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SCREENING OF MEDICINAL NATURAL EXTRACTS FOR THEIR ANTIBACTERIAL ACTIVITY AGAINST *SALMONELLA* SPECIES

AROOJ YOUSAF KHAN AND ZAKIA LATIF*

[1]*Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore-54590, Pakistan*
**Corresponding author: zakia.mmg@pu.edu.pk;umna123@yahoo.com*

Abstract

The present study was aimed to screen out natural crude extracts exhibiting antibacterial activity against *Salmonella* causing gastrointestinal problems in humans. Fifteen *Salmonella* species were isolated from uncooked chicken, polluted water, rotten potatoes, beef, rotten eggs etc. Aqueous plant extracts of *Allium sativum* (garlic), *Nigella sativa* (kalvanji), *Azadirachta indica* (neem), *Ficus carica* (anjeer), and *Trigonella foenum-graecum* (methi) were checked against *Salmonella* species by well plate method. In addition to plant extract, Honey was also used for antibacterial activity. Inhibition zones ranging from 2mm to 20mm were obtained with different concentration of plant extracts and honey. The antibacterial sensitivity pattern was in the order of kalvanji > garlic > honey > anjeer > methi > neem. The standard antibiotics such as Ceftriaxone and Ciprofloxacin were also used for comparison with natural extract for antibacterial activity. The extracts of *Allium sativum, Nigella sativa* and *Honey* were found to be more effective against *Salmonella* species for which even Ceftriaxone was found ineffective.

Key words: Antibacterial activity, *Salmonella*, Gastrointestinal, *Allium sativum, Nigella sativa*.

Introduction

Infectious diseases are the second major cause of death worldwide and in economically advanced countries; they are third leading cause of death. Bacterial pathogens are responsible for several serious diseases (Singh & Pandeya, 2011). A number of genera within the family Enterobacteriaceae (Graham *et al*., 2007) are human intestinal pathogens. These include *Shigella, Yersinia* and *Salmonella. Salmonella* are a group of motile rod-shaped bacteria living in the intestinal tracts of host and cause Salmonellosis in humans and are usually acquired through ingestion of contaminated foods or drinks (Omololu *et al*., 2009). They are potential enteric pathogens and transmission of *Salmonella* to a susceptible host usually occurs through consumption of contaminated foods. The most common sources of *Salmonella* include beef, poultry and eggs. Dairy products, vegetables, fruits and shellfish have also been implicated as sources of *Salmonella* (Alena & Mark, 2009). The genus *Salmonella* consist of over 2668 different serotypes and cause disease in both humans and animals.

According to the World Health Organization, approximately 10 million children die from diarrheal diseases each year before their fifth birthday (Clarke *et al*., 2002). Strains are getting resistant to antibiotics in clinical use (Anon., 2000; Chauhan *et al.,* 2010). Antibiotics resistance to antimicrobial agent has already become a serious issue in many areas of the world especially in developing countries (Shears, 2000). The ability of bacteria to deceive any kind of conventional therapy has become apparent and pathogens resistant to one or more antibiotics are emerging and spreading worldwide. Additionally unnecessary use of antibiotics has further stimulated this problem (Omololu *et al*., 2009). Bacterial resistance toward antibiotics cause significant increased in the occurrence of infectious diseases. In addition other adverse effects associated with the use of antibiotics on the host includes are hypersensivity, immuno-suppression and allergic reactions. Therefore, it is a need to develop alternative antibacterial drugs from medicinal plants and other natural extracts for the treatment of infectious diseases.

Natural products and natural extracts have been used as a major source for the development of new drugs (Dash *et al*., 2011). A wide range of medicinal natural extracts are used as raw drugs possess varied medicinal properties. Many other raw drugs are collected in larger quantities and traded in the market as the raw materials for herbal industries. While some of these raw drugs are collected in smaller quantities by the local communities and folk healers for local used (Uniyal *et al*., 2006). Plants used for traditional medicine contain a wide range of substances that can be used to treat infectious diseases caused by various pathogenic bacteria. Clinical microbiologists have great interest in screening of various medicinal natural extracts for new therapeutics development (Periyasamy *et al.,* 2010). Keeping in mind the adverse side effects of antibiotics used for the treatment of infectious diseases, *Salmonella* species from diverse sourced were isolated and biochemically characterized. Different natural extracts having strong antibacterial activity were screened against *Salmonella* species by well plate method.

