CHAPTER 4

Results and Discussion

4.1 Ethnobotanical survey

According to our ethnobotanical survey, traditional healers in ninety three villages in eleven subdistricts in Rong Kwang district in the northeastern part of Phrae province were interviewed with the questionnaire on their uses of medicinal plants with focus on four species of *Combretum*. Sixty one traditional healer, 37 men and 24 women, were interviewed on their traditional knowledge and medicinal plants used in their recipe. The ages of traditional healers ranged from 26 to 88.

The questionnaire (Appendix A) used for interviewing was divided into 4 parts. Part one was general information of the traditional healers. Part two was the detail on *Combretum* spp. used in traditional formula and their uses in folk wisdom. As results, the common occupation of the traditional healers is agriculturist. Plant species generally grown in the villages are rice, tobacco, corn, soybean and green beans. Medicinal plants used by traditional healers usually cultivated in their home gardens. Uses of the medicinal plants and also how to prepare the formula are the folk wisdom that has been passed from generation to generation. Techniques of traditional treatment are confidential. They believe that if the treatment of their patients presented to anyone, the treatment result will be unpleasant. Part three of the questionnaire was revealed that the villagers used Western medicines as well as herbal medicines for their health care. On the other hand, the traditional healers were familiar with herbal medicine more than Western medicines. The medicinal plants were collected from their home gardens, the forest nearby their community. Many species of medicinal plants were collected at the same time during daytime and kept the mature plants. Several remedies were imparted by the traditional healers for the treatment of cancer, hepatitis, diabetes, bacterial infection, diarrhea, anthelmintic, hemorrhoid, diseases of the skin, snake bites, abortions, muscle pain and bone fracture. Treatment of their patients was provided

service in their home or patient's place as appropriate. Part four was the specific part of data collection on *Combretum*; *C. deciduum* Coll. & Hemsl., *C. griffithii* Heur. & M.A., *C. latifolium* Bl. and *C. quadrangulare* Kurz, found in the northern Thailand. In folk wisdom, the identification of *Combretum* spp. used the difference of characters of fruits and leaves. While botanical identification of *Combretum* species, leaves are used as the most species-specific organs. Eighteen traditional healers (29.51 %) in the Rong Kwang District, Phrae Province were familiar with and used stem bark, roots, seeds and leaves of *C. quadrangulare* Kurz to treat diarrhea, anthelmintic infection, gonorrhea and muscle pain. So *C. quadrangulare* Kurz was the most popular species for medicinal purposes of all the *Combretum* species collected. The stems, roots and fruits of *C. latifolium* Bl. have been known and used by eight traditional healers (13.11 %) as a medicine for nourishing the blood, dysentery, dysmenorrhea, astringent, tonic, muscle relaxant and appetizer. Four traditional healers (6.56 %) knew and used the stem of *C. griffithii* Heur. & M.A. as a medicine for hepatitis. And four traditional healers (6.56 %) knew of *C. deciduum* Coll. & Hemsl., but did not use it as a medicine. For the indication of anthelmintic, they used their seeds by mixed with egg stir fry, and take single dose. And the other indication, they used several parts of the plants by preparing with decoction method, and continue to take them until the patients feel better. The results of the ethnobotanical survey of the four *Combretum* species related to the literature, the detail as shown in Table 4.1 and Table 4.2.

Table 4.1 Data on the traditional healers in Rong Kwang district, Phrae province, Thailand familiar with four *Combretum* species studied

Traditional healers known *Combretum* species	Number of traditional healers	Percent (%)
C. deciduum Coll. & Hemsl.	4	6.56
C. griffithii Heur. & M.A.	4	6.56
C. latifolium Bl.	8	13.11
C. guadrangulare Kurz	18	29.51
Not familiar with *Combretum* species	27	44.26
Total	61	100.00

Table 4.2 *Combretum* species growing in northern Thailand and traditional uses

Species	Vernacular name	Part used	Use	Literature
C. deciduum Coll. & Hemsl.	Naen Kruea	None	None	None
C. griffithii Heur. & M.A.	Kamin Krua, Naen	Stem	Treat hepatitis	Treat hepatitis
C. latifolium Bl.	Tua Pae Pao	Stem	-	Astringent, Nourishing blood and body, Dysentery, Dysmenorrhea
		Roots	Muscle relaxant	-
		Fruits	-	Nourishing blood, Appetizer
C. quadrangulare Kurz	Kon Kae, Chong Kae	Stem bark	Anticancer, Appetizer, Treat hemorrhoid	Diarrhea
		Roots	-	Anthelmintic, Gonorrhea
		Seeds	Anthelmintic	Anthelmintic
		Leaves	-	Muscle relaxant

4.2 Extraction of plant materials

The dried powdered leaves of four *Combretum* species were sequentially extracted with *n*-hexane, dichloromethane and methanol at room temperature (1.5 L, 24 h for each solvent). The *n*-hexane, dichloromethane and methanolic extracts were

concentrated in vacuum to obtain the crude *n*-hexane, dichloromethane and methanolic extracts, respectively.

The fresh leaves of four *Combretum* species were subjected to hydrodistillation for 8 h in a modified Clevenger-type apparatus. The volatile oils (light-colored) were collected, dried over anhydrous sodium sulfate and stored at 4 °C for further analysis.

The percentage yields of the crude extracts were calculated based on dry weight and the percentage yields of the volatile oils were calculated based on fresh weight. The results are shown in Table 4.3 and 4.4, respectively.

Table 4.3 Percentage yield (%, w/w) of dry leaf extracts from four *Combretum* species

Species and extracts	Dry weight (g)	Extract weight (g)	Yield (%)
C. deciduum Coll. & Hemsl.			
n-Hexane	402.31	13.19	3.28
Dichloromethane		5.31	1.32
Methanol		106.97	26.59
C. griffithii Heur. & M.A.			
n-Hexane	285.04	18.35	6.44
Dichloromethane		12.89	4.52
Methanol		38.11	13.37
C. latifolium Bl.			
n-Hexane	769.84	29.43	3.82
Dichloromethane		5.37	0.70
Methanol		134.95	17.53
C. quadrangulare Kurz			
n-Hexane	840.00	18.02	2.14
Dichloromethane		90.12	10.73
Methanol		123.98	14.76

Table 4.4 Percentage yield (%, w/w) of volatile oils from four *Combretum* species

Species and extracts	Fresh weight (g)	Volatile oil weight (mg)	Yield (%)
C. deciduum Coll. & Hemsl.	7341.10	51.50	0.0007
C. griffithii Heur. & M.A.	6087.80	49.20	0.0008
C. latifolium Bl.	5614.60	73.00	0.0013
C. quadrangulare Kurz	6189.30	30.90	0.0005

