Food Microbiology, 1997, 14, 603–608

ORIGINAL ARTICLE

Inhibitory effect of kiam (*Cotylelobium lanceotatum* craih). wood extract on gram-positive food-borne pathogens and spoilage micro-organisms

S. Chanthachum[1] and L. R. Beuchat[2*]

*Water extract from kiam (*Cotylelobium lanceotatum *craih.) wood was investigated for inhibitory and lethal activity against pathogenic bacteria and micro-organisms isolated from sugar palm sap. Bacteria representing six genera* Acetobacter, Flavobacterium, Lactobacillus, Leuconostoc, Micrococcus *and* Saccharomyces *isolated from sap were inhibited on agar media to which a 0·2% (wt/v) water extract of kiam wood saw dust was applied. Extract inhibited growth of* Listeria monocytogenes, *toxigenic* Staphloccoccus aureus *and enterotoxigenic* Bacillus cereus *but not enterohemorrhagic* Escherichia coli *0157:H7 or* Salmonella*. The behavior of* L. monocytogenes *inoculated onto shredded raw cabbage which was then treated with 0, 0·5 or 5% solutions of wood kiam extract and subsequently stored at 5°C for up to 5 days was determined. Populations of aerobic mesophiles and psychrotrophic micro-organisms were also monitored. Significantly (*P=0·05) reduced populations of L. monocytogenes *were detected in cabbage treated with 0·5 or 5·0% extract and held for 2 or 5 days. Treatment with kiam extract retarded the rate of growth of aerobic mesophiles and psychrotrophic micro-organisms naturally present on cabbage. Treatment with 5·0% extract had a sustained lethal effect during a 5 day test period. Further research is warranted to characterize the compound(s) in kiam wood extract responsible for inhibitory or lethal activity against spoilage micro-organisms and other pathogenic bacteria that may be present in foods.*

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Received: 18 February 1997

[1]Faculty of Agro-Industry, Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla, Thailand and [2]Center for Food Safety and Quality Enhancement, Department of Food Science and Technology, University of Georgia, 30223-1797 Griffin, Georgia, USA

Introduction

Bacteria capable of causing food-borne illness have been isolated from a wide range of salad vegetables (Beuchat 1996). The presence of *Listeria monocytogenes* on fresh produce has been documented by several researchers (Heisick et al. 1989, Gola et al. 1990, Vahidy 1992, Arumugaswamy et al. 1994). Its presence in pre-packed, ready-to-eat vegetable salads has also been reported (Sizmur and Walker 1988, Harvey and Gilmour 1993) and an outbreak of listeriosis linked to the consumption of shredded cabbage (coleslaw) has been documented (Schlech et al. 1983). *Listeria monocytogenes* can grow on shredded cabbage (Beuchat et al. 1986) and in cabbage juice (Conner et al. 1986) stored at 5°C.

Several antimicrobial compounds occur naturally in plants (Banks et al. 1986, Nychas 1994, Walker 1994) and are known to retard the growth or kill food-borne pathogens (Beuchat and Golden 1989). Essential

*Corresponding author.

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oils (Beuchat 1994) and juices (Beuchat and Doyle 1995) of plants are known to have antilisterial activity. Polyphenolic compounds in herbaceous and woody plants are known to have antimicrobial activity (Scalbert 1991). Pieces of wood from the kiam (*Cotylelobium lancetatum* craih.) tree have been traditionally submerged in sugar palm sap in Thailand to prevent or retard microbial fermentation. The study reported here was undertaken in order to determine if an aqueous extract of sawdust from kiam wood inhibits the growth of micro-organisms naturally found in palm sap, *L. monocytogenes* and four other food-borne pathogens.