Materials and Methods

Isolation of bacterial strains: The samples from poultry eggs, meat, sewerage mud, sewerage soil and from other environmental sources were collected in sterile specimen bottles and eppendorfs and stored in the refrigerator for further use. One gram of each sample was emulsified into 10 ml selenite broth and incubated at 37ºC on continuous shaking at 150 rpm. After incubation for 24 hours, test tubes were examined for growth of bacteria. The overnight cultures were streaked on Petri plates containing MacConkey's agar (Cheesbrough, 2002) as selective and differential medium. Sub culturing on Xylose Lysine Deoxycholate Agar (XLD) was performed to obtain pure bacterial cultures (Cheesbrough, 2002).

Characterization of bacterial strains: Morphological and biochemical characterization of purified bacterial species was done by following Cappuccino & Sherman (2007). The bacterial cultures were preserved as 20% glycerol stock at -20°C.

Collection of samples: Bulb of *Allium sativum* (garlic), seeds of *Nigella sativa* (kalvanji), leaves of *Azadirachta indica* (neem), *Trigonella foenum-graecum* (methi), fruits of *Ficus carica* (anjeer) and honey were purchased from local market.

Antibiotics used: The recommended antibiotics, Ciprofloxacin and Ceftriaxone of Bayer and Roche Company respectively were obtained from local pharmacy store. Both antibiotics were used in different concentration to check the antibacterial activity against *Salmonella* species.

Preparation of crude extracts: About 100 gm of each plant was washed thoroughly and homogenized with autoclaved distilled water (100ml) separately using a mortar and pestle. The homogenate was left overnight to allow the constituents to get dissolve in water and centrifuged at 12,000 rpm for 15 minutes. The supernatant was filtered through sterile filter assembly and preserved aseptically until further use. Honey purchased from the local market was considered as 100 %. The concentrations 25%, 50% and 75% were made by diluting the concentrated extract (100%) with appropriate volume of autoclaved water. (Bandelj *et al*., 2007; Durairaj *et al.,* 2009; Dubey *et al*., 2009).

Antibacterial activity of natural extracts: Antibacterial activity assay of each extracts was determined by zone of inhibition using well plate assay (Durairaj *et al.,* 2009). Strains of *Salmonella* were grown overnight in LB broth and about 100 µl of optical density 0.1 of the test cultures were spreaded separately on LB agar plates. The plates were left for 30 minutes to allow full embedment of the organisms. The plates containing agar medium were bored with the help of a sterile cork borer. Each extract of different concentration (100µl) was pipetted into the wells in assay plates as in Fig. 1. The plates were incubated in a upright position overnight at incubated 37°C. The sensitivity of the test organisms indicated by a halo around the wells and the diameter of the halo were taken as an index of the degree of sensitivity by measuring with a transparent plastic ruler at three different angles.

Antibiotics susceptibility test: Four concentrations (2.5 µg/ml, 5 µg/ml, 7.5 µg/ml and 10 µg/ml) of both antibiotics were prepared in sterile distilled water and 100 µl of each concentration was used for all *Salmonella* species in well plate method as described in antibacterial activity of natural extracts for comparison. Zones of inhibition were measured after overnight incubation of plates at 37°C.

