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Phytochemical Analysis and Effectiveness of Ripened *Spondias pinnata* Fruit Extracts against Antibiotic-resistant Mastitis-causing Bacteria

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ABSTRACT

Antibiotics are used to treat mastitis in dairy cows in Thailand's dairy industry. As a result, some antibiotics may persist in the milk. Furthermore, the usage of antibiotics may result in resistance to certain strains. As a result, the treatment's effectiveness is diminished, resulting in long-term health consequences for milk users. Nature's active substances have been explored to treat and prevent mastitis. This study aimed to investigate the antibacterial activity of *Spondias pinnata* fruit extracts against antibiotic-resistant mastitis. The ripened *S. pinnata* fruits were collected and extracted using the orderly polarity solvent hexane, ethyl acetate, isopropanol, and ethanol. The isopropanol (6) and ethanol (7) crude extracts were separated into two layers by liquid-liquid extraction (Hexane: Methanol: H_2O) combining ultrasonic. The Methanol: H_2O layer was named 6A* and 7A*, while 6B* and 7B* were the separating parts of hexane. The antibiotics and partial extracts were investigated for antimicrobial activity using a disc diffusion assay. Gas column chromatography-mass spectroscopy (GC-MS) analyzed the phytochemical profile. The results showed that the % yield of 6A*, 6B*, 7A*, and 7B* were 47.77, 1.01, 54.83, and 2.10%, respectively. Ciprofloxacin, amikacin, doxycycline and both extracts (6A* and 7A*) expressed the inhibiting growth of mostly mastitis-causing bacteria *Staphylococcus aureus* M007012, *Escherichia coli* M225012, *Staphylococcus epidermidis* M236021, *Mycoplasma pneumoniae* A2466, and *Enterococcus faecalis* A0522. Antimicrobial properties were identified as being associated with phytochemical types in 6A* and 7A*. Therefore, the dairy industry may develop or combine ripened *S. pinnata* fruit extract to prevent mastitis-causing bacteria.

Keywords: *Spondias pinnata*, biological activity, antibiotic-resistant microorganisms, mastitis, antimicrobial

INTRODUCTION

Mastitis is an infection of the mammary gland. This disease is very impact on breast-feeding animals which results in significant economic losses, usually caused by a bacterial infection. Mastitis negatively impacts milk production, cow health, and farm profitability (Aghamohammadi et al. 2018). Various bacteria, including *S. aureus*, Streptococcus spp. Mycoplasma spp, *Enterobacter* and *E. coli*, can cause mastitis in dairy cows (Acharya et al. 2022). The infection typically enters the udder through the teat canal, possibly due to improper milking practices, unhygienic conditions, or contaminated bedding (Ankita et al. 2023).

Mastitis has a detrimental impact on milk production and quality. Infected cows often experience a decrease in milk yield. The milk produced may contain bacteria, somatic cells, and inflammatory substances, leading to poor milk quality and reduced shelf life (Maia et al. 2018). The condition is characterized by atypical rise in the quantity of somatic cells and a decrease in the quality of milk. The Fluro optoelectronic approach is used to identify high somatic cell count (SCC) in mastitis cows. The threshold value for mastitis is approximately 200,000 cells/ml in the SCC (Acharya et al. 2022). Milk from cows with mastitis is typically discarded to prevent contamination of the bulk tank and maintain product quality. Mastitis in dairy cows is a significant challenge in

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the dairy industry. It affects milk production, cow health, and farm profitability. Through diligent management practices, timely detection, proper treatment, and preventive measures, the incidence and impact of mastitis can be minimized, resulting in improved cow health, milk quality, and overall productivity on dairy farms (Maia et al. 2018).

Most farms currently use antibiotics widely to treat mastitis in dairy cows. Excessive antibiotic use causes the presence of remains in unprocessed milk. However, the development of drug-resistant bacteria jeopardizes the efficacy of drugs for mastitis management (Li et al. 2023). Thailand has laws that limit the quantity of antibiotic residue in raw milk to a maximum of 0.004-0.2 mg/kg, according to the National Bureau of Agricultural Commodity and Food Standards (2010). Furthermore, there is concern over the presence of drug residue in raw milk, as well as the issue of antibiotic resistance among mastitis pathogens. The excessive and prolonged administration of antibiotics impacts the quality of unpasteurized milk. The presence of antibiotics in milk products poses significant risks for humans (Pașca et al. 2017). This encompasses the ongoing influence on those who ingest dairy products. In terms of mastitis infection, it is critical to discover novel and efficient treatment approaches that do not impact dairy products. Mastitis-causing bacteria has ability to produce biofilm. The biofilm matrix consists of polysaccharides, microbial cells, water, and other extracellular products. This structure supplies an environment of security and encouragement that promotes bacterial growth. As a result, bacteria are showing increased resistance to drugs and their hosts' immune systems (Caneschi et al. 2023). An alternative approach entails using botanical extracts or bioactive compounds, with the goal of eradicating bacteria that contribute to the development of mastitis. Gomes and Henriques (2016) have previously shown that some compounds found in medicinal plants, such as *Combretum molle* and *Commicarpus pediculosis* can kill bacteria that cause mastitis. The application of *Ocimum sanctum* leaf powder to cows with mastitis infections resulted in a decrease in somatic cell counts (Sharun et al. 2022). *Terminalia chebula* is beneficial for treating subclinical bovine mastitis. The protective effect of *T. chebula* extract at a dosage of 500 g/mL was comparable to that of amoxicillin (100 g/mL) against some bacteria, including *E. coli*, *S. aureus*, *Bacillus megaterium*, and *Pseudomonas aeruginosa* (Kher et al. 2019). However, there has been no research on the phytochemicals derived from *S. pinnata* fruit extracts to assess cattle's susceptibility to mastitis pathogens.

