International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
www.florajournal.com
IJHM 2021; 9(3): 09-17
Received: 04-02-2021
Accepted: 07-03-2021

Kumkum Sharma
Transcripotome Lab, Centre for Emerging Diseases, Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Sector- 62, Uttar Pradesh, India

Vibha Rani
Transcripotome Lab, Centre for Emerging Diseases, Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Sector- 62, Uttar Pradesh, India

Corresponding Author:
Vibha Rani
Transcripotome Lab, Centre for Emerging Diseases, Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Sector- 62, Uttar Pradesh, India

A comparative study of antioxidative and cardioprotective efficacy of raw and aged garlic extract

Kumkum Sharma and Vibha Rani

Abstract
Garlic (*Allium sativum* L.) has been used as a medicinal food for preventing disease and promoting heath. But consuming garlic to attain its therapeutic efficacy also has limitation because of the pungent smell and indigestion. Aged Garlic Extract (AGE) is a derivative of garlic prepared by aging the garlic cloves for twenty months. Aging of garlic converts reactive organosulfur compounds into stable compounds like S-allyl cysteine (SAC), Diallyl disulfide (DADS), S-allyl mercapto cysteine (SAMC) etc. Newly formed allyl compound believe to have exceptional therapeutic benefit as of the raw garlic, present study was designed to perform a comparison between antioxidant and cardioprotective potential of raw garlic and aged garlic extract. Phytochemical screening for raw and aged garlic extract was determined by qualitative methods. For antioxidative activity; DPPH, ABTS, Nitric oxide, Hydrogen peroxide activity was tested. Further, Ferric reducing antioxidant power (FRAP) assay and lipid Peroxidation activity by FTC and TBA was also performed. To determine the cardioprotective effect, cytotoxic dose of aged and raw garlic was optimized by MTT assay. Morphological analysis on below and above the cytotoxic dose was also done and cell viability was observed by trypan blue assay. This study suggest that aged garlic extract has higher antioxidative and cardioprotective activity compared to raw garlic.

Keywords: Reactive oxygen species**,** garlic, antioxidants, cardiomyocytes, stress

Introduction
Reactive Oxygen Species (ROS) are the by-product of cellular respiration and play a role in cell signalling [1]. However generation of excessive reactive oxygen species cause oxidative stress involved in the development of degenerative cardiovascular disease [2, 3]. Along with the free radical scavenging mechanism of body, Antioxidant phytochemicals present in food have been believed to be responsible for antioxidative activity and to reduce the oxidative damage [4]. *Allium Sativum* (Garlic) is a traditional medicinal food, originated from central Asia and some parts of southern Europe, northern Africa [5, 6]. Garlic contains dietary fibres, several nutrients, and traces of phytochemical. Also, garlic contains more than 30 organosulfur compounds which are responsible for its physiological effect involved in human health [7]. Anti-microbial, antioxidative, anti-cancer, antidiabetic, cardioprotective and antihypertensive activities are well known benefit of garlic consumption. Though garlic has numerous health effects, but consuming it daily in large amount to get its therapeutic benefits is also problematic because those reactive sulfur species produce pungent smell, [8] indigestion [9] and irritation to the gastrointestinal tract [10]. Studies suggested that consuming 4-5 garlic cloves daily for a long time can cause stomach ulcer [10, 11].
Aged garlic extract (AGE) is a derivative of garlic prepared by soaking the garlic cloves in aqueous solution for twenty months at room temperature. Aging converts the harsh odorous compound into stable and odourless organosulfur compounds such as allicin converts into antioxidant rich amino acids S-allylcysteine (SAC) [12]. S-allyl mercaptocysteine (SMAC), N-fructosyl arginine etc. The compound formed during aging does not have pungent smell and are comparatively safer than the reactive sulfur species present in garlic [13]. Also water solubility of these compounds improves the bioavailability and therapeutic potency [14]. Exempting the limitation of garlic makes aged garlic extract more preferable now a days [15]. But are the organosulfur present in aged garlic more effective then raw garlic is still a key question and need to be explored. This study investigates the antioxidative and cardioprotective activity of aged garlic and raw garlic. We comparative study with both the natural compound and seek which of the garlic derivative is more preferred and to be continued for further research.

Materials and methods
Chemicals
All the chemicals were purchased from Sigma-Aldrich, USA unless or otherwise mentioned.

Preparation of garlic extract
25g of raw and aged garlic was placed in a thimble and extracted with 200ml of methanol for 8 cycles in a Soxhlet apparatus separately. After 8 cycles, extract was filtered by whatman no.1 filter paper. Filtrates were then concentrated in a rotatory evaporator. The concentrated garlic extract were further kept at room temperature to dry completely for 2-3 days. Once the extracts dried, 1mg/ml solution was prepared in distilled water. Same concentration (1mg/ml) was also prepared for aged garlic in distilled water. Both the samples were again filtered and kept in clean bottles till further use.

Phytochemical analysis of developing and aged garlic
Raw and aged garlic was tested for the presence of bioactive compounds like tannins, flavanoids, alkaloids etc. by qualitative methods [16]. All the experiments were repeated in triplicates.

Detection for tannins
1% ferric chloride was drop wise added in 2 ml of raw and aged garlic methanolic extract. Black- brown precipitation showed the presence of tannins.

Detection for flavonoids
4 ml of diluted ammonia was added to 2 ml of both the extracts followed by few drops of conc. sulphuric acid. Appearance of yellow color confirmed the presence of flavonoids.

Detection for terpenoids
1 ml of chloroform and acetic anhydride were added in 1 ml raw and aged garlic extract, 2 ml of conc. sulphuric acid was added. The reddish- violet color confirmed the presence of terpenoids.

Detection of glycosides
Hydrochloric acid was added to raw and aged garlic extract in 1:1 ratio. The mixture was incubated in the water bath for 10 minutes. 1 ml of pyridine and few drops of sodium nitroprusside solution were then added. Conversion of pink to red color after addition of sodium hydroxide solution confirmed the presence of glycosides.

