RUHUNA JOURNAL OF SCIENCE
Vol 8: 90-102, December 2017
eISSN: 2536-8400
DOI: http://doi.org/10.4038/rjs.v8i2.30

Modification of tannin extracted from the bark of *Acacia auriculiformis* for the antibacterial activity and application of metal adsorption

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Received: 20th October 2017, Revised: 11th December 2017, Accepted: 30th December 2017

Abstract Tannins offer capability as a bio-derived chemical feedstock. Their current application is limited primarily to leather browning and wood board adhesives. However, proper derivatization may change both chemical and physical properties and thereby permit further utilization of polyphenols. This study focused on modification of tannin to obtain green polymeric materials. Tannin extracted from *Acacia auriculiformis* (Kaththi Karuvel) bark was modified by reacting with different aliphatic alkyl carboxylic acid (C_4 and C_{18}) to produce esterified tannin. Various weight percentage of alkyl chain lengths were introduced into the tannin by esterification. The resulted modified tannins were characterized by FT-IR and ATR spectroscopic techniques. Bivalent cations (Mg^{2+}, Cu^{2+} and Cd^{2+}) were used to evaluate the adsorption properties of both short and long chain fatty acid tannin esters. The esterified tannins were used to determine the inhibition effect against clinical isolates of *Staphylococcus aureus* and *Escherichia coli* aerobically using nutrient agar medium. Vulnerabilities were determined using standard agar well-diffusion method. Esterified tannins showed effectual ion exchange and antibacterial ability compared to that of virgin tannin. Partially esterified products showed higher exchange capacity compared to fully esterified tannin where C_{18}-carboxylic acid-ester product has shown the highest value of ion adsorption compared to C_4 carboxylic acid-ester product. The highest ion adsorption capacity was shown by the tannin stearates (1:1) for Cd^{2+} (0.020 mg/l), Mg^{2+} (13.852 mg/l) and Cu^{2+} (15.650 mg/l). In case of antibacterial activity, tannin stearates (1:6) possessed the highest inhibition against *Staphylococcus aureus* at 2.5 mg/ml concentration (LD_{50}:1.2mg/ml), and *S. aureus* was more susceptible to this extract compared to *E. coli*.

Keywords: *Acacia auriculiformis*, adsorption, antibacterial activity, tannin ester.

1 Introduction

Tannins are naturally occurring water-soluble polyphenolic subordinate metabolites of plants (Haslam 1989). Histologically, tannins themselves

originate predominantly in the bark, leaves and immature fruits of a wide range of plants (Hagerman 2002). Most noticeable activity of the tannins is their ability to interact with and precipitate proteins, including the proteins found in animal skin (Lees *et al*. 1992, Pizzi 2006). Tannins were initially used to tan animal hides and in leather industrial processes. The word "tannin" is used to express two classes of phenolic compounds with different chemical nature, namely hydrolysable tannins and condensed tannins (Arasaretnam and Karunanayake 2010).

Tannins have exceptional adsorption-desorption properties. Tannins' natural biomass contains high content of multiple adjacent phenolic hydroxyls in their molecules, and they have strong chelating ability towards many heavy metal ions thus can be used as alternative adsorbents (Luo *et al.* 2010). However, their high solubility in water limits their function as metal ion adsorbents in aqueous media. This problem can be overcome by immobilization of tannin by chemical modifications including esterification (Kumar *et al.* 1987). This is done by treating tannin with carboxylic acid to form tannin-esters.

Acacia auriculiformis (Kaththi Karuvel) is an evergreen tree in the Fabaceae Family. It is supposed to be native to the savannas of Papua New Guinea and Irian Jaya, the islands of the Torres Strait, and northern Australia (Santos-Buelga *et al*. 2000). Although this plant is not native to Sri Lanka, it has been introduced in the Batticaloa district (Eastern Province, Sri Lanka) by human intervention. The literature shows that tannin content of bark comprises adequate tannin (13-25 %) for commercial exploitation and contains 6-14 % of a natural dye (Santos-Buelga *et al.* 2000).

