

Evaluation of Anti-*Escherichia coli* K88 from Diarrhea Piglets and Phytochemicals of Galangal Rhizome Extracts

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Abstract

This study investigated the antimicrobial effect of galangal rhizome extracts against *Escherichia coli* K88 from diarrhea pigs. Galangal rhizome was extracted with 4 solvents: Water, ethanol, methanol and ethyl acetate. The extracts determination against *E. coli* K88 by disc diffusion method, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Results showed that inhibition diameters of galangal extract with ethyl acetate against *E. coli* K88 was significantly higher than that of galangal extracted with water, ethanol and methanol. However, the inhibition was significantly lower than that of streptomycin drug. The MIC and MBC values of ethyl acetate extracts against *E. coli* K88 were 0.39 and 0.78 mg/mL, respectively. Standard bacteria, pathogenic *S. aureus* ATCC6538, also used for testing antibacterial effect of galangal extract revealed the ethanol and methanol and ethyl-acetate extracts antibacterial effect against *S. aureus*. The phytochemicals tests performed on the extracts of galangal rhizome confirms the presence phenolic compounds exhibited antimicrobial activity. This study demonstrated antibacterial efficacy of galangal extracted with ethyl acetate to further develop as a substitute for antibiotics.

Keywords: *Escherichia coli* K88, Galangal rhizome, Phytochemicals, Piglets

Introduction

Piglet diarrhea due to enterotoxigenic *Escherichia coli* harbouring the K88 fimbriae (ETEC K88) is one of the most frequent diseases and cause detrimental impact on animal health and economic of pork production. This pathotype is characterized by the expression of an K88 fimbrial adhesin which can bind to specific F4 (K88) receptors located on the brush border of pig intestine and secretion of enterotoxins that cause diarrhea [1,2].

As an effort to control diarrhea and other gastro-intestinal disorders, farmers regularly added antibiotics to farm animal feeds, especially in swine rations. This practice in long term period may damage the animal health and it was proved that several *E. coli* isolates were resistant to commonly use antibiotics including ampicillin, streptomycin, trimethoprim and sulphamethoxazole [3]. Previous studies in countries of South and South East Asia region including Thailand have shown rapid increase of antimicrobials usage in livestock [4-6]. Globally, the average estimated annual consumption of antimicrobials in the swine 72 mg/kg and it is thought that global consumption of antimicrobials will increase 67 % by the year 2030 [2]. The antimicrobial residues typically occur due to indiscriminate and irrational use of antimicrobials in food animals without following the withdrawal period, extra-label dosages for animals, contamination of animal feed with the excreta of treated animals, and the use of unlicensed antibiotics [7].

The steadily increasing bacterial resistance to existing drugs is a serious problem resulting in the urgent need for exploration of new classes of antimicrobial agents. Antimicrobials of spice are found to use as alternative remedies to heal many infectious diseases and exhibit fewer side effects [8], with potential applications as natural food preservatives and natural alternatives to antibiotics in animal feeding [9]. Spice and their major components are generally recognized as safe with no historical records of detrimental impacts and with modern toxicological verification [10]. Moreover, plant-derived spices and extracts containing a mixture of active ingredients have not only for their effective antibacterial activity but also for the relative difficulty in developing resistance to them.

Alpinia galanga (L.) Willd (Zingiberaceae), commonly known as Galangal, belong to the ginger family and is native to Southern China and Thailand [11,12]. Galangal is a herbal medicine that contains a variety of chemical compounds. Phytochemicals found in the *Alpinia galanga* predominantly consisted of terpenes and phenolic compounds. Phenolic compounds include phenylpropanoids and their derivatives such as 1'S-1'-acetoxychavicol acetate (ACA) 1'-acetoxyeugenol acetic acid, and phydroxybenzoic acid. Terpenes, also known as terpenoids, and their derivatives such as monoterpenes, sesquiterpenes, and terpenes [13]. Numerous studies have investigated the pharmacological properties of galangal extracts as herbal medicines revealing various beneficial effects including antitumor [14], antiallergic [15], antiulcer [16], antifungal [17], antibacterial [18], antimycobacterial [19], antiviral [20], and antimalarial activities [21]. Traditionally, galangal has been used for treatment kidney disorders, diabetes, cough, tuberculosis, bronchitis, rheumatism, asthma, and heart diseases [11,18,22-24]. However, there is no previous research on the use of galangal extract as an alternative remedy for healing *E. coli* K88 infection in diarrhea piglets. Hence, the aim of the present study is to investigated the antimicrobial properties of galangal rhizome extracts against *E. coli* K88 isolated from diarrhea piglets and their phytochemicals by preparing galangal extract with different 4 solvents, water, ethanol, methanol and ethyl acetate and testing against *E. coli* K88.

