African Journal of Microbiology Research Vol. 6(29), pp. 5847-5851, 2 August, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.144
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Evaluation of antimicrobial activity of extracts of fresh and spoiled *Spinacia oleracea* against some mammalian pathogens

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Accepted 30 March, 2012

Many medicinal herbs have beneficial health effects. These have been used as flavoring agents in food, to prevent food from deterioration and antimicrobials against pathogenic microorganisms. The present study investigated the antimicrobial activity of aqueous, ethanolic, n-hexane and sonicated n-hexane extracts of fresh and spoiled spinach, *Spinacia oleracea* against mammalian pathogens through agar disc diffusion method. The common mammalian bacterial strains assayed for antimicrobial were *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli, Pasteurella multocida*, *Lactobacillus bulgaricus*, *Micrococcus luteus*, *Klebsiella pneumonia*, *Proteous vulgaris*, and *Staphylococcus epidermidis*. The aqueous extracts of both fresh and spoiled spinach inhibited the growth of all tested pathogens except for *S. typhimurium*. Similarly, the aqueous extract of fresh sample also had no effect on the growth of *M. luteus*. The ethanolic extracts significantly inhibited all the bacterial strains tested. It was also observed that the n-hexane extracts of fresh and spoiled vegetable were most effective against *S. typhimurium* and *S. aureus*, whereas the extracts of fresh and spoiled sonicated n-hexane only inhibited the growth of *S. typhimurium* as compared to other pathogens. This study show that spinach could be a potential source of new antimicrobial agents.

Key words: Antibacterial activity, agar disc diffusion method, bacterial pathogens, *Spinacia oleracea*.

INTRODUCTION

A lot of progress has been seen since several decades in the medicinal plant research focusing on isolation of new active principles. The medicinal plants have served as a source of many potent and powerful drugs (Srivastava et al., 1996). These plants are normally used in the form of herbal preparations and about 80% of the world population mainly relies on these traditional medicines for their primary healthcare needs (WHO, 1993). A number of researchers have reported the presence of antibacterial components in various medicinal plants. Due to the presence of wide range of therapeutic constituents, these medicinal herbs can be used to treat chronic as well as infectious diseases (Suffredini et al., 2006; Yasunaka et al., 2005; Gnanamani et al., 2003; Iauk et al., 2003; Palombo and Semple, 2002; Ali et al., 2001; Elegami et al., 2001;Brantner and Grein, 1994; Elegami et al., 2001; Gnanamani et al., 2003; Iauk et al., 2003; Palombo and Semple, 2002; Suffredini et al., 2006; Yasunaka et al., 2005).. The use of medicinal plants as traditional medicines is well known in rural areas of many developing countries like India (Shittu et al., 2007; Sandhu and Heinrich, 2005; Gupta et al., 2005). Clove extracts showed potent antimicrobial activity against *Escherichia coli* (Ayoola et al., 2008).

The question in our minds is whether the spoiled and discarded vegetative samples can provide a medicinally important active principle or not. This investigation is

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based on the hypothesis that the tissues of the spoiled vegetable samples may contain intermediary metabolites which may be harmful against some of the mammalian pathogens. So, the present study was carried out to evaluate the antibacterial activity of *Spinacia oleracea*, used as a model system and the literature has shown that no such work has already been done so, to the best of our knowledge, the work being reported is the first one along these lines.

MATERIALS AND METHODS

Collection of plant material and preparation of extracts

Fresh and spoiled sample of *S. oleracea* were collected from the super market, Islamabad, and studies were conducted in Biochemistry laboratory, Department of Chemistry, Azad Jammu and Kashmir University, Muzaffarabad, Pakistan. The aqueous, ethanolic, n-hexane and sonicated n-hexane extracts were prepared from both fresh and spoiled spinach (Satish et al., 1999; Harborne, 1998). Pieces of vegetable were crushed and soaked in different polar and nonpolar solvents such as ethanol and n-hexane for 24 h and blended in electric blender to form a fine solution. After 24 h, the extract was filtered and centrifuged at 4000 rpm for 30 min at 4°C. The extract was concentrated in evaporator at 40°C and stored at 4°C for further analysis. In case of sonicated n-hexane, the suspension of *S. oleracea* and solvent was sonicated by using 20 KHz ultrasonic generator having 70% amplitude for 5 min. The extracts from the spoiled *S. oleracea* were also prepared using same as the methods as for fresh sample.