Materials and Methods

Preparation of extract

Saw dust (20 mesh) from kiam wood was purchased from a farmer in Songkhla province in southern Thailand. Three procedures were used to prepare wood extract for further testing of antimicrobial activity. The first extraction procedure involved steeping kiam sawdust (200 g) for 16–18 h in 1 liter of distilled water at room temperature (26–28°C), followed by filtering through four layers of cheesecloth; the extract was then dried under vacuum at 60°C. The solid extract material was made into a powder using a mortar and pestle, dissolved in distilled water to give 0·2 and 1·0% (wt/v) solutions and filter (0·2 μ) sterilized. In the second procedure, kiam saw dust (200 g) was steeped in boiling water for 10 min instead of water at room temperature for 16–18 h. The procedure for preparing 0·2 and 1·0% solutions was the same. Extracts prepared by the first two extraction procedures were stored at 4°C and tested for their ability to inhibit growth of micro-organisms isolated from sugar palm sap. In the third procedure, which was used to prepare extract to test for activity against food-borne pathogens, wood dust (125 g) was steeped in 1 liter of deionized water at 60°C for 24 h, then separated from the extract by filtering through four layers of cheesecloth. The extract was dried in a forced-air oven at 60°C for 4 h. Solid extract material was made into a powder using a mortar and pestle. A 25% (wt/v) stock solution of extract powder in deionized water was filter (0·2 μ) sterilized and kept at 5°C until used within 24 h. These solutions are referred to as the ‘extracts’ in the following test.

Organisms and preparation for testing

Five bacterial isolates (one strain each of *Acetobacter, Flavobacterium, Lactobacillus, Leuconostoc* and *Micrococcus*) and one yeast isolate (*Saccharomyces*) were tested for their sensitivity to kiam wood extract. Isolates were identified only to the genus level.

Acetobacter, Flavobacterium, and *Micrococcus* were cultured in nutrient broth (Difco, Detroit, MI, USA), *Lactobacillus* and *Leuconostoc* were cultured in MRS broth (Difco), and *Saccharomyces* was cultured in YM broth (Difco). Inocula consisted of cells grown in broths for 24 h at 30°C.

Three strains each of four food-borne pathogenic bacteria were tested for sensitivity to kiam wood extract: *L. monocytogenes* V7, Brie-1 and LCDC 81-861; *Staphylococcus aureus* FRI 472, FRI 798 and FRI 1068 (all enterotoxigenic); *Bacillus cereus* F3802A/84, NVHIT and 1230-88 (all enterotoxigenic) and *Escherichia coli* 0157:H7 E105, CA-1 and 932. Three serotypes of *Salmonella* (*enteritidis, stanley* and *typhimurium*) were also tested. All pathogens were cultured in tryptic soy broth (TSB; Difco) at 30°C for 24 h before being used as inocula for screening experiments or, in the case of *L. monocytogenes*, to inoculate shredded raw cabbage.

Procedure for testing

The effect of kiam extract on growth of micro-organisms isolated from sugar palm sap was determined by a well method. A 24 h culture (0·1 ml) of each micro-organism was surface plated on an agar that consisted of the respective broth in which it had been grown plus 1·5% agar. Wells (6·2 mm diameter) were made in the agar plates (100 mm diameter) using a sterile cork borer. Fifty microliter of 0·2 or 1·0% kiam extract prepared by the first two methods described above was added to each well and plates were

incubated at 30°C for 48 h before measuring the diameter of zone of inhibition around wells.

The influence of kiam extract on growth of test pathogens was determined by two procedures. In one procedure, 0·1 ml of a 24 h culture was surface plated on tryptic soy agar (TSB supplemented with 1·5% agar) in 100 mm diameter petri plates. Twenty microliters of 0, 5, 15 and 25% kiam extract (pH 5·7), prepared by diluting the stock extract in sterile deionized water, was added to wells made as described above, and plates were then incubated at 30°C for 48 h before examining for zones of inhibition of growth of each pathogen. In the second procedure, sterile paper disks (6·2 mm diameter) (Sensi-disc, BBL, Becton Dickinson Co., Cockeysville, MD, USA) were dipped in 0, 5, 15 or 25% kiam extract solution (pH 5·7), blotted to remove excess solution and placed on the surface of TSA plates (100 mm diameter) that had been surface inoculated with 0·1 ml of 24 h cultures of test pathogens. The diameter of the zone of inhibition of growth around each disk was measured after incubating plates for 48 h at 30°C.