The objectives of the present study were to assess the antibacterial activity of modified tannins extracted from barks of *Acacia auriculiformis*, and to investigate the metal adsorption (Mg^{2+}, Cu^{2+} and Cd^{2+}) of esterified tannin. Antibacterial activity of tannin esters is analyzed using *Staphylococcus aureus* and *Escherichia coli*. *S. aureus* is Gram-positive, not always pathogenic yet it is a common cause of skin infections (Reed 1995, Anderson *et al.* 2012). The ability of *Staphylococcus aureus* to colonize surfaces contributes to its lifestyle as both a commensal and a pathogen. *Escherichia coli* are Gram-negative, non-spore forming bacilli. *E. coli* are common occupants of the terminal small intestine and large intestine of mammals (Makkar *et al.* 1993).

2 Material and Methods

2.1 Materials

Acacia auriculiformis was chosen as a suitable source of tannin for this study. Barks of *A. auriculiformis* were collected from Batticaloa district of Sri

Lanka. Tannins were obtained by solvent extraction from the bark using analytical grade reagents (AR). The plant was identified from the herbarium of Department of botany, Eastern University, Sri Lanka.

2.2 Pre-extraction of tannin

Pre-extraction of *Acacia auriculiformis* bark with petroleum sprit, diethyl ether and toluene were performed to separate hydrocarbons, waxes, carotenoids, terpenes and lipids. This method involved soxhlet extraction for 3 hours using petroleum sprit (500 ml) followed by toluene (500 ml) and diethyl ether (500 ml). The residual solvent was removed from the bark under reduced pressure and the yields were measured (Arasaretnam and Sesha Sainath 2016).

2.3 Extraction of tannin

Tannin was extracted from the bark of *Acacia auriculiformis*. The bark was ground to pass an 80 mesh screen, and 10 g of it was added into the extraction thimble and placed in the soxhlet apparatus. A small cone of thimble was placed to prevent any loss of the specimen. It was extracted (sequential extraction) using 200 ml of ethyl acetate, acetone, methanol and methanol-water (50:50) for 6-8 hours. After extraction with methanol-water solvents, the reaction mixture was filtered through Buchner funnel to remove the excess solvent with suction and washed the thimble with methanol. A gummy product was obtained after separating the extract from solvent in a rotary evaporator under reduced pressure at temperature of 50℃. After the removal of the solvent, 100 ml of diethyl ether and petroleum sprit were added respectively to the extracted product to remove any non-tannin compounds (Arasaretnam and Sesha Sainath 2016). The separated compound was analyzed by the FT-IR spectroscopy.

2.4 Quantitative analysis of tannin

The total phenolic content of the extracts was measured using tannic acid equivalent (Makkar *et al.* 1993).

Analysis of total phenolic content of the extracts

Gallic acid solutions in concentrations of 0.01, 0.015, 0.02, 0.025 and 0.03 $moldm^{-3}$ were used as the standards. One milliliter of aliquot of the Gallic acid solution was mixed with 5.0 ml of *Folin-Ciocalteau* reagent and allowed to react for 6-8 minutes. Thereafter, 4.0 ml of sodium carbonate solution (7.5% w/w) was added and the content of the test tube was mixed well. The test tube

was covered using an aluminum foil and kept at 30°C for 2 hours. Absorbance measurements were taken at 725 nm using UV spectrophotometer (Bio base, D580). A calibration curve was drawn using the data obtained.

Table 1. Esterification ratio and isolated yield of *Acacia auriculiformis* bark Tannin ester.

Reagent ratios (W/W)		Code name of ester
tannin	acid	
1	1	T_1B_1
1	2	T_1B_2
1	3	T_1B_3
1	4	T_1B_4
1	5	T_1B_5
1	6	T_1B_6
1	1	T_1S_1
1	2	T_1S_2
1	3	T_1S_3
1	4	T_1S_4
1	5a	T_1S_5
1	6	T_1S_6

