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Functional proteomic analysis revels that the ethanol extract of *Annona muricata* L. induces liver cancer cell apoptosis through endoplasmic reticulum stress pathway

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ABSTRACT

Ethnopharmacological relevance: *Annona muricata* L. is used to treat cancer in some countries. Extracts of *Annona muricata* have been shown to cause apoptosis of various cancer cells *in vitro*, and inhibit tumor growth *in vivo* in animal models. However, the molecular mechanisms underlying its anti-cancer and apoptotic effects of the herb remain to be explored.

Aim of study: The study investigated the molecular mechanisms underlying liver cancer cell apoptosis triggered by the ethanol extract of leaves of *Annona muricata* L.

Materials and methods: Liver cancer HepG2 cells were used as experimental model. MTT assay was employed to evaluate cell viability. Flow cytometry and TUNEL assays were performed to confirm apoptosis. We employed functional proteomic analysis to delineate molecular pathways underlying apoptosis triggered by the herbal extract.

Results: We showed that the extract was able to reduce viability and trigger apoptosis of the cancer cells. Proteomic analysis identified 14 proteins associated with the extract-elicited apoptosis, which included the increased expression levels of HSP70, GRP94 and DPI-related protein 5. Western blot analysis confirmed that the extract did up-regulated the protein levels of HSP70 and GRP94. Results from bioinformatic annotation pulled out two molecular pathways for the extract, which, notably, included endoplasmic reticulum (ER) stress which was evidenced by the up-regulation of HSP70, GRP94 and PDI-related protein 5. Further examinations of typical protein signaling events in ER stress using western blot analysis have shown that the extract up-regulated the phorsphorelation of PERK and eIF2α as well as the expression level of Bip and CHOP.

Conclusion: Our results indicate that the ethanol extract of leaves of *Annona muricata* L. causes apoptosis of liver cancer cells through ER stress pathway, which supports the ethnomedicinal use of this herb as an alternative or complementary therapy for cancer.

1. Introduction

Cancer is one of the most common non-communicable diseases word-wide; in China alone, one cancer patient is diagnosed in every six minutes. This casts great socio-economic burdens to societies. Surgery, chemotherapy and radiotherapy are common treatments for cancer. However, they often do not produce desirable outcomes and many patients are keen to seek alternative and complementary therapies. Hopefully, ethnomedicinal treatments can help in treating cancer as well as increase the efficacy and/or alleviate side effects of common clinical therapies (Smith et al., 2014; Wang et al., 2015).

Annona muricata L., also known as graviola, soursop and guanabana, is an evergreen tree that is seen in tropical and sub-tropical areas. The plant name of *Annona muricata* L. is verified by the World Checklist of Selected Plant Families (http://www.theplantlist.org/tpl1.1/record/kew-2640944) and is included in the Plant List (www.theplantlist.org). The plant is collected by different recognized herbariums such as the African Plant Database, the New York Botanical Garden Virtual Herbarium and Royal Botanic Gardens, Kew. The fruits of the plant are edible and are used to

Abbreviations: AGE, Annonaceous acetogenin; Bip, Heat shock protein family A member 5; CHOP, DNA damage inducible transcript 3 protein; DMEM, Dulbecco's Modified Eagle's medium; EGFR, Epidermal growth factor receptor; eIF2α, Eukaryotic translation initiation factor 2alpha; ER, Endoplasmic reticulum; GRP, Glucose-regulated protein; HSP, Heat shock protein; IEF, Isoelectric focusing; MTT, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PERK, Eukaryotic translation initiation factor 2 alpha kinase 3; PDI, Protein disulfide isomerase; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

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prepare syrups, candies, beverages, ice creams and shakes. The plant has long been used in different countries as ethnomedicinal treatment for various conditions which include cystitis, diabetes, hypertension, headaches, insomnia, rheumatism and parasite infection (Moghadamtousi et al., 2015a). More interesting, the plant is used in South America and tropical Africa as an ethnomedicine against tumor (Adewole and Ojewole, 2009). In keeping with the traditional use for anti-cancer treatment, Torres and co-workers have confirmed that extract of the plant can inhibit the viability and motility as well as induce apoptosis of pancreatic cancer cells *in vitro*, and suppress tumor growth and metastasis *in vivo* in mice model, which may be due to the extract-triggered down-regulation of metabolism and cell necrosis (Torres et al., 2012). In EGFR-over-expressing human breast cancer cells, extract of *Annona muricata* L. fruits inhibits the growth and cause apoptosis of the cancer cells *in vitro*, with no effect on non-tumorigenic human breast epithelial cells. In mice model, the extract reduces the protein level of EGFR in the cancer cells, and inhibits the tumor growth (Dai et al., 2011). Moghadamtousi et al., (2015b) used rat model with azoxymethane-induced colonic aberrant crypt foci to examine the *in vivo* chemopreventive potential of the ethyl acetate extract of the *Annona muricata* L. leaves, and found that their extract was able to reduce the formation of aberrant crypt foci. Apoptotic effect of the plant extracts appears to be an important mechanism for its anti-cancer activity, as it has been demonstrated under different experimental conditions. In addition to pancreatic cells (Torres et al., 2012), Pieme et al. extract the plant leaf, twigs or roots using hot 95% ethanol, and found all extracts could induce apoptosis of Human promyelocytic leukemia HL-60 cells (Pieme et al., 2014). Hexane extract of leaves causes colon cancer cell (HT-29 and HCT-116) apoptosis though the intrinsic pathway, as evidenced by phosphatidylserine externalization, mitochondria transmembrane potential drop, cytochrome c release and caspase activation. The extract also inhibits the cancer cell migration and invasion (ZorofchianMoghadamtousi et al., 2014). In lung cancer A549 cells, extract of the plant leave can suppress NF-κB activation, suggesting that NF-κB is involved in apoptotic signal transduction (Moghadamtousi et al., 2014).