4.3 Biological activities of the extracts

4.3.1 Antibacterial activity

Many studies on the biological activities of the *Combretum* species examined in the current research have been reported. Anthelmintic activity [Euswas, 1988], antibacterial activity [Nantachit, 2006], toxic activity [Nakornchai, 1994], hepatoprotective activity [Ferraro, 2000] and a pharmacognostic study in seeds of *C. quadrangulare* Kurz were published [Lengwahasatit, 1986]. Cytotoxic activity against lung cancer cells and antibacterial activity against *Mycobacterium tuberculosis* from the stem of *C. griffithii* Heur. & M.A. were found [Moosophon, 2011]. Banskota *et al*. [2003] reported that leaves, stem bark and seeds of *C. quadrangulare* Kurz are used in Vietnam and Thailand as traditional medicines against hepatitis, fever, dysentery and worm infections. Roots have been used for the treatment of abscesses, gonorrhea and helmintic infections. The literature and ethnobotanical survey were similar in terms of information, and justified our examination of possible antibacterial activity of the four *Combretum* species studied.

Antibacterial activity of the extracts (*n*-hexane, dichloromethane, and methanol) against three pathogenic bacteria : *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) are shown in Table 4.5. Methanolic extracts gave a higher yield than *n*-hexane and dichloromethane extracts. Of the four extracts, the methanolic extract showed the most potent antibacterial activity, compared with three conventional medicines (ampicillin, gentamicin and ceftriaxone). While, methanol and sterile water were used as negative controls. All the methanolic extracts were active against *S. aureus* and *P. aeruginosa*,

which supported the uses of these plants for treating infections, diarrhea and gastrointestinal disorders because *S. aureus* is a common cause of food poisoning. All the *n*-hexane extracts did not show any antibacterial activity and the antibacterial activity of dichloromethane extracts and hot water extracts were mildly active or inactive. The MIC values of all four *Combretum* species, were determined by observing the bacterial growth on agar plates at 24 h after incubation. Dichloromethane extracts of *C. griffithii* Heur. & M.A. and *C. quadrangular* Kurz showed the same MIC value against *S. aureus*, 2.50 mg/mL, and the MIC values of *C. griffithii* Heur. & M.A. against *P. aeruginosa* was 1.25 mg/mL. The MIC values of the methanolic extracts of all four *Combretum* species were 1.25-2.50 mg/mL (*S. aureus*) and 0.16 mg/mL (*P. aeruginosa*). All the four *Combretum* species extracts tested gave mildly active or inactive against *E. coli*.

Table 4.5 Antibacterial activity (MIC, mg/mL) of leaf extracts from four *Combretum* species

Species and samples	*S. aureus*	*E. coli*	*P. aeruginosa*
C. deciduum Coll. & Hemsl.			
n-Hexane	10.00	>10.00	>10.00
Dichloromethane	>10.00	>10.00	10.00
Methanol	2.50	>10.00	0.16
C. griffithii Heur. & M.A.			
n-Hexane	10.00	>10.00	>10.00
Dichloromethane	2.50	>10.00	1.25
Methanol	1.25	>10.00	0.16
C. latifolium Bl.			
n-Hexane	>10.00	>10.00	>10.00
Dichloromethane	10.00	>10.00	10.00
Methanol	1.25	>10.00	0.16
C. quadrangulare Kurz			
n-Hexane	>10.00	>10.00	>10.00
Dichloromethane	2.50	>10.00	10.00
Methanol	1.25	>10.00	0.16
Antibiotics			
Ampicillin	0.001	0.004	>0.032
Gentamicin	0.001	0.001	0.0005
Ceftriaxone	0.008	0.00006	0.032

Then antibacterial activity of the methanolic extracts from *Combretum* leaves was further investigated against an extended pathogenic bacteria (*Staphylococcus aureus* ATCC 25923, *S. aureus* MRSA (methicillin resistant), *S. aureus* MSSA sp 127 (methicillin sensitive, clinical isolate from human sputum), *S. aureus* MSSA 507 (clinical isolate from human), *S. aureus* MSSA bal 14 (clinical isolate from human bronchoalveolar lavage), *S. aureus* MSSA sp 116 (clinical isolate from human sputum), *S. aureus* MSSA sp 152 (clinical isolate from human sputum), *Escherichia coli* ATCC 25922, *E. coli* ESBL (-) (extended-spectrum β-lactamase negative), *E. coli* ESBL (+)

(extended-spectrum *β*-lactamase positive), *E. coli* ESBL (+) u 296 (clinical isolate from human urine), *E. coli* ESBL (-) sp 122 (clinical isolate from human sputum), *E. coli* ESBL (+) u 301 (clinical isolate from human urine), *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* MDS (multidrug-sensitive), *P. aeruginosa* MDR (multidrug-resistant), *P. aeruginosa* MDR u 281 (clinical isolate from human urine), *P. aeruginosa* MDS sp 149 (clinical isolate from human sputum), *Klebsiella pneumoniae* ESBL (-), *K. pneumonia* ESBL (+), *Enterobacter cloacae*, *Serratia marcescens*, *Acinetobacter baumannii* MDS and *A. baumannii* MDR), compared with three conventional medicines (Ampicillin, Gentamicin and Ceftriaxone). The results are shown in Table 4.6. The antibacterial activities and the antibacterial spectra (Table 4.5 and 4.6) were basically the same. The potency of antibacterial activity of methanolic extracts from the leaves of all four *Combretum* species were nearly the same. The antibacterial activity of *C. deciduum* Coll. & Hemsl. seemed to be a lightly weaker, indicating the similar usefulness of these plants for treating infectious diseases. All of the methanolic extracts showed potent antibacterial activity and the unique antibacterial spectrum. They were active against gram-positive (*S. aureus*) and gram-negative (*P. aeruginosa* and *A. baumannii*) bacteria, but were inactive against other gram-negative bacteria (*E. coli*, *K. pneumoniae*, *E. cloacae* and *S. marcescens*). The results support the ethnomedical use of these plants. The MIC values of *C. deciduum* Coll. & Hemsl., *C. griffithii* Heur. & M.A., *C. latifolium* Bl. and *C. quadrangulare* Kurz against *S. aureus*, were 2.50, 0.62-1.25, 0.62-1.25 and 0.62-1.25 mg/mL, respectively. The MIC values against *P. aeruginosa* were 0.16-1.25, 0.16-0.31, 0.16-1.25 and 0.16-0.31 mg/mL, respectively. The MIC values against *A. baumannii* were 0.62-2.50, 0.62, 1.25 and 0.62 mg/mL, respectively. Antibacterial activity of *C. deciduum* Coll. & Hemsl. is reported here for the first time. The antibacterial activity by *C. griffithii* Heur. & M.A. previously reported was against *M. tuberculosis* [Moosophon, 2011], and *C. latifolium* Bl. previously reported was against *S. aureus* and *E. coli* [Shrisha, 2011].

