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Research Article

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Comparative *in vitro* anti H_2O_2 activity and antibacterial efficacy of garlic and immature onion bulbs against selected human gammaproteobacterial pathogens

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ABSTRACT

Gastroenteric infections, an important public health problem and a principal cause of morbidity and mortality in both developing and developed countries as well. Many enteric infection causing organisms have adapted to the drugs designed to kill them and this has paved the way for alternative medicine from natural origin. Allicin, an antibacterial compound in Allium species like Garlic (Allium sativum) has extensive pharmacological effects against many pathogens. This compound is also present in other Allium species such as Onion (Allium cepa L.) which is known for its high flavonoid compound quercetin. This study focuses on anti H_2O_2 activity and antibacterial efficacy of Onion with Garlic. Antibacterial potentials of the extracts was assessed for four bacterial isolates which include Escherichia coli, Vibrio parahaemolyticus, Salmonella typhi, Enterobacter aerogenes associated with gastroenteric infections. All the bacterial strains showed varied sensitivity against the Garlic and Onion extracts. The maximum Zone of Inhibition observed are as follows: Onion extract showed 15mm,17mm,14mm,13mm against Enterobacter aerogenes, Escherichia coli, Salmonella typhi, Vibrio parahaemolyticus whereas the Garlic showed slightly higher Zones of Inhibition as 18mm,24mm,25mm&17mm respectively. The Time Kill curve shows the maximum kill has occurred for Methanolic extracts of garlic and immature onion bulbs when compared to the aqueous extracts. The maximum free radical scavenging took place for Methanolic garlic and onion extract which showed IC_{50} values - 132.12µg/ml and 414.89µg/ml respectively. The findings made in this study support the use of Onion as an alternative natural remedy against gastroenteric associated infections.

Keywords: Garlic, Onion, Gammaproteobacterial pathogens, Antibacterial activity, Anti H_2O_2 activity

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INTRODUCTION

Gammaproteobacteria is a group of bacteria comprised of facultative anaerobic and fermentative bacteria. It includes medically important, gastroenteric infections causing family such as Enterobacteriaceae and Vibrionaceae. Bacterial gastroenteritis is caused when an unhygienic food or undercooked food carrying pathogens are consumed. Enteric infections are normally treated with oral rehydration solution and antimicrobial agents like tetracycline, ampicillin, nalidixic acid, erythromycin and gentamycin. Antimicrobial resistance has developed against these antibiotics [1]. Resistance to more than one antibiotic is now common among the clinical isolates. Apart from the drug resistant enteric strains, research proves that antibiotics not only negatively affect our indigenous microbiota, but they can also essentially uphold the proliferation of enteric pathogens [2]. The onset of Irritable bowel syndrome (IBS) symptoms occurs after an acute gastroenteritis and the qualitative and quantitative changes of bacteria composition that occur in gut flora produce lasting bowel dysfunction [3]. As an alternative remedy for treatment of various infections, the natural products are found to be more successful with least side effects when compared to commercial antibiotics and so the research and practice of complementary and alternative medicine is now encouraged in developing countries [4].

Food-borne pathogens are widely distributed in the environment and may be a significant cause of mortality and morbidity in the population [5]. The mortality rate due to diarrheal infection is a robust indicator of the overall health status of population. According to WHO (2013) globally, there are nearly 1.7 billion cases of diarrheal disease every year. The most common enteric disease causing members of Gammaproteobacterial class belonging to family Enterobacteriaceae are *E.coli, Shigella, Salmonella, Proteus, Klebsiella, citrobacter, Enterobacter* and Vibrionaceae includes *Vibrio cholerae* and *V.parahaemolyticus* [6]. The garlic and onion which belongs to the genus allium has been used in the treatment of various ailments since ancient times. Many members of this genus possess antibacterial, antifungal, antiprotozoal, antioxidant and antihelmintic activities [7]. *Allium sativum* (Garlic) a member of the Liliaceae family has been used throughout history not only as food condiment but traditionally to treat a wide group of diseases namely; respiratory infections, ulcers, diarrhea and skin infections. Garlic has also been used to prevent wound infection and food spoilage [8]. Garlic extracts showed much better results compared to the commercial antibiotics on diarrheagenic pathogens [9]. A research proves that even the bacterial infections such as *Helicobacter* infections are found to be treated using a garlic derivative called Allitridi [10].

Allium cepa L; (Onion) is one of the important members of Allium genus and its bulbs are known for its medicinal assets. *A.cepa* has plentiful flavonoids and organosulfur compounds [11]. Earlier *in vitro* studies have revealed that the crude extract of the mature bulb of onion has many pharmacological properties including antibacterial activity against a variety of disease causing pathogens. Quercetin is an important flavonoid compound responsible for its antimicrobial property. Research shows that crude extracts of onion and garlic bulbs possess antagonistic activity against *E.coli, S.typhi, P.pyocaneus, B.subtilis* and fresh onion extracts showed inhibitory action against food-borne pathogens [12]. It has been found that raw onion extracts can completely sterilize the oral based infectious pathogens. Recent years have witnessed an increase in worldwide interest among biological research institutions and pharmaceutical institutions in the use of medicinal plants part or its bioactive constituent in health products and herbal remedies.