Materials and methods

Sample collection and *E. coli* isolation

Porcine feces samples were collected without giving any stress or harm to the animals. Samples were collected from diarrhea piglets in Lopburi province, Thailand. These specimens were later used for bacterial verification. A loop of feces sample was streaked onto plates of nutrient agar media (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 37 °C for 24 - 48 h. Suspected colonies were picked and examined microscopically in Gram-stained films before being transferred to semisolid agar to be subjected to further identification, including the Gram stain procedure and biochemical tests [25]. The polymerase chain reaction was used to identify *E. coli* K88. *Staphylococcus aureus* ATCC 6538, was obtained from stock culture of Department of Biology, Faculty of Science, Thepsatri Rajabhat University. Bacteria were sub-cultured on nutrient agar at 37 °C prior to being grown in nutrient broth overnight. All overnight cultures were standardized by matching to the McFarland 0.5 turbidity standard using sterile saline to produce approximately 10⁸ CFU/mL.

Preparation of crude extracts

Rhizomes of galangal was collected from a farm in Phu Khae, Saraburi province in January 2021. It was sliced and dried in a tray-dryer oven at 50 °C for 24 h, after which they were ground in a blender to make powder. Crude extraction was performed according to procedure modified from previous works [26,27]. Ten grams powder of the spices were extracted with 100 mL of water, ethanol, methanol or ethyl acetate, and left at room temperature for 24 h. The liquefied of the plants drug was filtered by Whatman paper filter (No.1). The extract was dried by distilling off the solvents under pressure using a rotary evaporator at a temperature of 40 - 50 °C. The galangal extract was stored as a crude extract after the solvent was evaporated. For testing its antibacterial activity, the extract will be dissolved using dimethyl sulfoxide (DMSO). The extract was dissolved with DMSO for concentration 100 mg/mL, filtered through 0.45 mm millipore filter and stored at 4 °C.

Phytochemical identification of galangal rhizome extracts

The compound and structure of some ingredients from major galangal ethanol extract were analyzed by gas chromatography-mass spectrometry (GC-MS) (GC 7890A Agilent -MSD5973C (EI) Agilent, Agilent Technologies, USA). A chromatographic column of 30 m × 0.25 mm ID × 250 mm film thickness was used (Agilent 7890A, USA). Chromatographic conditions were as follows: Split ratio 10:1; carrier gas: Helium; initial oven temperature 100 1C; injection volume 1 mL. Mass spectrometer condition (Agilent 5975C Network Mass selective detector): Ion source 230, threshold 100 eV. The percentage of each compound was calculated as the ratio of the peak area to the total chromatographic area. The identification of the major peak was compared to the reference mass spectra databases (W8N08 library John Wiley & Sons, Inc., USA).