Spondias pinnata, also known as wild olive, is a perennial botanical species indigenous to the Southeast Asian region of Thailand, a Southeast Asian country. The deciduous tree reaches a height of approximately 15–25 meters. The trunk is upright and has a round shape. The canopy is rounded, and branches tend to hang down. The bark of the tree is grey and thick, with a few small bumps. There have been previous research reports that have tested the resin extracts of *S. pinnata* for their antimicrobial properties and found that they can resist infection *Saccharomyces cerevisiae, Bacillus subtilis, E. coli, Enterobacter sakazakii* and *Acinetobacter baumannii* (Gupta et al. 2010). Moreover, *S. pinnata* extract can resist *Shigella dysenteriae, Salmonella typhi, P. aeruginosa, Bacillus megaterium, E. coli* and *S. aureus* (Uddin et al. 2011). However, research on inhibiting bacteria that cause mastitis, especially bacteria that show antibiotic resistance, is limited. There are just a few reports available.

This research aims to study the antimicrobial properties of ripened *S. pinnata* fruit extracts against mastitis pathogens (*S. aureus* strain M007012, *E. coli* strain M225012, *S. epidermidis* strain M236021, *M. pneumoniae* strain A2466, and *E. faecalis* strain A05224) compared with antibiotic drugs (ciprofloxacin, amikacin, sulfamethoxazole, doxycycline, and ampicillin). This study's data may be able to replace antibiotics in treating mastitis in dairy cows.

MATERIALS AND METHODS

Isolated strains of animal pathogens: *S. aureus* M007012, *S. epidermidis* M236021 (Gram-positive bacteria), *E. coli* M225012, *M. pneumoniae* A2466, *E. faecalis* A05224. The bacteria used in this experiment were obtained from the Department of Biology, Faculty of Science and Technology, Rajabhat Maha Sarakham University. The Animal Ethics Committee approved the research under the Animal Ethics Approval Certificate Number U1-04504-2559.

The extracts preparation

Ripened *S. pinnata* fruits are used as raw materials to extract active ingredients. The Northeastern Area of Thailand exhibit the ripened time program of *S. pinnata* fruit between September and February of next year. This study collected ripened *S. pinnata* fruits from Khok Yai Village, Ban Fang Subdistrict, Khon Kaen Province. The fruit materials were transferred under the low-temperature system using ice-cool packs. Then, ripened *S. pinnata* fruits were washed with cleaned water three times. After that, these materials were frozen at -20°C for 24 hours. This process is a technique that quickly collects the flesh and peels of *S. pinnata*. In this process, a homogenizer machine was used to reduce the surface area of *S. pinnata* flesh and peels. The samples were balanced and then placed into a homogenizer chamber. This machine was set to homogenize the sample at 3,000rpm in 5min. Next, the homogenate part was frozen dry by lyophilization. The powder of *S. pinnata'* s flesh and peels were kept at -20°C. This powder was balanced for 200g and then placed into extracted chambers. After that, the extraction process was performed according to the recent study by Patathananone et al. (2019). This study collected two extracts, WMRI_isopropanol and WMRI_Ethanol, to separate the bioactive agents using a mini-modified liquid-liquid extraction technique. Approximately 10g of each extraction part was placed into the extracted bottles and dissolved by hexane: methanol: water (4:1:0.001). The chambers were placed into the ultrasonicate machine and sonicated at 35kHz, 4°C for 60min. After that, the sonicated samples were subjected to a separation funnel and stood overnight at room temperature. The methanol layer of the separated WMRI_isopropanol sample was called 6A*, while 6B* was the hexane layer. In addition, methanol and hexane layers of WMRI_ethanol extract were

named 7A* and 7B*, respectively. All extracted parts (6A*, 6B*, 7A*, 7B*) were concentrated by an evaporator and then balanced, kept at -20°C.

