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Research Article

DETERMINATION OF ALLICIN IN *ALLIUM SATIVUM* USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND STUDY OF GENOTOXIC EFFECT ON HUMAN LEUKOCYTES

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ABSTRACT

Objectives: Allicin is an organosulfur compound featuring thiosulfinate functional group. The compound is formed in garlic after tissue damage, by the action of enzyme alliinase on alliin. This study involves extraction of allicin from garlic using high-performance liquid chromatography (HPLC) and study of it is the genotoxic effect on human leukocytes.

Methods: A simple and rapid reverse phase HPLC was used for the extraction of allicin. Quality allicin, in conjugation with chymosin, was used to study its genotoxic effect on leukocytes.

Results: Garlic oil and garlic extract showed similar retention time, and we measured the products using genotoxic effects in human leukocyte culture and it shows statistically not significant.

Conclusion: This study suggested that to take a lower concentration of garlic extracts benefits for health and these findings useful for further research.

Keywords: *Allium sativum*, Leukocytes, Chromosome, Genotoxic, High-performance liquid chromatography.

INTRODUCTION

Garlic (*Allium sativum*) possesses exquisite defense system and several therapeutical properties, contributed by several sulfur-containing compounds, mainly allicin. Allicin ($C_3H_5SS(O)C_3H_5$) is an organosulfur compound, first isolated and studied in the laboratory [1]. It is known as 2-propene-1-sulfinothioic acid S-2-propenyl ester, thio-2-propene-1-sulfinic acid S-allyl ester, diellyl disulfide-oxide, diallyl thiosulfinate, and percent composition: C - 44.4%, H - 6.21%, O - 9.86%, and S - 39.52% [2]. The interaction of non-protein amino acid alliin with the enzyme alliinase produces allicin [3]. Alliin (S-allylcysteine sulfoxide, percent composition: C - 40.66%, H - 6.26%, N - 7.90%, O - 27.08% and S - 18.09%) consists of a sulfoxide group, allyl group and the amino acid cysteine (contains SH rather than S=O). Alliin is synthesized from S-Allyl cysteine (deoxyalliin) [4]. It possesses a wide variety of medicinal properties including antimicrobial, bacteriostatic, anti-thrombotic, anti-fungal, anti-inflammatory, anti-cancer, and anti-atherosclerotic activities along with the capacity to lower serum lipid levels and ocular pressure [5-8]. On comminuting fresh garlic cloves, alliin is converted into diallyl thiosulfinate (allicin) in seconds. Allicin and the other thiosulfinates are unstable, but its stability can be greatly improvedby dissolving and diluting in water [9].

High-performance liquid chromatography (HPLC) has superior resolving power due to the high-pressure separation condition, making it the most preferred chromatographic technique for the separation of biological compounds. For the current study, the concentration of allicin in the isolated fraction is standardized by HPLC analysis. Sample analysis is carried out by extraction of garlic cloves with water and quantified against the isolated allicin external standard. Many researches are being carried out to improve the current scenario of cancer. However, until now less or no study has been done to check the genotoxic effect of allicin. This current work is an attempt to examine the effect of allicin on human blood leukocyte.

METHODS

Allicin extraction

The outer skin of the garlic cloves was peeled and crushed in a garlic press. The pressed garlic was then collected in a beaker and mixed thoroughly. 700-900 mg of the pressed mash was weighed and transferred to a 50 ml centrifuge tube. Using a volumetric pipette, 25 ml of cold water was delivered to the sample and immediately capped and shaken vigorously for 30 seconds. Heat transfer was avoided from hands by holding the tube cap while shaking. An additional 25 ml of cold water was added and shaken for 30 more seconds to dilute and mix the solution. Each sample is filtered through 0.45 µm glass filter into HPLC vial and capped for injection.

HPLC conditions

Column: Phenomex Prodigy™ ODS (3), 5 µm, 100 Å, 4.6 mm × 250 mm, mobile phase:methanol:water (50:50), flow rate: 1.0 ml/minutes, detector: 240 nm, injection Vol.: 25 µl, column temperature: 28°C, run time: 16 minutes.