The present study has investigated the comparative anti H_2O_2 activity and antibacterial activity of fresh aqueous, methanolic extracts of Garlic vs. immature onion bulbs against selected Gammaproteobacterial species.

EXPERIMENTAL SECTION

Procurement & Extraction of plant sample

Fresh Garlic bulbs and onion bulbs were procured from local markets of Chennai. The plant species was identified by a faculty of Centre for research in Botany from Ayya Nadar Janaki Ammal College, Sivakasi. The garlic cloves were separated and peeled to obtain the edible portion. Twenty grams of the edible portion of garlic and onion bulbs were washed thoroughly with distilled water and disinfected with 70% ethanol, chopped into pieces, crushed and homogenized with mortar and pestle using sterile distilled water for obtaining crude aqueous extract and methanol was used instead of water for obtaining methanolic extract. The extracts were filtered by passing it through a Whatmann No.1 filter paper to obtain crude extract. The filtrate was centrifuged at 5000 rpm using Centrifuge (REMI) for 10 minutes to obtain a centrifuged extract. The obtained extracts are considered to possess 100% concentration. The crude extracts having varying concentration such as 75%, 50% was prepared by diluting it with their respective solvent of extraction [13].

Procurement & maintenance of bacterial culture

The bacterial cultures were obtained from Marina Labs, Chennai. These include *E.aerogenes, E.coli, S.typhi* and *V.parahaemolyticus* which belongs to the medically important class of gammproteobacteria. The stock culture was sub cultured in Nutrient (*E.aerogenes, E.coli*) and Muller-Hinton (*S.typhi, V.parahaemolyticus*) agar plates using streak plate method and maintained at 4˚C.

Antibacterial Susceptibility test - Agar Disc Diffusion method

The *In vitro* antibacterial activity was assessed by the disc diffusion method with slight modifications [14]. 200 μL of bacterial culture suspended in nutrient and Muller-Hinton broth (HiMedia, Mumbai) adjusted to 0.5 McFarland standard (10^6 Colony Forming Units) was spread on Nutrient &Muller-Hinton agar plates evenly using a glass L-rod and allowed to dry for 15 minutes. The extract having varying concentration such as 100%, 75%, 50% was impregnated onto 6mm sterile discs (Whatmann No.1) with 150μL per disc. The discs were then placed on the surface of inoculated medium of the petriplates. After the incubation at 37˚C for 24 hours, the inhibition zones were examined and recorded. Gentamycin (HiMedia, Mumbai) was used as positive control for all the strains and the solvent used for extraction was used as negative control.

Time Kill Evaluation - Spectrophotometric method

This method is used in establishment of the rate at which a microorganism is killed and how effectively the growth

of a microbe has been affected. The preliminary time kill assay was performed by spectrophotometric method [15]. Initial OD (Optical Density) was measured for 16 hours old culture at 600nm (OD<1.2). To 1ml of bulb extracts, 2ml of respective broth (Muller-Hinton broth for *S.typhi*, *V.parahaemolyticus*; Nutrient broth for *E.coli*, *E.aerogenes*) was added followed by the addition of 1ml bacterial culture. OD was taken at initiation time, 0^{th} hr and every 2 hours for up to 8 hours. The Culture with Gentamycin was used as Positive control and the Culture without any antibiotic/extract was used as negative control.

Anti H_2O_2 activity

The hydrogen peroxide scavenging assay was carried out following the protocol by [16]. 4mM of H_2O_2 was prepared in 10ml of phosphate buffered saline (PBS) (0.045ml H_2O_2 in 10ml of PBS). 4ml of crude extract having varying concentration as 100%, 80%, 60%, 40%, 20% was prepared using distilled water and methanol. 0.6ml of H_2O_2 was added to each 4ml of extract and the readings were taken at 230nm after 10minutes incubation with PBS as blank solution for measuring OD values in UV visible Spectrophotometer (Elico, India). Ascorbic acid (4mg/ml) was used as standard control. The % scavenging activity was calculated using the formula (1) by substituting the observed OD values.

$$\% \text{ Scavenging activity} = [(Ac-As)/Ac] \times 100 \quad (1)$$

where, Ac – Absorbance of Control (Methanol, Water), As – Absorbance of sample(extract).

The Inhibition concentration, IC_{50} is the effective value at which 50% of antioxidant activity takes place. IC_{50} values for the extracts are calculated in Microsoft Excel worksheet using XY scatter plot (%scavenging vs. Concentration).