T: Tannin, B: Butyric acid, S: Stearic acid

2.5 Esterification of tannin

Bark tannin (1.5 g) was dispersed in acetone (10 ml) at 70°C. The corresponding amount of stearic acid was then added into the flask with stirring of the reaction mixture with con.H_2SO_4 (1 ml) as catalyst. Samples were taken throughout the reaction to monitor reaction progress, quenched with ethanol. After 24 hours, the reaction mixture was quenched with water (30 ml) and concentrated by rotatory evaporation. The reaction mixture was poured into petroleum sprit (20 ml), washed with saturated $NaHCO_3$ solution (200 ml), HCl (0.1 moldm^{-3}, 100 ml) water (50 ml), saturated NaCl solution (100 ml), and dried with $MgSO_4$. The solution was concentrated by rotary evaporation to yield a brown oily product. This was washed again using NaOH solution (0.5 moldm^{-3}, 200 ml). The above procedure was carried out using short chain fatty acid (Butyric acid) and added at various ratios of the acids (Table 1) to complete esterification of all OH groups of tannin unit in the presence of Con.H_2SO_4 as a catalyst (Arasaretnam and Sesha Sainath 2016). Tannin esters were analyzed by ATR-FTIR characterization after filtering.

2.6 Determination of ion exchange capacity of tannin esters

The total exchange capacity of the esters is the total number of exchanging sites available per unit mass of esters.

Determination of ion exchange capacity for Cd^{2+}

Cd^{2+} solution (10 ppm) was prepared (by dissolving 21 mg of $CdCl_2$ in 100 mL distilled water. Intermediate stock solutions containing 0.002 ppm, 0.010 ppm, 0.100 ppm and 1 ppm were prepared. Absorbance of the standard solutions was recorded by using Atomic Absorption Spectroscopy (AAS) (GBS SensAA-Dual) and calibration curve was plotted. The reduction of cadmium concentration in the tested water sample was measured using the calibration curve. The experiment was carried out in triplicates.

Determination of ion exchange capacity for Mg^{2+}

Mg^{2+} ion solution (100 ppm) was prepared (196 mg of $MgCl_2$ in 500 mL distilled water). Intermediate stock solutions containing 50 ppm, 10 ppm, and 1 ppm were prepared. Absorbance of the standard solutions was recorded by using the AAS and calibration curve was plotted. The reduction of magnesium concentration in water sample was measured using the calibration curve in the AAS. The experiment was carried out in triplicates.

Determination of ion exchange capacity for Cu^{2+}

Cu^{2+} ion solution (100 ppm) was prepared. . Intermediate stock solutions containing 50, 40, 30, 20, and 10 ppm were prepared. Absorbance of the standard solutions were recorded by using UV spectrophotometer (Bio base, D580) and calibration curve was plotted. The reduction of copper concentration in water sample was measured using the calibration curve in UV spectrophotometer (Bio base, D580). The experiment was carried out in triplicates.

2.7 Determination of antibacterial activity using agar well diffusion method

Identified two bacterial cultures were obtained from Department of pathology, Health care science, Eastern University, Sri Lanka. Antibacterial activity was examined by spread plate method for both *Staphylococcus aureus* and *Escherichia coli*. (Akiyama *et al*. 2001).

Nutrient agar was inoculated with the given microorganisms by spreading the bacterial inoculums (*S. aureus* and *E. coli*) on the media plates at 37^{o}C. Wells (5 mm diameter) were punched in the agar with a cork borer and filled with various concentration (0.5,1.0,1.5, 2.0, 2.5 mg/ml) of esterified products

dissolved in acetone and placed on the surface of agar plates seeded with bacteria (20 µl/ml). Control wells contained neat solvents (acetone-negative control) and standard antibiotic solution. The plates were incubated at 37˚C for 24 hours to obtain the maximum growth in the culture media. The antibacterial agent diffuses in the agar medium, and inhibition of the microbial strain was tested. For each extract, the diameter of the inhibition zone of growth minus the diameter of the well was measured to estimate the degree of antibacterial activity. Analyses were performed a total of three times per strain.

