HPLC-MTT assay: anti-cancer activity of aqueous garlic extract is from allicin

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Abstract

A strategy using RP-HPLC, TLC, MS, NMR, chemical synthesis, and MTT cell viability assay to identify allicin as the active anti-cancer compound in the aqueous extract of garlic (AGE) is described. Change the pH of the AGE from 7 to 5 eliminated interfering molecules and enabled a clean HPLC separation of the constituents in AGE. MTT assay of the HPLC fractions identify an active fraction. Further analysis by TLC, MS, and NMR verify the active HPLC fraction as allicin. Chemically synthesized allicin is used to provide further confirmation. The results clearly identify the active compound in AGE as allicin.

Keywords: Allium sativum; Garlic; Aqueous garlic extract; Allicin; Organosulfur compounds; Mouse colon cancer cell; MTT assay.

Garlic (*Allium sativum*) extracts (GEs) and garlic-derived compounds have recently received increasing attention due to their anti-proliferative activities against different types of cancer cells [1]. These activities are believed to be originated from induction of apoptosis [2-3], regulation of cell cycle progression [4], and signal transduction modification [5]. Data suggest that the active compounds may be the organosulfur compounds (OSCs) [6-7], which are released when garlic is processed. These OSCs appear to protect garlic plant from attack by bacteria, fungi, and animals. Gamma-glutamyl-S-allyl-L-cysteine is a naturally occurring (alkylation of glutathione followed by the cleavage of the glycine moiety) [8] major sulfur component in garlic. It hydrolyzes and oxidizes via S-Allyl cysteine (SAC) to form alliin (S-allyl-L-Cysteine sulfoxide) [9]. Another proposed route for alliin biosynthesis via SAC is the direct thioalkylation of serine as the starter followed by oxidation [10]. Alliin is a sulfoxide and accounts for approximately 80% of the cysteine sulfoxides in garlic and resides in the storage cells. Crushing, cutting, chewing, or otherwise processing the garlic releases alliinase (which makes up 10% w/w of total protein) from the bundle sheath cells, an enzyme that catalyzes alliin to allicin (3-prop-2-enylsulfinylsulfanylprop-1-ene). Allicin is a thioester of sulfenic acid or allyl thiosulfinate. It is an oily, colorless liquid that has a distinctively pungent smell. Allicin is relatively unstable, and decomposes into oil-soluble OSCs such as diallyl sulfide, diallyl disulfide, diallyl trisulfide, dithiins, and ajoene and some water-soluble OSCs such as SAC and S-allyl mercaptocysteine (SAMC) [11-13]. Although, allicin has been reported to inhibit the proliferation of cancer cells [3], other garlic-derived sulfur compounds, e.g., ajoene also has been shown to induce apoptosis of HL-60, MCF-7, U937, HEL, and OCIM-1 cancer cells and basal cell carcinoma [14-15]. In addition, ally sulfide, ally disulfide, ally trisulfide, and SAMC have all been reported to inhibit tumor proliferation *in vitro* and *in vivo* [16-18]. This makes the identification of the active

components in garlic extract (GE), which is vital for the understanding and further utilization of the GE for anti-cancer therapy, very difficult. Another complication is that the constituents and efficacy of GE can vary depend on the method of preparation and storage conditions [12,19-21]. Majority of the GE studies use ethanol or other organic solvents which further complicates the downstream analysis and can themselves be cytotoxic [19-21]. Heating during preparation, storage at higher temperature, or extreme pH can adversely affect the quality of the GE [1,12,21-24]. Data reported thus far on GE focus on the anti-proliferation effects of GE without identifying the active components. This is perhaps due to the lack of effective methods for isolation of the active compounds in their native forms and identify them. A need for an efficient technique to identify the active components in GE prevails, and will greatly facilitate the understanding of a specific type of GE preparation. This paper describes an efficient strategy to identify the active components of the AGE using a combination of techniques, including HPLC, MTT assay, TLC, MS, NMR, and chemical synthesis. A proof-of–concept study is presented that solely utilizes water to prepare the GE. Data shown here suggest that the active component of the GE is allicin and not the garlic-derived organic sulfides.