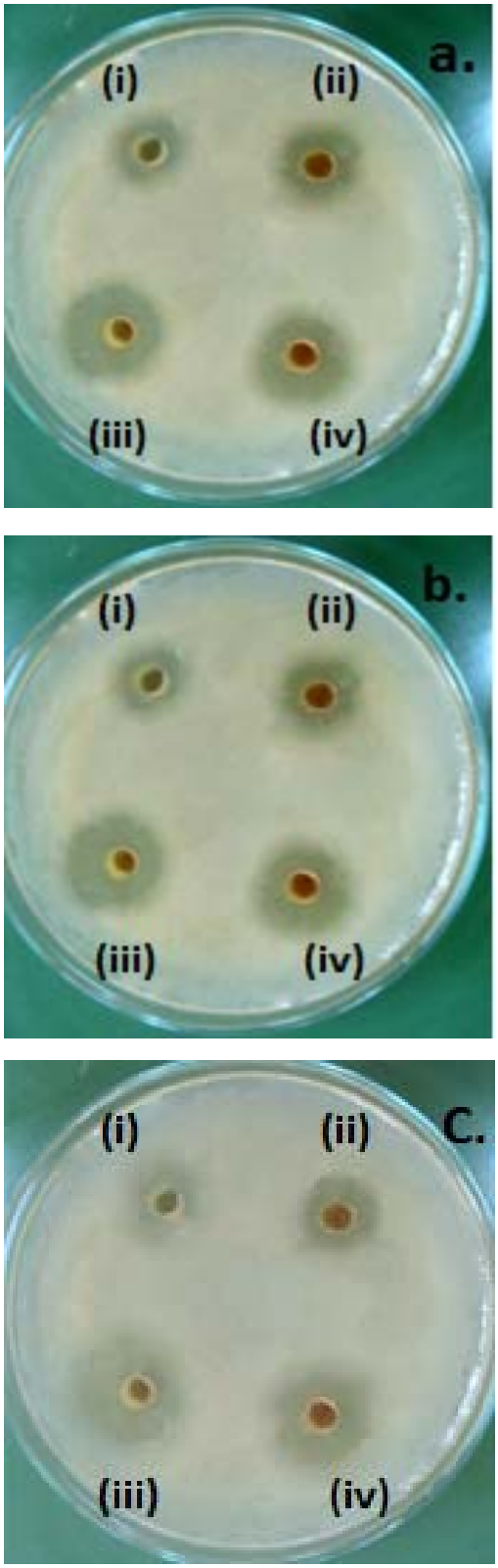

Fig. 1. Zones of inhibition of *Salmonella* speies by (a) *Nigella sativa* (kalvanji), (b) *Allium sativum* (garlic) and (c) Honey. Different concentrations of extracts, 25% (i), 50% (ii), 75% (iii) and 100% (iv), exhibiting different antibacterial activity in well plate assay.

Partial purification of antibacterial activity: Partitioning of antibacterial activity from black seeds (*Nigella sativa*) and garlic bulb (*Allium sativum*) was done by using organic solvents such as ethanol, ethyl acetate, acetone, and methanol (Chauhan *et al*., 2010). Crude extract (5ml) of each as prepared above was taken in separating funnel with double volume of each solvent separately. Mixture was mixed well by vigorous shaking with short intervals for about 30 minutes. Centrifuged the mixture at 10,000 rpm for 20 minutes. The supernatant was collected in a round bottom flask after filtration through whatsman filter paper No.1 and dried down in rotary evaporator (Heidolph Laborta 4000 Efficient). Solvent free content of the flask were resuspended in 5ml of autoclaved distilled water (100% v/v) as the crude extract of specimen and used within 24 hrs. for the evaluation of antibacterial activity by well plate assay as done earlier with crude extracts.

Results and Discussion

Characterization of bacterial strains: Morphological and biochemical characterization of isolated bacterial stains was performed (Table 1) and out of 75 bacterial strains, 15 were characterized as *Salmonella* species by following the method described by Cappuccino & Sherman (2007).

Antibacterial activity of natural crude extracts: The extracts of *Allium sativum* (garlic), *Nigella sativa* (kalvanji) and Honey out of six natural crude extracts were found to be effective against *Salmonella* species isolated from various habitat (Fig. 1). Size of inhibition zone varied according to the concentration of extract. In all cases 25% extract gave minimum zone size ranging from 2mm to 9mm. Maximum 9mm with 25% crude extracts was observed in case of *Allium sativum* and honey in well plate assay. The results clearly demonstrate that 100% crude extracts are more effective against few *Salmonella* species ranging from 8 to 20mm. Other showed complete resistant against 100% crude extract of *Allium sativum, Nigella sativa* seeds and honey. Mortality rate of 100% crude extract of *Allium sativum and Nigella sativa* seeds was approximately 87% where as in case of honey 76% against *Salmonella* species.

*Allium sativum (*garlic) crude extract showed maximum antibacterial activity exhibiting 20mm zone of inhibition in case of *Salmonella* species R2 and A2. Similar results were reported by Durairaj *et al*. (2009) as 36 mm zone of inhibition in case of *Salmonella mgulani* and 38 mm zones of inhibition in case of *Salmonella typhimurium*. Strain E shows 19 mm zone of inhibition against 100% honey (Fig. 2) while Omoya and Akharaiyi (2009) reported 32 mm zone of inhibition for *Salmonella typhimurium* using 7% honey extract. This variation in growth inhibition may be the due to the availability of low quality products.