Detection of phenols
1ml of Folin-Ciocalteu reagent and 0.5 ml of sodium carbonate were added to 1 ml of both the extract. Appearance of blue colour showed the presence of phenol.

Detection of alkaloids
2 ml hydrochloric acid was added to 4 ml of raw and aged garlic extract which was then filtered. 2 ml Dragendroff's reagent was added to both the filtrates. Formation of an orange or red precipitate confirmed the presence of alkaloids.

Detection of steroids
5 ml of chloroform was added to 1 ml of raw and aged garlic extract. 5 ml conc. sulphuric acid was then added drop wise to the mixture. Formation of the red brown ring confirmed the presence of steroids.

Detection of saponins
Olive oil were added to 2 ml of both the extracts drop wise. The formation of a soluble emulsion showed the presence of saponins.

Detection of reducing sugar
1 ml of raw and aged garlic extract was mixed with 1 ml of Fehling's solution (Fehling's A and B in equal volumes). Development of red precipitate on boiling for 5 min confirmed the presence of reducing sugar.

Detection of reducing monosaccharides
1ml of developing and aged garlic extract was mixed with 1 ml of Barfoed's reagent and heated for 2 min. Formation of reddish precipitate of cuprous oxide confirmed the presence of reducing monosaccharides.

Antioxidant activities of Raw and Aged garlic extract.
DPPH scavenging assay
Free radical scavenging activity of both the extracts was compared by DPPH assay. Aged and raw garlic extract at different concentration ranging from 0.2 -1 mg/ml were mixed equally with. 135 mM DPPH prepared in methanol [17]. The mixture was incubated at room temperature in dark for 30 minutes followed by measuring the absorbance at 517 nm using UV-VIS Spectrophotometer. Free radical scavenging activity was calculated using the following equation.

% inhibition = OD control - OD of Sample / OD of control * 100

Where OD control is the absorbance of DPPH + methanol, OD sample is the absorbance of DPPH radical + sample.

ABTS scavenging assay
For ABTS scavenging analysis, 7 mM ABTS solution and 2.4 mM potassium persulphate was mixed in equal proportion and incubated for 12-16 h at 25°C in the dark. Freshly prepared ABTS·+ solution (1 ml) was further added in the resulting mixture. Extracts were mixed separately with the mixture in 1:1 ratio [18]. After 10 min, the absorbance was measured at 734 nm. The inhibition capacity of the extracts for ABTS·+ was calculated by the equation as mentioned above.

NO scavenging activity
NO scavenging activity was determine by mixing 2 ml of 10mM sodium nitroprusside with different concentration of aged and raw garlic, the reaction was incubated for 2.5 hr at 25 C in dark. After incubation freshly prepared griess reagent was added in 1:1 ratio for 30 minutes at 25ºC in dark [19]. Absorbance was taken at 540 nm and nitric oxide scavenging activity was calculated using the equation of% inhibition.
% inhibition = OD control - OD of Sample / OD of control * 100

H_2O_2 scavenging activity
4 mM H_2O_2 solution (prepared in 0.1 M phosphate buffer, pH ~ 7.4) was mixed with different concentration of raw and aged garlic separately in equal proportion and incubated for 10 min at 25 °C. The absorbance was measured at 230 nm [20]. The percent inhibition of H_2O_2 by both the extracts was calculated using the% inhibition equation mentioned above.

Determination of reducing power potential by FRAP assay
Different concentrations of raw and aged garlic were added to 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide solution. The mixture was vortexed and incubated at 50 ºC for 20 minutes. After the

incubation 2.5 ml of 10% trichloroacetic acid (TCA) was added and centrifuged at 3,000 rpm for 10 min. The supernatant (2.5 ml) was mixed with 2.5 ml of deionised water and 0.5 ml of 0.1% ferric chloride. Absorbance was measured at 700 nm [21]. The reducing capacity of extract (Fe^{3+} to Fe^{2+}) was measured by the blue color intensity.

Lipid peroxidation assays

The antioxidant activity of aged and raw garlic was determined using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. The inhibition of lipid peroxidation was estimated by the% inhibition formula mentioned above.

FTC method

Different concentration of raw and aged garlic (.2 -1 mg/mL) was taken, 4.1 ml of 2.5% linoleic acid in 99.5% ethanol, 4 ml of 99.5% ethanol, 8.0 ml of 0.02 M phosphate buffer and 3.9 ml of distilled water was added to each concentartion and incubated in dark for 30 minutes and To measure the antioxidant activity, 9.7 ml of 75% (v/v) ethanol was added in the reaction mixture, followed by 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid [22]. After 5 min ferrous chloride was added and the absorbance was measured at 500 nm by UV-vis spectrophotometer.

TBA method

The similar reaction mixture used in FTC assay was taken for TBA assay. Then 20% trichloroacetic acid and 0.67% of thiobarbituric acid were added in similar ratio to both the extracts. Mixture was then incubated in water bath at 100ºC for 10 minutes, followed by centrifugation at 3000 rpm for 20 minutes [23].The absorbance activity of the supernatant was measured at 552 nm.

Cell cytotoxicity Assay

Cell metabolic activity under different concentration of raw and aged garlic was analyzed by MTT assay. Approximately 10,000 cells were cultured in 96 well plate for 24 hr. afterwards cell were seeded in 96 well plate and treatment of both the extract were given. After 48 hr. of treatment 20 µl MTT was added and incubated for 3 hr. in dark at 37 ºC. MTT absorbed in the cells and form a blue color crystal which was then dissolved in 100 µl DMSO. Absobance of sample was measured at 570 nm [24].

