Journal of Medicinal Plants Studies

www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating 2017: 3.53
JMPS 2017; 5(5): 111-116

Received: 14-07-2017
Accepted: 15-08-2017

Hepzibha Priya Dharshini S
PG & Research Department of Biotechnology, Women's Christian College, Chennai, Tamil Nadu, India

Anchana Devi C
PG & Research Department of Biotechnology, Women's Christian College, Chennai, Tamil Nadu, India

Correspondence
Anchana Devi C
PG & Research Department of Biotechnology, Women's Christian College, Chennai, Tamil Nadu, India

A study on extraction of Ajoene from *Allium sativum* and its applications

Hepzibha Priya Dharshini S and Anchana Devi C

Abstract
Allium sativum, commonly known as garlic, belongs to family *Allium*. Cooking and consumption of the plant and its partswere due to variety of flavours and textures of the species. It contains 0.1-0.36% volatile oil, responsible for most of the pharmacological properties.Ajoene one of the organosulfur garlic compounds was extracted from solo garlic and it's anti-microbial, anti-thrombotic and cytotoxicity were studied. Garlic extract was prepared with ethyl acetate using soxhlet apparatus and purified using chromatographic techniques. HPTLC analysis clearly confirmed the presence of Ajoene in the prepared extract. Antimicrobial assay proved the properties of Ajoene to inhibit microbial growth dose-dependently against various bacterial and fungal species. Antithrombotic activity was visually confirmed by reduction in size of the blood clot. Cytotoxicity testing proved non-toxicity of the extract to VERO cells.

Keywords: Garlic, Ajoene, antimicrobial, antithrombotic, cytotoxicity, HPTLC

Introduction
Allium sativum, commonly known as garlic, is a genus of monocotyledonous plants which includes a variety of other species such as onion, scallion, shallot,leek, chives and hundreds of other wild species (Block E., 2010) [1]. *Allium* species occur in temperate climates of Northern Hemisphere, except few species occurring in Chile (such as *A. juncifolium*), Brazil (*A. sellovianum*), and tropical Africa (*A. spathaceum*). Single clove garlic (also called pearl or solo garlic) originated in the Yunnan province of China.

Fig 1: Pearl/Solo garlic

Chemical compounds of solo garlic
Garlic contains 0.1-0.36% volatile oil generally considered to be responsible for most of the pharmacological properties of garlic. Sulphur compounds in garlic includealliin, allicin, ajoene, allylpropl, diallyl, trisulphide, allylcysteine, vinyldithiins, S-allylmercaptocystein, and others. Besides sulphur compounds garlic contains 17 amino acids and their glycosides, arginine and others. Minerals such as selenium and enzymes like allinase, peroxidases, myrosinase, and others. Sulphur compounds are responsible for the pungent odour and medicinal effects. (Block E., 2010) [1].

Ajoene
Ajoene is an organosulfur compound present in garlic. It is a colourless liquid that contains sulfoxides and disulfide functional groups. The name is derived from "ajo", the Spanish word for garlic. Several clinical trials and in vitro studies of Ajoene have demonstrated its best-known anti-thrombosis, anti-microbial and cholesterol lowering activities.

Structure of AJOENE and its isomers

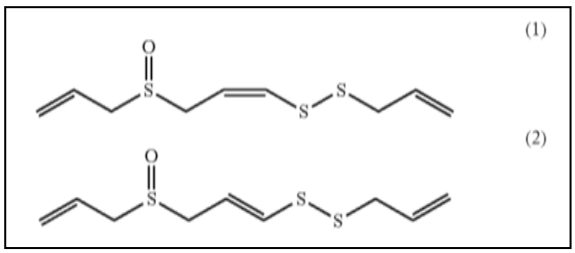

Fig 2: E and Z Isomers of Ajoene

Ajoene (E, Z-4, 5, 9-trithiadodeca-1, 6, 1 1-triene 9-oxide) is produced from pure allicin, and is chemically more stable than allicin. Ajoene exists in two isomeric forms, either as Z-ajoene (Cis form) of the chemical formula 1 or as E-ajoene (Trans form) of the chemical formula 2.

