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ARTICLES

Full Length Research Paper

Characterization of potential probiotic bacteria isolated from sorghum and pearl millet of the semi-arid tropics

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The present study was conducted to isolate and characterize probiotic properties of bacteria isolated from flour and batter samples of sorghum and pearl millet. A total of five different selective media including plate count agar, yeast glucose chloramphenicol agar, Bifidobacterium agar, actinomycetes isolation agar and de ManRogosa and Sharpe agar were used and the most prominent bacteria (which were found abundantly in the plate) were isolated and maintained on the respective media slants at 4°C for further studies. The bacteria were characterized for various traits including Gram staining, morphology (color, size, shape, elevation, margin, form and surface), biochemistry (urease, catalase, oxidase, hydrogen sulphide, nitrogen reduction, gelatin liquefaction, starch hydrolysis and carbohydrate utilization), IMViC tests (indole, methyl red, Voges Proskauer and citrate utilization), probiotic potentials [acid (pH 2, 3), bile (0.5%), NaCl (6 and 9%)], phenol tolerance [0.4%], antibiotic tolerance (tetracycline, streptomycin, kanamycin, chloramphenicol, ciprofloxacin, ampicillin, penicillin, erythromycin and vancomycin) and antimicrobial activity against human pathogens (*Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*). A total of nine probiotic bacterial isolates were short listed based on these traits. The sequences of 16s rDNA gene of the nine isolates were found matched with *Bacillus subtilis* (two isolates), *Bacillus cereus* (three isolates), *Bacillus pumilus* (one isolate), *Bacillus amyloliquefaciens* (one isolate), *Sphingobacterium thalpophilum* (one isolate) and *Brevibacterium* sp. (one isolate) in BLAST analysis. The sequences of the nine bacteria were submitted to NCBI and accession numbers obtained. This study indicated that the selected bacteria could be exploited to develop new probiotic foods.

Key words: Probiotics, prebiotics, sorghum, pearl millet, product development.

INTRODUCTION

Cereals such as rice (*Oryza sativa*) and wheat (*Triticum aestivum*) are presently the predominant staple food for millions across the world that lead not only to an array of emerging life style diseases but also challenges human health and nutrition. Thus, there is an urgent need for identifying and recommending diversity in diets through

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inclusion of other cereals in our diets in order to enhance the overall nutritional status as well as address malnutrition across the world. Two of ICRISAT's mandate crops, sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum)*, can serve this purpose. Sorghum and pearl millet are an important food for millions of people inhabiting the semi-arid tropics in Africa and Asia and are a major source of calories and vital component of food security in the semi-arid areas in the developing world (Amadou et al., 2013). Sorghum and pearl millet are often ground into flour and consumed as roti and/or porridge with milk and sometimes prepared as beverages (Obilana and Manyasa, 2002; Amadou et al., 2011). These crops can serve as the source of prebiotics (total dietary fiber, resistant starch, total oligosaccharides and β-glucan) for functional food (Awika and Rooney, 2004). Functional foods are referred as food ingredients or the food as such that influence beneficial effect on the host and/or reduce the risk of chronic diseases (Huggett and Schliter, 1996; Charalampopoulos et al., 2002). Probiotic microorganisms have been used for preparation of dairy food for thousands of years. Non-dairy based probiotic drinks utilizing cereals, including pearl millet, are also reported (Mridula and Sharma, 2015). Probiotic foods contain a single or mixed culture of probiotic microbes that improves the health of the host by improving intestinal microbial balance (Fuller, 1989). Information on the probiotic microorganisms associated with sorghum and pearl millet cultivars are inadequate (Badau, 2006). Hence, the present investigation was aimed to isolate and identify bacteria from flours and batter of sorghum and pearl millet cultivars and to further characterize these bacteria for their probiotic traits, in order to understand the probiotic potential of sorghum and pearl millet which are grown extensively in the Semi-Arid Tropics of the world.

METHODOLOGY

Collection of sorghum and pearl millet grain samples

A total of eight sorghum grain samples including K648 stress, K648 control, 6040 stress, 6040 control, R16 stress, R 16 control, K359 stress and K 59 control and two pearl millet grain samples including dual purpose hybrid (DPH) and high Fe hybrid were grown at ICRISAT and used for this study.

Collection of human pathogenic bacteria

Human pathogenic bacteria such as *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), and *Salmonella typhi* (ATCC 14028) were acquired from American Type Culture Collection (ATCC), USA.

Preparation of the grain samples

The grain samples of both sorghum and pearl millet were dried at room temperature (30 to 32°C) for 3 days and milled in Cyclotech[TM]

- mill. The ground samples were sieved through 0.2 mm sieve, in order to get fine flour. The batter samples were prepared, by mixing 5 g of flour in 5 ml of sterilized distilled water in a sterilized beaker and incubated/fermented at 28°C for 12 h. At the end of fermentation, the batter samples were immediately used for isolating the bacteria.

Isolation and maintenance of bacterial isolates

Ten grams of flour/batter from each of sorghum/millet grain sample was separately suspended in 90 ml of sterilized physiological saline (0.85% of NaCl in water) in a flask and placed on an orbital incubator shaker (at 120 rpm) for 30 min. At the end of shaking, the flour/batter samples were serially diluted up to 10^6 dilutions with physiological saline. Dilutions 10^4 to 10^6 were spread plated (0.1 ml) on five different selective media including plate count agar, yeast glucose chloramphenicol agar, Bifidobacterium agar, actinomycetes isolation agar and de ManRogosa and Sharpe (MRS) agar and incubated at 30°C for 24 h. Bacteria were enumerated and the most prominent colonies which were found abundantly in the plate isolated and maintained on the respective media slants at 4°C for further studies.

Morphological and biochemical characterization of bacterial isolates

The bacteria were streaked on respective media and incubated at 30°C for 24 h. At the end of incubation, the colonies were observed for its morphological traits including form, surface, texture, color, elevation and margin. Gram staining reaction of the bacteria was done as per the protocols of Pelczar et al. (2008). The bacteria were also characterized for their biochemical traits including hydrogen sulphide, urease, catalase, oxidase, nitrate reduction, gelatin liquefaction, starch hydrolysis and IMViC (Indole, Methyl red, Voges Proskauer and simmons Citrate) tests as per the methods of Holt (1984). Utilization of carbohydrates such as lactose and sucrose were determined as per Forouhandeh et al. (2010).

Antibiotic resistance pattern and antimicrobial activity of bacterial isolates

Antibiotic resistance pattern was conducted by disc diffusion method. The resistance or susceptibility to antibiotics of bacterial isolates to ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), erythromycin (15 μg), kanamycin (30 μg), penicillin (10 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (10 μg) (HiMedia, Mumbai, India) were determined according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Wikler, 2006). In brief, antibiotic discs were placed on the Muller-Hinton agar plates immediately after swab with actively grown bacterial cultures. The plates were incubated at 30°C for 24 h. At the end of incubation period, zone of inhibition was measured.

The antimicrobial activity of the bacterial isolates was done by ditch assay method as per the protocols of Aswathy et al. (2008) with slight modifications. In brief, the bacterial isolates were grown in Muller-Hinton broth at 30°C for three days. At the end of incubation period, the culture filtrates were collected by centrifugation at 10,000 × *g* for 20 min and concentrated on a rotary evaporator until one fifth of the original volume. The concentrated culture filtrate samples were filter-sterilized through 0.2 μm membrane filter. Human pathogens such as *E. coli*, *S. aureus* and *S. typhi*, grown separately on nutrient broth at 37°C for 24 h, were amended with sterilized nutrient agar (2.5%) at 45°C, poured on sterile Petri plates and allowed to solidify. Upon solidification, a ditch of 0.5 mm was cut in the Petri plate and filled with membrane-

filtered concentrated culture filtrate of bacterial isolate (0.2 ml). The plates were initially placed at 4°C for 1 h, for diffusion of metabolites present in the culture filtrate, and further incubated at 37°C for 18 h. At the end of incubation, zone of inhibition was noted.

Probiotic traits of bacterial isolates

The bacterial isolates were characterized for their probiotic traits including tolerance to acid (low pH), bile salt, phenol and NaCl as per the standardized protocols used at ICRISAT.

Acid tolerance

This test was done as per the protocols of Liu et al. (2007) for identifying the bacterial isolates which could tolerate simulated gut acidic conditions. In brief, the Muller-Hinton broth was adjusted to pH 2.0, 3.0 and 4.0, and inoculated with one ml of log phase bacterial isolate. The inoculated broth was adjusted to 0.6 OD at 620 nm in UV spectrophotometer and incubated at 37°C for 120 min. The samples were withdrawn at the interval of 30 min and absorbance was measured at 620 nm.

Bile tolerance

Bile tolerance test was determined based on Aswathy et al. (2008). Brain heart infusion (BHI) broth was amended with various concentrations of bile salt (0.3, 0.5 and 0.8%) and inoculated with one ml of log phase bacterial isolate. The inoculated broth was adjusted to 0.6 OD at 620 nm in UV spectrophotometer and incubated at 37°C for 120 min. The samples were withdrawn at the interval of 30 min and absorbance was measured at 620 nm.

NaCl tolerance

NaCl tolerance was done as per the methods of Graciela and Maria (2001). Muller-Hinton broth was adjusted to different concentration of NaCl (3, 6, 9, and 12%) and inoculated with 1 ml of log phase bacterial isolate. The inoculated broth was adjusted to 0.6 OD at 620 nm in UV spectrophotometer and incubated at 37°C for 120 min. The samples were withdrawn at the interval of 30 min and absorbance was measured at 620 nm.

Phenol tolerance

The phenol tolerance of the bacterial isolates was assessed using the protocols of Teply (1984) with slight modifications. Log phase of bacterial isolate was inoculated in Muller-Hinton broth containing 0.2 and 0.4% of phenol. Absorbance was measured at 620 nm using UV spectrophotometer initially at 0 h and after 24 h of incubation at 37°C.

Molecular identification of the bacterial isolates

Pure cultures of probiotic potential bacteria were grown until log phase and genomic DNA were isolated according to Bazzicalupo and Fani (1995). The amplification of 16S rDNA gene was done by using universal bacterial primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and 27F (5'-AGAGTTTGATCMTGGCTC AG-3') according to the conditions by Pandey et al. (2005). The polymerase chain reaction (PCR) product was sequenced at Macrogen Inc. Seoul, Korea. The sequences were compared with those from GenBank using the BLAST

program (Alschul et al., 1990), aligned using the ClustalW software (Thompson et al., 1997) and phylogenetic trees inferred using the neighbor-joining method (Saitou and Nei, 1987).

Submission of sequences to NCBI

The 16S rDNA gene sequences of potential probiotic isolates were submitted to NCBI and the GenBank accession numbers were obtained.

Compatibility of the nine probiotic potential bacteria

Compatibility study was done as per the published protocols of Gopalakrishnan et al. (2011). In brief, the nine probiotic potential bacteria were streaked on Luria Agar in 10 cm diameter plate in a specific pattern that was first drawn on a paper as template. The template was prepared as follows: A 5 cm long vertical line at a distance of 5 mm from the margin was drawn on one side of the circle. Five lines, each 5 mm away and as perpendicular to the verticalline, were drawn. Each perpendicular line is 5 cm long and 1 cm apart from each other. Keeping this as a template below the Petri dish, the nine test bacteria were streaked over the vertical line and over the perpendicular lines. Care was taken that the cultures did not touch wall of the Petri plate. The plates were incubated for 24 h and observed for compatibility. If zone of inhibition was found between the two bacteria both of them were considered as not compatible whereas if no zone was found, these were considered as compatible.

RESULTS

Isolation and morphological characterization of bacterial isolates

In the present investigation, a total of 218 bacteria were isolated from the five different selective media (plate count agar, yeast glucose chloramphenicol agar, Bifidobacteria isolation agar, actinomycetes isolation agar and MRS agar), of flour and fermented batter samples of sorghum and pearl millet. A total of nine probiotic bacterial isolates were short listed based on morphological and biochemical traits, IMViC tests, probiotic potential, resistance to antibiotics and antimicrobial activity against human pathogens. The selected nine bacteria (PHFB-22, PHFF-11, S6SF-44, S8CF-32, S8SF-4, SKSB-14, SKSF-55, SKSF-7 and SKSF-8) were found in different forms (circular/irregular), size (big/medium/punctiform), surface (rough/veined/glistening), texture (moist/mucoid/dry), color (cream/pink/pale-pink/translucent/yellow/white), elevation (flat/raised/umbonate), margin (entire/lobate) and Gram staining (Gram positive/Gramnegative; rod/cocci) (Table 1).

Biochemical characterization of the selected nine bacterial isolates

When the nine selected bacterial isolates were characterized for their biochemical traits, all were found

Table 1. Morphological characterizations of the nine probiotic potential bacterial isolates.

Isolate	Form	Size	Surface	Texture	Color	Elevation	Margin	Gram staining[*]
PHFB-22	Irregular	Medium	Rough	Moist	Cream	Flat	Entire	G$^+$ rod
PHFF-11	Irregular	Medium	Veined	Moist	Pale pink	Flat	Lobate	G$^+$ rod
S6SF-44	Circular	Medium	Glistening	Mucoid	Cream	Raised	Entire	G$^+$rod
S8CF-32	Circular	Big	Rough	Moist	Cream	Flat	Entire	G$^+$rod
S8SF-4	Circular	Punctiform	Glistening	Mucoid	Pink	Raised	Entire	G$^-$rod
SKSB-14	Circular	Punctiform	Rough	Mucoid	Translucent	Flat	Entire	G$^+$ rod
SKSF-55	Circular	Punctiform	Glistening	Mucoid	Yellow	Raised	Entire	G$^+$rod
SKSF-7	Circular	Medium	Veined	Moist	White	Flat	Entire	G$^+$rod
SKSF-8	Circular	Punctiform	Rough	Dry	Translucent	Umbonate	Lobate	G$^+$ rod

*G$^+$rod = Gram positive rod; G$^-$ rod= Gram negative rod.

Table 2. Biochemical characterization of the nine probiotic potential bacteria.

Isolate	HS	U	C	O	NR	GL	SH	I	MR	VP	Cit	Carbohydrate utilization test			
												L	LG	S	SG
PHFB-22	-	-	-	-	+	-	+	-	-	+	+	+	+	+	-
PHFF-11	-	-	-	-	+	-	+	-	+	+	-	+	+	+	-
S6SF-44	-	+	-	-	+	-	+	-	-	+	+	+	+	+	-
S8CF-32	-	+	-	+	+	-	+	-	-	-	+	+	+	+	-
S8SF-4	-	+	-	-	-	-	+	-	+	-	+	+	+	+	+
S6SF-44	-	+	-	-	+	-	+	-	-	+	+	+	+	+	-
SKCF-55	-	+	-	-	-	-	-	+	-	+	-	+	+	+	-
SKSF-7	-	-	-	-	+	-	+	-	+	+	-	+	+	+	-
SKSF-8	-	+	-	-	-	-	+	-	+	+	-	+	+	+	-

HS= Hydrogen sulfide test; U = urease test; C=catalase test; O= oxidase test; NR= nitrate reduction test; GL= gelatin liquefaction; SH = starch hydrolysis; I = indole test; MR = methyl red test; VP= Vogues Proskauer test; Cit= citrate utilization test; L= lactose; LG= lactose gas production; S= sucrose; SG= sucrose gas production.

positive for lactose utilization, lactose gas production, sucrose utilization, sucrose gas production (only for S8SF-4), urease test (except for PHDB-22, PHFF-11 and SKSF-7), nitrate reduction (except for S8SF-4, SKCF-55 and SKSF-8), starch hydrolysis (except for SKCF-55), Vogues Proskauer (except for S8CF-32 and S8SF-4), citrate utilization (except for PHFF-11, SKCF-55, SKSF-7 and SKSF-8), methyl red (only for PHFF-11, S8SF-4, SKSF-7 and SKSF-8) and iodine (only for SKCF-66). However, none of the isolates were positive for gelatin liquefaction, oxidase, catalase and hydrogen sulfide tests (Table 2).

Antibiotic resistance pattern and antimicrobial activity of the selected bacterial isolates

In the present investigation, the nine bacteria were evaluated for their antagonistic traits against human pathogens. It was noted that all the nine selected bacterial isolates were found to inhibit all the three tested pathogens, viz. *E. coli*, *S. aureus* and *S. typhi* (Table 3).

The selected nine isolates were also found resistance to tetracycline (except for S8SF-4) at 30 µg, streptomycin (except for S6SF-44 and S8SF-4) at 10 µg, kanamycin (except S8SF-4 and SKSB-14) at 30µg, chloramphenicol (except for S8SF-4) at 30 µg, ciprofloxacin (except for S8SF-4) at 10 µg, ampicillin (only for PHFB-22, S8CF-32 and SKSF-8) at 10 µg, penicillin (only for PHFB-22, S8CF-32 and SKSF-8) at 10 µg, erythromycin at 15 µg and vancomycin (except for S6SF-44 and SKSB-14) at 10µg (Table 3).

Probiotic traits and molecular identification of the selected bacterial isolates

When the selected nine bacterial isolates were tested for their probiotic properties, all of them were found to tolerate acidic pH (2-3), bile (at 0.3 to 0.5%), NaCl (3 to 9%) and phenol (0.2%; only for PHFB-22 and PHFF-11) (Table 4).

Phylogenetic analysis of 16S rDNA sequences of the seven of the nine probiotic potential bacterial isolates

Table 3. Antibiotic resistance pattern and antimicrobial traits (zone of inhibition in µg) of the nine probiotic potential bacteria.

Isolate	T	ST	K	Chl	Cip	Amp	Pen	Ery	Van	Antimicrobial activity		
										E. coli	*S. aureus*	*S. typhi*
PHFB-22	27	18	21	26	27	18	15	22	18	9	13	10
PHFF-11	23	22	22	20	27	0	0	21	18	11	10	21
S6SF-44	22	0	10	14	31	0	0	23	0	10	10	10
S8CF-32	27	26	22	27	27	12	10	28	20	11	10	11
S8SF-4	0	0	0	0	0	0	0	21	17	14	12	10
SKSB-14	14	14	0	13	21	0	0	21	0	11	10	14
SKSF-55	22	23	23	16	24	0	0	16	18	28	10	11
SKSF-7	20	20	18	17	24	0	0	19	15	11	11	22
SKSF-8	26	27	22	16	31	16	15	21	17	10	12	14
Mean	20	17	15	17	24	5	5	21	14	13	11	14
SE±	1.0***	0.6***	1.1***	0.6***	0.7***	1.0***	1.1***	0.8***	0.3***	0.5***	0.3***	0.4***
LSD (5%)	2.9	1.9	3.3	1.8	1.9	3.1	3.2	2.3	1.0	1.4	0.8	1.3
CV%	8	7	13	6	5	35	40	6	4	6	4	5

SE = Standard error; ***= Statistically significant at 0.001; LSD = Least significant difference; CV = Coefficient of variance; T = Tetracycline (30 µg); ST= Streptomycin (10 µg), K= Kanamycin (30µg), Chl= Chloramphenicol (30µg), Cip= Ciprofloxacin (10µg), Amp= Ampicillin (10 µg), Pen= Penicillin (10 µg), Ery= Erythromycin (15 µg), Van= Vancomycin (10 µg),*S. aureus*=*Staphylococcus aureus*, *S. typhi*= *Salmonella typhi*

Table 4. Probiotic properties, identity and NCBI accession numbers of the nine probiotic potential bacteria.

Isolate	Acid tolerance (pH)	Bile tolerance (%)	Phenol tolerance	NaCl tolerance (%)	Identified isolate	Accession number
PHFB-22	2	0.3	0.2%	9	*Bacillus subtilis*	–
PHFF-11	3	0.5	0.2%	6	*Bacillus cereus*	KM624626
S6SF-44	2	0.3	Nil	3	*Bacillus amyloliquefaciens*	KM624628
S8CF-32	2	0.5	Nil	9	*Bacillus subtilis*	KM624629
S8SF-4	2	0.5	Nil	3	*Spingobacterium thalpophilum*	KP326566
SKSB-14	3	0.3	Nil	3	*Brevibacterium* sp	KM817772
SKSB-55	3	0.3	Nil	6	*Bacillus cereus*	KM658265
SKSF-7	2	0.3	Nil	6	*Bacillus cereus*	KM658262
SKSF-8	3	0.3	Nil	9	*Bacillus pumilus*	KM658263

belonged to *Bacillus* but different species. The isolates PHFB-22 and S8CF-32 had maximum sequence similarities with *Bacillus subtilis*, PHFF-11, SKSB-55 and SKSF-7 showed maximum sequence similarities with *Bacillus cereus*; whereas S6SF-44 and SKSF-8 showed maximum sequence similarities with *Bacillus amyloliquefaciens* and *Bacillus pumilus*, respectively (Table 4 and Figure 1). The sequences of the other two isolates S8SF-4 and SKSB-14 were found to be similar to *Spingobacterium thalpophilum* and *Brevibacterium* sp., respectively (Table 4 and Figure 1).

Compatibility of the selected nine bacterial isolates

In the present study, when the probiotic potential bacteria were characterized for their compatibility there was no definite sign of suppression of four bacteria (S8SF-32, SKSF-55, SKSF-7 and SKSF-8) on any of the other bacteria while the remaining five bacteria showed inhibition over others, suggesting these were not compatible.

DISCUSSION

Fermentation of foods is traditionally carried out in every household in developing countries of Africa and Asia. The benefits of fermented foods include its increased nutritional value and keeping qualities. These products have a high content of probiotic bacteria with a large bio-diversity. For instance, Koko is a millet gruel produced in Northern Ghana and spontaneously fermented by lactic acid bacteria such as *Lactobacillus*
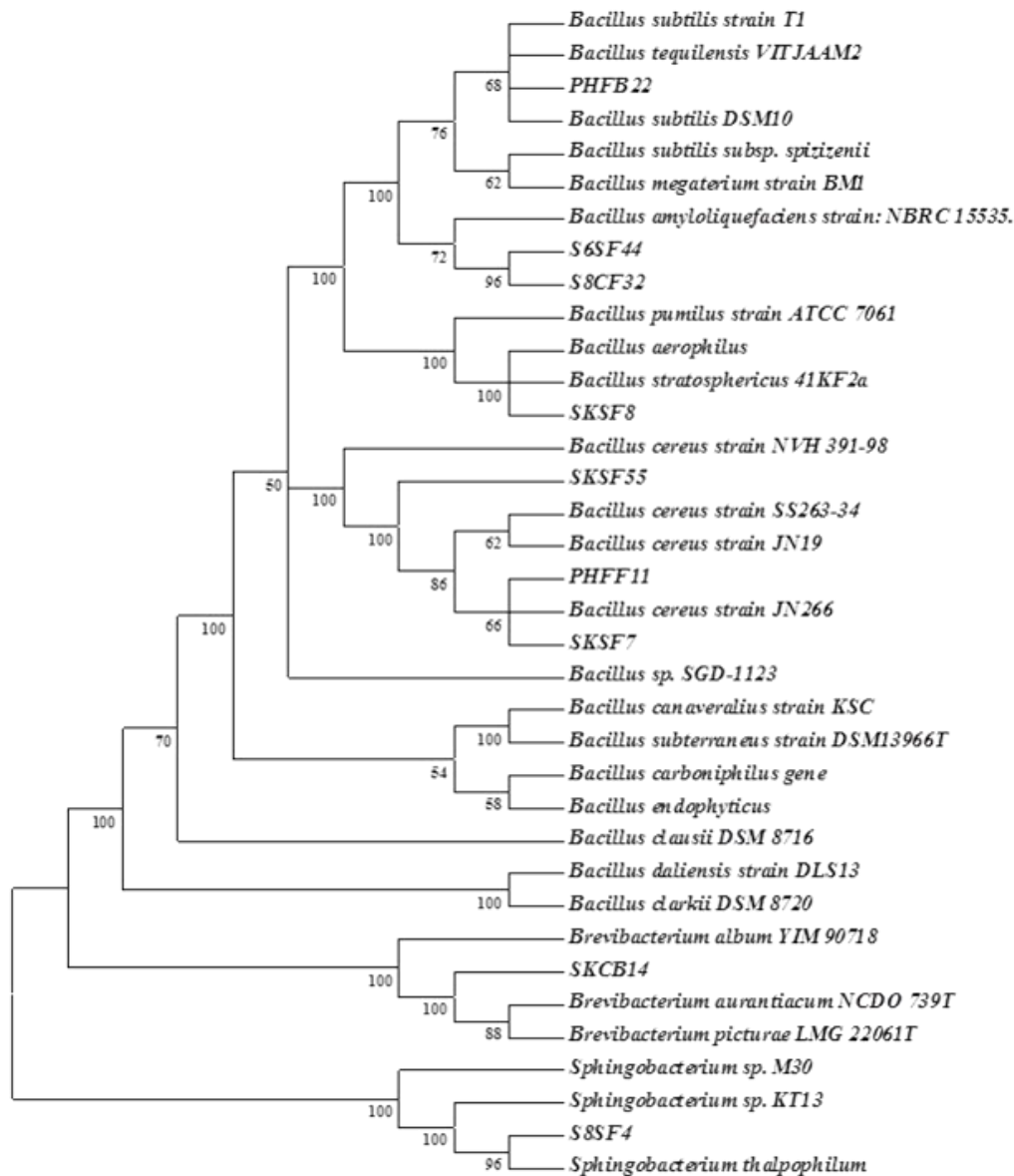
Figure 1. Phylogenetic relationship between the nine probiotic potential bacterial isolates and representative species based on full length 16 S rDNA sequences constructed using the neighbor-joining method.

fermentum and *Weissella confusa* (Lei and Jakobsen, 2004). Millets and sorghum are the two most important staple food for millions of poor people in Asia and Africa as it contains high carbohydrate energy and nutrition, thus making them useful components of dietary and nutritional balance in food. Probiotics help the existing microbial flora or repopulate the lost microbial flora in the colon due to antibiotics, disease and/or chemotherapy. Probiotic foods from millets and sorghum are rich in phytochemicals including phytic acid and phytates, which are known to lower cholesterol and reduce the risk of cancer, and are also reported to generate vitamins, fatty acids and other viral nutrients that improve the body's

resistance against human pathogens (FAO/WHO, 2001; Coulibaly et al., 2011; El-Salam et al., 2012). Hence, in the present investigation, millets and sorghum flour and fermented batter samples were used for isolation of probiotic bacteria.

