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BIO-ACTIVITIES AND PHYTOCHEMICAL INVESTIGATION OF *CNESTIS PALALA* (LOUR.) MERR.

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Abstract

Background: In traditional medicines, the root of *Cnestis palala* was used for the treatment of stomach ache, malaria, urinary track disorders and snakebite. The seed was used for poisoning rat and stray dogs. However, the bioactivities and chemical constituents have not been reported. So, this will be the first report.
Material and Methods: Biological investigations of *C. palala* extracts against bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Propionibacterium acnes, Escherichia coli, and Pseudomonas aeruginosa*), fungi (*Trichophyton mentagrophytes, Trichophyton rubrum* and *Microsporum gypseum*), yeast (*Candida albicans*), tuberculosis (*Mycobacterium tuberculosis*), malaria (*Plasmodium falciparum*) and cancer cell lines (MCF-7 and HT-29) were evaluated. The chromatographic and spectroscopic techniques were used for the isolation and identification of pure compounds.
Results: The results showed that the ethanol leaf and bark extracts were active against *S. aureus* and *S. epidermidis*. The hexane leaf and seed extracts and petroleum ether bark and root extracts showed strongly inhibition values against MCF-7. Moreover, the petroleum ether bark extract exhibited high inhibition value against HT-29. Seven compounds were isolated as *β*-sitosterol-glucoside (**CP1**), hydroquinone (**CP2**), *β*-sitosterol (**CP3**), mixture of fatty acids (**CP4**) and ethyl caffeate (**CP5**), scopoletin (**CP6**) and 2-nonenal (**CP7**). The **CP2** was strongly active against *S. aureus* and *S. epidermidis*, the MIC was showed 30.10 µg/ml and 15.05 µg/ml and the MBC was showed 15 µg/ml and 7.5 µg/ml, respectively.
Conclusion: These results suggested that *C. palala* is a great potential source of anti-microbial agents. Hence, this is the first study, which will be the database for chemical constituents and bioactivities of *C. palala*.

Keywords: *Cnestis palala*, Connaraceae, Phytochemistry, Bioactivities, Herbal medicines.

Introduction

Cnestis palala (Lour.) Merr., belongs to the Connaraceae family and in Thailand is commonly called Maa tai mi-phaak-laak and Ngonkai (local name in Thai: Kra phaak lak (Trat), Kalingping (Ratchabury), Mataimaithonglak (Chon Buri), Mataiwai or Masakkalat (Lampang), Mataiwai Ngonkai (Central), Ndonkipa, Kadoling (Nong Khai). In Peninsula, it is called Ngonkai nuai or Maa daeng (Smitinand, 2001). *C. palala* is a climbing shrub, laden with a velvety scarlet-orange fruit and has a wide repartition in South East Asia particularly in the Andaman Islands, Myanmar, Laos, Vietnam, Thailand, Malaysia, Sumatra, Borneo, Philippines and Sulawesi (Bunyapraphatsara and Lemmens, 2003).

In the past, some plants in *Cnestis* genus such as *C. glabra, C. polyphylla* and *C. ferruginea* exhibited interesting biological activities; anti-diarrheal (Akindele et al., 2006), anti-oxidant (Akindele et al., 2010), anti-plasmodia (Bero et al., 2009), anti-inflammatory (Akindele and Adeyemi, 2007), anti-bacterial and anti-malarial activities (Dan et al., 2006). In traditional medicines; the root of *C. palala* was used for treatment of stomach ache, malaria, urinary trouble and snakebite; the seed was used for poisoning rat and stray dog. However, the seeds were reported to be toxic and some Malays and Thais use the seed for poisoning stray dogs. From previous studies, 50% ethanol root extract and aerial parts were tested for poisoning in mice within which the mice died at 1000 mg/kg (Bhakuni et al., 1988). In Thailand, it has been reported about its poisoning fresh seed against students in Pangnga province. After 10 hours of eating fresh seed, the children developed symptoms like dizziness, headache, nausea and vomiting (Jiratchariyakul, 2001). In Japan it has been reported for acute toxicity that at 75% ethanol extracts of freshly harvested seeds in dogs present with the poisoning symptoms and subsequently died at 347 mg/kg dosage after 24-25 hr. An unusual amino acid, L-methionine sulfoximine has been isolated from fresh seeds and also reported its toxicity in dogs (Murakoshi et al., 1993). In addition, some plants in *Cnestis* genus presented with methionine sulfoximine (i.e. *C. glabra, C. polyphylla* and *C. ferruginea*), exhibited poisoning in various organism (Jeannoda et al., 1985a; Garon et al., 2007).

So, some biological activities such as anti-microbial, anti-cancer and anti-malarial activities of this plant has not been reported except for one compound, methionine sulfoximine from ethanol seed extract (Murakoshi et al., 1993). Thus, the study of biological activities and phytochemical investigation of this plant will be useful in getting more information and proof on the traditional uses of this plant in order to have a database for further study in new medicine from natural products.

This is the first report of the chemical constituents in all parts of this plant used in carrying out an experimental approach to assess the anti-microbial, anti-fungal, anti-malarial, anti-tuberculosis and cytotoxicity activities. Moreover, we described the isolation and structure elucidation, together with the evaluation of biological activities of isolated pure compounds.

Materials and methods

Plant material

C. palala was collected from Krabi Plant Production Materials Technical Service Center, Krabi Province and Rajjaprabha Dam, Surat

Thani Province, Thailand. The voucher specimen was identified by comparison with authentic sample and was deposited at the herbarium of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand with herbarium number SKP 053 03 16 01. After cleaning, plant materials were dried at 60°C in hot air oven and ground to powder and kept for extraction at room temperature.