Cell viability = Absorbance of sample - Blank / Absorbance of control – Blank

Morphological analysis

H9C2 cardiomyocytes were cultured in a six well plate for morphological analysis. ~70% confluent cells was trypsanised and centrifuged at 1500 RPM for 10 minutes. Cell pallet was resuspended uniformely and added in each of the six well and the cells were allowed to adhere for 24 hr. After 24 hr. cells were allowed to incubate on different concentrations of raw and aged garlic methanolic extract for 48 hr.

Giemsa staining

To study the cellular morphology after 48 hrs of treatments with different concentration of raw and aged garlic, cells were fixed with 100% methanol at −20 °C. Cells were then incubated with 20% giemsa stain diluted in 0.5% glacial acetic acid for 15 minutes at 25 °C and observed under inverted microscope at 40 X magnification [10].

Trypan blue dye exclusion assay

Cells death was examined by collecting the media from all the plates and incubated with 0.4% trypan blue stain in 1:1 ratio and kept at room temperature for 5 minutes. Dead cell were then examined and calculated using haematocytometer [11].

Statistical analysis

All the experiments were repeated three times in triplicates. The data was expressed as Mean ± SEM and significance was evaluated by T test and two-way ANOVA. P value was calculated on comparing the data where $p<0.05$.

Result

Raw and Aged garlic extract has similar phytochemical abundance

The qualitative analysis of phytochemicals present in aged and raw garlic indicates the presence of tannins, glycosides, phenol, alkaloids, reducing sugar and reducing monosaccharide (Table.1). However saponins were found to be present in aged garlic but not in raw garlic.

Table 1: Phytochemical screening of Methanolic extract of Developing and Aged garlic

Phytochemicals	Developing Garlic	Aged Garlic
Tannins	++	++
Flavonoids	++	++
Terpenoids	--	--
Glycosides	++	++
Phenols	++	++
Alkaloids	++	++
Steroids	++	--
Saponin	++	++
Reducing Sugar	++	++
Reducing Monosaccharides	++	++
Anthraquinones	++	--

[In the above table "++" indicate Presence and "—" indicate the absence of phytochemical.]

Aged garlic extract has better free radical scavenging potential

1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of aged garlic extract on 0.2, 0.4, 0.6, 0.8, 1 mg/ml concentration was found to be increased in concentration dependent manner 17% ±.04, 28% ±.01, 33% ±.02, 39% ±.01, 40 ±.12 respectively (Figure1). Where highest activity was observed at 1mg/ml concentration. While DPPH activity of raw garlic at the same concentration was found to be 17% ±.02, 28% ±.01, 28% ±.01, 28% ±.02, 28% ±.01. Which is significantly lower than aged garlic extract.

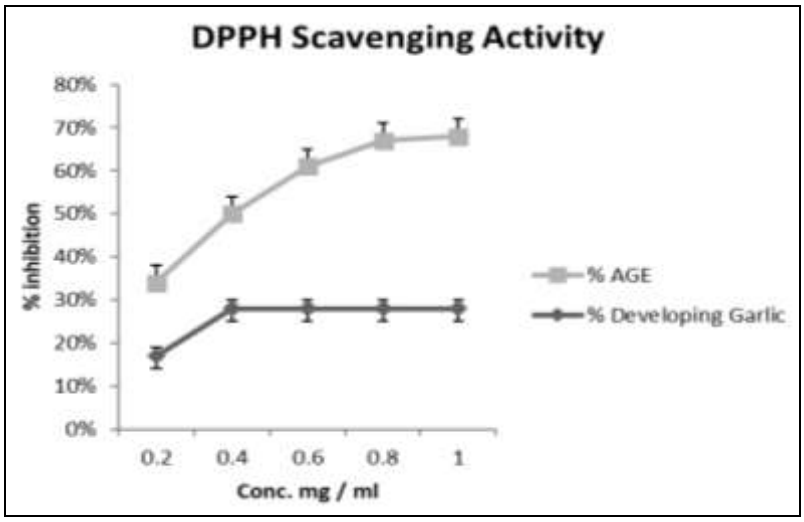

Fig 1: DPPH scavenging activity of Methanol extract of garlic and AGE, AGE has shown the highest antioxidative potential compared to developing garlic. Values are expressed as mean of percentage inhibition of DPPH radicals (n=3) ± SD. * represented the level of significance ($p \leq 0.05$).

ABTS activity of aged garlic was found to be 45%, 47%, 51%, 52% and 55% at 0.2, 0.4, 0.6, 0.8, 1 mg/ml respectively. However for raw garlic it was 39%, 40%, 41%, 42%, 43% on same concentration (Figure 2).

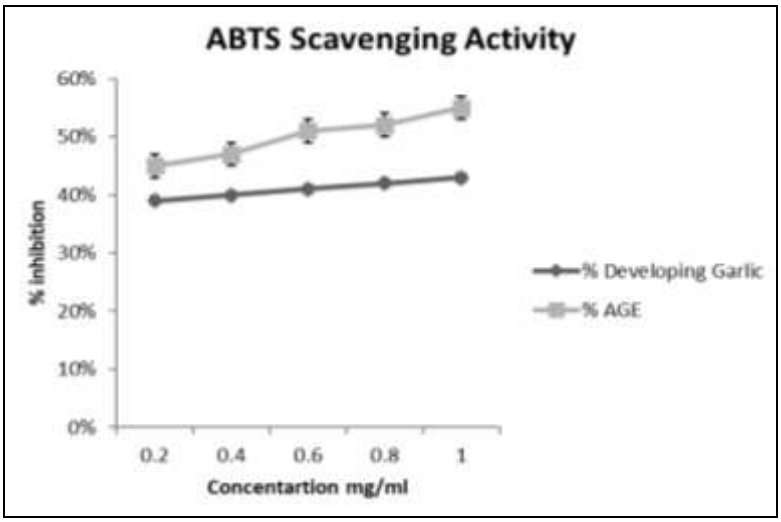

Fig 2: ABTS Scavenging Activity: AGE has shown higher ABTS scavenging activity compared to developing garlic. Values are expressed as mean of percentage of Reducing power potential (n=3) ± SD. * represented the level of significance ($p \leq 0.05$).