Table 4.6 Antibacterial activity (MIC, mg/mL) of methanolic leaf extracts of four *Combretum* species

Bacteria	CD	CG	CL	CQ	AMPC	GM	CT
S. aureus							
ATCC 25923	2.50	1.25	1.25	1.25	0.001	0.001	0.004
MRSA	2.50	1.25	1.25	1.25	0.001	0.001	>0.26
MSSA sp 127	2.50	1.25	1.25	1.25	0.0005	0.00025	0.13
MSSA 507	2.50	1.25	1.25	1.25	0.0005	0.00025	0.03
MSSA bal 14	2.50	1.25	1.25	1.25	0.0005	0.00025	0.13
MRSA sp 116	2.50	0.62	0.62	0.62	0.001	0.001	>0.26
MRSA sp 152	2.50	0.62	0.62	0.62	0.001	0.001	>0.26
E. coli							
ATCC 25922	>10.00	>10.00	>10.00	>10.00	0.004	0.001	0.00006
ESBL (-)	>10.00	>10.00	>10.00	>10.00	0.004	0.001	0.00003
ESBL (+)	>10.00	>10.00	>10.00	>10.00	0.004	0.001	>0.26
ESBL (+) u 296	>10.00	>10.00	>10.00	>10.00	0.004	0.001	>0.26
ESBL (-) sp 122	>10.00	>10.00	>10.00	>10.00	0.004	0.001	>0.26
ESBL (+) u 301	>10.00	>10.00	>10.00	>10.00	0.004	0.001	>0.26
P. aeruginosa							
ATCC 27853	0.16	0.16	0.16	0.16	>0.03	0.0005	0.03
MDS	1.25	0.31	1.25	0.31	>0.03	0.0005	>0.26
MDR	0.62	0.31	0.62	0.31	>0.03	0.002	>0.26
MDR u 281	0.16	0.16	0.16	0.16	>0.03	0.002	>0.26
MDS sp 149	0.16	0.16	0.16	0.16	>0.03	0.001	>0.26
K. pneumoniae							
ESBL (-)	>10.00	>10.00	>10.00	>10.00	>0.03	0.001	0.00002
ESBL (+)	>10.00	>10.00	>10.00	>10.00	>0.03	0.001	0.00003
E. cloacae	>10.00	>10.00	>10.00	>10.00	>0.03	0.002	>0.26
S. marcescens	>10.00	>10.00	>10.00	>10.00	>0.03	0.002	0.02
A. baumannii							
MDS	0.62	0.62	1.25	0.62	>0.03	0.002	0.13
MDR	2.50	0.62	1.25	0.62	>0.03	>0.03	>0.26

Note : CD = *C. deciduum* Coll. & Hemsl. ; CG = *C. griffithii* Heur. & M.A. ; CL = *C. latifolium* Bl. ; CQ = *C. quadrangulare* Kurz ; AMPC = Ampicillin ; GM = Gentamicin ; CT = Ceftriaxone

Eloff *et al*. [2008] reviewed the biological activity and chemistry of southern African Combretaceae, in which the authors stated that the *Combretum* species with the highest antibacterial activity, included: *C. erythrophyllum* (Burch.) Sond., *C. molle* R. Br. *ex* G. Don, *C. mossambicense* (Klotzsch) Engl., *C. padoides* Engl. & Diels, *C. paniculatum* Hook and *C. petrophilum* Retief. The MIC values of their extracts were between 0.10 and 6.00 mg/mL (average, 2.01 mg/mL). The antibacterial activities of methanolic extracts from the leaves of our four species against *P. aeruginosa* and *S. aureus* were comparable with those of the most potent species of *Combretum* in southern Africa. Eloff *et al*. [2008] also noted that extracts were generally active against four bacteria, eg. *S. aureus, E. coli, P. aeruginosa* and *Enterococcus faecalis*. Gram-positive bacteria were slightly more sensitive than gram-negative bacteria. In contrast, our results did not show any antibacterial activity against *E. coli*. Several studies reported that the extracts of *Combretum* were active against both *S. aureus* and *E. coli* [Eldeen, 2007 ; Elegami, 2007 ; Eloff, 1999]. Other studies have reported that the extracts of *Combretum* were active only against *S. aureus* and did not show any antibacterial activity against *E. coli* [Fyhrquist, 2002 ; Masika, 2002]. So it seems *Combretum* species are uniformly effective against *S. aureus*. Variation of the antibacterial activity based on extraction method has been reported [Angeh, 2007 ; Alexander, 1992 ; Banskota, 2003 ; Coulidiati, 2009 ; Elegami, 2007 ; Eloff, 1999 ; Eloff, 2005a ; Eloff, 2005b ; Fyhrquist, 2002 ; Katerere, 2003 ; Lima, 2012 ; Martini, 2004 ; Masika, 2002 ; McGaw, 2000 ; Nantachit, 2006 ; Udoh, 2012]. Eloff *et al*. [2005a] reported that *n*-hexane and dichloromethane extracts from the leaves of *C. woodii* Dümmer showed strong antibacterial activity against *S. aureus, E. coli* and *P. aeruginosa*, whereas *n*-hexane and dichloromethane extracts showed very little or no activity in our work. Acetone extracts of the leaves of *C. nioroense* Aubrév. *ex* Keay showed antibacterial activity against *S. aureus* and *E. coli*, but ethyl acetate extract did not show antibacterial activity against *E. coli* [Coulidiati, 2009]. Freshly prepared extract (70% aqueous acetone) from the leaves of *C. micranthum* G. Don was active against *S. aureus* and *E. coli*, but after 21 days of storage was active only against *S. aureus* [Alexander, 1992]. Eloff *et al*. [2005b] isolated an antibacterial compound (Combretastatin B5) from the leaves of *C. woodii* Dümmer which showed antibacterial activity only against *S. aureus*, whereas leaf extracts of this plant showed antibacterial

activity against both *S. aureus* and *E. coli*. Antibacterial activity mechanisms exhibited by *Combretum* species may include inactivation of microbial adhesives, enzymes and cell envelopes or transport proteins [Udoh, 2012]. Additional research is needed to explain the range of antibacterial activity exhibited by the different plant parts of *Combretum* species.