Antibiotic susceptibility test: Zones of inhibition exhibited by Ciprofloxacin were quite significant (Fig. 3) as compared to zones shown by six crude extracts. Strain S1 showed maximum sensitivity toward Ciprofloxacin, exhibiting 30 mm zone of inhibition while maximum antibacterial activity of garlic and kalvanji crude extract shown by strain A2, exhibiting 20 mm zone of inhibition for both extract. *Salmonella* Strain P1 did not show sensitivity against Ceftriaxone while showed sensitivity against kalvanji, garlic and honey crude extracts. It means crude extracts of kalvanji, garlic and honey are more significant and have more antibacterial potential than Ceftriaxone antibiotic, recommended nowadays by doctors. The results of antibacterial activity of two antibiotics in agar well plate assay showed significantly different response (Fig. 4). Ciprofloxacin and kalvanji antibacterial activity against some *Salmonella* strains was almost similar.

Table 1. Urease (Ure), Indole (Ind), Methyl red (M-r), Citrate utilization (Cit-u) and Voges-proskauer (V-p) tests for the biochemical characterization of *Salmonella* species.

S. No.	Strain name	Source	Ure	Ind	M-r	V-p	Cit-u
1.	C	Uncooked chicken meat	−	−	+	−	+
2.	P1	Polluted water	−	−	+	−	+
3.	P2	Polluted water	−	−	+	−	+
4.	P3	Polluted water	−	−	+	−	+
5.	P4	Polluted water	−	−	+	−	+
6.	S1	Standing rain water	−	−	+	−	+
7.	S2	Standing rain water	−	−	+	−	+
8.	B	Beef sample	−	−	+	−	+
9.	R1	Rotten potato	−	−	+	−	+
10.	R2	Rotten potato	−	−	+	−	+
11.	R3	Rotten potato	−	−	+	−	+
12.	R4	Rotten potato	−	−	+	−	+
13.	E	Rotten egg	−	−	+	−	+
14.	A1	Animal feces sample	−	−	+	−	+
15.	A2	Animal feces sample	−	−	+	−	+

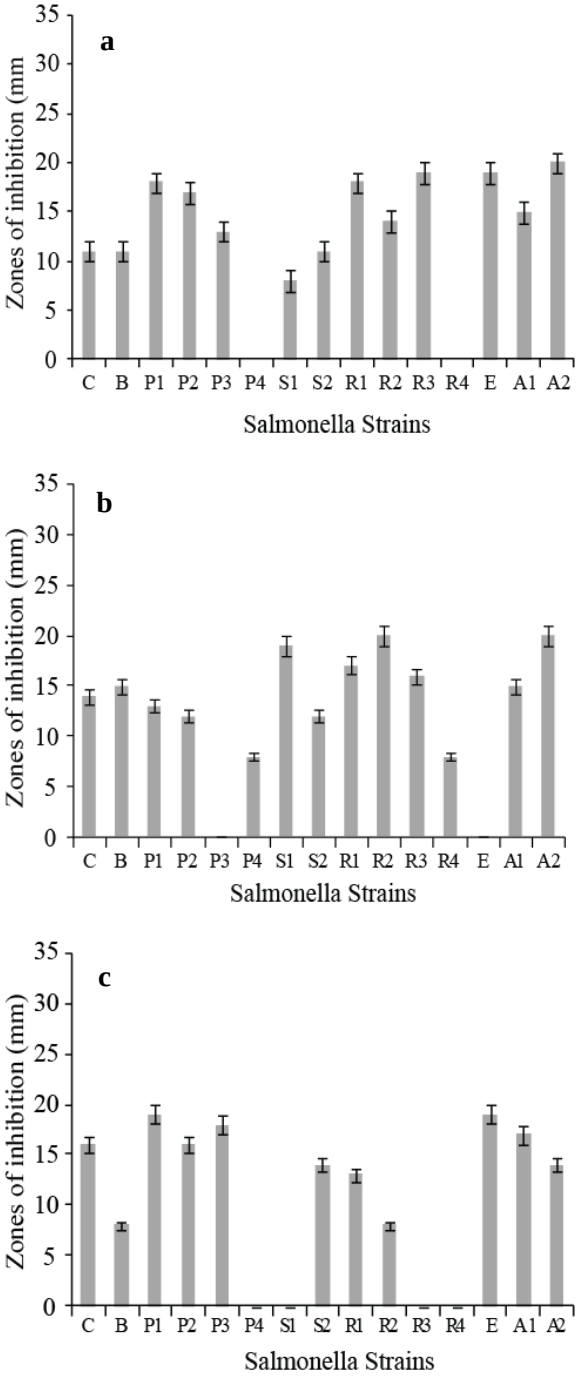

Fig. 2. Antibacterial activity of 100% (w/v) concentration of crude extracts of (a) kalvanji, (b) garlic and (c) pure honey against *Salmonella* species collected from various sources.

