In vitro Time-Kill and Antiradical Assays on Green Onion and Garlic Against Specific Diarrheagenic Pathogens

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Abstract

Enteric infection is one of the major public health problems in developing countries. The increasing prevalence of drug resistant bacteria has paved the way for novel drugs from natural sources. Even though researches have been conducted in various allium species to exploit their therapeutic applications, there still exist lacunae in the exploration of the medicinal properties of *Allium cepa* cultivars such as green onion varieties that could be a rich source of treasured phytocompounds. The present study is a comparative time-kill study of extract treated and non-treated bacterial cultures and antiradical activity of green onion with garlic. The time-kill assay was performed against specific diarrheagenic pathogens such as *Escherichia coli*, *Vibrio parahaemolyticus*, *Salmonella typhi* and *Enterobacter aerogenes*. The Time Kill curve showed that the maximum kill has occurred for methanolic garlic, onion extracts (100%, 75%) & aqueous garlic, onion extracts (100%, 75%). The maximum free radical scavenging took place for the maximal dose of extract (1000μg/ml) showing 89.9% scavenging activity for methanolic garlic & 77.1% scavenging activity for aqueous green onion extracts. The observations made in this study support the use of green onion as a natural remedy and as a low cost intervention in the enhanced therapy against gastroenteric related infections. It can be concluded that this study would definitely lead to the establishment of new and more potent drugs from cheaper native plants from natural origin.

Key words: Green onion, Garlic, Time-Kill assay, Hydrogen Peroxide Free Radical Scavenging assay, Diarrheagenic pathogens

List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. cepa cv.</td>
<td><em>Allium cepa</em> cultivar.</td>
</tr>
<tr>
<td>A. sativum</td>
<td><em>Allium sativum</em></td>
</tr>
<tr>
<td>H. pylori</td>
<td><em>Helicobacter pylori</em></td>
</tr>
<tr>
<td>E. aerogenes</td>
<td><em>Enterobacter aerogenes</em></td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>S. typhi</td>
<td><em>Salmonella typhi</em></td>
</tr>
<tr>
<td>V. parahaemolyticus</td>
<td><em>Vibrio parahaemolyticus</em></td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>Fig</td>
<td>Figure</td>
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<tr>
<td>%</td>
<td>Percentage</td>
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<td>°</td>
<td>Degree</td>
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<tr>
<td>μl</td>
<td>micro liter</td>
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<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>LIO</td>
<td>Locally Isolated Organisms</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MHA</td>
<td>Muller-Hinton Agar</td>
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<tr>
<td>MHB</td>
<td>Muller-Hinton Broth</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>ml</td>
<td>milli liter</td>
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<td>mM</td>
<td>milli molar</td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient Agar</td>
</tr>
<tr>
<td>NB</td>
<td>Nutrient Broth</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OSC</td>
<td>Organosulphur Compounds</td>
</tr>
<tr>
<td>pH</td>
<td>Hydrogen ion concentration</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations Per Minute</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible</td>
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</table>

Introduction

Diarrhea is characterized by increased frequency of bowel movement, wet stool and abdominal pain (Ezekwesili et al., 2004). According to the World Health Organization (WHO) and UNICEF, about 2 billion cases of diarrheal disease were reported every year worldwide, and in most of the developing countries, 1.9 million children younger than 5 years of age demise from diarrhea each year (Farthing et al., 2012). Infectious diarrhea is an episode of diarrhea that is caused by an infectious agent (Allen et al., 2010). The important bacterial pathogens causing foodborne bacterial diarrhea are are diarrheagenic *Escherichia coli*, *Salmonella*, *Campylobacter*, *Vibrio species*, *Enterobacter species*, *Shigella* and *Yersinia*. Infectious diarrhoea occurs mostly in developing countries than industrialized countries (Guerrant, 1990). They are the most common causes of morbidity and mortality in developing countries (Armstrong and Cohen, 1999). There has been an increasing interest in plants as a source of antimicrobial agents for the treatment of dysentery due to the emergence of multiple drug resistance strains of...
diarrheagenic pathogens (Munshi et al., 1987; Monroe and Polk, 2000). The use of Allium species for medicinal purposes was dated back at least 3500 years ago in the ancient Egyptian papyrus which documents their medicinal uses (Rivlin, 2001). Garlic and Onions displayed a broad antibiotic activity against both Gram positive and Gram negative bacteria (Whitemore and Naidu, 2000; Sridhar et al., 2011). The existence of high levels of organosulphur compounds (OSC) such as allicin and flavonoids are responsible for their therapeutic effects (Corzo-Martinez et al., 2007).