Despite the accumulated evidence for apoptotic effect of extracts of the plant on various cancer cells, the underlying molecular mechanisms remain to be explored further. The present study investigated whether the ethanol extract of *Annona muricata* L. leaves could cause apoptosis using liver cancer HepG2 cells as an experimental model. Functional proteomic analysis was performed to delineate the molecular pathways underlying the extract-induced apoptosis.

2. Materials and methods

2.1. Chemicals, reagents and antibodies

All chemicals, reagents, consumables and equipment for two-dimensional gel electrophoresis were purchased from GE Healthcare life sciences (Beijing, China). Chemicals for non-proteomic assays were from Sigma (St. Louis, MO, USA), unless otherwise specified. Cell culture medium (DMEM), penicillin-streptomycin, and L-glutamine were purchased from GIBCO (NY, USA). Fetal bovine serum was a product of Hyclone (Logan, UT, USA). Antibodies were purchased from Cell Signaling Technology (Denvers, MA, USA).

2.2. Preparation of plant extract

Annona muricata L. leaves were collected from Nanning city, Guangxi province of China. The taxonomic identity was verified by our botanist. Leaves were air dried and cut into pieces of approximately 1 cm × 1 cm in size. The leaf pieces were weighted and placed into glass bottles, and 70% ethanol was added to cover the leaf pieces. The bottles were placed in dark at room temperature for 10 days, after which the supernatants were collected by filtering through Whatman 3 mm filter paper and dried using a vacuum drier. The resulted powders were dissolved in DMSO and stored at room temperature in the dark till use.

2.3. Cell culture

Liver cancer HepG2 and colon cancer HCT116 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin in a humidified incubator with 5% CO_2 at 37 °C. Cell cultures at approximately 70% confluence were used for all experimental treatments.

2.4. Cell viability and apoptosis assays

MTT assay was employed to quantify cell viability (Lee et al., 2006, 2007). Approximately 7000 cells were seeded to each well of a 96-well plateand cultured for 24 h, followed by treatment for another 24 h (the control samples received DMSO alone). At the end of treatment, MTT reagent (5 mg/ml, 10 μl) was added to each well and cells were incubated for 3 h. Finally, medium was removed and 150 μl DMSO was added into each well and mixed by gentle shaking for 15 min, and optical density value of each well was determined using a plate reader at 490 nm. Apoptosis was evidenced and quantified using propidium iodide (PI) staining for DNA fragmentation by flow cytometry analysis (Lee et al., 2006, 2007). Cells were harvested in a 15-ml tube, fixed with 70% ethanol, and stained with PI. At least 10,000 events were analyzed by flow cytometry (FACS Calibur, Becton Dickinson, CA, USA) with the excitation set at 488 nm and emission at 610 nm. Data were analyzed using the built-in software package (Modfit). In Situ Cell Death Detection Kit from Roche Diagnostics (Penzberg, Germany) was used for TUNEL assay, which was to confirm treatment-induced apoptosis (Lee et al., 2010). Briefly, HepG2 cells were grown on Labtek chamber slides (Rochester, NY, USA), washed once with PBS, and fixed in 100% methanol at −20 °C for 10 min The fixed cells were blocked in blocking buffer (1% BSA and 0.05% Tween-20 in 1× PBS) for 1 h, followed by three washes with 1× PBS. The cells were then incubated with the TUNEL staining solution overnight at 4 °C and then washed three times with 1× PBS before mounting with UltraCruz Mounting Medium (Santa Cruz, CA, USA). The cells were immediately examined by fluorescent microscopy (Olympus IX73, Tokyo, Japan).

2.5. Proteomics

The two-dimensional gel electrophoresis procedure was detailed in previous publications (Lee et al., 2006, 2007). Briefly, IPG polyacrylamide strips with a linear pH gradient from 4 to 7 as the first-dimensional gels. IPG strips were rehydrated in re-swelling buffer for IEF using an IPGphor machine. Total cellular protein samples were applied using sample loading cup method. Focused IPG strips were frozen at −20 °C till use. Before the second-dimensional run, IPG strips were equilibrated twice in equilibration buffer, then were placedon top of 12% SDS-PAGE (Bio-Rad PROTEAN II xi cell) and sealed with 0.5% agarose containing trace amount of bromophenol blue in running buffer for the second-dimensional separation. Peptides separated on gels were visualized by silver staining method. Gel images were captured using an ImageScanner and analyzed using the software package ImagMaster 2D Platinum.