Procedure

Garlic Pearls, a soft gel of Ranbaxy, was taken as the standard. An extraction solvent blank of water/methanol was prepared. A single injection was made of the blank. A single injection of the reference standard was also made. A linearity curve for allicin was prepared, with the origin ignored, using peak versus concentration (µg/ml). Linear regression analysis on the data was performed. The coefficient of determination, r^2 must be ≥0.999. A single injection of the sample prepared was made. The percent allicin in the samples against allicin reference was calculated [10].

Leukocyte culture method

Chromosome preparations were obtained from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes following the modified method of Hungerford [11]. About 2 ml of venous blood sample was collected in a sterile heparinized syringe. 0.5 ml of the blood was

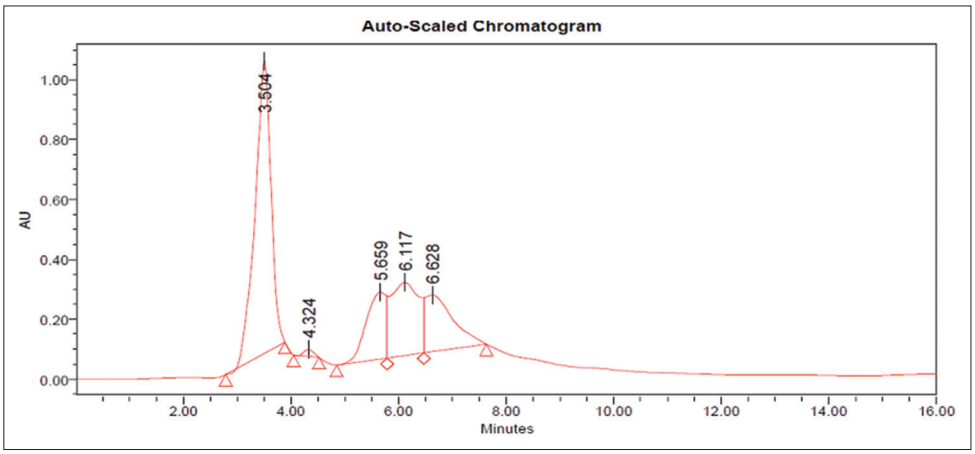

Fig. 1: High-performance liquid chromatography analysis of garlic pearls showing the retention times

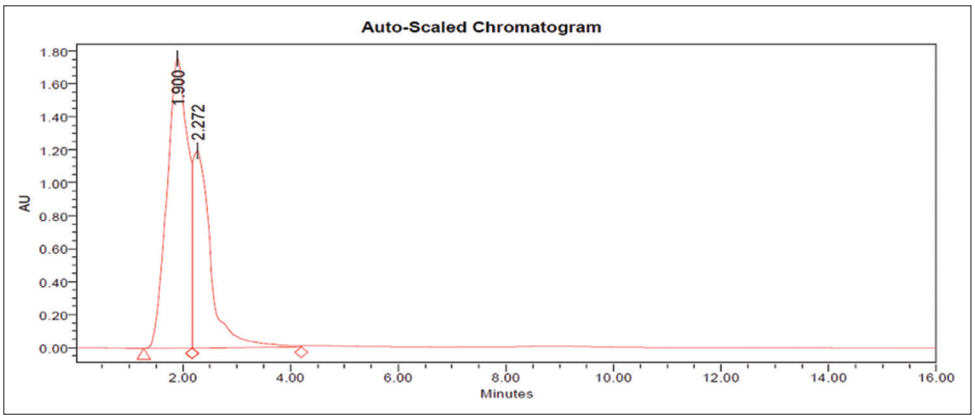

Fig. 2: High-performance liquid chromatography analysis of garlic extract showing the retention times

inoculated into the vials containing 5 ml of RPMI 1640 medium containing 1 ml of FB serum and 0.2 ml of PHA under aseptic condition. The culture vials were then placed in an incubator at 37°C. The cultures were shaken periodically, and carbon dioxide was released once every day.