Bacteria pathogens

Staphylococcus aureus, *Salmonella typhimurium*, *Escherichia coli, Pasteurella multocida, Lactobacillus bulgaricus, Micrococcus luteus, Klebsiella pneumonia, Proteous vulgaris*, and *Staphylococcus epidermidis* bacterial pathogens were taken from Microbial Biotechnology laboratory, Department of Zoology, Azad Jammu and Kashmir University, Muzaffarabad Pakistan.

Antibacterial activity through agar disc method

The antibacterial activity of different plant species was evaluated by agar disk diffusion method (Cappuccino and Sherman, 1999; Martinez-Vazquez et al., 1999). The microorganism viz., *S. aureus*, *S. typhimurium*, *E. coli, P. multocida, L. bulgaricus, M. luteus, K. pneumonia, P. vulgaris*, and *S. epidermidis* were activated by inoculating a loop full of the strain in the Nutrient broth medium (NBM; 25 ml) and incubated at 37°C on a rotary shaker for overnight. Next day, the old inoculated culture was mixed with Nutrient agar medium (NAM) when the temperature reaches up to 45°C and poured the sterilized plates. All plates were placed at room temperature under laminar flow to solidify. The discs of 5 mm were, soaked with 200 µl of a particular extract and then allowed to dry for the assay. Presoaked discs of sterilized filter paper were placed in the Petri dishes at their labeled position. The plates were incubated for 48 h at 37°C. Antibacterial activity was evaluated by measuring the diameters of zones of growth inhibition after 48h in mm. Chloroamphenicol was used as positive control. Each experiment was conducted thrice, and the mean of three results were taken for both the test and control.

RESULTS AND DISCUSSION

Successful prediction of botanical compounds is largely dependent on the type of solvent used in the extraction procedure. Traditional healers use primarily water as the solvent (Parekh et al., 2005) but in our studies we found that both fresh and spoiled extracts in organic solvent (ethanol) (Ahmed and Aqil, 2007) demonstrated more consistent antimicrobial activity compared to those extracted in water because ethanol is a highly polar solvent as compared to others (Figures 1 and 2). In this study, the antimicrobial activity of *S. oleracea* was calculated against nine bacterial strains, *S. typhimurium, E. coli, P. multocida, M. luteus, L. bulgaricus, S. aureus, K. pneumoniae, P. vulgaris and S. epidermis* through agar disc diffusion method and showed considerable results, although, the inhibitory activity was strain specific. Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, antimolluscal and anti-inflammatory properties of plants (Behera and Misra, 2005; Govindarajan et al., 2006). It was demonstrated that the spinach, traditionally a non-medicinal nutritive vegetable, does contain some active principles that inhibit growth of at least some of the mammalian pathogens. These results appear to be in contradiction with an earlier report that all green vegetables including spinach have no antibacterial activity against *S. epidermidis* and *K. pneumonia* (Lee et al., 2003). Growth of all the nine pathogens were used during the study was inhibited by one or the other extracts of spinach, respectively (Figures 1 and 2, Table 1).

Antimicrobial activity of aqueous extract

The fresh and discarded vegetable samples were used for their antimicrobial activities, both showing different results. The aqueous extract of fresh *S. oleracea* inhibited the growth of *E. coli, P. multocida, M. luteus, L. bulgaricus, S. aureus, K. pneumoniae, P. vulgaris* and *S. epidermis* as shown in the Table 1 and Figure 2. Chloroamphenicol used as a positive control, also inhibited the growth of all these bacteria (Data not shown). On the other hand, the aqueous extract of fresh *S. oleracea* has minimum effect on the *P. vulgaris, M. luteus* and *K. pneumonia*. However, the extract inhibited the activity of the rest of strains. Similarly, the aqueous extract of discarded *S. oleracea* had no affect on the growth of *S*. Typhimurium, *S. aurues* and *M. luteus* while the greater zone of inhibition was shown by *E. coli* as compared to other bacterial strains (Figures 1 and 2, Table 1).

