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ORIGINAL RESEARCH ARTICLE

Antibacterial Activity of Petung Bamboo (*Dendrocalamus Asper*) Leaf Extract Against Pathogenic *Escherichia coli* and Their Chemical Identification

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ABSTRACT

Petung bamboo (*Dendrocalamus asper*) is one of the abundant bamboos in Indonesia. Its shoot is nutritious and its wood is used for housing. Meanwhile, the leaves have been used as traditional medicine for remedy diarrhea in animals. Therefore, this research was conducted to compare the antibacterial activity of leaf extracts against diarrheagenic *Escherichia coli*. The extracts were prepared using methanol, ethanol, and the mixture thereof at the ratio 1:1, followed by solvent evaporation, to obtain methanolic, ethanolic, and methanol-ethanolic extracts, respectively.

The antibacterial activity of all extracts was assayed by well, disc diffusions and micro-dilution and their effect were compared to tetracycline as positive control. The well and disc diffusion results were not obvious, but micro-dilution assay showed that ethanolic extract of *D. asper* leaves with yield of 28.47±3.76% (w/w) was the most effective to inhibit all tested *E. coli* strains. The chemical identification using pyrolisis-GC/MS showed that fatty acids, together with esters, long chain alcohols, and aldehydes were the major compounds in ethanolic extract of *D. asper* leaves. Further research to improve the effectivity of ethanolic extract as antidiarrheal agent is necessary. Application of the semi pure extract to animal models is also important.

Key words: Petung bamboo, antibacterial activity, microdilution, pyrolisis-GC/MS, fatty acids.

INTRODUCTION

Bamboo, a group of large woody grasses belonging to the family Poaceae and subfamily Bambusoideae, is one of forest plants which has been used extensively, especially its shoot and wood. The shoot can be processed into various health foods and medicines [1,2], while the wood contributes mainly to housing and furniture [3]. Using the wood in a big amount yields leaves in a big amount too. Utilization of bamboo leaves was reported by some previous research. The ash of bamboo leaves is potential for stabilizing lateritic soils in highway construction [4].

Bioactive compounds are normally accumulated in all parts of plants, but their concentration varies according to the part of plants. The bark and leaves of neem (*Azadirachta indica* L.) and the leaves of Tulsi (*Ocimum sanctum* L.) possessed antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli* [5]. Kumaizasa bamboo leaves showed immunopotentiating activity, radical scavenging effects and suppression of tumor growth [6]. Ogunjinmi *et al*. [7] reported that dysentery, diarrhea, pile, and epilepsy could be treated by *Bambusa vulgaris* leaf extract.

From more than 1,000 species of bamboo in the world, there were about 60 species of bamboo that could be found in Indonesia. In Indonesia, Petung bamboo (*Dendrocalamus asper*), was abundantly available, together with three other species, i.e. apus bamboo (*Gigantochloa apus*), andong bamboo (*G. pseudoarundinacea)*, and black bamboo (*G. atroviolaceae*) [8]. *D. asper* is also one of the most bamboo species growing in Thailand and India [9,10].

The shoot of *D. asper* has higher calcium and potassium content than some other species, such as *Bambusa bambos*, *B. tulda*, and *D. strictus*, but their vitamin C and magnesium content are similar [9]. *D. asper* wood has 10-18 cm diameter in size and 11-18 cm thickness. It is widely used for housing while the leaves have been used as

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traditional medicine for remedy of diarrhea in poultry, calves, piglets, and rabbit.

Beauchamp and Sofos [11] reported that food-borne outbreaks caused by diarrheagenic *E. coli* were primarily associated with beef products. As much as 50 avian pathogenic *E. coli* (APEC) from broiler chickens with colisepticemia showed their high resistance (≥ 80%) against common antibiotics, such as nalidixic acid (100%), lincomycin (100%), erythromycin (97%), oxytetracycline (95%), chlortetracycline (95%), tetracycline (94%), flumequine (94%), tiamulin (91%), doxycycline (88%), difloxacin (83%), neomycin (81%), streptomycin (81%), trimethoprim-sulphamethoxazole (80%) [12]. As much as 56-100% of *E. coli* isolates from human fecal were resistant to tetracycline, ampicillin, chloramphenicol, and streptomycin [13]. Sharma [14] stated that if the antibiotic resistance could not be overcome, there will be no cure for tomorrow.