Medicinal uses: Ajoene has many medicinal uses. It functions as an antioxidant. Ajoene also has antithrombotic properties, which helps prevent platelets in the blood from forming blood clots, potentially reducing risk of heart disease and stroke in humans. Ajoene has shown potential virucidal properties against a number of viruses including vesicular stomatitis, vaccinia, human rhinovirus parainfluenza, and herpes simplex. Ajoene has broad-spectrum antimicrobial (antibacterial and antifungal) properties (Torres. J. Romero, 2012) [2]. Ajoene has been found to decrease basal-cell carcinoma tumor size by inducing apoptosiswhile it has also been shown effective in inhibiting tumor cell growth by targeting the microtubule cytoskeleton of such cells and by other mechanisms. Ajoene inhibits genes controlled by quorum sensing (Givskov M; *et al*, 2012) [5].

Materials & Methods

Sample Collection: One Kg solo garlic was collected from a local market in Parry's, Chennai, Tamil Nadu. This was stored for further use throughout the study. By a process described by Yoshida *et al.*, (1987) [6], raw garlic was fractionated into Allicin and Ajoene. Garlic was homogenated with water and half of the homogenate was steam distilled using Soxhlet extractor as explained below.

Soxhlet extraction: Mashed garlic pulp was packed using a filter paper, and then loaded into the main chamber. Soxhlet extractor was placed onto a flask containing ethyl acetate. Soxhlet is then equipped with a condenser, inlet and outlet pipe. The solvent is heated to reflux at 40 °C. Solvent vapour travels up a distillation arm, and floods into chamber housing the thimble of solid. The chamber containing solid material slowly fills with warm solvent. When Soxhlet chamber is almost full, chamber was automatically emptied by a siphon side arm, with the solvent running back down to distillation flask. After 6 cycles, solvent was removed to obtain extracted compound. Non-soluble solid portion of the extracted compound in the thimble was discarded.

Column chromatography: A slurry is prepared with stationary phase (silica gel 100*200 mesh), and poured into the column without air bubbles. Stationary phase is protected by securing the bottom of the column with cotton. After stationary phase settled uniformly in the column, crude garlic extract was added, and filled with eluent on top. Extraction was done under pressure using Ethyl acetate and water (1:1) as mobile phase. 3 Fractions were collected, and compared with standard garlic oil.

Thin layer chromatography: A small drop of sample was placed on silica plate. Ethyl acetate and water (1:1) was used as mobile phase. Silica plate was placed in the developer tank containing mobile phase and left undisturbed. After spreading, plate was air dried (5-10 min) by heating at 100˚C.Detection agent (0.1mg vanillin in 10 ml Conc. Sulphuric acid) was poured onthe silica plate. Brown spots indicated presence of Ajoene. Results are quantified using formula given below:

$$\text{Retention factor } (R_f) = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}} \times 100$$

High performance thin layer chromatography: Silica gel 60 F254 having a pore size of 6 mm with fluorescent indicator (coat material) was used. Plates are washed and conditioned at 120 °C for 15-20 mins. Then sample spot was applied (1 mm).Linear development method is most familiar technique in HPTLC, where the plate is placed vertically in solvent system. Solvent is fed by capillary action and chromatogram can be developed from both the sides. Spots are detected by spraying inert gas in chamber. Developed HPTLC plates were scanned at selected UV regions wavelength by the instrument, and detected spots are seen on computer in the form of peaks, which can be recorded as percentage on the printer (Sethi P D., 1996) [3].

Applications of AJOENE

Antimicrobial activity: Agar well diffusion method is most widely used method to evaluate antimicrobial activity of plants or microbial extracts. The agar plate surface was inoculated by spreading a volume of microbial inoculum over the entire agar surface. Extract solution (20–100 µl) are added to the wells at different concentrations. Extract diffuses in agar medium, and inhibits growth of microbial strain tested. (Balouiri M, *et al*., (2015) [4].

Antithrombotic activity: Blood sample was collected, and allowed to clot. Blood clot (5 mg) was washed using sterile water, mixed with garlic extract, and observed after 1 week.