Oxidative stress caused due to cell damage by the action of free radicals, leads to the development of chronic diseases (Pham-Huy et al., 2008). Antioxidants are compounds that protects against oxidative stress (Diplock, 1994; Poppel, 1997; Papas, 1999; Tamimi, 2002; Willcox, 2004). Gastric cancer is the second leading cause of cancer death and the fourth most common cancer in the world (Ferlay, 2004). More than 80% of the gastric cancer is associated to Helicobacter pylori (H.pylori) infection, although the etiology of gastric cancer is multifactorial (Nagini, 2012). Generation of oxidative stress was identified as a virulence factor in H.pylori-infected hosts (Suzuki et al., 2012). A study across 10 European countries found a positive correlation between high intake of dietary antioxidants and reduced risk of gastric cancer (Seraphin et al., 2012). The epidemiological and experimental study results indicate a major influence of antioxidants in the prevention of gastric carcinoma (Gonzalez and Riboli, 2010). The tumor inhibitory properties exhibited by allium vegetables in laboratory studies are chiefly due to the richness in flavonoids and OSC (Fleischauer et al., 2000; Milner, 2001; Fenwick et al., 1985; Fukushima et al., 1997; Welch et al., 1992; Ali et al., 2000). A study carried out in China revealed a significant reduction in gastric cancer risk with increasing consumption of allium vegetables in an area where gastric cancer rates were high (You et al., 1989).

Onions can be largely classified into spring onion and storage onions. Green onions or spring onions are grown in warm weather climates and have characteristics mild taste (Yang et al., 2004). Numerous cultivars have been developed economically for various parameters such as size, form, color, storability, and climatic adaptations. Cultivars are divided into the Common Onion Group (A. cepa var. cepa), which contains most of the economically important varieties (that includes the cultivars grown for green or salad onions) and the Aggregatum Group, which includes shallots and potato onions (Brewster et al., 1994).

The present study aimed to evaluate the comparative bactericidal and antitumor properties of Allium sativum (Garlic) and Allium cepa (Green Onion) against Enterobacter aerogenes, Escherichia coli, Salmonella typhi and Vibrio parahaemolyticus. The comparative study was carried out to understand the inhibitory properties exhibited by allium vegetables in laboratory studies. A study across 10 European countries found a positive correlation between high intake of dietary antioxidants and reduced risk of gastric cancer (Seraphin et al., 2012).

Materials and Methods

Procurement of Plant Samples

Garlic (Allium sativum) bulbs were procured from the supermarkets around Chennai while the organically cultivated whole plants of Green Onions (Allium cepa cultivar) were collected from the rural farms in Thiruvallur district, Tamil Nadu. The plant specimens were identified by the senior authority, Centre for Research & PG Studies in Botany, Ayya Nadar Janaki Ammal College, Sivakasi, Tamil Nadu.

Collection of Bacterial Pathogens

Clinical human strains which are gastrointestinal tract pathogens were all locally isolated organisms (LIO) obtained from the stock culture collection of the Department of Microbiology, SK Hospital, Trivandrum, kerala. These include Enterobacter aerogenes, Escherichia coli, Salmonella typhi and Vibrio parahaemolyticus.

Chemicals and Reagents

All the chemicals were purchased from Hi-Media lab. Ltd., Mumbai, India.

Growth and Maintenance of Microorganisms

Pure cultures of E.aerogenes and E.coli was streaked on sterile Nutrient Agar (NA) plates. Pure cultures of S.typhi and V.parahaemolyticus was streaked on Muller-Hinton Agar (MHA) plates. The agar plates were kept in the incubator for 24hrs at 37°C and stored at 4°C.

Preparation of Inoculum

Figure 1. Allium cepa cultivar (Green Onion)

Figure 2. Allium sativum (Garlic) bulbs
The bacterial species of *E. aerogenes* and *E. coli* was cultured in Nutrient Broth (NB) and *S. typhi* and *V. parahaemolyticus* in Muller-Hinton Broth (MHB) prior to the time-kill assays. This was done by inoculating a loopful of the bacterial mother culture into the respective broths. The conical flasks containing the inoculum were kept at 37°C overnight for 24 hours in rotary shaker incubator at 150 rpm.

