Ethyl acetate fraction of garlic (Allium sativum) inhibits the viability of MCF7 and HepG2 through induction of apoptosis and G2/M phase cell cycle arrest

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ABSTRACT

Background: Garlic (Allium sativum) with its main component organosulfur compounds (OSCs) has an anticancer effect against a large variety of cancer cells. This anticancer effect was studied on individual garlic components, rather than fractions. Methods: Herein, we investigated the anticancer effect of different garlic fractions on the MCF7 and HepG2 cells and studied the underlying mechanism. Results: Different garlic fractions, extracted by methylene chloride (MC), petroleum ether (PE), ethyl acetate (EtOAc) and butanol (B) solvents, each alone exhibited significant dose-dependent anti-proliferative activity on MCF7 and HepG2 cells with best results for EtOAc with IC₅₀ values 21.32 and 26.22 μg/ml, respectively, as compared to vehicle-treated cells. HPLC analysis revealed the presence of 14 components in EtOAc fraction with highest concentrations for linoleic acid (LA) and S-allylcysteine (SAC). EtOAc fraction inhibited cancer cells proliferation through induction of apoptosis (revealed by a significant increase in mRNA levels of apoptotic markers, Caspase 3 and Bax and a significant decrease in mRNA levels of the anti-apoptotic marker, Bcl2) and cell cycle arrest in G2/M phase (indicated by increase in number of MCF7 and HepG2 cells in this phase). Conclusions: These in vitro results suggest that garlic EtOAc fraction or its main component could be used as an adjuvant to anticancer drug or can help in the development of new anticancer drugs based on components of this fraction.

INTRODUCTION

Despite advanced therapeutic strategies and approaches for cancer, it remains the most devastating disease that ultimately leads to death (de Mestiquita et al., 2009). According to the American Cancer Society, about 3.5 million people died annually from cancer (Fouché et al., 2008). Although the actual mechanism for cancer induction is still incompletely known, exposure to toxins, radiation, toxic chemicals, some oncogene viruses are the most relevant predisposing factors for this disease. The currently used anticancer drugs do not only target the cancer cells but they also kill healthy cells. This non-specific targeting along with the development of drug resistance are the main side effects of chemotherapy. Looking for a highly efficient, safer anticancer drug becomes a necessary need to overcome this horrible disease, but to date, such drug was not evolved yet. Alternatively, for several years great effort has been devoted to find suitable extract(s) from the medicinal plant(s) that has an anticancer activity to be used as a co-adjuvant with anticancer drugs (Shah et al., 2013). Among these plants, garlic (Allium sativum) with its major ingredients, S-allylcysteine and S-allylmercapto-L-cysteine organosulfur compounds (OSCs), have long been known to have medicinal qualities (Petrovska and Čekovska, 2010) and anticancer effect against pancreas, colon, stomach, liver and breast cancer (Fleischauer and Arab, 2001; Li et al., 2004; Chan et al., 2005;...
Setiawan et al., 2005; González et al., 2006). The OSCs induce apoptosis in cancer cells by altering Bax/Becl2 ratio and stimulating cell cycle arrest in the G2/M phase (Ariga and Seki, 2006; Herman-Antosiewicz et al., 2007). Among garlic, other active constituents with anticancer activity are allicin and its derivatives Diallyl sulfide (DAS) and Diallyl disulfide (DADS). DAS has the ability to induce apoptosis of HeLa human cervical cancer cells in vitro (Wu et al., 2011), while DADS reduces the risk of colon carcinogenesis through inhibition of DHUG and DHDG genes in colon cancer cells (Huang et al., 2011). Data stated above showed the effect of each individual component of garlic on the cancer cells, however, the anticancer effect of different garlic fractions has not investigated yet. Therefore, this study aimed to compare the anticancer effect of different garlic fractions on the MCF7 and HepG2 cells and determine the underlying mechanism.