Inhibition of human pathogenic microbes and resistance to antibiotics by probiotic microbial strains are advantageous traits. Hence, in the present study, the nine selected bacterial isolates were found to inhibit all the three tested pathogens, viz. *E. coli*, *S. aureus* and *S. typhi* (Table 3). Similar studies were conducted by Oluwajoba et al. (2013). Lactic acid bacteria isolated from millet and sorghum grains and fermented products were

demonstrated to inhibit human pathogens such as *S. aureus* 25923, *E. coli* 25922, *Pseudomonas aeruginosa* 27853 and *Enterococcus faecalis* 29212 (Oluwajoba et al., 2013). Probiotic microbial strains such as *L. fermentum*, *Bifidobacterium* sp. and *W. confusa* are also reported to help in preventing and treating acute diarrhea (Lei et al., 2006). The major aim of using these probiotic strains should be to affect beneficially the gut microbial composition and activities. Hence, in the present study, when the selected nine bacterial isolates were also tested for their antibiotic resistance pattern, these were found resistance to tetracycline (except S8SF-4), streptomycin (except S6SF-44 and S8SF-4), kanamycin (except S8SF-4 and SKSB-14), chloramphenicol (except S8SF-4), ciprofloxacin (except S8SF-4), ampicillin (only PHFB-22, S8CF-32 and SKSF-8), penicillin (only PHFB-22, S8CF-32 and SKSF-8), erythromycin and vancomycin (except S6SF-44 and SKSB-14). It is concluded that the selected nine isolates have good antagonistic potential as well as antibiotics resistance pattern.

The selection criteria of suitable probiotic microbial starter should be its ability to survive the acidic environment of the final fermented product and the adverse conditions of the gastrointestinal tract. Although, differences exist between species and specific strains, Lactobacilli (for instance, *L. casei* and *L. plantarum* showed longer shelf life than *L. acidophilus*) are generally considered to be intrinsically resistant to pH values higher than 3.0 (Hood and Zottola, 1988; Lee and Salminen, 1995). Hence, in the present investigation, the bacterial isolates were characterized for their probiotic properties including acid tolerance, bile tolerance, phenol tolerance and NaCl tolerance and all of them were found to tolerate acidic pH (2-3), bile (up to 0.5%), NaCl (up to 9%) and phenol (0.2%). Hence, it is concluded that the selected nine isolates have good probiotic properties.

In order to determine the identity of the nine potential probiotic bacteria, its 16S rDNA was sequenced and analyzed. A neighbor joining dendrogram was generated using the sequence from the nine potential probiotic bacteria (1400 bp) and representative sequences from the databases which revealed seven of the nine probiotic potential bacterial isolates belonged to *Bacillus* but different species whereas the other two isolates were found to be similar to *Spingobacterium thalpophilum* and *Brevibacterium* sp., respectively. Millet and sorghum flour have been used for isolation of lactic acid bacteria such as *Lactobacillus plantarum*, *L. cellobiosus*, *L. pentosus*, *Leuconostoc mesenteroids* and *Pedicoccus pentosaceus* (Okoronkwo, 2014). Mridula and Sharma (2015) reported a non-dairy probiotic drink from the sprouted cereals, legumes and soy milk using lactic acid bacteria. As found in the current investigation, *Bacillus subtilis* was reported to be isolated from the un-malted pearl millet grains in addition to *Lactobacillus plantarum*, *Streptococcus lactis* and *Torulopsis glabrata* (Badau, 2006) and reported to

have probiotic potential (Kamgar et al., 2013). Microbes such as *Lactobacillus* sp., *Bifidobacterium* sp. and *Weissella* sp. are reported widely as probiotic microbes. *Lactobacillus acidophilus* was used to ferment a food mixture containing sorghum flour, whey powder and tomato pulp (Jood et al., 2012). However, in the present study, we report the isolation of *Bacillus* sp., *Sphingobacterium* sp. and *Brevibacterium* sp. as potential probiotic cultures. Hence, these cultures also can be exploited for making functional foods.

For any probiotic products/functional food, the ability of the probiotic microbial strain to attain high cell population is of primary importance. The probiotic microbial cell population of about 10^7 cells ml^{-1} at the time of consumption is considered functional (Gomes and Malcata, 1999). Probiotic foods obtained using a single microbial strain are not welcomed by the consumers due to rather sour and acidic taste and therefore probiotic strains are mixed (Saarela et al., 2000). Keeping this in mind, in the present investigation, all the probiotic potential bacteria were characterized for their compatibility so that these can be used in consortia. Compatibility studies between the nine probiotic potential bacteria revealed that there was no definite sign of suppression of four bacteria (S8SF-32, SKSF-55, SKSF-7 and SKSF-8) on any of the other bacteria while the remaining five bacteria showed inhibition over others, suggesting these were not compatible. Hence, it is concluded that the four of the bacterial isolates (S8SF-32, SKSF-55, SKSF-7 and SKSF-8) can be used as consortia in functional food product development.

Conclusion

Cereals such as sorghum and millets are not only valuable sources of prebiotics and bioactive compounds useful for help in production of functional foods but also are sources of probiotic microbial cultures, which should be exploited for the production of new and innovative functional foods. Cereals and pseudocereals like Sorghum and pearl millet contain good quantities of potential prebiotic components, such as, water-soluble fibre (includes β-glucan and arabinoxylan), oligosaccharides (includes galacto- and fructo-oligosaccharides) and resistant starch important for the probiotic concept (Charalampopoulas et al., 2002). Hence, the multiple beneficial effects of cereals, especially the pseudo cereals such as sorghum and millets, can be used in association with good probiotic microbial cultures also isolated from these cereals in various ways to design novel cereal-based functional foods targeting different consumer segments having specific health requirements. Some of the important traits that need to be considered while selecting the cereals and the probiotic microbial culture include the composition of cereal grains, the substrate formulation,

the growth capability of the starter probiotic culture, the stability of probiotic culture during storage, the organoleptic properties and the nutritional value of the final functional food product (Charalamoppulos et al., 2002). Cereals not only have the ability to grow probiotic microbes such as lactic acid bacteria in the human gut but also contain potential prebiotic compounds, whose functionality needs to be explored. However, the information available on the effects of cereal composition on the growth of probiotic microbes is limited. Therefore, the importance of substrate composition in conjunction with the nutritional requirements of the specific probiotic microbial strain is absolutely essential in order to make good quality functional food with validated health claims. In summary, the present work has demonstrated that the functional foods based on cereals, such as sorghum and millets, in association with good probiotic microbial stains can be exploited for designing novel cereal based functional foods for addressing food and nutritional security for millions of malnourished people living in the poorest of the poor countries of Africa and Asia.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Abbreviations

PHFB, Pearl millet high Fe batter; **PHFF,** Pearl millet High Fe flour; **S6SF,** Sorghum 6040 Stress flour; **S8CF,** sorghum K 648 control flour; **S8SF,** sorghum K 648 stress flour; **SKSB,** sorghum K 359 stress batter; **SKSF,** sorghum K 359 stress flour.

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Full Length Research Paper

Comparison of phenolic and volatile profiles of edible and toxic forms of *Detarium senegalense* J. F. GMEL

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In Senegal, *Detarium senegalense* J.F. Gmel. (ditax in Wolof) is one of the most important forest fruit species. However, exploitation of the edible fruit is based on local people's knowledge. Only trees whose fruits are consumed by animals are exploited. To identify them, a chemical comparison of edible and toxic forms was done in order to highlight differences between both forms. Dichloromethane leaf extracts from toxic and edible trees were analyzed by gas chromatography. Phenolic profile and volatile compounds from fruits extracts were studied respectively by High Performance Liquid Chromatography-mass spectrometry (HPLC-MS) and Gas Chromatography Mass Spectrometry (GC-MS). Cytotoxicity effect of fruits extracts was evaluated on murine macrophage cells J774 A1. GC-analysis of dichloromethane leaf extracts revealed the presence of lupenone and lupeol only in toxic extracts. 6'-O-galloyl-epiheterodendrin and isovaleronitrile were detected in toxic pulp. However, no cytotoxic effect was found in our conditions. This study has given the opportunity to identify within the same species, compounds which could differentiate both edible and toxic forms. Nevertheless further studies are needed to better understand which compounds are responsible for toxicity in the toxic form.

Key words: Ditax, *Detarium senegalense*, toxicity, lupeol, lupenone, cyanogenic glycoside, isovaleronitrile.

INTRODUCTION

In Senegal, *Detarium senegalense* J. F. Gmel generates a great economic activity in the central and southern regions. The fruit called "ditax" in Wolof, is a globulous

drupe from 3 to 8 cm in diameter, with a large central core surrounded by a farinaceous, greenish, fibrous, acidulated and sweetened pulp (Kerharo and Adam,

1974; Haddad, 2000; Arbonnier, 2002). The fruits are very appreciated and widely consumed as nectar or fresh (Diop et al., 2010). However, in some areas, toxic and edible trees cohabit with a great difficulty to identify and differentiate them morphologically. By describing *D. senegalense* species, Guillemin et al. (1830), observed that this tree could produce edible or toxic fruit. Cases of intoxications have been reported one century and half ago but the compounds responsible for this toxicity have not been identified (Heckel and Schlagdenhauffen, 1889; Paris and Moyse-Mignon, 1947; D'Almeida, 1984; Imbert and Teyssier, 1986; Adam et al., 1991; Berthelot et al., 2000). Among previous studies which have been done on this field, we can mention Sambuc who did the first study on chemistry of *D. senegalense* fruits by giving an alcohol toxic fruits extract to dogs but no side effect was observed (Sambuc, 1887; Diatta, 1995; Cavin, 2007). Heckel and Schlagdenhauffen (1889) studied also toxicity of *D. senegalense*. According to them, toxicity of *D. senegalense* fruit is due to a volatile compound. In 1947, Paris and Moyse-Mignon have succeeded to isolate from toxic fruits, a bitter compound which is responsible for the toxicity according to them. They concluded that this compound appears to depress the central nervous system after transiently excited.

In Senegal, the distinction is based on the knowledge of the local population that collects only trees whose fruits are consumed by animals. Moreover, there are no objective methods to differentiate the two forms. In order to highlight difference between toxic and edible form of *D. senegalense* and to find objective parameters for their distinction, a chemical study was done by comparing their composition.

MATERIALS AND METHODS

Leaves and fruits

Leaves and fruits from edible and non-edible varieties of *D. senegalense* harvested in 2009 (from 1 adult toxic tree and 1 adult edible tree) and 2012 (from 5 different adult toxic and edible trees) in Ziguinchor were studied. Leaves were dried between two papers at room temperature and stored away from light. Fruits were frozen and stored at -18°C just after harvesting.

Gas chromatography analysis of leaf extracts

Leaves from toxic and edible old trees (1.4 g) were crushed and macerated in 10 ml of dichloromethane (Merck, Darmstadt, Germany) under agitation for 1 h in an ultrasounds water bath (88154, Bioblock Scientific - Fisher, Illkirch, France). Extracts (three replicates) were filtered, then solvent was evaporated for 10 min at 35°C with a centrivap concentrator (Labconco, Kansas City, USA). Extracts were stored for 48 h at 4°C before analysis by gas chromatography using a Trace GC Thermo Quest chromatograph / Mass detector Trace ms plus (Courtaboeuf, France). Helium was used as carrier gas at 1mL/min. Separation was carried out on a capillary column RTX 5 (30 m X 0.25 mm I.D., 0.25 μm, Restek, France) with the following temperature program: 50°C for 5 min then slope of 5°C/min up to 270°C and held for 15 min. Injector

temperature was 250°C, in splitless mode. Tentative identification of peaks was done by comparing mass spectra with those of the National Institute of Standards and Technology (NIST) data base. Standards of lupenone and lupeol (Sigma Aldrich & Fluka, Saint-Quentin Fallavier, France) were injected each one separately, then after mixing together. Toxic and edible leaf extracts with and without lupenone and lupeol standards were also analyzed by GC-MS.

Phenolic profile of fruit pulp

Edible fruit pulp was extracted from cores and fiber network surrounding the core. Toxic fruit pulp was extracted at the same way. Phenolic compounds were extracted from each toxic and edible pulp fruit by stirring for 10 min, 500 mg of pulp in 20 ml of acetone. After filtration and evaporation, extract was diluted in 2 ml of a mixture of methanol – water 50/50 then filtered at 0.45 μm. Pulp extracts were then analyzed by High Performance Liquid Chromatography – Mass Spectroscopy (HPLC-MS) using an HPLC SURVEYOR, equipped with a DAD detector UV 6000 LP and coupled with a mass spectrometer LCQ (THERMO FINNIGAN, San José, USA). Separation was done on an ACE C-18 column (250 mm X 4.6 mm, 5 μm, France HAS) thermostated at 30°C in reversed phase. Mobile phases were water / formic acid (0.1%) for eluant A and water / acetonitrile / formic acid (19.9/80/0.1) for eluant B. The following binary gradient was used with a flow of 0.7 mL.min^{-1} : T = 0 min : 5% B ; T = 50 min : 35% B ; T = 55 min : 50% B; T = 60 min : 80% B ; T = 65 min : 100% B ; T = 70 min : 100% B ; T = 72 min : 5% B ; T = 85 min : 5% B. Volume injection was 10 μl. Detection was carried out at 280, 330 and 360 nm. The electro-nebulization was carried out in negative mode.

Volatile profile of fruits by solid phase micro extraction (SPME) and gas chromatography (GC) / mass spectrometry (MS) analysis

Eight edible whole fruits and 8 toxic whole fruits were set in glass jars with a septum in the top. Micro extraction was carried out for 2 h at ambient temperature using a polydimethylsiloxane-divynilbenzene 65 μm fiber (StableFlex ^{TM} SPME fiber, Supelco). A tandem gas chromatograph 6890 / MSD 5973 / Gerstel Multipurpose Sample MPS-2 was used for volatiles compounds analysis (Agilent Technologies, Palo Alto, USA). After trapping, injection was carried out in splitless mode at 250°C for 180 s for desorption. Separation was done on a DB-WAX polar column (J&W, 30 m x 0.25 mm x 0.25 μm). The following temperature program was used: 40°C for 1 min; 3°C/min from 40°C to 170°C, then 10°C/min up to 240°C and held for 10 min. Mass spectra were recorded in Electron Ionization impact (EI+) mode at 70 eV within 40 to 300 Da. Source temperature was 250°C and helium was used as carrier gas at 1 mL/min. Isovaleronitrile standard was used as internal standard for identification and quantification in toxic pulp extract.

Cellular toxicity evaluation of fruit extracts by MTS/PMS assay

The cellular toxicity of pulp and husk from edible and toxic fruits was evaluated using a J774 A1 cells of murine macrophages obtained from the American Type Culture Collection (ATCC, TIB67, Rockville, MD) by the technique of MTS/PMS as previously described by Dussossoy et al. (2011).

Preparation of fruits extracts

Methanol extracts were prepared from toxic and edible fruits.

Briefly, 10 g of toxic pulp and 10 g of toxic husk were stirred 10 min with 5 ml of water and 40 ml of methanol. The liquid phase was filtered on paper and the residue was extracted again with 20 ml of methanol for 10 min. The methanol phase was evaporated under vacuum and the crude extract was taken up in 2 ml of methanol. Ten ml of diethyl ether were added to remove chlorophyll pigments. For edible extracts, 5 g of pulp and 5 g of husk were used.

After that, 40 ml of phosphate saline buffered (PBS) were added to each methanol extracts, which were filtered through 0.45 μm and diluted with the culture medium at the concentration of 20 mg/ml. The following concentrations (mg/ml) were tested on the cells: 10 – 5 – 1 - 0.5 - 0.1 - 0.05 -0.01 - 0.005 - 0.001 - 0.0005 and 0.0001. Each concentration was repeated 6 times. The toxicity of isovaleronitrile standard was also evaluated on J774 A1 cells at 1-0.5 –0.1 – 0.05 – 0.01 – 0.05 – 0.001 – 0.0005%.

Cells preparation and cytotoxicity evaluation

Briefly, 1 x 10^5 cells/well were seeded in a 96-well plate in RPMI (*Roswell Park Memorial Institute medium* 1640 with Glutamax® supplemented with 100 μg/ml of streptomycin, 100 Units/ml of penicillin and 10% heat inactivated fetal bovine serum, Gibco Life Technologies, Grand Island, NY, USA) and incubated with various concentrations of extracts under 200 μl for 20 h at 37°C in a humidified incubator containing 5% CO_2. After incubation, 20 μl/well of a tetrazolium salt MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, Promega Corporation, 2007), mixed with an electron-coupling reagent (PMS, phenazine methosulfate) diluted in PBS, was added. The plate was incubated for another 4 h at 37°C. Dehydrogenase enzymes found in metabolically active cells accomplish the conversion of MTS into soluble formazan. The quantity of formazan product was measured by the amount of the absorbance at 490 nm, which is directly proportional to the living cells in culture.

RESULTS AND DISCUSSION

### Gas chromatography analysis of leaf extracts

Dichloromethane leaf extracts from edible and toxic adult trees were analyzed by gas chromatography in order to highlight presence or absence of at least one compound, which could be used as criterion of differentiation between the two forms. Mass spectra of some unidentified compounds eluted between 55 and 60 min, let think that these compounds could belong to the triterpenes family like lupeol and lupenone. Lupenone and lupeol standards were analyzed each one separately and then together (Figure 1). Mass spectra of lupenone and lupeol were shown in Figure 2. Leaf extracts of edible and toxic trees were then analyzed by GC-MS, without standards and with standards. Retention time of lupenone and lupeol were 56.5 and 57.0 min respectively with interval time ranging between 56 and 58 min (Figure 3). Lupenone and lupeol were detected only in toxic leaf extracts (Figure 3c and d). It is the first time that lupenone and lupeol were found in *D. senegalense*. Lupeol is a triterpenic alcohol present in various families of plants. It is one of the most active compounds of several medicinal plants. Lupeol is found in grape, hazel nut, olive oil, cocoa butter, cabbage (Gallo and

Sarachine, 2009), mango pulp (Duke, 1992) and tamarind (Imam et al., 2007). Lupeol has many pharmacological activities in particular anti-protozooairy (Fotie et al., 2006; Hoet et al., 2007; Ajaiyeoba et al., 2008), anti-inflammatory (Theophile et al., 2006; Rocha et al., 2008; Vasconcelos et al., 2008) and antitumor (Saleem et al., 2008; Cmoch et al., 2008; Prasad et al., 2008; Saleem, 2009). Even if toxicity of lupeol is not well established (Imam et al., 2007; Fotie et al., 2006; Hoet et al., 2007), it could be used to identify toxic and edible forms because they are present only in toxic leaves. Nevertheless, their presence or absence in toxic and edible fruits has to be done in perspective.

Phenolic profile of fruit pulp

Profiles of phenolic compounds from edible and toxic fruit pulps were compared on the basis of UV-VISIBLE spectrum, mass of molecular ions and ions fragments as well as using data from the literature. Figure 4 shows chromatograms of pulp extract from edible and toxic fruit recorded at 280 nm. Table 1 presents phenolic compounds identified in edible and toxic fruit pulps of *D. senegalense*. The identified compounds are primarily flavanols, dimers of catechine and epicatechine as well as derivatives of catechine and catechine gallate. Galloyl derivatives of shikimic acid were also observed. The gallic acid was also present. This result agrees with those of Haddad (2000) who found 7.05 mg/kg of gallic acid in the edible pulp of ditax. The main difference between phenolic profiles of the two chromatograms was the presence of a phenolic compound eluted at 36.74 min only in the toxic fruit pulp extract. This compound, characterized by a $λ_{max}$ of 274 nm and a m/z of 412.11 is very close to the 6'-O-galloyl-(R)-epiheterodendrin ($λ_{max}$ 275 nm; m/z 412) found by Cavin (2007). According to this author, this compound is formed by a β-glucose linked with isovaleronitile and a gallic acid in esterified form (Figure 5). When isovaleronitrile is linked in 1' by a β-glucose, the molecule is named heterodendrin or epiheterodendrin, according to the absolute configuration S or R, respectively of C-2 (Jaroszewski, 1986; Lechtenberg et al., 1996; Nielsen et al., 2002). Our results seem to confirm the implication of 6'-O-galloyl-(R)-epiheterodendrin in the toxicity of *D. senegalense* fruit as previously noticed by Cavin (2007). Indeed, this compound seems to belong to the class of cyanogenic glycosides. Cyanogenesis is the ability of some plants to synthesize cyanogenic glycosides, which when enzymatically hydrolyzed, release cyanohydric acid (HCN) (Francisco and Pinotti, 2000; Harborne, 1972). ß-glucosidase enzyme is responsible in most cases, for the hydrolysis reaction, which produces sugars and a cyanohydrin that spontaneously decomposes to HCN and a ketone or an aldehyde (Figure 6). Hydroxynitrile lyase enzyme can also catalyze the HCN release reaction from cyanohydrin (Harborne, 1993). However, according to
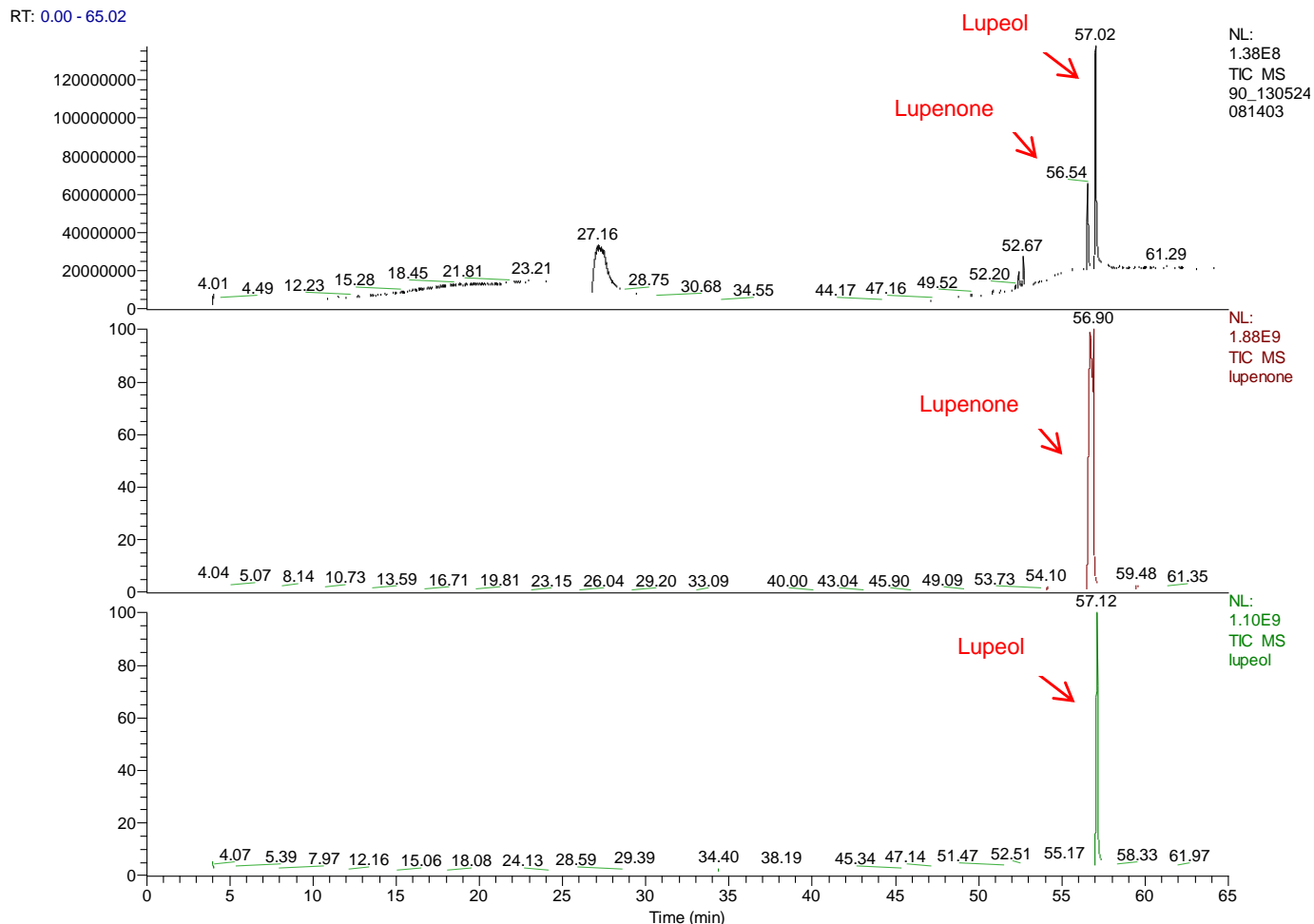
Figure 1. Chromatograms of standards: (a) lupenone and lupeol together; (b) lupenone alone; (c) lupeol alone.

Francisco and Pinotti (2000), HCN of cyanogenic glycoside is not release enzymatically in some cases like in *Rapanea umbellata* plant. Cyanogenic glycosides are formed from amino acids, and are classified according to the nature of them (Cavin, 2007). According to Lechtenberg et al. (1996), isovaleronitrile linked to glucose of 6'-O-galloyl epiheterodendrin probably comes from to L-leucine as presented in Figure 7.

The description of a bitter almond odor, which would be released from the toxic fruits related by Berthelot et al. (2000), seems to confirm the involvement of 6'-O-galloyl-(R)-epiheterodendrin because cyanogenic glycosides have a bitter almond odor (Paris and Moyse-Mignon, 1947; D'Almeida, 1984). Moreover, the release of HCN was confirmed by Cavin (2007) by Prussian blue formation.

Volatile profile of fruits

Since animals, in particular monkeys, do not consume toxic fruits because of their bitter odor (Berthelot et al., 2000), volatile compounds from whole fruits were extracted by solid phase micro-extraction and analyzed by GC-MS to seek an eventual difference between toxic and edible fruits. Figure 8 shows volatile profiles of the two extracts and the principal difference between the two profiles is the detection of isovaleronitrile at 7.12 min retention time only in the whole toxic fruits. Presence of isovaleronitrile in toxic fruit pulp could explain the bitter almond odor release from toxic fruits and related by some authors (Berthelot et al., 2000; Adam et al., 1991). Chromatogram of whole fruits extracts between 6 and 7.3 min as well as the mass spectrum of isovaleronitrile are presented in Figure 8b. Thereafter, isovaleronitrile was sought in toxic and edible pulps with a standard of isovaleronitrile. Isovaleronitrile was detected only in toxic pulp extracts at 37.3 nmol/mg per fresh fruit pulp.