Nitric oxide scavenging activity of raw garlic at different concentration (0.2-1 mg/ml) was found to be 3% ±.01, 4% ±.01, 4.3% ±.02, 5.4% ±.01, 6% ±.02 while aged garlic had 7% ±.02, 7%±.01, 8% ±.02, 14%±.02, 19% ±.03 signifies three fold higher antioxidative activity at 0.8 and 1 mg/ml concentration (Figure 3).

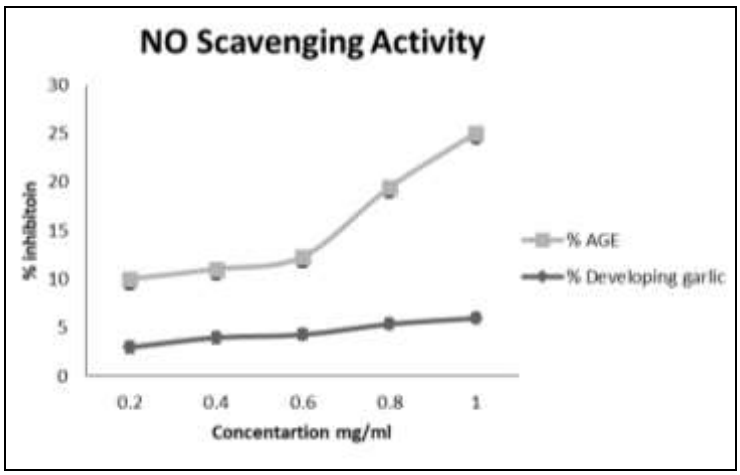

Fig 3: NO scavenging activity AGE has shown the greater NO scavenging potential compared to developing garlic. Values are expressed as mean of percentage inhibition of nitric oxide radicals (n=3) ± SD. * represented the level of significance ($p \leq 0.05$).

Hydrogen peroxide scavenging activity of raw garlic was found to be 2% ±.04, 5% ±.03, 6% ±.01, 7% ±.02, 8% ±.02. whereas aged garlic extract was found to be 20% ±.01, 40% ±.02, 45% ±.02, 50% ±.02, 54% ±0.4 on the same concentration conformed the efficacy of aged garlic extract is comparatibly better than raw garlic (Figure 4).

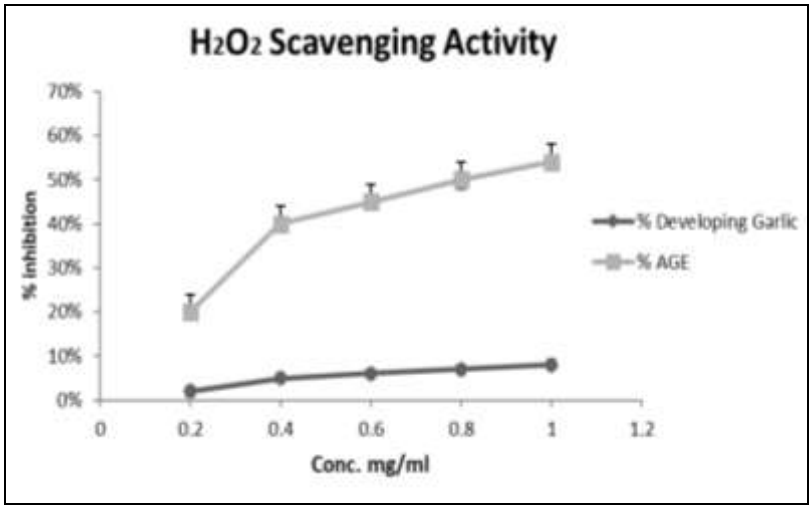

Fig 4: H2O2 scavenging activity AGE has shown highest hydrogen peroxides scavenging compared to developing garlic. Values are expressed as mean of percentage inhibition of hydrogen peroxide (n=3) ± SD. * represented the level of significance ($p \leq 0.05$).

In the next experiment, reduction potential of antioxidants present in both the extract was determined by FRAP method. It is a spectroscopic method and higher absorbance's represent higher electron donating capacity of antioxidants. The result showed increase in absorbance with increasing concentration (0.2-1 mg/ml) of aged garlic extract 0.131, 0.137, 0.14, 0.15, and 0.16. while the absorbance of raw garlic was found to be 0.123, 0.129, 0.132, 0.139, 0.141 (Figure 5).

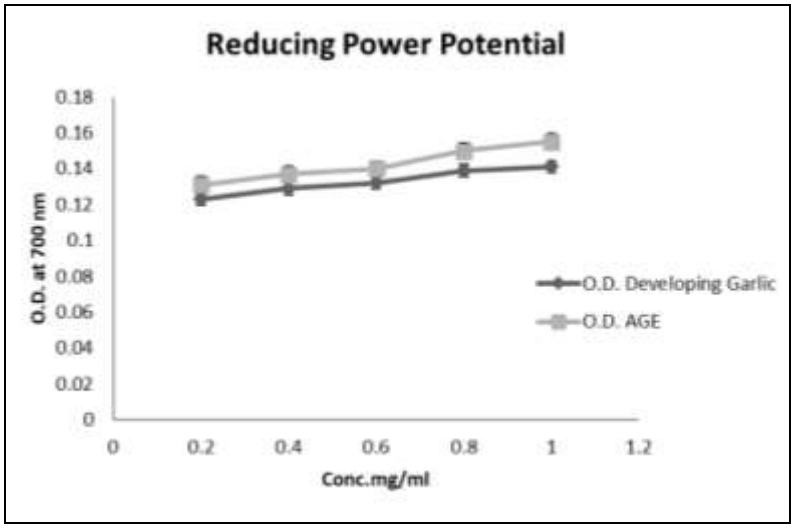

Fig 5: Reducing power potential AGE has shown higher Reducing power potential compared to developing garlic. Values are expressed as mean of percentage of Reducing power potential (n=3) ± SD. * represented the level of significance ($p \leq 0.05$)