**Extraction of Plant Samples**

The bulbs of garlic were peeled to remove the skin and the bulbs of green onions were separated from its aerial part. The bulbs were surface sterilized using 75% ethanol and dried. The plant parts were washed thoroughly with distilled water and chopped into small pieces. 10g of each garlic and green onion bulbs were ground separately in sterile mortar and pestle to yield a finely ground paste. The paste of each plant bulbs were mixed with 10ml of distilled water and methanol separately. It was then filtered through Whatmann filter paper No 1. and stored at 4°C until use.

**Preparation of Extract Concentrations**

The aqueous and methanolic crude extracts of garlic and green onions bulbs were considered as 100% concentration. 75% and 50% concentration were made by diluting the concentrated extracts with appropriate volumes of the solvents (Srinivasan et al., 2009, Mukhtar et al., 2012). The three concentrations were prepared prior to the time-kill assay.

Different concentrations (250µg/ml, 500µg/ml, 750µg/ml and 1000µg/ml) of the aqueous and methanolic bulb extracts of the two plants were prepared using the appropriate solvents prior to the antiradical assay.

**In vitro Bacterial Time-Kill Assay**

The turbidity measurement of a cell suspension using instrument such as spectrophotometer estimates the increase in both total cell mass and cell number (Dalgaard and Koutsoumanis, 2001). A bactericidal effect is defined as a 99.9% kill over a specified time (Wolfe et al., 1997). According to National Committee for Clinical Laboratory Standards (NCCLS) 1999, the bactericidal activity can be either concentration-dependent or time-dependent.

A bacterial time-kill assay was performed to monitor the best time for the extracts to kill or reduce the bacterial population and the time-kill curve was plotted. The assay was performed by following the method of Olama et al., 2014 with minor modifications. A 16 hrs culture was harvested by centrifugation. The suspension was adjusted using the McFarland standard to achieve approx. 1.5x10^8 CFU/ml. 1 ml of each extracts (50%, 75% & 100%) was added to aliquots of 2ml of MHB (*S. typhi* & *V. parahaemolyticus*) and 2ml of NB (*E. coli* & *E. aerogenes*).

Then 1ml of the inoculum was added to each test tube containing the extracts and broth. Further the samples were taken from each test tube to monitor bacterial growth by measuring the absorbance at 600nm at time intervals 0, 2, 4, 6, 8 and incubated at 37°C. The culture without the plant extract was taken as negative control and the culture with Gentamycin was treated as positive control. A graph was plotted between Absorbance vs Time (in hours).

**In vitro Antiradical Activity Assay**

The main characteristic of an antioxidant is its ability to trap free radicals. The antiradical activity was performed using Hydrogen Peroxide Free Radical Scavenging Assay. Hydrogen peroxide may enter into the human body through inhalation of vapor or mist and through eye or skin contact. H₂O₂ is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals that can initiate lipid peroxidation and cause DNA damage in the body (Alam et al., 2013).

The ability of the extracts to scavenge hydrogen peroxide was determined by the method of Ruch et al., 1989. A solution of hydrogen peroxide (H₂O₂) (40 mM) was prepared in phosphate buffer (pH 7.4). 4 ml of each extracts (250-1000µg/ml) was added to 0.6ml of H₂O₂ solution. The absorbance of H₂O₂ at 230 nm was determined after 10 min against a blank containing phosphate buffer without H₂O₂ using UV-Vis Spectrophotometer (IR 513D). The control used here was Ascorbic Acid (1mg/ml). The percentage of H₂O₂ scavenging by the extracts was calculated using the formula

\[
\% \text{Scavenged} (\text{H}_2\text{O}_2) = \left(\frac{\text{AC} - \text{AE}}{\text{AC}}\right) \times 100
\]

Where AC was the absorbance of the control and AE was the absorbance of the extract.