Treatment with standard garlic pearls

At the 71st hrs of incubation, the cultures were treated with different concentration of standard Garlic Pearls (10, 20, 40, 80 µg/ml) for 1 hrs. After 1 hrs of incubation, the culture was thoroughly washed by centrifuging the content at 1000 rpm for 10 minutes, and supernatant were discarded carefully and 6 ml of fresh RPMI 1640 medium was added to the pellet and mixed well with Pasteur pipette.

Treatment with extracted allicin

At the 71st hrs of incubation, the cultures were treated with different concentration of extracted allicin (10, 20, 40, 80 µg/ml) for 1 hrs. After 1 hrs incubation, the culture was thoroughly washed by centrifuging the content at 1000 rpm for 10 minutes, and supernatant were discarded carefully and 6 ml of fresh RPMI 1640 medium was added to the pellet and mixed well with Pasteur pipette.

Culture harvesting

After washing process, the dividing cells were arrested at metaphase by adding two drops of 0.001% colchicine solution to each culture vial. The cultures were incubated further for 20 minutes at 37°C. The contents of the vials were then transferred to 15 ml centrifuge tubes and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the cells were resuspended in a small amount of solution left behind by gently tapping the tube. 6 ml of pre-warmed (37°C) hypotonic solution (0.075M KCl) was added to the tubes, and the contents were mixed gently using a Pasteur pipette. It was then incubated for 5 minutes at 37°C after which it was centrifuged for 6 minutes. The supernatant was carefully removed, and the cells were fixed with 6 ml of filtered carnoys fixative (3:1 methanol: acetic acid). The tubes were left at room temperature for 2 hrs. One change of fixative was given prior to slide preparation.

Slide preparation

The cell pellet was suspended in a small quantity of freshly prepared fixative. A test slide was prepared by gently placing a drop of the cell suspension on a cleaned glass slide and dried immediately on a hot plate (40°C). The test slide was examined under the microscope for cell density and metaphase spreads. Other slides were prepared after suitable modifications.

Staining procedure

The slides were stained in 4% Giemsa solution for 4 minutes and washed in distilled water for 1 minutes and then air-dried.

Microscopic analysis

Scoring of well-spread and stained cells was performed under oil immersion (×100) of the light microscope (Leica ATC 2000). The well spread metaphases were photographed using Olympus microscope with the camera.

RESULT AND DISCUSSION

The chromosomal aberration analysis is one of the widely used parameters for testing the protective effects of natural compounds on the drug and chemical induced toxicity. The modulatory effect of natural compounds on the chromosomal aberration induced by various kinds of chemicals and drugs are well established [12-15].

HPLC technique was applied for the present research work. In 2012 Rahman *et al*. [9] reported that except HPLC none other technique is reliable for the study of allicin. A study showed that the retention time of allicin seemed to vary from 3 to 3.7 minutes/ml in several garlic varieties [16]. Optimum conditions were applied for extraction of allicin from Indian garlic, and garlic oil was used as a standard. HPLC result showed that the retention time of standard garlic pearls is 3 minutes/ml and that of garlic extract was noted to be 2.2 minutes/ml (Fig. 1). The garlic extract had also shown similar retention time essentially depicting the presence of allicin at a higher level in the extract (Fig. 2). Although the analysis of allicin is difficult due to its instability, and the current study was an attempt to determine the presence of allicin in garlic extract and to check its genotoxic effect on human blood leukocytes. The methanol-garlic extract and standard were applied to the metaphase state of *in vitro* cultured human blood leukocyte and the chromosomal aberrations were found to be not statistically significant.

Allicin is widely used as a therapeutic agent for cancer, but no work has been done on its impact on normal blood leukocyte culture. The current study shows many chromatid and chromosomal aberrations in treated metaphase spread (Fig. 3). Hence, there is no significant difference observed between control, extract and standard samples in the following frequency: The percentage of chromosomal aberrations were 1%, 5%, 6%, respectively, (Tables 1-3 and Fig. 4).

In the market, a lot of garlic based products are available. They can be usually oil, powder or extract form. As compared to other garlic preparations, dehydrated garlic powder is thought to retain the same ingredients as raw garlic, both water-soluble and organic soluble, although the proportion and amount of various constituents may differ significantly [17,18]. Allicin liquid forms thiosulfinate compounds. These components are difficult to measure or detect without HPLC. Hence, in this study we used high HPLC for determination of allicin in garlic. This study seems to be the one of the best methods to measure genotoxic effects in human leukocytes.