Extraction, Isolation and Identification:
Extraction procedure

Dried leaf, wood, pod, bark, root and seed powders weighing 6, 3.5, 0.30, 1.5, 1.48 and 0.052kg, respectively were used. They were macerated at room temperature for three days with petroleum ether, hexane and ethanol consecutively (3 times). The filtrates obtained from each step were concentrated under reduced pressure, afforded the petroleum ether, hexane and ethanol extracts, respectively.

Isolation procedure of plant material
Isolation of ethanol extract of *C. palala* leaf

Ethanol extract from leaf (40 g) was isolated with silica gel column (vacuum liquid chromatography, VLC). The column was eluted with the mixture of methylene chloride (CH_2Cl_2) and ethyl acetate (EtOAc). The ratios between CH_2Cl_2 and EtOAc were 100:0 to 0:100. The solvent polarity was increasing until it yielded 42 fractions (500 ml/each). Fractions were pooled as guided with TLC fingerprint and evaporated to dryness under reduced pressure to give the following fraction L1-6. The fraction L5 (6.654 g) from VLC added on a silica gel column and eluted by the mixture of $CHCl_3$, acetone and MeOH with the ratio 100:0:0 to 50:40:10, respectively. The fractions were combined into six major groups depending on their TLC profiles, to furnish compound **CP1** 137.5 mg (The %yield = 0.022 % based on dried weight of crude leaf extract).

The fraction L1 (1.207 g) was further isolated by silica gel column. The column was eluted with the mixture of CH_2Cl_2 and EtOAc using the ratio 100:0 to 60:40, respectively. The fractions were combined into 10 fractions (H1-H10) based on TLC fingerprint. The fractions H1-H10 was submitted to flash column chromatography, being eluted with petroleum ether and EtOAc, in increasing polarity afforded compound **CP3** (25.7 mg; % yield = 0.064% based on dried weight of crude leaf extract) and compound **CP4** (7.28 mg; % yield = 0.018% based on dried weight of crude leaf extract).

The fraction L3 were pre-fractionated by column chromatography (CC) on silica gel eluting with the mixture of chloroform ($CHCl_3$) and methanol (MeOH) with the ratio 100:0 to 50:50 to get 54 fractions. The fractions were combined into 8 fractions (F1-F8). After crystallization (F8) in $CHCl_3$ and MeOH, compound **CP2** (220.7 mg; %yield = 0.55% based on dried weight of crude leaf extract) was obtained as white needle. According to TLC analysis, the **CP2** was a major component of ethanol leaf extract. The fraction F5 (297.5 mg) was purified by silica gel column and eluted by the mixture of hexane and EtOAc with the ratio 100:0 to 60:40 to get 24 fractions (Le1-24). The Le10-18 (100.8 g) was subjected to Sephadex® LH-20 column eluted with MeOH 100% to furnish 25 fractions (Le1a-Le25a). Fractions Le2a (40.23 mg) were subjected to RP-18 CC eluted by the mixture of MeOH and H_2O with the ratio 50:50. Preparative TLC was used to provide **CP5,** 8.84 mg (The %yield = 0.022 % based on dried weight of crude leaf extract).

Isolation of ethanol extract of C. palala wood

The EtOH extract (20 g) was dissolved in the mixture of EtOH and H_2O by the ratio 2:8 and further extracted by partition with hexane (3 x 250 ml) to get hexane and aqueous extracts. Aqueous extract was diluted with H_2O (250 ml) and then extracted with $CHCl_3$ (3 x 250 ml), EtOAc (3 x 250 ml), and *n*-BuOH (3 x 250 ml), respectively. Solvent extracts were then evaporated to dryness under reduced pressure to give hexane extract (2.468g), $CHCl_3$ extract (698.2 mg), EtOAc extract (5.973 g), *n*-BuOH extract (8.312g) and remaining aqueous extract (3.447g).

The $CHCl_3$ fraction (698 mg) was subjected to RP-18 column in the mixture of MeOH and H_2O by the ratio 5:5 and the fractions were subjected to silica gel in the mixture of $CHCl_3$ and MeOH by the ratio 100:0 to 50:40 and obtaining 8 main fractions (WCC A-H). The WCC A fraction (138.1 mg) was further fractionated by CC on silica gel using increasingly polar in the mixtures of hexane and EtOAc by the ratio 100:0 to 60:60. The fractions were combined into 4 major groups (WCC A1-4) depending on their TLC profiles and elution with hexane and EtOAc. The WCC A2 fractions were combined and applied to Sephadex® LH-20 column eluted with 100% MeOH, affording compound **CP6** 3.48 mg (The %yield = 0.01% based on dried weight of crude extract wood). However, **CP1** and **CP3** were also isolated from hexane and EtOAc wood extracts.

Isolation of ethanol extracts of C. palala seed

Ethanol seed extract (5 g) was isolated in the mixture of $CHCl_3$, acetone, MeOH by the ratio 100:0:0 to 20:70:10, respectively. The extracts were evaporated under reduced pressure to gain 64 fractions and combined into 15 major groups (SE1-15), the fraction SE2 to provide compound 7 (**CP7**) 80 mg (the % yield = 6.0 % based on dried weight of crude seed extract). **CP1** (25 mg) was also isolated from fraction SE6. So, **CP1** could be isolated from leaf, wood and seed of this plant.

Biological studies
Assay of anti-microbial activity

Agar disc diffusion method; determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were followed using Lorian (2005); Yasunaka et al., (2005); Phongpaichit et al. (2006); Kuete et al. (2007); Niyomkam (2007); Kummee and Intaraksa (2008); Ghalem and Mohamed (2009). The gram-positive bacteria, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Propionibactelium acnes* and gram-negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa* were obtained from the culture collection of the Thailand Institute of Scientific and Technology Research. The dermatophytes employed in this study,

Trichophyton mentagrophytes, *Trichophyton rubrum*, *Microsporum gyseum* and *Candida albicans* were obtained from the culture collection of the Specialized Centre for Medical Mycology, Federal University of the Department of Medical Science, Ministry of Public Health, Thailand.