Spots of interests were excised with a scalpel for identification determination through peptide mass finger printing that involved in-gel tryptic digestion, tryptic peptide mass determination by mass spectrometry. Peptide masses were used for identification search using the MASCOT search engine 2.2 (Matrix Science, Ltd.) embedded into GPS-Explorer Software 3.6 (Applied Biosystems) on the NCBI database with the following parameter settings: 100 ppm mass accuracy, trypsin cleavage one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionine was allowed as variable modification, MS/MS fragment tolerance was set to 0.4 Da. GPS Explorer protein confidence index $\geq$95% were used for further manual validation.

2.6. Bioinformatics

For pathway annotation, amino acid sequences of proteins of interests were retrieved from Uniprot (http://www.uniprot.org/) and submitted to KEGG Automatic Annotation Server (Ver. 2.0; http://www.genome.jp/tools/kaas/) to pull out pathways. We used default parameter except that we selected BLAST for search program, text data for query sequences, and single-directional best hit for assignment method.

2.7. Western blot analysis

Western blot analysis was performed as described previously (Lee et al., 2006, 2007). Cell lysates were subjected to 10% polyacrylamide gel electrophoresis then transferred onto PVDF membranes. After blocking overnight with 5% dry milk in TBST solution (50 m MTris/ HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20), membranes were washed three times using TBST, exposed to primary antibody overnight at 4 °C with gentle shaking, washed with TBST three times, exposed to secondary antibody for 1 h, washed three times again in TBST, and eventually subjected to chemiluminescence detection using the SuperSignal Substrate Western Blotting Kit.

2.8. Statistical analysis

All experiments were performed in triplicate. Data were expressed as mean $\pm$ SD, and were analyzed by one way ANOVA analysis using the statistical software package SPSS. A $p < 0.05$ was considered to be statistical significant.

3. Results

3.1. Ethanol extract of Annona muricata L. leaves inhibited the viability of liver cancer HepG and colon cancer HCT116 cells.

We first examined the effect of the extract on cell viability using MTT assay. Clearly, the extract was able to inhibit the viability of the liver cancer HepG2 (Fig. 1(A)) and colon cancer HCT116 (Fig. 1(B)) cells in a dose- and time-dependent manner. The values of IC50 values were estimated to be approximately150 μg/ml for both HepG2 and HCT116 cells.

3.1.1. Ethanol extract of Annona muricata L. leaves triggered apoptosis of liver cance HepG cells

Next, we analyzed cell cycle of the control and treated HepG2 cells using PI staining in combination flow cytometry. Results showed that the herbal extract increased the amount of sub-G1cells in a dose-dependent manner. It produced approximately 33% of sub-G1 cells at the concentration of 120 μg/ml (Fig. 2 (A) and (B)). We then performed TUNEL assay to confirm the apoptotic effect of the extract, which showed that the extract significantly increased the number of cells positive for TUNEL staining (Fig. 2(C)).

3.1.2. Proteomic analysis identified 1 proteins associated with the herbal extract-induced apoptosis

To look for molecular pathways underlying the extract-evoked apoptosis, we performed traditional proteomic analysis to identity proteins responsive to the treatment by the extract. As shown in Fig. 3(A) and (B), the extract caused changes in 14 peptide spots on the two dimensional gels. Subsequently, we obtained the identities of the 14 proteins of interests through peptide mass finger printing analyses (Table 1). They are proteins candidates of signal transduction mediators for the extract-elicited apoptosis.

3.1.3. Bioinformatic annotation reveled that endoplasmic reticulum stress was the main pathway underlying the extract-induced apoptosis

We used the 14 identified proteins for bioinformtic annotation for molecular pathways. Totally, 2 pathways were retrieved by KEGG annotation. One is estrogen signaling pathway which had included 2 of the 14 proteins, HSP70 and HSP90 (figure not shown). The other was endoplasmic reticulum (ER) stress pathway that had included 3 proteins (Fig. 4(A)), which were GRP94, HSP70 and PDI-related protein. Western blot confirmed that the extract did increase the expression levels of GRP94 and HSP70proteins (Fig. 4(B)). The effect of the extract on the protein level of PDI-relate protein was not examined by Western blot, as we had found no commercial antibody specific to this protein.