Using natural antimicrobial agent had some benefits. Extracts of *Caryophyllus aromaticus* and *Syzygyum joabolanum* were tested and the most potent antimicrobial agent was observed. Those extracts had higher activity against antibiotic-resistant bacteria than against susceptible bacteria. Furthermore, association of antibiotics and plant extracts showed synergistic antibacterial activity against antibiotic-resistant bacteria [15]. However, not all natural antimicrobial agents could inhibit *E. coli*. A recent investigation reported that *E. coli* was the most resistant bacteria to the natural antimicrobial agent from edible mushroom (*Dictyophora indusiata*) [16].

The antibacterial activity of the bamboo leaves has been studied previously. Singh *et al.* [34] reported that fresh leaves of bamboo were more effective in inhibiting *S. aureus* than penicillin. Additionally, leaves of bamboo from local area of Sultanpur (*Bamboosa arundinaceae*) were reported to inhibit *S. aureus, E. coli, Pseudomonas aeruginosa,* and *Bacillus* sp.

Therefore, this research was designed to investigate the activity of *D. asper* leaves against some *E. coli* strains, which cause diarrhea in poultry, piglets, and humans, and to identify the bioactive compounds in the selected extract. If the result is positive, it will not only improve the value of the bamboo leaves, but also decrease the use of synthetic antibiotic in humans and animals, and minimize the economic loss caused by the mortality of the neonatal poultry and piglets due to *E. coli* infections.

MATERIALS AND METHODS

The leaves of *D. asper* were freshly harvested from Ende (Mataram). The solvents (methanol and ethanol) and Tween-20 were reagent grade (Merck) and NaCl was obtained from Oxoid. The media for antibacterial assay were Nutrient Agar (NA), Nutrient Broth (NB), and Mueller Hinton Agar (MHA), all were obtained from Oxoid. Four strains of *E. coli* used as tested bacteria were two strains isolated from diarrheal livestock (piglets from BBALITVET Bogor and poultry from UGM Yogyakarta) and *E. coli* O157:H7 (strain JB4 from well water and AT9 from river water, Microbiology and Fermentation Laboratory, Faculty of Biotechnology Atma Jaya Catholic University, Jakarta). The *E. coli* serotypes 0157:H7 were chosen because they also cause diarrheal outbreaks [18].

Sample preparation,

The fresh bamboo leaves were cut into small pieces, oven dried at 50^o for 2 days, milled to become flour. Finally, the flour was sieved using 40 mesh siever to obtain the powdered leaf. The powdered leaf of *D. asper* was extracted at concentration of 6% (w/v) using methanol, ethanol, and the mixture thereof with a volume ratio 1:1. The mixture was filtered to remove the insoluble matter and the solventless extract was obtained by evaporating the solvent using vacuum rotary evaporator (Buchi R-215) and kept at 4^o for further use. The density of solventless extract was measured using calibrated picnometer. All extraction were done triplicate. Each solventless extract was used for antibacterial activity assay and the assay were carried out duplo. The yield was calculated as follows:

$$yield\ \left(\%\frac{w}{w}\right) = \frac{weight\ of\ solventless\ extract}{weight\ of\ fresh\ leaf} x\ 100\%$$

Preparation of *E. coli* for antibacterial activity assay,

E. coli from diarrheal poultry and piglets were grown in NA at 37^o for 24 h, then 5 colonies were put into 5 mL NB and reincubated at 37^o to reach 0.5 McFarland turbidity [19].

Antibacterial activity assay,

The antibacterial activity assay was conducted using three methods, i.e. well and disc diffusions [20] and microdilution [2]. The first and second methods were conducted using MHA as the media and the tested bacteria were grown in saline solution. The microdilution method was established by mixing 10-40 µl of extracts with 5 ml of NB, then adding 10 µl *E. coli*. The concentration of extract could be calculated using the equation:

$$[extract](\%\frac{v}{v}) = \frac{V_{extract}\ (\mu l)}{V_{extract}\ (\mu l) + V_{media}\ (\mu l) + V_{E.coli}\ (\mu l)}\ x\ 100\%$$