Cytotoxic activity

Sulforhodamine B (SRB) assay was used for cytotoxic activity determination and was supported by Assist. Prof. Dr. Supreeya Yuenyongsawad (Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University). In this study, the human breast adenocarcinoma cells (MCF-7), human colon adenocarcinoma cell line (HT-29) and normal cell (human gingiva fibroblasts cell) were obtained from National Cancer Institute, Bangkok, Thailand.

Anti-malarial activity

The anti-malarial activity assay was tested by National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The micro-culture radioisotope technique based on the method described by Desjardins et al. (1979) was used in this assay. *Plasmodium falciparum* (K1, multidrug resistant strain) was used for evaluation.

Anti-tubercular activity

The anti-tubercular activity assay was tested by the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The Green fluorescent protein (GFP)-based fluorescent detection assay as described by Changsen et al. (2003) was used for this assay. *Mycobacterium tuberculosis* strain H37Ra was used for evaluation.

Results

Extraction

The dried powder of leaf, stem, fruit, bark, root and seed from *C. palala* were exhaustively extracted with petroleum ether, hexane and ethanol by maceration, respectively.

Screening of biological activities
Activity against gram negative bacteria

The gram negative bacteria were treated with *C. palala* crude extracts by disc diffusion method; all crude extract did not show activity against *E. coli* and *P. aeruginosa* (at 2.5 mg/ml). While, negative standard norfloxacin was presented inhibition zone against *E. coli* and *P. aeruginosa*, 30.8±0.5 mm and 25.10±0.9 mm, respectively.

Activity against gram positive bacteria

The crude extracts of fruit, root and seed were not tested for activity against *S. aureus* and *S. epidermidis*. Although, all parts of crude extract were not tested for activity against *P. acnes*, the crude ethanol leaf, stem and bark extracts showed low effect against *S. aureus* and *S. epidermidis* as showed in Table 1.

Screening of anti-fungal and yeast activities

The results as showed in Table 2 indicated that some parts of the ethanol extract produced the inhibition zones against all fungi and yeast strains. The ethanol seed extract was observed strongly against *T. rubrum*, *T. mentagrophytes* and *M. gypseum*. The ethanol leaf, stem and bark extracts showed the inhibition zones against *C. albicans.*

Determination of Minimum Inhibitory Concentration (MIC) and
Minimum Bactericidal Concentration (MBC) for gram positive bacteria

From the screening of the activity against gram positive bacteria the ethanol leaf, stem and bark extracts displayed an inhibition zone against *S. aureus* and *S.epidermidis as* indicated. So, MIC and MBC were determined as showed in Table 3.

Table 1: Screening of anti-gram positive bacteria activity from *C. palala* crude extracts.

Plant part	Average of growth inhibition zone (mm) at sample concentration 2.5 mg/ ml/ disc								
	Petroleum ether extract			Hexane extract			Ethanol extract		
	S. aureus	***S. epidermidis***	***P. acnes***	***S. aureus***	***S. epidermidis***	***P. acnes***	***S. aureus***	***S. epidermidis***	***P. acnes***
Leaf	-	-	-	-	-	-	7.96±0.70	13.83±0.90	-
Stem	-	-	-	-	-	-	7.05±0.43	7.00±0.21	-
Bark	-	-	-	-	-	-	10.9±0.60	13.42±0.67	-
Fruit	-	-	-	-	-	-	-	-	-
Root	-	-	-	-	-	-	-	-	-
Seed	-	-	-	-	-	-	-	-	-
Oxacillin[♣]	19.0±0.17	29.33±0.70	29.85±0.90	19.0±0.17	29.33±0.70	29.85±0.90	19.0±0.17	29.33±0.70	29.85±0.90

[♣]Standard for gram positive bacteria, concentration 1 µg/disk

Table 2: Screening of anti-fungi and yeast activities from *C. palala* crude extracts.

Plant part	Average of growth inhibition zone (mm) atsample 2.5 mg/disc											
	Petroleum ether extracts				Hexane extracts				Ethanol extracts			
	TR[✦]	**TM[✦]**	**MG[✦]**	**CA[✦]**	**TR[✦]**	**TM[✦]**	**MG[✦]**	**CA[✦]**	**TR[✦]**	**TM[✦]**	**MG[✦]**	**CA[✦]**
Leaf	-	-	-	-	-	-	-	-	-	-	-	-
Stem	-	-	-	-	-	-	-	-	-	-	-	12.71 ± 0.56
Bark	-	-	-	-	-	-	-	-	-	-	-	19.66 ± 0.56
Fruit	-	-	-	-	-	-	-	-	-	-	-	-
Root	-	-	-	-	-	-	-	-	-	-	-	-
Seed	-	-	-	-	-	-	-	-	54.98 ± 0.38	49.20 ± 0.85	45.74 ± 0.45	33.20 ± 0.14
amphotericin B[*]	-	-	-	16.40 ±0.65	-	-	-	16.40±0 .65	-	-	-	16.40 ± 0.65
ketoconazole[✱]	58.80 ± 0.35	43.75 ± 0.66	40.43 ± 0.75	-	58.80 ± 0.35	43.75 ± 0.66	40.43 ± 0.75	-	58.80 ± 0.35	43.75 ± 0.66	40.43 ± 0.75	-

[✦]TR = *T. rubrum*, TM = *T. mentagrophytes*, MG = *M. gypseum*, CA = *C. albicans*,
[*]standard for *C. albican*s, [✱]standard for *T. rubrum*, *T. mentagrophytes* and *M. gypseum*.

