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Inhibition of Pathogenic Bacteria Isolated from Nuts and Seeds by Natural and Commercial Coumarins

تثبيط البكتيريا المسببة للأمراض المعزولة من بعض بذور المكسرات بواسطة الكومارين الطبيعية وبعض الكومارين التجاري

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ABSTRACT

The antibacterial potential of a natural coumarin compound that was extracted from the root of Lotus lalambensis Schweinf and several commercial coumarin compounds (herniarin, xanthotoxin, umbelliferone and scopoletin) were investigated against five pathogenic bacteria (Staphylococcus epidermidis, Bacillus mycoides, Escherichia coli, Staphylococcus aureus and Bacillus cereus) that were isolated from different nut products. The natural coumarin showed the best antibacterial potential against the most sensitive bacteria (Staphylococcus aureus and Bacillus cereus) with a minimum inhibitory concentration of 62.5 µg ml-1. The antibacterial mechanism of different coumarin compounds was investigated by assessing the effect of coumarin on the membrane potential to quantify the alkaline phosphatase and lactate dehydrogenase activities of tested bacteria. The membrane potential of Staphylococcus aureus was higher in natural coumarin than in the control and commercial coumarin, followed by Bacillus cereus. The concentration of extracellular protein and the activity of lactate dehydrogenase and alkaline phosphtase were evaluated, and the bacteria that was treated with the natural coumarin had the highest antibacterial activity. The results showed the significant potential of coumarin in isolated DNA cleavage reactions. Our data also showed that the addition of natural coumarin and commercial coumarins resulted in a higher discharge of membrane potential in the supernatant. The conclusion revealed that the natural coumarin of Lotus lalambensis is potentially a significant source of useful molecules for the expansion of new antibacterial agents and the promising manipulation of natural coumarin to protect contaminated nuts and other foods against the pathogenic bacteria.

الملخص

تم فحص الإمكانية المضادة للجراثيم لمركب كومارين الطبيعي مستخرج من جذر لوتس لالامبينسيس شوينف وبعض مركبات الكومارين التجارية (هيرنارين، زانثوتوكسين، أومبيليفيرون، وسكوبوليتين) ضد خمسة بكتيريا (ستاف ايبيدرمس، باسيلس مايكويد، ايشيريشيا كولاي، ستاف اوريوس، باسيلس سيريوس) معزولة عن منتجات الجوز المختلفة. أظهر الكومارين الطبيعي أفضل إمكانات مضادة للجراثيم ضد أكثر أنواع البكتيريا حساسية (المكورات العنقودية الذهبية والبكتريا العصوية) مع قيم MIC تبلغ 62.5 ميكروغرام مل -1. تمت دراسة الآلية المضادة للجراثيم لمركبات الكومارين المختلفة من خلال قياس الفوسفاتيز القلوي، وإزالة الهيدروجين اللاكتات، وعمل اختبار للغشاء. أظهر تركيز البروتين خارج الخلوي، ومستويات ديهيدروجينيز اللاكتات، وانزيم فوسفاز القلوي في مسببات الأمراض البكتيرية المعالجة بالكومارين الطبيعية نشاطه المضاد للميكروبات. تكشف الاستنتاجات التي تم التوصل إليها أن الكومارين الطبيعي لوتس لالمبينسيس هو مصدر مهم للجزيئات المفيدة لتوسيع العوامل المضادة للجراثيم الجديدة باستخدام الكومارين الطبيعي لحماية المكسرات الملوثة وغيرها من المواد الغذائية ضد البكتيريا المسببة للأمراض.

1. Introduction

Nuts are generally viewed as delicacies across the world due to their high nutritional value and taste. Different nut products are considered to be raw materials in several industries, as well as suitable for direct consumption. Nut seeds have higher a content of proteins, fats and minerals and contain little water content; therefore, they are highly vulnerable to microbial attacks, especially bacterial attacks (Bhat and Siruguri. 2003). Although nuts are extensively sold and consumed, there is scarce information available about their microbiological quality. In the last stage of processing, the levels of water activity become lower, which makes microbial growth difficult. Nuts can become polluted with foodborne microbes at any stage of manufacture, such as during harvesting, processing or consumption. Proper food safety measures should be adopted to prevent contamination issues.