RESULTS AND DISCUSSION

Agar Disc Diffusion method

All the test gram negative bacteria showed sensitivity for the onion & garlic bulb extracts. The varying susceptibility of the test gammaproteobacteria for varying concentrations (50%, 75%,100%) of the aqueous and methanolic extracts is shown in Figure 1 & 2 respectively. The activity was a linear function of concentration showing the inhibitory action in a concentration dependent manner.

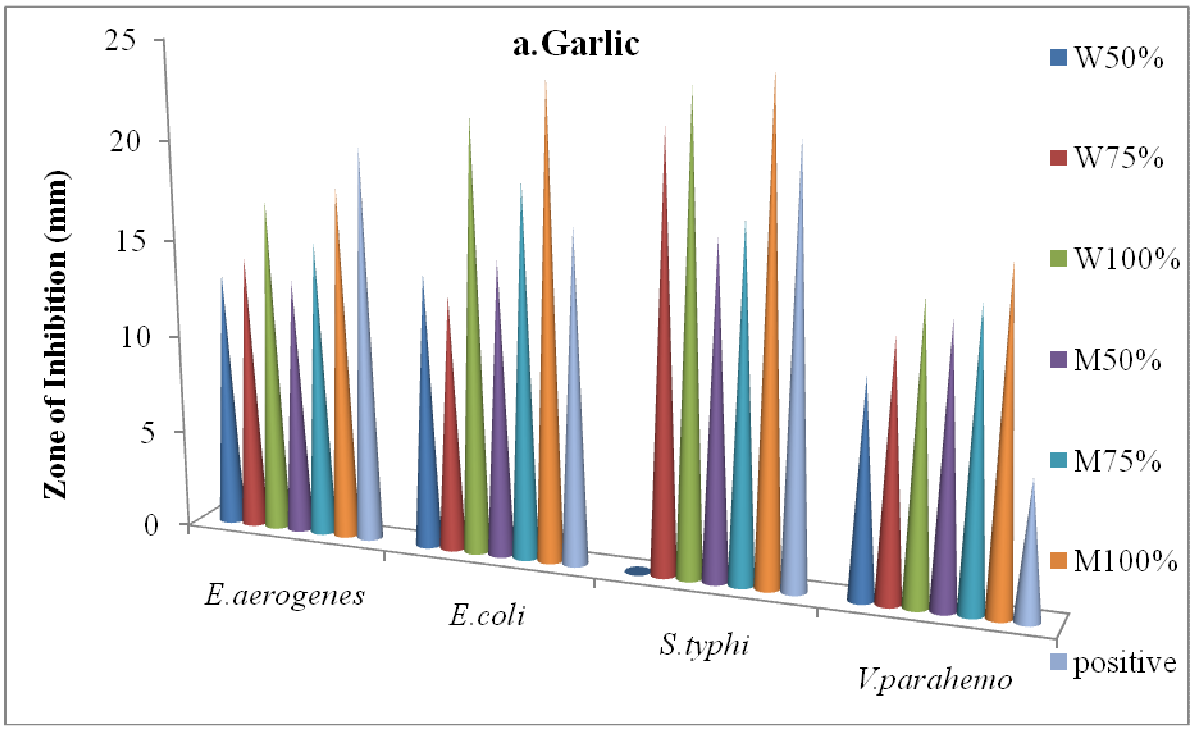

Figure 1: Inhibitory activity of different concentration of Garlic extract against test bacteria. W-Water extracts; M-Methanol extracts