Preparation of bacterial cell cultures

The isolated strained of mastitis bacteria (*S. aureus* strain M007012, *E. coli* strain M225012, *S. epidermidis* strain M236021, *M. pneumoniae* strain A2466, *E. faecalis* strain A05224 were streaked on the nutrient agar solid medium, incubate at 37°C for 24–48 hours. The 3–5 single colonies of bacterial cells were picked and then incubated in sterile nutrient broth flasks. The cultured flasks were incubated at 37°C for 16–18 hours. The bacterial cell growth in nutrient broth was collected and inoculated with 1X PBS or 0.85% normal saline solution until the turbidity was equal to McFarland no. 0.5 showed the bacterial cells of 1.5 x 10^8 CFU/mL.

Antibacterial activity assay by disc diffusion technique

Position the discs along the intended location to pour the solution and let it dry. Next, transfer a solution containing mature *S. pinnata* fruit extract 6A* at 150µg/mL and 7A* at 91µg/mL concentration onto a 10µL disc and allow it to dry. Use 1X PBS as a negative control and ciprofloxacin, amikacin, sulfamethoxazole, doxycycline, and ampicillin as positive controls. Incubate the samples at a temperature of 37°C for a duration of 24 hours. The measurement was taken of the diameter of the resultant clean zone.

Analysis of phytochemical types using gas column chromatography-mass spectroscopy

The compounds in 6A* and 7A* were determined using gas column chromatography (Agilent 7890B). The samples were separated using HP-5 MS 25m x 250µm x 0.25µm column, flow rate 1.2mL/min, and helium was set as the carrier gas. The initial temperature was 250°C, split less mode 0.75 min, and the MSD transfer line 250°C. The time program was performed as follows. In the initial step, the oven temperature was performed at 60°C, hold times for 3mins, and then run for 3mins. The oven temperature of Ramp1 was 80°C and ran for 23mins. Next, the oven temperature was increased to 120°C and then managed to 36.33mins. Finally, the temperature was increased to 240°C, and the time program was stopped at 66.33mins. The mass analyzer (Agilent 5977B) was investigated using an EI type that fixed electron energy 70 eV. The scan mode started at mass 45.00 to 500.00, with ion source temperature 230°C and quadrupole temperature 150°C.

Statistical analysis

The antibiotics and 6A* and 7A* extracts could eliminate microbes, as evidenced by the mean diameter of the inhibitory clearance zone and the standard deviation (mean±SD).

RESULTS

% Yield of the separated fractions

The powder of *S. pinnata* extracted by hexane, ethyl acetate, isopropanol, and ethanol were named WMRI Hexane, WMRI Ethyl acetate, WMRI Isopropanol, and WMRI Ethanol, respectively. The potential of biological activities was expressed in WMRI Isopropanol and WMRI Ethanol. Thus, both extracts were collected and separated using the liquid-liquid extraction technique. WMRI Isopropanol was fractionated into two layers: methanol layer and hexane layer. Both extracted layers were named 6A and 6B, respectively. Additionally, WMRI Ethanol was separated by using the same process, and the partial fraction was named 7A* and 7B*, respectively. The % yield of each fraction is represented in Table 1. The polarity solvent system used to extract phytochemical agents from the powder of *S. pinnata* also showed the capacity to dissolve the high polarity of compounds in WMRI Isopropanol and WMRI Ethanol extracts. Therefore, the partial fractions 6A* and 7A* represented % a yield higher than 6B* and 7B*.

Table 1: Yield (%) of the partial fractions collected from the liquid/liquid extraction technique.

Solvent layer	Extract's name	% Yield
Methanol: H_2O	6A*	47.77
Hexane	6B*	1.01
Methanol: H_2O	7A*	54.83
Hexane	7B*	2.10

Antibacterial activities of the phytochemical in extracts (6A*, 7A*)

Ripened *S. pinnata* fruit extract 6A* concentration 150µg/mL, 7A* concentration 91µg/mL, and 1X PBS pH 7.4 were used as a negative control, and the antibiotics ciprofloxacin, amikacin, sulfamethoxazole, doxycycline and ampicillin as a positive control, which showed that the substances Extracted from ripened *S. pinnata* 6A*, 7A* could inhibit the growth of bacteria. *S. aureus* strain M007012, *E. coli* strain M225012, *S. epidermidis* stain M236021, *M. pneumoniae* stain A2466 and *E. faecalis* stain A05224, while the bacteria is resistant to antibiotics as shown in Fig. 1.