Salmonella is considered a target organism for dry foods, including tree nuts and peanuts, because of its long-term persistence and high heat resistance in dry foods (Uesugi *et al.* 2007); (Kimber *et al.* 2012; Beuchat *et al.* 2013; Brar *et al.* 2015). The high levels of fat contribute to the enhanced resistance of pathogens in nuts (Beuchat and Mann. 2010). Infiltration of *Salmonella* into in-shell pecans is higher at 21°C and 35°C than at four °C and −20°C (Beuchat and Mann. 2010). Infiltrated pathogens demonstrate a higher thermal resistance than pathogens that are present on the surface (Beuchat and Mann. 2011). Other bacterial foodborne pathogens associated with dry foods, including tree nuts and peanuts, are *B. cereus*, *L. monocytogenes*, *C. botulinum*, *E. coli*, *C. perfringens* and *S. aureus* (Beuchat *et al.* 2013). *Enterococcus* and coliform microorganisms are the predominantly identified bacteria on nut surfaces (Hyndman. 1963). The major bacterial genera that have been identified are *Streptococcus, Staphylococcus, Bacillus, Xanthomonas, Achromobacter, Pseudomonas, Micrococcus* and *Brevibacterium* (King *et al.* 1970; Al-Moghazy *et al.* 2014). Although infectious bacteria, such as *L. monocytogenes*, *E. coli* and *Salmonella*, cannot regenerate on nut seeds, they can grow on these products for a year (Beuchat and Scouten. 2002; Blessington *et al.* 2013). *Salmonella* has also been identified in other nut products, such as raw almonds (Danyluk *et al.* 2005; Eglezos *et al.* 2008).

Traditional antibacterial therapy has been unsuccessful due to the cumulative resistance to the current agents (Ojala *et al.* 2000). Nevertheless, the plant domain constitutes a source of novel phytochemicals that might be important for use in medicinal applications (Alice *et al.* 1991). Coumarin is a promising heterocyclic molecule for the expansion of biologically active compounds for positive intervention in several infectious situations (Dua *et al.* 2011). It has aromatic and aliphatic characteristics because it contains a benzene ring attached to a pyrone ring. It is one of the oldest and most active groups of biologically active compounds with numerous compounds established for extensive biological action (Dua *et al.* 2011). Preceding work of diverse derivatives of coumarin discovered an extensive variety of anti-inflammatory (Curini *et al.* 2004), antibacterial (Basanagouda *et al.*, 2010); (Al-Rifai *et al.* 2012) (Li *et al.* 2015), antifungal (Dekic *et al.* 2010); (Arshad *et al.* 2011) anticoagulant (Manolov *et al.* 2006), anti-HIV ((Shikishima *et al.* 2001), antinociception (Manolov *et al.* 2006) and DNA-binding

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(Sarwar *et al.* 2015) properties.

The membrane's potential alteration is an early indication of damage in bacteria, and it can be evaluated by measuring the fluorescent intensity of DiBAC4(3). DiBAC4(3) is an anionic, voltage-sensitive, fluorescent probe that crosses the cytoplasmic membrane according to the transmembrane potential, with low intracellular fluorescence indicating the hyperpolarisation of a cell. Numerous studies have reported that phytochemicals possess a membrane-active mechanism that causes severe membrane damage through the disruption of the membrane integrity (Wu *et al.* 2016)

The present work reports the evaluation of the antibacterial potency of a series of commercial coumarins compared to a natural coumarin compound extracted from the root of *Lotus lalambensis.*

2. Material and Methods

2.1. Sampling:

Samples of various nuts, such as pistachios, hazelnuts, cashews, peanuts, almonds, walnuts and chestnuts (Table 1), were collected from various stores in Al Ahsa in Saudi Arabia in five replicates over the period of 2015–2016. The samples were taken using sterilised spatulas and collected in sterile, closed polythene bags. Fifty grams of each sample were collected. The samples were transported to the laboratory where they were maintained at room temperature for microbiological studies.

2.2. Microbiological Analysis:

Fifty grams of the sample were sterilised using a 0.4% sodium hypochlorite solution for three minutes. Following that, the supernatant solution was thrown away, and the sample was washed in sterilised distilled water and allowed to dry. The sample was then grinded, and one gram was added to 100 ml of sterilized distilled water and agitated.

2.3. Isolation of Bacteria:

2.3.1. Total viable count

The total viable count was carried out in line with the modified method, as described by Tassew & Seifu, 2011. All spreads were counted using the colony counter (Stuart Scientific Colony Counter, UK). Each colony was kept as slant cultures at five °C for further studies.

2.3.2. Total and faecal coliforms

The most probable number method was carried out to evaluate the total and faecal coliforms in each sample, according to the method described by Tassew and Seifu (2011).

2.3.3. Isolation and identification of *Escherichia coli*

From the tubes that gave positive results, a drop was transmitted to a test tube that contained trypton water and incubated at 44°C for 24 hours. All tubes in which a red ring colour appeared represented the formation of indole and were documented as positive for *E. coli*. The counts per gram were also calculated (Hood *et al.* 1983).

2.3.4. Isolation and identification of other bacteria

All the colonies were examined based on the morphology of colonies and cells, gram staining and additional biochemical tests. The identification of species was done by examining the cultures in the analytical profile index (API) galleries, API 50CHL (bioMérieux, Marcy L'Etoile, France), and using API 20E (Analytab Products, Inc., Plainview, N.Y.).