Antibacterial assay of partially purified compounds from garlic and black seeds: Different solvent extractions of garlic and black seeds were subjected to antimicrobial assay by well plate assay as done earlier with crude extracts. The inhibitory effect was observed by the zone size on the plates around the well. Solvent extraction of black seeds and garlic in ethyl acetate showed some antibacterial activity but to a much lower extent as compared to crude extract. Strain S1showed 19 mm zones of inhibition when garlic extract in ethyl acetate was used. Strain R1 exhibit 22 mm zone of inhibition by black seeds extract partitioned in ethyl acetate. The inhibitory activity of different solvent extracts of garlic and black seeds is presented in Fig. 5.

The maximum zone of inhibition of kalvanji crude extract is 20 mm for strain A2 (Fig. 1) while maximum zone of inhibition of kalvanji extract in Ethyl acetate is 22mm shown by strain R1 (Fig. 6). The results appear widely conflicting, when reports of the antibacterial activity of different natural extracts used in different studies for evaluation of their antibacterial activities, were compared. It concluded that such discrepancies could perhaps be attributed on occasion to different assays being used (Basile *et al*., 2000). Such inconsistencies may be due to variations within each assay. For example, different groups using the agar dilution technique have used different sizes of bacterial inoculums (Ng *et al*., 1996; Kim *et al.,* 1999). Many different assays are employed in extracts antibacterial activity evaluation research, including Agar dilution technique, Paper disk diffusion assay, Hole-plate diffusion method, Broth macro-dilution or micro-dilution technique and Cylinder diffusion method (Rauha *et al*., 2000). In particular, assays relying on diffusion of test extract may not give a reliable qualitative activity against microbes because a potent antibacterial extract may have a low rate of diffusion (Zheng *et al*., 1996). According to the report by the National Committee for Clinical Laboratory Standards (NCCLS), inoculums size was considered the single most important variable in susceptibility testing (Anon., 2000). However, it will remain necessary to consider carefully additional variables such as the solvent used to dissolve test extract. It has previously been shown that precipitation occurs when selected extracts are dissolved in organic solvents and diluted with neutral polar solutions (Cushnie & Lamb, 2005).

Infections with *Salmonella* species continues to be a public health problem worldwide as it is the most frequently reported pathogen associated with gastroenteritis. Most outbreaks of human Salmonellosis are caused by contaminated food products such as egg and egg products, poultry, other meat products and dairy products. Besides poultry and poultry products, *Salmonella* is also transferred by fresh vegetables, spices and cheese. *Salmonella enteritidis* is known to have a wide range of animal reservoirs, high potential spread and the ability to survive in the environmental water or polluted water (Levinson, 2008). Resistance to antimicrobial agents has become an increasingly important now a days and pressing global problem. Of the 2 million people who acquire bacterial infections in US hospitals each year, 70% of cases involve strains that are found to be resistant to at least one drug (Singh & Pandeya, 2011). As already mentioned finding healing powers in the natural products is an ancient idea. People on all continents have long applied infusions of hundreds, if not thousands, of indigenous plants and natural extracts.