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for gram positive bacteria

Sample & standard drug	MIC & MBC (µg/ml)			
	S. epidermidis		*S. aureus*	
	MIC	MBC	MIC	MBC
Leaf	**500**	**1000**	**1000**	**1500**
Stem	2500	2500	1250	5000
bark	**250**	**500**	**250**	**1000**
oxacillin	0.126	0.54	0.24	0.97

In the present study, it can be observed that all microorganisms were susceptible to the ethanol leaf and bark extracts, with a variation in the MIC values from 0.03-4 µg/ml. We determined through the broth dilution method carried out in triplicate that the MIC cut point was established at 500 µg/ml. The results suggested that ethanol extract from bark was found to be more effective than the ethanol extract from leaf against all the organisms.

Determination Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) for fungi and yeast

From the screening of the activity of *C. palala* crude extract against fungi and yeast displayed strong inhibition zone. Consequently, we have tested MIC and MFC of ethanol stem, bark and seed extracts for fungi and yeast as showed in Table 4. From the results, the MIC and MFC showed that the crude extracts were low potency against fungi and yeast.

Table 4: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) for fungi and yeast

Sample ethanol extract & Standard	MIC & MFC							
	C. albicans		*T. rubrum*		*T. mentagrophytes*		*M. gypseum*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
stem[†]	3.75	15	-	-	-	-	-	-
bark[†]	1.00	>30	-	-	-	-	-	-
seed[†]	1.25	>40	5.00	>40	10	>40	5	>40
amphotericin B[*]	0.5	15	-	-	-	-	-	-
ketoconazole[*]	-	-	0.5	15	0.25	25	0.25	15

[†]= mg/ml, [*, *] = µg/ml

Screening anti-malarial and anti-tuberculosis activities

In traditional medicine, the decoction of *C. palala* root was used for the treatment of malaria (Murakoshi et al., 1993). Therefore the ethanol root extract of *C. palala* was investigated for anti-malarial activity. The results showed that the ethanol root extract of *C. palala* was inactive against *P. falciparum* at maximum concentration 10 µg/ml.

The result of anti-tubercular activity showed that the ethanol root extract was inactive against *Mycobacterium tuberculosis* H37Ra at the maximum concentration 50 µg/ml.

Screening of cytotoxic activity

The results of cytotoxic activity against MCF-7 and HT-29 of the extracts from *C. palala* were depicted in Table 5. The cytotoxic activities against MCF-7 from hexane extract of leaf and seed; petroleum ether extract of bark and root showed % inhibition values of 79.97±6.09, 83.91±13.74, 93.58±1.87 and 83.36±7.44, respectively. Pronounced cytotoxic activity of HT-29 was exhibited by the petroleum ether bark extracts with % inhibition value 92.16±2.38 and other fractions showed low effect to both cell lines. In addition, the results of both cell lines showed that the most active extract was non-polarity fraction, because of the petroleum ether and hexane extracts were shown to be highly active against MCF-7 and HT-29 cell lines. This result suggested that the active constituents should be non-polarity compounds. Although several members of the *Cnestis* genus have already been reported as having cytotoxic activity in methanol, ethanol and aqueous extracts (Jeannoda et al., 1985a; Garon et al., 2007). It was found that the L-methionine sulfoximine was the potent cytotoxic compound in *Cnestis* (Jeannoda et al., 1985b; Garon et al., 2007). Some species such as *Cnestis ferruginea* is abundant in West Africa and in root applications, used in traditional medicine to treat diverse conditions were studied from sub-chronic toxicity in methanol root extract. The results showed that possible side effects identified as induced delayed platelet anomaly, anemia in females, weight reduction and sterility in males (Ishola et al., 2012).

From the screening of cytotoxic activity against MCF-7 and HT-29 cell lines possessed a high activity accordingly. Our study involved the investigation of cytotoxic activity against normal cells as presented in Table 5. The petroleum ether and hexane extracts not being active against normal cells suggested that they were not toxic against normal cells in high concentration (25 µg/ml). Consequently, these fractions were investigated in isolation from the active compound.

Structure evaluations

This study was the first report on the chemical investigation of *C. palala* except for those reported in the ethanol seed extract, which was found L-methionine sulfoximine, which was very toxic in organism from pervious study (Murakoshi et al., 1993; Garon et al., 2007). The isolation of pure compound through chromatography techniques and characterized by spectroscopy techniques. In the first study, the active components in ethanol extract of leaf and bark against gram positive bacteria were isolated (bioassay guided fractionation) and in the second, the components of all several parts of *C. palala* were investigated also.

Chemical compounds from ethanol extract of leaf and bark

Compound 1-5 (**CP1-5**) were isolated from the leaf extract and in this study could not purify the compound from the bark

Structure determination of compound 1 (CP1)

The compound 1 (**CP1**) was isolated as white powder but presented violet color after spraying with anisaldehyde-sulphuric acid reagent and 50 % sulphuric acid reagent and heating on an electric oven at 110°C. The UV spectrum in methanol demonstrated absorption maxima (λ_{max}, MeOH) 204, 230 and 244 nm and the structures according to FT-IR presented functional group to be as follows: 3600-3400 cm^{-1} (-OH stretching), 2934 cm^{-1} (-C-H stretching), 2850 cm^{-1} (=C-H sp^2 stretching), 1640 cm^{-1} (-C=C- stretching), 1450 and 1379 cm^{-1} (-C-H bending), 1073 and 1023 cm^{-1} (-C-O- stretching). The EI mass spectrum exhibited a molecular ion at 396 *m/z*, consistent with formula of $C_{29}H_{49}O_6$.

From the ^{1}H-NMR spectrum (500 MHz, pyridine-d_5), the high field region indicated six methyl groups at δ 0.68 (3H, *s*), δ0.88 (3H, *d*, *J* = 6.6 Hz), δ 0.87 (3H, *d*, *J* = 6.9 Hz), δ 0.90 (3H, *m* , *J* = 7.3 Hz), δ 0.96 (3H, *s*), δ 0.99 (3H, *d* , *J* = 6.5 Hz) and the low field region showed methine proton at δ 3.95 (1H, *m*), δ 4.01 (1H, *t* , *J* = 7.9 Hz), δ 4.25 (1H , *m*), δ 4.22 (1H , *m*), δ 4.52 (1H, *dd*, *J* = 11.6 Hz, 2.1 Hz, β), δ 4.38 (1H, *dd* , *J*=11.7 Hz, 5 .2 Hz,α), respectively and one olefinic proton at δ 5.36 (1H, broad *s*).