Table 1: The chromosome aberration in control sample

Sample code	Number of cells scored	Chromosome aberration	Total number of aberration
		Breaks	N (%)
Control 01	50	1	1 (2)
Control 02	50	0	0 (2)
Control 03	50	1	1 (2)
Control 04	50	0	0 (0)
Total	200	2	2 (1)
Mean±SD		0.50±0.58	

SD: Standard deviation

CONCLUSION

Although the analysis of allicin is difficult due to its instability, the current study was an attempt to determine the presence of allicin

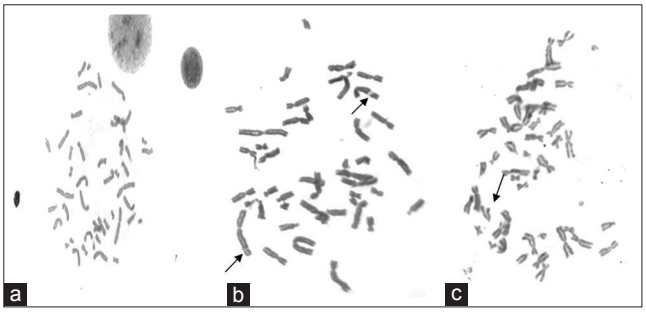

Fig. 3: The representative images of metaphase chromosomes control and treated with allicin, (a) control, (b) chromosome breaks, (c) chromosome deletion

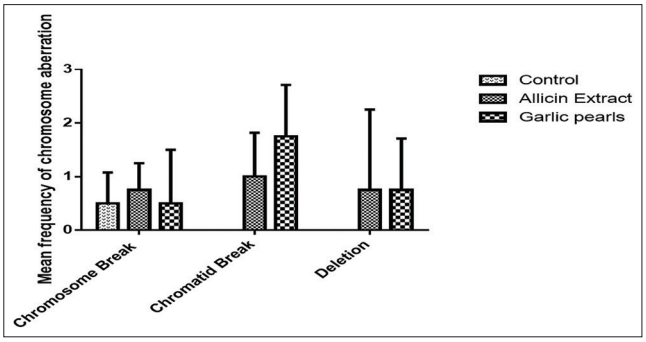

Fig. 4: Different types of chromosomal aberrations compared with control, garlic pearls and extracted allicin

Table 2: The chromosome aberration in treated with extracted garlic product

Concentration	Number of cells scored	Chromatid break		Chromosome aberration	Total number of aberration
		Breaks	Deletion	Breaks	N (%)
10 µl	50	1	0	0	1 (2)
20 µl	50	0	0	1	1 (2)
40 µl	50	2	0	1	3 (6)
80 µl	50	1	3	1	5 (10)
Total	200	4	3	3	10 (5)
Mean±SD		1±0.82	0.75±1.50	0.75±0.50	

SD: Standard deviation

Table 3: The chromosome aberration in treated with standard garlic pearls

Concentration	Number of cells scored	Chromatid break		Chromosome aberration	Total number of aberration
		Breaks	Deletion	Breaks	N (%)
10 µl	50	1	0	0	1 (2)
20 µl	50	1	0	0	1 (2)
40 µl	50	2	0	2	4 (8)
80 µl	50	3	2	1	6 (12)
Total	200	7	2	3	12 (6)
Mean±SD		1.75±0.96	0.75±0.96	0.50±1	

SD: Standard deviation

in garlic extract and to check its genotoxic effect on human blood leukocytes. Rahman *et al.* [9] reported that except HPLC none other technique is reliable for the study of allicin. In reference to different literatures, HPLC technique was applied for the present research work. From the HPLC result, we conclude that the garlic oil used as a standard for allicin showed a retention time of 3 minutes/ml. The garlic extract had also shown similar retention time essentially depicting the presence of allicin in a higher level in the extract. The methanol-garlic extract and standard were applied on the metaphase state of *in vitro* cultured human blood leukocyte, and the chromosomal aberrations were found to be statistically not significant.

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