2.3.5. Natural coumarin

A natural coumarin compound 7-(5-Hydroxy-3,7-dimethylocta-2,6-dienyloxy)-chromen was extracted from the roots of *Lotus lalambensis,* which was kindly provided by Dr. Enas M. Ali, Department of Biological Sciences, Faculty of Science, King Faisal University.

2.3.6. Extraction

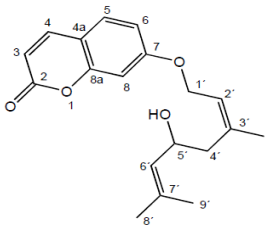
The extraction was carried out using petroleum ether at room temperature. After evaporation till dryness, the extract was obtained in yields (w / w) of 5.55.

2.3.7. Isolation

A sample from the petroleum ether extract (15.25 g) was applied to a Sephadex LH-20 column that was equilibrated with PE:MeOH:CHCl3 (2:1:1). After the comparison withthin layer chromatography TLC, the following five fractions were obtained: 1: 0.4076 g, 2: 1.66 g, 3: 4.25 g, 4: 0.734 g and 5: 0.0813 g.

Fraction 3 (four g) was applied to a Sephadex LH-20 column. After the comparison with TLC, the fractions were combined, which resulted in five new fractions, as follows: 1: 0.0055 g, 2: 0.0661 g, 3: 0.2234 g, 4: 0.5321 g and 5: 0.7221 g. From fractions 1–5, a pure compound (85.66 mg) was obtained. The compound was identified as 7-(5-Hydroxy-3,7-dimethylocta-2,6-dienyloxy)-chromen-2-one, depending on the (Nuclear magnetic resonance spectroscop NMR data that was compatible with those obtained by Ngadjui *et al.* (1989).

Figure 1. Chemical structure of isolated and purified coumarin compounds



2.3.8. Commercial coumarin compounds

Bergapten (Fluka Chemie AG, Switzerland), herniarin, xanthotoxin and umbelliferone (Carl Roth GmbH, Germany) and scopoletin (Sigma, St. Louis, MO) were dissolved in methanol (or acetone) to give a concentration of one mg ml^{-1}.

2.3.9. Antibacterial susceptibility testing

The culture was diluted with a sterile saline to a final density of five × 10^5 CFU mL^{-1}. The antibacterial potential was assessed using the agar dilution method. The positive control was vancomycin (VA, 30 µg, Oxoid, Basingstoke-Hampshire, UK). The Petri dishes were incubated for one day at 37ºC. The minimum inhibitory concentration (MIC) was defined as the lowermost concentration of compounds that did not show visible growth of bacteria. Extracts and compounds with MICs of less than or equal to 250 µg / mL were considered of concern Golus *et al.* (2016).

2.3.10. Determination of the effect of coumarin compounds on alkaline phosphatase activity of *Staphylococcus aureus* and *Bacillus cereus* screening of Alkaline Phosphatase- (ALP) producing bacteria

The isolates of Staphylococcus *aureus* and *Bacillus cereus* were screened by cultivating them on the heart infusion agar (Difco) containing 0.01% phenolphthalein bisphosphate tetrasodium salt (Sigma) and ten per cent NaCl (w / v), using the method described by Barber and Kuper (1951). After incubation at 37 °C for one to two days, all pink colonies were selected as potential ALP-producing strains. The selected isolates were separately confirmed for their ability to produce ALP using the following procedure: a loopful of the selected ALP-producing strain was inoculated into five ml of JCM

no.377 broth and incubated on a rotary shaker at 37 °C (150 rpm) for 24 hours for the seed culture. The seed culture broth (0.5 ml) was transferred into 50 ml of modified JCM No. 377 broth in a 250 ml Erlenmeyer flask (duplicate) and incubated in the above conditions. The supernatant was obtained after centrifugation of the cultures at 10,000 rpm (13,300 g) and four °C for ten minutes and was used as a crude enzyme for ALP activity detection.

2.3.11. Alkaline phosphatase activity assay

The ALP activity assay was carried out using the method described by Helianti *et al.* (2007). The reaction mixture that comprised one ml of ten mM p-nitrophenylphosphate (pNPP) (Sigma) in 0.2 M Tris-HCl buffer pH 10.0 with five mM MgCl2 and 0.1 ml of the crude enzyme was incubated at 37 oC for 15 minutes. The reaction was stopped with one ml of 1M NaOH, and its absorbance was measured at 405 nm. One unit of ALP was defined as the amount of the enzyme that yielded one µM of p-nitrophenol within one minute \ mg protein under the assay conditions. The protein concentration was estimated using the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as the standard effect of some coumarin compounds on the lactate dehydrogenase activity of *Staphylococcus aureus* and *Bacillus cereus*.