Aged garlic extract has higher lipid Peroxidation inhibition activity as compared to raw garlic

The antioxidative activity of aged and raw garlic extract on inhibition of lipid Peroxidation at different concentration (0.2-1 mg/ml) was determined by ferric thiocyanate (FTC) method. antioxidative activity of aged and raw garlic extract has been represented as percent inhibition formula mentioned above. The percent inhibition of aged garlic was found to be 48% (0.2mg), 55% (0.4mg), 60% (0.6mg), 65% (0.8mg), 72% (1mg), while the percent inhibition of raw garlic was 48% (0.2mg), 50% (0.4mg), 51% (0.6mg), 53% (0.8mg), 57% (1mg), which was lower than the aged garlic extract (Figure 6). In the next experiment we determined malondialdehyde (MDA) inhibition which is a marker of lipid Peroxidation by thiobarbituric acid method (Figure 7). Aged garlic extract was found to have 46% (0.2mg), 47% (0.4mg), 56% (0.6mg/ml), 60% (0.8mg), 62% (1mg) which was significantly higher than the raw garlic extract 5% (0.2mg), 14% (0.4mg), 19% (0.6mg), 35% (0.8mg), 52% (1mg).

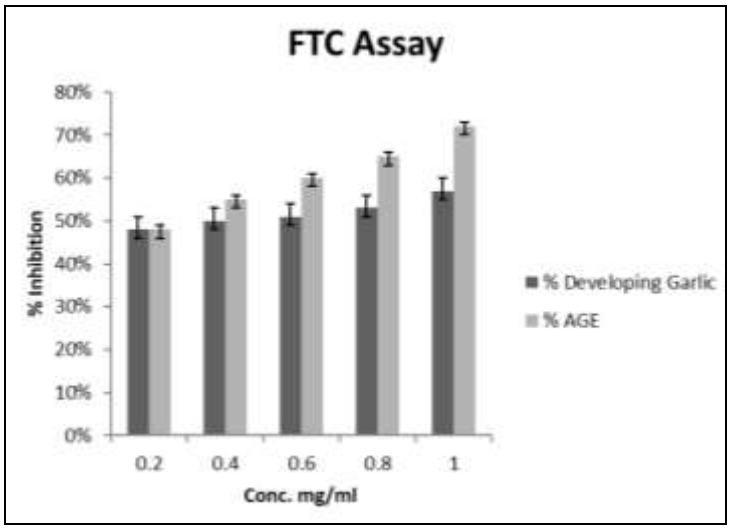

Fig 6: Lipid peroxides inhibition by FTC assay AGE has shown higher Lipid peroxides inhibition by FTC assay compared to developing garlic. Values are expressed as mean of percentage inhibition lipid peroxidation (n=3) ± SD. * represented the level of significance ($p \leq 0.05$)

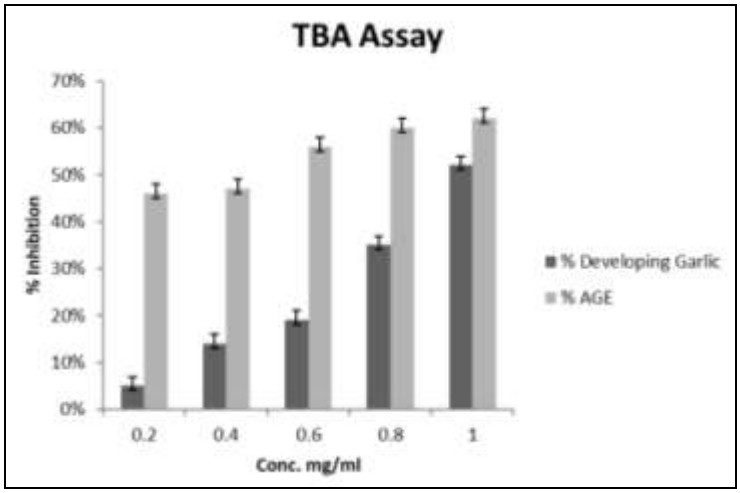

Fig 7: Lipid peroxides inhibition by TBA assay AGE has shown higher Lipid peroxides inhibition by TBA assay compared to developing garlic. Values are expressed as mean of percentage inhibition of lipid peroxidation (n=3) ± SD. * represented the level of significance ($p \leq 0.05$)

Aged garlic extract has less cytotoxicity of cardiomyocytes as compared to raw garlic

MTT assay was carried out to determine the cytotoxicity of raw and aged garlic extract. The results indicated that after 25 mM concentration of raw garlic cell viability start to decrease. whereas aged garlic extract excerpt less toxicity even on higher concentration (Figure 8). Morphological analysis of H9C2 cells at different concentration of raw and aged garlic showed variation in their morphology (Figure 9), which was further validated by cell death assay for both the extract. Percent Cell death for aged garlic at 10mM, 30Mm, 50mM was found to be 4%, 16% and 25% respectively. While percent cell death for raw garlic was found to be 7%, 31%, 42% (Figure 10).