Cytotoxicity testing: Vero cells (1×10^5/well) were plated in 24-well plate, and incubated at 37 °C with 5% CO_2 condition. After cell reaches confluence, media was removed from wells without disturbing the cells. Various concentrations of samples (100 µl) were added and incubated for 24 hrs. After incubation, sample was removed, and washed with phosphate buffered saline (pH-7.4) or MEM without serum.100µl/ well (5mg/ml) of 3-(4, 5- dimethyl- 2-thiazolyl)-2, 5-diphenyl-tetrazonium bromide (MTT) was added and incubated for 4 hours. After incubation, 1 ml of DMSO was added in all the wells. Absorbance at 570 nm was measured with ELISA reader. The % cell viability was calculated using the following formula:

$$\%\text{Cell viability} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100$$

Results and Discussions

Sample collection: Garlic was peeled, weighed (25 grams) and homogenated to prepare garlic extract. (Fig 3)

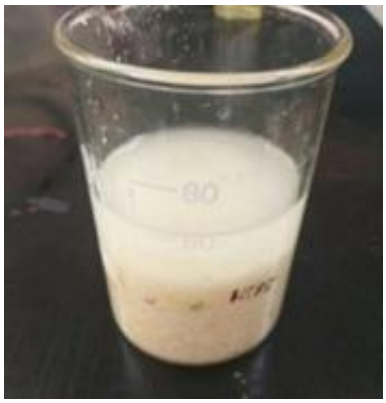

Fig 3: Garlic extract

Extraction using Soxhlet apparatus

Fig 4a): Soxhlet apparatus

Fig 4b): Garlic extract obtained by Soxhlet extraction

Soxhlet extraction was done with reference to standard procedures (Harwood, Laurence M *et al.*, 1989) [7]. In Fig. 4(a). Solvent was heated to reflux, vapours travel up the distillation arm, and floods into the chamber with homogenated sample. Condenser cools solvent vapours which also drips back into the chamber with sample. Chamber is emptied by siphon, when it is almost filled. Crude garlic extract was collected from bottom of the flask (Fig 4b). Solvent was evaporated, and subjected to silica gel column for purification. From 25 grams of garlic, about 10 mg of crude garlic extract was obtained.

Column Chromatography: It is a type of adsorption chromatography, in which individual chemical compounds are purified from mixture of compounds. Since the plant extract is bulk and individual components are to be separated, this method is best to separate them. The purified fractions of the extract were collected, separated in sterile vials Fig. 5(b) then subjected to TLC.

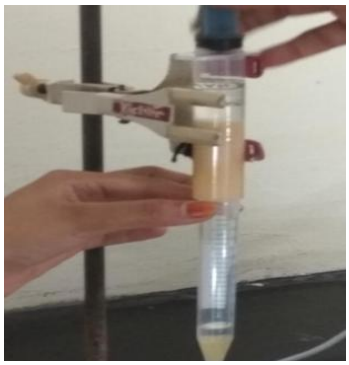

Fig 5a): Column chromatography

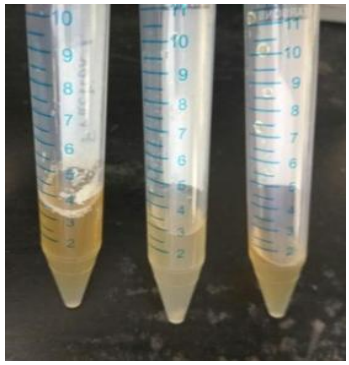

Fig 5b): Purified fractions

Thin Layer Chromatography: It is a technique used to separate non-volatile mixtures, performed on a sheet of glass, plastic or aluminium foil, which is coated with an adsorbent material such as silica gel, alumina or cellulose, which is the stationary phase (Harry W. Lewis & Christopher J. Moody., June 1989) [7]. TLC of the purified fractions, crude garlic extracts and standard showed the presence of Ajoene, detected by brown color spots (Fig 6b) and identified using retention factor (Rf) values (Gitin L *et al*., 2014) [9]. Rf value was found to be 0.46 which is closer to the value obtained by HPLTC analysis (Yoo M *et al.*, 2012) [22].