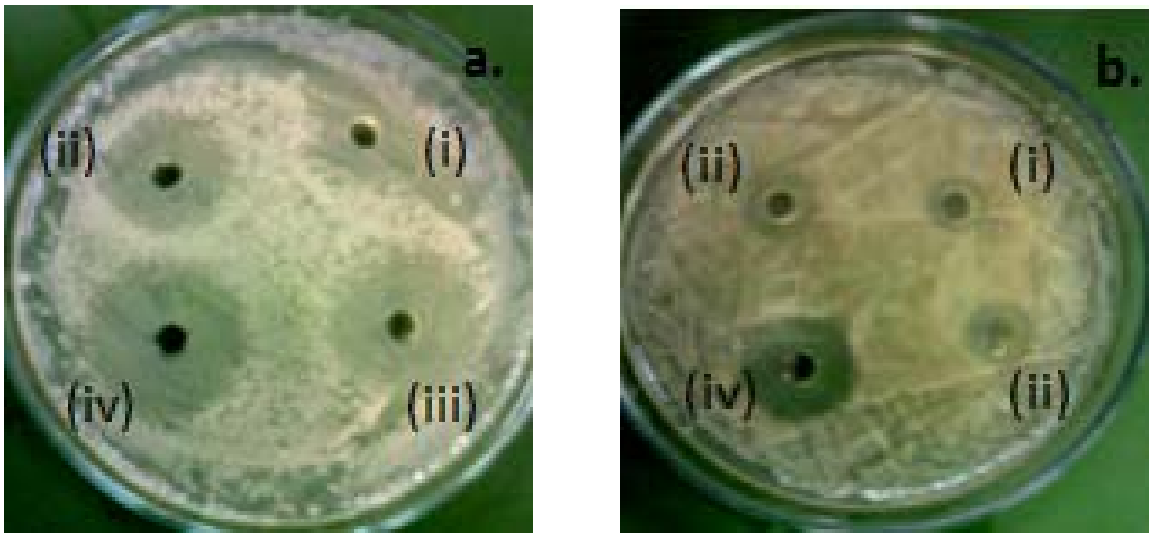

Fig. 3. Zones of inhibition of *Salmonella* species against (a) Ciprofloxacin and (b) Ceftriaxone (2.5 µg/ml (i), 5 µg/ml (ii), 7.5 µg/ml (iii) and 10 µg/ml).

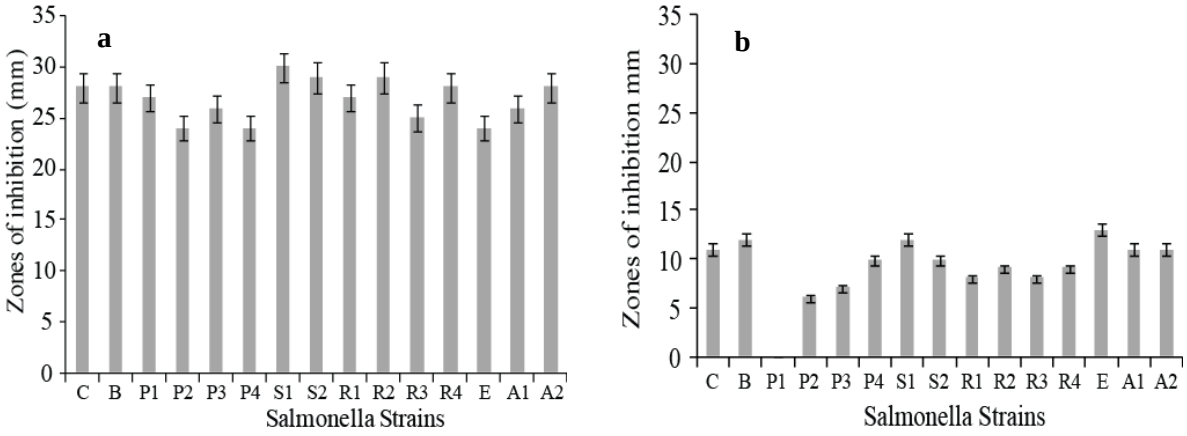

Fig. 4. Zones of inhibition by 10 µg/ml of antibiotics, a) Ciprofloxacin b) Ceftriaxone against different strains of *Salmonella*.