Detection of isovaleronitrile only in toxic fruit pulp seems to confirm presence of 6'-O-galloyl-(R)-epiheterodendrin in toxic fruits. Moreover, according to Heckel and Schlagdenhauffen (1889), it is a volatile
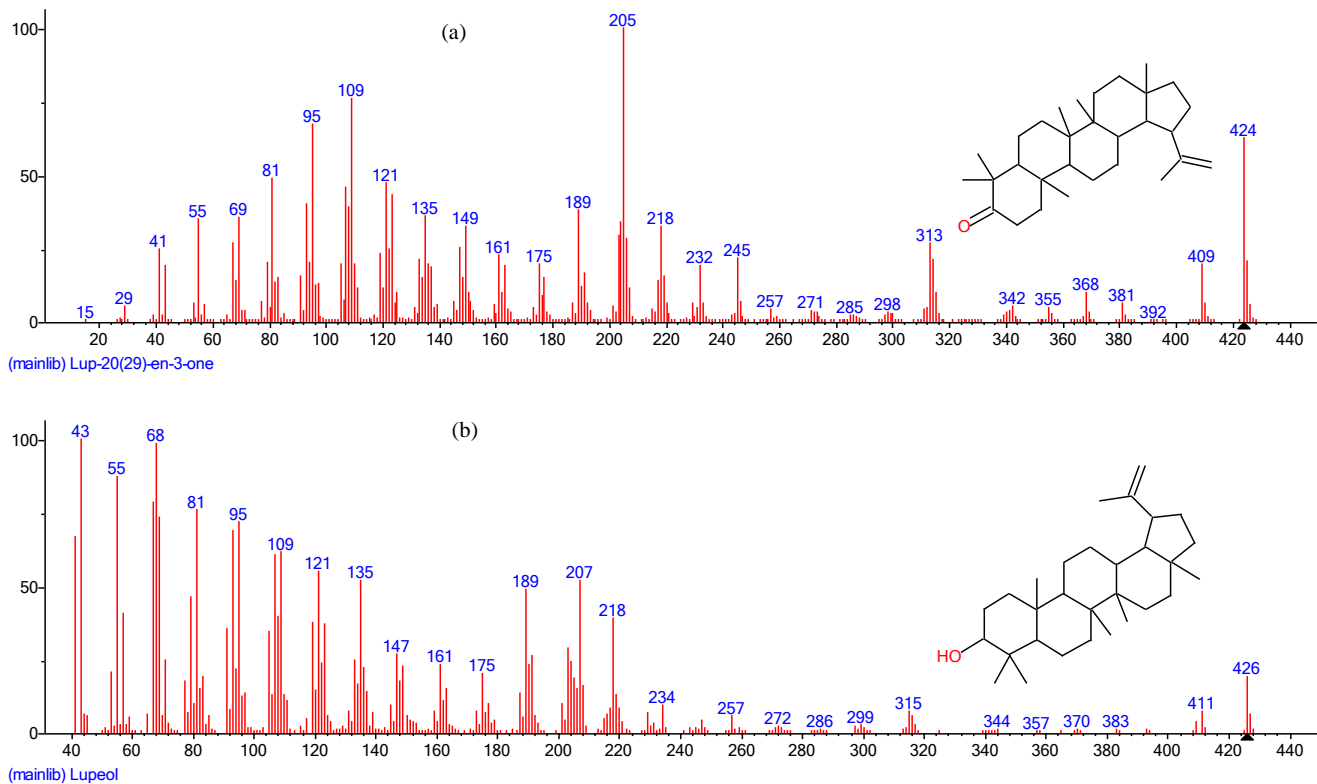
Figure 2. Mass spectra of standards: (a) lupenone; (b) lupeol.
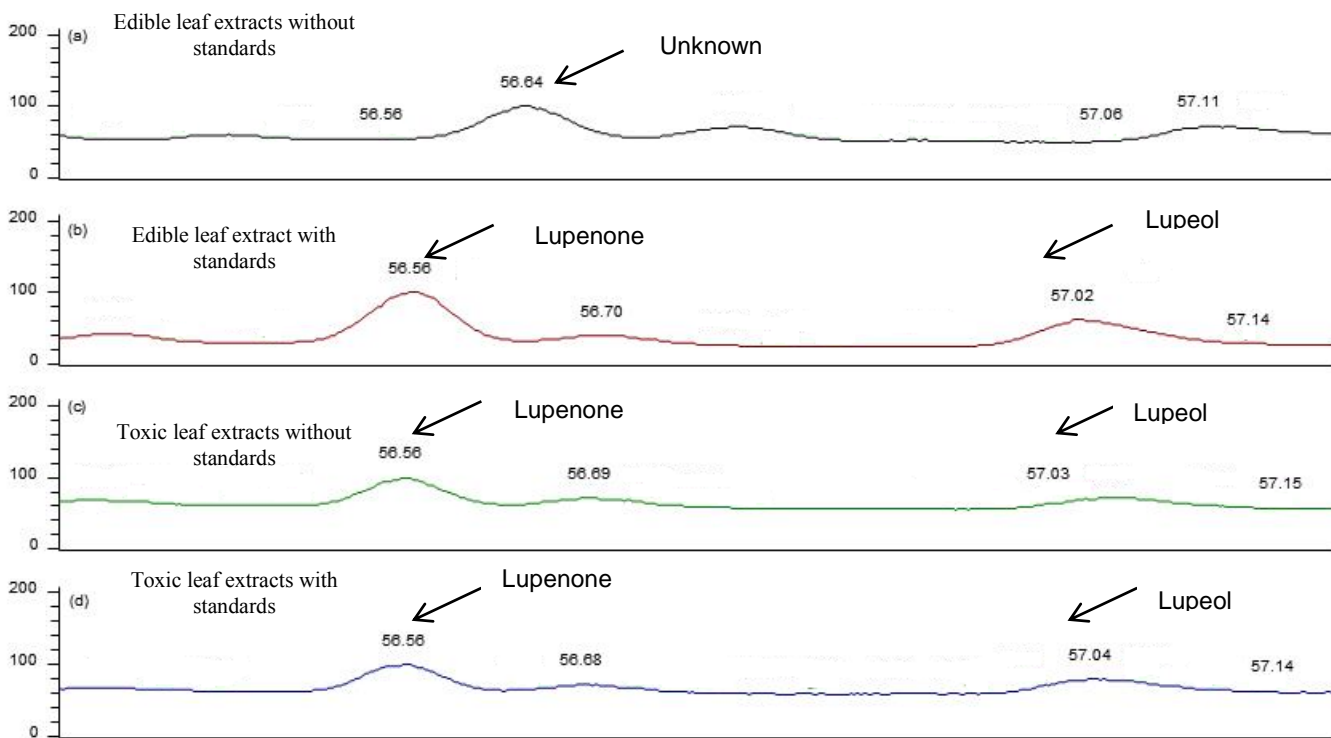
Figure 3. Chromatograms of dichloromethane leaf extracts from *Detarium senegalense* between retention times 56 to 58 min: (a) dichloromethane extract of edible leaves; (b) dichloromethane extract of edible leaves with lupenone and lupeol add as standards; (c) dichloromethane extract of toxic leaves; (d) dichloromethane extract of toxic leaves with lupenone and lupeol add as standards.
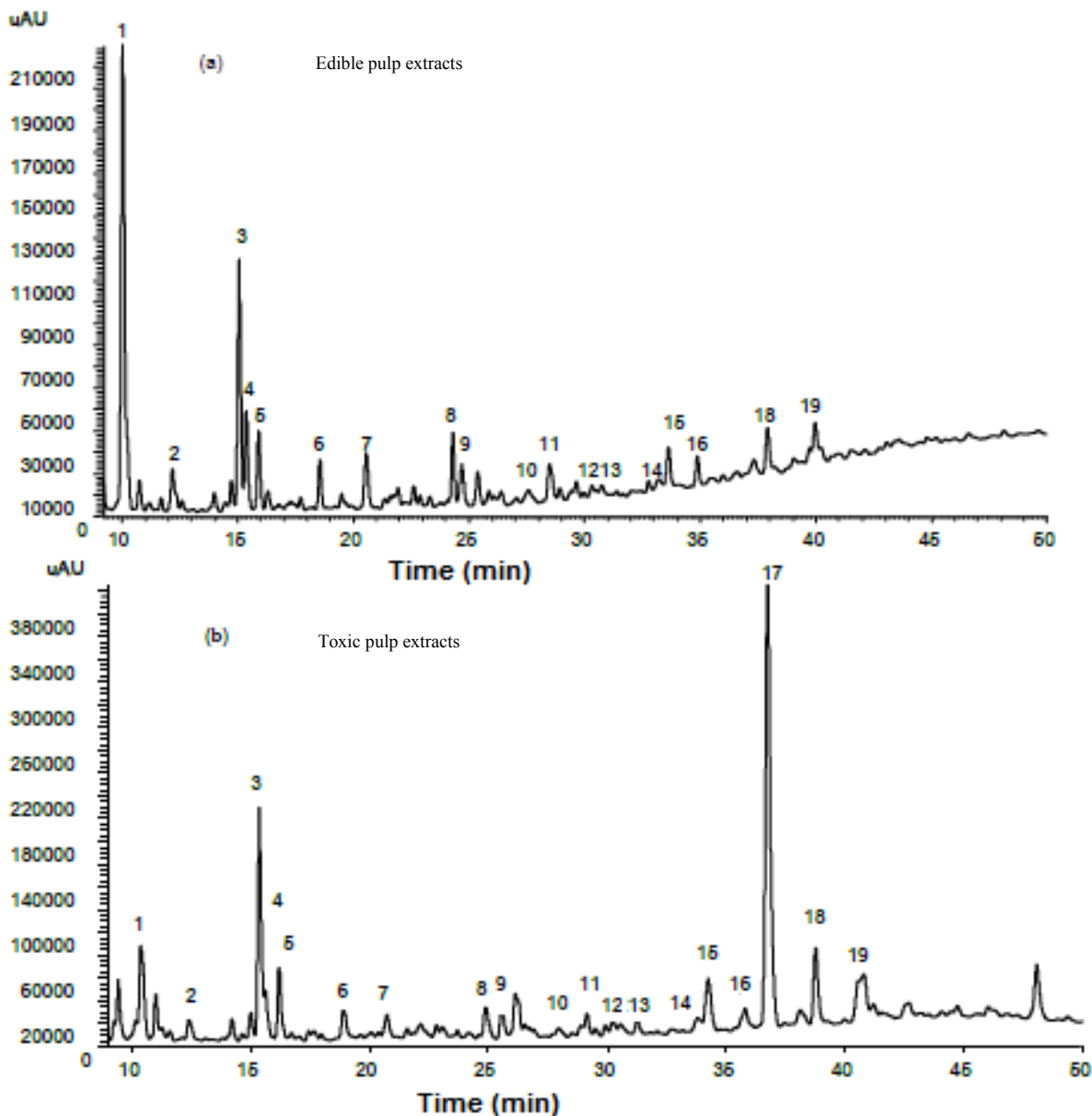
Figure 4. Chromatogram HPLC of phenolic compounds (detection at 280 nm) of edible (a) and toxic (b) pulp extracts of *Detarium senegalense*. (cf. Table 1 for peaks identification).

compound that would be responsible for toxicity.

Evaluation of cellular toxicity

Our study revealed that both toxic and edible fruit extracts had no toxic effect on viability of murine cells J774 (Figure 9). In the same way, isovaleronitrile did not have toxic effect on cell viability even if a slight decrease of absorbance was noticed (from 1.27 to 1.13) between 0.1 and 1% (Figure 10). These extracts have now to be tested on other cellular models especially on human cells

to evaluate their potential toxicity in human. Nevertheless, this test is an in vitro test, which gives an indication of cytotoxicity but in any case not presage an in vivo toxicity in humans after ingestion.

Conclusion

Our results highlighted differences between the edible and toxic forms of *D. senegalense*. Analysis of phenolic compounds revealed the presence, only in the toxic form, of a cyanogen glycoside: the 6'-O-galloyl epiheterodendrin.

Table 1. Phenolic compounds identified in edible and toxic pulp of *D. senegalense* fruits.

Pics	Tr (min)	UV	MS	MS^2	Tentative of identification
1	10-10.3	271	169		Gallic acid
2	12.2	273	493		n.i.
3	15.1-15.3	275	325	169	Galloyl of shikimic acid
4	15.4-15.6	274	325	169	Galloyl of shikimic acid
5	15.9-16.2	275	325	169	Galloyl of shikimic acid
6	18.6-18.9	273	487	443,407, 271	n.i.
7	20.6-20.7	278. 290sh	519	473,451, 443	n.i.
8	24.3-24.6	270	483	331,271, 169	Digalloyl glucose
9	24.7-24.8	278	289	245, 205, 179	Catechine (cat)
10	27.6-27.9	278	577	509,425, 407	Procyanidine dimere
11	28.5-29.1	256sh. 278	481	437, 313,169	n.i.
12	30.3-30.5	278	289	245, 205, 179	Epicatechine (épicat)
13	30.8-31.2	274	729	577, 407, 289	Cat-cat gallate
14	32.8-33.2	274	635	591, 483, 331	Trigalloyl glucose
15	33.6-34	278	729	577, 407, 289	Epicat-Cat-gallate
16	34.9-35.6	272	609	565, 457, 271	n..i
17	*36.7*	*274*	*412*	*313, 169*	*6'-O-galloyl-(R)- epiheterodendrin*
18	37.9-38.7	278	881	729, 577,289	Cat-gallate/cat-gallate
19	40-40.7	278	441	289	Cat-gallate

n.i. : unidentified.
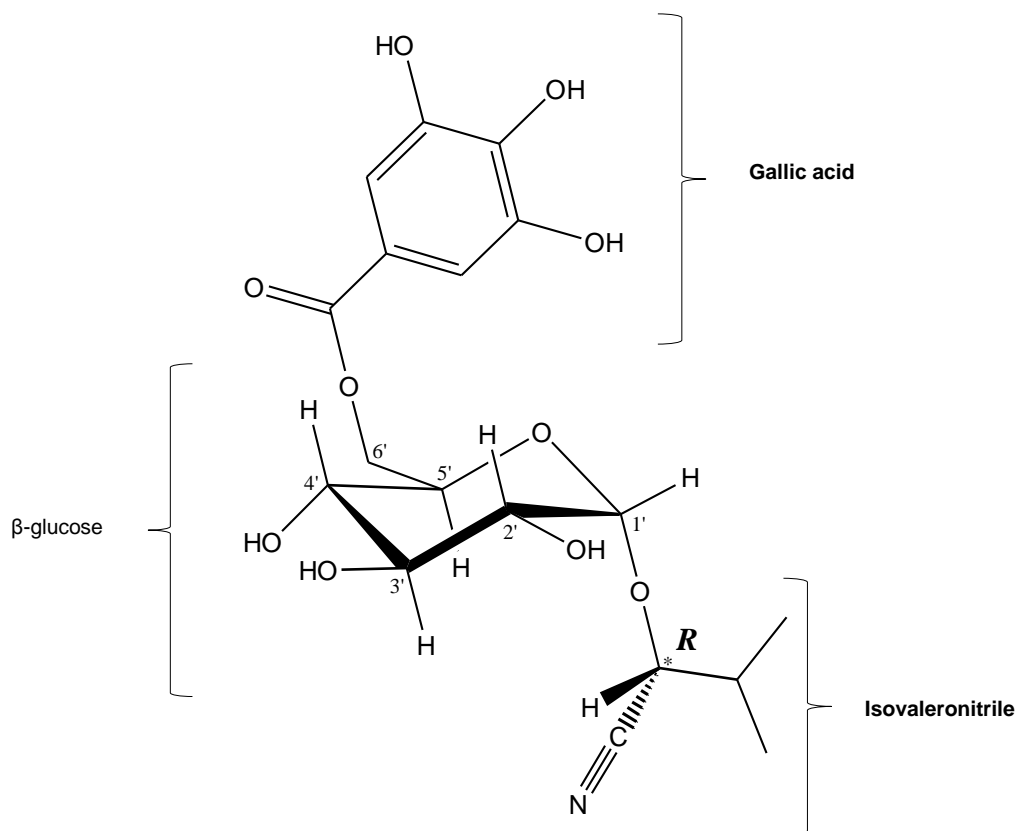
Figure 5. Chemical structure of 6'-O-galloyl-(R)-epiheterodendrin isolated from toxic fruit pulp of *D. senegalense* by Cavin (2007).
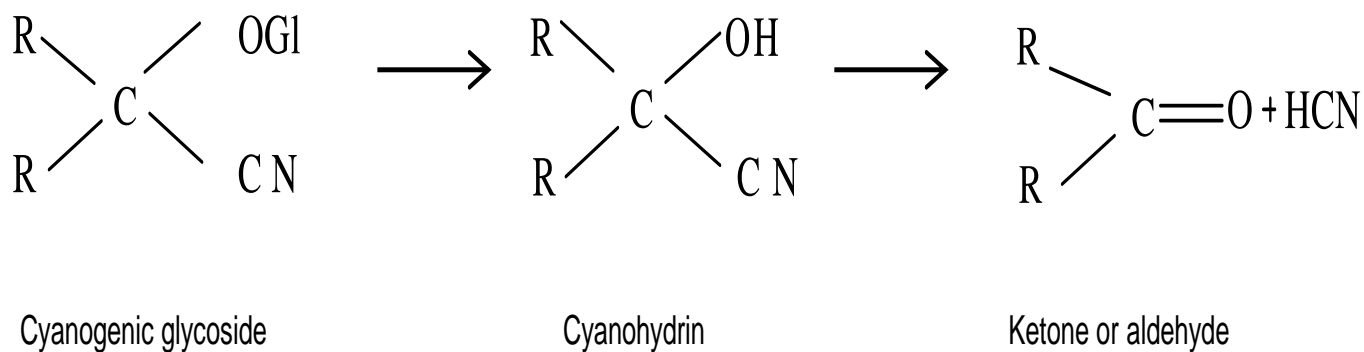
Figure 6. Pathway of HCN by cyanogenic glycoside plants (Francesco and Pinotti, 2000).
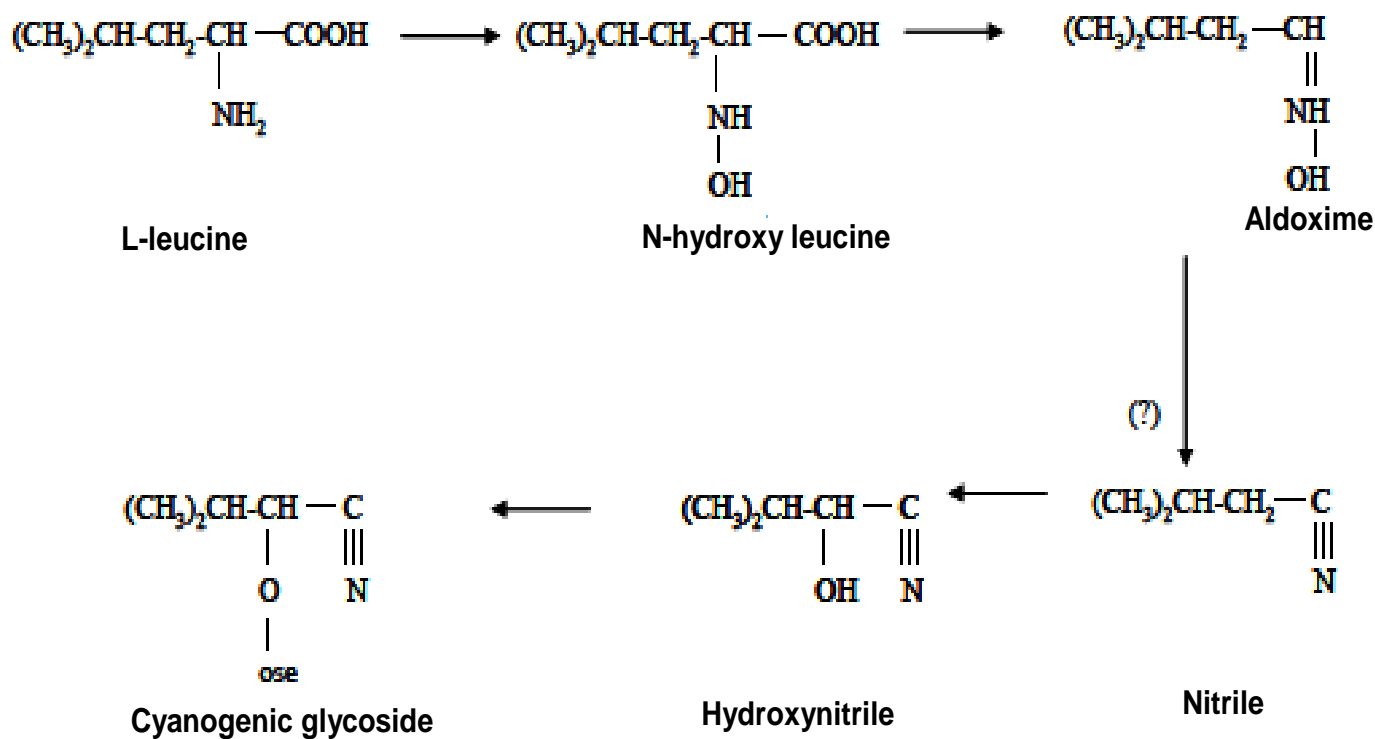
Figure 7. Biogenetic pathway of 6'-O-galloyl-(R)-epiheterodendrin formation (Cavin, 2007; Lechtenberg et al. 1996; Bruneton, 1999).

Lupeol and lupenone were also detected only in the toxic leaf extracts. Quantification of 6'-O-galloyl epiheterodendrin, lupeol and lupenone in fruits and leaves could be used to distinguish the edible and toxic forms. The development of a rapid method to detect isovaleronitrile, lupeol or lupenone could be considered in order to quickly classify fruits. Nevertheless, it would be interesting to enlarge tree sampling with different geographical origins and to validate reproducibility of the results. Complementary work would be carried out in order to identify the compounds responsible for the toxicity.

Conflict of Interests

The authors have not declared any conflict of interests.
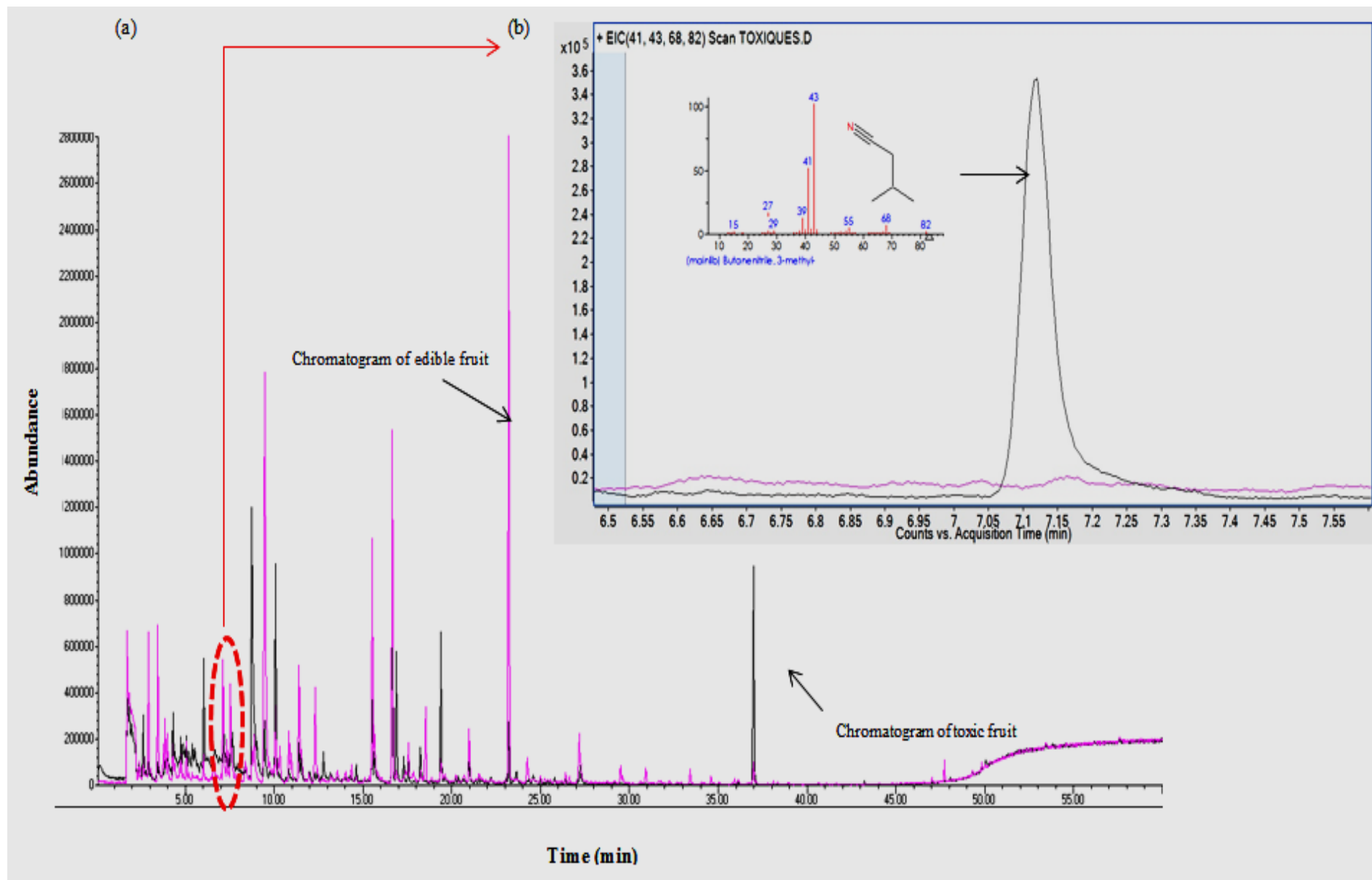
Figure 8. Comparison of volatiles compounds (a) extracted by solid phase micro extraction (SPME) on whole toxic fruits (black line) and whole edible fruits (pink line) of *D. senegalense* ; (b) zoomed chromatograms between 6.5 and 7.5 min with the characteristic ions of isovaleronitrile (41,43,68,42).
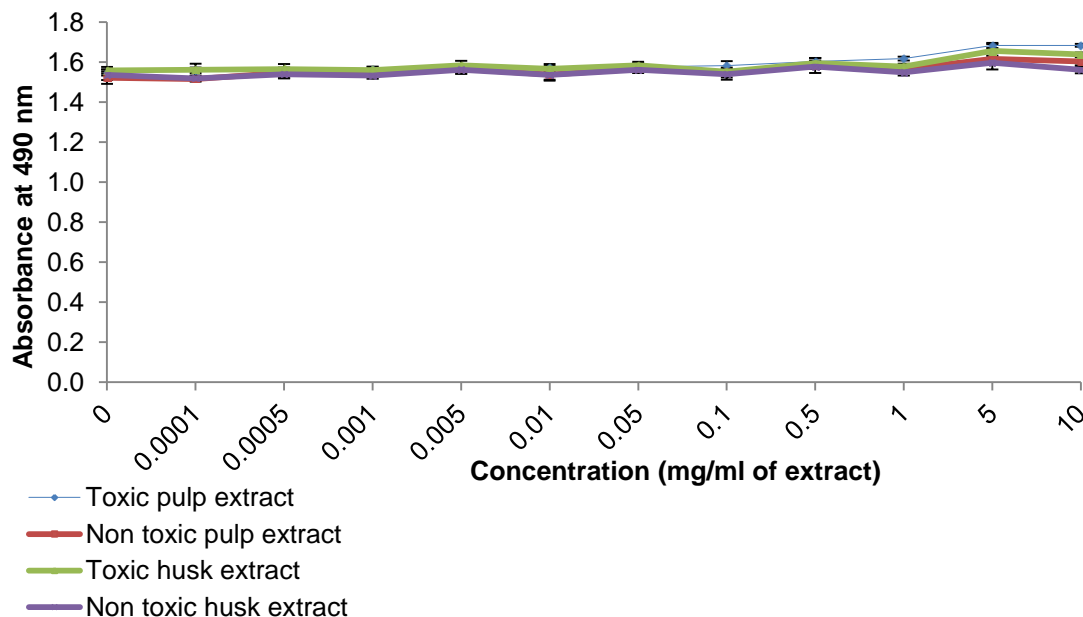
Figure 9. Evaluation of cellular toxicity of toxic and non-toxic methanolic pulp and husk extracts from *D. senegalense* on J774 A1 cells of murine macrophages after 6 h treatment (means and standard deviation for 6 assays).
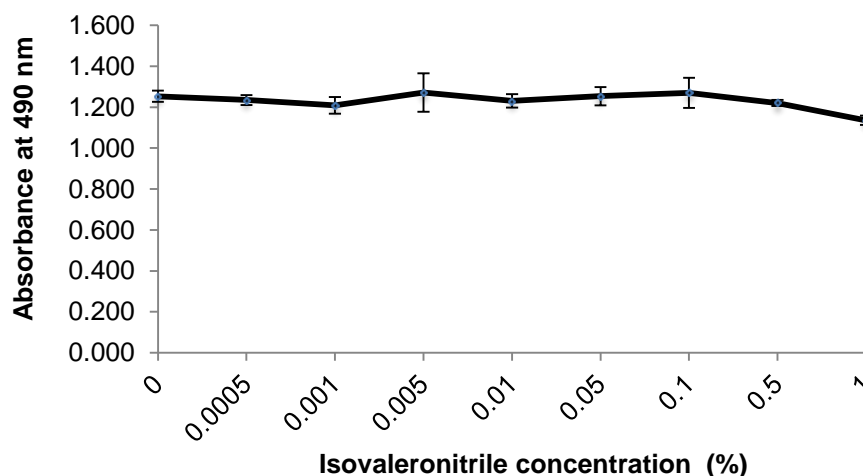
Figure 10. Evaluation of cellular toxicity of isovaleronitrile pure standard on J774 A1 cells of murine macrophages after 6 h treatment (means and standard deviation for 6 assays).