The antibacterial activity of the leaf volatile oils of four *Combretum* species were investigated using the agar disc diffusion method against three bacterial strains (*S. aureus, E. coli* and *P. aeruginosa*) was assessed by determination of the inhibition zone, compared with three conventional medicines (ampicillin, gentamicin and ceftriaxone). Methanol and sterile water were used as negative controls. The results are shown in Table 4.7. The oils of four *Combretum* species showed antibacterial activities against gram-positive bacterium (*S. aureus*). The leaf volatile oils of two species (*C. latifolium* Bl. and *C. quadrangulare* Kurz) exhibited antibacterial activity against gram-negative bacterium (*E. coli*) with inhibition zones of 9.33±0.06 and 8.00±0.10 mm, respectively. While, *C. deciduum* Coll. & Hemsl. and *C. griffithii* Heur. & M.A. did not show antibacterial activity against gram-negative bacteria (*E. coli*). And gram-negative bacteria (*P. aeruginosa*) resisted all extracts. This is the first reported that describes the chemical constituents of the leaf volatile oils of these *Combretum* species and their antibacterial activities. The leaf volatile oils of *C. latifolium* Bl. showed potent antibacterial activity against selected strains of bacteria (*S. aureus* and *E. coli*) than *C. quadrangulare* Kurz. These results suggest that the volatile oils of the leaves of *C. latifolium* Bl. and *C. quadrangulare* Kurz could be used for treatment of infectious diseases caused by *S. aureus* and *E. coli*. And further research is needed to get more information on other biological activities of these oils. So these two volatile oils of the leaves of *C. latifolium* Bl. and *C. quadrangulare* Kurz were analysed by GC-MS.

Table 4.7 Antibacterial activity of the leaf volatile oils of four *Combretum* species

Samples	Zone of inhibition in (mm)[a]		
	Pathogen		
	S. aureus	*E. coli*	*P. aeruginosa*
C. deciduum Coll. & Hemsl.	7.83 ± 0.06	7.67 ± 0.06	6.33 ± 0.06
C. griffithii Heur. & M.A.	7.17 ± 0.03	6.83 ± 0.08	6.00 ± 0.00
C. latifolium Bl.	8.50 ± 0.05	9.33 ± 0.06	6.33 ± 0.03
C. quadrangulare Kurz	7.50 ± 0.05	8.00 ± 0.10	6.33 ± 0.03
Ampicillin[b]	56.67 ± 0.15	25.50 ± 0.05	6.50 ± 0.00
Gentamicin[c]	30.50 ± 0.05	37.00 ± 0.10	27.83 ± 0.08
Ceftriaxone[d]	34.50 ± 0.05	35.50 ± 0.05	26.00 ± 0.00

Note : [a]Diameter of inhibition zones (mm) including the diameter of disc (6 mm) ; value are given as mean ± SD of triplicate experiment ; tested volumn = 5 µg/disc ; [b,c,d]Antibiotic used as positive controls.

4.3.2 Antifungal activity

Antifungal activity test of the extract of four *Combretum* species (*C. deciduum* Coll. & Hemsl., *C. griffithii* Heur. & M.A., *C. latifolium* Bl. and *C. quadrangulare* Kurz), the results are shown in Table 4.8. The agar well diffusion method was used to evaluate the antifungal activity by measuring the inhibition zone against the test microorganisms. The antifungal studies were carried out *in vitro* against both gram- positive and gram-negative organisms. Ketoconazole was used as positive controls. This is the first report of the antifungal activity from these four plants. The results indicated that the all crude four *Combretum* species extracts possessed antifungal activity against all used fungi strains (*Aspergillus flavus, Candida albicans* and *Trichophyton mentagrophytes*). All crude extracts showed antifungal activity against *A. flavus*, but did not inhibit the growth of *T. mentagrophytes*, with their respective diameter of inhibition zones. While, all three extracts of *C. deciduum* Coll. & Hemsl. and only methanolic extract of *C. quadrangulare* Kurz showed antifungal activity against *C. albicans*. The antifungal activities of these plants seem to be mildly active.

Table 4.8 Antifungal activity of four *Combretum* species leaf extracts

Samples	Zone of inhibition in (mm)[a]		
	Pathogen		
	A. flavus	*C. albicans*	*T. mentagrophytes*
C. deciduum Coll. & Hemsl.			
n-Hexane	12.00 ± 0.00	15.00 ± 0.00	Inactive
Dichloromethane	11.00 ± 0.00	10.50 ± 0.71	Inactive
Methanol	12.67 ± 0.58	12.00 ± 0.00	Inactive
C. griffithii Heur. & M.A.			
n-Hexane	11.00 ± 0.00	Inactive	Inactive
Dichloromethane	11.67 ± 0.58	Inactive	Inactive
Methanol	12.33 ± 0.58	Inactive	Inactive
C. latifolium Bl.			
n-Hexane	11.33 ± 0.58	Inactive	Inactive
Dichloromethane	11.33 ± 0.58	Inactive	Inactive
Methanol	11.67 ± 0.58	Inactive	Inactive
C. quadrangulare Kurz			
n-Hexane	11.67 ± 0.58	Inactive	Inactive
Dichloromethane	11.67 ± 0.58	Inactive	Inactive
Methanol	12.67 ± 0.58	12.00 ± 0.00	Inactive
Antibiotic			
Ketoconazole[b]	25.00 ± 0.00	37.00 ± 0.50	16.00 ± 0.00

Note : [a]Diameter of inhibition zones (mm) including the diameter of well (9 mm) ; tested volumn = 5 mg/well ; [b]antibiotic used as positive with concentration 0.25 mg/mL

4.3.3Anti-tuberculosis activity

This is the first report of the anti-tuberculosis activity from the leaves of *C. deciduum* Coll. & Hemsl., *C. griffithii* Heur. & M.A., *C. latifolium* Bl. and *C. quadrangulare* Kurz. The anti-tuberculosis activity of the methanolic extracts were selected to evaluate against *M. tuberculosis* H37Ra strain showed no activity. Rifampicin, streptomycin, isoniazid, ofloxacin and ethambutol were used as positive

controls, and 0.5% DMSO was used as negative control. The all extracts and MIC of positive controls were shown in Table 4.9.

Table 4.9 Anti-tuberculosis activity of methanolic leaf extracts of four *Combretum* species

Species and standards	IC_{50} (µg/mL)
C. deciduum Coll. & Hemsl.	Inactive
C. griffithii Heur. & M.A.	Inactive
C. latifolium Bl.	Inactive
C. quadrangulare Kurz	Inactive
Rifampicin	0.02
Streptomycin	0.62
Isoniazid	0.09
Ofloxacin	0.39
Ethambutol	0.47

4.3.4 Antioxidant activity

The antioxidant activities of the extracts were determined by using ABTS free radical-scavenging method and DPPH radicals scavenging method.