3.2. Typical molecular events in ER stress appeared after cells were treated by the herbal extract

Finally, we investigated typical protein signaling events in ER stress to confirm the finding from the bioinformatic annotation,

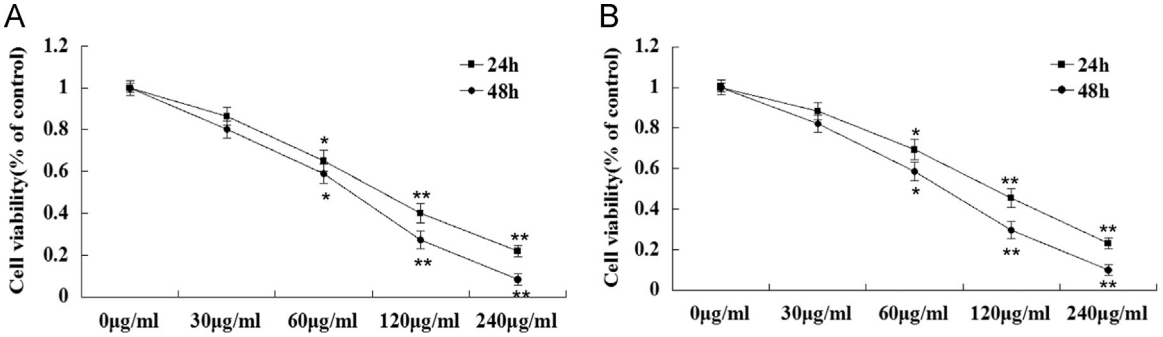

Fig. 1. The extract of *Annona muricata* leaves inhibited the viability of HepG2 and HCT116 cells. (A). Liver cancer HepG2 cells; (B). Colon cancer HCT116 cells.

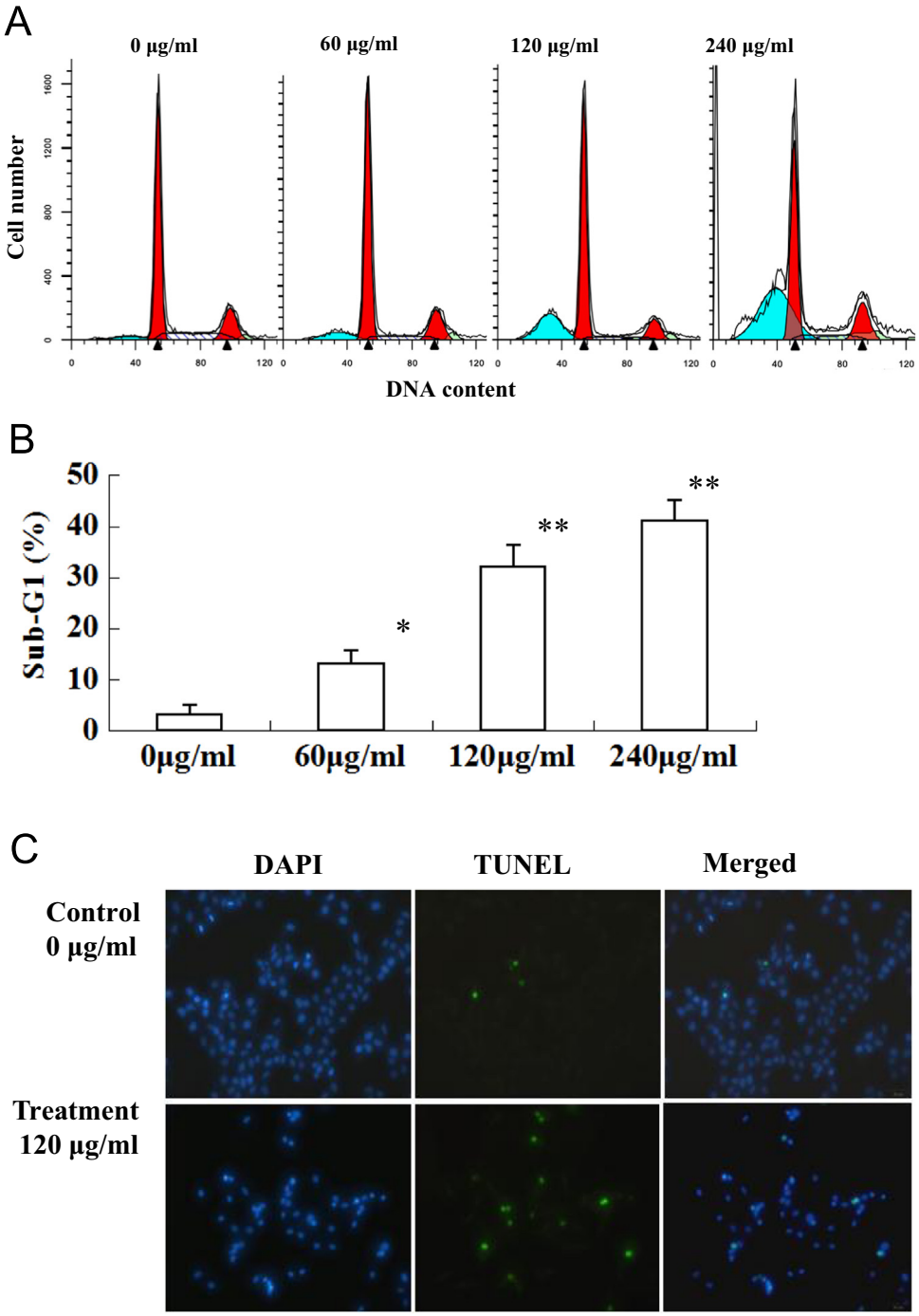

Fig. 2. The extract of *Annona muricata* leaves evoked apoptosis of liver cancer HepG2 cells. (A). Flow cytometry analysis outcome; (B). Percentage of sub-G1 cells from flow cytometry analysis; (C). TUNEL assay outcome.

which included the increased expression levels of Bip and CHOP as well as increased level of phosphorylations of PERK and eIF2α. As shown in Fig. 5, the herbal extract did increase the protein levels of Bip and CHOP, and enhanced the phosphorylation of PERK and eIF2α.