The absorbance of samples at 630 nm after being incubated at 37^o for 24 h was measured. Solvents for extraction were used as negative controls. The percentage of inhibition by extract was calculated using the equation:

$$\%\ \boldsymbol{inhibition} = \mathbf{100} - \left(\frac{\boldsymbol{A_{E.coli+NB+extract} - A_{NB+extract}}}{\boldsymbol{A_{E.coli+NB+solvent} - A_{NB+solvent}}}\boldsymbol{x100}\right)$$

Tetracycline was used as positive control and its inhibition was determined using microdilution by mixing 5-10 µl of tetracycline (10 mg/ml) with 5 ml of NB, then adding 10 µl *E. coli*. The concentration of tetracycline could be calculated using the equation:

$$[\boldsymbol{Tetracycline}]\left(\frac{\boldsymbol{mg}}{\boldsymbol{ml}}\right) = \frac{\boldsymbol{V_{tetracycline}\ (\mu l)}}{\boldsymbol{V_{tetracycline}\ (\mu l) + V_{media}\ (\mu l) + V_{E.coli}\ (\mu l)}}\ \boldsymbol{x\ 10mg/ml}$$

The concentration unit of extract was converted from % (v/v) to mg/ml by multiplying with its density. The dosage-inhibition percentage graphics of extracts and tetracycline were developed to calculate the concentration of either extract or tetracycline to inhibit 99.9% of bacteria. Finally, the effectivity of extract to inhibit *E. coli* growth compared to tetracycline could be calculated-using the equation:

$$\boldsymbol{Effectivity\ of\ extract}\ (\%) = \frac{[\boldsymbol{tetracycline}]_{I=99.9}}{[\boldsymbol{extract}]_{I=99.9}}\boldsymbol{x100}\%$$

Identification of bioactive compounds,

The selected extract with the highest antibacterial activity was identified using pyrolisis-GC/MS (QP 2010 Shimadzu) using the method reported by previous research [21].

RESULTS AND DISCUSSIONS

The yield of extracts depended on the polarity of solvents which were used for extraction. The results showed that the yield of methanolic, ethanolic, and methanol-ethanolic extracts were 35.01±8.40, 28.47±3.76, and 29.01±1.18 % (w/w), respectively. The density of all solventless extracts was 0.87, 0.81, and 0.86 g/ml, respectively.

Antibacterial activity assay by well diffusion and disc diffusion methods resulted in ambiguous data. The clear zone around the well or the disc could not be seen obviously. There were two possibilities that cause these results. Firstly, the extract did not have antibacterial activity against the tested *E. coli.* Secondly, while diffusing in the media, the extract caused the turbid zone due to formation of colloid products. Therefore, we conducted the third method to validate the results.

The result of microdilution assay showed that both ethanolic and methanol-ethanolic extracts of *D. asper* leaves could inhibit the growth of all tested *E. coli*, but methanolic extract did not (**Table 1**). It seemed that the ethanolic extract of *D. asper* leaves was more effective to inhibit *E. coli*, especially *E. coli* O157:H7 from well water (JB4). The data of methanolic extract were inaccurate because the absorbance were too large (more than 1.5), eventhough their concentration was the same as other extracts. According to Altman [22], the spectrophotometer will not give accurate results if the absorbance is either too high (>1.5) or too low (<0.02). This research is also in accordance with Scorzoni *et al.* [23] which reported that microdilution was more sensitive than agar diffusion. Broth microdilution is the most widely used method in clinical laboratories [19]. The data in Table 1 were used to develop a linear regresion in order to determine the required dosage of the antimicrobial agent to inhibit the growth of *E. coli* by 99.9% so that the effectivity of extract to inhibit bacterial growth could be compared to that of tetracycline [24].

(**Table 2**) shows the inhibition growth of all tested *E. coli* when administered by tetracycline. The results showed that the percentage inhibition of *E. coli* depended on the strain of *E. coli* but not on the solvent used to dissolve the antibiotic. It seemed that *E. coli* isolated from diarrheal piglets was the most sensitive to tetracycline.

(**Table 3**) showed that ethanolic extract was more effective than methanol-ethanolic extract to inhibit all tested pathogenic *E. coli*. Among all strains, *E. coli* O157:H7 from well water seemed the most sensitive to the extract.