1) ABTS method

Trolox and vitamin C were used as standard controls. The series of standard solutions containing: 0.50-5.50 mg/mL of Trolox and 1.00-11.00 mg/mL of vitamin C solutions were prepared, repectively. The absorbance of each solution was measured at 734 nm. The percentage inhibitions were plotted against standard solution of Trolox and vitamin C concentrations to give linear equation (y = 18.49x - 3.827 and y = 36.54x - 3.827, respectively). This calibration curve was used for the ABTS screening method (Figure 4.1 and Figure 4.2). The percentage inhibition of each standard solution was calculation as shown in Table 4.10 and 4.11.

Table 4.10 The percentage inhibition of Trolox concentration series for ABTS method

Concentration of Trolox (mg/mL)	% Inhibition
1.00	4.86
2.00	16.08
3.00	29.64
4.00	45.24
5.00	60.85
6.00	84.77
7.00	102.48
8.00	119.03
9.00	143.73
10.00	168.85
11.00	190.42

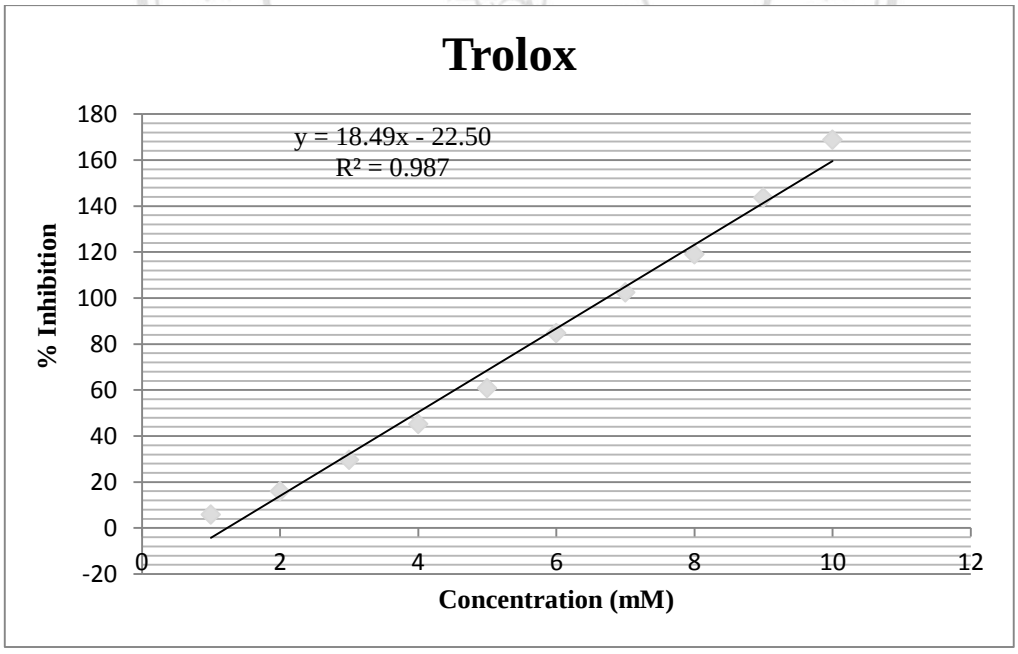

Figure 4.1 Concentration-response curve for the absorbance at 734 nm of ABTS method as a function of the concentration of standard Trolox

Table 4.11 The percentage inhibition of vitamin C concentration series for ABTS method

Concentration of vitamin C (mg/mL)	% Inhibition
0.50	15.92
1.00	36.68
1.50	50.12
2.00	61.74
2.50	87.75
3.00	109.61
3.50	126.45
4.00	143.22
4.50	158.99
5.00	175.76
5.50	200.45

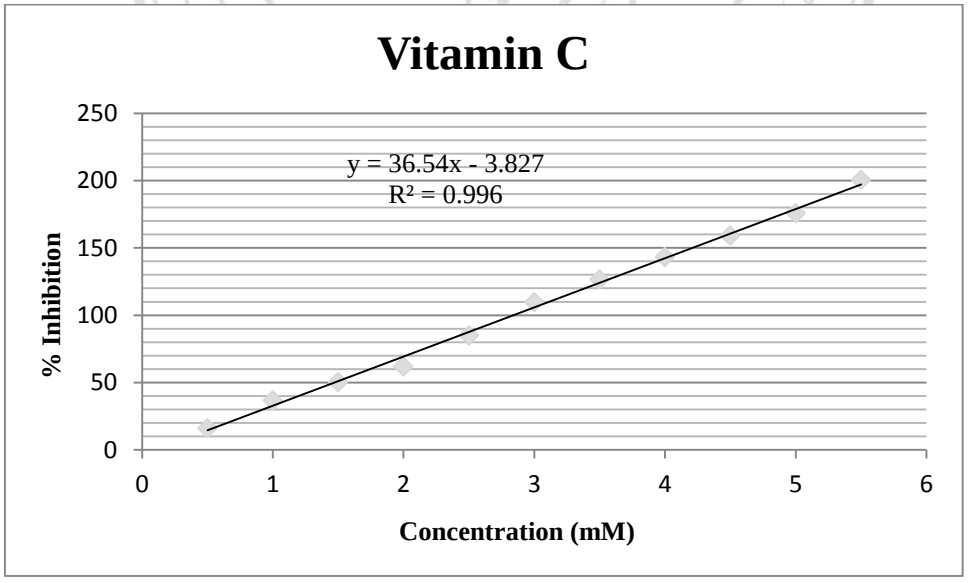

Figure 4.2 Concentration-response curve for the absorbance at 734 nm of ABTS method as a function of the concentration of standard vitamin C

For the ABTS method, the highest antioxidant activity was obtained with methanolic extracts (5.37-10.74 mM Trolox/mg of extracts and 2.21-4.92 mM vitamin C/mg of extracts) followed by dichloromethane extracts (3.65-5.36 mM

Trolox/mg of extracts and 1.33-2.20 mM vitamin C/mg of extracts) and *n*-hexane extracts (2.86-4.91 mM Trolox/mg of extracts and 0.94-1.97 mM vitamin C/mg of extracts). The results were summarized in Table 4.12.