4. Discussion

As summarized by Cassileth and Deng (Cassileth and Deng, 2004), a Data Monitor Survey shows that up to 80% of patients have used alternative and/or complementary therapy in fighting cancer. The visits of cancer patients to alternative and complementary medicine practitioners outnumbered the visits of cancer patients to U. S. primary care physicians. Medicinal plants provide a useful resource for alternative and complementary therapy. This shows that alternative and complementary medicine is highly valued by patients fighting against cancer. *Annona muricata* L. extracts can inhibit proliferation and cause apoptosis of various types of cancer cells in vitro as well as in mice models in vivo, and can suppress tumor metastasis (Torres et al., 2012; Dai et al., 2011; Pieme et al., 2014; Moghadamtousi et al., 2014, 2015), which shows that *Annona muricata* L. provides an option for cancer ethnomedicinal therapy.

Our results confirm that leaf extract can cause apoptosis of liver cancer cells, as evidenced by data from flow cytometry and TUNEL

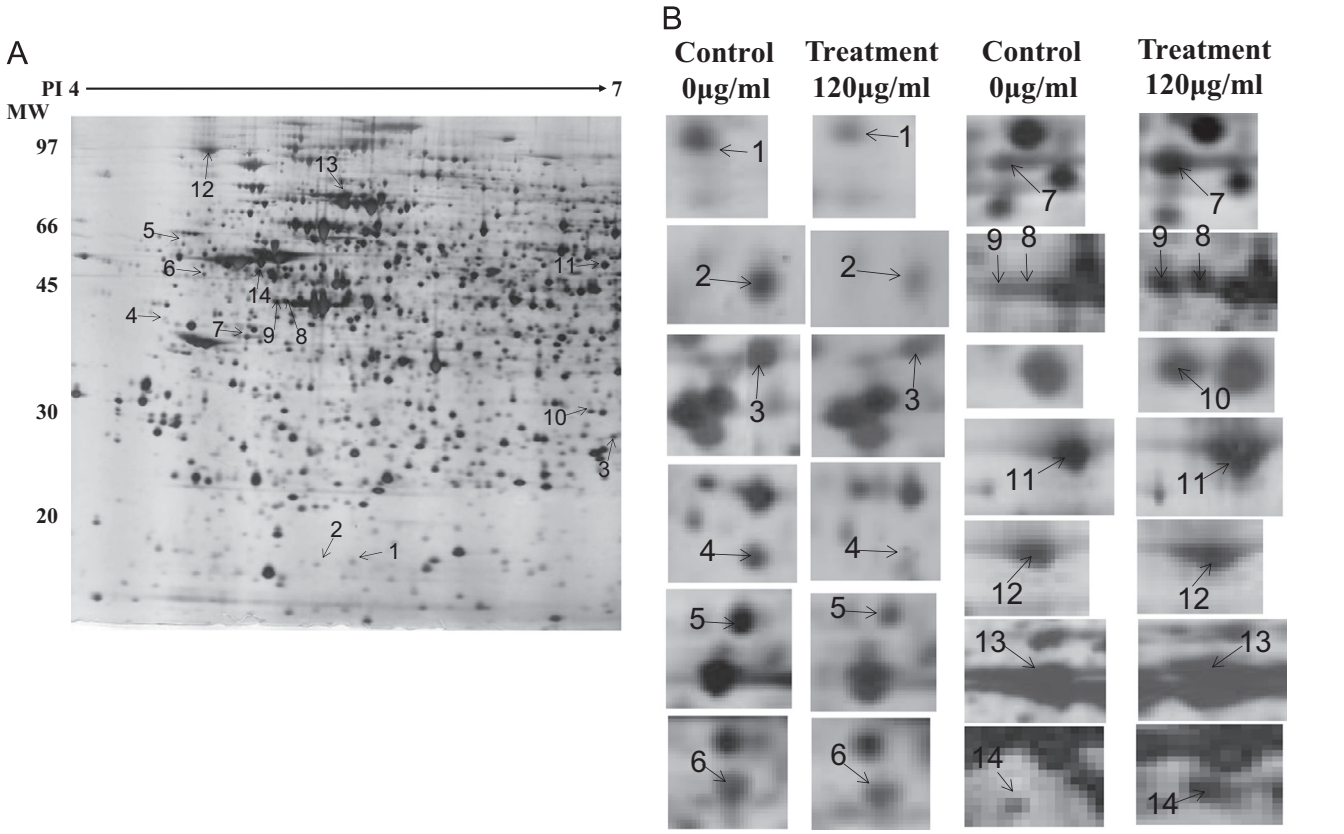

Fig. 3. Proteomic analysis identified 14 peptides associated with the treatment by the extract of *Annona muricata* leaves. (A). Global view of the peptide spots on the two dimensional gels. The spots responsive totreatment by the extract of *Annona muricata* leaves were labeled; (B). Localized view of the peptide spots on the two dimensional gels. The spots changed by treatment by the extract of *Annona muricata* leaves were labeled.