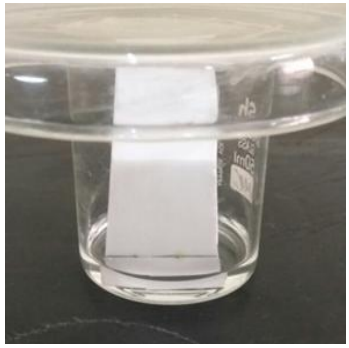

Fig 6a): Thin layer chromatography

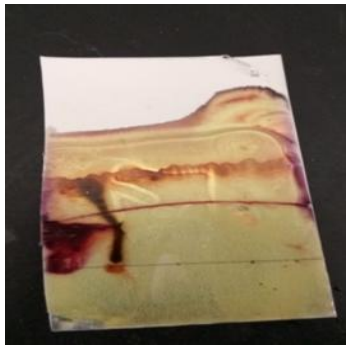

Fig 6b): TLC plate showing brown spots

High Performance Thin Layer Chromatography: It is an advanced formof TLC. Use of piezoelectric devices and inkjet printers for applying the sample is a recent approach to automation, which is useful to overcome the uncertainty in size and position of droplet when sample is applied to the TLC plate. Spot capacity can be increased by developing plate with two different solvents. Widely used instrument for HPTLC is from CAMAG, which provides automated sample loading and plate development, detection and documentation (CAMAG. Retrieved 2014-08-16). HPTLC is preferred over the conventional TLC as it has the following advantages: More resolving power per unit distance, faster development times and reduced solvent consumption. Sample was run using a mobile phase of ethyl acetate: water (1:1), and was found to have an Rf value of 0.48 which coincides with the value obtained by (Yoo, M *et al*., 2012) [22]. (Fig 7)

Standard Sample

Fig 7a): HPTLC plate's image captured using CAMAG visualizer: 170503

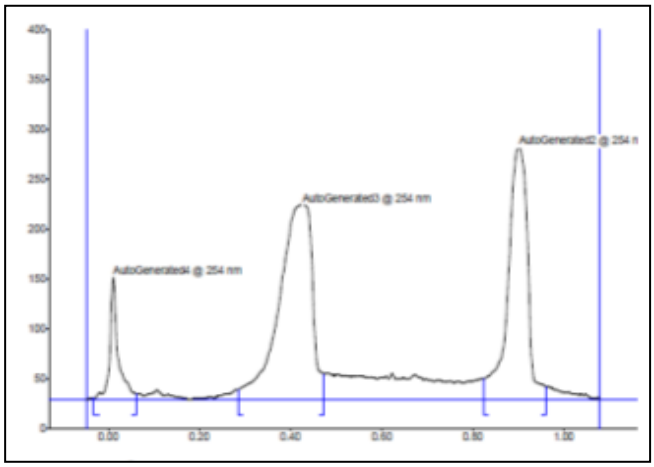

Fig 8: HPTLC profile of garlic extract.

Applications of AJOENE

Antimicrobial activity: The antimicrobial activity of the purified garlic extract was tested against the following microbial species- bacteria (*Klebsiella* spp, *Proteus* spp, *Bacillus* spp, *Staphylococcus* spp, *E.coli)* and fungi (*Candida albicans* and *Aspergillus niger).*The MHA & SDA plates were observed after 24 hrs, the inhibition was very less against *Bacillus spp, Staphylococcus* spp, *Klebsiella* spp, *E.coli* and *Candida albicans.*

Antimicrobial activity of Ajoene was found to be very less (Table-1). The inhibition was less because of the partially purified sample, after purifying the sample with a high grade technique with suitable conditions, its activity can confirmed.

Table 1: Antimicrobial activity of purified Ajoenefrom solo garlic

S. No	Organisms	Zone of inhibition (in mm)				
	Concentration Of Sample (in µl)	**20**	**40**	**60**	**80**	**100**
1.	*Klebsiella*spp	2	3	4	5	7
2.	*Proteus*spp	2	3	4	6	9
3.	*E. coli*	-	2	3	4	7
4.	*Bacillus* spp	-	2	3	5	6
5.	*Staphylococcus* spp	2	3	3	4	7
6.	*Aspergillusniger*	2	3	4	5	7
7.	*Candidaalbicans*	3	4	5	6	7
	Control	No growth				