Antimicrobial activity of ethanolic extract

It was observed that almost all the bacterial strains were

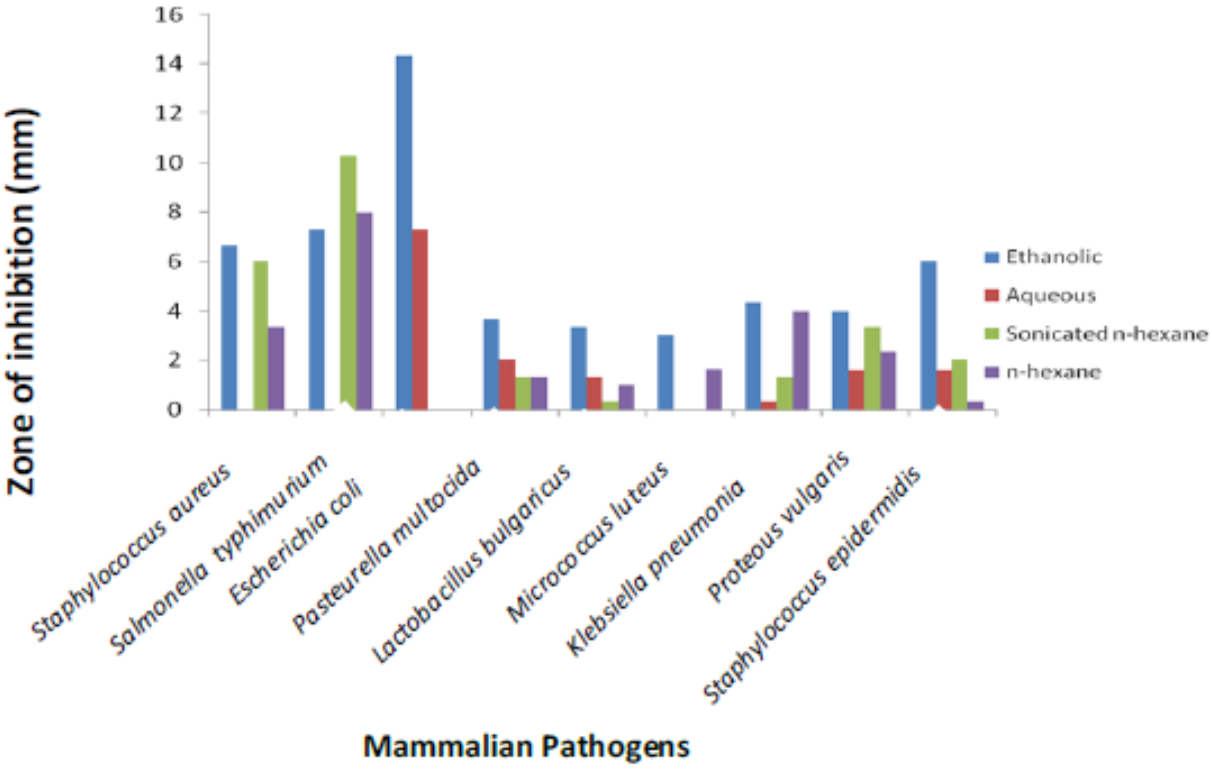

Figure 1. Antimicrobial activity of extracts of spoiled samples of *S. oleracea* against mammalian pathogens.