Cultures were grown aerobically with 0.5 to one litre of medium on a shaker at 80 cycles per min and incubated at 37 °C. Next, it was harvested by centrifugation at 2,500 X g for ten minutes at four °C. The cell debris was removed by centrifugation. The lactate dehydrogenase activity of the extracts were determined according to the method of Garrard and Lascelles (1968).

2.3.12. Determination of the effect of coumarin compounds on the lactate dehydrogenase activity of *Staphylococcus aureus* and *Bacillus cereus* Growth and the harvesting of organisms.

Cultures were grown aerobically in 2.5-liter flasks, which contained 0.5 to one litre of medium, on a reciprocal shaker at 80 cycles per minute. All cultures were incubated at 37 °C. The cultures were harvested in the late exponential phase of growth by centrifugation at 2,500 X g for ten minutes at four °C. The cells were washed once with 0.05 M potassium phosphate buffer (pH 6.5) and stored at -20 °C.

2.3.13. Preparation of cell-free extracts

The cells were suspended in approximately 20 volumes of 0.05 M potassium phosphate buffer (pH 6.5) and disrupted by three passages through a precooled French pressure cell. The cell debris was removed by centrifugation at 27,000 X g for 15 minutes at four °C. The extracts that were prepared in this manner generally contained approximately 0.3 mg of protein per ml.

2.3.14. Lactate dehydrogenase assay

Extracts that were prepared as above were assayed immediately for lactate dehydrogenase (LDH) activity. The complete assay mixture contained the following components in a final volume of one ml: potassium phosphate buffer (pH 6.5), 50 µmoles; sodium pyruvate, 2.5 µmoles; reduced nicotinamide adenine dinucleotide (NADH2), 0.136 µmoles; and from one to ten µg of protein, depending on the activity of the extract. After measuring the endogenous NADH2 oxidase activity, reactions were initiated by adding pyruvate. The decrease in optical density at 340 mA, resulting from the oxidation of NADH2, was followed with a Zeiss M4 QIII spectrophotometer (Carl Zeiss, Inc., New York, N.Y.) and equipped with a Varicord model 43 recorder. Assays were conducted at 24 °C. Specific activity is expressed as micromoles of NADH2 oxidised per minute per milligram of protein.

2.3.15. Determination of the effect of coumarin compounds on the membrane potential of *Staphylococcus aureus* and *Bacillus cereus*

The experiment was carried out according to the method of Sánchez, García, and Heredia, 2010 with some modifications. In brief, the overnight cultures of *S. aureus* and *B. cereus* were diluted with fresh nutrient broth to obtain a cell density of one × 10^7 CFU / mL. The bacterial suspensions were subjected to treatment with the samples at 25 °C for ten minutes. Then, the treated bacterial suspensions were further incubated with 0.5 µg / mL of the membrane potential-sensitive fluorescent probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3); Life Technologies, Eugene, OR, USA) in the dark for five minutes. After incubation, the fluorescence intensity of DiBAC4(3) was measured using a fluorescence spectrophotometer (Cary Eclipse G9800A, Agilent technologies trading Co., Ltd., Shanghai, China) at excitation and emission wavelengths of 492 nm and 515 nm, respectively. Background fluorescence resulting from CHQA that was added to the medium was determined and corrected.

2.4. DNA cleavage experimentation

2.4.1. Extraction of DNA

The DNA extraction method presented in this paper is an improved method of the standard phenol / chloroform method by Neumann *et al.* (1992). We eliminated the lysis step that used SDS / lysozyme or proteinase K and lysed the cells directly by phenol. To extract the DNA from the bacteria, one ml cell suspension was centrifuged at 8000 g for two minutes. After removing the supernatant, the cells were washed with 400 µl STE buffer (100 mM NaCl, 10 mM Tris / HCl, one mM EDTA, pH 8.0) twice. Then, the cells were centrifuged at 8000g for two minutes. The pellets were resuspended in a 200 µl TE buffer (ten mM Tris / HCl, one mM EDTA, pH 8.0). Following that, 100 µl Tris-saturated phenol (pH 8.0) was added to these tubes, followed by a vortex-mixing step of 60. The samples were subsequently centrifuged at 13,000 g for five minutes at four ºC to separate the aqueous phase from the organic phase. The 160 µl upper aqueous phase was transferred to a clean 1.5 ml tube. The 40 µl TE buffer was added to make 200 µl and mixed with 100 µl chloroform and centrifuged for five minutes at 13,000 g at four ºC. The lysate was purified by chloroform extraction until a white interface was no longer present; this procedure might have to be repeated two to three times. The 160 µl upper aqueous phase was transferred to a clean 1.5 ml tube. The 40 µl TE and five µl RNase (at ten mg / ml) were added and incubated at 37 ºC for ten minutes to digest the RNA. Then, 100 ll chloroform was added to the tube, mixed thoroughly, and centrifuged for five minutes at 13l000 g at four ºC. The150 µl ll upper aqueous phase was transferred to a clean 1.5 ml tube. The aqueous phase contained purified DNA and was either directly used for the subsequent experiments or stored at 20 ºC. The purity and yield of the DNA were assessed spectrophotometrically by calculating the A260 / A280 ratios and A260 values to determine the protein impurities and DNA concentrations.