Table 4.12 Antioxidant capacities of the leaf extracts of four *Combretum* species

Extracts	ABTS % inhibition/mg of extracts	ABTS mM Trolox/mg of extracts	ABTS mM vitaminC/mg of extracts
C. deciduum Coll. & Hemsl.			
n-Hexane	30.37± 0.0015	2.86	0.94
Dichloromethane	44.97± 0.0076	3.65	1.33
Methanol	78.41± 0.0006	5.46	2.25
C. griffithii Heur. & M.A.			
n-Hexane	57.70± 0.0029	4.34	1.68
Dichloromethane	54.35± 0.0015	4.16	1.59
Methanol	76.86± 0.0012	5.37	2.21
C. latifolium Bl.			
n-Hexane	62.61± 0.0015	4.60	1.82
Dichloromethane	53.11± 0.0108	4.09	1.56
Methanol	176.06± 0.0000	10.74	4.92
C. quadrangulare Kurz			
n-Hexane	68.20± 0.0021	4.91	1.97
Dichloromethane	76.62± 0.0040	5.36	2.20
Methanol	154.59± 0.0006	9.58	4.33

2) DPPH method

A series of standard solutions containing: 0.03-0.07 mg/mL of Trolox, were prepared. The absorbance of each solution was measured at 540 nm and the percentage inhibition of each concentration was calculated. Results are presented in Table 4.13. The standard curve of Trolox was constructed by plotting the percentage inhibitions and the concentration of standard solution to give linear equation (y = 761.80x + 0.36) as shown in Figure 4.3. This calibration curve was used for DPPH

screening method. Trolox, vitamin C and quercetin were used as positive controls. The IC_{50} values denoted the concentration of the standard which were required to scavenge 50% of DPPH free radicals.

Table 4.13 The percentage inhibition of each concentration of the Trolox standard solution for DPPH method

Concentration of Trolox (mg/mL)	% Inhibition
0.03	23.88
0.04	29.41
0.05	38.63
0.06	47.39
0.07	52.97
0.08	61.32

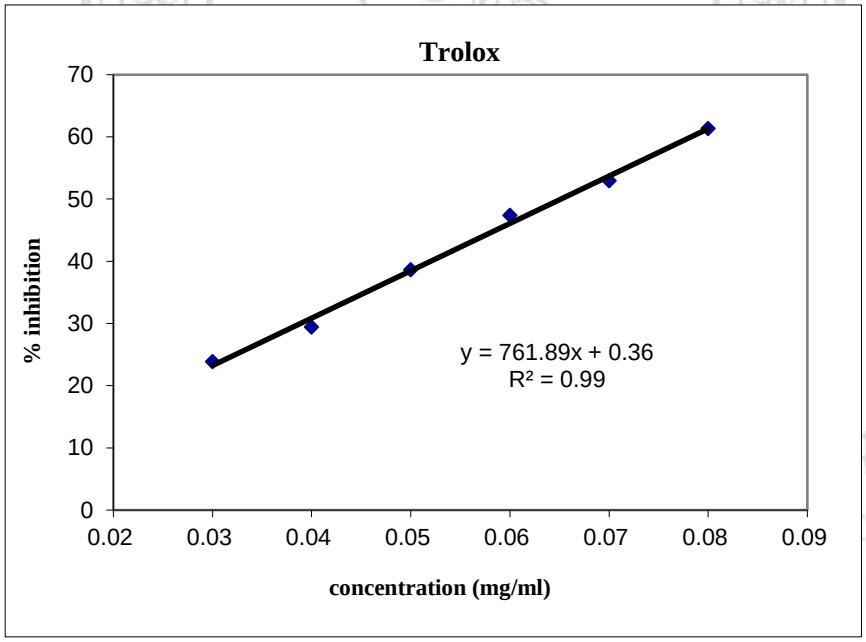

Figure 4.3 Calibration curve for the absorbance at 520 nm of DPPH method as a function of the concentration of Trolox standard solution

For the DPPH method, the IC_{50} values of the *n*-hexane extracts were 0.52-6.53 µg/mL, the dichloromethane extracts were 1.52-5.93 µg/mL and the methanolic extracts were 0.13-0.81 µg/mL. The three standards (Trolox, vitamin C and

quercetin) showed antioxidant activity with the DPPH method with IC_{50} values of 0.06, 0.07 and 0.05 µg/mL, respectively. The results were summarized in Table 4.14.

Table 4.14 DPPH radical scavenging activity of leaf extracts of four *Combretum* species

Extracts and standard	IC_{50} (µg/mL)
C. deciduum Coll. & Hemsl.	
n-Hexane	1.76± 0.02
Dichloromethane	1.52± 0.03
Methanol	0.81± 0.02
C. griffithii Heur. & M.A.	
n-Hexane	0.52± 0.02
Dichloromethane	5.93± 0.03
Methanol	0.23± 0.01
C. latifolium Bl.	
n-Hexane	2.79± 0.02
Dichloromethane	3.57± 0.02
Methanol	0.13± 0.02
C. quadrangulare Kurz	
n-Hexane	6.53± 0.02
Dichloromethane	5.16± 0.02
Methanol	0.23± 0.02
Standards	
Trolox	0.06± 0.02
Vitamin C	0.07± 0.02
Quercetin	0.05± 0.03

In these antioxidant activities studied, methanol extracts had the most potent antioxidant activity followed by *n*-hexane extracts and dichloromethane extracts. The antioxidant activity of these four species is newly reported here. Methanolic extracts of the four species displayed the most potent antioxidant activity against both $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals. Methanolic extracts of *C. latifolium* leaves showed the highest

antioxidant activity, suggesting that this extract is a rich source of antioxidants. The antioxidant activity of *C. quadrangulare*, *C. griffithii* and *C. deciduum* followed. To study antioxidant activity, it is recommended to use at least two methods, as we did here. Our results showed that extracts with different polar compounds exhibit different antioxidant activities.

Lima *et al*. [2012] reviewed the bioactivities of the genus *Combretum*, in which the authors stated that ethanolic leaf extract of *C. decandrum* showed antioxidant activity with IC_{50} value of 0.75 g/kg by ferrous ion oxidation-xylenol orange method in rats. And ethanolic leaf extracts of *C. duarteanum* possess a strong antioxidant potential by using thiobarbituric acid reactive species (TBARS), hydroxyl radical–scavenging and scavenging activity of nitric oxide assays.

Our findings related to the previous research in antioxidant properties of the extracts from the genus *Combretum*. These results indicated that the properties of *Combretum* species might be further explored in the search for new antioxidant compounds.

4.3.5 Anticancer activity

The anticancer activity of the selected active extracts (methanolic extracts) of these four *Combretum* species leaves were performed using the resazurin microplate assay (REMA). The methanolic extracts of four *Combretum* species were subsequently investigated for anticancer activity. The methanolic extracts of all four *Combretum* species exhibited significant anticancer activity against KB (epidermoid carcinoma of the oral cavity, ATCC CCL-17), MCF7 (breast adenocarcinoma, ATCC HTB-22) and NCI-H187 (small cell lung carcinoma, ATCC CRL-5804) cell lines with IC_{50} (Table 4.15). The methanolic extract of *C. deciduum* Coll. & Hemsl. inhibited KB-oral cavity and MCF7-breast cancer cell lines with an IC_{50} value of 34.34 µg/mL and 28.84 µg/mL, respectively. The methanolic extract of *C. latifolium* Bl. inhibited MCF7-breast cancer cell lines with an IC_{50} value of 26.63 µg/mL. Methanolic extract of *C. quadrangulare* Kurz inhibited KB-oral cavity and NCI-H187-small cell lung cancer cell lines with IC_{50} values of 26.76 µg/mL and 46.88 µg/mL, respectively. However, methanolic extract of *C. griffithii* Heur. & M.A. was inactive against all three cell lines. Ellipticine, Doxorubicin and Tamoxifen were used as standard compounds. The

cytotoxicity of the selected active extracts was performed using Green Fluorescent Protein (GFP) detection methodology. All methanolic extracts were non-cytotoxic against primate cell lines (Vero cells).