Antibacterial assay of galangal rhizome extracts

The antibacterial activity of galangal rhizome extracted with water, ethanol, methanol and ethyl acetate was tested against the studied bacteria. The antibacterial activity of the extracts was carried out by disc diffusion method using 25 µL of the standardized bacterial suspension of the tested bacteria (10⁸

CFU/mL) spread on plates. The sterilized discs (6 mm in diameter) were then impregnated with 10 μ L of a single 100 mg/mL galangal rhizome extracts (100 mg/discs) followed by air drying. The prepared discs containing the various extracts were placed on tested bacteria plates using sterilized forceps according to Wiegand *et al.* [28]. The disc with solvent alone (water or DMSO) was the negative control and an antibiotic disc (Streptomycin, 10 μ g/disc) was the positive control. The plates were incubated at 37 °C for 24 h. The zone of growth and inhibition of the bacteria by the test extracts was recorded. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone around the disc as mean \pm standard deviation (SD) of the triplicates of each condition.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of different samples of galangal rhizome extracts was determined by the broth dilution assay described by the National Committee for Clinical Laboratory Standards [29]. The MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms. Serial dilution of 100 mg/mL of the extracts were separately done to achieve 50, 25, 12.50, 6.25, 3.12, 1.56, 0.78, 0.39, 0.195 and 0.097 mg/mL concentration were used for MIC determination. Briefly, 100 μ L of varying concentrations of samples were added into the test tubes separately, containing 9 mL of the standardized suspension of tested bacteria (10^8 CFU/mL). The test tubes were incubated at 37 °C for 24 h. Controls were used with the test organisms, using distilled water instead of the plant extract. The least concentration of the samples with no visible growth was taken as the MIC. The lowest concentration of the treatment that prevented the growth of the organism after subculture on tryptic soy agar (TSA) following serial dilution and plating was taken as the MBC. Triplicate samples were included for each treatment.

Statistical analysis

All data were subjected to statistical analysis including the calculation of the mean and standard deviation (SD) of the triplicate samples of each condition. Significant differences between the means of different extracts with the same isolate were evaluated using one-way analysis of variance (ANOVA), followed by post-hoc analysis (Duncan's test) using the statistical package SPSS 22.0. The significance level was $p < 0.05$.

Results and discussion

***E. coli* K88 solation**

The *E. coli* K88 was identified by the Gram staining, biochemical test and polymerase chain reaction. These bacteria obtained from diarrhea piglets in Lopburi province, Thailand. The Gram-negative bacteria were smooth, flat, circular and medium-sized colonies. The biochemical tests conducted on the Gram-negative bacteria showed positive results for the Indole test and methyl red test, while the Voges-Proskauer test and citrate test yielded negative results. Moreover, the presence of *E. coli* K88 was confirmed through the polymerase chain reaction. *E. coli* K88 refers to a specific strain of *E. coli* that possesses antigen K88-fimbriae. These fimbriae enable the bacteria to bind to receptors present on the brush border of the small intestinal wall of pigs. The secretion of enterotoxins by these bacteria can result in diarrhea in piglets [2].

Galangal extraction yield and phytochemicals identification

The yield of each individual component depended on solvent applied. Totally, ethanolic extract contained the highest yield of crude extract (17.66 %) followed by methanolic extract (16.85 %) and aqueous extract (16.30 %), while ethyl acetate extract showed the lowest yield (5.50 %). The results of the phytochemicals analysis by gas chromatography-mass spectrometry (GC-MS) indicated different types of active constituents in aqueous, ethanolic, methanolic and ethyl acetate extracts of galangal rhizome (**Figures 1 to 4** and **Table 1**). The presence of the 4 solvents extracts showed a positive effect qualitatively on secondary metabolite compounds such as compounds known as phenylpropanoids, cholestanoids and sesquiterpenoids. The phenylpropanoids were observed in aqueous extract containing 12.54 % area of 4-(3-hydroxy-1-propenyl) phenol and 18.07 % area of (4-[(1e)-3-hydroxy-1-propenyl] phenol. The highest compound of ethanolic and methanolic galangal extract were cholestanoids, cholest-5-en-3-ol,23-ethyl-, (3.β.,23S) (3.12 % area) and cholest-5-en-3-ol, 23-ethyl-, (3.β.,23S) (3.49 % area), respectively. Among 4 extracts, ethyl acetate extract revealed the highest phenol, 4-(2-propenyl), acetate (0.81 % area) and 3 sesquiterpenoids presented only in ethyl acetate extract as 3-(1,5-dimethyl-4-hexenyl)-6-methylene-1-cyclohexene; and 1,5-dimethyl-8-(methylethylidene)-1,5-cyclododecadiene and (-)-5-oxatricyclo[8.2.0.0(4,6)] dodecane, 12-trimethyl-9-methylene; and 3-phenyl-1,4(e)-dodecadiene.