Table 1.
Identification of proteins of interests using peptide mass finger printing.

Spot no.	Protein description	Accession no.	Protein	MW (kDa)		pI value (pH)		Type of change[b]
			Score C. I.%[a]	Calib-rated	Theo-retical	Calib-rated	Theo-retical	
1	Stathmin isoform a	gi44890050	100	15–20	17	5–6	5.76	–
2	Chain A, Solution Structure Of Oxidised Erp18	gi238828062	99	15–20	17	5–6	5.66	–
3	PGAM1	gi49456447	100	25–30	28	6–7	6.67	–
4	RecName: Full=Reticulocalbin-1;Flags: Precursor	gi2493462	100	35–40	38	4–5	4.86	–
5	Tumor rejection antigen (gp96)1 variant, partial	gi62088648	100	65–70	66	4–5	5.08	–
6	vimentin variant, partial	gi62896523	100	50–55	54	4–5	5.06	–
7	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	gi14249959	100	30–40	32	4–5	5.00	+
8	Actin, cytoplasmic 2	gi316659409	100	40–45	42	5–6	5.31	+
9	Actin, cytoplasmic 1	gi46397333	100	40–45	42	5–6	5.29	+
10	Proteasome (prosome, macropain)subunit, alpha type1	gi119588884	100	25–30	30	6–7	6.51	+
11	Heterogeneous nuclear ribonucleoprotein H isoform ×1	gi767938356	100	50–55	51	6–7	6.33	+
12	Heat shock protein 90 kDa beta (Grp94)	gi119618130	100	90–95	92	4–5	4.76	+
13	Heat shock 70 kDa protein 8 isoform 1 variant, partial	gi62897129	100	70–75	71	5–6	5.28	+
14	Protein disulfide isomerase-related protein 5, partial	gi1710248	100	45–50	46	4–5	4.95	+

[a] Protein Score C. I.% is the confidence interval of identified proteins calculated from MS data.
[b] (+), protein level was up-regulatied; (−), protein level was down-regulated; compared with those in the control samples.

assays (Fig. 2). Proteomic analysis revealed 14 proteins responsive to the treatment by the leaf extract (Fig. 3; Table 1). Bioinformatic annotation outcome suggests that ER stress is the pathway underlying the extract-induced apoptosis, which is evidenced by the increase in the protein levels of GRP94, HSP70 and PDI-related protein (Fig. 4). These three proteins are ER chaperones that are involved in the regulation of ER stress (Sozen et al., 2015). So far, several different ER stress pathways have been delineated (Sozen et al., 2015). One of the well characterized ER stresssignal transduction route is Bip-PERK-eIF2α-CHOP pathway: PERK dissociates from Bip and becomes phosphorylated. This leads to phosphorylation of eIF2α, which eventually results in the up-regulation of CHOP expression. CHOP activates downstream proteins to cause apoptosis (Sozen et al., 2015). Upon treatment by our *Annona muricata* L. leaf extract, phosphorylations of PERK and eIF2α as well as the protein levels of Bio and CHOP were all up-regulated