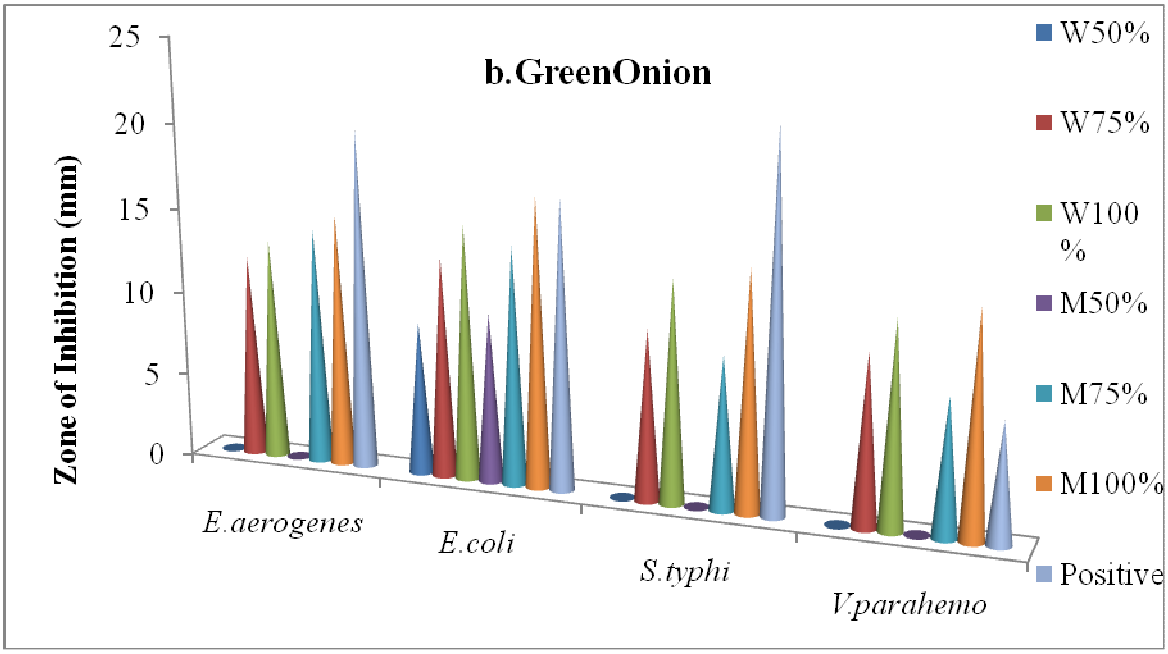

Figure 2: Inhibitory activity of different concentration of immature Onion extract against test bacteria. W-Water extracts; M-Methanol extracts

The Garlic extracts showed excellent antibacterial activity at concentrations 100% & 75%, whereas Onion bulbs showed intermediate to low antibacterial activity for 100% & 75%, and no activity was shown for 50% concentration (Figure 2). The centrifuged extracts showed antagonistic activity against test bacteria because of the removal of plant debris removal and isolation of low molecular weight sulfur containing compounds which are responsible for antibacterial action.

The methanolic extract of *A.sativum* bulbs showed maximum activity for maximal concentration against *S.typhi* (25mm), *E.coli* (24mm), *E.aerogenes* (18mm) & *V.parahaemolyticus* (17mm) The antimicrobial activity of the garlic has been attributed to its phytochemical components, allicin (a thiosulfinate) whose removal completely renders garlic ineffective against microorganisms [17]. The immature *A.cepa* L.cutivar methanolic bulb extracts exhibited higher activity than aqueous extracts against *E.coli* (17mm), *E.aerogenes* (15mm), *S.typhi* (14mm) and *V.parahaemolyticus* (13mm). These results are slightly lesser than the findings by [18]. He reported that methanol extract of mature *Allium cepa* bulbs showed higher activity than aqueous extracts against *E. aerogenes*, *E.coli*, *Salmonella* species [19].

[20] Stated that plant extraction using organic solvents like methanol has much significant antimicrobial property than water extraction. This is due to the higher amounts of saponins and flavonoids like quercetin & polyphenols which could be extracted with polar solvent like methanol. The flavonoids inhibit contractions caused by spasmogens, stimulate normalization of the deranged water transfer across the mucosal cells, and inhibit gastrointestinal release of acetylcholine [21]. The saponins act as a good antimicrobial and anti-diarrheal compound by inhibiting the release of acetylcholine, prostaglandins & histamine release in intestines [22]. Allicin exhibit its antimicrobial activity by partially inhibiting DNA and protein synthesis but main target of inhibition is RNA synthesis The Allium species generally possess low antibacterial activity towards Gram-negative bacteria [23]. The structural differences of the bacterial strains play a role in the bacterial susceptibility to garlic constituents [24]. The varying inhibitory effect of the onion bulb extracts could be accredited to the presence of antibacterial compounds and to their dissolving ratios in the solvents and concentration doses [18]. Garlic had high antagonistic activity due to the presence of high allicin compound which is comparatively low in other allium species [25]. The weak antibacterial effect of Onion is due to high water content of the plant and less concentration of sulfur compounds present in immature bulbs [26].

Time kill evaluation by Spectrophotometric method

Time kill anti-bacterial study has been used to investigate numerous antibacterial agents and they are also often used as the basis for *in vitro* investigations for pharmacodynamic drug interaction [27]. The results from this study shows that extract had both bactericidal and bacteriostatic effects as it shows a concentration dependent killing. At higher concentration and longer duration of time, more bacteria were killed but not completely (Figure3-6).