2.4.2. Agarose gel electrophoresis

The products of cleavage were investigated according to the method of agarose gel electrophoresis. The samples (one mg ml^{-1}) were dissolved in Dimethylformamide (DMF). The samples (25 µg) were then added to the DNA that was isolated from the selected bacteria. The test samples were then incubated for 2two hours at 37 °C, and 20 µl of DNA was loaded into the wells of the electrophoresis chamber accompanied by a standard DNA marker that contained the TAE buffer. The gel was run at a constant 50 V for 30 minutes. The gel was detached and stained with ten mg / ml ethidium bromide for 15 minutes, and the bands were detected using the Vilberlourmate gel documentation system and photographed to evaluate the degree of DNA cleavage. Finally, the obtained results were compared with a standard DNA marker.

2.4.3. Statistical analysis

The data was described as mean ± standard error (SD). The data also analysed statistically using a one-way analysis of variance (ANOVA), and the differences between the means were evaluated for significance at $P \leq 0.05$ using Duncan's multiple range test (SPSS, 16.1 Chicago, USA).

3. Results

3.1. Isolation and Identification of Bacteria:

The data recorded in Table 2 shows the bacterial populations of different nut products that were gathered from different shops in Al Ahsa in Saudi Arabia. The total viable count (Table 3) ranged from 2.0 to 5.3 log CFU g^{-1}. The total viable count values varied significantly across the nut products. The uppermost count of the total coliforms (3.5 log CFU g^{-1}), faecal coliforms (0.95 log CFU g^{-1}) and *E. coli* (0.66 log CFU g^{-1}) were found in peanuts. The chestnut samples showed the lowest values. The isolation of other microbes (Table 3) showed that the number of isolates differed among each sample. The highest number of isolates (92) occurred in pistachios, while the lowest (36) was recorded in hazelnuts.

Depending on the morphological features, biochemical tests and the grouping of API 20E and API 50CHL, *Staphylococcus epidermidis, Staphylococcus aureus*, *Bacillus mycoides*, and *B. cereus* were identified. Some isolates did not show a reaction on the API 20E and 50CH (Table 3). The percentage of each isolate varied among different samples. For example, the percentage of *B. cereus* was 32.6, 41.66, 39.58, 31.4, 39.68, 74.22 and 35% in pistachio, hazelnut, cashews, peanut, almond, walnut and chestnut, respectively.

Table 1: English and scientific names of tested nuts

No.	English name	Scientific name
1	Pistachio	Pistacia vera
2	Hazelnut	Corylus avellana
3	Cashews	Anacardium occidenta
4	Peanut	Arachis hypogaea
5	Almond	Prunus dulcis
6	Walnut	Juglans regia
7	Chestnut	Castanea

Table 2: Count expressed as log CFU / 100 ml indicating the microbial quality of different nuts

parameter	Log CFU/100 ml							
	Pistachio	Hazelnuts	Cashews	Peanuts	Almonds	Walnuts	Chestnuts	
Total coliforms	$2.5^{e} \pm 0.3$	$1.95^{c} \pm 0.1$	$1.9^{c} \pm 0.2$	$3.3^{f} \pm 0.5$	$2.3^{d} \pm 0.7$	$1.5^{b} \pm 0.4$	$1.2^{a} \pm 0.3$	
Faecal count	$0.83^{e} \pm 0.11$	$0.71^{d} \pm 0.21$	$0.44^{b} \pm 0.15$	$0.95^{g} \pm 0.2$	$0.90^{f} \pm 0.26$	$0.63^{c} \pm 0.19$	$0.33^{a} \pm 0.27$	
Total viable count	$5.3^{f} \pm 0.6$	$3.2^{d} \pm 0.8$	$2.4^{b} \pm 0.4$	$6.1^{g} \pm 0.5$	$4.6^{e} \pm 0.8$	$2.9^{c} \pm 0.3$	$2.0^{a} \pm 0.7$	
Escherichia coli	$0.48^{d} \pm 0.11$	$0.33^{b} \pm 0.21$	$0.36^{bc} \pm 0.15$	$0.66^{g} \pm 0.21$	$0.53^{e} \pm 0.26$	$0.56^{ef} \pm 0.2$	$0.22^{a} \pm 0.19$	

Values are mean (n = 3) ± SE. The means followed by different superscript letters in the same raw are significantly different according to ANOVA and Duncan's multiple range tests.