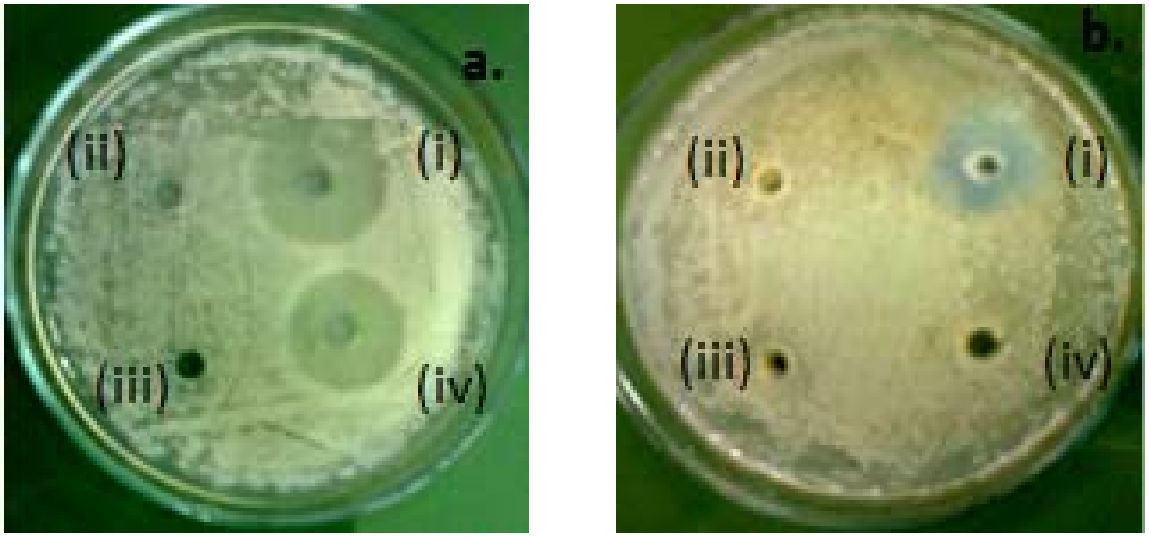

Fig. 5. Zones of inhibition by (a) kalvanji and (b) garlic antibacterial activity against *Salmonella* strains partitioned by organic solvents; Ethyl acetate (i), Acetone (ii), Ethanol (iii) and Methanol (iv).

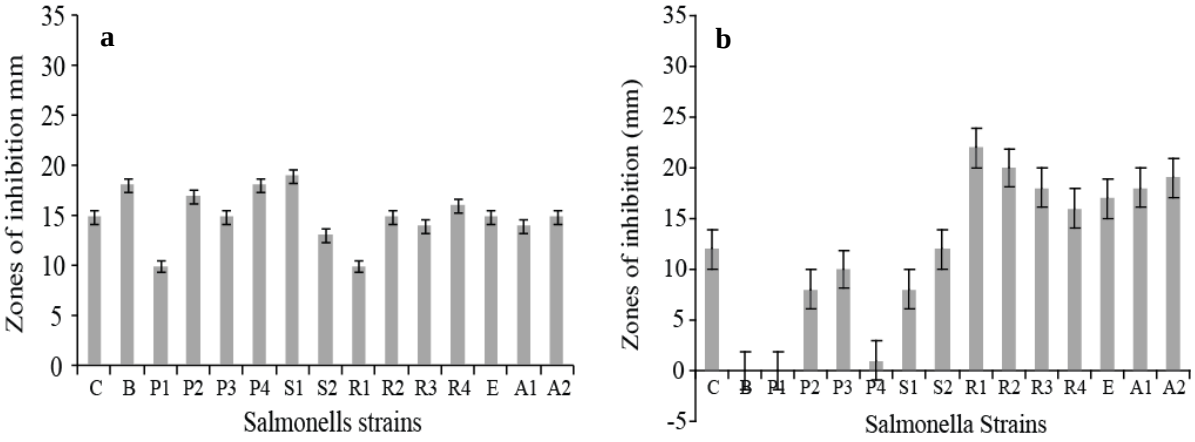

Fig. 6. Antibacterial activity of (a) garlic and (b) kalvanji extracts partitioned in Ethyl acetate against different strains of *Salmonella*.

Bacterial resistance toward antibiotics is a major medical problem nowadays, because it seriously limits the usefulness of many antibiotics (Parekh *et al*., 2005). Natural medicinal Extracts are remarkable source of remedy for various diseases caused by pathogens (Cowan, 1999). An authentic saying of the Prophet Muhammad (Peace be upon him) about black seeds is quoted in Al-Bukhari as: Abu Huraira (Allah be pleased from him) narrated that Allah's Apostle (peace be upon him) said "Use the black seed, which is a healing for all diseases except 'As-Sam" and As-Sam is Death". Kalvanji, garlic and honey extract could be the alternative of antibiotics as no resistance mechanisms against natural medicinal extract has been reported in *Salmonella* species.

Conclusion

Major reason for decline in the popularity of antibiotics is the presence of resistance among various pathogenic bacteria. In addition over prescription and misuse of traditional antibiotics favors the survival rate of pathogen. Many people preferred to have more autonomy over their medical care so there is the need of alternate source of remedy that would allow cheap and wholesome way to cope with infectious diseases. A call has therefore been made for the development of new classes of drug alternate. Researchers are getting close to the end game in terms of parent structure alterations of antibiotics (Singh & Pandeya, 2011).

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