GC-MS data

The GC-MS results of the biological compounds in 6A* and 7A* are represented in Table 2 and 3, respectively. Approximately eleven substances were detected in 6A*, such as 1H-Tetrazole,1-methyl-, 2-Furancarboxylic acid, hydrazide, 5-Amino-3H-[1,2,3]triazole-4-carboxylic acid, hydrazide, 2-Cyclohexylethyl ethylphosphonofluoridate, Oxirane, diethylboryloxymethyl-, ethanol,1-(2-methyl-2H-tetrazol-5-yl)-2-[(thiophen-2-ylmethyl)amino]-Phosphoric acid, diethyl dodecyl ester, Tetradecanoic acid, Decanoic acid, silver (1+) salt, Methyl 12,13-tetradecadienoate and beta-sitosterol. Twenty-five types of biological agents were found in 7A* and exhibited a higher number than 6A*. Antibacterial and antimicrobial activities are shown by various identified compounds such as 3-Furaldehyde, 1H-Tetrazole,1-methyl, 2-Furancarboxylic acid, hydrazide, Methyl 12,13-tetradecadienoate, 3,3-dimethoxypropane-nitrile, 1H-Tetrazole,1-methyl-, cyclohexane-carboxylic acid, 2-ethoxyethyl ester, Methyl 12,13- tetradecadienoate, n-Hexadecanoic acid, Methyl 12,13-tetradecadienoate.

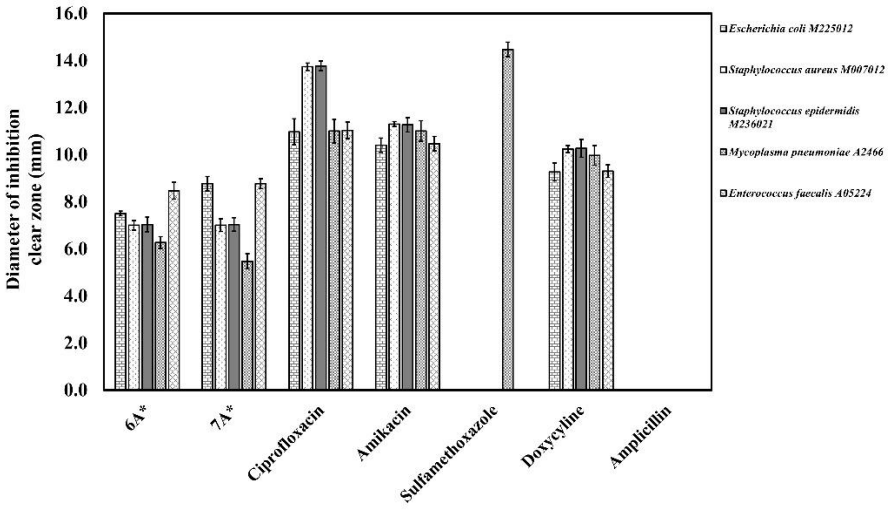

Fig. 1: The graph shows the inhibition of *S. aureus* M007012, *E. coli* M225012, *S. epidermidis* M236021, *M. pneumoniae* A2466 and *E. faecalis* A05224 with the antibiotic drugs and ripened *S. pinnata* fruit extract (6A*, 7A*) using a disc diffusion assay technique.