There were 20 organic compounds in methanol-ethanolic extract of *D. asper* leaves which had been identified using py-GC/MS, and 15 of them could be identified with similarity index ≥90%. They could be grouped as fatty acids and the esters (54.72%), long chain alcohols (30.11%), derivatives of phenol (2.52%), aliphatic hydrocarbon (1.04%), and cyclic hydrocarbon (0.83%). Py-GC/MS predicted that there were 32 organic compounds in ethanolic extract of *D. asper* leaves, and 21 of them could be identified with similarity index ≥90%. They could be grouped as fatty acids and the esters (74.21%), nitrogenous compounds (7.09%), aliphatic hydrocarbons (3.21%), cyclic hydrocarbons (2.62%), long chain alcohols (2.50%), derivatives of phenol (1.38%), long chain aldehydes (0.89%), and long chain ketone (0.36%) (**Table 4**). Singh *et al.* [17] also reported that ethanol was the best solvent to extract bamboo leaves to obtain antibacterial agent, including against *E. coli*, compared to several tested solvents, i.e. petroleum

ether, benzene, chloroform, ethyl acetate, and water.

We hypotesized that fatty acids in ethanolic and methanol-ethanolic extracts play an important role in inhibiting the growth of *E. coli*. According to Desbois and Smith [25], free fatty acids possessed broad spectrum antibacterial properties, but their activity is influenced by their structure and shape. Esters also had antibacterial properties because they would be hydrolyzed by the lipase secreted from the sebaceous glands. Furthermore, it was reported that unsaturated fatty acids tend to have greater potency than saturated fatty acids with the same length carbon chain. This report is in accordance with our result. The ethanolic extract contained more fatty acids and esters than the methanol-ethanolic extract of *D. asper* leaves (74.21% vs 54.72%). In addition, the ethanolic extract also contained more unsaturated fatty acids and esters than the methanol-ethanolic extract (35.38% vs 28.94%).

Fatty acids from other plant extracts have also been reported about their antibacterial and antifungal activities [26]. The mixture of 14 fatty acids (mostly palmitic, lauric, and linolenic acids) in blind-your-eye mangrove (*Excoecaria agallocha*) leaf extract was 0.03-0.20% effective as ciprofloxacin. It seemed that *E. coli* was more sensitive to fatty acids than *K. pneumoniae* and *P. aeruginosa*.

Khaliq-Uz-Zaman *et al.* [27] reported that methanolic extract of *Chara coralline* var wallichii (A. Br.) R.D. Wood (Charophyta) could inhibit *E. coli, Corynebacterium diphtheriae, P. aeruginosa, Salmonella typhi, S. aureus, Streptococcus pyrogenes, Vibrio cholerae, Allescheria boydii, Aspergillus niger, Curvularia lunata, Microsporum canis, Nigrospora oryzae, Pleurotus austreatus, Stachyobotrys atra,* and *Trichophyton mentagrophytes,* but were not active to *C. hoffmannii, K. pneumoniae, Shigella flexneri, Candida albicans,* and *Dreschslera rostrata*. That extract contained 36.45% saturated, 35.17% dienoic, 15.06% trienoic, and 13.24% monoenoic FAMEs, such as lauric, heptadecanoic, palmitic, stearic, linoleic, and linolenic ones.

Abitogun and Badejo [28] reported that the percentage of fatty acids in ginger oil were linoleic (23.91), oleic (22.09), palmitic (20.96), lauric (8.93), linolenic (5.64), capric (4.14), myristic (3.75), stearic (3.50), lignoceric (2.28), caprylic (1.30), and arachidonic (0.19). This extract could inhibit *E. coli, S. aureus, K. pneumoniae,* and *P. aeruginosa*.