Table 3: Identification of bacterial species isolated from different nuts

Bacterial species	Pistachio		Hazelnut		Cashews		Peanut		Almond		Walnut		Chestnut	
	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Bacillus cereus	30	32.6	15	41.66	19	39.58	22	31.4	25	39.68	17	74.22	14	35
Bacillus mycoides	25	27.17	9	25	13	27.0	18	25.7	19	30.15	ND	0	11	27.5
Staphylococcus epidermidis	24	26.0	8	22.22	11	22.91	19	27.14	10	15.87	16	44.44	12	30
Staphylococcus aureus	12	13.04	ND	0	ND*	0	8	11.42	8	12.69	2	5.55	3	7.5
Unidentified species	1	1.08	2	5.55	5	10.4	3	4.28	1	1.58	2	2.77	ND	0
Total bacteria	**92**	-	**36**	-	**48**	-	**70**	-	**63**	-	**37**	-	**40**	-

* Not determined

%= No. of each isolate in a sample/ total bacteria in a sample× 100

3.2. Antibacterial Activity of Coumarin:

The structure of the natural coumarin compound is shown in Fig. 1. This is the first study to report the presence of this compound in *Lotus lalambensis* Schweinf. Therefore, we assessed the antibacterial potential of natural coumarin compared with five different coumarin derivatives available commercially, including herniarin, Scopoletin, Bergapten, umbelliferone and xanthotoxin. The natural and commercial coumarin compounds showed antibacterial activities with both bacteriostatic and bactericidal capabilities (Table 4). However, some variances were detected amongst the action of the five compounds and the natural coumarin. The natural coumarin was the most active, while herniarin and umbelliferone showed the lowest activity. Interestingly, as natural coumarin was the most active compound, this suggests that natural coumarin is responsible for the antibacterial activity that was observed in the coumarin extract of *Lotus lalambensis*.

Table 4: Antibacterial effect of different coumarin compounds and (MIC / MBC µg / ml)

Bacteria	Compound						Reference drug
	NC*	Herniarin	Bergapten	Umbelliferone	Scopoletin	Xanthotoxin	Vancomycin
Staphylococcus epidermidis	250/125	>250	125/250	>1000	500/500	>1000	62.5/125
Bacillus mycoides	125/250	>250	500/500	>1000	>500	>1000	>250
Escherichia coli	250/250	>250	>1000	>1000	>250	>500	125/250
Staphylococcus aureus	62.5/125	>125	125/250	>1000	250/250	>250	62.5/125
Bacillus cereus	62.5/250	>125	62.5/500	>1000	125/125	750	62.5/125

NC* natural coumarin

3.3. Effect of Coumarin on Lactate Dehydrogenase, Alkaline Phosphatase Activities and Membrane Potential:

To define the antibacterial mechanism of coumarin, the effect of coumarin on the membrane potential of the tested bacteria was assessed. The results showed that the addition of natural coumarin and commercial coumarins resulted in a higher discharge of membrane potential in the supernatant. Amongst the infectious bacteria, the membrane potential of *Staphylococcus aureus* was higher in the natural coumarin than the control and commercial coumarin, followed by *Bacillus cereus*. The effect is significantly different in *Staphylococcus aureus* only. Xanthotoxin and bergapten control coumarin provided the highest broken membrane potential compared to the other commercial coumarins in *Staphylococcus aureus* and *Bacillus cereus* (Table 5).

The quantification of the LDH and enzyme activity suggested that treatment with coumarin powerfully affected the cell wall of the bacteria. The results showed an increase in the enzymes activities of the media containing the natural coumarin compared with that that was supplemented with commercial coumarin (Table 5). Among the commercial coumarin, bergapten had the highest effect on ALP. LDH enzyme activity was higher in *S. aureus,* and this increase was significant. The commercial coumarin xanthotoxin had the best effect and showed high LDH activity, while umbelliferone produced the lowest activity of LDH enzyme activity in both *Staphylococcus aureus* and *Bacillus cereus* (Table 5).

Table 5: ALP activities (nmol min^{-1} (mg protein) $^{-1}$ and membrane potential in *S. aureus* and *B. cereus*