Table 2: GC-MS analysis used to identify bioactive compounds in 6A*

RT	Compounds	Molecular Formula	Molecular weight	Area (%)	Biological activity	References
3.706	3-Furaldehyde	$C_5H_4O_2$	96	3.80	antiviral, antioxidant, antitumor, antihistaminic and fungicides	Makhoahle (2022)
5.640	1H-Tetrazole,1-methyl	$C_2H_4N_4$	84	3.46	Antifungal activity, Antibacterial activity	Verma et al. (2013)
6.982	3-Aminopyrazine 1-oxide	$C_4H_5N_3O$	111	2.18	No activity reported	
7.076	3-Aminopyrazine 1-oxide	$C_4H_5N_3O$	111	3.82	No activity reported	
8.518	N(1)-[1-[4- Chlorophenyl]-1Htetrazol-5-yl]-N(2) ,N(2)- dimethyl-1,2- propanediamine	$C_{12}H_{17}ClN_6$	280	1.84	No activity reported	
14.237	2-Furancarboxylic acid, hydrazide	$C_5H_6N_2O_2$	126	1.18	Anti-cancer, Antimicrobial	Verma et al. (2013)
14.756	1-(Dimethyl(prop-2- enyl)silyloxy)butane	$C_9H_{20}OSi$	172	2.11	No activity reported	Makhoahle (2022)
19.369	4H-Pyran-4-one, 2,3- dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	144	2.43	Anticancer, Antiinflammatory, antioxidant	Reddy et al. (2020)
20.349	3-Aminopyrazine 1-oxide	$C_4H_5N_3O$	111	1.45	Nucleotides and their derivatives	Liu et al. (2022)
24.663	2-Cyclohexylethyl ethylphosphonofluoridate	$C_{10}H_{20}FO_2P$	222	10.32	No activity reported	
25.722	Oxirane,diethylboryloxymethyl-	$C_7H_{15}BO_2$	142	3.97	No activity reported	
26.634	d-Ribonic acid, .gamma.- lactone, cyclic 2,3-(ethylboronate)	$C_7H_{11}BO_5$	186	1.19	No activity reported	
28.238	1-(2-methyl-2H-tetrazol-5-yl)-2-[(thiophen-2-ylmethyl)amino]-ethanol	$C_9H_{13}N_5OS$	239	12.36	No activity reported	
29.523	Phosphoric acid,diethyl dodecyl ester	$C_{16}H_{35}O_4P$	322	2.94	No activity reported	
31.913	1-(2,5-diethyl4-methyl-1,3,2- dioxaborolan-4-yl)-ethanone	$C_9H_{17}BO_3$	184	2.03	No activity reported	
44.012	Methyl 2-isothiocyanato4-(methylthio)butyrate	$C_7H_{11}NO_2S_2$	205	100	No activity reported	
44.117	Methyl 2-isothiocyanato4-(methylthio)butyrate	$C_7H_{11}NO_2S_2$	205	12.02	No activity reported	
45.244	Methyl 2-isothiocyanato4-(methylthio)butyrate	$C_7H_{11}NO_2S_2$	205	2.99	No activity reported	
45.605	Methyl 2-isothiocyanato4-(methylthio)butyrate	$C_7H_{11}NO_2S_2$	205	3.34	No activity reported	
45.857	Quinoline, 2-methyl-, 1- oxide	$C_{10}H_9NO$	159	6.58	No activity reported	
46.339	Quinoline, 2-methyl-, 1- oxide	$C_{10}H_9NO$	159	2.27	No activity reported	
46.638	Quinoline, 2-methyl-, 1- oxide	$C_{10}H_9NO$	159	3.35	No activity reported	
46.842	1,3-Xylyl-18-crown-5, 2- (diethylboryl)-	$C_{20}H_{33}BO_5$	364	1.00	No activity reported	
46.989	alpha-d-Mannofuranose, 2,3-5,6- di-O-phenylboranediyl	$C_{18}H_{18}B_2O_6$	352	1.72	No activity reported	
49.804	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	4.36	Anti-inflammatory, Antioxidant properties	Vijayan (2017)
54.758	Decanoic acid,silver(1+)salt	$C_{10}H_{19}AgO_2$	279	1.01	No activity reported	
57.940	Methyl 12,13-tetradecadienoate	$C_{15}H_{26}O_2$	238	1.27	Antimicrobial, Antioxidant	Makhoahle (2022)
63.449	Methyl 8,9-octadecadienoate	$C_{19}H_{34}O_2$	294	1.69	No activity reported	