The main antimicrobial effect of Ajoene is due to its chemical reaction with thiol groups of various enzymes, e.g. alcohol dehydrogenase, thioredoxin reductase, and RNA polymerase, which can affect essential metabolism of cysteine proteinase activity involved in the virulence of *E. histolytica* (Ankri S and Mirelman D., 1999) [10]. The growth of both *Aspergillus niger* and *Candida albicans* was inhibited by Ajoene at <20, ug/ml (Yoshida S *et al*., 1987) [6]. It is reasonable to conclude, therefore, that the wide spectrum antimicrobial effects of Ajoene are due to the multiple inhibitory effects it may have on various thiol-dependent enzymatic systems. Some enzymes such as the thiol proteases, which cause severe damage to the host tissues, may be inhibited at the lowest concentrations. At low concentrations the inhibition of these enzymes may not be lethal, but sufficient to block the microbe's virulence. At slightly higher concentrations other enzymes such as the dehydrogenases or thioredoxin reductase may be affected, and even partial inhibition of these enzymes could be lethal for the microorganism (Ankri S and Mirelman D, 1999) [10].

Antithrombotic activity: Ajoene was reported as a potent antithrombotic agent among other garlic compounds and is a potent inhibitor of platelet aggregation. Antithrombotic activity was determined by incubating the clotted blood sample in garlic extract and then it was observed after 7 days. The activity was confirmed by the change in size of the blood clot. Thus Ajoene in the garlic extract was found to have antithrombotic activity (Fig 9).

Fig 9: Antithrombotic activity

A great deal of research has been completed and published on the anticoagulant and antithrombotic activities of garlic (*Allium sativum*). The beneficial effects of garlic include lowering of plasma cholesterol, decrease of fibrinogen, coupled with increased fibrinolytic activity, and inhibition of platelet activity (Agriga and Seki, 2006; Bordia, 1978; Mohammad and Woodward, 1986; Rivlin, 2006; Srivastava, 1986) [11, 12, 13, 16, 14]. The therapeutic actions of garlic and its constituents have been well documented. These include antithrombotic and anticancer effects (Mohammad and Woodward, 1986; Srivastava, 1986; Makheja and Bailey, 1990) [13, 14, 15]. These beneficial effects result in the improvement of blood fluidity. However, as garlic significantly enhances fibrinolytic activity, it is theoretically possible that its over-activity could cause platelets to aggregate through the release of fibrinogen degradation products (FDP) because it has been reported that excessive fibrinolysis is associated with the release of FDP (Bordia., 1978) [12]. The inhibitory effect of processed garlic on human platelet aggregation has been known since 1978 (Lawson *et al*., 1993) [18]. Antithrombotic activity of Ajoene was assessed by Justin A *et al.,* 2000) and the results establish that antithrombotic activity is associated with disulfides directly attached to a phenyl ring and is further enhanced by an α-sulfonyl group.

Cytotoxicity assay: Ajoene possesses a broad spectrum of biological activities that include anticancer activity. It's Cytotoxicity towards cancer cells are postulated to occur via an apoptotic mechanism involving activation of the mitochondrial-dependent caspase cascade. (Catherine H, *et al*, 2014) [17]. Cytotoxicity assay helps to infer the anticancer activity of Ajoene. Cytotoxicity assay of Ajoene showed that it is non- toxic to the cells as the VERO cells were not killed. (Fig 10)

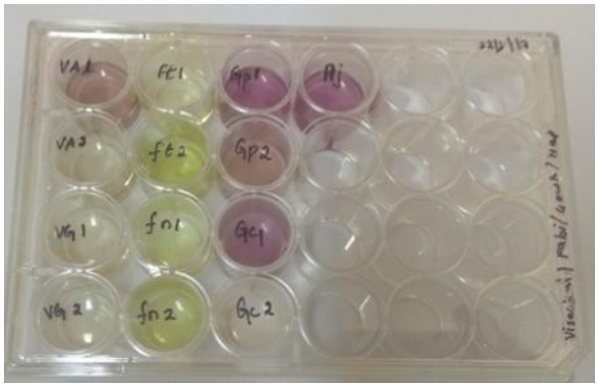

Fig 10: Cytotoxicity test for Ajoene

Calculation

$$\%\text{Cell viability} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100$$

= 0.160/0.232
= 69%

Since the value of cell viability is more than 50% the sample is non-toxic to normal cell and may be toxic for cancerous cells.