**Results**

**In vitro Bacterial Time-Kill Assay**

The bacterial Time kill assay performed against *E. aerogenes* showed that, 100% aqueous extracts of green onion bulbs displayed maximum bacterial kill and its kill rate is slightly higher than the kill rate by positive control, whereas the 100% methanolic extracts of garlic showed a maximum kill rate but less than that of positive control (Fig. 3 & 4). For *E. coli*, the 100% methanolic extracts of both garlic and green onions bulbs showed a significant decrease in graph with consecutive increase in time but slightly less than that of positive control gentamycin. The 100% aqueous bulb extract of green onions did not exhibit an activity similar to that of the methanolic extracts (Fig. 5 & 6). In the time-kill curve for *S. typhi* all the extracts of garlic did not show a significant difference when compared with the positive control and negative control, that is the curves for all the extracts lies in between the two controls, whereas, for green onion bulbs, both aqueous and methanolic extracts at 100% concentration showed a decrease in OD value indicating a decline in the bacterial culture (Fig. 7&8). For *V. parahaemolyticus*, methanolic extracts of garlic at 100% concentration showed a decrease in the bacterial population indicating that the extracts showed a reduction in the OD values than that of gentamycin. The plot displayed by green onion bulbs showed that maximum bacterial kill was by 100% methanolic extracts and its kill rate is slightly higher than the kill rate of positive control (Fig. 9&10).

**In vitro Hydrogen Peroxide Free Radical Scavenging Assay**

The extracts of *Allium sativum* and *Allium cepa* cultivar were capable of scavenging hydrogen peroxide in a concentration-dependent manner. All the extract concentrations of garlic and green onion bulbs exhibited varied levels of free radical scavenging activity against hydrogen peroxide radicals (Fig.11&12). Significantly higher scavenging activity was observed in the methanolic extracts of garlic bulbs with 89.9% activity and 77.1% for the aqueous extracts at 1000µg/ml when compared to the reference compound Ascorbic Acid that showed 70.8% of
Figure 3. Graph showing the Time-Kill plot of Garlic bulb extracts for \textit{E. aerogenes}.

Figure 4. Graph showing the Time-Kill plot of Green Onion bulb extracts for \textit{E. aerogenes}.
**Figure 5.** Graph showing the Time-Kill plot of Garlic bulb extracts for *E. coli*

**Figure 6.** Graph showing the Time-Kill plot of Green Onion bulb extracts for *E. coli*
Figure 7. Graph showing the Time-Kill plot of Garlic bulb extracts for *S.typhi*

Figure 8. Graph showing the Time-Kill plot of Green onion bulb extracts for *S.typhi*
Figure 9. Graph showing the Time-Kill plot of Garlic bulb extracts for *V.parahaemolyticus*

Figure 10. Graph showing the Time-Kill plot of Green onion bulbs for *V.parahaemolyticus*
Research Article

Table 1. \( \text{H}_2\text{O}_2 \) Free Radical Scavenging Activity of aqueous & methanolic extracts of \textit{A. sativum} (Garlic) bulbs

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>%Scavenging (aqueous extract)</th>
<th>%Scavenging (methanolic extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>38.3</td>
<td>66</td>
</tr>
<tr>
<td>500</td>
<td>68.3</td>
<td>79</td>
</tr>
<tr>
<td>750</td>
<td>74.1</td>
<td>83.8</td>
</tr>
<tr>
<td>1000</td>
<td>77.1</td>
<td>89.9</td>
</tr>
</tbody>
</table>

Ascorbic acid standard concentration 1mg/ml, % scavenging activity-70.8%

Table 2. \( \text{H}_2\text{O}_2 \) Free Radical Scavenging Activity of aqueous & methanolic extracts of \textit{A. cepa} cultivar (Green onion) bulbs

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>%Scavenging (aqueous extract)</th>
<th>%Scavenging (methanolic extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>28.5</td>
<td>24.7</td>
</tr>
<tr>
<td>500</td>
<td>36.5</td>
<td>37.1</td>
</tr>
<tr>
<td>750</td>
<td>46.3</td>
<td>51.8</td>
</tr>
<tr>
<td>1000</td>
<td>65.1</td>
<td>62.7</td>
</tr>
</tbody>
</table>

Ascorbic acid standard concentration 1mg/ml, % scavenging activity-70.8%

**Figure 11.** %Scavenging of aqueous garlic & green onion bulb extract with reference to Ascorbic acid scavenging activity (Table 1). When compared with the scavenging activity of garlic and ascorbic acid standard, the extracts of green onion bulbs exhibited lesser % of scavenging. The maximum value was showed by the aqueous extract at 100% concentration with an activity of 65.1% followed by the 100% methanolic extract with 62.7% of scavenging activity (Table 2).