Annamalai *et al*. [29] reported that the inhibition activity of caprylic acid toward *E. coli* and total anaerobic bacteria in bovine rumen fluid depended on pH. Its bactericidal activity toward those bacteria was more efficient and faster at pH 5.6 than at pH 6.8. In addition, Nair *et al.* [30] reported that caprylic acid and monocaprylin could inactivate common mastitis pathogens, including *S. agalactiae, S. dysgalactiae, S. uberis, S. aureus,* and *E. coli* and potential as alternatives or adjuncts to antibiotics as intra-mammary infusion to treat bovine mastitis. Caprylic compound, such as caprylic glycol has been known about its antimicrobial activity against *S. aureus, E. coli, P. aeruginosa,* and *C. albicans* [31]. Pubmed [32] reported that lauric acid itself had no antibacterial activity, but its combination with gentamicin or imipenem was synergistic against methicillin-resistant *S. aureus* (MRSA). Lauric acid, together with its monoglyceride, capric acid, myristic acid, and linoleic acid, were reported as the most potent inhibitors across MRSA, but has not been tested against *E. coli*. The antimicrobial activity of liposomal lauric acids against *Propionibacterium acnes* had also been proved [33].

However, thorough investigation about the inhibiting activity of other fatty acids and esters against diarrheagenic *E. coli* shoud be conducted. Marounek *et al.* [34] reported that among C_2-C_{18} fatty acids, only caprylic acid and capric acid had significant antimicrobial activity toward *E. coli*. The same research team also reported that the sensitivity of the same species of bacteria varied according to the strain. Only medium-chain fatty acids (C_8 to C_{14}), oleic acid, and linoleic acid inhibited *C. perfringens* [35].

In addition, the methanol-ethanolic extract also contained alcohols and aldehydes, namely 9,12,15-octadecatrienal, myristaldehyde, 4,8-dimethyl-1-nonanol, hexahydrofarnesol, phytol, and 9,12,15-octadecatrien-1-ol. Those compounds could be oxidized to become its acid form which possessed antibacterial activity.

Stigmast-5-en-3-ol, oleat might have bacteriostatic activity. It could be hydrolyzed as oleic acid and stigmasterol. While the first was fatty acid, the latter was sterol which was active toward several bacteria and fungi, such as *E. coli, S. typhi,* and *S. pyogenes, S. aureus, V. cholerae, A. boydii, C. lunata, M. canis, N. oryzae, P. austreatus, S. atra,* and *T. mentagrophytes,* but no active against *C. diphtheriae, C. hoffmannii, K. pneumoniae, P.*

aeruginosa, S. flexneri. A. niger, C. albicans, and *D. rostrata* [27].

Pubmed [36[reported that guaiacol could inactivate the spores and vegetative forms of harmful microorganisms and it was not permanently absorbed in the blood but could be eliminated rapidly from the tested rats. The ethanolic extract contained phenol which antimicrobial activity was similar to guaiacol [37].

Long chain alkane might possess antibacterial activity, but not toward *E. coli*. Yayli *et al*. [38] reported that essential oil of air-dried *Minuartia meyeri* (Boiss.) Bornm possessed moderate antibacterial activity against *Yersinia pseudotuberculosis, Enterococcus faecalis,* and *S. aureus,* but the activity of *E. coli, K. pneumoniae, Serratia marcescens, B. subtilis, C. albicans,* and *C. tropicalis* was not significantly inhibited by the presence of the extract. Some of chemical constituents in the extract were also present in our ethanolic extract, such as n-tetradecane, pentadecane, phytol, n-hexadecane, and n-docosane. In addition, the methanol-ethanolic extract also contained a well known antioxidant, namely butylated hydroxyanisole.

Purification might be a good strategy to increase the effectivity of extracts to inhibit the growth of pathogenic *E. coli*. Some important physical characteristics of bioactive compounds in both extracts had been predicted using ACDLABS 12.0 (**Table 5**) so that the appropriate purification strategy could be considered.

The physical properties of bioactive compounds (fatty acids, alcohols, aldehydes, and esters) and ethanolic extract of *D. asper* leaves were quite different from those of its impurities; therefore purification based on those physical properties might be feasible. Theoretically, the yield of purified extract based on their refractive index seemed to be the highest but the lowest impurities could be achieved by purification based on the density. Regarding the yield of crude ethanolic extract, the profile of purified ethanolic extract was depicted in (**Figure 1**). It can be seen that the purification should be based on the surface tension or density to obtain the least impurity among the four purification principle. The possible impurities in each purified extract was shown in (**Table 6**).