**METHODS**

**Extraction and fractionation**

The air-dried garlic powder (1 Kg) was extracted by cold percolation with 70% ethanol till exhaustion. The ethanol extract was evaporated under reduced pressure for 16 h to give the dry residue of reddish-brown garlic ethyl extract (GEE). An aliquot of GEE was suspended in 400 ml of distilled water and partitioned successively with methylene chloride (MC), petroleum ether (PE), ethyl acetate (EtOAc) then butanol (B) saturated with water and evaporated under reduced pressure to yield 0.3527, 0.3283, 0.1087 and 0.9045 g dry residues of MC, PE, EtOAc and B fractions, respectively.

**Analysis of total constituents in EtOAc by HPLC/QQQ/MS**

HPLC separation of the components of garlic ethyl acetate fraction was analyzed in comparison to that reported by (Farag et al., 2017). The HPLC/QQQ/MS system from Agilent Co. Agilent type: 6420 was composed of G4204A Quat. pump, G1316A column temperature controller, G4226A HiP Sampler, Quadrupole mass spectrometer with ESI ion source, and ACQUITY UPLC BEH Shield RP 18 1.7 um 2.1 × 150 mm column. A binary solvent system of water (A) and methanol (B) in 50:50 v/v ratio under isocratic conditions. The column temperature was at 45°C, the flow rate of 0.3 mL/min and detection at 208 nm. A single quadrupole mass spectrometer with ESI positive mode was used to determine the mass spectrum of each compound using the following condition: scanning range 100-1500 m/z, drying gas flow 7 L/min, nebulizer pressure 45 psi, dry gas temperature 350°C, vaporizer temperature 250°C, capillary voltage 2500 V, charging voltage 2000 V, corona current 4 μA and fragmentor voltage 135 V (Chen et al., 2014) and the MS2 fragmentation pattern and TIC type of chromatogram.

**Cell viability by MTT assay (determination of IC50)**

The anti-proliferative activity of the garlic fractions by MC, PE, EtOAc and B on human hepatocellular carcinoma HepG2 and breast adenocarcinoma MCF7 cells (purchased from American Type Culture Collection; ATCC, NY, USA) were evaluated against the standard anticancer drugs tamoxifen and cisplatin using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Molecular Probes, Eugene, Oregon, USA; Cat.no.V-13154)] as previously described (El-Magd et al., 2018). In brief, before addition of garlic fractions, the cancer cells were seeded in a 96-well plate [1 × 10^4 cells/well, 100 μl/well] containing DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (all obtained from Gibco, New York, USA), and 2% L-glutamine (Invitrogen, New York, USA) and incubated at 37°C for 24 h under 5% CO2, 95% air until reaching a confluence of 70-80%. The anticancer standards and the four garlic fractions were applied separately at different concentrations to the wells to achieve final concentrations ranging from 0 to 200 μg/ml and the cells were cultured for 24 h. The cells were incubated with 5 mg/ml of MTT (Sigma) for 4 h and then the medium was replaced with 100 μl DMSO (Sigma Aldrich, St. Louis, MO, USA) to dissolve purple formazan crystal formed at the bottom of the wells and vortexed for 20 min. A negative control of 10 μl of the MTT stock solution added to 100 μl of medium alone was included. Absorbance was recorded at 570 nm using microplate reader (StatFax-2100, Awareness Technology, Inc., USA). The proportion of surviving cells was calculated as (OD of the treated sample – OD of blank)/(OD of control – OD of blank) × 100%. The concentration of anticancer standards and the four garlic fractions inhibiting 50% of cells (IC50) was calculated using the sigmoidal curve using GraphPad (Prism) statistic software.

Following determination of IC50, all garlic fractions were added separately, at a dose equal to their IC50 values, into MCF7 and HepG2 cells at a confluence of 70-80% and the cells were incubated in a CO2 incubator for 24 h. The cells were harvested by trypsinization and were immediately processed for mRNA isolation and flow cytometric analysis.

