. H. (2009). Synergistic effect of apple extracts and quercetin 3-β-d-glucoside combination on antiproliferative activity in mcf-7 human breast cancer cells in vitro. Journal of Agricultural & Food Chemistry, 57(18), 8581.

Yuan, Z., Kim, D., Kaneko, S., Nicosia, S. V., & Cheng, J. Q. (2005). Akt/pkb interaction protein apαb mediates akt and pak1 cell survival signal. Cancer Research, 65.

Zhou, J. R., Gugger, E. T., Tanaka, T., Guo, Y., Blackburn, G. L., & Clinton, S. K. (1999). Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumour angiogenesis in mice. Journal of Nutrition, 129(9), 1628-1635.