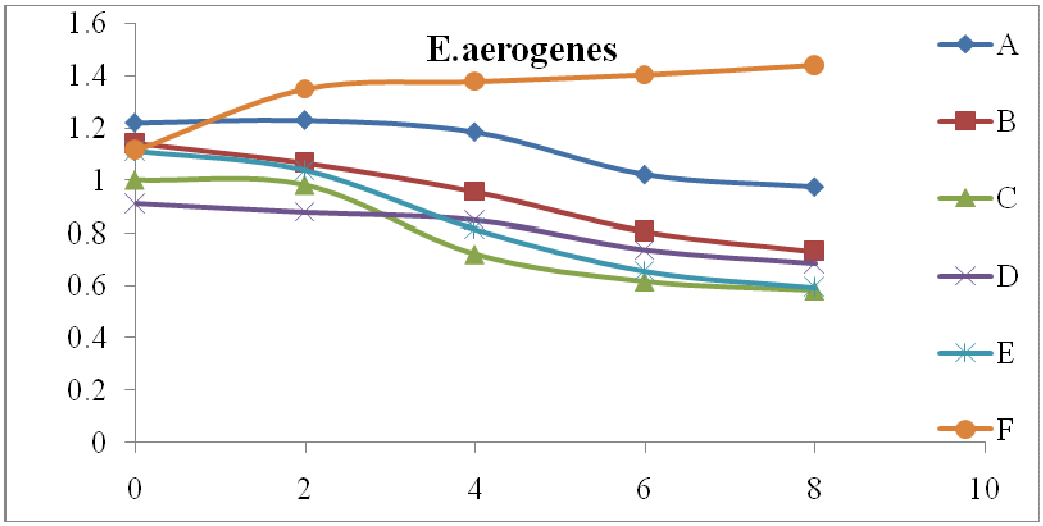

Figure 3: Effect of Garlic & immature Onion bulb extract on the growth curve model of test bacteria. (*E.aerogenes*) A- Aqueous garlic extract, B- Methanolic garlic extract, C- Aqueous Onion extract, D- Methanolic Onion extract, E-+ve control, F:-ve control

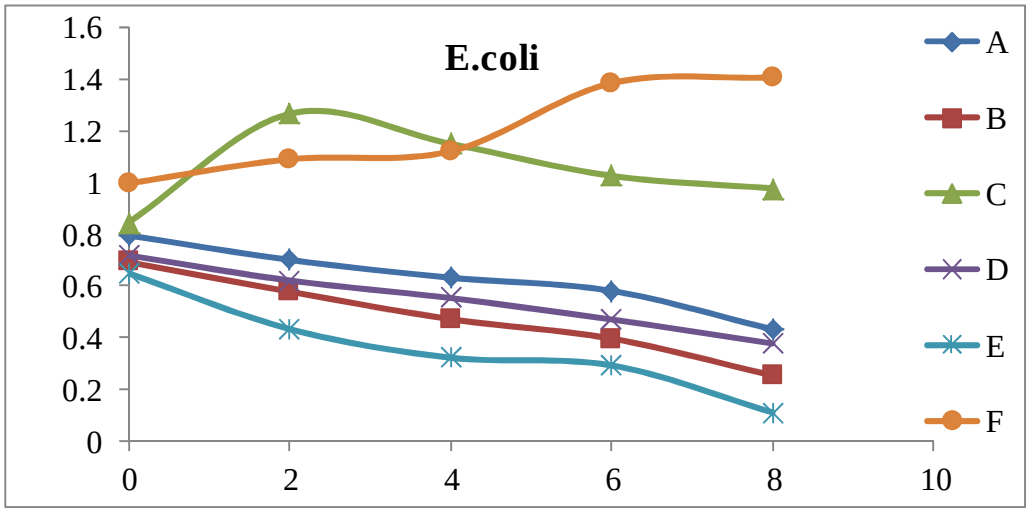

Figure 4: Effect of the Garlic and immature Onion bulb extract on the growth curve pattern of test bacteria (*E.coli*). A- Aqueous garlic extract, B- Methanolic garlic extract, C- Aqueous Onion extract, D- Methanolic Onion extract, E-+ve control, F:-ve control

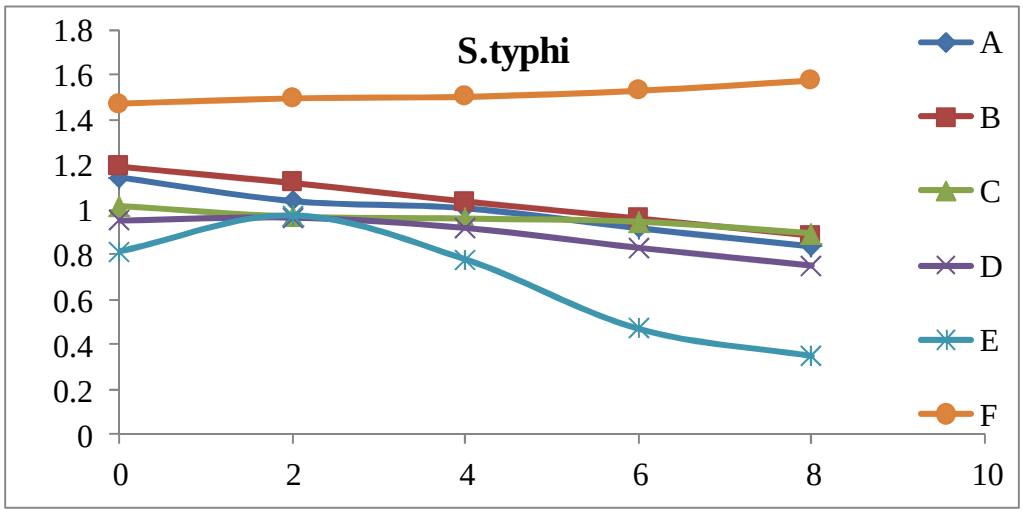

Figure 5: Effect of the Garlic and immature Onion bulb extract on the growth curve pattern of test bacteria (*S.typhi*). A- Aqueous garlic extract, B- Methanolic garlic extract, C- Aqueous Onion extract, D- Methanolic Onion extract, E-+ve control, F:-ve control