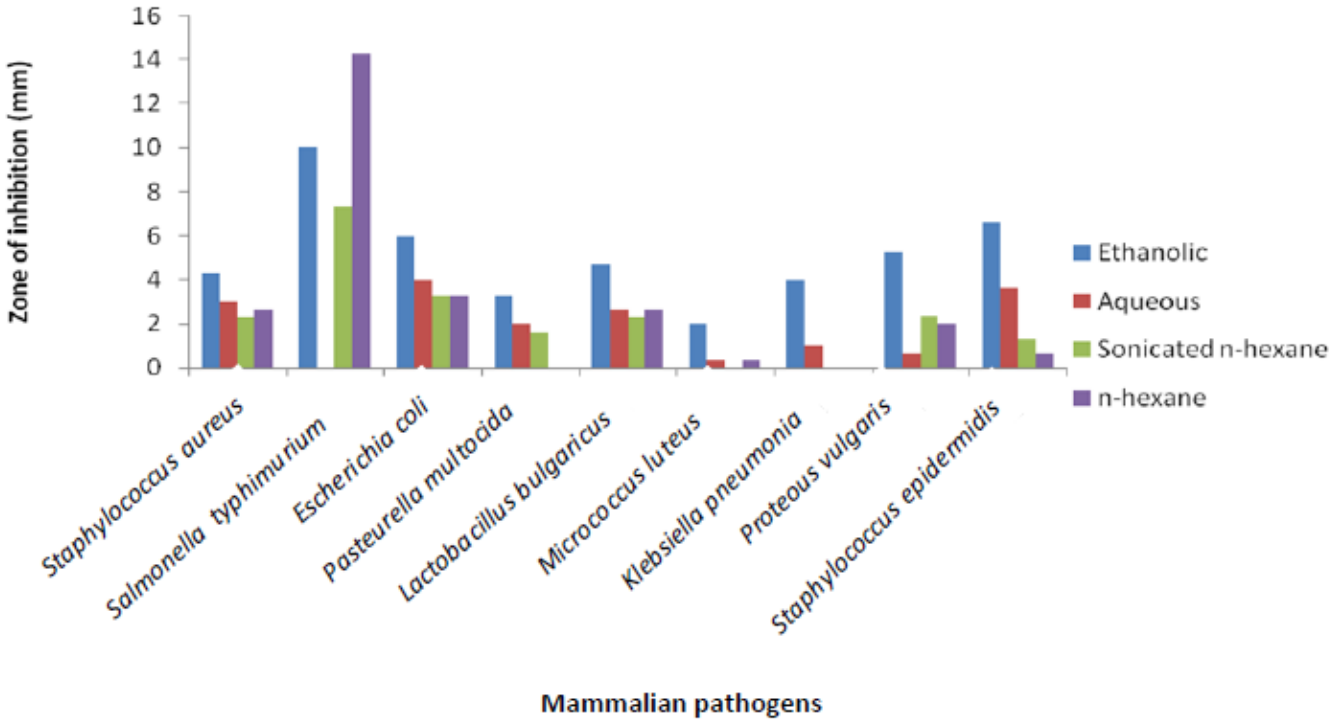

Figure 2. Antimicrobial activity of extracts of fresh samples of *S. oleracea* against mammalian pathogens.

Table 1. Antibacterial activity of extracts of fresh and spoiled *S. oleracea* against mammalian pathogens.