Inoculation of cabbage

Application of kiam wood extract to shredded raw cabbage for the purpose of determining its effect on survival and growth of *L. monocytogenes* was investigated. Cabbage was purchased from a local grocer. The outer leaves and core were removed and the remaining portion was shredded to yield pieces c. 1·5 cm thick wide.

A suspension of *L. monocytogenes* (strain LCDC 81-861) was prepared by combining 24 ml of a 24 h TSB culture with 2400 ml of sterile 0·1 M potassium phosphate buffer (pH 7·0). Inoculation was achieved by dipping 1200 g of shredded cabbage into 2425 ml of *L. monocytogenes* suspension for 1 min. After draining the excess suspension, 250 g of cabbage was dipped into 250 ml of 0 (deionized water), 0·5 or 5% kiam wood extract (pH 5·7) for 1 min. Treated cabbage was then drained, placed in polyethylene bags, sealed and stored at 5°C for 0, 2 or 5 days before analysing for populations of *L. monocytogenes*, aerobic mesophilic micro-organisms and psychrotrophic micro-organisms.

Microbiological analyses

In tests to determine the antimicrobial effect of kiam wood extract on palm sap isolates and pathogens using the well and disc methods, populations in inocula were determined by serially (1:10) diluting cultures in sterile 0·1% peptone water and surface plating duplicate 0·1 ml aliquots on appropriate enumeration media. Colonies were counted after plates were incubated at 30°C for 48 h.

Triplicate samples (25 g) of un-inoculated or inoculated shredded cabbage were analysed for populations of *L. monocytogenes*, aerobic mesophilic micro-organisms and psychrotrophic micro-organisms. Samples were combined with 50 ml of sterile 0·1% peptone and pummelled at medium speed in a Stomacher for 1 min. The wash solution was serially diluted in sterile 0·1% peptone and surface plated in duplicate (0·1 ml) or quadruplicate (0·25 ml) or modified Oxford medium (Monk and Beuchat 1995). Presumptive colonies of *L. monocytogenes* that developed within 48 h at 30°C were counted. Confirmation was done by microscopic examination and by using appropriate biochemical tests (Golden et al. 1988).

Diluted samples were also surface plated on plate count agar (Difco). Populations of aerobic mesophilic micro-organisms were determined by incubating plates at 48 h at 30°C, whereas populations of psychrotrophic micro-organisms were determined by incubating plates for 7°C for 10 days.

Statistical analysis

Experiments were done in triplicate and a minimum of two samples were analysed at each sampling time. Data were subjected to analysis of variance and Duncan's multiple range test (SAS Institute, Cary, NC, USA) to determine whether significant differences (P=0·05) existed between mean values of treatments and control.

Results and Discussion

The range in population in inoculum (0·1 ml) of the micro-organisms isolated from sugar palm sap was plated on agar media in order to determine that the inhibitory activity of kiam extract was 10^5–10^6 CFU. Zones of inhibition of *Saccharomyces, Leuconostoc, Micrococcus, Acetobacter, Flavobacterium*, and *Lactobacillus* were, respectively, 15·0, 14·5, 11·0, 9·5, 8·6 and 8·4 mm in diameter around wells to which 50 μl of a 0·2% solution was added; zones resulting from the addition of 1·0% extract were 19·7, 18·2, 18·8, 12·4, 13·8 and 15·9 mm, respectively. There was essentially no difference in the diameter of zones produced using extracts made by the first two extraction methods. Apparently, the application of heat during the steeping process does not result in higher amounts of active components in the extract, nor are the active components heat labile.

The mean population of three strains of *L. monocytogenes* in 0·1 ml culture surface plated on TSA plates for the purpose of determining inhibitory activity of kiam wood extract was $3{\cdot}3\times10^6$ CFU; mean populations of three strains of *S. aureus* and *B. cereus* were $9{\cdot}4\times10^6$ and $3{\cdot}1\times10^6$ CFU, respectively. The mean population of *E. coli* 0157:H7 strains was $2{\cdot}4\times10^7$ CFU and the mean population of *Salmonella* serotypes was $2{\cdot}3\times10^6$ CFU. The diameters of zones of inhibition on TSA plates seeded with *L. monocytogenes* were 16·6, 19·7 and 22·1 mm, respectively, around wells to which 20 μl of a 5, 15 or 25% solution of kiam wood extract was added. Zones of inhibition of *S. aureus* were 15·7, 19·9 and 22·2 mm around wells containing 20 μl of 5, 15 or 25% extract, respectively, whereas zones for *B. cereus* were 16·8, 20·9 and 22·2 mm. Growth of *E. coli* 0157:H7 and *Salmonella* was not inhibited by any of the test concentrations of extract. Likewise, growth of the five pathogens was unaffected by water controls.