The phytochemical analysis in present study revealed active ingredients in galangal extracts as the previous report containing Acetoxychavicol acetate (ACA), p-coumaryl diacetate, palmitic acid, acetoxyeugenol acetate, eugenol, β -bisabolene, β -farnesene, and sesquiterpenoids [30]. The main component of ACA is the ester structure of acetic acid. Such substances cause changes in the charge of the cell membrane, and causes a change in the pH within the cells, resulting in protein deterioration in cells. Therefore, galangal extract ruptures the outer membrane of bacteria, leaking cellular elements, and also cause intracellular elements to precipitate [13,31]. Trichophytonmentagrophytes.24 1'S'-1'- acetoxychavicol acetate obtained from dried rhizome of *Alpinia galanga* was reported to act as an efflux pump inhibitor which provokes resistance in *Mycobacterium* [32].

Antibacterial activity of galangal rhizome extracts

The antibacterial activity of aqueous extract, (2) ethanolic extract, (3) methanolic extract, and ethyl acetate extract of galangal rhizome was shown in **Table 2**. Among the different extracts of galangal rhizome, ethyl acetate extract displayed a pronounceable better antibacterial effect against *E. coli* K88 (23.4 ± 0.66 mm). However, the ethyl acetate extract of galangal rhizome showed the most pronounced activity with inhibition zones on *S. aureus* ATCC 6538 (24.63 ± 0.72 mm). The aqueous extracts showed low antibacterial effect on *E. coli* K88 and *S. aureus*. There was no inhibition of ethanolic and methanolic extracts against all *E. coli* K88.

The ethyl acetate extract of galangal rhizome had MIC and MBC against *E. coli* K88 at 0.39 and 0.78 mg/mL, respectively (**Table 3**). The ethyl acetate extract showed the same potent against *S. aureus* ATCC 6538. From result of MIC and MBC values, antibacterial activity of ethyl acetate extract against *E. coli* K88 and *S. aureus* ATCC 6538 was the same as Streptomycin drug.

Similarity to presented study, there was report showed that galangal extract had the ability to inhibit the growth of *S. aureus* and *E. coli*. The minimum bactericidal concentration of galangal extract for inhibition of all pathogens was 106.25 mg/mL. Galangal extract can inhibit Gram-positive bacteria (*S. aureus*, *Pediococcus* spp. and *L. plantarum*) were more effective than Gram-negative bacteria (*E. coli*) [33,34].

The data obtained from present study demonstrated that the inhibitory activity against *E. coli* K88 of galangal extract with ethyl acetate was found as the same as streptomycin drug, which was consistent with the MIC and MBC extract values. This test result of galangal extract extracted with ethyl acetate as antibacterial efficacy is consistent with the report on the pharmacological properties of galangal for its antibacterial properties [18]. The antibacterial effect of galangal extracted with ethyl acetate against *E. coli* K88 and *S. aureus* was related to the highest presence of phenylpropanoid, 4-(2-propenyl), acetate and sesquiterpenoids that was presented only in ethyl acetate extract, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-1-cyclohexene; and 1,5-dimethyl-8-(methylethylidene)-1,5-cyclodecadiene and (-)-5-oxatricyclo[8.2.0.0(4,6)] dodecane, 12-trimethyl-9-methylene; and 3-phenyl-1,4(e)-dodecadiene. Since, these compounds were reported as substances destroy the cell membranes of bacteria and also reduce intracellular ATP which corresponds to the report of their antimicrobial property [32].