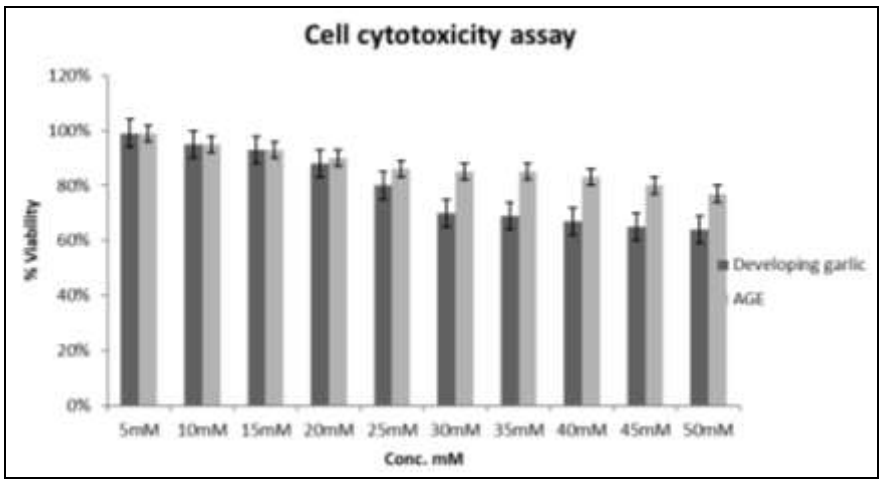

Fig 8: MTT assay, dose optimization of developing garlic and AGE showed AGE has higher viability compared to developing garlic. Values are expressed as mean of percentage cell viability (n=3) ± SD. * represented the level of significance ($p \leq 0.05$)

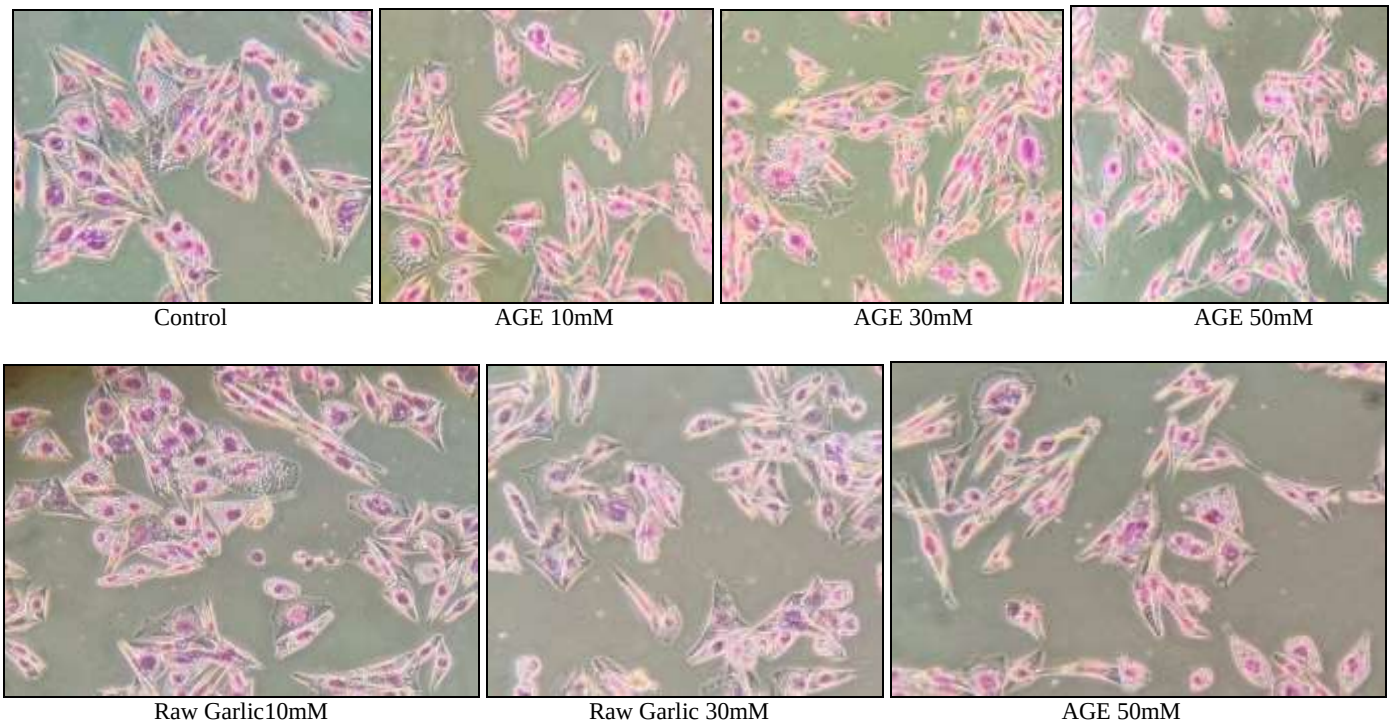

Fig 9: Morphological analysis of H9C2 cells at different concentration of Raw and Aged garlic

Discussion

There is a considerable interest in association of reactive oxygen species with cardiovascular diseases now days. Once the homeostasis between Reactive oxygen species and antioxidant start to fail, oxidative stress become apparent and leads to cellular damage [27]. Besides this free radicals are involve in several signaling pathways in balanced state and overproduction of these free radical unstable these pathways [28]. In recent time antioxidants obtained from plant have gain a substantial interest because of their potential therapeutic value and garlic through their antioxidative activity have extensively been reported to provide defense against free radical damage in body. But due to some limitation like bioavailability and bed odor its consumption to attain its therapeutic potential is limited. However aged garlic extract modifies the antioxidative property of garlic by stabilizing highly unstable compound to stable water soluble organosulfur molecule. And in this study we found that aged garlic extract has higher antioxidative activity and lower cell toxicity.

Phytochemicals like flavanoids, phenols and alkaloids are extensively reported to have antioxidative effect and provide health benefits through cell signaling pathway. However tannins and glycosides are reported to have physiological effect in cardiovascular system like accelerate blood clotting and lowering the blood pressure. In this study, phytochemical analysis of both raw aged garlic methanolic extract showed the presence of several phytochemical except saponin which were present in aged garlic extract abundantly but not present in raw garlic. High level of saponine contributes to the antioxidative activity of aged garlic extract they have also been reported to have lowering blood glucose response making aged garlic a natural antiglycative agent [29].