Zones of inhibition observed using the disc method ranged from 6·5 to 7·8 mm for the gram-positive pathogens when discs had been dipped in 25% kiam extract stock solution. No zones of inhibition were observed around discs on TSA seeded with *E. coli* 0157:H7 or *Salmonella*. Thus, it would seem that from the limited number of micro-organisms tested, the inhibitory activity of kiam extract is restricted to gram-positive species.

The mechanism of action responsible for antimicrobial activity of phenolic compounds present in herbaceous and woody plants has not been fully defined, although activity has been attributed to inhibition of extracellular enzymes, deprivation of substrates required for growth, inhibition of oxidative phosphorylation or iron deprivation (Scalbert 1991). Sensitivity to tannins and other phenolic compounds varies greatly among micro-organisms. Some, including *E. coli* (Lewis and Starkey 1969) and *Pseudomonas fluorescens* (Basaraba 1966), both gram-negative species, are capable of growing on tannins as a source of carbon. Whether the strains of *E. coli* 0157:H7 and *Salmonella* tested in our study are capable of metabolizing kiam wood tannins or other components is not known. The gram-negative bacteria investigated in our study do not appear to be sensitive to kiam extract, whereas the gram-positive micro-organisms were sensitive, suggesting that differences in sensitivity may be associated with cell wall structure or function.

Preliminary work to purify and identify water-soluble phenolic compounds in kiam wood that may be responsible for the antimicrobial activity observed in our studies has revealed four major fractions. One of the four fractions consists of dichloro-1,2-benzene, cineole-1,8- nonanol-dimethyl-1,2- benzene, naphthaline, α-terpeneol, hydrocarbon sesquiterpenes and hexadeconal. The level of antimicrobial activity of each of these compounds has not been determined.

Listeria monocytogenes has been isolated from numerous salad vegetables (Beuchat 1996). Its ability to grow on raw vegetables at refrigeration temperature and its association with coleslaw in an outbreak of listeriosis (Schlech et al. 1983) led us to choose shredded cabbage as a model food to determine the effects of treatment with kiam extract on the survival and growth of the pathogen. *Listeria monocytogenes* was not detected in the shredded cabbage used in this study. The population of *L. monocytogenes* in the inoculum

suspension was $2{\cdot}4\times10^6$ CFU/ml. Populations of *L. monocytogenes* and aerobic mesophiles on cabbage were $7{\cdot}7\times10^4$ CFU/g and $1{\cdot}1\times10^5$ CFU/g, respectively, after dipping in the inoculum.

Populations of *L. monocytogenes*, aerobic mesophilic micro-organisms and psychrotrophic micro-organisms on shredded cabbage immediately after treatment with kiam extract (0 day) and after storage for 2 or 5 days at 5°C are shown in Fig. 1. A 1-min dip in 5% extract caused an immediate significant (P=0·05) reduction in populations of *L. monocytogenes* and aerobic mesophiles but not psychrotrophs. After 2 and 5 days at 5°C, significantly lower populations of *L. monocytogenes*, aerobic mesophiles and psychrotrophs were detected in cabbage treated with 0·5 or 5% extract compared to populations in control cabbage. With the exception of populations of psychrotrophic micro-organisms after 2 days, further significant reductions in populations of *L. monocytogenes*, aerobic mesophiles and psychrotrophs occurred in cabbage that had been treated with 5% extract and stored for 2 or 5 days.