Substituting conventional antimicrobial agents to fatty acids may be realistic and beneficial [25]. Fatty acids are also active against methanogens in the guts of ruminants, so the emissions of methane could be reduced. Pigletss treated with lipids and a lipolytic enzyme showed improved weight gain and feed conversion. In summary, among the three tested solvents, ethanol is the best to extract the antidiarrheal agent from *D. asper* leaves and the bioactive compounds in the extract was fatty acids and their relatives, such as alcohols, aldehydes, and esters. This promising result should be continued with further research to improve the effectivity of ethanolic extract. Application of the selected extract to animal models is also important.

Table 1: Inhibition of the growth (%) of *E. coli* at several concentration of extract

Extract		Me[1]	Et[2]	Mix[3]	Me[1]	Et[2]	Mix[3]
µl	(% v/v)	***E. coli* from diarrheal poultry**			***E. coli* from diarrheal piglets**		
10	0.199	8.6	43.0	46.8	30.7	57.0	10.8
20	0.398	0	55.6	56.5	25.1	80.9	72.8
40	0.792	0	60.6	54.0	15.1	93.9	86.5
		E. coli O157:H7 from water (JB4)			*E. coli* O157:H7 from water (AT9)		
10	0.199	0	34.1	41.5	0	17.3	15.3
20	0.398	0	89.0	36.1	0	53.7	44.8
40	0.792	0	100	41.8	0	85.0	82.0

[1]Methanolic extract, [2]Ethanolic extract, [3]Methanol-ethanolic extract

Table 2: Percentage inhibition of *E. coli* in the presence of tetracycline

Tetracycli		Me[1]	Et[2]	Mix[3]	Me[1]	Et[2]	Mix[3]
µl	mg/ml	***E. coli* from diarrheal poultry**			***E. coli* from diarrheal piglets**		
5	0.0100	44.4	39.3	37.1	8.1	36.4	6.3
8	0.0159	48.8	44.2	42.2	44.9	61.9	43.8
10	0.0199	50.7	46.2	44.3	62.0	73.7	61.2
		E. coli O157:H7 from water (JB4)			*E. coli* O157:H7 from water (AT9)		
5	0.0100	13.4	19.3	15.07	32.9	45.7	42.4
8	0.0159	15.4	21.2	17.0	49.7	59.3	56.8
1	0.0199	18.3	23.9	19.8	52.5	61.5	59.2

[1]Me = tetracycline dissolved in methanol, Et[2] = tetracycline dissolved in ethanol, Mix[3]= tetracycline dissolved in methanol-ethanol (1:1)

Table 3: Effectivity of extracts in inhibiting *E. coli* compared to tetracycline (%)

Sources of *E. coli*		Et[1]	Mix[2]
Animals	Diarrheal poultry	0.54	0.20
	Diarrheal piglets	0.39	0.37
Water	Well (JB4)	3.30	0.09
	River (AT9)	0.58	0.52
Average		1.20	0.30

[1]Ethanolic extract, [2]Methanol-ethanolic extract

Table 4: Organic compounds in methanol-ethanolic and ethanolic extracts of *D. asper* leaves