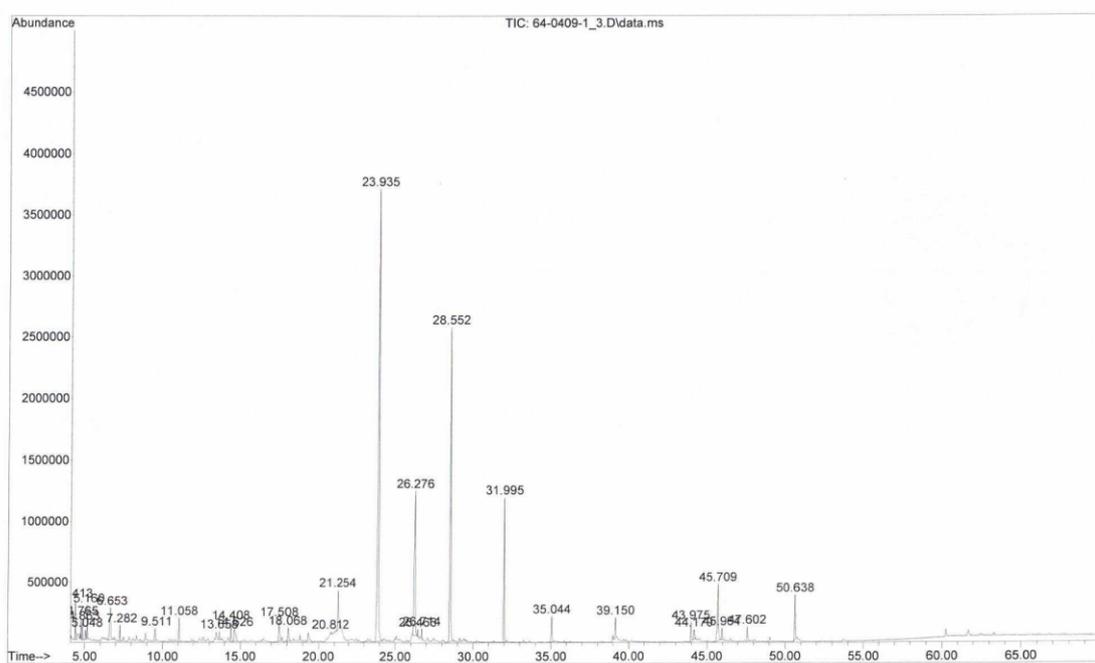


Figure 1 Chromatogram of positive active compound from aqueous extract of galangal rhizome after GC-MS.