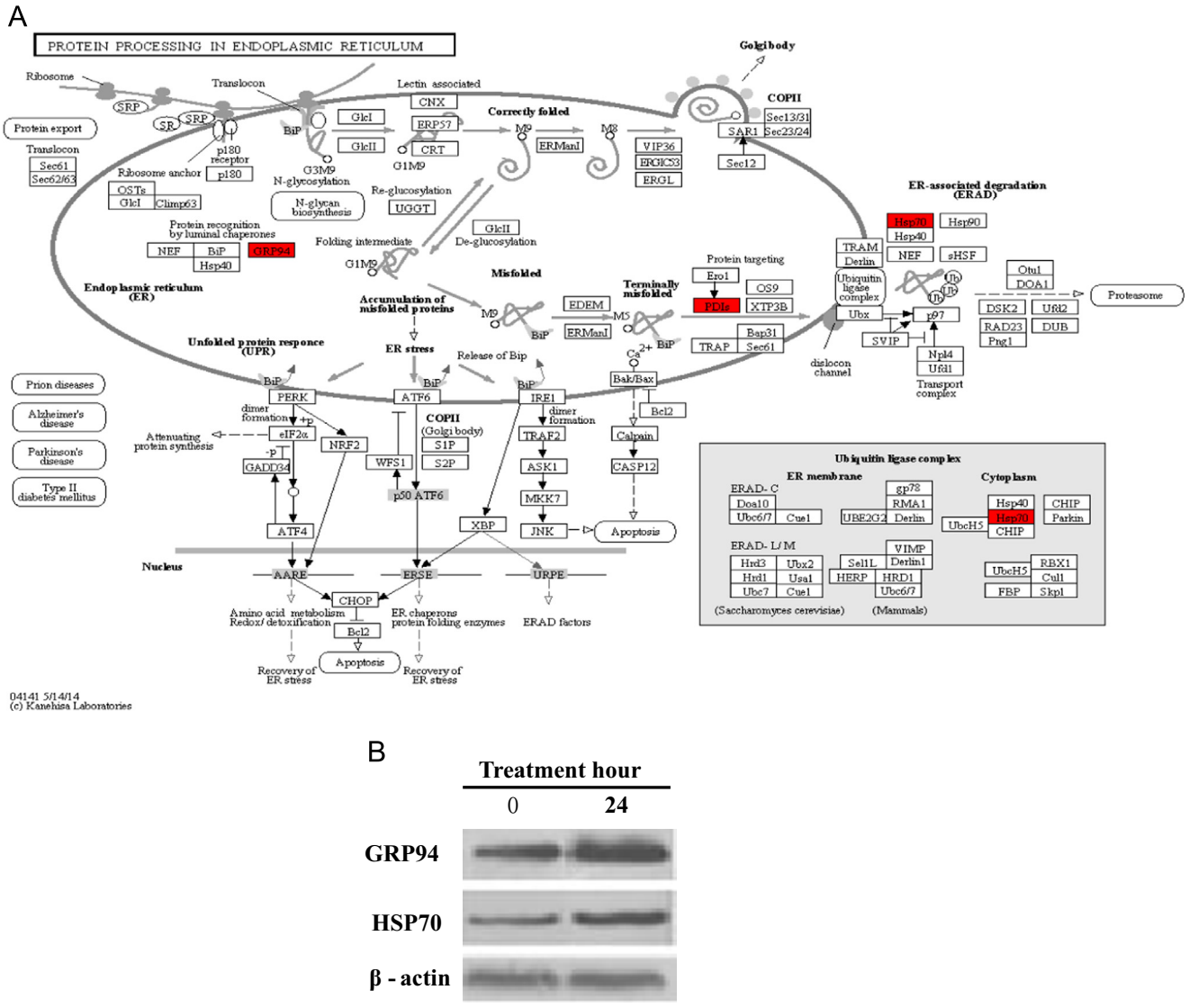

Fig. 4. Western blot analysis confirmed that the protein levels of HSP70 and GRP94 were up-regulated after treatment by the extract of *Annona muricata* leaves.

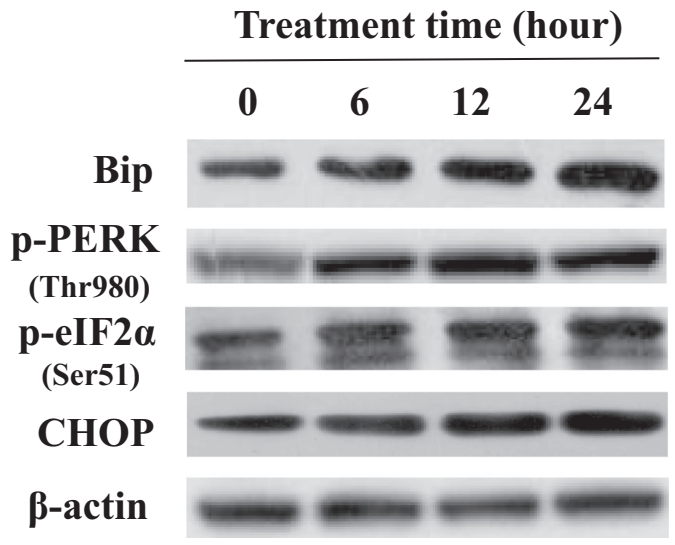

Fig. 5. Western blot analysis showed that phorsphorelation of PERK and eIF2α as well as the protein levels of Bip and CHOP were all up-regulated after treatment by the extract of *Annona muricata* leaves.

(Fig. 5), indicating that the extract causes ER stress by Bip-PERK-eIF2α-CHOP pathway. Since ER stress is a recognized pathway to cause cell death including apoptosis (Verfaillie et al., 2013), it is thus suggested that the ethanol extract of *Annona muricata* L. leaves triggers the liver cancer cell apoptosis through ER stress. Better understanding of the molecular pathways underlying the herb-induced apoptosis is helpful for beneficial use of the plant as anti-cancer ethnomedicine.