Populations of micro-organisms, as affected by the storage time, were also analysed for significant differences. The population of *L. monocytogenes* in cabbage dipped in water (control) did not change during the first 2 days of storage but significantly decreased after 5 days; populations in cabbage treated with 0·5 or 5·0% extract significantly decreased after 2 days and, again, after 5 days storage. Populations of aerobic mesophiles in control cabbage increased significantly after 2 days and again after 5 days, but decreased significantly after 2 and 5 days when cabbage was treated with 5% extract. The inhibitory activity on microflora occurring naturally on cabbage that was dipped in 0·5% extract was significant after 2 days; however, populations of aerobic mesophiles and psychrotrophs increased significantly between 2 and 5 days, indicating that some species were not adversely affected or may have adapted to the lower concentration (0·5%) of extract in the dip, and subsequently grew.

It is clear that treatment of shredded cabbage with kiam wood extract has a lethal

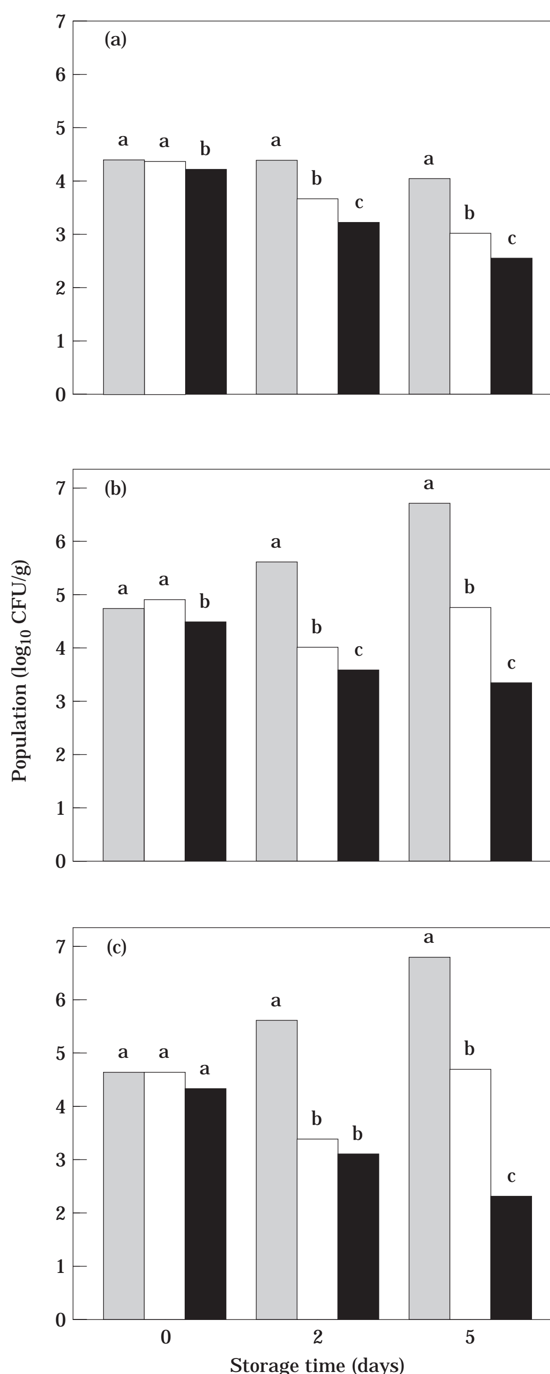

Figure 1. Populations of (a) *Listeria monocytogenes*, (b) aerobic mesophilic micro-organisms and (c) psychrotrophic micro-organisms in shredded cabbage stored at 5°C for 0, 2 and 5 days. Cabbage was dipped in deionized water ▒, 0·5% kiam wood extract □ or 5·0% kiam wood extract ■ for 1 min before storage was initiated. Within storage time and type of micro-organism, bars with the same letter are not significantly different (P=0·05).

effect on *L. monocytogenes* during storage for up to 5 days at 5°C. Furthermore, treatment with extract initially inhibits the growth of micro-organisms occurring naturally on cabbage; the duration of inhibition being affected by the concentration of extract applied. Specific tannins or other natural phenolic compounds responsible for these antimicrobial activities should be determined. Their application to cabbage and perhaps other foods for the purpose of controlling the growth of gram-positive pathogenic bacteria as well as spoilage micro-organisms deserves continued research attention.

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