The ^{13}C-NMR spectrum (125 MHz, pyridine-d_5) showed thirty-five carbons at δ 12.04, 12.22, 19.09, 19.32, 19.47, 19.99, 21.38, 23.54, 24.57, 26.66, 28.57, 29.65, 30.35, 32.19, 32.25, 34.36, 36.44, 37.58, 37.03, 39.46, 40.08, 42.60, 46.22, 50.51, 56.41, 56.96, 63.02, 71.91, 75.39, 78.49, 78.31, 78.66, 102.70, 141.07 and 121.92 ppm. Based on the information obtained from the HMQC spectrum all protonated carbons of **CP1** were assigned. The long-rang C-H correlation of **CP1** could observed from HMBC spectrum.

From ^{1}H-NMR spectrum in high field region at δ 0.68-2.75 ppm to the same class of phytosterols and in the region low field region at δ 3.95-5.03 was sevens protons of the sugar moiety. The EI-Mass spectrum was proposed 396 (m/z) [Aglycone (sterol) + H – H_2O)] that suggested molecular ion peak of an only aglycone of sterol that the sugar was hydrolyzed. The HMBC spectrum showed correlations of the long rang coupling between ^{1}H and ^{13}C. The **CP1** was melting point at 275–277°C.

On the basis of IR, ^{1}H-NMR,^{13}C-NMR, 2D-NMR spectral data of **CP1** and the comparison of its spectral data with the previous report (Moghaddam et al., 2007), it was identified as ***β*-sitosterol-D-glucoside (Daucosterin).**

Structure determination of compound 2 (CP2)

The compound 2 (**CP2**) is colorless prismatic needles and it presented brown spot on TLC (chloroform: methanol (9:1, R_f= 0.49). The UV spectrum in methanol revealed absorption maxima (λ_{max}, MeOH) at 224 and 294, the FT-IR spectrum absorptions bands provided at 3172 cm^{-1} (-OH stretching), 3030 cm^{-1} (=CH stretching), 2012-1651 cm^{-1} (phenyl ring substitution overtones), 1517 and 1467 cm^{-1} (aromatic ring stretching), 1193 and 1092 cm^{-1} (-C-O- stretching), 833 and 760 (phenyl ring substitution bands). The EI mass spectrum performed a molecular ion at 110 *m/z* (62.9, 64, 68.9, 81, 82, 83, 94.9, 107.9, 108.9, 109.9, 110.9 and 111.9), suggesting a molecular formula as $C_6H_6O_2$. The two signals of ^{1}H NMR spectrum of **CP2** exhibited resonances (acetone-d_6) at δ 6.65 ppm (2H, s) δ 7.61 ppm (1H, s). The ^{13}C-NMR spectrum provided two carbons at δ 116.59 ppm and 151.18 ppm.

From ^{1}H NMR and ^{13}C-NMR spectrum of **CP2** suggested aromatic derivative that the symmetry in the chemical structure. Its UV, IR, MS, ^{1}H-NMR, ^{13}C-NMR, spectral data and the comparison with previous report (Sabrahanian et al., 1973) suggested that **CP2** was hydroquinone.

Structure determination of compound 3 (CP3)

The compound 3 (**CP3**) is a white crystalline compound. It displayed a positive anisaldehyde-sulphuric and 50 % sulphuric acid reagent as shown through the violet color. Its melting point was at 93-95 °C. The UV spectrum in chloroform showed absorption maxima (λ_{max}, $CHCl_3$) 216 and 243 nm, the FT-IR spectrum absorptions bands appeared at 3423 cm^{-1}(-OH stretching), 2936.8 cm^{-1}(-CH_2 stretching), 2867.38 cm^{-1}(=CH stretching), 1710.1 cm^{-1}(-C=C-), 1465.9 cm^{-1} (-CH_2 bending), 1382.5 cm^{-1} (-CH_3 bending) 1053.6 cm^{-1} (-C-O- stretching). The EI mass spectrum exhibited a molecular ion at 414 *m/z* (396, 381, 353, 329, 303, 272, 255, 231, 213, 199, 173, 163, 161, 145, 133, 107, 95, 85 and 71), corresponding to the molecular formula $C_{29}H_{50}O$.

Table 5: Determination of cytotoxic activity with MCF-7, HT-29, and Normal cell lines.

Plant part	% inhibition (at 25 µg/ml)								
	MCF-7 cell line			HT-29 cell line			Normal cell		
	Petroleum ether extract	Hexane extract	Ethanol extract	Petroleum ether extract	Hexane extract	Ethanol extract	Petroleum ether extract	Hexane extract	Ethanol extract
Leaf	60.98±16.42	**79.97±6.09**	44.67±19.51	1.89±14.52	5.877±5.63	35.76±8.45	N	17.04±6.09	N
wood	31.48±13.77	60.67±20.08	27.36±46.63	56.45±4.76	34.75±6.83	41.09±8.26	N	N	N
Bark	**93.58±1.87**	66.33±11.60	38.14±20.92	**92.16±2.38**	38.05±9.57	25.33±7.26	2.66±0.8	N	N
Fruit	35.88±13.35	1.65±5.99	-2.64±14.86	29.17±10.57	26.25±37.21	41.09±24.99	N	N	N
Seed	27.42±12.51	**83.91±13.74**	36.24±20.96	45.93±17.96	41.00±10.22	37.99±24.16	N	20.68±10.76	N
Root	**83.36±7.44**	57.13±8.77	40.05±15.07	43.65±0.08	53.01±11.64	59.45±2.9	N	N	25.3±4.47

N = not active against normal cell.