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Full Length Research Paper

Production and characterization of β-glucosidase from *Gongronella butleri* by solid-state fermentation

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Among the enzymes of the cellulolytic complex, β-glucosidases are noteworthy due to the possibility of their application in different industrial processes, such as production of biofuels, winemaking, and development of functional foods. This study aimed to evaluate the production and characterization of β-glucosidase from the filamentous fungus *Gongronella butleri,* recently isolated from Cerrado soil and cultivated in agro-industrial residue substrates. The highest production of β-glucosidase, about 215.4 U/g of dry substrate (or 21.5 U/mL), was obtained by cultivation of the microorganism on wheat bran with 55% of the initial moisture, for 96 h at 30°C. This β-glucosidase showed higher catalytic activity at pH 4.5, and a temperature of 65°C. The original enzymatic activity was recovered in a pH range of 3.0-7.5 after 24 h of incubation. The enzyme retained 80% of its catalytic activity when incubated for 1 h at 50°C. The enzyme was strongly inhibited by glucose, an effect that was completely reversed by increasing substrate concentration in the reaction mixture, which is typical for competitive inhibition. High catalytic activity was observed in solutions containing up to 20% ethanol, allowing the application of this enzyme in processes with high alcohol concentrations (for example beverages and biofuels). The significant production of β-glucosidase by the selected strain, along with these enzyme characteristics, highlights the biotechnological potential of the fungus *G. butleri*.

Key words: Microbial enzyme, biofuels, agro-industrial residues, cellulases, hemicellulases.

INTRODUCTION

Agro-industrial residues are usually discarded in the environment, yet their composition allows the use of such materials as nutrient sources in fermentation processes.

In this context, solid-state fermentation is essential in the use of residues as substrates for the synthesis of various compounds of industrial interest, in addition to being

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beneficial to the environment (Deswal et al., 2011; Pereira et al., 2015a).

Lignocellulosic biomass is the most abundant renewable source found in nature, and is mainly composed of cellulose, hemicellulose and lignin (Pauly and Keegstra, 2010). Cellulose is a linear homopolysaccharide that constitutes of glucose molecules linked through β-glycosidic bonds of the type $(1 \rightarrow 4)$ (Huber et al., 2006; Thakur and Thakur, 2014). For efficient conversion of cellulose into fermentable sugars, an enzymatic system constituted of at least three groups of enzymes is necessary: (1) endoglucanases (EC 3.2.1.4), which internally hydrolyze cellulose chains, resulting in a rapid reduction in the degree of polymerization; (2) exoglucanases (EC 3.2.1.91), which hydrolyze glycosidic bonds at the ends of chains, releasing principally cellobiose; and (3) β-glucosidases (EC 3.2.1.21), which finalize hydrolysis, converting cellobiose to glucose. The glucose from lignocellulosic biomass can be converted into ethanol through fermentation by microorganisms, which has aroused great interest in biofuel industries (Bansal et al., 2012).

β-Glucosidase has the ability to increase the overall yield of fermentable sugars (Rani et al., 2014) while reducing the cellobiose inhibitory effect on other cellulolytic enzymes, promoting continuity of enzymatic hydrolysis process (Rani et al., 2014; Borges et al., 2014). These enzymes have many applications in industrial processes, including the conversion of isoflavone glycosides into aglycones, which have antioxidant activities and are more easily absorbed by the human intestine. Studies have shown that the use of these aglycones confer health benefits, including the prevention of certain types of cancer and a reduction in the risk of cardiovascular disease, osteoporosis, menopausal symptoms and diabetes, among others (Leite et al., 2007; Singhania et al., 2013). The addition of microbial β-glucosidase in winemaking processes promotes the release of volatile terpenes (deglycosylated by the action of the enzyme), contributing to the aromatic composition of the wine (Alves-Prado et al., 2011).

The high cost of enzymes is a major factor limiting the use of these biocatalysts on a large scale. Increasing productivity while reducing the cost of production are crucial factors for the industrial application of an enzyme (Romero et al., 2007; Oliveira et al., 2015). In this context, this study aimed to optimize the parameters in solid-state fermentation of the filamentous fungus *Gongronella butleri*, aimed at producing β-glucosidase. The biochemical characteristics of β-glucosidase and the catalytic profile of enzymatic extract produced were also assessed.

MATERIALS AND METHODS

Microorganism

In this study, the filamentous fungus mesophilic *Gongronella butleri*

was used. The fungus was isolated from soil samples collected from the Cerrado biome, located in Dourados-MS (Brazilian Midwest-22°10'49.2''S 54°56'57.4W). Soil samples were serially diluted (10^{-1}-10^{-4}) in a solution of sodium chloride (0.9%) and plated on Sabouraud Dextrose Agar (dextrose 4%, mycological peptone 1% and agar 1.5%), aiming to obtain purified strains. The selected strain was identified by Brazilian Collection of Microorganisms of Industry and Environment (Coleção Brasileira de Microrganismos de Indústria e Meio Ambiente – CBMAI), at UNICAMP, Campinas, SP. The fungus was cultivated at 28°C in Sabouraud Dextrose Agar and maintained at 4°C.

β-Glucosidase production by solid-state fermentation

Inoculum

The fungus was cultivated in 250 mL Erlenmeyer flasks containing 40 mL of Sabouraud Dextrose Agar, and incubated for 48 h at 28°C. Microorganism suspension was obtained by scraping the surface of the culture medium using 25 mL of nutrient solution (0.1% ammonium sulfate, 0.1% magnesium sulfate heptahydrate and 0.1% ammonium nitrate, w/v) (Merheb-Dini et al., 2009). The fungus was inoculated in the agro-industrial residues by transfer of 5 mL of the microbial suspension (10^5 spores/g of dry substrate). The inoculum volume was considered to calculate the initial moisture in solid-state fermentation processes.

Solid-state fermentation

Several agro-industrial residues were tested for β-glucosidase production: wheat bran, soy bran, rice peel, corn cobs and corn straw. Prior to inoculation with microorganism, all substrates were washed with distilled water and dried at 50°C for 48 h. Fermentation was performed in 250 mL Erlenmeyer flasks containing 5 g of substrate sterilized at 121°C for 20 min. Initially, all substrates were moistened to 60% with nutrient solution (previously described). After inoculation of the microorganism, Erlenmeyer flasks were incubated at 28°C for 96 h. The substrate that showed the best β-glucosidase production by the fungus was selected for the evaluation of other fermentation parameters (e.g., moisture, temperature and time of cultivation) whose optimized values were adopted in subsequent assays. All the assays were performed in duplicate. The results were expressed as U/g refer to enzymes units per gram of dry substrate.

Enzyme extraction

Extraction of the enzyme from the fermented substrate was carried out by adding 50 mL of distilled water, and then shaking constantly at 150 rpm for 1 h. This mixture was then filtered through synthetic tissue (nylon) and centrifuged at 1500 ×g for 5 min at 5°C. The supernatant was used for the enzymatic assays.

Determination of β-glucosidase activity

β-Glucosidase activity was determined with 50 μL of enzymatic extract, 250 μL of 0.1 M sodium acetate buffer (pH 4.5), and 250 μL of 4 mM p-nitrophenyl β-D-glucopyranoside (pNPβG, Sigma), incubated for 10 min at 50°C. The enzymatic reaction was stopped by addition of 2 mL of 2 M sodium carbonate, and the liberated product was spectrophotometrically quantified at 410 nm using a 4-nitrophenol standard curve. One unit of β-glucosidase was defined as the amount of enzyme that releases 1 μmol of nitrophenol per minute of reaction (Palma-Fernandez et al., 2002).

Protein determination

Protein content was measured, following the method of Hartree (1972), using bovine serum albumin (BSA) as standard.

Biochemical characterization of β-glucosidase

Effect of pH and temperature

The optimum pH was determined by measuring the enzyme activity in 0.1 M citrate-phosphate buffer solution at 50°C and pH values of 3.0-8.0. The optimum temperature was determined by measuring the enzyme activity at temperatures from 35 to 70°C, at the optimal pH of the enzyme. The pH stability was determined by incubating the enzyme for 24 h at 25°C at different pH values (3.0-10.5). The buffers used (0.1 M) were McIlvine buffer (pH 3.0-8.0), Tris-HCl (pH 8.0-8.5) and Glycine-NaOH (pH 8.5-10.5). Thermostability was studied by incubating the enzyme for 1 h at different temperatures (35-70°C) at McIlvine buffer pH 4,5. After incubation, aliquots were withdrawn and cooled on ice bath. Residual activities were measured under optimal conditions of the enzyme.

Effect of ethanol and glucose on β-glucosidase activity

Enzymatic activities were quantified with the addition of glucose or ethanol at different concentrations in the reaction mixture (0-35 mM glucose or 0-30% ethanol). Assays were performed under optimal conditions of pH and temperature.

Catalytic potential of the enzymatic extract

Carboxymethylcellulase (CMCase) and xylanase activities were determined by adding 100 µL of enzyme extract in 900 µL of 0.1 M sodium acetate buffer (pH 4.5) containing 3% carboxymethyl-cellulose (Sigma C5678) and 0.5% xylan (Sigma Birch -Wood), respectively. After 10 min of reaction, the reducing sugar released was quantified by the DNS method (3,5-dinitrosalicylic acid) (Miller, 1959) using glucose or xylose standard curve, respectively. β-Xylosidase activity was measured with the synthetic substrate p-nitrophenyl β-D-xylopyranoside (4 mM; Sigma), following the methodology previously described for β-glucosidase. Assays were performed in 0.1 M sodium acetate buffer (pH 4.5) at 50°C. One unit of enzymatic activity was defined as the amount of enzyme capable of producing 1 µmol of product per minute of reaction.

RESULTS AND DISCUSSION

β-Glucosidase production by solid-state fermentation

Different agro-industrial residues were tested for β-glucosidase production by the fungus *G. butleri*, for which other fermentation parameters were fixed, including moisture (60%), temperature (28°C) and cultivation time (96 h). The highest enzyme production (213.8 U/g dry substrate) and specific activity (6.39 U/mg of protein) was obtained using wheat bran as substrate. Other residues evaluated in this study showed no significant production when compared with wheat bran (Table 1). A previous study confirmed the potential for β-glucosidase production by microorganisms of the genus *Gongronella*, when

Table 1. β-Glucosidase production by *G. butleri* on agro-industrial residues by solid-state fermentation, 60% moisture, 28°C for 96 h of cultivation. The data presented were averages of two experiments.

Substrate (Residues agro-industrial)	β-glucosidase (U/g dry substrate)	Specific activity (U/mg of protein)
Rice peel	0.13±0.02	0.034±0.007
Wheat bran	213.6±0.16	6.39±0.39
Soy bran	9.76±0.11	0.6±0.05
Corn straw	2.26±0.06	0.24±0.02
Corn cob	2.00±0.27	0.108±0.003

grown in wheat bran (Fang et al., 2014).

The increased production of the enzyme on wheat bran can be attributed to the fact that this residue is rich in proteins, cellulose, inorganic salts, nitrogen and carbon. Wheat bran acts as a source of nutrients and support for mycelial adhesion of the filamentous fungi, being similar to their natural habitat (Abdella et al., 2014). The nutritional complexity of this residue favors the growth of many microorganisms, and production of several enzymes of industrial interest (Haque et al., 2002). Considering the results shown in Table 1, wheat bran was selected as substrate for subsequent optimization of parameters that affect enzymatic production.

Among the evaluated temperatures, the optimal production of β-glucosidase by the fungus *G. butleri* was obtained in cultivations at 30°C, reaching about 211.7 U/g (Figure 1A). The optimum temperature for enzyme production does not always correspond to the temperature of the natural habitat of the microorganism (Kheng and Omar, 2005). High or very low temperatures can slow down the growth of a microorganism, and thereby the formation of product. The low thermal conductivity of agro-industrial residues used in solid-state fermentation processes can hinder the dissipation of metabolic heat generated by microbial growth (Pandey et al., 2003). Thus, analysis of the cultivation temperature is essential to delineate a bioprocess to produce enzymes, considering that the biological catalysts are denatured at high temperatures.

The fungus *G. butleri* was also grown on wheat bran with different moisture levels. As seen in Figure 1B, the optimal production of β-glucosidase was obtained in cultivations containing 55 or 60% initial moisture (w/v). In cultures containing 80% moisture, enzyme production by the microorganism was near zero (Figure 1B), indicating that higher levels of moisture were unfavorable for enzyme production.

Moisture content has key role in the success of solid-state fermentation processes, and may vary according to the substrate and the microorganism used (Kalogeris et al., 2003). The moisture of the medium must contribute to the growth of the microorganism, which does not occur at very low levels. However, excess moisture reduces the porosity of the medium and hampers gas exchange,

A

B

C
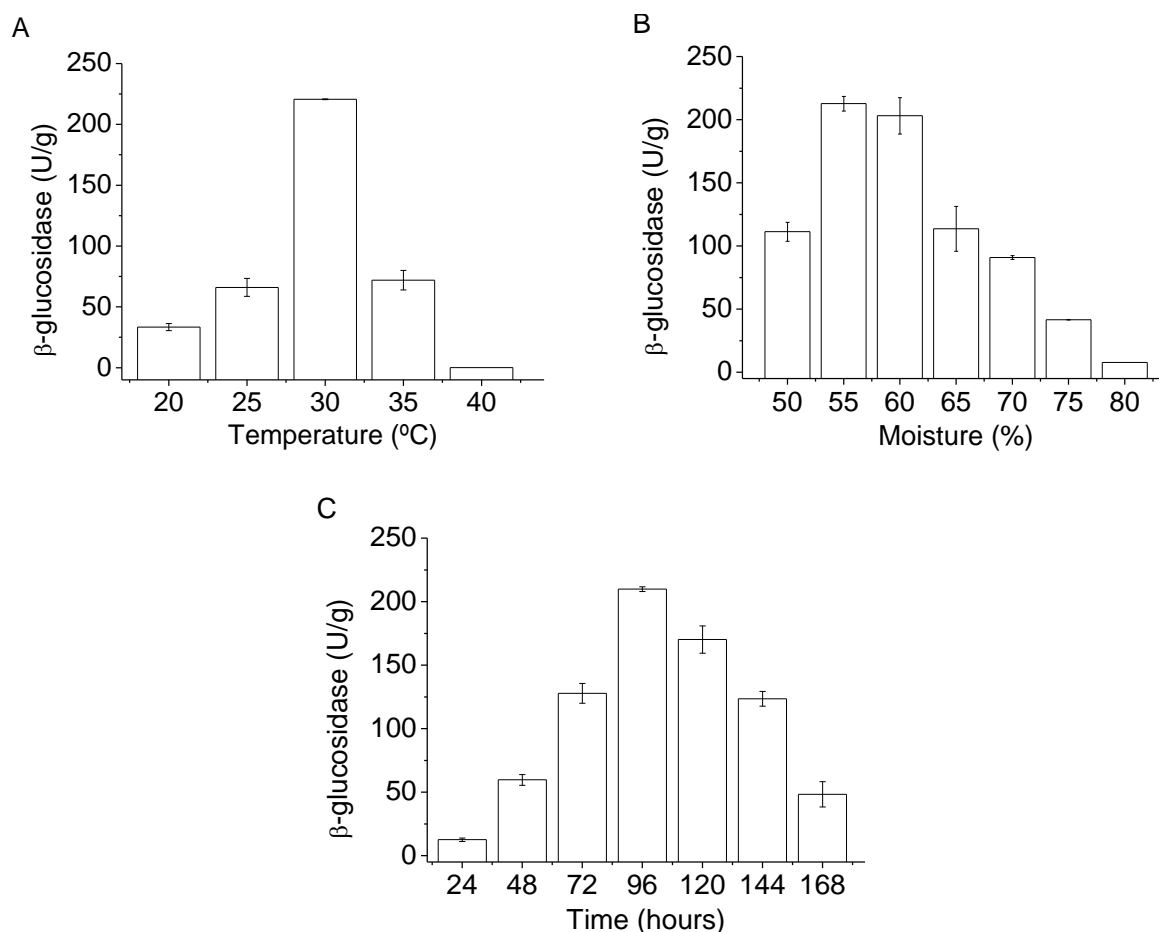
Figure 1. β-Glucosidase production by *G. butleri* on solid-state fermentation using wheat bran as substrate. **(A)** Influence of cultivation temperature. **(B)** Influence of substrate moisture. **(C)** Influence of cultivation time. The data presented are averages of two experiments.

resulting in the reduction of microbial activity and consequently the production of enzymes (Bansal et al., 2012).

Cultivation time was the last parameter evaluated. Production of β-glucosidase was highest at 96 h of cultivation, reaching about 215.4 U/g dry substrate (or 21.5 U/mL). After this period, enzymatic activity fell considerably (Figure 1C); the decline in enzyme production after 120 h of cultivation can be related to the reduced amount of nutrients and metabolic changes of fungus, resulting in the denaturation of enzymes (Bon et al., 2008).

Higher enzyme production was observed with only 96 h of cultivation, confirming the reduced time to obtain enzymes of microbial origin, when compared with plant and animal enzymes, which highlight the trend of the use of microbial enzymes in industrial processes (Oliveira et al., 2006). When compared with other fungal strains, the optimum cultivation time for the production of β-glucosidase by the fungus *G. butleri* is considerably lower. For example, Pereira et al. (2015b) reported maximum enzyme production after 192 h of solid-state

cultivation of the fungus *Thermomucor indicae-seudaticae* N31. Leite et al. (2008) had higher production of β-glucosidase at 120 h of solid-state cultivation by the yeast *Aureobasidium pullulans*.

Production of β-glucosidase by the fungus *G. butleri* was extremely high, about 215.4 U/g of dry substrate, as compared to previously reported data (Table 2). Considering the low incidence of studies using this fungal species for the production of industrial enzymes, the β-glucosidase produced by *G. butleri* was biochemically characterized and, the catalytic profile of enzymatic extract was valued.

Biochemical characterization of β-glucosidase

Effect of pH and temperature

β-Glucosidase produced by the fungus *G. butleri* showed optimal activity at pH 4.5 and 65°C (Figure 2A and B). However, the half-life ($t_{1/2}$) of the enzyme was

Table 2. β-Glucosidase production by different microorganisms on solid-state fermentation using agro-industrial residues as substrates.

Microorganism	Substrate	β-Glucosidase (U/g)	Authors
Gongronella butleri	Wheat bran	215.4	This study
Thermoascus aurantiacus	Wheat straw	61.6	Xin and Geng (2010)
Aspergillus niger KK2	Rice straw	94	Kang et al. (2004)
Aureobasidium pullulans	Wheat bran	13	Leite et al. (2008)
Aspergillus terreus M11	Corn residues	119	Gao et al. (2008)
Lichtheimia ramosa	Wheat bran	172.6	Gonçalves et al. (2013)
Lichtheimia ramosa	Pequi	0.61	Silva et al. (2013)
Gongronella sp. W5	Wheat bran	49.9	Fang et al. (2014)
Thermomucor indicae-seudaticae N31	Soybean meal and rice husk	41.8	Pereira et al. (2015b)

approximately 36 and 12 min., at 60 and 65° C, respectively (Figure 2E), suggesting the enzyme employment at 60°C. The enzyme retained its original activity after 24 h of incubation over a pH range of 3.0-7.5 (Figure 2C). After incubation for 1 h at 50°C, about 80% of the catalytic potential was recovered. When incubated at 55°C for the same period, the enzyme maintained 57% of its original activity (Figure 2D).

The characteristics of the β-glucosidase produced by the fungus *G. butleri* are similar to those described in the literature for β-glucosidase produced by mesophilic and thermophilic fungi. Leite et al. (2008) reported optimal pH values of 4.5 and 4.0 for β-glucosidase produced by the fungi *A. pullulans* and *Thermoascus aurantiacus*, respectively. Lin et al. (1999) obtained the same optimum temperature for β-glucosidase produced by the fungus *Thermomyces lanuginosus* SSBP. Garcia et al. (2015) reported optimal activity at pH 5.5 and 65°C for β-glucosidase produced by the fungus *Lichtheimia ramosa*; further, the enzyme retained its catalytic activity after 1 h at 55°C. Camassola et al. (2004) showed that the highest activity of β-glucosidase produced by *Penicillium echinulatum* occurred between 55 and 60°C. Yun et al. (2001) reported the production of extracellular β-glucosidase by the fungus *Trichoderma harzianum*, and demonstrated that the enzyme was stable at temperatures below 55°C for 15 min, keeping only 36% of the initial activity after 15 min at 60°C. Delabona et al. (2013) described the stability of β-glucosidase produced by the fungus *Aspergillus fumigatus* P40M2 at temperatures of 40-60°C and pH levels 3.0-5.5. The β-glucosidase from *Penicillium funiculosum* NCL1 showed optimum activity at pH 4.0 and 5.0, optimum temperature of 60°C, and exhibited a half-life of 1 h at 60°C (Ramani et al., 2012).

Effect of ethanol and glucose on β-glucosidase activity

As shown in Figure 3A, ethanol concentrations near 5% potentiated the enzymatic activity as compared to the control, increasing levels to approximately 40%. The enzyme showed catalytic activity similar to the control in solutions containing 15% ethanol, yet when the ethanol concentration was increased to 25%, only 62% of the original enzymatic activity was recovered (Figure 3A).

β-Glucosidases can be applied in industrial processes that contain alcohol (e.g., production of biofuels and beverages), which makes ethanol tolerance an extremely valuable feature for this group of enzymes (Sun and Cheng, 2002). The characteristics described in this study allow us to infer that the β-glucosidase produced by *G. butleri* can be applied in processes which have alcoholic fermentation as their central metabolic route, considering that the final ethanol concentration in fermented broths obtained by traditional processes is about 10%. Alcoholic concentrations greater than this are generally harmful to the fermenters organisms (Gu et al., 2001).

The enzyme was strongly inhibited by glucose, showing only 50% of its catalytic activity in solutions containing 10 mM glucose (Figure 3B). However, when the concentration of substrate (pNPβG) was increased to the same concentration of the inhibitor (glucose), inhibition of the enzyme was completely reversed (Table 3), indicating that the inhibitor and the substrate compete for the same binding site, typical characteristic of competitive inhibition (Leite et al., 2008).

Most microbial β-glucosidases are inhibited by glucose, which may be competitive or non-competitive (Leite et al, 2008; Sonia et al., 2008). Competitive inhibition can be controlled by periodic addition of substrate to keep it at a concentration exceeding the levels of the inhibitor (product of the enzymatic action); alternatively, the product formed by the action of the enzyme can be removed to maintain it at concentrations below the substrate (Bon et al., 2008; Garcia et al., 2015). The characteristics described for the β-glucosidase of fungus *G. butleri* (ethanol tolerance and competitive inhibition of glucose) enable its application in simultaneous saccharification and fermentation processes, with the aim of obtaining ethanol from vegetal biomass. In this type of process, the glucose liberated by enzymatic hydrolysis of
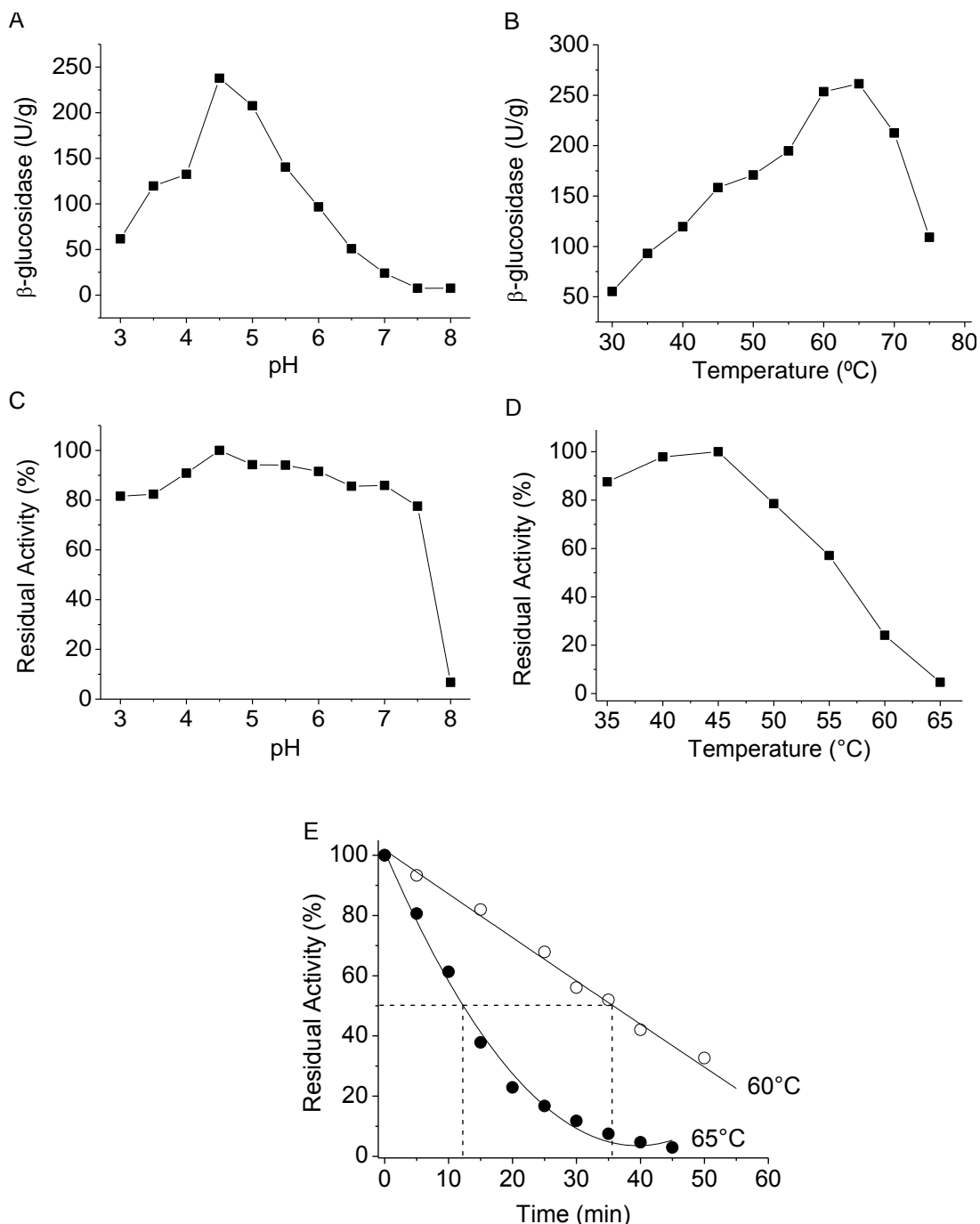
Figure 2. Biochemical characterization of β-glucosidase produced by *G. butleri*. (A) optimum pH. (B) optimum temperature. (C) pH stability. (D) Temperature stability.

cellulose is simultaneously converted to ethanol by microbial fermentation, minimizing inhibition of the enzymes of the cellulolytic complex (Scott et al., 2013).