**Quantitative real-time PCR (qPCR)**

Total RNA was isolated from HepG2 and MCF7 cells using an RNA extraction kit, including DNase I digestion to eliminate residual genomic DNA, following the manufacturer’s protocol (Thermo Scientific) and as previously described (Saad et al., 2018). The integrity of total RNA was assessed by inspection of the ribosomal RNA bands (18S and 28S) in ethidium bromide-stained 1.5% agarose gels under ultraviolet (UV) light. The concentration and purity of RNA were determined by nanodrop (Quawell Q5000/USA). RNA (5 μg) was reverse transcribed into complementary DNA (cDNA) using Quantscript reverse transcriptase (Thermo Scientific) that contains Revert Aid H minus Reverse Transcriptase. Real-time PCR with SYBR Green was used to measure the expression of mRNAs of target genes in MCF7 and HepG2 cells, with β-actin as an internal reference. The isolated cDNA was amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer protocol (Thermo scientific) and gene-specific primers. The primers used in the amplification are shown in Table 1. The web-based tool Primer 3 was used to design these primers based on published human sequences. At the end of the last cycle, temperature was increased from 60 to 95°C to produce a melt curve. The housekeeping gene (β actin) represented as normalize that used to calculate the relative gene expression or fold change in the target gene. The thermal cycling conditions, melting curves temperatures, and calculation of relative expression using 2^ΔΔCt were done as previously described (El-Magd et al., 2017a).
Cell cycle analysis using flow cytometry

The cells were digested with warm Trypsin-EDTA + warm PBS-EDTA (0.25%) (500 µl + 500 µl) with incubation for 10 min at 37°C. The mixture was centrifuged at 4500 rpm for 5 min, then the supernatant was carefully removed. The mixture was washed twice in warm PBS and the cell pellet was resuspended in 500 µl warm PBS, centrifuged and the supernatant was removed. A volume of 150 µl PBS + 350 µl ice-cold 70% ethanol was added and incubated at 4°C for 1 h to fix the cells. To remove ethanol, the mixture was centrifuged at 3500 rpm for 10 min and then the supernatant was carefully removed. The mixture was washed twice in warm PBS and the cells were resuspended in 500 µl warm PBS, centrifuged and the supernatant was removed. The cells were resuspended in 100 µl PBS and stored at 4°C. In the darkness, the cells were stained with 100 µl of PI solution + 50 µl RNase A solution (100 µg/ml) and incubated in darkness for 30-60 min. The stained cells were read in Attune flow cytometer (Applied Bio-system, US).

Statistical analysis

Statistical analysis was performed using student t-test using the Graph Pad Prism 6 (Graph Pad Software, Inc., LaJolla, CA, USA). Significant differences among means were estimated at P < 0.05. The results were expressed as mean ± standard error of mean (SEM).

RESULTS

Effect of garlic fractions on MCF7 and HepG2 cell viability

MTT assay was performed to evaluate the effect of garlic fractions by MC, PE, EtOAc, and B on the proliferation of human MCF7 and HepG2 cells. The cells were treated with varying concentrations of each fraction. The four garlic fractions each alone exhibited significant dose-dependent anti-proliferative activity on MCF7 and HepG2 cells with best results for EtOAc with IC₅₀ values 21.32 and 26.22 µg/ml, respectively, as compared to vehicle-treated cells (Figure 1). Consequently, EtOAc garlic fraction was selected for further qPCR and cell cycle experiments.

Identification of Allium sativum constituents via HPLC/QQQ/MS

Phyto-constituents of A. sativum were analyzed via reversed-phase HPLC/QQQ/MS, using a gradient mobile phase consisting of water and methanol. Complete elution of metabolites was achieved within a short time (ca. 30 min). In the current study, a total of 14 metabolites were detected according to (Farag et al., 2017) (Table 2, Figure 2). Among these metabolites, Linoleic acid (LA) and γ-Glutamyl-S-allylthiocysteine (SAC) showed the highest concentrations.