3 Results and Discussion

The soxhlet extraction technique is an important method to show extraction of tannin effectively with non-polar solvents (Tan *et al*. 2013). The tannins could not be extracted optimally by using a single solvent. Therefore, extraction of tannins is carried out using a mixture of polar and less polar solvents (Tan *et al.* 2013). Methanol has been found to be the most commonly used polar solvent for the extraction of tannins rather than other organic solvents (FAO 2000), as the polarity of methanol helps to form strong interactions with polar groups of tannins. But, it is reported that although tannins are highly soluble in water, water proved to be an ineffective extraction solvent for tannins. This could be due to the formation of tannin-protein complexes. Previous studies stated that mixture of alcohols and water as the solvent to extract phenolic compounds was shown as a better solvent when compared to mono-solvents such as pure water. Thus extraction was performed using mixed solvent system of methanol-water (50:50). This mixed solvent boils at relatively lower temperatures (50˚C) under reduced pressure in order to avoid degradation or decomposition of phenolic compounds. However, excessive extraction time would be unnecessary as the solvent and sample would be in final equilibrium after certain duration. This is based on Fick's second law of diffusion (Cobzac *et al*. 2005, Dent *et al*. 2013). Based on the yield of the extraction, the polyphenolic body content of the bark of *Acacia auriculiformis* was found to be 13.7 % (w/w) on a dry mass basis.

FT-IR spectrum of extract of *Acacia auriculiformis* bark in range of 400-4000 cm^{-1} is shown in Figure 1. The strong broad band centered at 3318 cm^{-1} is due to -OH group and aromatic C-H bands at 3000 cm^{-1} which is overlapped with wide band of hydroxyl group. Vibration of aromatic ring (C-C) is located in area of 1612 cm^{-1}. The stretching vibration of methyl group is observed at 1443.88 cm^{-1}. The symmetrical and asymmetrical vibration of C-O appears at 1322 cm^{-1} and 1064 cm^{-1} respectively.

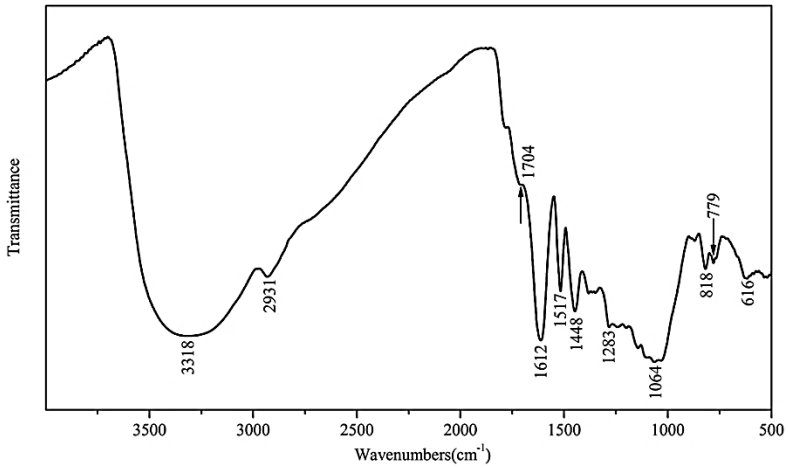

Fig 1. FT-IR spectrum of condensed tannin of *Acacia auriculiformis.*

ATR-FTIR initially provided evidence of successful ester formation at 1716 cm^{-1}, and indication of the extent of product esterification was shown in Figure 2.

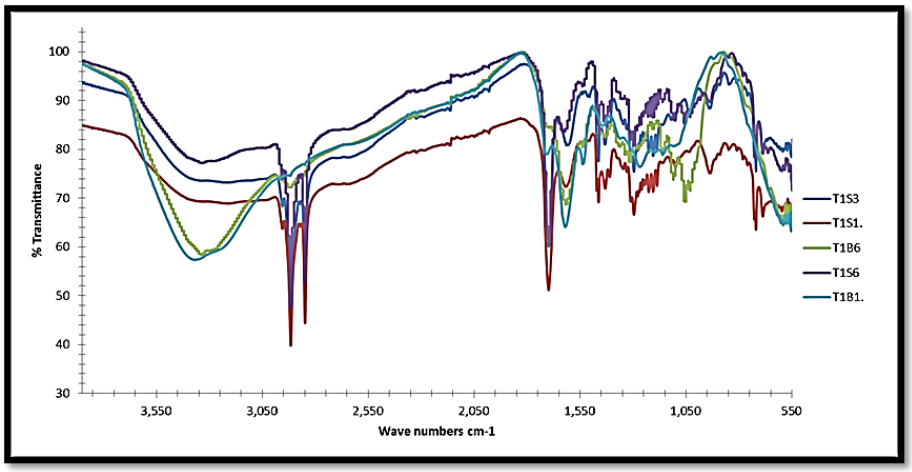

Fig 2. ATR- FTIR spectrum of esterified tannin of *Acacia auriculiformis*.