Table 4.15 Anticancer activity of methanolic leaf extracts of four *Combretum* species

Species and standards	IC_{50} (µg/mL)			
	Human cancer cell lines			Vero cells
	KB	MCF7	NCI-H187	
C. deciduum Coll. & Hemsl.	34.34	28.84	Inactive	Non-cytotoxic
C. griffithii Heur. & M.A.	Inactive	Inactive	Inactive	Non-cytotoxic
C. latifolium Bl.	Inactive	26.63	Inactive	Non-cytotoxic
C. quadrangulare Kurz	26.76	Inactive	46.88	Non-cytotoxic
Ellipticine	1.19	NT	1.11	NT
Doxorubicin	0.38	9.04	0.07	NT
Tamoxifen	NT	9.61	NT	NT

Note : Ellipticine, Doxorubicin and Tamoxifen were used as positive controls ; 0.5% DMSO was used as negative control ; IC_{50} = concentration that killed 50% of cell lines ; IC_{50} > 50 µg/mL = inactive ; NT = not tested.

The leaf extracts of four *Combretum* species showed anticancer activities, which suggests the presence of anticancer compounds in the leaves. Anticancer activity on KB, MCF-7 and NCI-H187 cell lines of *C. deciduum* and *C. latifolium* are initially reported here. As traditional medicinal plants associated with anticancer uses might be potential sources of potent natural antioxidant, we also investigated these extracts for anticancer activity. The methanolic extracts from the leaves of *C. deciduum*, *C. latifolium* and *C. quadrangulare* exhibited anticancer activity against cancer cell lines (KB, MCF7 NCI-H187). However, the methanolic extract from the leaves was inactive against all three cell lines. All methanolic leaf extracts were non-cytotoxic against Vero cell lines. It is generally accepted that free radicals react with biological molecules, leading to the possible development of cancer. Considerable laboratory evidence from chemical, cell culture and animal studies indicates that antioxidants may slow or possibly prevent cancer by stabilizing biological molecules and preventing damage to cells. The study confirmed that the methanolic extracts are the most potent in terms of

their antioxidant, suggesting that the polar compounds residing in these extracts may be responsible. However, the anticancer activity of the plants could not be associated with these polar extracts, which have shown strong radical scavenging activity. These results suggest that free radical scavenging activity may not be the only mechanism preventing development of cancer.

Lima et al. [2012] stated that ethanolic extracts of the leaves, root and stem of *C. duarteanum* showed anticancer activity against KB cells. Methanolic and ethanolic extracts of dried airial parts of *C. collinum* exhibited anticancer activity against squamous carcinoma KB with IC_{50} value of 20.00 μg/ml and methanolic extracts of leaves and root exhibited anticancer activity against MCF7 breast cancer with IC_{50} value of 25.00 μg/ml. Methanolic extracts of leaves and root of *C. apiculatum* subsp *apiculatum*, *C. fragrans*, *C. micranthum*; methanolic extracts of stem bark and root of *C. padoides*; methanolic extracts of stem bark of *C. hereroense* and *C. psidioides*; and methanolic extracts of root and fruits of *C. zeyheri* inhibited MCF7 breast cancer with IC_{50} value of 25.00 μg/ml.

Our study related to the previous report. These results indicate that properties of *Combretum* species might be further explored in the search for new anticancer compounds. This work could be extended by testing other parts of the four species studied here, or expanding to additional species.

4.4 Chemical constituents of the volatile compounds

The qualitative and quantitative analysis of the volatile compounds of *C. latifolium* Bl. and *C. quadrangulare* Kurz were performed by GC-MS (Figure 4.4 and 4.5 ; Table 4.16 and 4.17, respectively). GC-MS analysis successfully detected nine and fifteen components, with 93.50% and 99.99%, respectively of the chromatographic components. The components of the volatile compounds were identified by their retention indices (RI) relative to *n*-alkanes indices on a HP-5 column and by a comparison of mass spectra from Wiely7n.1 and NIST libraries, as well as by comparison of the fragmentation patterns of the mass spectra with the data reported in the literature [Mancini, 2009 ; Radulovic, 2011 ; Zito, 2010]. The components identified

from the volatile compounds with their retention time (RT), percentage composition (%), and retention indices (RI) were summarized in Table 4.16 and Table 4.17.

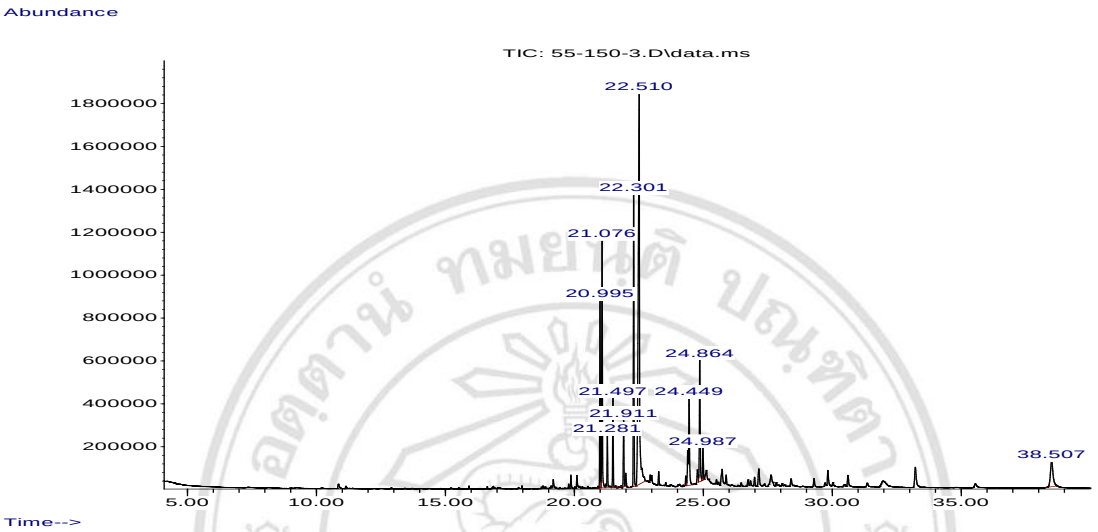

Figure 4.4 GC chromatogram of leaf volatile compounds of *Combretum latifolium* Bl.