Catalytic potential of the enzymatic extract

The catalytic potential of enzymatic extract obtained

under optimal conditions of cultivation was evaluated. Among the enzymes of the cellulolytic and hemicellulolytic complexes that were evaluated, high xylanase production by the fungus *G. butleri* was noted, reaching about 4303 U/g dry substrate (or 430.3 U/mL) (Table 4). The xylanase values obtained in this study are considerably high when compared with those previously reported. For example, Maciel et al. (2008) reported the production of
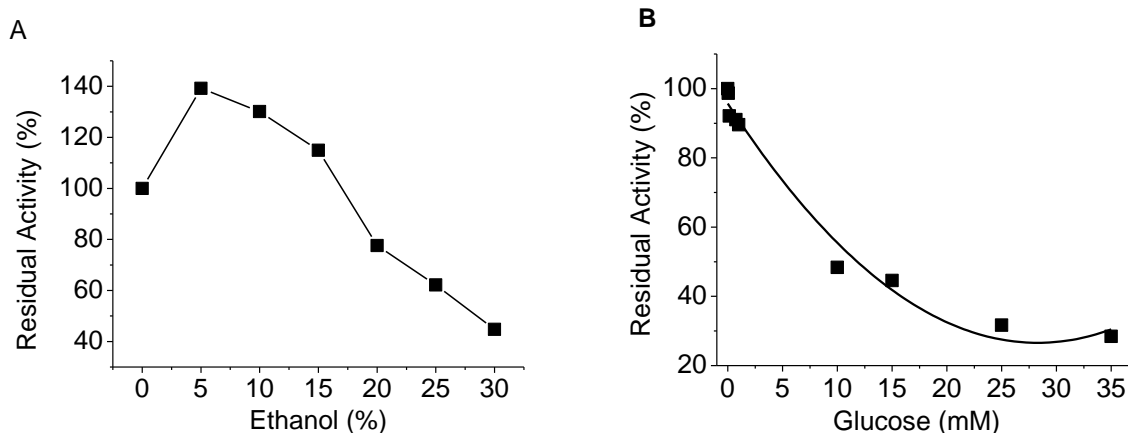
Figure 3. Effect of ethanol (A) and glucose (B), on the activity of β-glucosidase produced by *G. butleri*.

Table 3. Residual activity for different concentrations of substrate (pNPβG) and inhibitor (glucose).

Enzyme	Residual activity (%) pNPβG – 2mM	Residual activity (%) pNPβG – 2 mM Glucose- 10 mM	Residual activity (%) pNPβG – 10 mM; Glucose – 10 mM	Inhibition type
G. butleri	100	48.68	107.78	Competitive

Table 4. Catalytic potential of the enzymatic extract produced by *G. butleri* in wheat bran, 55% of moisture, 30°C for 96 h of cultivation.

Enzyme	U/mL	U/g
CMCase	1.390	13.90
β-glucosidase	21.54	215.4
Xylanase	430.39	4303.9
β-xylosidase	2.84	28.4

xylanase by the fungus *A. niger* LPB 326 in solid-state fermentation, to be about 3099 IU/g of substrate. Rezende et al. (2002) obtained 288 U/mL of xylanase by solid-state fermentation of the fungus *T. harzianum* in sugar cane bagasse and Da-Silva et al. (2005) produced 107 U/mL xylanase by the fungus *T. aurantiacus* using corncob as substrate. Sadaf and Khare (2014) reported xylanase production of 1025 U/g of substrate by the fungus *Sporotrichum thermophile* by solid-state fermentation.

Significant values for CMCase and β-xylosidase were not obtained in optimized cultivation conditions (Table 4). The catalytic profile of enzymatic extract described in this work is extremely desirable for application within the cellulose and paper industries. Enzymatic extract with high concentrations of xylanase and reduced cellulase activity can be used for bleaching Kraft pulp, in order to obtain white paper. The presence of cellulases can reduce the quality of paper because they degrade cellulose fibers. Hydrolysis of xylan facilitates the removal of residual lignin from pulp, thereby reducing the use of conventional chemical agents that contribute to the environmental damage caused by the effluents of the paper industry (Xin and He, 2013).

Conclusions

The results obtained in this study highlight the fungus *G. butleri* as an excellent β-glucosidase producer, especially when cultivated on wheat bran. The characteristics of this β-glucosidase enable the application of this enzyme in different industrial processes, such as hydrolysis of cellulose for production of second-generation ethanol, or in the food and beverage industry. The enzymatic extracts obtained under optimal growing conditions showed high xylanase activity with reduced cellulolytic potential, making them highly applicable for improvement of cellulose pulp bleaching processes.

Conflict of interest

The authors declare that there is no conflict of interests.

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Full Length Research Paper

Comparison of the properties of collagen extracted from dried jellyfish and dried squid

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With increased recent interest in the utilization of industrial by-products, finding different sources, optimizing extracting conditions and characterization of collagen extracts have recently become important research topics. This study addresses the isolation of acid-soluble and pepsin-soluble forms of collagen from dried jellyfish and squid, and their partial characterization. The properties of these proteins have been studied and a comparison made of the protein patterns of collagen extracted from marine organisms with those from other organisms, to determine which collagen subtypes are present, and in what proportions. Pepsin-soluble collagen (PSC) from dried jellyfish and dried squid contained a collagen form classified as type I, of molecular composition comparable with that of collagen type I from rat tail. Peptide maps of collagens digested by achromopeptidase were slightly different, indicating some differences in amino acid sequence or conformation. The collagen showed high solubility at acidic pH (4-5) but its solubility markedly decreased in the presence of sodium chloride (NaCl) up to 2%. Collagen type I from dried jellyfish and dried squid could be a useful alternative to mammalian collagen, with potential use in the biomedical, pharmaceutical and nutraceuticals industries.

Key words: Collagen, pepsin-soluble form, acid-soluble form, partial characterization.

INTRODUCTION

Collagen is a major class of structural proteins in bone, skin, cartilage and connective tissue (Liu et al., 2007; Ogawa et al., 2003; Bateman et al., 1996). It plays an important role in tissue development and is the most abundant protein in vertebrates, constituting about 30% of the total. Collagen finds a wide range of applications in the food, cosmetic, biomedical, pharmaceutical, leather

and film industries. The collagens from different tissues vary considerably in polypeptide chain composition, amino acid composition and physiochemical characteristics, thereby meeting the specific functional requirements of the tissues. Collagen is unique in its ability to form insoluble fibres ('fibrils') of high tensile strength (Gelse et al., 2003). Some 28 distinct vertebrate

collagen types have been identified (Gordon and Hahn, 2010; Birk and Bruckner, 2005), although not all of these form fibrils. The structure of mature collagen, known as tropocollagen, consists of three polypeptide chains, called α-chains, intertwined in the so-called collagen triple-helix, each chain being coiled in a left-handed helix, and wound around two others to form a right-handed triple super-helix. After secretion from cells, collagen fibrils are further polymerized through the subsequent formation of covalent cross-links. The major collagenous component of vertebrate bones and tendons is type I, of cartilage type II, of skin type III and of basement membranes type IV; in each case several minor collagen types are also present (Kimura et al., 1976; Mayne and Zettergen, 1980). With the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot and mouth disease (FMD), there has been an increased demand for non-mammalian collagen for food markets, as well as for a wide range of other applications. As a consequence, alternative sources of collagen, especially from aquatic animals (Nagai and Suzuki, 2000; Sathivel et al., 2003), including freshwater and marine fish, have received increasing attention.

Many reports focus on the isolation and determination of the biochemical and physiochemical properties of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) extracted from sea organisms such as squid (Mario et al., 2010; Ilona et al., 1999; Mingyan et al., 2009; Thanonkaew et al., 2006; Nagai et al., 2001), bigeye snapper fish (Nalinanon et al., 2007), jellyfish (Sourour et al., 2011; Nagai et al., 2000; Miura and Kimura, 1985; Zhuang et al., 2009; Krishnan and Perumal, 2013; Thanonkaew et al., 2006; Addad et al., 2011) and others (Zelechowska et al., 2010; Byun and Kim, 2001; Je et al., 2007). Squid and jellyfish have been used as food for thousands of years, and represent a renewable marine resource with a high nutritional value. If a suitable extraction procedure can be developed, squid could potentially become a significant source of collagen. From the medical point of view, the analysis of collagens is of great importance; the use of collagen in biochemical applications has also grown rapidly and expanded into biomaterials with biocompatibility and biodegradability. From the analytical point of view, there are several methods for determining collagen and collagen types, the most common being based on the quantitation of hydroxyproline, which accounts for approximately 10% of the collagen molecule. The amino acid composition can also be analyzed by High Performance Liquid Chromatography (HPLC). Determination of the molar ratios of particular collagen types involves separation of the peptide mixture produced by enzymatic digestion, using various separation methods, such as Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) or HPLC, and their detection by Mass Spectrometry (MS) or

Liquid chromatography/electrospray ionization-mass spectrometers (LC/ESI-MS) (Guifeng et al., 2006) and High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) (Pataridis et al., 2008) which enable the analysis of marker peptides in peptide mixtures produced by cyanogen bromide/trypsin digestion. Another analytical method involves the radioactive labeling of proline and enzyme immunoassay by collagen-specific antibodies (Van der et al., 1994).

The aim of the present study is to compare the acid-soluble and pepsin-soluble isolated forms of collagen from dried jellyfish and dried squid, and their properties. It should be possible to determine which collagen subtypes are present in collagen extracted from dried jellyfish and dried squid, and the functional properties of these proteins will be studied and a comparison made of collagen extracted from marine organisms with those from other organisms, as marine collagens could be used as alternative sources of high quality collagen in applications within the pharmaceutical and nutrition industries.

MATERIALS AND METHODS

Experimental samples

Dried squid and dried jellyfish bought from a local market, Bangsean, Chonburi, Thailand were used in this study.

Preparation of collagen

Extraction of acid-soluble collagen (ASC)

Duplicate samples were washed in distilled water at 4°C with continuous stirring for 3 days, and the flesh cut into small pieces and soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) at 4°C for a further 3 days. The mixture was filtered through two layers of cheesecloth and the residue re-extracted under the same conditions. The two filtrates were combined. Collagen was precipitated by adding 0.9M NaCl with 0.05M Tris (hydroxymethyl) aminomethane buffer, pH 7.0. The resultant precipitate was collected by centrifugation at 20,000xg for 60 min, then dissolved in a minimal volume of 0.5 M acetic acid and dialyzed against 50 volumes of 0.1 M acetic acid for 3 days, followed by the dialysis against the same volume of distilled water for another 3 days. The dialysate was freeze-dried and is referred to as acid-soluble collagen (ASC). The yield of ASC was calculated from the dry weight of collagen extraction in comparison with the wet weight of the initial fresh squid used.

Extraction of pepsin-soluble collagen (PSC)

The undissolved residue obtained after ASC extraction was used for further extraction with the gastric proteinase pepsin. The residue was soaked in 0.5M acetic acid with a solid/solvent ratio of 1:15 (w/v) and pepsin (20 U/g residues) added. The mixture was continuously stirred at 4°C for 3 days, followed by filtration through two layers of cheesecloth. The filtrate was subjected to precipitation and the pellet dialyzed, as previously described for ASC. The dialysate was freeze-dried and is referred to as pepsin-soluble collagen (PSC). The yield of PSC was calculated in the same

manner as ASC. Additionally, the accumulated yield of collagen was calculated from the total yield of ASC and PSC.

Protein quantitation and electrophoresis

Quantitation of total protein

The protein contents of the clear protein samples were determined using the Coomassie Plus (Bradford) protein assay kit (Thermo scientific, USA), with bovine serum albumin as a standard (Bradford, 1976).

Molecular weight determination

All samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), 5 □g of each protein sample being separated according to the method of Laemmli (1970). The separation was performed with a 10% separating gel and a 5% stacking gel using the miniVE Vertical electrophoresis system (GE Healthcare, U.S.A). The relative molecular mass (M_r) of each protein was determined using a standard curve generated from standard set of 12 pre-stained proteins in the range 3.5-260 kDa (Novex Sharp Protein Standard, Invitrogen, USA).

The gels were visualized with staining with Colloidal Coomassie Brilliant Blue G-250 (CBB) as described by Neuhoff et al. 1988. After gel electrophoresis, the gel was transferred into a fixative solution (50% ethanol, 2% Phosphoric acid in ddH$_2$O) for 1 h at room temp. The gel was stained with CBB (0.2% w/v) for 12-24 h by agitation on a shaker overnight. The staining solution was then removed. The gel was destained with several changes of ddH$_2$O.

Peptide mapping of collagen

Peptide mapping of ASC and PSC was performed according to the method of Kittiphattanabawon et al. (2005) with slight modification. A collagen sample (6 mg) was dissolved in 1 ml of 0.1 M sodium phosphate, pH 7.2 containing 0.5% (w/v) SDS, the mixture preheated at 45°C for 3 h and 300 μl of the preparation transferred to test tubes for digestion. To initiate the digestion, 20 μl of lysyl endopeptidase from *Achromobacter lyticus*, (5.0 μg/ml) was added to the mixture, which was then incubated at 37°C for 5 min. Proteolysis was stopped by boiling for 3 min. SDS-PAGE was performed by the method of Laemmli (1970) in 10% acrylamide NuPAGE Bis-Tris Mini Gels with MOPS buffer, followed by staining and destaining as previously described.

Collagen solubility

Collagen was dissolved in 0.5 M acetic acid to a final concentration of 3 mg/ml and the mixture stirred at 4°C until completely solubilized (Kittiphattanabawon et al., 2005).

Effect of pH on solubility

Four ml collagen solution (3 mg/ml) was transferred to 15 ml centrifuge tubes and either 6 N NaOH or 6 N HCl added to obtain the final pH, ranging from 1 to 10. The volume of solution was made up to 10 ml with deionized water previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 20,000 g for 30 min and the protein content of the supernatant determined. Protein solubility was calculated using the following equation.

Solubility= (protein content of the supernatant)/(total protein content of the sample)

Relative solubility= (solubility at given pH)/(highest solubility in the range of pH)

Effect of NaCl on solubility

Four ml collagen solution (3 mg/ml) was mixed with 1 ml of NaCl in 0.5 M acetic acid to give final concentrations of 0 to 12%. The mixture was stirred for 30 min, followed by centrifugation at 20,000 g for 30 min. Protein content in the supernatant was measured and the relative solubility calculated as previously described.

Statistical analysis

All experiments are replicated three times, and the results are presented as means ± standard deviation (SD). Analyses of variance (ANOVA) were performed and mean comparison was done by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Electrophoretic characterization of collagen is shown in Figure 1. The electrophoretic patterns of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) were examined by SDS-PAGE using 10% acrylamide Bis-tris gels. In the case of collagen extracted from jellyfish, the ASC fraction contained no high-molecular weight bands, although there was a band of molecular weight about 21 kDa, which was also found in the PSC fraction and bands of α_1 and α_2 chains were visible near 116 kDa in the PSC fraction. Bands below 116 kDa represent the products of enzymatic hydrolysis of collagen. In the case of squid, the PSC showed the typical SDS-PAGE pattern of type I collagen with two different α bands, α_1 and α_2. It also contains β and γ chains as well as other higher molecular weight cross-linked components, together with some higher molecular weight protein bands. In ACS extracts α_1 and α_2 chains were found. The band intensity of α_2 was approximately 2-fold higher than that of α_1, suggesting a $[\alpha_2]_2\alpha_1$ in collagen triple helix. We suggest that the collagen in both marine organisms are different type I collagens.

Peptide mapping

The peptide maps of both forms of extracted collagen from dried jellyfish and squid, after digestion by Achromopeptidase (37°C for 5 min), are shown in Figure 2, with collagen type I from rat tail for comparison. The band intensities of molecular weight cross-linked components β and γ component, of rat tail collagen (control collagen) slightly decreased with the appearance of 116 kDa peptide fragment. The results suggested that in both ASC and PSC, the α_1, α_2, β and γ chains were more resistant to digestion by Achromopeptidase than rat-tail collagen. It appears that more stringent conditions or different proteases will be required to degrade these
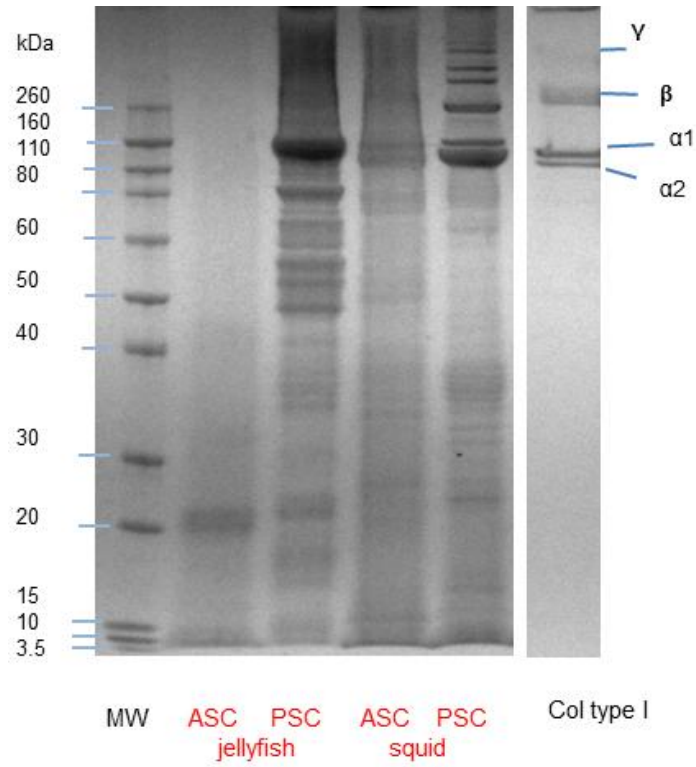
Figure 1. SDS-PAGE profiles of ASC and PSC extracted from jellyfish and squid.
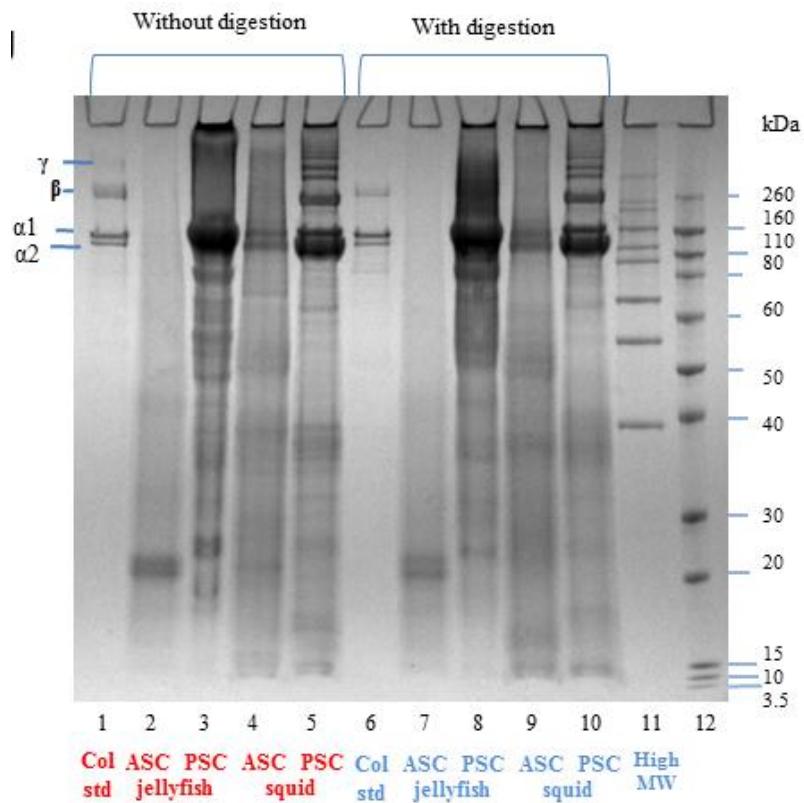
Figure 2. Comparison of jellyfish and squid collagen with/out digested.
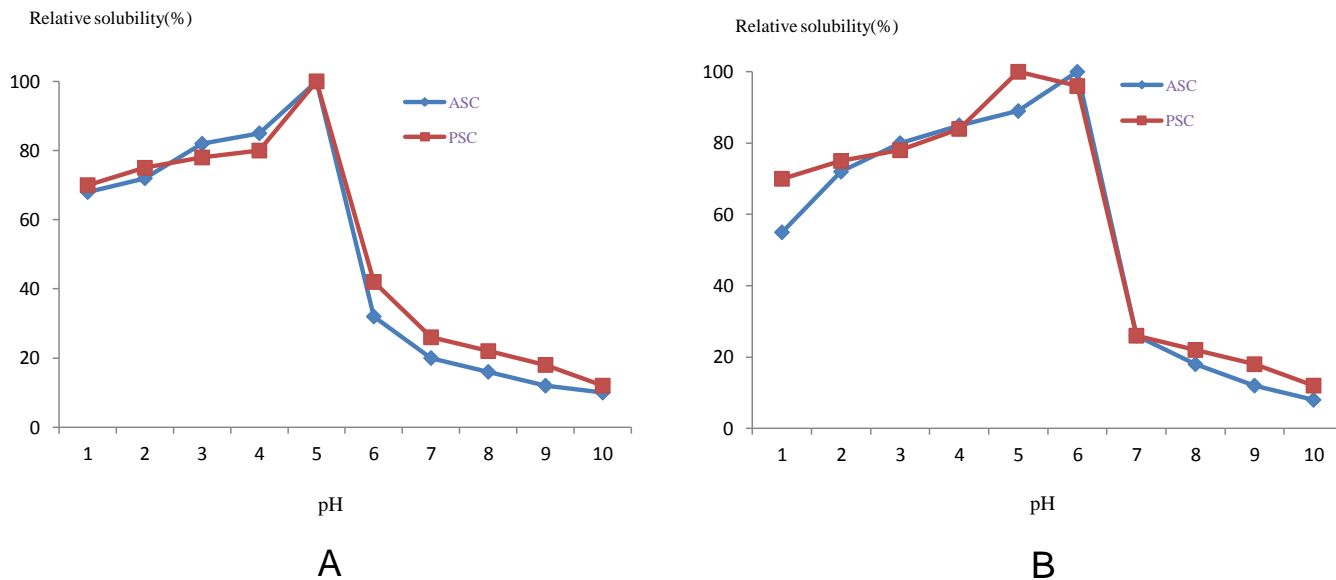
Figure 3. Relative solubility (%) of ASC and PSC extracted from jellyfish (A) and (B) at different pH values.
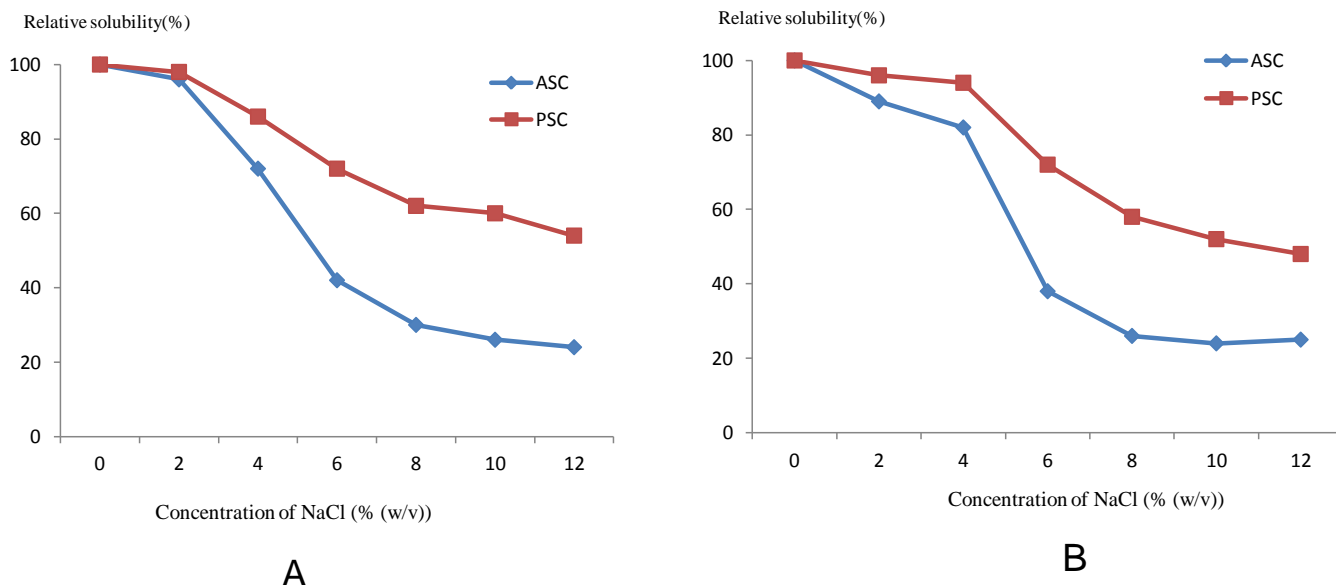
Figure 4. Relative solubility (%) in 0.5M acetic of ASC and PSC extracted from the Jellyfish (A) and squid (B) in the presence of NaCl at different concentration.

collagens. Thus, it was presumed that primary structures of collagens from the organism were quite similar in term of amino acid composition.

Solubility of collagens

The effects of pH and NaCl concentrations on collagen solubility are shown in Figures 3 and 4. The highest solubility of ASC and PSC from jellyfish and PSC from squid was at pH 5, whereas ASC from squid had a higher solubility at pH 6. A sharp decrease in solubility was observed at neutral pH. However, solubility was also slightly decreased at very acidic pH values. The solubility in 0.5 M acetic acid of both collagen fractions from jellyfish was maintained in the presence of NaCl up to 2%, whereas ASC and PSC fraction of squid maintained its solubility up to 4%. A marked decrease in solubility

was observed with increasing NaCl concentration, the well-known salting out effect. From these data, collagen from squid was more tolerant to salt than collagen from jellyfish.