Acacia auriculiformis tannin butyrate (T_1B_6) showed a peak around 1716 cm^{-1} which indicates the presence of ester carbonyl as shown in Figure 2. T_1B_1

showed a broad absorption at 3335 cm^{-1} attributable to hydroxyl stretching vibrations. *A. auriculiformis* tannin stearates (T_1S_6) fully esterified product has three strong absorptions at 2917cm^{-1}, 2847 cm^{-1} (ν CH_2), and 1695 cm^{-1} (ν C=O) cm^{-1} attributable to the stearate ester carbonyl.

The broad absorption between 2500 cm^{-1} and 3700 cm^{-1} attributable to hydroxyl stretching vibrations was also observed to decrease. *Acacia auriculiformis* tannin stearates (T_1S_1) esterified product has three strong absorptions at 2908 cm^{-1}, 2851 cm^{-1} (ν CH_2), and 1698 cm^{-1} (ν C=O) cm^{-1} attributable to the stearate ester carbonyl. The broad absorption between 2500 cm^{-1} and 3700 cm^{-1} attributable to hydroxyl stretching vibrations . The spectra revealed that presence of ester C-O bond because of the peaks obtained in the 1300-1000 cm^{-1} region. Tannin stearates (T_1S_3) partially esterified product has three strong absorptions at 2900 cm^{-1}, 2847 cm^{-1} (ν CH_2), and 1691cm^{-1} (ν C=O) cm^{-1} attributable to the stearate ester carbonyl. The broad absorption between 2500 cm^{-1} and 3700 cm^{-1} attributable to hydroxyl stretching vibrations.

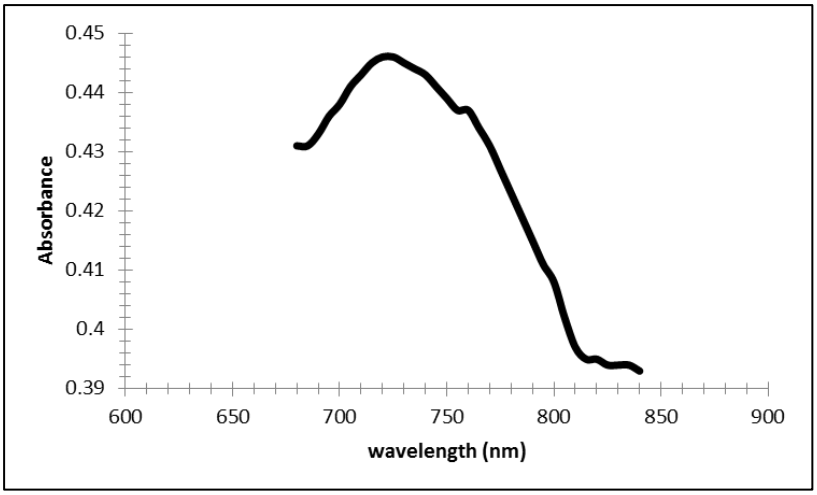

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Fig 3. Absorption maximum of Gallic acid at 725 nm

The amount of total phenolic content in extracts was determined with the *Folin-Ciocalteu* reagent. The quantitative result attested that 0.44 g of Gallic acid equivalent of condensed tannin was present in the tannin powder of the sample. Standard curve used for the determination of total phenolic content was prepared using absorption maximum of Gallic acid as in the Figure 3.

Tannins have strong chelating capacity towards many heavy metal ions thus they can possibly be used as alternative, effective, and efficient adsorbents for

the regaining of metal ions. However, tannins are water-soluble compounds, thus, when they are used as an adsorbent for recovery of metals from aqueous systems, they have the disadvantage of being leached to water (Hagerman 2002). To overcome this difficulty, attempts have been made to immobilize tannins into various water-insoluble esters. The results obtained for metal adsorptions are given in Tables 2 and 3.

Table 2. Metal adsorption of untreated water.