**Discussion**

The bactericidal activity of \textit{Allium sativum} (Garlic) bulb and \textit{Allium cepa} cv. (Green Onion) leaves extracts against the test enteric isolates was determined using a time–kill assay, as this method, allows determination of the speed of bactericidal activity of the extract (Aiyegoro et al., 2009). The extracts exhibited maximum bactericidal effect at 1 mg/ml (100%) concentration against all the test bacteria. A significant decrease in population of test organisms was observed at each interval. In the present investigation, a comparative study was done to evaluate the antibacterial activity of the most promising bioactive extracts (Garlic and Green Onion) against the diarrheagenic pathogens versus gentamycin (Positive control). The growth of bacterial cells without the addition of the extract (Negative control) showed a normal growth curve pattern.

The time-kill assay more closely simulates to determine the interaction between antimicrobial agents and strain of bacteria. This assay correlates with the pharmacodynamics and pharmacokinetic effects of the \textit{in vivo} conditions. The antimicrobial activity of allium
is due to the presence of an array of phytochemicals, but most importantly due to the activity of a flavone named quercetin. The flavone may act directly on microorganisms and result in growth inhibition by disrupting cell membrane synthesis or synthesis of essential enzymes. The antibacterial activity of onion juice can be attributed to the presence of flavonoids and polyphenols which has been reported to have broad spectrum of antibacterial activity. Polyphenols from plants have been reported to have antibacterial activity (Ani et al., 2006). It was reported that the antibacterial activity of flavonoids is related to damage of the bacterial membranes, causing an increase in the permeability of the inner bacterial membrane, and dissipation of the membrane potential (Cushnie and Lamb, 2005). The low catechin susceptibility of Gram-negative bacteria may be partially attributable to the presence of lipopolysaccharide acting as a barrier (Milane, 2006). There are so far no reports of time-kill assays performed on the bulbs of green onions and its comparative activity with garlic against diarrheagenic pathogens. A report is available which investigated the antimicrobial activity of the phenolic compound extracts of common onion including green onion and garlic which showed that the activity of green onion was significant and was concentration-dependent (Benkeblia et al., 2005a).

The present study on Antiradical activity shows that the extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Scavenging activity of the methanol extract of Garlic caused a strong dose-dependent inhibition of hydrogen peroxide, whereas the bulbs of Green Onion showed lesser scavenging activity. The methanol extract showed good scavenging activity compared to the Ascorbic acid and this may be due to the higher amounts of saponins and flavonoids like quercetin and polyphenols which could be extracted only with non polar solvent like methanol. The lesser scavenging activity of Green Onion is because, the immature bulbs exhibits lower levels of flavonoids. (Mccutcheon et al., 1992; Ela et al., 1996).

Onion is one of the richest sources of quercetin (flavonol) in the human diet (Sellappan et al., 2002), with quercetin constituting 45% of the total polyphenol content. Many researches showed that phytochemical constituents such as flavonoids and other phenolic compounds which have been reported to have multiple biological effects such as antioxidant activity, anti-inflammatory actions, inhibition of platelets aggregation and antimicrobial activities (Venkatanarayan et al., 2010). Flavonoids and phenolic compounds have good antioxidant potentials and mechanism of action of flavonoids is through scavenging or chelation (Yildirim et al., 2000), while phenolic compound are important because of their hydroxyl groups which posses scavenging ability (Cook and Samman, 1996). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals. It was reported that the flavonoids present in green onions benefit health by preventing the development of certain cancer. The flavonoid in green onions Quercitin, will slow the formation of cancer cells and precancerous lesions in the colon (Saha, 2013). Only a few literatures have been reported for the antioxidant activity of green onion, one such report is the investigation of various in vitro antioxidant assays against four types of onions. It was showed that the antioxidant properties were correlated to total phenolics content which was high in red, purple onions and garlic and quite less in green onion (Benkeblia et al., 2005b).
Conclusion

It can be concluded from the present study that Garlic and Green Onion can act as a good ant diarrheal agent that helps in hindering the spread of bacterial growth and helps in betterment of bowel movements during and after infection in affected individuals. From the above results and discussion it can be concluded that the methanol extract of Green Onion and Garlic possesses the potent substances which may be responsible for its bactericidal and antiradical activity and as well as justify the basis of using this plant's extract in folkloric remedies.

The preliminary bactericidal activities exhibited by this plant suggest it as a potential candidate in bioprospecting for drugs and the isolation and the identification of the active principles of the plants will be a step forward in drug discovery. Since methanol is highly toxic when ingested by human system, in vivo toxicity studies would provide a good basis or support for the intake of methanolic extracted compounds. However, further isolation of bioactive compounds would assist to ascertain its potency and safety as a lead antioxidant candidate for pharmaceutical uses.

Acknowledgments

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