All chemicals were purchased from Sigma-Aldrich Chemical Company (Saint Louis, MO) and used without further purification. Distilled water was purified by a Milli-Q system (Milford, MA). Garlic (Spice World, Orlando, FL) was purchased from the local market and the aqueous garlic extract was prepared from 23g of skinned garlic cloves in 40 ml water. The mixture was crushed in a blender (NutriBullet, Los Angeles, CA) for 10 seconds at one pulse per second, left to rest for 10 minutes, and then crushed at one pulse per second again for 10 more seconds. The crushed garlic was pressed and the extract solution was filtered through a cheesecloth. The filtrate was aged overnight to settle down any particulate. On next day, the AGE was spun at 896

x g for 4 minutes, and then spun again at 9878 x g for another 4 minutes. Finally, the supernatant was filtered through a sterilized 0.22µm syringe-driven filter (Becton Drive, Franklin Lakes, NJ) to clean out any residual precipitate and bacteria. The clear supernatant was aliquoted and stored at -80 ^{o}C until use.

The anti-cancer effects of AGE were determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay (Wallert and Provost Laboratory, Minnesota State University, Moorhead, MN). This assay is based on the conversion of MTT to MTT-formazon by mitochondrial enzymes. MTT assay was performed as follows: First, roughly 7500 *Mus musculus* colon carcinoma cells CT26.WT (ATCC, Rockville, MD) were added into each well in a 96 well plate and incubated overnight. Then, 8 µl of GE at varying concentrations was added. The CT26.WT cell line was used because it can be easily inoculated subcutaneously into BALB/c mice for later *in vivo* studies and it is believed that garlic consumption is beneficial for the suppression of colon cancer. Cells were re-incubated at 37^{o}C in a humid environment containing 5% CO_2 for 72 hrs., then 30 µl of MTT solution (5 mg/ml in PBS) was added to each well. After incubated for additional 3.5 hrs., the media was removed and 150 µL of MTT solvent (4 mM HCl, 0.1% Nondet P-40 all in isopropanol) was added to each well. The cells were agitated with an orbital shaker for 20 minutes, and then the absorbance was read at 545 nm with a reference filter of 630 nm. The absorbance correlated with % cell viability. The IC_{50} was calculated using a four parameter logistic curve of the SigmaPlot software (Richmond, CA). A control group (medium without AGE) and a blank group (without cells or medium) were also included. Interestingly, an aqueous onion extract is completely inactive and can be used as a control. Experiments were performed in triplicates. Data were presented as means ± SDs. Where appropriate, one-way ANOVA or Student paired t-test was performed using SigmaPlot. *P*-values

less than 0.05were considered statistically significant. Figure 1 shows the dose-response curve of AGE on viability of *Mus musculus* colon carcinoma cells CT26.WT assessed by MTT assay. Results show clear evidence of cytotoxicity of the AGE against the CT26.WT in a dose dependent manner, compared to the control. Shrinkage, granulation of cytoplasm, and detachment were visible in treated cells. The IC 50, dilution of the AGE to produce fifty percent inhibition of cells *in vitro*, is calculated to be 1:340 water dilution of the AGE.

PD10 desalting column (GE Healthcare Biosciences, Piscataway, NJ) was used to separate low-molecular weight compounds from high-molecular weight (> 5000 Da) biomolecules and then PD10 fractions were checked by MTT assay. PD10 fractions were prepared by loading 2.5 ml of AGE onto a PD10 column, and then water was used to elute the components into 3.5 ml and five 2 ml fractions. MTT assay indicates that the third fraction has the activity. 1-D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 4-12 % (w/v) polyacrylamide (Invitrogen, Carlsbad, CA) with MES running buffer was used to check each PD10 fractions for proteins. 1-D SDS-PAGE gel shows many proteins in AGE and no protein in PD10 fraction 3. These results indicate that the active component is a low molecular weight compound.