Table 1. Metabolites identified via HPLC/QQQ/MS in ethyl acetate fraction of A. sativum using positive ionization mode.

<table>
<thead>
<tr>
<th>NO</th>
<th>MS</th>
<th>Formula</th>
<th>Metabolite</th>
<th>RT. Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>191.0196</td>
<td>C₅H₁₀O₂</td>
<td>Citric acid</td>
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<tr>
<td>2</td>
<td>305.0799</td>
<td>C₅H₁₀O₂S</td>
<td>Jasmonic acid-hydroxy-O-sulfate</td>
<td>6847.74</td>
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<tr>
<td>3</td>
<td>265.1477</td>
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<td>Trimethylmonosulphate</td>
<td>5742.27</td>
</tr>
<tr>
<td>4</td>
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<td>Stearic acid</td>
<td>6808.24</td>
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<tr>
<td>5</td>
<td>255.2329</td>
<td>C₅H₁₀O₂</td>
<td>Palmitic acid</td>
<td>5077.67</td>
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<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
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<tr>
<td>8</td>
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<tr>
<td>9</td>
<td>321.0587</td>
<td>C₅H₁₀N₃O₂S</td>
<td>γ-Glutamyl-S-allylthiocyesteine</td>
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<tr>
<td>10</td>
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<td>Oleic acid</td>
<td>1766.83</td>
</tr>
</tbody>
</table>

Effect of garlic EtOAc fraction on the expression of apoptosis-related genes

Our results revealed significant (P ≤ 0.05) increase in mRNA levels of apoptotic markers, Caspase 3 and Bax in MCF7 and HepG2 cells treated by garlic EtOAc fraction as compared to vehicle-treated cells (Figure 3). In contrast, a significant decrease in mRNA levels of the anti-apoptotic marker, Bcl2 was noticed in garlic EtOAc fraction-treated cells relative to those treated by vehicle.

Effect of garlic EtOAc fraction on cell cycle in MCF7 and HepG2 cells

The effect of garlic EtOAc fraction on MCF7 and HepG2 cell cycle was determined by flow cytometry using PI. The obtained data exhibited a significant elevation in the number of MCF7 and HepG2 cells in G2/M phase after treatment with garlic EtOAc fraction as compared to vehicle-treated cells (Figure 4). However, this treatment resulted in a significant reduction in the number of MCF7 and HepG2 cells in G0/G1 phase and no significant changes in cell number in S phase.
Fig. 1: Cytotoxic effect of the isolated garlic fractions on MCF7 and HepG2 cells. Cells were treated with various concentrations of the four fractions (200, 100, 50, 25, 12.5, 6.25, 3.125, and 0 μg/mL) for 24 h and the cell viability was determined by MTT assay. The results are expressed as a percentage of cell growth relative to vehicle-treated control cells.

DISCUSSION

Garlic (Allium sativum) is rich in sulfur compounds which responsible for garlic odor, flavor and were used for medicinal purposes as an antihypertensive, antibiotic, and anticancer agent (Ross et al., 2006). Garlic also can be used as a preventive agent in some cancers such as gastric, pancreatic, intestinal, and breast cancer (González et al., 2006). However, little is known about the in vitro anticancer effect of different garlic fractions. Hence, we first investigated the cytotoxic effects of the main four fractions of Allium sativum extracted by MC, PE, EtOAC and B on human
breast cancer (MCF-7) and human hepatocellular carcinoma (HepG2) cells using MTT assay and found anticancer activities for the four fractions with best effect for EtOAc fraction which had the lowest IC\(_{50}\) values 21.32 µg/ml in MCF7 and 26.22 µg/ml in HepG2. Next, we analyzed the different components of this fraction using HPLC and obtained 14 components (Farag et al., 2017) with highest concentrations for linoleic acid (LA) and the organosulfur compound, S-allylthiocysteine (SAC). Inconsistent results were obtained regarding the effect of LA, especially its conjugated form, on breast cancer. In support to our finding, some studies revealed anticancer effect for LA (Voorrips et al., 2002; Zheng et al., 2013) through inhibition of estrogen receptors and subsequent limitation of ER-positive cells proliferation (Durgam and Fernandes, 1997). Other studies showed either carcinogenic effect (Chajes et al., 2003) or no relationship with breast cancer incidence, metastasis, or progression (Chajes et al., 2003). Notably, LA also has a powerful inhibitory effect on HepG2 cells (Lu et al., 2015). The anticancer effect of garlic EtOAc fraction may be also due to the second ingredient of this fraction, SAC. Indeed, previous studies demonstrated anticancer activity for SAC against pancreas, colon, stomach, liver, and breast cancer (Fleischauer and Arab, 2001; Li et al., 2004; Chan et al., 2005; Setiawan et al., 2005; González et al., 2006).