From the ^{1}H-NMR spectrum (500 MHz, chloroform-*d*), its presence was indicated six methyl groups at δ 0.72 (3H, s), δ 0.85 (3H, *d*, *J* = 6.7 Hz), δ 0.87 (3H, *d*, *J* = 6.7 Hz), δ 0.89 (3H, *t*, *J* = 7.4 Hz), δ 0.96 (3H, *d*, *J* = 6.5 Hz), 1.05 (3H, s) and methine proton at δ 3.56 (1H, *m*), and one olefinic proton at δ 5.39 (1H, broad *m*), respectively.

The ^{13}C-NMR spectrum (125 MHz chloroform-d_5), showed twenty nine carbons at δ 11.86, 11.99, 18.79, 19.04, 19.40, 19.83, 21.09, 23.07, 24.31, 26.07, 28.26, 29.16, 31.60, 31.65, 31.93, 33.95, 36.15, 36.50, 37.02, 39.88, 42.30, 42.31, 45.83, 50.13, 56.06, 56.77, 121.70 and 140.70.

From ^{1}H-NMR and ^{13}C-NMR spectra pattern were similar to that of *β*-sitosterol glucoside (**CP1**), but the chemical shift at 3.95-5.03 (the region of sugar moiety) does not appear. The comparison of its ^{1}H-NMR and ^{13}C-NMR spectra with previously report (Patra et al., 2010), **CP3** was identified as ***β*-sitosterol**.

Structure determination of compound 4 (CP4)

Compound 4 (**CP4**) was white powder; it showed violet color when spayed with 50% sulphuric acid and showed one spot on TLC.

The ^{1}H-NMR spectrum of **CP4** displayed the mixtures of fatty compounds, the^{1}H-NMR spectrum directed δ (Chloroform-*d*); δ 3.62 ppm (1H, *t*), 1.54 ppm (2H, *m*), the large methylene (CH_2) proton signal δ 1.20-1.33 ppm (29H, *m*), 0.85 ppm (2H, *t*).

From ^{1}H-NMR spectrum suggested that **CP4** are long-chain carbon, when analyzed of long-chain carbon by GC-MS, they performed **the mixture of two major saturated fatty acids**, as hexadecanoic acid (72.16%), octadecanoic acid (32.83%) and unknown (5.74%).

Structure determination of compound 5 (CP5)

The compound 5 (**CP5**) was a yellowish powder, with UV-visible absorption in methanol at λ_{max} 207, 299, 300, 328 nm, the structures according to FT-IR spectra presented functional group to be as follows: 3399 cm^{-1} (-OH stretching), 2927 cm^{-1} (-CH_2 stretching), 2850 cm^{-1}(=CH stretching), 2298 cm^{-1} (Overton of aromatic ring), 1699 cm^{-1} (-C=O bending), 1602 cm^{-1} (-C=C- of aromatic ring), 1447 cm^{-1} (-CH_2 bending), 1369 cm^{-1} (-CH_3 bending), 1264 1117, 1034 and 1180 cm^{-1} (-C-O- stretching) and 981-701 cm^{-1} (signal of aromatic). The EI-MS spectrum of **CP5** showed the molecular ion peak at m/z 208, corresponding to $C_{11}H_{12}O_4$.

The ^{1}H -NMR spectrum of **CP5** showed one methyl group at δ 1.22 ppm (3H, *t*), one methylene group at δ 4.15 ppm (2H, *q*) and offered olifinic proton at δ 6.25 ppm, (1H, *d*, *J* = 16.06 Hz), δ 6.88 ppm (1H, *d*, *J* = 8.3 Hz), δ 6.99 ppm (1H, *dd*, *J* = 2.07 Hz, *J* = 8.03 Hz), δ 7.03 ppm (1H, *d*, *J* = 2.07 Hz) and δ 7.55 ppm (1H, *d*, *J* = 16.81 Hz).

The ^{13}C-NMR spectrum of **CP5** displayed eleven signals at the δ 14.40, 59.8, 114.15, 114.91, 115.0, 121.47, 125.60, 145.13, 145.74, 148.61 and 166.68 ppm.

Moreover, the coupling constants in the ^{1}H NMR spectrums exhibited the coupling of olefinic proton in side chain proton, presented *trans* form, the internal molecule showed coupling aromatic ring, the presented J_{ortho} coupling at 8.03 Hz, J_{meta} coupling at 2.07 Hz and J_{para} coupling was not shown in the ^{1}H NMR.

Its ^{1}H and ^{13}C NMR spectra when compared with previous report (Chiang *et al.*, 2005) suggested that **CP5** was **ethyl caffeate**. It is a derivative of caffeic acid. From literature, it presented an anti-inflammatory and anti-oxidant activity (Chiang et al., 2005).

Chemical compounds from other extracts *of C. palala*
Compound from ethanol wood extract

Ethanol wood extract of *C. palala* showed the weakest activity for microorganisms but was active against snake-bite in preliminary study (Jiratchariyakul, 2001). So, this part was investigated for chemical constituents. From the experiment, we noted that a major compound in this part was *β*-sitosterol-glucoside. However, compound 6 (**CP6**) was also isolated from ethanol wood extract.

Structure determination of compound 6 (CP6)

CP6 was obtained as a yellow needle, the UV-wavelength at 365 nm showed violet color on the TLC plats. This compound **CP6** presented UV absorbance (λ_{max}, methanol) at 207, 223, 297 and 348 nm, the FT-IR spectrum of **CP6** showed absorption bands at 3436 cm^{-1} (due to a hydroxyl group), 1683 cm^{-1} (corresponding to carbonyl group (δ-lactone)), 1615 cm^{-1} (corresponding to CH=CH group) 1507 and 1424 cm^{-1} (corresponding to aromatic benzene ring), 1365 cm^{-1} (-CH_3 bending) and 1020 cm^{-1} (-C-O bending). The GC-MS presented molecular ion peak at m/z 192.1 (42, 51.1, 69, 79, 92, 105.1, 121.1, 149.1, 164.1, 177, 192.1), corresponding to $C_{10}H_8O_4$.