In conclusion, PSC isolated from dried jellyfish and dried squid contained a collagen form classified as type I, of molecular composition comparable with collagen type I from rat tail. Squid collagen is a heteropolymer of α_1, α_2, β and γ chains, but, jellyfish PSC constitute mainly α_1 and α_2 chains. Peptide mapping of these collagens by digestion with Achromopeptidase was attempted but was largely unsuccessful, indicating some differences in amino acid sequence or conformation from rat-tail collagen. The marine collagens showed high solubility at acidic pH (4 to 5) and the solubility markedly decreased in the presence of NaCl up to 2%. Type I collagen has also been extracted from skin, bone, fins, and scales of fresh water and marine fishes, chicken skin and different marine animals such as squid, octopus, jellyfish, starfish and fish (Swatschek et al., 2002; Sadowska et al., 2003; Nagai et al., 2004; Falguni et al., 2010). Collagens from these sources were evaluated for their potential applications as alternatives to mammalian collagen. However, the properties of collagen vary markedly with the type of marine organism, indicating a need for characterization of this protein from different sources (Nagai and Suzuki, 2002; Kittiphattanabawon et al., 2005). In future work, collagen from jellyfish and squid will separated by 2DE following development of the extraction protocols. Then the protein spots of interest will excised from the polyacrylamide gels, digested with trypsin and analyzed by Matrix-assisted-laser-desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS). In the final step, the acquired peak lists will analyzed by searching NCBI database with Mascot software.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Chemical composition, antioxidant effects and antimicrobial activities of some spices' essential oils on food pathogenic bacteria

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Thymus vulgaris, Cinnamomum zeylanicum* and *Ocimum gratissimum* are spices widely used as aroma enhancers and food preservatives. This work assessed the chemical composition, antioxidant and antimicrobial effect of their essential oils on some food pathogenic bacteria, namely, *Staphylococcus aureus, Citrobacter freundii, Enterobacter cloacae, Morganella morganii, Escherichia coli, Salmonella typhimurium, Proteus vulgaris* and *Shigella flexineri*. After chemical analyses of the essential oils by gas chromatography and gas chromatography coupled to mass spectroscopy, the antimicrobial effects were subsequently assessed by disk and microdilution methods, while the antioxidant evaluations were performed by free radical scavenging activity. *T. vulgaris* essential oil composed of p-cymene (45.90%) and thymol (23.72%) which exhibited the highest inhibitory diameters of 20.33±0.58 and 18.00±1 mm, respectively, on the growth of *S. aureus* and *C. freundii*. *O. gratissimum* essential oil with thymol as major compound (47.11%) was more active to inhibit the growth of *C. freundii* and *S. flexineri* with respective inhibitory diameters of 18±1.73 and 16±2 mm. Essential oil from dry leaves of *C. zeylanicum* containing cinnamaldehyde (82.23%) and linalool (12.12%) was found to have the lowest values for minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) (≤3.53 mg/mL) considering the growth of *S. flexineri, C. freundii* and *E. cloacae*. Regarding the antioxidant effect, *C. zeylanicum* and *T. vulgaris* essential oil showed the most scavenging effect with half-maximal DPPH scavenging concentration (SC_{50}) of 2.5×10^{-5} and 6.5×10^{-5} mg/ml, respectively. Their antioxidant effects were higher than conventionally used antioxidants in food products, butylhydroxyltoluene (BHT) and vitamin C.

Key words: Spices, essential oils, chemical composition, antibacterial and antioxidant effects.

INTRODUCTION

Spices are used as flavour and aroma in many processed and cooked food. Different amounts of spices are used by different populations all over the world. Chemical components mainly belonging to the essential oil (EO)

fraction are responsible for the flavour and aroma of spices. Some of these compounds have medicinal properties and can exert bactericidal and bacteriostatic effects (Nwinyi et al., 2009). These antimicrobial effects

are provided by the presence of peptides, alkaloids and EO compounds, which are the major components in these plants (Okigbo and Igwe, 2007). Associated to these antimicrobial effects, spices have been attributed antioxidant properties. In fact, these activities due to compounds that, when present at low concentrations compared to that of an oxidizing substrate, markedly delay or prevent its oxidation (Cavero et al., 2005). Hence, food or supplements containing spices or their compounds may contribute to alleviate the pathologies associated with the presence of reactive oxygen species (ROS) originating from oxidative processes in human cells (Nwinyi et al., 2009). The whole plant, part of it, flowers or fruits are generally used as spices. Added to this, the different agro-ecological environment where the spices are produced can influence the composition and therefore their biological activity. Applications of spices for their antimicrobial and antioxidant properties have been proposed by some authors (Hernández-Ochoa et al., 2014; Burt, 2004; Essia Ngang et al., 2014) and these properties have been related to the composition of the spices (Hyldgaard et al., 2012). In this perspective, it is important to evaluate the effectiveness of the antimicrobial and antioxidant properties of spices typical for certain locality, because of important differences in activity that may occur as a result of the different chemical composition. This is the rational that brought us to assess the chemical composition, the antimicrobial and antioxidant properties of EOs from *Thymus vulgaris, Cinnamomum zeylanicum* and *Ocimum gratissimum*.

T. vulgaris is the most widely cultivated species of the Laminaceae family (Alonso, 2004; Al-Bayati et al., 2008). It is used as spice and traditional drug for antispasmolytic, antibacterial and antifungal illnesses (Özgüven and Tansi, 1998). Regarding *C. zeylanicum*, many species are reported throughout the world, but the most used are those from Sri-Lanka (Richard and Multon, 1992). Its EO is rich in cinnamaldehyde and eugenol and has shown antimicrobial activities on food borne pathogens (Paranagana et al., 2003; Chang et al., 2001). On the other hand, *C. zeylanicum* that belongs to the family of Leguminocaeae, has its origin in Asia (Ndoye, 2001). In many parts of Africa, the plant is used to cure upper respiratory tract infections, diarrhoea, pneumonia, teeth, and gum disorder (Nwinyi et al., 2009).

MATERIALS AND METHODS

Plant

Samples of *T. vulgaris, O. gratissimum* and *C. zeylanicum* were collected from spice sellers in Bafoussam (West region), Yaounde (Center region) and Lolodorf (South region) of Cameroon,

respectively. After collection, the spices were identified by the Cameroon National Herbarium and a voucher specimen deposited with the following codes N° 25746/SRF/Cam, 5817/SRF/Cam, and 22309/SRF/Cam for *T. vulgaris, O. gratissimum*, and *C. zeylanicum*, respectively. For *C. zeylanicum*, based on the work of Richard and Multon (1992), who attributed these activities to different parts of the plant, leaves and stem barks were separated for the study.

Preparation of EOs

The EOs of the spices were extracted by hydro distillation using a Clevenger apparatus. Oils recovered were dried over anhydrous sodium sulphate and stored at +4°C until usage. The extraction yield was calculated as the ratio of oil recovered mass over the spices mass used multiplied by 100 (Ndoye, 2001).

Chemical analyses of EOs

Gas chromatography (GC)

GC analyses were performed on a Varian gas chromatograph, model CP-3380, with flame ionization detector containing two silica capillary columns: HP5 J&W Agilent (5%-Phenyl-methylpolysiloxane) capillary column (30 m × 0.25 mm i.d. × 0.25 µm film) and Supelcowax 10 (polyethylene glycol) fused capillary column (30 m × 0.25 mm i.d. × 0.25 µm film); N_2 was the carrier gas at 0.8 ml/min; injection type 0.1 µl of pure sample, split ratio 1:100; injector temperature 220°C, detector temperature 250°C; temperature program 50 to 200°C at 5°C/min, then kept at 200°C for 10 min. The linear retention indices of the components were determined relative to the retention times of a series of *n*-alkanes.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed using a Hewlett–Packard GC 5890 series II equipped with a HP5 (5 % Phenylmethylpolysiloxane) fused silica column (30 m × 0.25 mm; film thickness 0.25 µm) and a DB-Wax fused silica column (30 m × 0.25 mm; film thickness 0.25 µm) interfaced with a quadrupole detector (Model 5972); temperature program (50 to 200°C at 5°C/min); injector temperature, 220°C; MS transfer line temperature, 180°C; carrier gas, helium at a flow rate of 0.6 ml/min; injection type, split, 1:10 (1 µl 10:100 CH_2Cl_2 solution); ionization voltage, 70 eV; electron multiplier 1460 eV; scan range 35 to 300 amu; scan rate, 2.96 scan/s.

Qualitative analysis

The identification of the constituents was based on comparison of their relative retention times and mass spectra with either that of authentic samples or with published data in the literature (Adams, 2007).

Preparation of test microorganism

Microbial strains of *Staphylococcus aureus, Salmonella*

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tiphymurium, Proteus vulgaris, Enterobacter cloacae, Shigella flexneri, Morganella morganii, Escherichia coli, and *Citrobacter freundii* were obtained from the culture collection of the Microbiology Laboratory of the Department of Microbiology, University of Yaoundé I, Cameroon. Strains, originally stored at –80°C were sub-cultured twice in Brain Heart Infusion (BHI) at 37°C for 24 h and conserved on Muller Hinton slants at 4°C throughout the work. These strains were chosen for their implication in food borne diseases outbreaks. Prior to each test, a loop of the strain was inoculated in Mueller Hinton broth and incubated at 37°C for 24 h. Based on preliminary assessments, the overnight cultures were diluted in order to use about 10^6 cells/ml for the antimicrobial susceptibility test.

Antimicrobial activities of the EOs

The disk method was performed according to NCCL (2009) method with some adaptations. Briefly, 100 µl of an inoculum of 10^6 cells/ml were spread on sterile Mueller Hinton agar plates and let to dry at ambient temperature. 10 µl of the EO solubilised in 10% Tween 20 solution in order to obtain a concentration of 500 mg/ml were deposited on a 6 mm diameter disk and placed at the centre of the plate containing the microorganism. A disk containing 10 µl of 10% Tween 20 solution was used as control, while disk containing 10 µg of gentamicin was adopted as reference. Inhibition diameters (intended as the zone surrounding the disk with no bacterial growth) were measured after incubation at 37°C for 24 h on each of the three repetitions performed and expressed as mean ± standard deviation. The inhibition diameter was calculated as the difference between the test and the control.

For minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), microdilution method was used according to Bajpai et al. (2008) and Yèhouenou et al. (2010) in a 96 wells plate with single well covers. EOs diluted in 10% dimethyl sulfoxide (DMSO) were added to one of the series of wells containing the strain culture at 10^6 cells/ml and 0.02 mg/L of phenol red in order to have a concentration of 452.5 mg/L. Subsequently, series of 2 fold dilutions were prepared until a final concentration of the EO of 0.022 mg/L. A well containing the culture and phenol red without EO was used as positive control, while another well without the strain was used as negative control. After 24 h of incubation at 37°C, wells where the phenol red had changed to yellow were considered as having microbial growth, hence, indicating that the EO concentration used was not active. The MIC was considered as the lowest concentration where the phenol red did not change the initial red colour. Gentamicin used as reference antimicrobial was tested with the same methodology up to a concentration of 1600 µg/L.

For the MBC, 100 µl of the wells without visible bacterial growth were inoculated in a fresh Mueller Hinton broth and incubated at 37°C for 24 h. Following this, the lowest concentration of the EO which did not permit any growth was considered as MBC. Results for the MIC and MBC were reported as mean of the three repetitions and their standard deviation.

Free radical scavenging activity determination

Stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was used for the determination of the free radical scavenging activity of the EOs according to Brand-William et al. (1995) adapted to EO as described by Agnaniet et al. (2004). Briefly, 100 µl of the EO methanolic solution was added to 1900 µl of an ethanolic solution of 0.04 mg/ml of DPPH and the optical density (OD) read after 30 min on a Jenway 6305 Spectrophotometer at 517 nm. Four successive concentrations of the methanolic solutions were prepared for each EO. The negative control consisted of 1900 µl of ethanolic solution

of DPPH and 100 µl of methanol and the positive control made of 1900 µl of ethanolic solution of DPPH and 100 µl of methanolic solution of BHT (various concentrations) or vitamin C (various concentrations) were used as reference. The DPPH scavenging activity (%) was calculated as follows:

DPPH Scavenging activity (%) = [(OD control − OD sample) / OD control] ×100

SC_{50} (concentration of the tested substance that provides 50% scavenging activity) was determined graphically on the best curve fitting the experimental points representing the percentage scavenging activities as a function of the antioxidant concentration. The data was represented as mean of the three replicates and their standard deviation.

RESULTS

EO extraction and analyses

The hydro distillation of the different parts of *C. zeylanicum* plant gave different yield ranged from 1.05 to 1.10%, while those of *T. vulgaris* and *O. gratissimum* were 0.16 and 0.48%, respectively. Regarding the composition of the EOs, Table 1 shows the percentage content of the different compounds identified. The lowest percentage of compounds identified was 98.89% obtained with the EO of *O. gratissimum*, while the highest was 100% for the EO of *C. zeylanicum* fresh leaves. Concerning the composition; *T. vulgaris* was mainly composed of p-cymene (45.90%), thymol (23.72%), sabinene hydrate (5.90%), while some popular antimicrobial compounds like carvacrol and eugenol were detected in low levels of 2.02 and 0.4%, respectively. In *O. gratissimum* EO, thymol was the major compound (47.11%) followed by ɣ-terpinene and p-cymene at 16.60 and 14.06%, respectively. The percentage of carvacrol was very low, 0.62% in this EO, as compared to *T. vulgaris* EO. Regarding *C. zeylanicum*, it can be observed that the composition variability was less than that of the previous two EOs. In fact, only 9 compounds were identified. Among the different plant material, the EO of dry and fresh leaves of *C. zeylanicum* was quite similar in composition, demonstrating that the physical state did not affect the composition. On the other hand, *C. zeylanicum* stems EO demonstrated a quite different composition with respect to the leaves. As it can be observed, the EO of the leaves was mainly composed of cinnamaldehyde (82 to 84%) and linalool (11 to 12%) with traces of thymol and carvacrol (between 0.57 and 1.10%). *C. zeylanicum* stem EO was composed of 68.11% α-terpinene, about 10% of thymol and 10% of carvacrol. Moreover, it contained 8.47% of α-guaiene which was not found in *Thymus* and *Ocimum* species.

Antibacterial activities of EOs

Disk diffusion method was used to assess the inhibitory

Table 1. Chemical composition of the essential oils tested.

S/N	Compounds names	RI * (HP-5)	T. vulgaris (%)	O. gratissimum (%)	C. zeylanicum (%)		
					Dry leaves	Fresh leaves	Dry stems
1	α-thujene	928	1.20	-	-	-	-
2	α-pinene	937	1.56	4.10	0.80	0.86	0.68
3	camphene	954	1.79	1.22	0.42	0.46	0.45
4	β-pinene	976	0.55	0.67	0.34	0.37	0.37
5	myrcene	981	0.29	0.37	-	-	-
6	α-phellandrene	991	1.49	3.76	-	-	-
7	α-terpinene	1021	-	0.80	1.11	1.11	68.11
8	p-cymene	1031	45.90	14.06	-	-	-
9	limonene	1034	0.51	1.05	-	-	-
10	trans- β-ocimene	1038	-	0.53	-	-	-
11	cis- β-ocimene	1049	-	0.31	-	-	-
12	Cis- Sabinene hydrate	1064	5.90	-	-	-	-
13	γ-terpinene	1066		16.60	-	-	-
14	linalool	1094	3.96	1.56	12.12	11.26	0.46
15	borneol	1154	2.82	0.49	-	-	-
16	p-menth-1,5-dièn-8-ol	1174	1.25		-	-	-
17	terpinen-4-ol	1184	1.24	1.08	-	-	-
18	α -terpineol	1194	-	0.44	-	-	-
19	(E)-cinnamaldehyde	1278	-	-	82.23	84.16	0.30
20	thymol	1300	23.72	47.11	0.98	0.57	10.40
21	carvacrol	1307	2.02	0.62	1.10	0.88	10.44
22	eugenol	1365	0.4	-	-	-	-
23	α-copaene	1389	-	0.35	-	-	-
24	(E)-β-caryophyllene	1435	1.53	0.59	-	-	-
25	α-guaiène	1440	-	-	0.41	0.33	8.47
26	δ-cadinene	1504	0.25	0.96	-	-	-
27	α -cadinene	1514		0.34	-	-	-
28	caryophyllene oxyde	1536	0.43	0.25	-	-	-
29	eudesmol	1606	2.47	1.63	-	-	-
30	Epi- α-eudesmol	1658	0.33	-	-	-	-
	% compounds identified		**99.61**	**98.89**	**99.51**	**100**	**99.68**
31	NI	1286	0.36	-	-	-	-
32	NI	1781	-	-	0.49	-	0.31

*According to Adams (2007).

effect of the EOs on the growth of selected pathogens. Table 2 shows the diameters of inhibition obtained from *T. vulgaris, O. gratissimum* and different parts of *C. zeylanicum* on tested bacterial species. The absence of inhibition zone was observed when the EO of *C. zeylanicum* dry stem bark was tested against *S. aureus*, *S. typhimurium* and *E. coli*. On the other hand, the highest sensitivity to the EOs was observed for *S. aureus* in the presence of *T. vulgaris* EO (20.33±0.58 mm), followed by *C. freundii*, 18.00±1 mm and 18.00±1.73 mm in the presence of *T. vulgaris* and *O. gratissimum* EOs, respectively. There was no statistical difference between the inhibition diameters of *C. zeylanicum* fresh and dry

leaves EOs against the microorganisms tested. In general, the EOs that were tested at 500 mg/L (this means 50 µg of the EO deposited on the disks) gave lower or comparable inhibition zone compared to those obtained with gentamicin whose disk contained 10 µg of the antibiotics.

The MIC and MBC of EOs and that of gentamicin used as reference were assessed by the microdilution method. The results presented in Table 3 are expressed in mg/L for the EOs and µg/L for gentamicin. Due to the results obtained with the disk method, *C. zeylanicum* fresh leaves were not tested since it was demonstrated to have the same composition and antimicrobial potential as *C.*

Table 2. Diameters of inhibition of the essential oils of *T. vulgaris, O. gratissimum, C. zeylanicum* (dry leaves, fresh leaves and dry stems) and gentamicin on selected microorganism using the disk method.

Strain	*T. vulgaris*	*O. gratissimum*	*C. zeylanicum*			Gentamicin
			Dry leaves	Fresh leaves	Dry stems	
			Diameter (mm)			
S. aureus	20.33±0.58	13.67±2.31	12±1.73	9.33±1.53	0	22.67±0.58
S. typhimurium	12±2.65	11.33±2.52	9±1.00	10±1.00	0	18.33±0.58
S. flexineri	14.67±2.08	16±2.00	13.33±0.58	12.33±2.08	18±2	25.33±1.53
C. freundii	18±1.00	18±1.73	15±2.65	14.33±1.53	14.67±1.53	19±00
E. cloacae	11.17±2.08	15.33±3.51	10±1.00	12±3.61	14.33±3.21	15±0
E. coli	16±1.00	13±1.00	10.67±2.08	10.67±0.58	0	29.67±0.58
M. morgana	13.67±1.53	16.33±1.53	12.67±1.15	14±00	14±1.00	21.33±3.06

Table 3. Minimal inhibitory (MIC) and bactericidal concentrations (MBC) of the essential oils of *T. vulgaris, O. gratissimum, C. zeylanicum* (dry leaves and dry stems) and gentamicin on selected microorganism.

Strain	*T. vulgaris* (mg/ml)		*O. gratissimum* (mg/ml)		*C. zeylanicum* (mg/ml)				Gentamicin (µg/ml)	
					Dry leaves		Dry stems			
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	28.28	28.28	7.07	14.14	-	-	-	-	0.10	0.4
S. typhimurium	56.56	113.12	7.07	28.28	-	-	-	-	50	50
S. flexineri	14.14	56.56	14.14	28.28	1.77	1.77	226.25	452.5	100	100
C. freundii	56.56	452.50	56.56	452.5	0.88	3.53	452.5	-	100	200
E. cloacae	-	-	14.14	28.28	3.53	3.53	28.28	452.5	400	400
E. coli	14.14	28.28	7.07	14.14	-	-	-	-	100	1600
M. morgani	3.53	7.07	14.14	28.28	7.07	14.14	-	-	50	50

-No activity up to the highest concentration tested. *MIC and MBC of EO are expressed in mg/ml for the EOs and in µg/ ml for gentamicine and the standard deviations were all zero.

zeylanicum dry leaves. *T. vulgaris* and *O. gratissimum* EOs showed the most potent antimicrobial activity. In fact, it was possible to obtain a MIC and MBC with the EOs of these spices on all the microorganisms tested except on *E. Cloacae,* which were not sensitive to *T. vulgaris* up to 452.5 mg/ml. Gentamicin chosen as reference antibacterial compound was more active than the EOs. The MIC and MBC reported in Table 3 were the lowest when *S. aureus* was exposed to it (to what? gentamicin or EO; according to Table 3 it should be gentamicin). Among the Gram negative bacteria, *S. typhimurium* and *M. morgani* were the most sensitive to this antibiotic. Regarding the EOs, *C. zeylanicum* dry leaves EO was the most active to inhibit the growth of *S. flexneri*, *C. freundii* and *E. cloacae* than the other EOs tested. *C. zeylanicum* dry stem bark EO was the less active EO in general. Moreover, *C. zeylanicum* EOs, no matter the part chosen were not active on *S. typhimurium* and *E. coli* up to 452.5 mg/L.

Antioxidant properties of the EOs

The antioxidant properties of the EOs were assessed using the DPPH scavenging method. Table 4 represents the DPPH scavenging percentages obtained with EOs and the reference antioxidants BHT and vitamin C. From these data, a comparison index, namely, the SC_{50} was obtained as described in material and methods and also reported in the same table. The higher the SC_{50}, the lower the scavenging activity of the substance. It can be observed that *C. zeylanicum* EOs obtained from dry and fresh leaves had the best scavenging properties (2.5 × 10^{-5} mg/ml) even compared to the reference compounds. *T. vulgaris* EO also demonstrated a good antioxidant property as its SC_{50} was 6.5 × 10^{-5} mg/ml. These values are lower than that of BHT and vitamin C which exhibited SC_{50} of 245 × 10^{-5} and 11 × 10^{-5} mg/ml, respectively. In general, among the substances tested, BHT was the less efficient in scavenging the DPPH radicals.

DISCUSSION

Testing and proving of biological activity of EOs is one of the major aims of the study of natural substances. It is now accepted by all the scientific community that these studies should pay attention to the variability of the

Table 4. DPPH scavenging percentage (DPPH %) of the essential oils of *T. vulgaris, O. gratissimum, C. zeylanicum* (dry and fresh LEAVES), BHT and vitamin C at different concentrations (mg/ml).

| | *O. gratissimum* | | *T. vulgaris* | | *C. zeylanicum* | | | | BHT | | Vitamin C | |
| | | | | | Dry leaves | | Fresh leaves | | | | | |
	Conc.	DS%	Conc.	DS%	Conc.	DS%	Conc.	DS%	Conc.	DS%	Conc.	DS%
	2.25×10^{-5}	25	2.15×10^{-4}	29	4.75×10^{-5}	75.2	4.75×10^{-5}	72	2×10^{-3}	15	1×10^{-4}	18.8
	4.5×10^{-5}	42	4.3×10^{-4}	54.5	9.5×10^{-5}	86	9.5×10^{-5}	83.5	3×10^{-3}	64	1.5×10^{-4}	82
	9×10^{-5}	60	6.45×10^{-4}	61	19×10^{-5}	91.2	19×10^{-5}	87	5×10^{-3}	86	2.5×10^{-4}	96
	13.5×10^{-5}	63.5	10.75×10^{-4}	76.5	28×10^{-5}	97.5	28×10^{-5}	99.9	10×10^{-3}	89	5×10^{-4}	100
SC_{50}	6.5×10^{-5}		47×10^{-5}		2.5×10^{-5}		2.5×10^{-5}		245×10^{-5}		11×10^{-5}	

Where, Conc. = concentration of essential oils, BHT or Vitamin C in mg/ml; DS% = %DPPH scavenging activity, and SC_{50} = the concentration necessary for the scavenging of 50% DPPH radical from solution.

biological material concerned. In fact, it is known that the composition of EOs can be different due to the species, the agro-ecological factors and the part of the plant that is being analysed (Bruneton, 1999; Brada et al., 2007). In this specific study, the EOs tested showed different composition considering the species. Within the same species, dry and fresh leaves EOs of *C. zeylanicum* had roughly the same composition, but different from that of dry stems. The compositions of the EOs of these different parts of *C. zeylanicum* were also different from that of the same plant studied in Asia and Mid Orient. Richard and Multon (1992) and Cuvelier (1997) observed that EOs of leaves of *C. zeylanicum* had eugenol and campheme as their major components whereas the barks were rich in cinnamaldehydes. Later on, Yèhonenou et al. (2010) when analysing EOs of leaves of the same plant reported similar composition to that of this work, confirming that the agro-ecological factors can be determinant to the composition of EOs of the same species. To support these findings, in some countries *C. zeylanicum* is a big tree while those harvested in Cameroon are very small plants with a stem that cannot be easily dissociated from the bark.

Regarding *T. vulgaris* EO, the composition obtained in this work indicated that the major components were p-cymene and thymol. These results are similar to those of Moghtader et al. (2012), but differ in the relative percentages of the compounds. In Moghtader et al. (2012), thymol was the major component followed by p-cymene, which is opposite to the findings in this work. Many chemo-types of *T. vulgaris* have been identified in literature. Thymol, carvacrol, thymol and even borneol were identified as chemo-types for *T. vulgaris* EOs collected from different parts of Morocco (Chebli et al., 2003). Few years ago, Imelouane et al. (2009) discovered a camphene chemo-type in Eastern Morocco. The *T. vulgaris* from Bafoussam, Cameroon, can be considered as a p-cymene chemo-type.

O. gratissimum EO analysed in this work had thymol as the major compound and also contained p-cymene and γ-terpinene. This is in accordance with the conclusions of Orwa et al. (2009) who observed that eugenol, thymol and carvacrol are the major compounds generally found in the EO of this plant.

The antimicrobial properties of the EOs tested in this work were assessed by disk diffusion and microdilution methods. The first method can be considered as an explorative assessment that helps in a preliminary screening. In fact, this permitted to assess that dry leaves and fresh leaves EOs of *C. zeylanicum* had the same activity and hence only the dry leaves EOs were used to assess the MIC and MBC. EO from the leaves of *C. zeylanicum* was the most active and that of the dry stem was the less active, in particular on *S. aureus*, *S. typhimurium* and *E. coli*. In fact, this EO contains 68% of α-terpinene, 10% of carvacrol and 10.4% of thymol. Among these compounds, carvacrol and thymol are mostly endowed with antimicrobial properties (Burt, 2004; Chiasson et al., 2005). The high content in cinnamaldehyde (>80%) of the leaves EO of this plant is probably the main reason of its antimicrobial activity. As a matter of fact, cinnamaldehyde antimicrobial properties according to Walsh et al. (2003) acts on bacteria by inhibiting the biosynthetic enzymes and can also, according to Oussalah et al. (2006) contribute in the reduction of bacteria intracellular pH. Hadri et al. (2014) observed that EO from *C. zeylanicum* barks with 91% cinnamaldehyde inhibited the growth of *S. aureus* and *E. coli*, while this was not the case in our study with an EO containing more than 80% of the same compound. These different results could be explained by the additional 8.5% of cinnamaldehyde acetate of the EO or by the strain variability.