In the present study, treatment by garlic fraction rich in LA and SAC induced apoptosis in MCF7 and HepG2 cancer cells as revealed by qPCR results which exhibited a significant upregulation in the apoptotic genes, Bax and caspase3, and a significant downregulation in the anti-apoptotic gene, Bcl2. This suggests that the inhibitory effect of garlic EtOAc fraction on MCF7 and HepG2 cancer cells may be, at least in part, mediated via induction of apoptosis. This also indicates that the apoptosis-dependant anticancer effect of this garlic EtOAc fraction is not restricted to a single cancer cell type. In agreement with our results, LA also induces apoptosis in ER+ breast epithelial cells and MCF7 via similar molecular pathway (Wang et al., 2008). Similarly, LA can also induce HepG2 apoptosis through upregulation of mitochondrial dependant Bax-caspase3 pathway (Lu et al., 2015). Another possible mechanism for LA-induced apoptosis is LA ability to stimulate genes implicated in cholesterol efflux which leads to cholesterol cell deprivation and results in hindrance of cell division and initiation of apoptosis (El Roz et al., 2013). In addition, LA can target caveolae, a specialized cell membrane structure that modulates cancer cell function, leading to apoptosis of cancer cells (Huo et al., 2010). As a second major constituent of garlic EtOAc fraction, SAC was also reported to induce apoptosis in cancer cells through the down-regulation of Bcl2 and the up-regulation of Bax (Ariga and Seki, 2006; Herman-Antosiewicz et al., 2007). SAC induces mitochondrial membrane collapse which further improves cytochrome c release from the mitochondria into the cytoplasm that finally triggers apoptosis through activation of caspase 9 and then caspase 3 (Trio et al., 2014).
Fig. 3: Real-time quantitative PCR analysis of the *caspase 3*, *Bax*, and *Bcl2* genes expression in MCF7 and HepG2 cells. Analyses were performed in triplicate. Values (columns and error bars) are expressed as mean ± SEM. ****P < 0.0001.