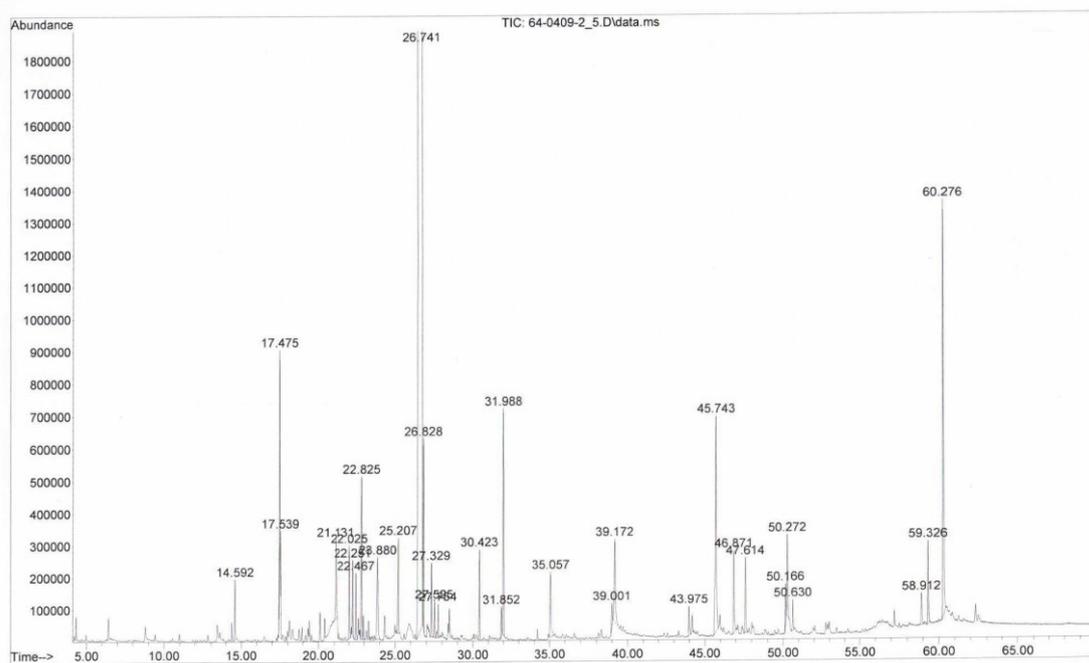


Figure 2 Chromatogram of positive active compound from ethanolic extract of galangal rhizome after GC-MS.

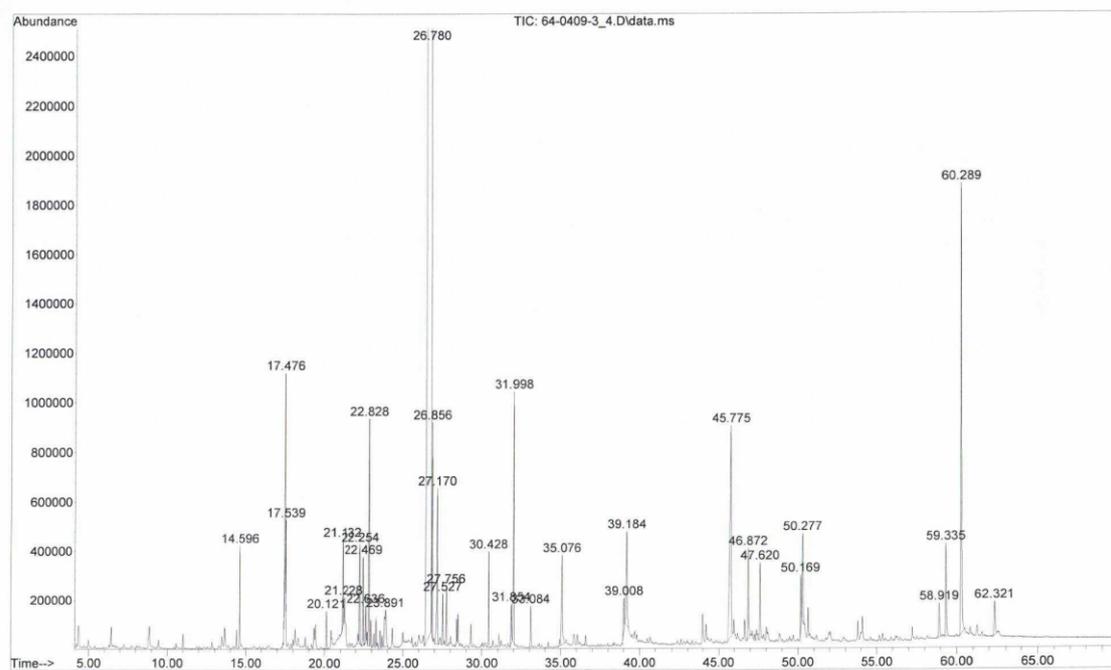


Figure 3 Chromatogram of positive active compound from methanolic extract of galangal rhizome after GC-MS.