Treatment	Staphylococcus aureus			Bacillus cereus		
	ALP	LDH	Membrane potential	ALP nmol min-l (mg protein) -1	LDH	Membrane potential
Control	a7.53b±0.06	a9.26b±0.08	a-8.56b±0.05	a5.13a±0.05	a7.52a±0.05	a-7.22a±0.07
Herniarin	d13.45b±0.17	d22.87b±0.14	c-84.11b±0.11	c8.23a±0.04	c10.11a±0.11	c-64.11a±0.16
Bergapten	f17.23b±0.07	e27.56b±0.16	e-117.15b±0.17	f12.56a±0.02	d16.17a±0.08	f-101.17a±0.03
Umbelliferone	b11.35b±0.12	b14.73b±0.03	b-59.19b±0.04	b6.61a±0.08	a7.56a±0.14	b-37.16a±0.12
Scopoletin	c13.71b±0.13	c20.62b±0.15	d-92.25b±0.17	d8.43a±0.01	b9.33a±0.22	d-66.33a±0.21
Xanthotoxin	e16.51b±0.22	f32.32b±0.11	f-119.77b±0.15	e11.32a±0.06	e21.11a±0.09	e-99.11a±0.05
NC*	g21.33b±0.66	g46a±0.29	g-133a±0.23	g16.22a±0.36	f33.11a±0.43	f-126b.11a±0.33

NC*: Purified natural coumarin

One unit of ALP was defined as the amount of the enzyme yielding one micromole of *p*-nitrophenol within one minute per milligram protein under the assay conditions.

Values are mean (n = 3) ± SE. The means that it is followed by the same superscript letter in the same row, and those followed by the same subscript letter in the same column are not significantly different according to ANOVA and Duncan's multiple range test.

3.4. Agarose Gel Electrophoresis

The DNA extracted from *S. aureus* and treated with natural coumarin (NC) (Fig. 2) was in dimer form. This indicates degradation, which is a characteristic that is not found in untreated DNA. The results showed the significant potential of coumarin in the isolated DNA cleavage reaction. From the obtained results, we concluded that the coumarin can act as a powerful nuclease agent. When the compound was found to cause DNA cleavage, it could be determined that the compound caused inhibition of the pathogenic microbe by cleaving its genome.

Figure 2. Cleavage of S. aureus DNA. M: standard molecular weight marker; C: control DNA of S. aureus ; NC: S. aureus DNA treated with natural coumarin.

4. Discussion

Food products with low-moisture levels, such as nuts, are considered a low risk for foodborne pathogens because they are consumed in a dry state. This may lead to the misconception that the low levels of pathogenic bacteria in nuts are not a food safety concern. Therefore, the incidence of bacterial contamination in nuts has been infrequently described. However, many foodborne pathogens can cause infections, even when present at very low levels. Bacterial diseases threaten nut products all over the world. The occurrence of *E. coli* in nut seeds may be a consequence of contamination from irrigation water, processing containers, the people taking part in the agricultural processes and occasionally from the processing environment.

Our results are compatible with those found by (Freire and Kozakiewicz. 2005), who found that while *Bacillus* spp. were the most common isolated organisms from cashews, other diverse genera of bacteria were also detected. Coliforms, such as *E. cloacae*, *E. sakazakii* and *K. pneumonia* were also found, along with *B. cereus* and *S. aureus*, which can cause food poisoning. Although pathogenic bacteria may be present on cashews, they are mostly either saprophytic (making them incapable of producing enterotoxins) or isolated in low numbers. They also may be destroyed later during processing due to the heat and salt. Our outcomes are in accordance with those of (Blessington *et al.* 2013), who described the occurrence of *S. enterica, E. coli* and *L. monocytogenes* on walnuts during storage. Other researchers successfully isolated and identified some strains of *Xanthomonas arboricola pv.juglandis* in walnuts growing in areas of Turkey (Ozaktan *et al.* 2012). Nut products have been recognized as a vector for salmonellosis. (Zhang *et al.* 2017) estimated the occurrence and level of infection of *Salmonella* in cashews, pecans, walnuts, hazelnuts and pine nuts at markets in the United States. *E. coli* O157:H7 has been isolated from peanuts, pecans and walnuts, while *L. monocytogenes* has been isolated from peanuts, pecans and mixed nuts (Brar *et al.* 2015)

In the present work, a new phytochemical coumarin and some commercial coumarin derivatives were assayed for their antibacterial potential on the growth of some bacterial species. We have detected that all coumarin derivatives had an excellent to moderate interfering effect on bacterial growth. The antimicrobial activity of some prenylated coumarins and prenylated furanocoumarins (Schinkovitz *et al.* 2003; Stavri and Gibbons, 2005), in addition to the antimicrobial potential of furanocoumarins and prenylated furanocoumarins, have been described before (Walasek *et al.* 2015). Our results were parallel with those of (M de Souza *et al.* 2005), who reported the antimicrobial potential of coumarin *per se* and other coumarin derivatives against *B. cereus*, *S. aureus*, *P.aeruginosa* and *E. coli*. They found that osthenol was the most active against gram-positive bacteria with MIC (125 and 62.5 µg / ml). Our data is also compatible with those of (Tan *et al.* 2017) who found that the *Prangos hulusii* extract and its prenylated coumarins derivatives display antimicrobial potential against different bacterial strains at concentrations between five and 125 µg / ml. The novel coumarin, 40-senecioiloxyosthol, exhibited MIC of five µg / ml against *B. subtilis,* while MIC of murraol and auraptenol has a 63 µg / mL value against *K. pneumoniae* and *B. subtilis*. (Das *et al.* 2018) studied the effect of 18 new coumarin derivatives for antibiofilm activity against *S. aureus* and *P. aeruginosa*. They detected that all the coumarin derivatives displayed major effects in controlling activities against bacteria.