When cells are exposed to stress, signal transduction pathways known as checkpoints are switched on in the G1/S or G2/M phase, leading to cell cycle arrest (Pietenpol and Stewart, 2002). Most of the apoptotic event is associated with cell cycle disruption. This prompts us to investigate whether apoptosis induced by garlic EtOAc fraction is accompanied by cell cycle arrest. Expectedly, we found cell cycle arrest in G2/M phase (indicated by the elevated number of cells at this phase) in MCF7 and HepG2 cancer cells treated by garlic EtOAc fraction as compared to vehicle-treated cells. However, this treatment resulted in a significant reduction in the number of MCF7 and HepG2 cells in G0/G1 phase and no significant changes in cell number in S phase. In agreement with our findings, previous studies demonstrated cell cycle arrest in G2/M phase (Xiao et al., 2005), no change in S phase (Malki et al., 2009; Ma et al., 2014) and reduction in cell number in G0/G1 phase (Xiao et al., 2009a; Xiao et al., 2009b; Ma et al., 2014) in a large number of cancer cell types following treatment by garlic or its main constituents. The absence of cell cycle arrest in S phase may be due to the effect of LA content of this garlic fraction because previous studies have shown an inhibitory effect for LA on fatty acid synthase (FAS), an enzyme necessary for the feeding of the cancer cells (Song et al., 2012). The increased activity of this enzyme dysregulates the cell cycle by preventing the induction of apoptosis in S phases (Zhou et al., 2003). Further confirmation of our results, SAC also stimulate cell cycle arrest in the G2/M phase of cancer cells (Ariga and Seki, 2006; Herman-Antosiewicz et al., 2007; Omar and Al-Wabel, 2010). OSCs activity is related to sulfur chain length. OSCs affect the early mitotic arrest by making changes in the microtubule network which result from increasing the reaction of sulfur atoms against thiol groups of microtubules in the cell, so OSCs in garlic used as chemoprevention and chemotherapy (Cerella et al., 2011).
Fig. 4: Effect of EtOAc fraction of garlic on % of cells in the three phases of cell cycle in MCF7 and HepG2 cells as measured by flow cytometer. (A) Cell cycle histograms of MCF7 and HepG2 cells after treatment with EtOAc fraction of garlic. The X-axis represents the propidium iodide (PI) fluorescence based on the DNA content and the Y-axis represents the number of cells in each phase. (B) Graphical presentation of % of cells in G0/G1, S and G2/M cell cycle phases in MCF7 and HepG2 cells. Analyses were performed in triplicate. Values (columns and error bars) are expressed as mean ± SEM. ***P < 0.001, ****P < 0.0001.
Herbal extracts and natural products improve the efficacy of chemotherapeutics through not only induction of apoptosis, but also by inhibition of cancer cell metastasis and angiogenesis (Desai et al., 2015; Banjerdpongchai et al., 2016; Deng et al., 2016; El-Magd et al., 2017a). It is, therefore, crucial to investigate the in vitro and in vivo combined effect of garlic EtOAc fraction and an anticancer drug on cancer cell growth, invasion, and angiogenesis. Finally, we also recommend further studies to be conducted on each component of this fraction to determine which component(s) is(are) responsible for this anticancer effect as it is possible to find other valuable constituents, other than LA and SAC, with anticancer effect. Studying the structure-activity relationship (SAR) of the major identified compounds in EtOAc garlic fraction via molecular docking may also be of great value to investigate the actual underlying mechanism.

The obtained in vitro results on MCF7 and HepG2 cells suggest anticancer effect for garlic EtOAc fraction with focus on the role of its main two constituents, LA and SAC, which could be used as an adjuvant to anticancer drug or can help in the development of new drugs. However, further investigations are required to determine whether it is safe to give high-dose of garlic fractions or their components to breast and liver cancer patients as some of the garlic components, such as S-methylcysteine (SMC), induced cardiomyocyte toxicity in rats when given in a large dose (El-Magd et al., 2017b).

CONCLUSION

The present investigation demonstrated that the ethyl acetate fraction of garlic has comparable stronger anti-proliferative activities than the other fractions on MCF7 and HepG2 cells. This anticancer effect may be attributed to its major constituents LA and SAC and probably mediated by induction of apoptosis and cell cycle arrest in the G2/M phase of MCF7 and HepG2 cancer cells.

LIST OF ABBREVIATIONS

B: Butanol
EtOAc: Ethyl acetate
GE: Garlic Ethyl Extract
HepG2: Hepatocellular carcinoma
MC: Methylene chloride
MCF7: Human breast cancer cell
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OSCs: organosulfur compounds.
PE: Petroleum ether.

CONFLICT OF INTEREST

Authors do not have a financial or personal relationship with other people or organizations that could inappropriately influence the content of this paper.

DATA AVAILABILITY

All original data will be available upon request.

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