Different parts of *Annona muricata* L., leaf, twigs or roots, have active apoptotic chemical components. Leaf and root extracts have comparable anti-proliferative activities, which are higher than twig extract (Moghadamtousi et al., 2014). Fruit extract appears to be more potent, which shows an IC_{50} value of 4.8 μg/ml in proliferation inhibition (Dai et al., 2011). However, leaves are considered to be a better raw material for extraction, as they are more abundant and available. Using 95% hot ethanol as solvent, the resulting leaf extract can inhibit the proliferation of HL-60 cells at an IC_{50} of less than 12 μg/ml (Moghadamtousi et al., 2014). The values of IC_{50} for hexane extract are 11.43 ± 1.87 μg/ml and 8.98 ± 1.24 μg/ml against HT-29 and HCT-116 cells respectively (Moghadamtousi et al., 2014). In various cancer cells lines that included MCF-7, MDA-MB-231, A549, HepG2 and WRL-68, the IC_{50} of ethyl acetate extract was the lowest compared with those of

hexane and methanol extracts (Moghadamtousi et al., 2014). In our study, 75% ethanol as solvent was used to prepare extract at the room temperature, and he IC_{50} of proliferation inhibition was 150 μg/ml for liver cancer HepG2 cells after 24 h of treatment (Fig. 1). This shows that 75% ethanol is also good solvent for the extraction of anti-cancer ingredients from the herb. However, compared amongst the solvents ever used for extraction, ethyl acetate seems to be the best.

Natural products provide a useful resource for drug development. In the area of cancer, from 1940s to December 2010, 48.6% anti-cancer small molecule drugs developed are from natural products or their derivatives (Newman and Cragg, 2012). The development of natural products for cancer treatment was not favored over a short period of time, in particular in 1990s, due to the emergence of targeted therapies which rely on synthetic compounds and antibodies. However, the natural products strike back soon, since the drawbacks of targeted therapy start to appear after years in use. Since the approval of rapamycin in 2007, 12 novel natural product derivatives have been brought to market for treating cancer so far (Basmadjian et al. 2014). Since extracts of *Annona muricata* L. have potent anti-cancer activities, it is logical to look for active chemical components from extracts, which may be further developed into mainstream drugs for the management of cancer. As reviewed by Moghadamtousi et al. (2015a), *Annona muricata* L. has alkaloids, megastigmanes, flavonol triglycosides, phenolics, cyclopeptides, essential oils and notably annonaceous acetogenin (AGE) compounds. More than 100 AGEs have been identified from this plant. Some AGEs are found to have toxicity against various types of cancer cells. For examples, annomuricins A, B, C and E from the plant leaves are toxic to lung A549, breast MCF-7, colon HT-29 or pancreatic MIA PaCa-2 cancer cells (Wu et al., 1995a, 1995b; Kim et al., 1998a; Jaramillo et al. 2000). Murihexocin C and muricoreacin, also from the plant leaves, have toxicity against prostate adenocarcinoma (PC-3) and pancreatic carcinoma (PACA-2) cells (Kim et al., 1998b). Other AGEs, some of which are shown to be toxic to cancer cells, are listed in the review by Moghadamtousi and co-workers (Moghadamtousi et al. 2015a).

There is evidence that AGEs, in addition to their toxicities to cancer cells, have unacceptable toxic side effects such as neurotoxicity, which may cause neurodegeneration (Champy et al. 2005). Thus, AGEs are not good drug candidates, unless chemical modification can maintain the apoptotic activity and reduce that neurotoxicity (Kojima et al., 2015). The anti-cancer effect and mechanism of extracts may be different from that of a single chemically defined compound, as active components in extracts may have combinational effects (Chan et al., 2008). Isolation of active single compound from extracts may not only compromise the therapeutic efficacy but also render toxicity. To this end, Yang et al., (2015) compared the anti-cancer activity of extract of the plant leaves, flavonoid-enriched and AGE-enriched fractions, and found that the extract was more potent in inhibitingthe proliferation, viability and clonogenic capacity of prostate cancer cells *in vitro*, compared to flavonoid-enriched fraction. Oral administration of the extract also showed a higher tumor growth-inhibitory efficacy thanthe flavonoid-enriched fraction *in vivo* in human prostate tumor xenografts. AG-enriched fraction, although was superior in cancer cell toxicity, caused death of mice. This implicates that the action of extract is complex, and, extract may be better than currently isolated chemically defined single constituents. It further suggests extracts of the plant leaves deserve further development into therapeutic use.

5. Conclusion

In conclusion, our results have shown that the ethanol extract of *Annona muricata* L. leaves causes apoptosis of liver cancerHepG2 cells, promote phosphorylation of PERK and eIF2α, and up-regulate the protein level of Bip and CHOP. The results indicate that the herbal extract triggers the cancer cell apoptosis through ER stress pathway. Out data support the use of *Annona muricata* L. leave extracts as ethnomedicine for the management of cancer.

6. Conflict of interests

The authors declare that there are no conflicts of interests.

7. Contributions of the authors

Na Liu (naliu@sxu.edu.cn) and Hua Li Yang (1,564,072,701@qq.com) participated in all experimental work; Pu Wang (xyq641160@outlook.com) contributed to flow cytometry and proteomic analyses; Yu Cheng Lu was involved in bioinformatic annotation; Ying Juan Yang (yangyingjuan911@hotmail.com) contributed to cell TUNEL assay and Western blot analysis; Lan Wang (lanwang@sxu.edu.cn) and Shao Chin Lee (lee_shao@hotmail.com) applied for grants, designed the experimental protocols and prepared the manuscript.

8. Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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