To simplify the detection of the active component, majority of the proteins were eliminated by changing the pH of the GE from 7 to 5. This is accomplished by adding 50 µl 1M HCl to 950 µl AGE and then spun at 9878 x g for 4 minutes. 1-D SDS-PAGE gel shows very little proteins in AGE at pH5. AGE at pH 5 shows the same activity as at pH 7. Reversed-phase HPLC is used to further separate and purify the active component (Figure 2a). The HPLC fractions are passed through a sterilized syringe filter and used as such in MTT assay. A set of the same fractions by injecting H_2O is used as a control (none of the fractions show any activity).

The active fraction at 18.3 min is identified as allicin by TLC, MS, and NMR and further confirmed using a pure synthetic allicin. The synthesis of allicin is based on the oxidation of allyl disulfide with hydrogen peroxide in acidic media [23]. Figure 2b shows the HPLC profile of the allicin reaction mixture. MTT assay indicates that the fraction at 18.3 min of the allicin reaction mixture has the activity. It is important to note that the HPLC trace of the PD10 fraction 3 shows a major peak at 18.3 min.

For the identification of the HPLC active fraction at 18.3 min as allicin, various techniques, such as TLC, MS, and ^{1}H-NMR were used. The presence of Allicin was identified on aluminum backed silica gel with F254 indicator plate (Dynamic Adsorbents, Norcross, GA). Pure Allicin was used as a control for comparison. Hexane-isopropanol (92:8) was used as the mobile phase and compounds were detected at 254 nm. Rf value was calculated to be 0.44. MS (MALDI-TOF, CHCA as matrix, MH^{+1}; Insert in Figure 2a): m/z 163. ^{1}H NMR (Bruker 900 MHz, D_2O, δ / ppm; DSS as an internal standard; Insert in Figure 2b): δ 3.82-4.02 (2 H, $CC\mathbf{H}_2SO$) + (2 H, $SC\mathbf{H}_2C$), 5.24-5.53 (4 H, $C\mathbf{H}_2$), 5.94-6.00 (2 H, $CH_2C\mathbf{H}CH_2$).

In conclusion, we present here an effective strategy to identify the active components in GEs. In a proof-of–concept study, allicin has been identified as the sole compound that is responsible for the anti-cancer activity of our AGE. Several factors in this method that facilitate the identification of the active components are as follows: First, the use of a simple PD10 desalting column allows determination of the molecular size of the active components. Second, changing the pH slightly can greatly simplify the HPLC separation by eliminating interfering molecules but still retain the activity. Third, by taking advantage of the extremely high resolution power of the RP-HPLC, active components can be separated and purified. Fourth, using MTT assay directly on the individual HPLC fractions, active fractions can be identified. Lastly, many

effective techniques such as TLC, MS, NMR, and informatics are available nowadays to quickly reveal the compounds in the active HPLC fractions. Since majority of the garlic-derived compounds are known and have been investigated extensively using various techniques. For example, the oil-soluble DADS has been investigated extensively and reported to be more effective than allicin in suppressing breast cancer *in-vitro* and *in vivo* [25]. It is felt that, the strategy described here provides a successful method for obtaining and identifying intact active GE components that will be very useful in analyzing different varieties of garlic preparations.

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Figure caption

Figure 1. Dose-response curve of aqueous garlic extract on viability of *Mus musculus* colon carcinoma cells CT26.WT assessed by MTT assay. Dashed line is the predicted data from a four parameter logistic curve fitting (SigmaPlot Software).

Figure 2. HPLC chromatograms of water extract of garlic (a) and reaction mixture for allicin synthesis (b). HPLC was done under the following conditions: Instrument; Hewlett Packard 1100 (Agilent Technologies, Santa Clara, CA). Column; Kinetex RP-C18 (Phenomenex, Torrance, CA), 5 µm, 250 x 4,6 mm. Solvent A: 0.1% TFA in H_2O. Solvent B: 0.1% TFA in acetonitrile. Flow rate: 1 ml/min. Elution: A gradient from 5% to 100% solvent B over 30 min. Detection wavelength; 254 nm. Insert at the up right hand corner is the MALDI-TOF MS of HPLC fraction at 18.3 min. Insert at the bottom left hand corner is the NMR of HPLC fraction at 18.3 min.