Groups	Chemical constituents	Formula	Mix[1]		Et[2]	
			%	SI	%	SI
Phenol derivatives	Guaiacol	$C_7H_8O_2$	0.43	94	-	-
	Maltol	$C_6H_6O_3$	0.68	92	0.33	91
	Phenol	C_6H_6O	-	-	0.29	82
	Phenol, 4-ethenyl-2-methoxy-	$C_9H_{10}O_2$	0.41	94	-	-
	2,6-Dimethoxyphenol	$C_8H_{10}O_3$	0.77	95	0.76	85
	Butylated hydroxyanisole	$C_{11}H_{16}O_2$	0.23	73	-	-
Cyclic hydrocarbons	l-Limonene	$C_{10}H_{16}$	0.36	95	1.48	96
	Corylon	$C_6H_8O_2$	-	-	0.85	96
	p-Menth-1-ene	$C_{10}H_{18}$	-	-	0.29	87
	(-)-m-menth-1(7)-ene	$C_{10}H_{18}$	0.47	79	-	-
Aliphatic hydrocarbons	n-Pentadecane	$C_{15}H_{32}$	0.20	96	0.44	96
	n-Hexadecane	$C_{16}H_{34}$	-	-	0.23	91
	n-Tetradecane	$C_{14}H_{30}$	-	-	0.57	90-96
	n-Docosane	$C_{22}H_{46}$	-	-	0.60	97
	Squalene	$C_{30}H_{50}$	-	-	0.34	95
	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	$C_{20}H_{40}$	0.84	93	1.03	92
Fatty acid and the ester	Lauric acid	$C_{12}H_{24}O_2$	0.38	87	0.45	93
	11,14,17-Eicosatrienoic acid, methyl ester	$C_{21}H_{36}O_2$	0.32	88	-	-
	Menthol acetate	$C_{12}H_{22}O_2$	-	-	0.60	79
	Acetic acid 3,7,11,15-tetramethyl-hexadecyl ester	$C_{22}H_{44}O_2$	0.16	94	-	-
	Methyl palmitate	$C_{17}H_{34}O_2$	6.18	95	2.82	97
	Palmitic acid	$C_{16}H_{32}O_2$	11.24	94	7.69	96
	Methyl heptadecanoate	$C_{18}H_{36}O_2$	0.63	96	-	-
	Allyl stearate	$C_{21}H_{40}O_2$	0.35	81	0.44	86
	Methyl linoleate	$C_{19}H_{34}O_2$	-	-	4.37	93
	Methyl stearate	$C_{19}H_{38}O_2$	-	-	1.95	89
	Stigmast-5-en-3-ol, oleat	$C_{47}H_{82}O_2$	-	-	0.99	85
	Methyl linolenate	$C_{19}H_{32}O_2$	28.62	95	29.44	92-94
	Ethyl linoleate	$C_{20}H_{36}O_2$	-	-	0.58	90
	Dioctyl adipate	$C_{22}H_{42}O_4$	6.84	90	24.88	84
Long chain aldehyde	9,12,15-Octadecatrienal	$C_{18}H_{30}O$	-	-	0.52	88
	Myristaldehyde	$C_{14}H_{28}O$	-	-	0.37	82
Long chain ketone	2-heptadecanone	$C_{17}H_{34}O$	-	-	0.36	95
Long chain alcohol	9,12,15-Octadecatrien-1-ol	$C_{18}H_{32}O$	22.26	90	-	-
	Phytol	$C_{20}H_{40}O$	7.85	94	1.77	93
	4,8-Dimethyl-1-nonanol	$C_{11}H_{24}O$	-	-	0.45	90
	Hexahydrofarnesol	$C_{15}H_{32}O$	-	-	0.28	93
Nitrogenous compounds	Ammonium carbamate	CH_3NO_2	-	-	4.87	100
	Cyclopentolate	$C_{17}H_{25}NO_3$	-	-	1.41	88
	Trimethylamine	C_3H_9N	-	-	0.81	98

SI = similarity index (%),[1] Methanol-ethanolic extract, [2]Ethanolic extract

Table 5: Some physical properties of bioactive compounds in methanol-ethanolic and ethanolic extracts predicted using ACDLABS 12.0.