Metal ion	Metal ion concentration of untreated water (mg/l)
Cd^{2+}	0.050
Mg^{2+}	17.521
Cu^{2+}	32.306

Based on the results obtained from ANOVA test (MINITAB 14), significant differences were present in ion exchange capacities between the tannin esters and tannins ($p< 0.001$). Based on the results obtained for tannin butyrate and tannin stearates on copper ion adsorption, the tannin stearates have shown a significant reduction ($p<0.05$) in metal concentration. T_1B_1 and T_1S_1 have shown a high yield of 47.62% and 51.55% respectively. Furthermore, T_1S_1 has shown a significant reduction in the metal concentration compared to esters of tannin butyrate.

Table 3. Metal adsorption of tannin esters of *Acacia auriculiformis*

Code for Tannin – ester	Metal ion adsorption capacity (mg/l)						Percentage of metal adsorption (%)		
	Cd^{2+}		Cu^{2+}		Mg^{2+}				
	Mean	SD	mean	SD	mean	SD	Cd^{2+}	Cu^{2+}	Mg^{2+}
T_1B_1	0.025	0.0015	16.781	0.1876	14.33	0.1681	46	47.62	18.87
T_1B_2	0.031	0.002	18.753	0.2045	14.77	0.1709	38	42.58	15
T_1B_3	0.033	0.002	25.545	0.0395	15.96	0.0051	34	21.06	8.88
T_1B_4	0.042	0.002	28.522	0.0375	16.76	0.1573	20	11.84	3.84
T_1B_5	0.049	0.002	31.789	0.1760	17.32	0.0037	6	1.28	1.13
T_1B_6	0.0496	0.002	31.906	0.0549	17.36	0.0020	4	1.06	0.89
T_1S_1	0.019	0.0015	15.555	0.0970	13.74	0.1383	60	51.55	20.94
T_1S_2	0.025	0.0025	16.718	0.1340	13.92	0.0664	54	47.97	20.17
T_1S_3	0.026	0.0015	23.549	0.2722	15.76	0.1709	44	27.76	9.52
T_1S_4	0.032	0.0015	25.867	0.0291	16.69	0.0948	32	19.82	4.44
T_1S_5	0.043	0.0015	31.645	0.1215	17.47	0.0653	14	2.22	0.604
T_1S_6	0.047	0.0015	32.470	0.3773	17.51	0.0643	2	0.631	0.131

According the results obtained for tannin butyrate and tannin stearates on cadmium ion adsorption, the tannin stearates have shown a significant reduction in metal concentration. T_1B_1 and T_1S_1 have shown a high yield of 46% and 60% respectively. T_1S_1 has shown a significant reduction ($p<0.05$) in the metal concentration compared to esters of tannin butyrate. The results obtained for tannin butyrate and tannin stearates on magnesium ion adsorption, the tannin stearates have shown a significant reduction in metal concentration.T_1B_1 and T_1S_1 have shown a high yield of 18.87% and 20.94% respectively. T_1S_1 has shown a significant reduction in the metal concentration compare to esters of tannin butyrate.

Antibacterial activity was shown from the ratio of 1:4 of tannin esters. The results obtained for the tannin esters were tabulated in the Table 4 and 5.

Table 4. Antibacterial activity for tannin butyrates against *Staphylococcus aureus* and *Escherichia coli*

Sample	Concentration (mg/ml)	Average diameter of well (mm)	Average inhibition zone (mm) after 48 hours (mean ± SD)	
			S. aureus	*E. coli*
T_1B_4	0.5	5	0	0
	1.0	5	0	0
	1.5	5	1 ± 0.57	0
	2.0	5	2 ± 1.15	1 ± 1
	2.5	5	4 ± 1.15	2 ± 1
T_1B_5	0.5	5	0	0
	1.0	5	3 ± 0.57	2 ± 1
	1.5	5	4 ± 0.57	3 ± 1.15
	2.0	5	6 ± 1	5 ± 1
	2.5	5	6 ± 1	5 ± 1
T_1B_6	0.5	5	4 ± 1	2 ± 0.57
	1.0	5	5 ± 1.15	4 ± 0.57
	1.5	5	6 ± 1	5 ± 1
	2.0	5	8 ± 0.57	6 ± 1
	2.5	5	8 ± 0.57	6 ± 1.15

Acetone alone was used as the control (20 µl) during the experiment. After 24 hours there was no inhibition zone observed in control plates. Hence, it is concluded that acetone does not show any antibacterial activity. But, the inhibition was obtained only from the esterified tannin products. The results pointed out that these tannin esters possessed the highest antibacterial activity against the bacteria *Staphylococcus aureus* compared to *Escherichia coli.* The tannin stearates (T_1S_6) were shown higher activity than tannin butyrate for *Staphylococcus aureus* as well as *Escherichia coli*. This may be attributed to

the fact that these two groups differ by its cell wall component and its thickness (Chung *et al*. 1998, Akiyama *et al*. 2001).