T. vulgaris did not show antimicrobial activity on the growth of *E. cloacae*. The antimicrobial activity of this EO has been generally attributed to the high content in thymol and carvacrol (Sokovic et al., 2007; Khodaei Motlagh et al., 2014). In our study, thymol was present at 23% and p-cymene at 46%, while carvacrol was only 2%. The synergy between thymol and carvacrol may be the

base of the antimicrobial properties of this EO. Knowing that thymol is a more potent antimicrobial than p-cymene, the inversion of the percentage contains those of these compounds in *O. gratissimum* EO (thymol 47% and p-cymene 14%) gave way to a more important antimicrobial activity. It was observed that MIC and MBC of *O. gratissimum* EO were generally lower than those obtained with *T. vulgaris* for the same microorganism. Beside the fact that antimicrobial activities of thymol are well known, the interaction of all the molecules can have a synergic effect that may potentiate the activity of the whole EO. In this regard, a synergic activity has been reported for many compounds (Jayaprakasha et al., 2003; Matasyoh et al., 2007). The EOs activities are attributed to their disorganising effect of the molecules on the cell membrane, leading to a non control of the exchange between cytoplasm and the external medium (Bouhdid et al., 2009; Gardini et al., 2009). After entering into the microorganism's cytoplasm, other mode of action could be the acidification (Wright, 2002) and the destruction of the genetic material (Brackman et al., 2008; De Martino et al., 2009).

The antioxidant activity of the EOs was also assessed and compared to BHT, a primary antioxidant with the ability to inactivate free radicals by transferring a proton (Cuvelier, 1997) and vitamin C as a secondary antioxidant that blocks oxygen and metals that can faster the oxidation (Cuvelier, 1997). In the case of this study, *C. zeylanicum* leaves EO was the most active in scavenging the DPPH followed by the EO of *T. vulgaris*. This may be due to the presence of linalool in larger extent than that of cinnamaldehyde. Although an *in vivo* antioxidant property have been reported for cinnamaldehyde in rat kidney (Gowder and Devaroy, 2006), this aromatic aldehyde has a low proton transferring property than linalool. On the other hand, thymol's percentage is higher in *O. gratissimum* EO compared to *T. vulgaricus*. The latter gave the best scavenging properties. The presence of 4% linalool and 45% p-cymene may be the reason for this activity. De Oliveira et al. (2015) observed that p-cymene has an antioxidant potential *in vivo* as well as a neuroprotective agent in the brain. The reason behind the good scavenging performances of the EOs with respect to the reference compounds may be the interactivity of all the compounds composing the EOs. It can be observed from these results that these EOs can well replace BHT in the limitation of oxidation in food and tissues.

Conclusion

EOs of *T. vulgaris, O. gratissimum* and *C. zeylanicum* showed a high composition variability depending on the species, and within the same species of *C. zeylanicum*, between leaves and stem. This variability was also highly influenced by the agro-ecological factors as demonstrated by comparison of the results with those in the literature. Moreover, the studied antimicrobial properties were of large spectra for *T. vulgaris* and *O. gratissimum* EOs, while that of *C. zeylanicum* leaves was more active to inhibit the growth of *S. flexneri* and *C. freudii* than the other EOs. These EOs demonstrated to be good natural antioxidants that may be used in food industries and in the prevention of oxidative degenerative diseases.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Growth promotion and elicitor activity of salicylic acid in A*chillea millefolium* L.

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Salicylic acid (SA) is a phenolic compound present in plants and has multiple functions, including hormonal effect on stimulus of plant growth and development and induction of plant defense responses under conditions of biotic and abiotic stresses. Studies related to SA's elicitor action on the synthesis of secondary metabolite in medicinal plants have been conducted in order to increase the economic value of these species. The objective of this study was to determine the effect of SA foliar application on biomass production and the synthesis of secondary compounds in yarrow (*Achillea millefolium* L. - Asteraceae). The experiment was conducted in potted plants under greenhouse conditions. The SA application was done at concentrations of 0, 0.25, 0.50 and 1.00 mM 20 days after transplanting the seedlings to pots. The effect of SA on the metabolism of yarrow plants was evaluated through biometric parameters of growth and biochemical parameters. The SA at 0.50 mM resulted in linear increases in biomass accumulation of roots, total dry mass, ratio root/shoot and chlorophyll *a* and chlorophyll *a+b* content in yarrow plants.The application of SA at 0.50 and 1.00 mM was most effective in eliciting the production of essential oils and total phenols, with a consequent improvement of the antioxidant activity of the plant extract. It can be concluded that SA application constitutes an advantageous management practice for commercial production of *Achillea millefolium*, increasing the nutraceutical and medicinal values of this species.

Key words: Photosynthetic pigments, essential oil, phenolic compounds, antioxidant activity.

INTRODUCTION

Achillea millefolium L. (Asteraceae), known as yarrow is a perennial species used in folk medicine against various diseases, including skin inflammation and gastrointestinal and hepatobiliary disorders. In addition to the traditional use, yarrow is also used as raw material in tea-producing industries (Chandler et al., 1982; Benedek and Kopp,

2007). Among the active compounds of yarrow, the presence of essential oil with terpenes, tannins, mucilages, coumarins, resins, saponins, steroids, fatty acids, alkaloids and bitter principle can be highlighted (Simões et al., 1998).

Salicylic acid (SA) is a phenolic compound, benzoic

acid produced by the plants with important roles in plant defense responses to biotic and abiotic stress (Popova et al., 1997; He et al., 2002; Noreen et al., 2009; Hayat et al., 2005). SA also acts as a regulator of many physiological processes related to growth and development of plants, this is the reason why this compound was referred to as a plant hormone (Hegazi and El-Shrayi, 2007; Singh and Usha, 2003; Liu et al., 2011).

Recently, the emphasis on SA elicitor activity has been gaining attention in the literature. Elicitors can be defined as substances that initiate or increase the biosynthesis of secondary compounds in plants, both in medicinal plant species and the species categorized as functional foods (Taguchi et al., 2001; Radman et al., 2003). The SA is classified by the Food and Drug Administration as "substance generally recognized as safe", which enables its use in commercial cultivation of medicinal species targeting the market of phytomedicines (Poulev et al., 2003; Divya et al., 2014).

Marigold plants (*Calendula officinalis* L.) treated with SA (0, 0.25, 0.50 and 1.00 mM) showed linear increases in the accumulation of biomass, number of inflorescences and flavonoid content; without changes in photosynthetic activity and transpiration however (Pacheco et al., 2013). In mint (*Mentha piperita*), the foliar application of SA (2 mM) resulted in increases in growth parameters and changes in the metabolic profile of carbohydrates and amino acids of the resulting infusions, therefore lower concentrations increased content of phenolic compounds in leaves (Pérez et al., 2014). The concentration of betacyanin and total phenols in *Alternathera tenella* leaves cultivated *in vitro* increased after 36 h of treatment with SA. In contrast, the increase in exposure time caused a slight decrease in the contents of total flavonoids and decreased antioxidant activity (Brandão et al., 2014). Divya et al. (2014) studied the application of different levels of methyl jasmonate and salicylic acid on pre-harvest of coriander (*Coriandrum sativum* L.). Plants treated with SA 0.5 mM showed increases of 5.4 and 3.5% in the levels of carotenoids and phenolic compounds, besides additional increases in compounds such chlorophyll and lutein. There have been no reports in the literature on the exogenous application of SA in *Achillea milefolium* so far. Thus, the aim of this study was to evaluate the effect of different concentrations of SA in *A. millefolium* in order to promote growth and simultaneously increase the synthesis of secondary compounds in this medicinal species.

MATERIALS AND METHODS

Study site and plant material

The experiment was conducted under controlled greenhouse conditions (temperature of 26°C and 70% of humidity) in Presidente Prudente (22°7'39" S, 51°23'8" W, 471 ma.s.l.), São Paulo, Brazil. The seedlings were obtained from mother plants of

yarrow (*Achillea millefolium* L.) from the Garden of Medicinal Plants of UNOESTE. Botanical identification was performet at the Universidade Federal de Uberlândia Herbarium (voucher specimen number HUFU 46844).

The seedlings were propagated by clump division and after the training period (30 days) they were planted in 18 L pots containing soil. The soil was corrected as Boletim 100 (IAC) recommendations for perennial herbaceous species. Dolomitic lime 85% PRNT (31.86 g.pots[-1]), potassium chloride (2.7 g.pots[-1]) and simple super phosphate (18 g.pots[-1]) were added. For fertilization with micronutrients, FTEBR12® (S: 3.9%; B: 1.8%; Mn: 2.0% and Zn: 9.0%) (0.9 g.pots[-1]) was added. The pots were irrigated by sprinklers twice daily at 6 a.m. and 18 p.m. The irrigation blade was 6.4 mm to maintain high soil humidity during all the period of the experiment.

The application of salicylic acid (SA) was performed 20 days after transplanting the seedlings to pots, with three consecutive applications of SA solutions (40 mL per plant) in the early hours of the morning. The different concentrations of SA (0.00, 0.25, 0.50 and 1.00 mM) were prepared from a master solution of 1.00 mM. The product, as in powder formulation (Sigma Aldrich, PM= 138.1 g) was weighed in analytical balance, dissolved in 10 mL ethanol 90° and finally dissolved in distilled water 1000 mL. SA treatments were carried out by spraying the aerial part of the plants with waterbased solutions supplemented with Agral® (50 µL.L^{-1} of solution) until drip point. Control plants were sprayed with only distilled water (1000 mL) mixed with ethanol (10 mL).

Plants were harvested at 120 days after transplanting the seedlings to pots, picking up youth and adult leaves. The leaves were dried in an oven with air circulation at 40°C until they achieved constant weight to determine the dry mass.

Plant growth

The effect of SA on the plants was evaluated using the following variables: total leaf area (LA - cm[2]), number of leaves per plant (NL), dry weight of leaves and roots (g.plant[-1]), leaf area/number of leaves ratio and biomass partitioning.

Total leaf area was determined with a LI-3000A portable area meter (LI-COR, Lincoln, NE, USA) in five plants per treatment. Leave number per plant was determined considering the young and adult leaves. Leaf and root dry mass (g.plant[-1]) was measured after drying samples at 40 (leaves) and 60°C (roots) for 72 h until they achieved constant weight. The biomass partitioning in the plants was determined for the root/shoot and leaf area/number of leaves ratios.

Pigments and secondary metabolites content

The levels of chlorophylls, carotenoids and anthocyanins (µmol.g^{-1}) were determined spectrophotometricly following extraction on TRIS-acetone buffered solution (hydroximetil-aminomethan), according to the method of Sims and Gamon (2002).

The determinations of the content of total phenolic compounds (µg.mL) and antioxidant capacity (IC$_{50\%}$) were made from ethanol extract of the leaves. A mass of 50 g of dried and crushed leaves was subjected to extraction with 1.5 L of ethanol at room temperature and protected from light. The process of maceration and filtration of the supernatant was carried out in three consecutive stages and each extraction lasted 30 for min. The extracts obtained were combined and concentrated by evaporation *in vacuo*. Extracts were then dried at 30°C in a forced air circulation oven (Costa, 2002; Simões et al., 2007). The total phenolics content was determined according to Folin-Ciocalteu reagent (Singleton et al., 1999) with modifications, using gallic acid as a standard in ethanol, and sodium carbonate solution. The antioxidant capacity was
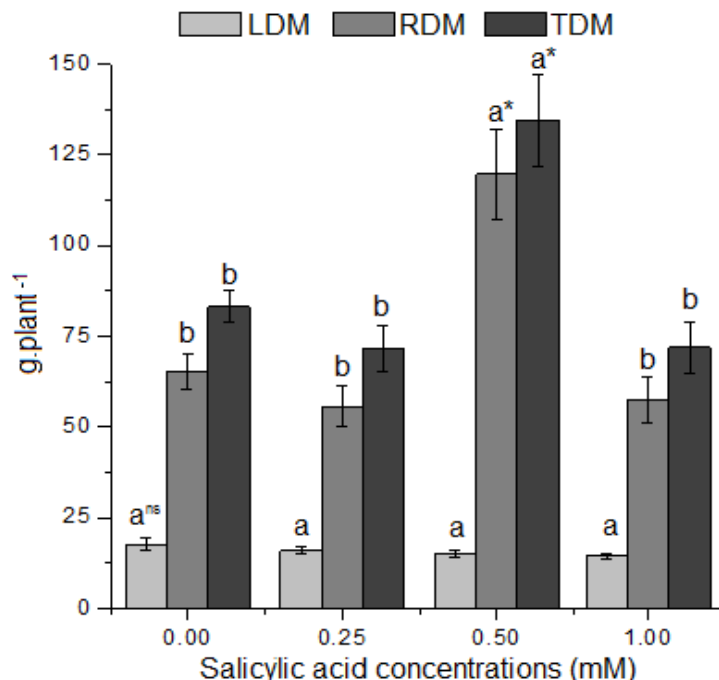
Figure 1. Determination of the leaf (LDM), root (RDM) and total (TDM) dry mass of *Achillea millefolium* L. plants, under different concentrations of salicylic acid. Different letters indicate significant differences by Tukey's test (p ≤ 0.05) and ns (not signicant) (p=0.1736, p=0.0001, p=0.0001).

assessed via free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The amount of antioxidants required to decrease the initial concentration of DPPH by 50%, termed "inhibitory concentration (IC_{50})" (Brand-Williams et al., 1995) was determined. The absorbance values at all concentrations tested were also converted to percentage of antioxidant activity (% AA), determined by the equation: AA (%) = [($A_{control}$ - A_{sample})/$A_{control}$] x 100 (Moreira et al., 2005).

Essential oil content (%) and yield (g) were estimated by hydrodistillation in Clevenger apparatus, from 10 g of dried leaves samples. Oil mass was obtained after removal of the salt (anhydrous magnesium sulfate) and evaporation of the solvent (dichloromethane), according to Brant et al. (2009).

Data analysis

The experiment was arranged in a completely randomized design with four treatments (SA concentrations) and 10 replicates (individual plants) per treatment. ANOVA was performed on all experimental data using the statistical program ASSISTAT (Silva, 2010) and mean differences were assessed at a 5% level by Tukey's test. Regression equations were used to express the behavior of the variables as a function of increased AS doses just when a high correlation coefficient (R^2) value was observed.

RESULTS AND DISCUSSION

Plant growth

The exogenous application of SA did not affect significantly the shoot dry mass of the yarrow plants (Figure 1). However, an increment of 83.11% in the dry mass of roots was observed in plants treated with 0.50 mM SA, which in turn contributed to the higher total dry mass of the plants (up to 61.93% as compared to control plants). Parashar et al. (2014) reported increases in both root dry mass (26%) and the shoot (51%) in *Brassica juncea* plants treated with 0.01 mM SA. In our case, the SA promoted changes in mass distribution between the different organs of the plant, prioritizing the root system over the shoots.

It is well known that SA promotes both cell division and elongation (Hayat et al., 2005; Ahmad et al., 2014). SA has been reported to increase plant growth parameters (number of branches, number of leaves and leaf area) in other medicinal plant species, like marigold (Pacheco et al., 2013), marjoram and oregano (Gharib, 2007). In this study, the number of leaves and leaf area in yarrow plants treated with 0.25 and 1.00 mM SA did not differ from control plants (Figures 2A and B). However, the application of the SA at 0.50 mM resulted in a significant reduction in the number of leaves per plant (28%) and in the total leaf area (46.2%). It is tempting to speculate that this result may be a consequence of a higher export of carbohydrates to the large roots system exhibited by plants treated with this SA concentration.

There was an increase in the root/shoot ratio of yarrow plants in the concentration of 0.50 mM SA (Figure 3A). In
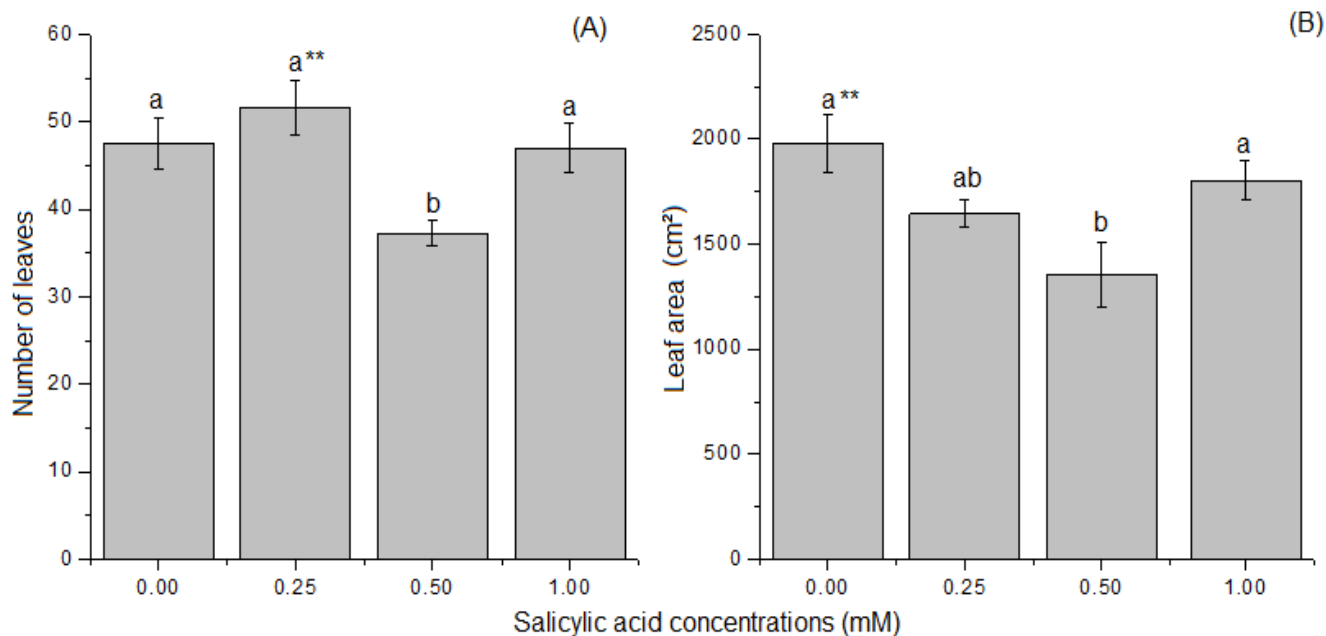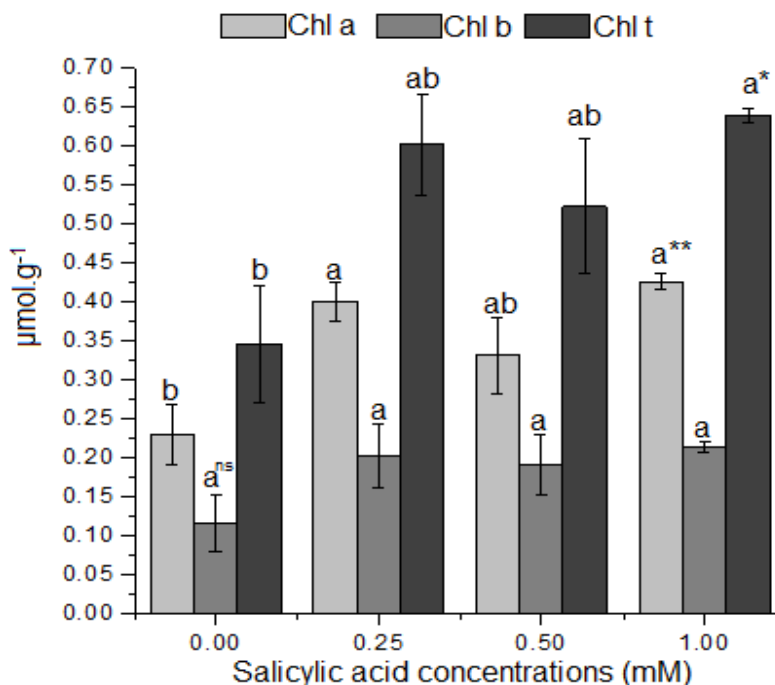
Figure 2. Number of leaves and leaf area of *Achillea millefolium* L. plants under different concentrations of salicylic acid. Different letters indicate significant differences by Tukey's test (p ≤ 0.01) (p=0.0191, p=0.0082).
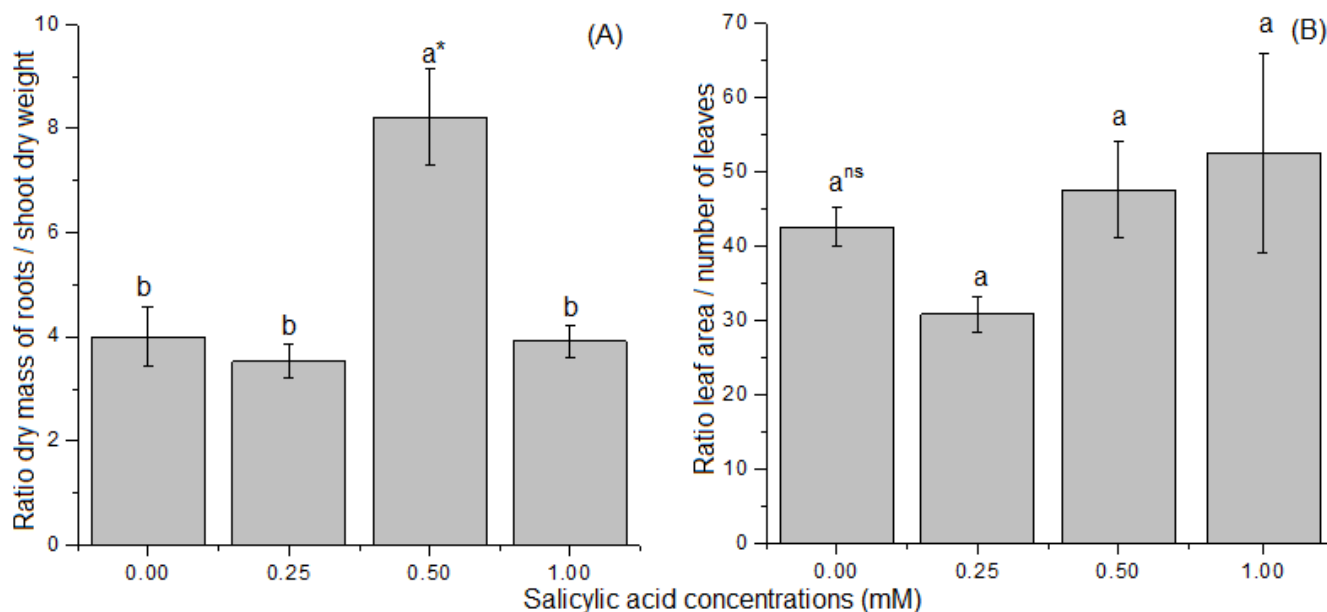
Figure 3. Root/shoot and leaf area/number of leaves ratios of *Achillea millefolium* L. under different concentrations of salicylic acid. Different letters indicate significant differences by Tukey's test (p ≤ 0.05) and ns (not significant) (p=0.0001, p=0.2516).

contrast, the leaf area/number of leaves ratio observed in SA treated plants did not differ significantly from the control plants (Figure 3B). The root/shoot ratio seems to be controlled by a functional balance between water uptake by roots and photosynthesis by the shoot. In this way, the shoot can eventually grow to its full size until the

water uptake by the roots becomes limiting and the roots until the demand for shoot photosynthates is equal to the supply. This functional balance is changed if the water supply decreases (Blum, 2005) or by the application of plant growth regulators, as occurred in this experiment. However, the observation of a higher root/shoot ratio was

Figure 4. Chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (Chl t) contents of *Achillea millefolium* L. under different concentrations of salicylic acid. Different letters in each column indicate significant differences by Tukey's test (p ≤ 0.01 and p ≤ 0.05) and (ns - not significant) (p=0.0036, p=0.2134, p=0.0287).

not repeated in the highest SA concentration applied (1.00 mM SA). Different results were found in maize plants treated with 20 and 40 mg.L^{-1} SA, which presented lower root/shoot ratio than the untreated plants (Ahmad et al., 2014). Thus, our results confirm that the SA exerts species-specific and concentration-dependent responses in plants (Divya et al., 2014; Pérez et al., 2014).

Pigments content

The chlorophyll content is an important physiological index directly related to the photosynthetic performance of plants (Abdollahi et al., 2011; Parashar et al., 2014). The *Chl a* content (Figure 4) observed in yarrow plants treated with 0.25 and 1.00 mM SA was significantly higher (74.46 and 85.77%, respectively) as compared to control plants, although no effect was seen for *Chl* b content (Figure 4). The total chlorophyll content (*Chl t*) significantly increased up to 84.83% in 1.00 mM SA treated plants when compared with the control plants. Similar results were observed in *Coriandrum sativum* plants treated with 0.50 mM SA (Divya et al., 2014).

Increased levels of chlorophylls after SA applications observed in both stressed and the control plants and were explained by the positive action of this hormone in the plant nutrient uptake (Shakirova and Sakhabutdinova, 2003) as greater contents of Fe, Mg and Ca can

stimulate the biosynthesis of chlorophylls (Parashar et al., 2014). Further, the increase of photosynthetic pigments can be a result of SA stimulatory effect on the activity of the Rubisco enzyme and photosynthesis (Idrees et al., 2010).

Higher contents of other pigments, such as carotenoids and anthocyanins, in response to SA application have been reported in recent studies (Divya et al., 2014; Baenas et al., 2015). However, here we did not observe significant differences in these two pigments classes between the SA-treated and control yarrow plants (Figure 5).