The great antibacterial activity of coumarin might be attributed to its lipophilic properties and planar molecular structure, which increases its penetration into the cell membranes of bacteria (M de Souza *et al.* 2005). The suppressive action of coumarins was influenced by the patterns of substitution. Substitution of a less polar group (OMe, Me) at C6 by an OH group reduced the antibacterial activity against all of the tested bacteria (Jurd *et al.* 1971). The addition of an OH group at C7 of the coumarin *per se* decreased the antibacterial potential against all the tested bacteria. These conclusions suggest that the antimicrobial action of oxygenated coumarins are influenced by the position of both polar and less polar groups at the aromatic ring of the coumarin (de Souza *et al.* 2005). The antimicrobial potential of coumarins might be related to the number of oxygen substituents. These results revealed that the extent of substitution can be considered one of the most important factors in the activity of coumarins, while polarity can be considered another important factor. From the examination of the groups of coumarins, it was found that a very close relationship exists between structure and activity (Kayser and Kolodziej. 1999). Coumarin displayed high antibacterial potential that might be suggestive of passive diffusion, which is enabled by its lipophilic properties and its planar molecular structure. It is recommended that simplicity of aromatic substitution and evasion of bulky side chains helps with diffusion into the bacterial cell wall (Rauckman *et al.* 1989). Therefore, the mechanism of action is attributable to interactions with the cell membrane of bacteria.

It illustrated that the diffusion of the coumarin through the bacteria could be prohibited due to the occurrence of the peptidoglycon barriers and another components (Nazzaro *et al.* 2013). Furthermore, (Arokiyaraj *et al.* 2014) found that plant compounds interrupted cells and damaged the bacterial cell membrane potential (Arokiyaraj *et al.*

2014). The data of protein is in agreement with the statement of (Sang Sung and Gun Lee. 2010), who found that the phytochemical compositions of the medicinal plants generally resulted in modifications to the cell walls of the gram positive and gram negative bacteria by influencing the membrane (Sang Sung and Gun Lee. 2010).

Reports have demonstrated that pathogenic bacteria release ALP and LD enzymes when exposed to stressful conditions and throughout their time of sporulation (Arokiyaraj *et al*. 2014). The increase in enzymes concentration showed that the coumarin created a bad environment for bacterial growth, which results in the release of the enzymes. To the best of our knowledge, the effect of coumarin on the activity of LDH and ALP was never understood. However, the results of the membrane damage activity in this study show a low membrane damage activity. The LDH release activity values of different compounds are generally high, which shows that the mode of action of the coumarin compounds are through membrane damage of the bacteria cells. Increased permeability of the cell membrane or leakage of cell contents could be caused byreactive oxygen species ROS.

Our results showed that coumarin was able to cleave isolated DNA, and from this, we conclude that coumarin can act as a powerful nuclease agent. Its ability to cleave DNA could cause the inhibition of pathogenic microbes by cleaving its genome. The coumarin's fluorogenic properties and reactivities in DNA were rarely reported and unclear, which limits its bio applications due to possible side reactions towards biomolecules. Similarity, (Jadhav *et al*. 2010) showed that tetrahydroisoquinoline and protoberberine alkaloids (coumarin analogues) were very active against the DNA of *S. aureus* and the fungus *A. niger*. Similarly, our results mirror those of (Ma *et al*. 2004), who prepared a root extract of *Mallotus resinosus* and found that the extract displayed substantial $Cu^{(2+)}$ dependent DNA strand scission activity. They also identified that scopoletin, which is a coumarin derivative, was the major compound accountable for the cleavage of DNA in the extract. Coumarin derivatives can serve as perfect DNA cross-linking agents, alkylation agents for site-specific labelling and fluoroprobes for a single nucleotide polymorphism (SNP) analysis. They provided a novel insight of biotoxicity of coumarin in the biological system and are a novel bioanalytical tool (Sun, 2015).

5. Conclusion

In this research, we have examined the antibacterial action of purified coumarin, 5'-hydroxy-aurapten, from the roots of *Lotus lalambensis* against different bacteria isolated from nuts. Purified natural coumarin was shown to significantly damage the membrane potential and increase the production of lactate dehydrogenase and alkaline phosphatase enzymes of both *S.aureus* and *B. subtilis*. Therefore, *Lotus lalambensis* is a new source of natural coumarin that could be established as an antibacterial agent to protect nuts and other food products against contamination from pathogenic bacteria in the food agriculture and industry.

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