The antioxidative activity of raw and aged garlic extract was determined by the ability to scavenge DPPH, which is a violet colored compound and a free radical method to determine the hydrogen atom donating capacity of natural compounds and measure by the extent of colorless complex produced after reaction causing the decreased absorbance. The DPPH scavenging activity of aged garlic extract was found to be three fold higher than raw garlic (Figure1). Which is due the presence of stable organosulfur compounds and their redox property? Garlic has also shown DPPH scavenging activity, which is might be because of the phytochemicals present in it [30]. But the ability to reduce was much more lesser then aged garlic extract and these results were in agreement with those of previous work reported. While DPPH assay is conducted in organic solvents. ABTS assay is conducted in aqueous condition. The method is based on the ability of antioxidants to scavenge radical cation produced by reacting ABTS solution with a strong oxidizing agent, which is reduced by hydrogen donating antioxidants compounds. The hydrogen donating capacity of raw and aged garlic was evaluated by decrease in the absorbance of blue green color ABTS radical reaction. The ability of garlic and aged garlic to scavenge ABTS was compared with ascorbic acid. The highest ABTS scavenging activity of aged garlic extract was found to be 56.3% at 1mg/ml concentration while the activity of raw garlic was 43% at the same concentration (Figure 2). These results indicate that aged garlic extract has better reduction potential in organic as well as aqueous phase.

Among the reactive oxygen species associated with cardiovascular disease nitric oxide and hydrogen peroxide are considered to be chemically more stable than other ROS like superoxide etc [31] high level of these species in blood increase the contribution to vascular pathophysiology. Increased concentration of NO is highly reactive which cause oxidative stress and tissue damage. In this study we found that aged garlic extract have five fold higher NO scavenging activity than raw garlic which is mainly due to the stable organosulfur compound present in aged garlic extract (Figure 3). Also H_2O_2, which is a non free radical species have high toxicity in presence of metal ions [32] aged garlic extract scavenges hydrogen peroxide by 57% at 1 mg/ml concentration which is approximately ten times higher than raw garlic extract (5%) on same concentration (Figure 4). Therefore a high NO and H_2O_2, scavenging activity of aged garlic extract might be useful for protection against oxidative toxicity.

In the Ferric reducing antioxidant power potential **(**FRAP) which is a widely used colorimetric assay to determine the electron donating capacity of antioxidant rather than the hydrogen atom transfer. The FRAP reaction is conducted at low pH decreases the ionization potential that drives hydrogen atom transfer and increase the redox potential. The reduction of Fe^{3+} to Fe^{2+} occurs in the presence antioxidants accompanied by the formation of blue colored complex (Absorbance at 593 nm) which is related to the degree of hydroxylation [33]. Higher absorbance indicates higher electron donating capacity of antioxidant. Unexpectedly, aged garlic extract and raw garlic showed similar electron donating capacity which indicates that the metal chelating ability of aged and raw garlic extract depends on the phytochemicals especially phenolic compounds rather than organosulfur compounds (Figure 5). Though the result indicated that aged garlic extract has a slightly higher side of FRAP activity which showed that the phenolic content in it is also high.

Lipid Peroxidation and its association with cardiovascular disease like atherosclerosis is a major cause of morbidity [34]. The ability of garlic and aged garlic to reduce lipid Peroxidation was determined by two methods. The Ferric thiocyanate (FTC) method was used to determine the amount peroxide produced at the beginning of lipid Peroxidation. In this method peroxides reacts with ferrous chloride to form ferric ion the ferric ion is then combined with ammonium thiocynate and produce ferric thiocyanate which is red in color. Production of peroxide at the beginning of reaction is inversely proportional to the antioxidative activity of sample. Aged garlic extract was recorded to have highest activity (70%) at 1 mg/ml concentration while the highest activity of raw garlic was found to be 53% at same concentration (Figure 6). Subsequently at the later stage of lipid oxidation, peroxide decomposes to malondialdehyde (MDA) which is an end product of lipid Peroxidation measured by thiobarbituric acid or TBA method, a highly sensitive method based on reactivity of free MDA with thiobarbituric acid. Which was found to be relatively higher in aged garlic extract compared to raw garlic extract (Figure 7). The lipid Peroxidation activity of raw garlic was decreased at the second stage unexpectedly which represent that aged garlic extract is more stables and exhibits high antioxidative property on both the stages of lipid Peroxidation.

Furthermore, cell cytotoxic dose of both the extract was determined by MTT assay and the dose optimized for raw garlic was found to be 25 mM while for aged garlic it was 35mM. Also after 25mM to 50mM concentration percent viability was reduced by 20% with raw garlic and by 5% with aged garlic extract (Figure 8). The results were further validated by analyzing the cells morphologically (Figure 9) and trypan blue assay also showed the similar result (Figure 10). With these results we can conclude that aged garlic extract is a safer derivative of raw garlic because of the stability of compounds present in it.

Conclusion

The aging of garlic converts reactive organosulfur compounds into stable water soluble compounds like SAC, SAMC. Aging of garlic may modify its antioxidative activity. The high antioxidative activity of aged garlic extract might be due to the antioxidant compound other than phenolic compound. The study demonstrates that both protect against oxidative stress, but the scavenging activity of aged garlic extract is significantly higher than the raw garlic, Also aged garlic extract has found to be a safer derivative for cardiovascular disease. Our findings together with previous data suggest that consuming aged garlic as a dietary supplement might be beneficial for preventing cardiovascular disease. It is important to identify all the organosulfur compound present in aged garlic and their beneficial role. Further research is needed to elucidate whether aged garlic extract attenuate the oxidative stress *in vivo*.

Acknowledgement

We acknowledge Jaypee Institute of Information and Technology, Noida, India for providing the infrastructure and literature support.