Table 3: GC-MS analysis used to identify bioactive compounds in 7A*

RT	Compounds	Molecular Formula	Molecular weight	Area (%)	Biological activity	References
3.166	3,3- dimethoxypropanenitrile,	$C_5H_9NO_2$	115	8.40	Surfactant, Antifungal Antibacterial Antioxidant, Anticancer, Anticonvulsant	Ayodele et al. (2020)
3.722	3-Furaldehyde	$C_5H_4O_2$	96	21.20	Hepatoprotective	Vandayar and Pushpam (2020)
4.261	Methylphosphonic acid	CH_5O_3P	96	1.06	No activity reported	
5.640	1H-Tetrazole,1-methyl-	$C_2H_4N_4$	84	13.77	Antifungal activity	Vijayan (2017)
7.035	3-Aminopyrazine 1-oxide	$C_4H_5N_3O$	111	41.18	Main base found in DNA,RNA	Dave et al. (2018)
7.333	2-Furancarboxaldehyde,5-methyl-	$C_6H_6O_2$	110	2.84	Antibacterial, preservative, inflammatory, anti anticancer, antiasthma diuretic, hepatoprotective and antioxidant properties	Aina and Fagbemi (2022)
8.072	3H-Pyrazole-3-carboxylic acid, 4,5-dihydro-5,5-dit-butyl-, ethyl ester	$C_{14}H_{26}N_2O_2$	240	2.56	No activity reported	
8.838	Iron, tricarbonyl[(O,1,2,3- .eta.)-methyl 2- propenoate]-	$C_7H_6FeO_5$	227	1.32	No activity reported	
11.254	Propanoic acid	$C_3H_6O_2$	74	4.44	Preservative	Pucot et al. (2021)
12.402	Pyridine,4-methoxy-1-oxide	$C_6H_7NO_2$	125	4.93	Diuretic, Antidiabetic	Sivalingam (2021)
14.473	Furyl hydroxymethyl ketone	$C_6H_6O_3$	126	5.43	Anti-inflammatory activity and inhibits DNA polymerase γ and is nephrotoxic	Aina and Fagbemi (2022)
14.730	5-Trimethylsilanyl-3H- [1,2,3]triazole-4-carbaldehyde	$C_6H_{11}N_3OSi$	169	10.34	No activity reported	
16.470	Cyclohexane, 1-isopropyl-1-methyl	$C_{10}H_{20}$	140	2.49	No activity reported	
17.073	5-Amino-3H-[1,2,3]triazole-4-carboxylic acid,hydrazide	$C_3H_6N_6O$	142	35.76	No activity reported	
18.462	diethyl-borinic acid	$C_4H_{11}BO$	86	1.11	Acidifier, Arachidonic acid Inhibitor, Increases Aromatic Amino acid decarboxylase activity, Inhibits the production of uric acid, Urine acidifier	Perumal et al. (2021)
19.243	4H-Pyran-4-one, 2,3- dihydro-3,5-dihydroxy-6- methyl	$C_6H_8O_4$	144	14.18	Antioxidant, ameliorative, anti-inflammatory	Vijayan (2017)
23.657	Cyclohexanecarboxylic acid, 2-ethoxyethyl ester	$C_{11}H_{20}O_3$	200	40.12	Antimicrobial activity, anti diabetic, anti-viral	Vijayan (2017)
24.354	2-Cyclohexylethyl ethylphosphonofluoridate	$C_{10}H_{20}FO_2P$	222	26.20	No activity reported	
25.308	Aluminum, triethyl-	$C_6H_{15}Al$	114	2.47	No activity reported	
26.561	d-Ribonic acid, gamma- lactone, cyclic 2,3- (ethylboronate)	$C_7H_{11}BO_5$	186	3.79	No activity reported	
26.949	2-Cyclohexyl-1-tetrazol-2-yl-ethanone	$C_9H_{14}N_4O$	194	1.93	No activity reported	
27.735	1-(2-methyl-2H-tetrazol-5-yl)-2-[(thiophen-2-ylmethyl)amino]-ethanol	$C_9H_{13}N_5OS$	239	100	No activity reported	
29.460	Phosphoric acid,diethyl dodecyl ester	$C_{16}H_{35}O_4P$	322	15.87	No activity reported	
29.759	d-Ribonic acid,gamma-lactone, cyclic 2,3-(ethylboronate)	$C_7H_{11}BOS$	186	27.12	No activity reported	
30.236	trans-2-methyl-4-npentylthiane, S,S-dioxide	$C_{11}H_{22}O_2S$	218	2.30	No activity reported	
31.494	6-Methyl-2-mercaptopyridine-1-oxide	C_6H_7NOS	141	1.10	No activity reported	
31.908	6-Methyl-2- mercaptopyridine-1- oxide	C_6H_7NOS	141	84.61	No activity reported	
34.692	1-Hydroxy-4-isopropyl2,2,5,5-tetramethyl-3- imidazoline-3-oxide	$C_{10}H_{20}N_2O_2$	200	3.91	No activity reported	
36.479	Butylphosphonic acid, di(3-(2-methoxyethyl)nonyl) ester	$C_{28}H_{59}O_5P$	507	2.54	No activity reported	
36.952	1,3,2-Oxazaborolane-4- carboxylic acid, 2-butyl-, methyl ester	$C_8H_{16}BNO_3$	199	1.69	No activity reported	
37.412	2-Butoxy-4-methyl- [1,3,2]dioxaborinane	$C_8H_{17}BO_3$	172	1.79	No activity reported	
40.342	trans-2-methyl-4-n-pentythiane,S,S-dioxide	$C_{11}H_{22}O_2S$	204	1.68	No activity reported	
41.842	Decanoic acid, silver(1+) salt	$C_{10}H_{19}AgO_2$	279	17.46	No activity reported	