Bioactive compounds	MW	RI	ST (dyne/cm)	ρ (g/ml)
Lauric acid ($C_{12}H_{24}O_2$)	200.32	1.447 ± 0.02	33.2 ± 3.0	0.905 ± 0.06
Palmitic acid ($C_{16}H_{32}O_2$)	256.42	1.453 ± 0.02	33.3 ± 3.0	0.892 ± 0.06
Methyl palmitate ($C_{17}H_{34}O_2$)	270.45	1.441 ± 0.02	30.2 ± 3.0	0.865 ± 0.06
Methyl linolenate ($C_{19}H_{32}O_2$)	292.46	1.475 ± 0.02	31.6 ± 3.0	0.895 ± 0.06
Phenol (C_6H_6O)	94.11	1.553 ± 0.02	40.9 ± 3.0	1.071 ± 0.06
Ethyl linoleate ($C_{20}H_{36}O_2$)	308.50	1.465 ± 0.02	31.3 ± 3.0	0.882 ± 0.06
9,12,15-octadecatrienal ($C_{18}H_{30}O$)	262.43	1.476 ± 0.02	31.3 ± 3.0	0.863 ± 0.06
Myristaldehyde ($C_{14}H_{28}O$)	212.37	1.435 ± 0.02	29.3 ± 3.0	0.826 ± 0.06
9,12,15-octadecatrien-1-ol ($C_{18}H_{32}O$)	264.45	1.485 ± 0.02	32.6 ± 3.0	0.869 ± 0.06
Guaiacol ($C_7H_8O_2$)	124.14	1.534 ± 0.02	38.6 ± 3.0	1.109 ± 0.06
11,14,17-Eicosatrienoic acid, methyl ester ($C_{21}H_{36}O_2$)	320.51	1.475 ± 0.02	31.8 ± 3.0	0.891 ± 0.06
Menthol acetate ($C_{12}H_{22}O_2$)	198.30	1.441 ± 0.02	28.2 ± 3.0	0.917 ± 0.06
Acetic acid 3,7,11,15-tetramethyl-hexadecyl ester ($C_{22}H_{44}O_2$)	340.58	1.445 ± 0.02	29.1 ± 3.0	0.858 ± 0.06
Methyl heptadecanoate ($C_{18}H_{36}O_2$)	284.48	1.442 ± 0.02	30.4 ± 3.0	0.864 ± 0.06
Allyl stearate ($C_{21}H_{40}O_2$)	324.54	1.452 ± 0.02	30.9 ± 3.0	0.868 ± 0.06
Methyl linoleate ($C_{19}H_{34}O_2$)	294.47	1.464 ± 0.02	31.2 ± 3.0	0.884 ± 0.06
Methyl stearate ($C_{19}H_{38}O_2$)	298.50	1.444 ± 0.02	30.5 ± 3.0	0.863 ± 0.06
Stigmast-5-en-3-ol, oleat ($C_{47}H_{82}O_2$)	679.15	1.509 ± 0.03	38.0 ± 5.0	0.950 ± 0.1
Dioctyl adipate ($C_{22}H_{42}O_4$)	370.57	1.451 ± 0.02	33.1 ± 3.0	0.929 ± 0.06
4,8-Dimethyl-1-nonanol ($C_{11}H_{24}O$)	172.31	1.435 ± 0.02	28.4 ± 3.0	0.826 ± 0.06
Hexahydrofarnesol ($C_{15}H_{32}O$)	228.41	1.443 ± 0.02	29.0 ± 3.0	0.831 ± 0.06
Phytol ($C_{20}H_{40}O$)	296.53	1.459± 0.02	29.8± 3.0	0.845± 0.06

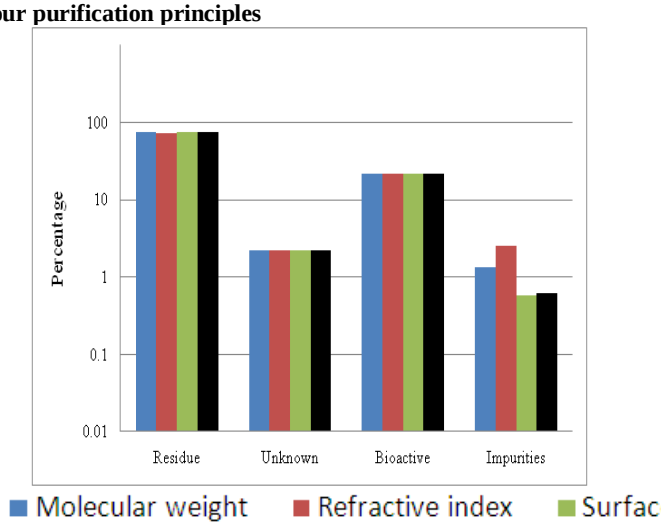
MW: molecular weight, RI: refractive index, ST: surface tension, ρ: density

Table 6: Prediction of impurities in purified ethanolic extract of *D. asper* leaf

	Purification strategy			
Impurities	**MW**	**RI**	**ST**	**ρ**
Ammonium carbamate	-	+	-	-
l-Limonene	-	+	-	+
p-Menth-1-ene	-	+	-	-
2,6-Dimethoxyphenol	-	-	+	-
n-Tetradecane	+	-	-	-
n-Pentadecane	+	-	-	-
n-Hexadecane	+	-	-	-
2-heptadecanone	+	+	+	+
2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	+	+	-	-
Cyclopentolate	+	-	-	-
n-Docosane	+	+	+	-
Squalene	-	+	+	+

+ Present ; - Absent

Fig 1: Profile of purified ethanolic extract of *D. asper* leaf using four purification principles



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