The organosulfur compound Ajoene, a constitutent of garlic, has been shown to induce apoptosis in a leukemic cell line as well as in blood cells of a leukemic patient. The mechanisms of action of Ajoene, however, are unknown (VM Dirsch *et al*., 1998) [19]. Wong *et al.,* studied the structural requirements of synthetic organosulfur compounds to induce apoptosis in human leukemia cells. They found that a disulfide moiety seems to be necessary to trigger cell death whereas adjacent groups contribute to the specificity of cell killing **(**Wong *et al.,* 2000) [20]. Cytotoxicity testing proved that Ajoene is non-toxic to the normal cells and it might be toxic against cancer cell lines. Since the garlic extract used for this study is a partially purified extract, it will have a combination of other compounds. Thus it can be used as a therapeutic agent, after purification and stabilization under proper conditions.

References

1. Block E. Garlic and other Alliums: The Lore and the Science. Royal Society of Chemistry Wiley-VCH Verlag GmbH & Co KGA, Weinheim. 2010, 454.
2. Torres J, Romero H. In vitro antifungal activity of Ajoene on five clinical isolates of *Histoplasma capsulatum var. capsulatum*". Revista Iberoamericana de Micologia. 2012; 29(1):24-28.
3. Sethi PD. High performance thin-layer liquid chromatography. Edition. 1996, 4-28.
4. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. 2015; 6(2):71-79.
5. Givskov M, *et al*. Ajoene a sulfur rich molecule from garlic, inhibits genes controlled by quorum sensing". Antimicrobial agents and chemotherapy. 2012; 56(5): 2314-25.
6. Yoshida S, Kasuga S, Hayashi N, Ushiroguchi T, Matsuura H, Nakagawa S. Antifungal activity of Ajoene derived from garlic. Applied and Environmental Microbiology. 1987; 53(3):615-617.
7. Harwood Laurence M, Moody Christopher J. Experimental organic chemistry: principles and practice, 1989.

8. Harry Lewis W, Christopher Moody J. Experimental organic chemistry. Principles and Practice. Wiley Blackwell. 1989, 159-173.
9. Gitin L, *et al.* Sulpfur compounds identification and quantification from Allium spp. fresh leaves. Journal og food and drug analysis. 2014; 22(4):425-430.
10. Ankri S, Mirelman D. Antimicrobial properties of allicin from garlic. Microbes and Infection, © Elsevier, Paris edition. 1999, 125-129.
11. Agriga T, Seki T. Antithrombotic and anticancer effects of garlic derived sulfur compounds. A Review Biofactors. 2006; 26:93-03.
12. Bordia A. Effect of garlic on human platelet aggregation *in vitro*. Atherosclerosis. 1978; 30:355-360.
13. Mohammad SF, Woodward SC. Characterization of a potent inhibitor of platelet aggregation and release reaction isolated from *Allium sativum* (Garlic). Thromb. Res. 1986; 44:793-806.
14. Srivastava KC. Evidence for the mechanism by which garlic inhibits platelet aggregation. Prostaglandins Leukot. Medicine. 1986; 22:313-321.
15. Makheja AN, Bailey JM. Antiplatelet constituents of garlic and onion. Agents Actions. 1990; 29:361-363.
16. Rivlin RS. Is garlic alternative medicine? J. Nutr. 2006; 136:7135-7155.
17. Catherine Kashula H, Hunter R, Parker MI. Garlic-derived anticancer agents: Structure and biological activity of Ajoene. Biofactors. 2010; 36(1):78-85.
18. Lawson LD, Wang ZL. Prehepatic fate of the organosulfur compounds derived from (*Allium sativum*). Planta Medica. 1993; 59(5):688-689.
19. Dirsch VM, Gerbes AL, Vollmar AM. The American Society for Pharmacology and Experimental Therapeutics. 1998; 53:402-407.
20. Wong WW-L, Macdonald S, Langler RF, Penn LZ. Novel synthetic organosulfur compounds induce apoptosis of human leukemia cells. Anticancer Res. 2000; 20:1367-1374.
21. Harry Lewis W, Christopher Moody J. Experimental organic chemistry. Principles and Practice. Wiley Blackwell. 1989, 159-173.
22. Yoo M, Lee S, Kim S, *et al*. Validation of HPLC Method for Determination of E- and Z-Ajoene in Oil-macerated Garlic Juice Food Sci Biotechnol. 2012; 21:107.