From the ^{1}H NMR spectrum (500 Mz, DMSO-d_6) was indicated one methoxy groups at δ 3.80 (3H, *s*), the four olefinic proton at δ 6.20 (1H, *d*, *J* = 9.08 Hz), 6.76, (1H, s), 7.2, (1H, s) and 7.88 (*d*, 1H, *J* = 9.08 Hz). The ^{13}C-NMR spectrum showed eleven carbons at δ 56.1, 102.9, 109.7, 110.5, 111.6, 144.6, 145.5, 149.7, 151.6 and 160.8. Based on the information obtained from the HMQC, all protonated carbons of **CP6** were assigned.

The comparisons of ^{1}H NMR and ^{13}C-NMR spectra of **CP6** with previous report (Silva et al., 2001), from spectroscopy techniques suggested that the **CP6** was **scopoletin (7-(hydroxy-6-methoxycoumarin)**, it showed its melting point as 203-204^{o}C. It is simple coumarin group. It has been shown to exert several biological activities, such as: anti-cholinesterasic (Orhan et al., 2008), anti-nociceptive (Ribas et al., 2008), anti-inflammatory (Kim et al., 2004), anti-thyroid, anti-oxidant, anti-hyperglycemic (Panda and Kar, 2006), hypouricemic (Ding et al., 2005) and anti-tumor activities (Liu et al., 2001).

Compound from ethanol seed extract

Ethanolic seed extract from *C. palala* showed a chemical constituent likely comparable with the compound in leaf and wood extract. The chemical structures were presented *β*-sitosterol, *β*-sitosterol-glucoside and Compound 7 (**CP7**).

Structure determination of compound 7 (CP7)

The **CP7** presented white powders and showed violet color after spay with anisaldehyde-sulphuric acid reagent on the TLC plats. The FT-IR spectra of **CP7** showed absorption bands at 2919 cm^{-1} (-C-H stretching), 2851 cm^{-1} (=C-H sp^2 stretching), 1738 cm^{-1} (-C=O- stretching), 1471, 1417, 1392, 1281, 1266, 1247, 1221, 1197, 1179 and 1113 cm^{-1} (-C-H bending).

The ^{1}H -NMR spectrum of **CP7** showed one terminal methyl group at δ 0.84–0.86 ppm, (3H, *m*), the signal of the methylene (CH_2) protons at δ 1.2–1.3 ppm ((CH_2)$_m$, *m*), at δ 1.56–1.59 ppm ((CH_2)$_2$, *m*),), protons attached to the allylic carbons at δ 2.0–2.1 ppm, protons attached to the bis-allylic carbons at δ 2.2–2.3 ppm, δ 4.10–4.13 ppm (1H, *dd*, *J* = 6 Hz), at δ 4.25–4.28 ppm (1H, *dd*, *J* = 4.5 Hz) and olefinic protons at δ 5.2–5.3 ppm (2H, *m*). From ^{1}H -NMR spectrum of **CP7** showed a signal of the mixture with long chain hydrocarbon. Therefore, GC-MS was used for structure determination of this compound.

The gas chromatography presented many peaks on the chromatogram, and MS library predicted a compound in the GC-chromatogram at molecular ion peak 139 m/z, corresponding to $C_{10}H_8O_4$, that showed as 2-Nonenal. From all data, **CP7** was concluded as **2-nonenal**.

Determination of anti-bacterial activity of isolated compounds

All isolated compounds from ethanol leaf extract obtained in the present study as **CP1-5** were studied for activity against gram positive bacteria (*S. aureus* and *S. epidermidis*), the results are showed in Table 6.

Table 6: Anti-gram positive bacterial activity of isolated compounds from *C. palala*

Pure compounds & Standard	**MIC & MBC (µg/ml)**			
	S. aureus		***S. epidermidis***	
	MIC	**MBC**	**MIC**	**MBC**
β-sitosterol-glucoside (CP1)	500	>500	500	>500
Hydroquinone (CP2)	**15.05**	**30.10**	**7.25**	**15.05**
β-sitosterol (CP3)	500	>500	500	>500
Mixture of fatty acids (CP4)	N	N	N	N
Ethyl caffeate (Le11)	500	>500	250	>500
Oxacilline (Std.)	0.25	1.00	0.25	1.00

N = inactive against *S.aureus* and *S.epidermidis*

The anti-microbial activities of substances were assayed against two gram-positive bacteria (*S. aureus* and *S. epidermidis*). The MIC obtained in the initial assessment of the **CP2** demonstrated a very promising activity from the ethanolic leaf extract against *S. aureus* and *S. epidermidis*. It also exhibited strong activity against *S. epidermidis* which presented MIC and MBC as 7.25µg/ml and 15.05µg/ml, respectively. Conversely, **CP2** exhibited activity against *S. aureus* that showed MIC and MBC as 15.05µg/ml and 30.10µg/ml respectively. The other compounds showed weak activity against the *S. aureus* and *S. epidermidis* when displayed with the MIC and MBC showing a high concentration.