42.738	d-Galactono-1,4-lactone, 5,6-O-(ethylboranediyl)-	$C_8H_{13}BO_6$	216	16.92	No activity reported	
43.456	d-Galactono-1,4-lactone, 5,6-O-(ethylboranediyl)-	$C_8H_{13}BO_6$	216	4.71	No activity reported	
44.353	1-Aza-4- phosphacyclohexane, 1- butyl	$C_8H_{18}NP$	159	17.21	No activity reported	
44.725	1-(3,4- dihydro-1H-isoquinolin-2- yl)-2-(1-methyl-1Htetrazol-5-ylsulfanyl)-ethanone	$C_{13}H_{15}N_5OS$	289	14.86	No activity reported	
44.929	1-Aza-4- phosphacyclohexane, 1- butyl	$C_8H_{18}NP$	159	11.73	No activity reported	
45.495	1-(3,4- dihydro-1H-isoquinolin-2- yl)-2-(1-methyl-1Htetrazol-5-ylsulfanyl)-ethanone	$C_{13}H_{15}N_5OS$	289	8.49	No activity reported	
46.109	alpha-D-Glucopyranoside, 1-Omethyl-2,3-Odiethylboryl-4,6-Ooctylidene	$C_{23}H_{46}B_2O_6$	440	25.50	No activity reported	
46.580	4,5,6,7- Tetrahydroxydecyl isothiocyanate	$C_{11}H_{21}NO_4S$	263	1.84	No activity reported	
46.795	4,5,6,7- Tetrahydroxydecyl isothiocyanate	$C_{11}H_{21}NO_4S$	263	1.81	No activity reported	
46.921	alpha-D-Glucopyranoside, 1-Omethyl-2,3-Odiethylboryl-4,6-Ooctylidene	$C_{23}H_{46}B_2O_6$	440	13.05	No activity reported	
47.330	alpha-D-Glucopyranoside, 1-Omethyl-2,3-Odiethylboryl-4,6-Ooctylidene	$C_{23}H_{46}B_2O_6$	440	4.30	No activity reported	
47.728	alpha-D-Glucopyranoside, 1-O-methyl-2,3-O-diethylboryl-4,6-O-octylidene-	$C_{23}H_{46}B_2O_6$	440	1.94	No activity reported	
49.228	4,5,6,7- Tetrahydroxydecyl isothiocyanate	$C_{11}H_{21}NO_4S$	263	1.24	No activity reported	
49.343	4,5,6,7- Tetrahydroxydecyl isothiocyanate	$C_{11}H_{21}NO_4S$	263	4.27	reported No activity	
49.773	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	95.03	Anti-inflammatory, Antioxidant properties	Shahin et al. (2022)
52.845	Methyl 12,13- tetradecadienoate	$C_{15}H_{26}O_2$	238	1.59	Antimicrobial	Shahin et al. (2022)
53.820	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	5.49	Antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, anti androgenic, flavor, hemolytic-5-α reductase inhibitor.	Ayoola et al. (2020)
54.748	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	21.61	Antibacterial properties, Antioxidant, hypocholesterolemic nematicide, pesticide, Anti androgenic, flavor, hemolytic 5-alpha reductase inhibitor	Shahin et al. (2022)
55.481	Phosphorothioic acid, O,O-diethyl O-[2-(ethylthio)ethyl] ester	$C_8H_{19}O_3PS_2$	258	2.49	reported activity No	
57.788	Methyl 12,13-tetradecadienoate	$C_{15}H_{26}O_2$	238	2.80	Antimicrobial, Antioxidant	Shahin et al. (2022)
57.940	Methyl 12,13- tetradecadienoate	$C_{15}H_{26}O_2$	238	9.26	Antimicrobial	Shahin et al. (2022)
58.805	Methyl 12,13- tetradecadienoate	$C_{15}H_{26}O_2$	238	4.78	Antimicrobial	Shahin et al. (2022)
59.324	Methyl 12,13- tetradecadienoate	$C_{15}H_{26}O_2$	238	1.08	Antimicrobial	Shahin et al. (2022)
59.444	Nona-2,3-dienoic acid, ethyl ester	$C_{11}H_{18}O_2$	182	7.29	Pharmacological activities, Pharmacokinetics	Vijayan (2017)
64.524	Methyl 12,13- tetradecadienoate	$C_{15}H_{26}O_2$	238	26.64	Antimicrobial	Shahin et al. (2022)
64.524	Methyl 12,13- tetradecadienoate	$C_{15}H_{26}O_2$	238	26.64	Antimicrobial	Shahin et al. (2022)

DISCUSSION

The findings from the experiment investigating the impact of preventing the growth of bacteria responsible for mastitis using extracts derived from the ripened *S. pinnata* fruit, specifically extracts 6A* and 7A*, indicate that a concentration of 150µg/mL for extract 6A* and 91µg/mL for extract 7A* shown significant effects. Exhibits antimicrobial properties the strains used in this study are *S. aureus* (M007012), *E. coli* (M225012), *S. epidermidis* (M236021), *M. pneumoniae* (A2466) and *E. faecalis* (A05224). Evidently, the application of antibiotic treatment results in the emergence of a distinct zone in all samples. The observed resistance of these bacteria to antibiotics, namely sulfamethoxazole and ampicillin, aligns with previous studies that have investigated the efficacy of ethanol-extracted *S. pinnata* fruit extracts in inhibiting bacterial growth. The certification and study of phytoconstituents are consistently advancing, as they include numerous powerful medications. Gas chromatography and mass spectrometry (GC-MS) have been confirmed as valuable techniques for the exploration of plant bioactive chemicals. In the present study, a total of 34 compounds were identified in 6A* and 63 compounds in 7A*. A high percentage of the compound contains Methyl 2-isothiocyanato4-(methylthio)butyrate, 3-Furaldehyde, 3-Aminopyrazine 1-oxide, 5-Amino-3H-[1,2,3]triazole-4-carboxylic acid, hydrazide, Cyclohexanecarboxylic acid, 2-ethoxyethyl ester, 2-Cyclohexylethyl ethylphosphonofluoridate, 1-(2-methyl-2H-tetrazol-5-yl)-2-[(thiophen-2-ylmethyl)amino]-ethanol, d-Ribonic acid,gamma-lactone, cyclic 2,3-(ethylboronate), 6-Methyl-2- mercaptopyridine-1- oxide, alpha-D-Glucopyranoside, 1-Omethyl-2,3-Odiethylboryl-