Table 5. Antibacterial activity for tannin stearates against *Staphylococcus aureus* and *Escherichia coli*

Sample	Concentration (mg/ml)	Average diameter of well (mm)	Average inhibition zone (mm) after 48 hours (mean ± SD)	
			S. aureus	*E. coli*
T_1S_4	0.5	5	0	0
	1.0	5	1 ± 1	0
	1.5	5	2 ± 1	1 ± 1
	2.0	5	4 ± 1.15	3 ± 1
	2.5	5	4 ± 1.15	3 ± 1
T_1S_5	0.5	5	0	4 ± 1.15
	1.0	5	6 ± 1	5 ± 0.57
	1.5	5	7 ± 1	6 ± 1
	2.0	5	8 ± 1	7 ± 0.57
	2.5	5	9 ± 0.57	7 ± 0.57
T_1S_6	0.5	5	7 ± 1.15	5 ± 1
	1.0	5	9 ± 1	6 ± 1
	1.5	5	10 ± 1.15	8 ± 1
	2.0	5	12 ± 0.57	9 ± 0.57
	2.5	5	12 ± 0.57	9 ± 0.57

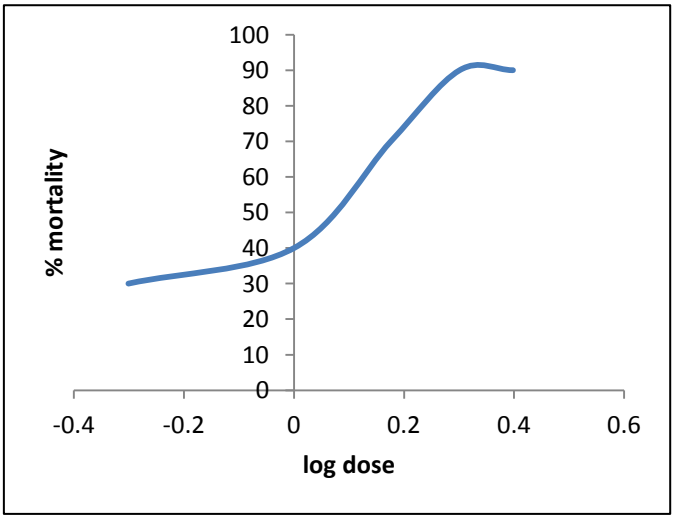

Fig 4. Plot of percentage mortality of *Staphylococcus aureus* against *log* dose of 2.5 mg/ml tannin stearates (T_1S_6).

The inhibition of tannin esters isolated in this study against the test organisms was shown the highest activity at 2.5 mg/ml of concentration (LD_{50}: 1.2) (Figure 4).

4 Conclusions

The mixture of highly polar solvents (methanol-water 50:50) has shown higher amount of yield from the raw material compared to other non-polar/medium polar solvents. The total polyphenolic body content of the bark of *Acacia auriculiformis* was found to be 13.7 % (w/w) on a dry mass basis. Depending on the qualitative tests and the quantitative test, the extracted polyphenol was shown clearly to be a type of condensed tannin. The quantitative results attested that 0.44 g of Gallic acid equivalent of condensed tannin present in the tannin powder of the sample. The highest ion adsorption capacity was shown by the ester T_1S_1 for Cd^{2+} (0.020 mg/l), Mg^{2+} (13.852 mg/l) and Cu^{2+} (15.650 mg/l). Furthermore, among partially esterified products (C_{18} carboxylic acid) long chain carboxylic acid ester products have shown highest value of ion adsorption compared to short chain (C_4 carboxylic acid) acid-ester products. In antibacterial activity, tannin stearates (1:6) possessed the highest activity against the bacteria *Staphylococcus aureus* at 2.5 mg/ml concentration, and these esters owned higher antibacterial activity against *Staphylococcus aureus* compared to *Escherichia coli*.

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