The known anti-microbial mechanisms associated to each group of chemical to which the isolated compounds belong may explain the anti-microbial potency of the crude extracts from leaf of *C. palala*. The mechanisms of hydroquinone against microorganism could be suggested as having likely mechanisms action of quinone group (Stern et al., 1996; Kim et al., 2010) which actions are known to be complex irreversibly with nucleophilic amino acids in proteins, often leading to the inactivation of proteins and loss of function membrane. The influence of the position of hydroxyl groups on the aromatic ring, which seemed to play an important role to the agglutination of organism, was examined. From previous study, Himejima et al., (2004), studied agglutination and adherence of hydroquinone against *Streptococcus mutans* that its action mechanism was same as *S. aureus* and *S. epidermidis*. Hydroquinone altered the sucrose-induced agglutination of *S. mutans* by forming the hydroquinone bridge, while neither resorcinol nor catechol exhibited any activity, presumably because of their steric hindrance. The result clearly indicates that the para-dihydroxy substitution is essential in making bridges between *S. mutans* cells. This study is the first report of the activity of hydroquinone against *S. aureus* and *S. epidermidis*.

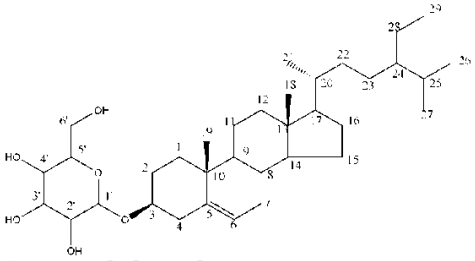
Discussion

Cnestis palala (Connaraceae Family) was said to be adaptive for poisonings in dogs, rats and other organism. In this study, the phytochemical investigation and screening of biological activities of leaf, wood, bark, fruit, seed and root in petroleum ether, hexane and ethanol extracts from *Cnestis palala* were done. The eighteen fractions were screened by biological activities against gram negative and gram positive bacteria, fungi, yeast and cytotoxicity on cancer cell lines. The ethanol root extract was screened with anti-malarial and anti-tuberculosis activities followed as traditional uses. From the results obtained, the ethanol leaf and bark extracts presented activity against gram positive bacteria (*S. epidermidis* and *S. aureus*) and all extracts were inactive against the gram negative bacteria, fungi and yeast. The results of cytotoxic activity with MCF-7 cancer cell lines demonstrated that hexane extract of leaf and seed; petroleum ether extract of bark and root showed % inhibition values of 79.97±6.09, 83.91±13.74, 93.58±1.87 and 83.36±7.44, respectively. Then petroleum ether bark extract exhibited the potential cytotoxicity to HT-29 cancer cell line with % inhibition value as 92.13±2.38 and other fractions showed low potency to both cancer cell lines.

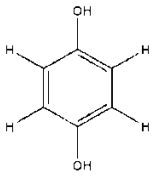
In the phytochemical study, the isolation of compounds was done by chromatography techniques and characterization by spectroscopy techniques. The active components in ethanol leaf and bark extracts against gram positive bacteria were investigated and the components of all several parts from *C. palala* were investigated also. The ethanol leaf extract was performed *β*-sitosterol-glucoside (**CP1**) 0.022 %, hydroquinone (**CP2**) 0.55%, *β*-sitosterol (**CP3**) 0.064%, the mixture of fatty acids (**CP4**) 0.018% and ethyl caffeate (**CP5**) 0.022 %, respectively (Fig 1). From the

results of **CP1-5** against *S. aureus* and *S. epidermidis* were suggestive that hydroquinone (**CP2**) showed the strongest inhibition against *S. aureus* and *S. epidermidis*, the MIC and MBC of *S. aureus* and *S. epidermidis* were presented as 7.5 µg/mland 15 µg/ml and 15.05 µg/mland 30.10 µg/ml, respectively. This is the first report of anti-bacterial activity of hydroquinone against *S. aureus* and *S. epidermidis*. Conversely, other compounds **CP1**, **CP3**, **CP5** and the mixture of fatty acids displayed the lowest activity against *S. epidermidis* and *S. aureus*.

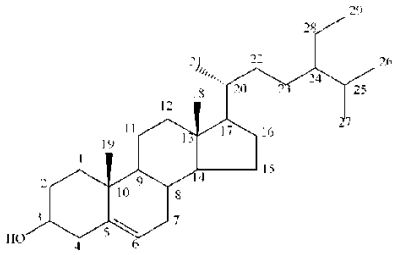
Furthermore, in the results we proposed the phytochemical study from other parts of *C. palala*, wood, fruit and seed extracts. The ethanol wood extract provided the scopoletin (**CP6**), the ethanol seed extract was including *β*-sitosterol, *β*-sitosterol-glucoside and the mixture of long chain hydrocarbon (the GC-MS library was predicted as 2-Nonenal).



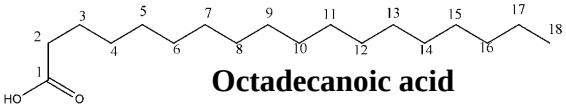
***β*-sitosterol-glucoside; Daucosterin (CP1))**

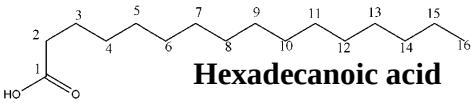


Hydroquinone (CP2)

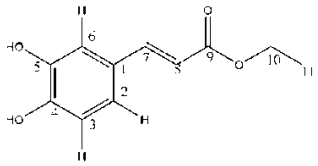


***β*-sitosterol (CP3)**

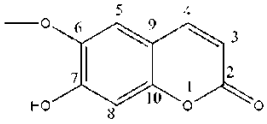




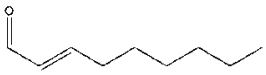
The mixture of fatty acids (CP4)



Ethyl caffeate (CP5)



Scopoletin (CP6)



2-Nonenal (CP7)

Figure 1: Chemical structure of isolated compounds from *C. palala*.

Conclusions

This study is the first report on the biological activities and phytochemical investigation in all parts of this plant, whose plant extracts have great potential as anti-microbial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by microorganisms. Moreover, the synergistic effect from the association of antibiotics with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotics when it is no longer effective by itself during therapeutic treatment. In addition, this study will provide a database for *C. palala* as touching the contents of chemical constituents and biological activities.

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