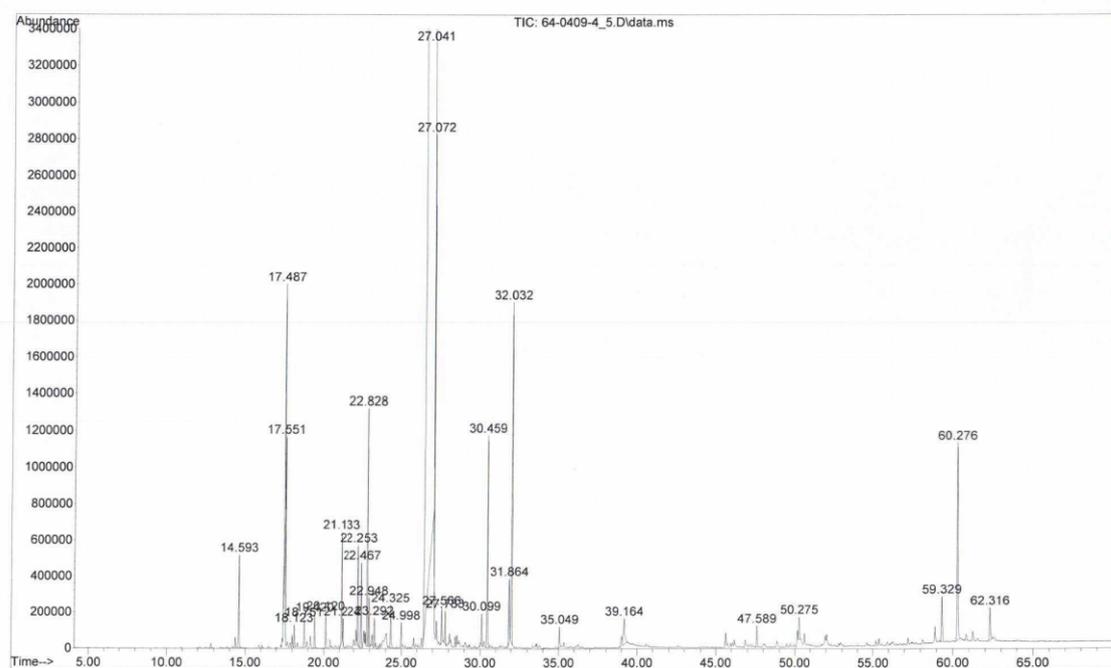


Figure 4 Chromatogram of positive active compound from ethyl acetate extract of galangal rhizome after GC-MS.

Table 1 Chemical composition of galangal rhizome extracts.

Chemical constituents	Aqueous extract		Ethanollic extract		Methanolic extract		Ethyl acetate extract	
	R.T.min	%Area	R.T.min	%Area	R.T.min	%Area	R.T.min	%Area
4-cyclopentene-1,3-dione	4.413	1.00						
2(5H)-furanone, 5-methyl	5.163	1.02						
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	11.062	1.08						
2-furancarboxaldehyde, 5-(hydroxymethyl)	13.660	0.51						
(1RS, 4SR, 5RS)-1,3,3-trimethyl-2-oxabicyclo [2.2.2]octan-5-ol	14.409	0.86						
2-oxabicyclo [2.2.2]octan-6-ol, 1,3,3-trimethyl-, acetate							18.123	0.1
4-allylphenol	14.627, 17.511	1.17, 1.28	14.592	0.37	14.598	0.62	14.592	0.40
4-allylphenyl acetate			17.533	0.53				
Phenol, 4-(2-propenyl)-,acetate					17.539	0.67	17.551	0.81
Benzaldehyde, 4-hydroxy-	18.071	0.80						
Benzene, 1,2-dimethoxy-4-(2-propenyl)							19.422	0.15
Cryophyllene					20.120	0.24	20.120	0.16
1,6,10-dodecatriene, 7,11-dimethyl-3-methylene			21.133	1.57	21.132	0.56	21.138	0.52
2,6,6,9-tetramethyl-1,4,8-cycloundecatriene					21.230	0.19	21.224	0.14
7-isopropenyl-4a-methyl-1-methylenedecahydronaphthalene			22.254	0.48	22.254	0.62	22.254	0.45
2-isopropenyl-4a-dimethyl-12,3,4,4a,5,6,8a-octahydronaphthalene			22.466	0.42	22.471	0.53	22.466	0.38
Pentadecane					22.637	0.25		
1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-1-cyclohexene			22.826	0.95	22.826	1.36	22.832	1.04
Phenol, 2-methoxy-4-(2-propenyl)-acetate							22.952	0.22
3-(1,5-dimethyl-4-hexenyl)-6-methylene-1-cyclohexene Phenol, 2-methoxy-4-(2-propenyl)-acetate							23.296	0.12
1,5-dimethyl-8-(methylethylidene)-1,5-cyclodecadiene							24.326	0.21
(-)-5-oxatricyclo[8.2.0.0(4,6)]dodecane, 12-trimethyl-9-methylene							25.001	0.10
Phenol, 4-(3-hydroxy-1-propenyl)	26.277	12.54						
(1R)-(-)-fenchone formaldehyde azine					27.169	1.06		
(6Z, 9E)-heptadeca-6, 9-diene			27.524	0.22	27.530	0.31	25.570	0.20
8-heptadecene			27.753	0.22	27.759	0.35	27.782	0.14