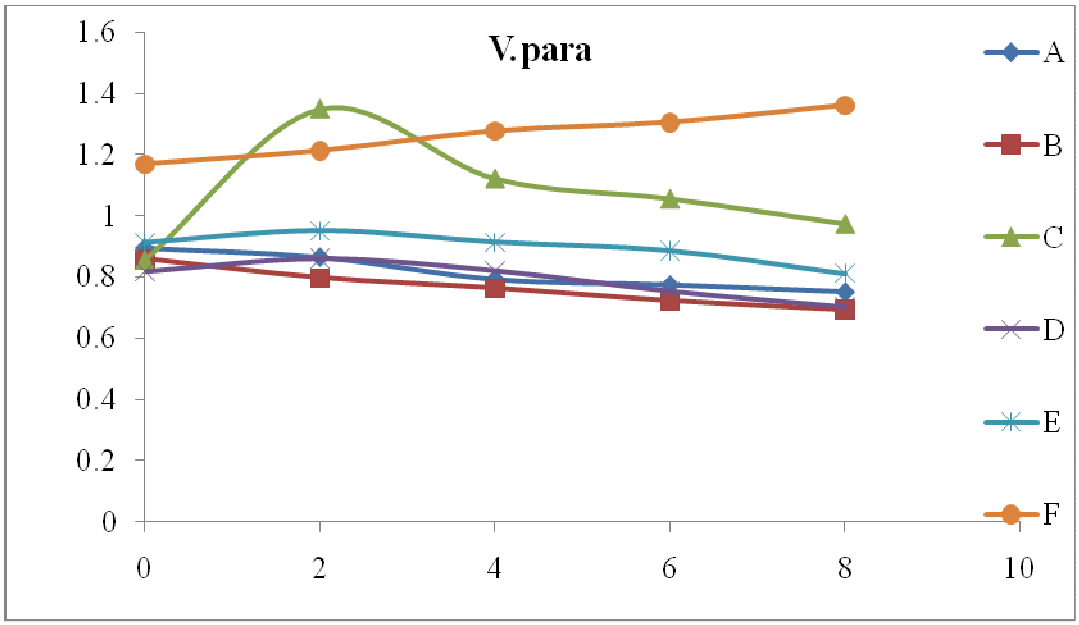

Figure 6: Effect of the Garlic and immature Onion bulb extract on the growth curve pattern of test bacteria (*V.parahemolyticus*). A- Aqueous garlic extract, B- Methanolic garlic extract, C- Aqueous Onion extract, D- Methanolic Onion extract, E-+ve control, F:-ve control

The current study shows that methanolic and aqueous extracts showed maximum kill rate. Of all the bacterial species used, *E.coli* showed maximum susceptibility towards the extracts and maximum kill has occurred in 8 hours (Figure 4). The antimicrobial activity of Allium is due to the presence of phytochemical which is a flavone named quercetin. [28] Reported that the antibacterial activity of flavonoids is associated to damage of the bacterial membranes, causing dissipation of the membrane potential by increasing the permeability of the inner bacterial membrane, and dissipation of the membrane potential. In the case of the flavonols (quercetin, fitesin, rutin and hesperidin) it was shown that they caused an increase of the permeability of the inner bacterial membrane and dissipation of the membrane potential [29].