S/No.	Pathogen	Zone of inhibition (mm) of extracts of fresh *S. oleracea*			
		Ethanolic	**Aqueous**	**Sonicated n-hexane**	**n-hexane**
1.	*S. aureus*	4.3**	3.0	2.3	2.66
2	*S. Typhimurium*	10.0****	0.00	7.3***	14.3****
3	*E. coli*	6.0**	4.0	3.3	3.3
4	*P. multocida*	3.3	2.0	1.6	0.0
5	*L. bulgaricus*	4.66**	2.66	2.3	2.66
6	*M. luteus*	2.0	0.33	0.0	0.33
7	*K. pneumoniae*	4.0	1.0	0.0	0.0
8	*P. vulgaris*	5.3**	0.66	2.33	2.0
9	*S. epidermidis*	6.6**	3.66	1.3	0.66
		Zone of inhibition (mm) of extracts of discarded *S. oleracea*			
		Ethanolic	**Aqueous**	**Sonicated n-hexane**	**n-hexane**
1.	*S. aurues*	6.66**	0.00	6.0**	3.33
2	*S. Typhimurium*	7.3***	0.0	10.3****	8.0***
3	*E. coli*	14.3****	7.3***	0.00	0.00
4	*P. multocida*	3.66	2.0	1.3	1.3
5	*L. bulgaricus*	3.33	1.33	0.33	1.0
6	*M. luteus*	3.0	0.00	0.00	1.66
7	*K. pneumoniae*	4.33**	0.33	1.33	4.0
8	*P. vulgaris*	4.0	1.6	3.33	2.33
9	*S. epidermidis*	6.0	1.6	2.0	0.33

****, Highly significant; ***, moderately significant; **, slightly significant. The results are expressed as the mean of the triplicate in mm.

strongly inhibited by the ethanolic extract as compared to the other extracts. *S. typhimurium* was inhibited more significantly by the fresh ethanolic spinach extract. The zone of inhibition was exactly 10 mm. Similarly, the spoiled extract of spinach showed a good zone of inhibition for *E. coli* as it has 14.3 mm as compared to the *S. typhimurium* 7.3 mm. The inhibition activity of fresh ethanolic extracts against other mammalian pathogens was momentous (Table 1, Figures 1 and 2).

Antimicrobial activity of n-hexane and sonicated n-hexane extracts

Similarly, in the current scenario, n-hexane extracts of both fresh and discarded spinach did not show any significant results as compared to the ethanolic and aqueous extracts (Ahmad et al., 1998, Ahmed and Aqil, 2007; Parekh et al., 2005). It was also analyzed that n-hexane extracts of the fresh and spoiled vegetable were most effective only against *S. typhimum* and *S. aureus,* whereas the sonicated n-hexane extracts indicated the significant inhibition of *S. typhimum* as compared to other tested pathogens. The differences observed could be due to the filtration of the extracts, which might have led to removal of the antibacterial components. Hexane is a nonpolar solvent, and has a high preferential interaction with nonpolar compounds while ethanolic and aqueous solvent has a higher affinity for polar compounds as compared to hexane. In turn, a predominantly non-polar solvent will have low solubility in the aqueous phase.

For significant results, the n-hexane extract was sonicated with ultrasonic generator (Misonix S3000) and it was observed that the sonicated n-hexane extract of both fresh and spoiled samples significantly inhibited the growth of *S. typhimurium* 10.3 and 7.3 mm, respectively (Table 1, Figure 1 and 2). It was also shown that sonicated n-hexane extract of both fresh and spoiled samples had no affect on *M. luteus, K. pneumoniae* and *E. coli*, respectively. Other selected strains of the bacteria were less inhibited by this extract as shown in Figures 1 and 2.

Interestingly, it was analyzed that *E. coli, L. bulgaricus, P. vulgaris, S. aureus* and *S. epidermidis* were inhibited by all the four extracts of fresh spinach, whereas *P. multicida, L. bulgaricus, K. pneumonia, P. vulgaris* and *S. epidermidis* were inhibited by all the four extracts of spoiled spinach. The positive result of recent study indicated that the tissues of the fresh and spoiled vegetable may contain intermediary metabolites, and may be harmful against some of the mammalian pathogens as we hypothesized. On the basis of the

present experimental study, it is concluded that there exists a great potential in the search of new and more potent antimicrobial substances from the natural sources and vegetable plants. The results obtained from current studies confirmed the therapeutic potency of spinach used in traditional medicine.

ACKNOWLEDGMENT

Authors are especially thankful to Dr. Hafiz-u-din, (Pathologist) and Mr. Raja Saleem (Lab Assistant) of Microbiology Laboratory of Combined Military Hospital (CMH), Muzaffarabad, Pakistan for the identification of used bacterial pathogens.

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