Chemical constituents	Aqueous extract		Ethanolic extract		Methanolic extract		Ethyl acetate extract	
	R.T.min	%Area	R.T.min	%Area	R.T.min	%Area	R.T.min	%Area
4-[(1e)-3-hydroxy-1-propenyl]phenol	28.554	18.07						
Farnesyl acetate 3			31.856	0.20				
Farnesol					31.855	0.29	31.867	0.33
Acetamide, 2-(diethylamino)-n-(2,6-dimethylphenyl)					33.086	0.27		
n-hexadecanoic acid	35.043	1.33	35.060	0.52	35.077	0.84		
9-octadecenoic acid	39.151	1.59	50.275	1.05	39.185, 50.280	1.41, 1.19	39.163	0.26
9,12-octadecenoic acid (Z, Z)-			39.002, 50.166	0.30, 0.37	39.014, 50.172	0.45, 0.48		
6-octadecenoic acid (Z, Z)-			39.174	1.14				
2-hydroxy-1-(hydroxymethyl)ethyl palmitate			46.870	0.59	46.876	0.65		
3-phenyl-1,4(e)-dodecadiene							50.280	0.27
Campesterin			58.915	0.29	58.920	0.34		
Stigmasterol			59.327	0.60	59.332	0.70	59.327	0.25
Cholest-5-en-3-ol, 23-ethyl-, (3.β.,23S)-			60.277	3.12	60.288	3.49		
Stigmast-5-en-3-ol							60.277	1.11
Stigmastan-3,5-diene					62.319	0.32	62.314	0.22

Table 2 Zones of inhibition against the tested bacteria of galangal rhizome extracts.

Bacteria	Zone of inhibition)mm((Mean ± SD)				
	Aqueous extract	Ethanolic extract	Methanolic extract	Ethyl acetate extract	Streptomycin
<i>E. coli</i> K88	0.49 ± 0.44 ^{a1}	-	-	23.4 ± 0.66 ^{a2}	25.85 ± 0.04 ^{a3}
<i>S. aureus</i> ATCC 6538	1.49 ± 0.58 ^{b1}	18.36 ± 1.41 ²	19.94 ± 0.84 ³	24.63 ± 0.72 ^{a4}	25.13 ± 0.01 ^{a4}

Mean ± SD of triplicates; - = no inhibition; ^{a, b} Significant differences between means with different letter superscripts in the same column; ^{1, 2, 3, 4} Significant differences between means with different letter superscripts in the same row ($p < 0.05$)

Table 3 MIC and MBC of ethyl acetate extract of galangal rhizome against *E. coli* K88 and *S. aureus* ATCC 6538.

Bacteria	MIC mg/mL		MBC mg/mL	
	Ethyl acetate extract	Streptomycin	Ethyl acetate extract	Streptomycin
<i>E. coli</i> K88	0.39	0.39	0.78	0.78
<i>S. aureus</i> ATCC 6538	0.39	0.39	0.78	0.78

Conclusions

The phytochemicals tests performed on the extracts of galangal rhizome confirms the presence phenolic compounds exhibited antimicrobial activity. Our results showed that the galangal rhizome extracted with ethyl acetate have a strong antimicrobial effect against *E. coli* K88 causing diarrhea in piglets. Thus, galangal rhizome extract could be used as an alternative source of production of antibiotic drug against the diarrhea in piglets for reducing cost and resistance to conventional medicine.

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