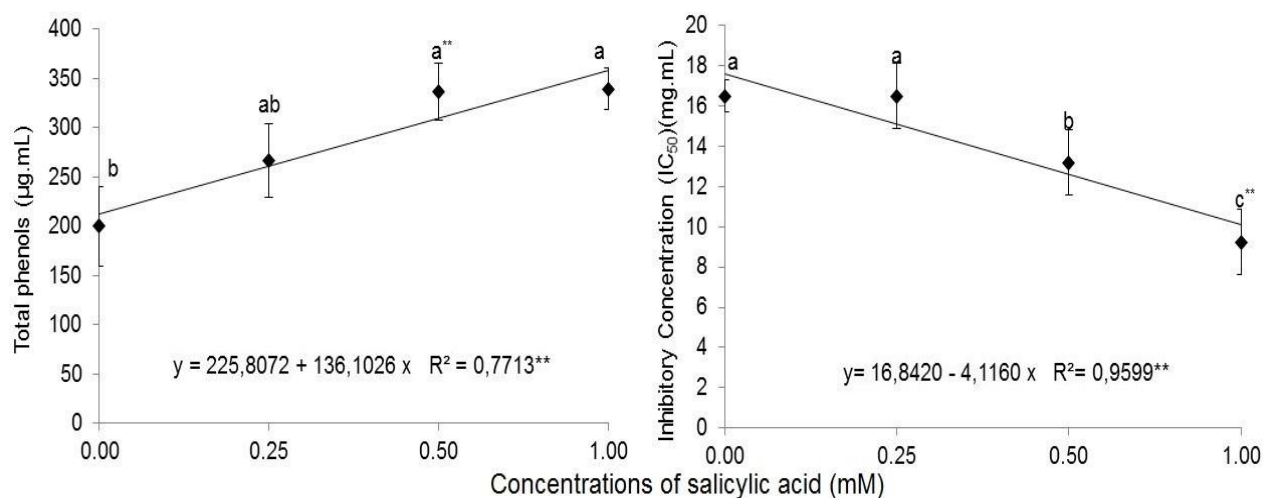**Essential oil, phenols and antioxidant activity**

In plants, SA also acts as a chemical elicitor which can enhance the production of different groups of secondary metabolites, such as terpenes, alkaloids, flavonoids, phenolic compounds and phytoalexins (Ali et al., 2006; Silva et al., 2014). In this study, the application of 0.50 mM SA resulted in significant increases in the content (94.86%) and yield (58.24%) of essential oil in the yarrow plants as compared to the control (Figure 6). Nourafcan et al. (2014) reported similar results with *Lippia citriodora,* as they showed that the application of 10 mM SA caused an improvement in the yield and quality of the essential oil.
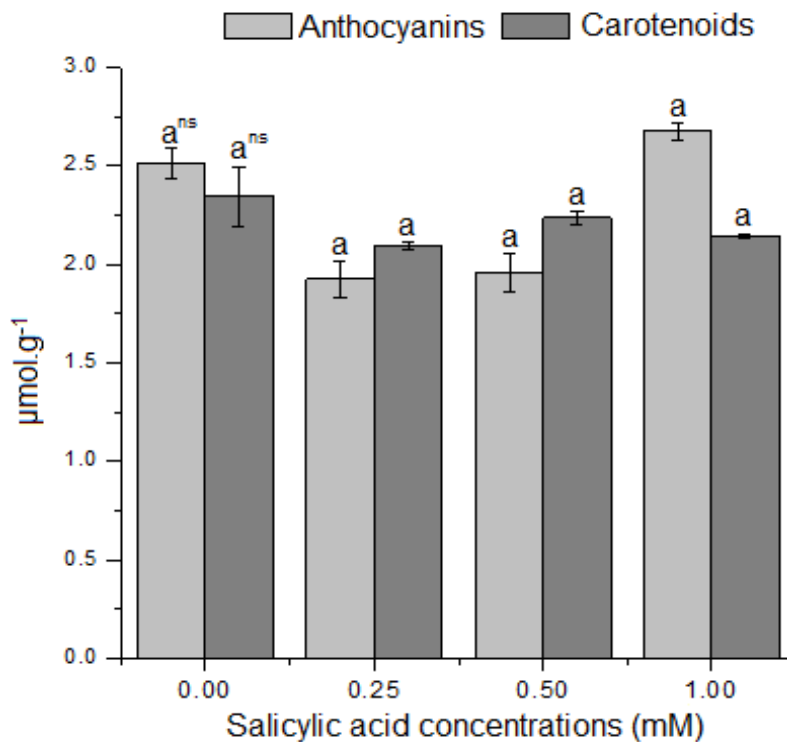
Figure 5. Anthocyanins and carotenoid contents of *Achillea millefolium* L. under different concentrations of salicylic acid. Equal letters in each column indicate no significant differences by Tukey's test ($p=0.2189$, $p=0.8311$).
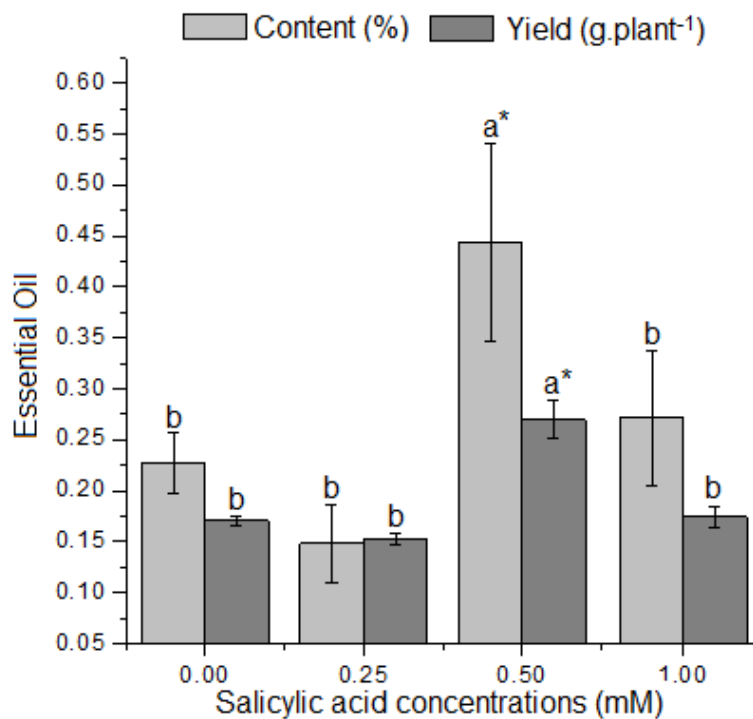
Figure 6. Content and yield of essential oil of *Achillea millefolium* L. under different concentrations of salicylic acid. Different letters in each column indicate significant differences by Tukey's test ($p \leq 0.05$) ($p=0.0308$, $p=0.0104$).

Figure 7. Content of total phenols and antioxidant activity of *Achillea millefolium* L., under different concentrations of salicylic acid. Different letters indicate significant differences by Tukey's test (p ≤ 0.05) (p=0.0246, p=0.0001).

Regarding phenolic compounds, it was observed that exogenous application of SA exerted significant effects on these secondary metabolites, as shown by the positive linear relationship between phenolic content and SA concentrations (Figure 7). Plants treated with 0.50 and 1.00 mM SA showed a marked increase in the total phenolic content (68.11 and 69.74%, respectively) as compared to control plants. The results of this study are in agreement with those obtained by Perez et al. (2014) in mint plants treated with SA, where increases up to 65% in the total phenolic content were detected, depending on the applied SA concentrations.

Enhancement of the synthesis of various phenolic compounds in response to elicitors application, especially SA, may be explained by the induction of a state of oxidative stress in plants (Perez et al., 2014); as exogenous SA, even at low concentrations, interacts with signaling mechanisms of stresses. One example is the concentration-dependent inhibition of the antioxidant enzymes ascorbate peroxidase and catalase by SA, generating an increase in the cellular content of H_2O_2 (Moharekar et al., 2003; Askari and Ehsanzadeh, 2015). Thus, the applied concentration and duration of treatment with elicitors' substances must be carefully considered in order to avoid harmful effects to the plant (Cheeseman, 2007). Still, biotic and abiotic elicitors may have different effects on different and even the same plant species, making it also necessary to consider the type of elicitor to be used (Brandão et al., 2014).

The antioxidant activity of the leaf extract of yarrow plants was analyzed according to the IC_{50} values (Figure 7B), where lower values indicate high antioxidant activity (Sousa et al., 2007). The concentrations of 0.50 and 1.00 mM SA resulted in significant reductions in IC_{50} values

relative to control plants (25 and 79%, respectively). Similar results have been reported in mint plants (*M. piperita*) treated with SA (Perez et al., 2014), where the concentrations of 0.5 and 1.0 mM SA resulted in significant decrease in IC_{50} values as compared to control plants. According to these authors, this effect can be attributed to the increase of phenolic compounds (such as flavonoids and hydroxycinnamic acids) in plants treated with SA, which are considered the major antioxidant compounds in the plant. Phenolic compounds act in the removal of singlet oxygen and other free radicals in cells, and contribute to the stabilization of oxidative stress (Rice-Evans et al., 1995). Antioxidant properties of phenolic compounds are mainly due to its ability to donate hydrogen from hydroxyl groups positioned along the aromatic ring, in order to avoid oxidation of lipids and other biomolecules (Foti et al., 1994). However, other organic compounds whose levels in the plant are increased by the SA application may also act as antioxidants, such as carbohydrates (Bohnert and Jensen, 1996), betacyanins (Brandão et al., 2014) and terpenes such as carotenoids and tocopherol (Janda et al., 2014).

Yarrow plants treated with 0.50 and 1.00 mM AS showed higher antioxidant activities for the same ethanol extracts quantities as compared to control plants (Figure 8). It is suggested that increased endogenous levels of SA in treated plants can trigger cell signaling responses that regulates the expression of defense genes encoding enzymes related to phenylpropanoids production pathway (Ruiz-Garcia and Gómez-Plaza, 2013). The increase in the activity of key enzymes in this metabolic pathway, such as phenylalanine ammonia lyase and chalcone synthase, is as a result of the SA application
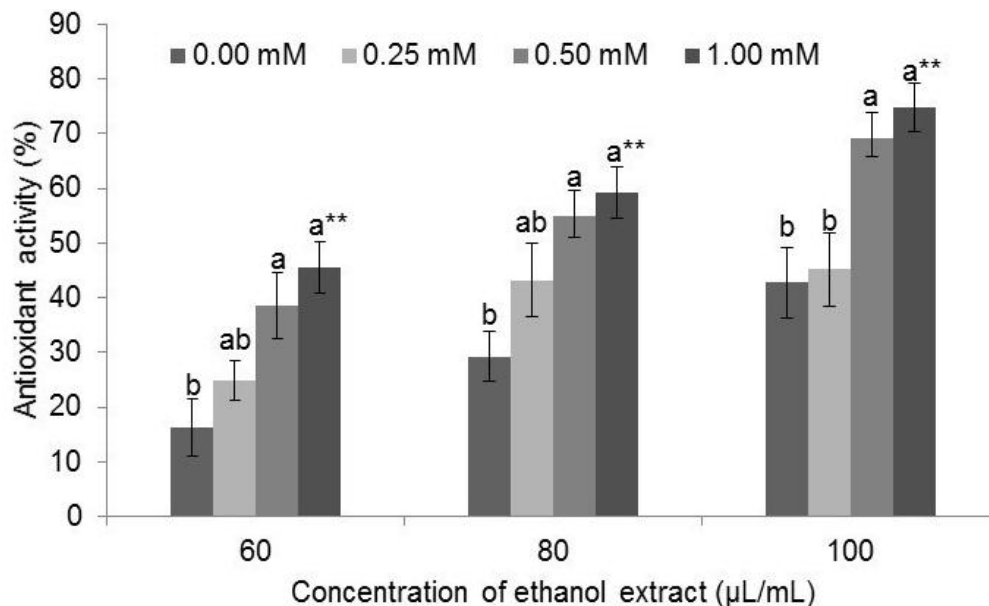
Figure 8. Percentage of antioxidant activity of ethanol extracts of *Achillea millefolium* L., under different concentrations of salicylic acid. Different letters indicate significant differences between the salicylic acid concentrations for each extract concentration, by Tukey test (p≤0.01) (p=0.0055, p=0.0054, p=0.0020).

(Ghasemzadeh et al., 2012; Obinata et al., 2003). Another mode of action of SA would be through its interference in the activity of antioxidant enzymes, where inhibition of the enzyme catalase (CAT) would cause an increase in cellular levels of H_2O_2, which in turn can increase the production of secondary metabolites in the plant for acting as a second messenger (Askari and Ehsanzadeh, 2015).

Conclusions

The global market along with the use of medicinal plants has been growing in recent years and generating an alternative income source for small farmers. Given the results obtained in this study, it can be concluded that the application of SA constitutes an advantageous management practice for commercial production of *A. millefolium*. Plants treated with 0.50 mM SA presented increased root growth, enabling producers to cultivate a greater number of plants per area which can generate better economic results than that obtained with lower density. In relation to the synthesis of secondary metabolites, the application of SA at 0.50 and 1.00 mM was most effective in eliciting the production of essential oils and total phenols, with a consequent improvement of the antioxidant activity of the plant extract.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Antibacterial activity of six indigenous Indian plants: *Acacia nilotica* (Fabaceae), *Albizia saman* (Fabaceae), *Azadirachta indica* (Meliaceae), *Carica papaya* (Caricaceae), *Cymbopogon citratus* (Poaceae) and *Mangifera indica* (Anacardiaceae)

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Plants have been used as major source of active compounds with medicinal importance since human civilization. These naturally occurring pharmacologically active agents have least or no toxicity to the host. The antibacterial activity of extracts (water, acetone and methanol) from six indigenous Indian plants: *Acacia nilotica* (Fabaceae), *Albizia saman* (Fabaceae), *Azadirachta indica* (Meliaceae), *Carica papaya* (Caricaceae), *Cymbopogon citratus* (Poaceae) and *Mangifera indica (*Anacardiaceae) were determined against the pathogenic bacteria (*Staphylococcus aureus:* ATCC 25923; *Escherichia coli:* ATCC 25922 and *Klebsiella pneumonia:* ATCC 700603). The antimicrobial study was carried out by the gel diffusion method and the results show that as compared to aqueous extract, methanolic and acetone extracts were more effective. Of all the studied plants, the methanolic extract of *A. saman* leaves inhibited the growth of all the three test organisms.

Key words: Antibacterial activity, pathogenic bacteria, indigenous plants.

INTRODUCTION

According to World Health Organization, plants are the preferred source of a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds acquired from plants (Selvamohan et al., 2012). Therefore, immense research is required for better understanding of properties, safety and efficiency of these plants. There is a huge variety of plants, rich in secondary metabolites [which may be potential sources of drugs] and essential oils of therapeutic importance (Dipankar et al., 2011). These products are known by their pharmacologically active agents, for example, the organic compounds which are

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part of the essential oils, as well as in tannin. In general, bacteria have the genetic ability to transmit and acquire resistance to various environmental factors viz. metals, drugs, which are utilized as therapeutic agents (Singh et al., 2015). The increasing prevalence of multidrug resistant strains of the bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the spectre of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies.

Plants like *Acacia nilotica* (babool), *Albizia saman* (jungle jalebi), *Azadirachta indica* (neem), *Carica papaya* (papaya), *Cymbopogon citratus* (lemon grass), *Mangifera indica* (mango), etc. possess many medicinal properties. Apart from these, various other plants have been successfully used against several ailments. Studies have been conducted to isolate the medicinal ingredients from different parts of the plants which are effective against many diseases produced by various pathogenic and antibiotic resistant bacteria.

Therefore, researchers are increasingly turning their attention to traditional methods, looking for novel molecules to synthesize better drugs against microbial infections sources of drugs and essential oils of therapeutic importance. Despite the existence of potent antibiotic and antifungal compounds, resistant microorganisms are rapidly appearing (Byarugaba, 2005), posing immediate need for a permanent search and development of new drugs, which is safe, more dependable than costly drugs and which have no adverse side effects. The present study aimed to evaluate the antimicrobial activity of the above mentioned common plants against three pathogenic bacteria.

MATERIALS AND METHODS

Preparation of plant extracts

Fresh leaves were collected from six common indigenous plants, namely *A. nilotica* (Babool), *A. saman* (Jungle jalebi), *A. indica* (Neem), *C. papaya* (Papaya), *C. citratus* (Lemon grass) and *M. indica* (Mango) from the Amity University gardens and its surroundings. These were washed thoroughly, first with tap water (two to three times) and then with distilled water to remove the dust particles and mid-ribs of the leaves were removed. These were wiped by means of a tissue paper, kept in dark till they were completely dry and crushed into very fine powder using a pestle-mortar. Five grams powder of each plant was equally divided into three parts, each of which was kept in tarson tubes having acetone, distilled water and methanol, separately (10-12 ml). These tarson tubes were kept in a dark and hygienic place for 24 h and then in a water bath for 1 h at 60°C. This helped the powder to get dissolved in the solvent. Sterile 90 mm Petri dishes (Tarsons) were taken, weighed on the electric balance and labeled accordingly- plant names with their respective solvents. Then, the extract in the tarson tube was filtered using filter papers into these empty Petri plates, separately. The liquid in these plates was kept for air drying. When completely dried, the plates were taken out and weighed on the balance again. The weight of the empty Petri plates was subtracted from the weight of Petri plates with extracts. Extracts were dissolved in dimethyl sulfoxide (DMSO; 4 times the amount of

extract). These tubes were kept in the freezer for future use.

Test organisms

Three organisms: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603) were included in the study. The organisms were made as stock by mixing 100 µl of suspension in 10 ml of sterile nutrient broth and grown overnight. The organisms were maintained by subculturing them on nutrient agar at regular intervals and used throughout the study.
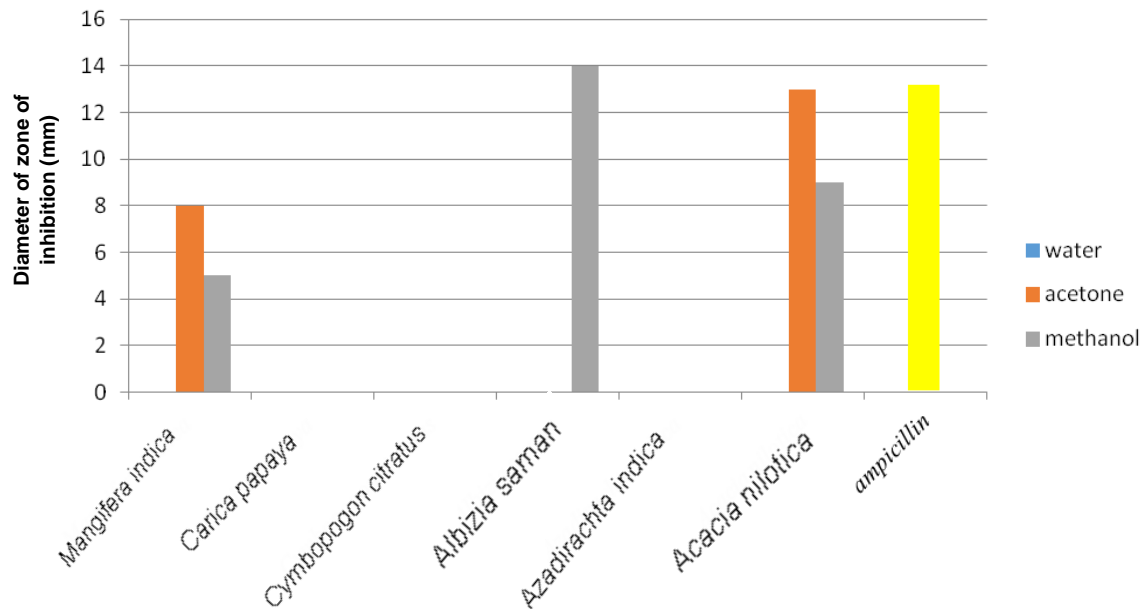
Antimicrobial activity assay

The microbial susceptibility test was done by using gel diffusion method. Nutrient agar media was prepared as per the supplier's instruction and sterilized by autoclaving at 121°C for 15 min. After preparing nutrient agar plates, four wells of 0.5 mm width and 0.5 mm depth were made at equal distance on each plate aseptically. Separate plates were inoculated with 50 µl of *E. coli, S. aureus* and *K. pneumonia*. For each bacteria, 50 µl of each extract prepared from same plant part, were loaded in the wells and plates were incubated at 37°C for 24 and 48 h. Antibacterial activity of each extract was expressed in terms of zone of inhibition (mm). Each experiment was repeated three times and average of all values was taken.

RESULTS AND DISCUSSION

Six locally available plants were selected randomly and the antimicrobial study was carried out. In this work, the objective was to discover an efficient alternative against the pathogenic bacteria which was free of the disadvantages of antibiotics. Extracts of six different plants viz. *A. nilotica* (babool), *A. saman* (jungle jalebi), *A. indica* (neem), *C. papaya* (papaya), *C. citratus* (lemon grass) and *M. indica* (mango) were made in three different solvents (acetone, distilled water and methanol) and tested for antimicrobial activity against *E. coli, S. aureus* and *K. pneumoniae*. Figure 1 shows zone of inhibition obtained against *E. coli* in the different extracts of plants. *A. saman* (in methanol), *M. indica* (acetone and methanol extracts) and *A. nilotica* (in acetone and methanol) showed good inhibition against *E. coli,* with methanolic extract of *A. saman* giving maximum zone of inhibition. Figure 2 represents the activity pattern of extracts against *K. pneumonia*, with *A. saman* having maximum inhibitory zones with all solvents in which *A. indica* showed least activity. The activity pattern for *S. aureus* is given in Figure 3. It shows maximum zone of inhibition with *A. saman*. Of all the studied plants, extracts of *A. indica*, *C. citratus* and *A. saman* were found to be most active against all test organisms, whereas *C. papaya* was effective only on Gram positive tested strains, that is, *S. aureus.*

Although antibiotics have been greatly of help in conquering a large number of microbial pathogens, these benefits have reduced significantly over the last couple of years since antibiotics have become less efficient against
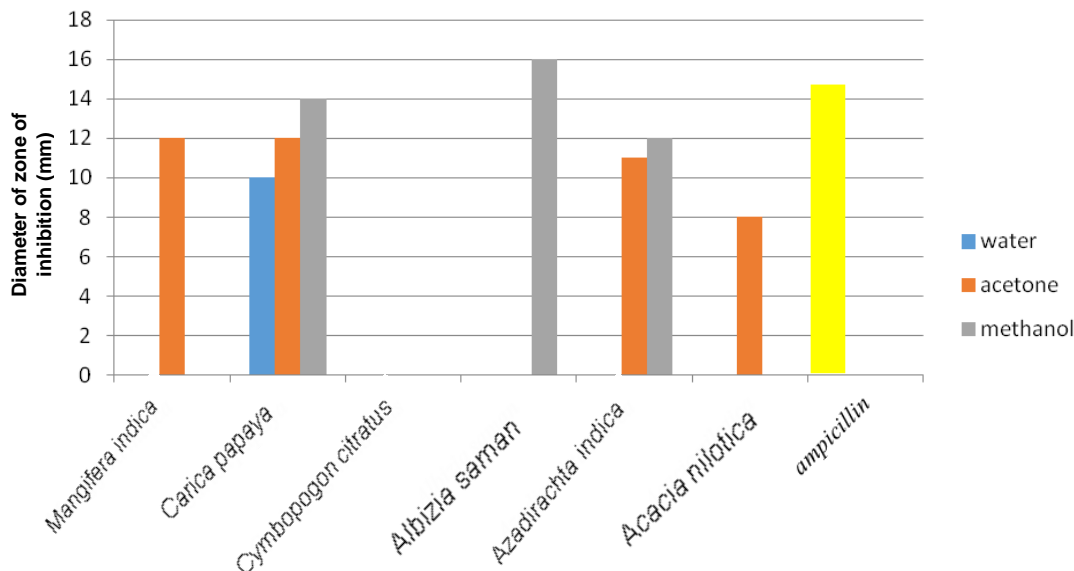
Figure 1. Antimicrobial activity of leaf extracts of test plants measured for *E. coli.*
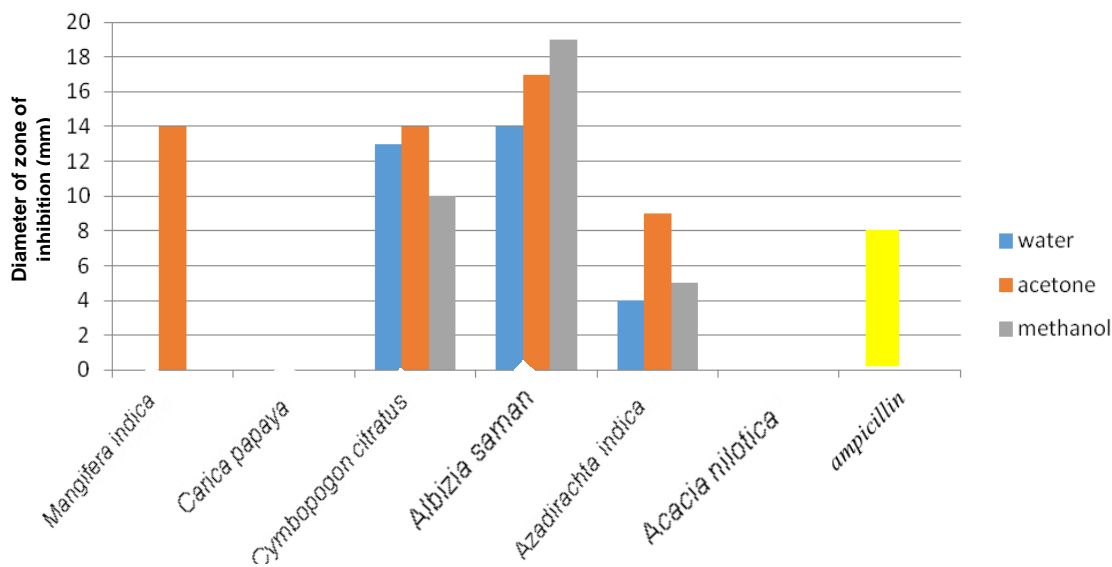
Figure 2. Antimicrobial activity of leaf extracts of test plants measured for *K. pneumoniae.*

various pathogenic bacteria. The main reason for this is the development of disease-resistant bacterial strains. Bacteria possess the ability to acquire as well as transmit resistance against antibiotics which are prepared synthetically. This is a genetic quality of bacteria- they recognize a therapeutic agent and develop resistance against it with time. Another reason which is contributing to the downfall of antibiotics is that some of them cause adverse effects to human health, the most common being unhealthy and deteriorating effects on some organs (Towers et al., 2001).

Many of these plant-extracts showed significant results. Prominent zones of inhibition show that these plants have a lot of potential to serve as effective antimicrobial drug alternatives against many antibiotics. These plants not only possess antimicrobial properties but are also 'human friendly', that is, they do not cause any harmful effects on the body (Trivedi et al., 2013). However, further studies are required for efficient evaluation of the potential effects of the plant-extracts as antimicrobial agents (Farnsworth, 1993). The results of this study and those of the previous researches (Mathur et al., 2013) will form the base for the

Figure 3. Antimicrobial activity of leaf extracts of test plants measured for *S. aureus.*

selection of other plants for investigations to be carried out in the future. This investigation will prove to be a milestone in this field of discovering new compounds having antimicrobial activities (Lam, 2007). This is a very promising and beneficial field; a lot of researches should be initiated for the betterment of mankind.

Conclusion

With this serious problem of antibiotic resistance in bacteria, there is a need to look for alternative antimicrobial agents other than synthetic antibiotics (Parmar and Rawat, 2012). Plants have valuable resources of secondary metabolites within them (Hada and Sharma, 2014). Various plant extracts have been known to have medicinal applications. Studies have been conducted all over the world to establish that plant extracts are indeed highly efficient against many pathogenic bacteria (Chowdhury et al., 2013). Therefore, the results justify that antibiotics from plants extracts are promising agents in treating these pathogenic strains.

Conflict of Interests

The authors have not declared any conflict of interests.

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