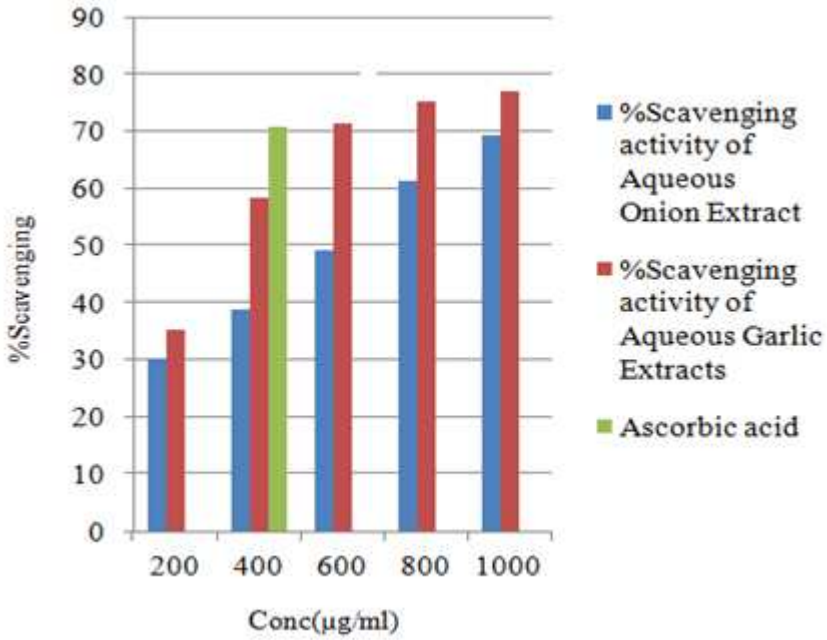

Figure 7: %Scavenging activity of aqueous *A.sativum* & immature *A.cepa* bulb extract with reference to Ascorbic acid

Anti H_2O_2 activity

The phytochemical constituents such as flavonoids and other phenolic compounds have been accounted to have numerous biological effects such as antioxidant activity, anti-inflammatory actions, inhibition of platelets accumulation and antimicrobial activities [30].

The present study shows that the methanolic bulb extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Scavenging activity of the methanolic extract of Garlic presented a strong dose-dependent inhibition of hydrogen peroxide comparable to that of activity shown by ascorbic acid (Figure 7&8), whereas the Onion showed comparatively lesser scavenging activity.

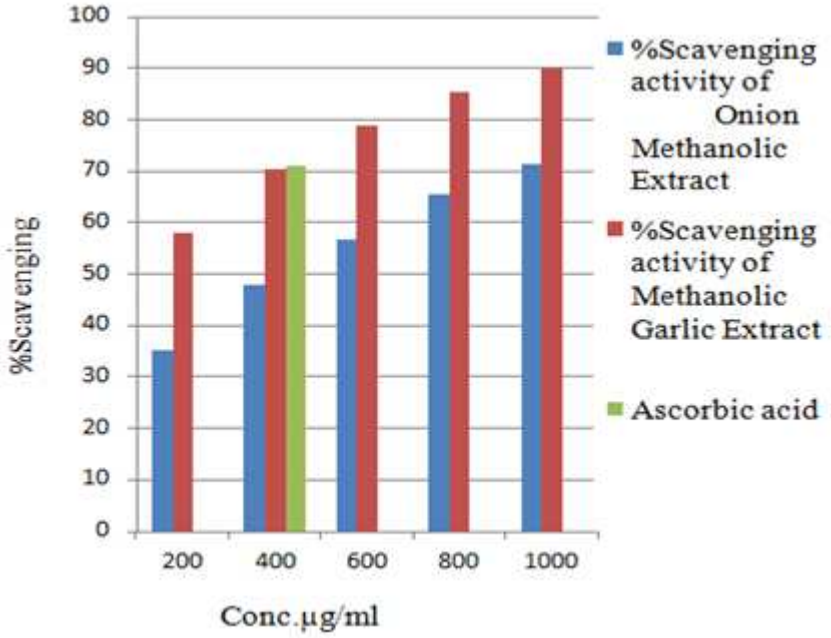

Figure 8: %Scavenging activity of methanolic *A.sativum* & immature *A.cepa* bulb extract with reference to Ascorbic acid

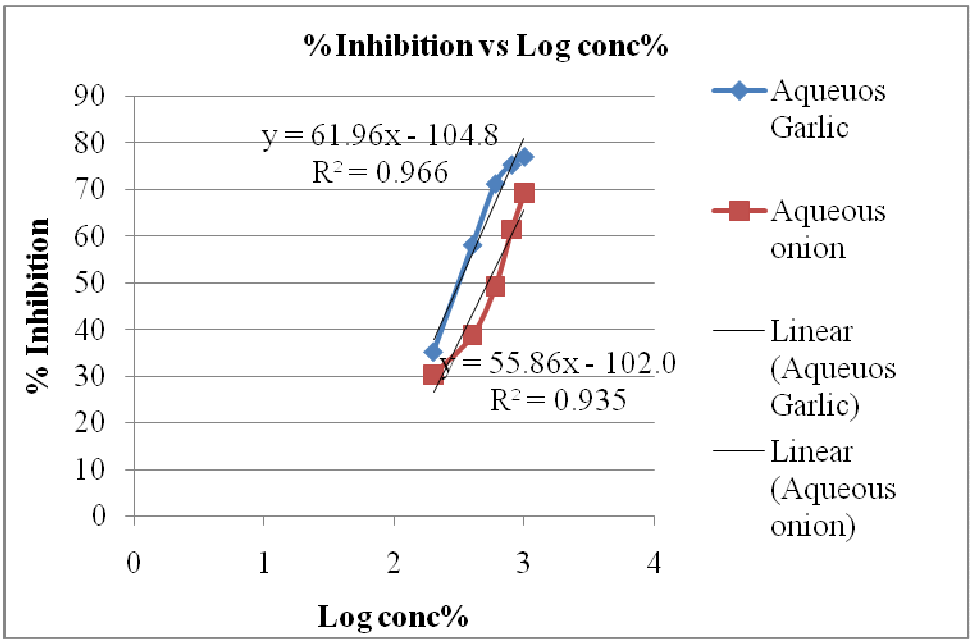

Figure 9: Graph showing Trend line for H_2O_2 inhibition of aqueous garlic & immature onion extracts