References

1. Bashan N, Kovsan J, Kachko I, Ovadia H, Rudich A. Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. Physiol. Rev. 2009;89:27-71.
2. Madamanchi N, Vendrov R, Runge AMS. Oxidative stress and vascular disease. Arterioscler., Thromb. Vasc. Biol 2005;25:29-38.
3. Park JGOh, GT. The role of peroxidases in the pathogenesis of atherosclerosis. BMB Rep. 2011;44:497-505.
4. Esterbauer H, Dieber-Rotheneder, Striegl M, Waeg G. Role of vitamin E in preventing the oxidation of low-density lipoprotein. Am. J. Clin. Nutr 1991;53:314s-321s.
5. Zou P. Traditional Chinese Medicine, Food Therapy, and Hypertension Control: A Narrative Review of Chinese Literature. The American Journal of Chinese Medicine. 2016;44(08):1579-1594.
6. Krishnakumar P. Indian garlic shipments zoom thanks to output shrinkage in China 2019.
7. Hall E, Tschulik K, Batchelor-McAuley C, Compton R. Electrochemical bromination of organosulfur containing species for the determination of the strength of garlic (*A. sativum*). Food Chemistry 2016;199:817-821.
8. Blumenthal M *et al*. The Complete German Commission E Monographs-Therapeutic Guide to Herbal Medicines. Boston, MA: Integrative Medicine Communications; Austin, TX: American Botanical Council 2015;14(4):54-59.
9. Lawson L, Wang Z. Pre-Hepatic Fate of the Organosulfur Compounds Derived from Garlic (*Allium sativum*). Planta Medica 1993;59(S 1):A688-A689.
10. Freeman F, Kodera Y. Garlic Chemistry: Stability of S-(2-Propenyl)-2-Propene-1-sulfinothioate (Allicin) in Blood, Solvents, and Simulated Physiological Fluids. Journal of Agricultural and Food Chemistry 1995;43(9):2332-2338.
11. Amagase H, Petesch B, Matsuura H, Kasuga S, Itakura Y. Intake of Garlic and Its Bioactive Components. The Journal of Nutrition 2001;131(3):955S-962S.
12. Kodera Y, Ushijima M, Amano H, Suzuki J, Matsutomo T. Chemical and Biological Properties of S-1-Propenyl-L-Cysteine in Aged Garlic Extract. Molecules 2017;22(4):p.570.
13. Jang H, Lee H, Yoon D, Ji D, Kim J, Lee C. Antioxidant and antimicrobial activities of fresh garlic and aged garlic by-products extracted with different solvents. Food Science and Biotechnology 2017;27(1):219-225.
14. Elosta A, Slevin M, Rahman K, Ahmed N Aged garlic has more potent antiglycation and antioxidant properties compared to fresh garlic extract *in vitro*. Scientific Reports 2017;7(1).

15. Cervantes M, de Oca Balderas P, de Jesús Gutiérrez-Baños, *et al*. Comparison of antioxidant activity of hydroethanolic fresh and aged garlic extracts and their effects on cerebral ischemia. Food Chemistry 2013;140(1, 2):343-352.
16. Munir KM, Chandrasekaran S, Gao F, Quon MJ. Mechanisms for food polyphenols to ameliorate insulin resistance and endothelial dysfunction: therapeutic implications for diabetes and its cardiovascular complication. American Journal of Physiology-Endocrinology and Metabolism, 2013;305:E679-E686.
17. Sun J, Tsuang Y, Chen I, Huang W, Hang Y, Lu F, An ultra-weak chemiluminescence study on oxidative stress in rabbits following acute thermal injury. Burns. 1998;24(3):225-231
18. Brand-Williams W, Cuvelier M, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT-Food Science and Technology 1995;28:25-30.
19. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 1999;26(9, 10):1231-1237.
20. Garratt D, The quantitative analysis of drug, in The Quantitative Analysis of Drugs, ed: Springer 1964, 1-669.
21. Nishikimi M, Rao NA, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen, Biochemical and Biophysical Research Communications 1972;46:849854.
22. Oyaizu M. Studies on products of browning reactions: antioxidant activities of products of browning reaction prepared from glucosamin, Journal of Nutrition 1986;44:307-315.
23. Kikuzaki H, Usuguchi J, Nakatani N. Constituents of Zingiberaceae I. Diarylheptanoid from the rhizomes of ginger (Zingiber officinale Roscoe, Chem Pharm Bulletin 1991;39:120-122.
24. Plumb JA, Milroy R, Kaye SB. Effects of the pH dependence of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromideformazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. Cancer Res 1989;49(16):4435-4440.
25. Barcia JJ. The giemsa stain: its history and applications, Int. J. Surg. Pathol 2007;15:292-306.
26. Cummings B, Wills L, Schnellmann R. Measurement of Cell Death in Mammalian Cells. Current Protocols in Pharmacology 2012;56(1):12.8.1-12.8.24.
27. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha M *et al*. Heart disease and stroke statistics—2014 update: A report from the american heart association. Circulation 2014;129:e28-e292.
28. Sies H. Oxidative stress: A concept in redox biology and medicine. Redox Biol 2015;4:180-183.
29. Vinson JA, Su X, Zubik L, Bose P. phenol antioxidant quantity and quality in food: Fruits. J. Agric Food Chem 2001;49:5315-21.
30. Ide N, Lau BHS, Ryu K, Matsuura H, Itakura Y. Antioxidant effects of fructosyl arginine, a Maillard reaction product in aged garlic extract. J. Nutr. Biochem 1999;10(6):372-376.
31. Stone JR, Yang S. Hydrogen Peroxide. A signaling messenger. Antioxid Redox Signal 2006;8:243-270.
32. Bienert GP, Schjioerring JK, Jahn TP. Membrane transport of hydrogen peroxide. Biochim Biophy Acta. In press
33. Prior RL, Standardized method for the determination of antioxidant capacity and phenolics in food and dietary supplements. J. Agric Food Chem 2005.
34. Barry Halliwell, Lipid Peroxidation, antioxidants and cardiovascular disease: how should we move forward. Cardiovascular Research. 2000;47(3):410-418.