4,6-Ooctylidene, Tetradecanoic acid, n-Hexadecanoic acid, Methyl 12,13- tetradecadienoate, such compound have properties in antimicrobial, antibacterial, anti-inflammatory, antioxidant and antiviral. In a study conducted by Muhammad et al. (2011), it was observed that *S. aureus, S. epidermidis, P. aeruginosa*, and *Salmonella typhi* exhibited resistance against these bacteria when exposed to a concentration of 500µg/disc. Additionally, extracts from *S. pinnata* fruit were obtained at a concentration of 500µg/disc. According to the findings of Manik et al. (2013), the utilization of n-hexane has demonstrated resistance against many bacterial strains, including *Shigella boydii, B. subtilis, Salmonella typhi, Salmonella paratyphi, P. aeruginosa*, and *E. coli*. According to Chai et al. (2013), the antibacterial activity of this substance may be attributed to its significant levels of furfural, α-terpineol, and γ-terpineol, which could hinder the growth of Salmonella bacteria and *B. subtilis*. The antimicrobial action of Spondias species extracts is likely attributed to the previously established antibacterial activity of quercetin and rutin, as shown by Cushnie and Lamb (2005). The existence of tetradecenoic acid in Cassia angustifolia has been shown to have medicinal, anti-inflammatory, and antioxidant properties. The existence of n-hexadecanoic acid has been found in both the leaf and root extracts of *S. khasianum*. Other organic substances found in leaf extract that contribute to its widespread usage in healthcare uses include dodecanal, which is known to exhibit one of the most potent antibacterial properties. Furthermore, it provides antioxidant, hypocholesterolemic nematicide, pesticide, anti-androgenic, flavor, and hemolytic 5-alpha reductase inhibitor (Faridha et al. 2016). Beta-sitosterol plays a role in the production of several hormones, such as progesterone, androgens, estrogens, and corticoids. Additionally, it provides antimicrobial, anti-inflammatory, anticancer, antiasthma, diuretic, and hepatoprotective (Kaur et al. 2011; Karthikeyan et al. 2016). 2-Furancarboxaldehyde,5-methyl- is effective in treating antibacterial, preservative, anti-inflammatory, anticancer, antiasthma diuretic, hepatoprotective, and antioxidant properties (Park et al. 2003). 8-methylnonanoic acid, ethyl ester, exhibits efficacy in the treatment of conditions such as antioxidant, antibacterial, COX-1 and COX-2 inhibition, antiviral, hypocholesterolemic, and candidicidal effects (Karthikeyan et al. 2016). Methyl 13,14-octadecadienoate demonstrates effectiveness in treating illnesses such as anti-inflammatory, hypocholesterolemic, cancer preventive, and hepatoprotective (Rani and Kapoor, 2019). 1H-Tetrazole,1-methyl exhibits effective antifungal properties against various fungal cultures, including Candida species, *Cryptococcus neoformans*, and Aspergillus species (Deepa et al. 2011). 2-Furancarboxylic acid, hydrazide has anticancer and antimicrobial properties (Aksharadevi et al. 2022). Methyl 12,13-tetradecadienoate shows antimicrobial and antioxidant properties (Shahin et al. 2022; Nursanty et al. 2023).

The researcher should be informed that using extracts derived from mature *S. pinnata* effectively suppressed the mastitis pathogens employed in the experimental study. In prospective research endeavors, examining extracts derived from mature *S. pinnata* has been promised as a potential therapeutic intervention for mastitis in dairy cows. These extracts may be explored as viable alternatives to antibiotics, potentially administered through udder inserts or other related products.

Conclusion

All of the data in this research can conclude that the ripened *S. pinnata* fruit extracts (6A* and 7A*) displayed antimicrobial activity in *vitro*. Both extracts (6A* and 7A*) inhibited the growth of mastitis-causing microorganisms such as *S. aureus* M007012, *E. coli* M225012, *S. epidermidis* M236021, *M. pneumoniae* A2466, and *E. faecalis* A05224 at the treated concentration of 150 and 91 µg/mL, respectively. The diameter of the clear zone is shown in Fig. 1. All the tested mastitis-causing microorganisms showed resistance to ampicillin. Additionally, *S. aureus* M007012, *E. coli* M225012, *S. epidermidis* M236021, and *E. faecalis* A05224 can resist sulfamethoxazole, as evidenced by the absence of a clear zone around the test cards. Phytochemical types found in 6A* and 7A* are related to antimicrobial properties. Therefore, bioactive compounds in 6A* and 7A* may substitute antibiotics for mastitis treatment in dairy heifers.

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