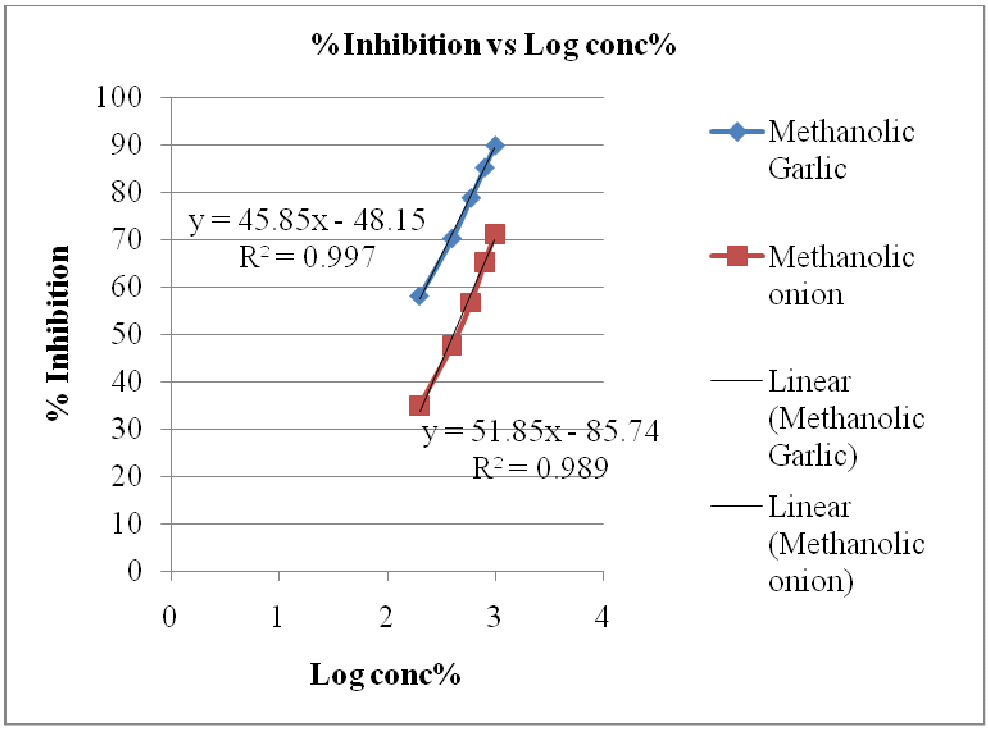

Figure10: Graph showing Trend line for H_2O_2 inhibition of methanolic garlic & immature onion extracts

The IC_{50} values are calculated from the trend line equation from Figure 9&10. For methanolic extracts of garlic and onion the IC_{50} values are 132.12 µg/ml and 414.89 µg/ml respectively. The IC_{50} value for aqueous extracts of garlic is 315.05 µg/ml and for onion is 526.12 µg/ml. The methanolic bulb extracts showed good scavenging activity with respect to aqueous bulb extracts. This is because of the presence of the phenolics present in the extract. This could be attributed to the solvent of extraction i.e. methanol [31] which donate electrons to H_2O_2, thus neutralizing it to water. The lesser scavenging activity of onion bulbs is because of the flavonoid content which is higher only in the mature bulbs [32]. The cell mediated immune functions (activated macrophages and phagocytes) endogenously generates reactive oxygen species (ROS) during microbial infections [33]. The generated ROS directly or indirectly through its reduced hydroxyl product acts as initiator for the synthesis of inflammatory mediators involved in the pathogenesis of Gastrointestinal illness including certain types of Gastrointestinal cancers after the infection [34]. Thus by the consumption of antioxidant rich source like onion, the epithelial mucosa cell integrity and homeostasis are protected from the harmful effects.

CONCLUSION

It is concluded that Garlic and Onion act as an antagonist to the gammaproteobacterial pathogens studied here and if experimented on the synergistic action of both these bulbs active compounds, it might provide good results in future. The methanolic extracts showed better results because polyphenols groups are extracted with polar solvent like methanol and purity of extract is also maintained. Further investigation of individual phenolic compounds and *in vivo* antioxidant mechanisms are required. Garlic intolerant people can consume immature Onion as an alternative healthy food.

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