Table 4.16 Chemical constituents of leaf volatile compounds of *Combretum latifolium* Bl.

Plants	Peak No.	Compounds	RA[a]	RT[b]	RI[c]	RI[d]	References
C. latifolium Bl.	1	Neophytadiene	7.71	20.99	1812	1841	[Radulovic, 2011]
	2	Hexahydrofarnesyl acetone	11.54	21.08	1819	1845	[Zito, 2010]
	3	Unidentified	2.94	21.91	1889		
	4	Isophytol	13.47	22.30	1921	1951	[Radulovic, 2011]
	5	Palmitic acid	37.05	22.51	1936	1957	[Mancini, 2009]
	6	Phytol isomer	7.17	24.45	1947	2122	[Zito, 2010]
	7	Unidentified	7.12	24.86	1964		
	8	Unidentified	1.82	24.99	1969		
	9	*n*-Nonacosane	4.68	38.51	2850	2900	[Zito, 2010]
Group components							
Oxygenated diterpene			28.35				
Ketones			11.54				
Carboxylic acids			37.05				
Hydrocarbon			4.68				
Unidentified (3 in total, area > 0.5%)			11.88				
Total			**93.50**				

Note : [a]Relative area in % (peak area relative to total peak area) ; [b]Retention Time (min) ; [c]Retention Indices determined in this study ; [d]Retention Indices of Kovat index or references.

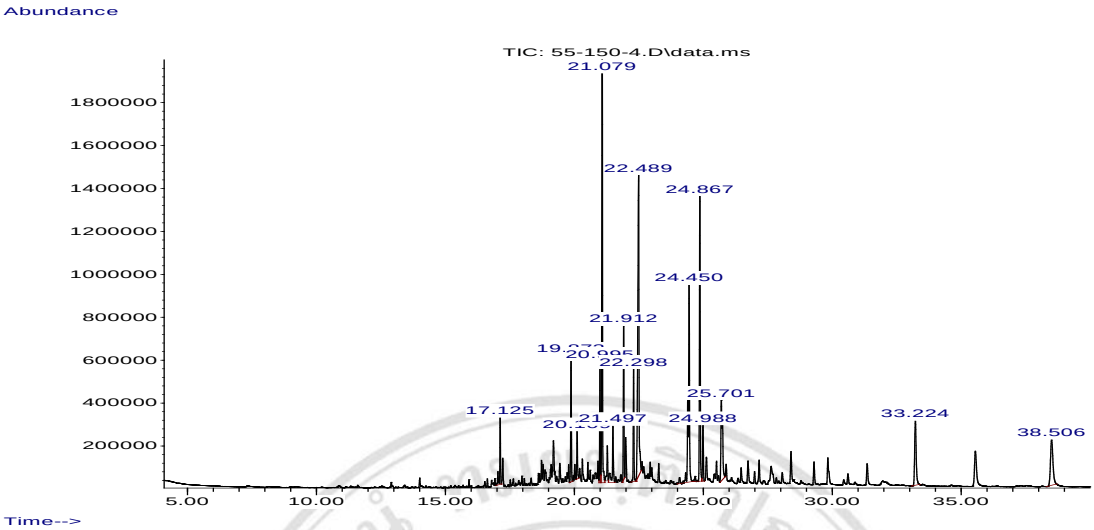

Figure 4.5 GC chromatogram of leaf volatile compounds of *Combretum quadrangulare* Kurz

Table 4.17 Chemical constituents of leaf volatile compounds of *Combretum guadrangulare* Kurz

Plants	Peak No.	Compounds	RA[a]	RT[b]	RI[c]	RI[d]	References
C. quadrangulare Kurz	1	*β*-Selinene	2.46	17.13	1479	1475	[Mancini, 2009]
	2	Unidentified	4.16	19.87	1712		
	3	Tetradecanoic acid	1.54	20.11	1733	1766	[Zito, 2010]
	4	Neophytadiene	3.52	20.99	1812	1841	[Radulovic, 2011]
	5	Hexahydrofarnesyl acetone	17.36	21.08	1819	1849	[Zito, 2010]
	6	Unidentified	2.88	21.50	1854		
	7	Unidentified	5.12	21.91	1889		
	8	Isophytol	3.71	22.30	1920	1951	[Radulovic, 2011]
	9	Palmitic acid	17.74	22.49	1935	1957	[Mancini, 2009]
	10	Phytol	11.52	24.45	2091	2122	[Mancini, 2009]
	11	Unidentified	11.40	24.87	2064		
	12	Unidentified	2.15	24.99	2069		
	13	Unidentified	6.31	25.70	2098		
	14	*n*-Heptacosane	4.75	33.22	2411	2500	[Zito, 2010]
	15	*n*-Nonacosane	5.37	38.51	2850	2900	[Zito, 2010]
Group components							
Oxygenated sesquiterpene			2.46				
Oxygenated diterpene			18.75				
Ketones			17.36				
Carboxylic acids			19.28				
Hydrocarbon			10.12				
Unidentified (3 in total, area > 0.5%)			32.02				
Total			**99.99**				

Note : [a]Relative area in % (peak area relative to total peak area) ; [b]Retention Time (min) ; [c]Retention Indices determined in this study ; [d]Retention Indices of Kovat index or references.

The major components of *C. latifolium* Bl. and *C. quadrangulare* Kurz in volatile portions were Palmitic acid (37.05% and 17.74%), Hexahydrofarnesyl acetone (11.54% and 17.36%), Isophytol (13.47% and 3.71%), Neophytadiene (7.71% and 3.52%) and *n*-Nonacosane (4.68% and 5.37%), respectively. Other major compounds for *C. latifolium* Bl. oil were Phytol isomer (7.17%), and other compounds for *C. quadrangulare* Kurz oil were *β*-Selinene (2.46%), Tetradecanoic acid (1.54%), Phytol (11.52%) and *n*-Heptacosane (4.75%).

Most of the components of the volatile compounds were carboxylic acids (Palmitic acid and Tetradecanoic acid), oxygenated diterpene (Phytol and Isophytol) and ketones (Hexahydrofarnesyl acetone).

Palmitic acid seems to be a major fatty acid. Similar results were reported in *Arthrocnemum indicum* (Willd.) Moq., *Suaeda maritima* (L.) Dumort. and *Suaeda monoica* Forssk. The mesocarp and seed of *Hippophae rhamnoides* L. and *Myrtus communis* L. have been reported to be rich in Palmitic acid. In the previous report higher amount of Palmitic acid, was recorded in some mangroves from the Pichavaram mangrove forest [Chandrasekaran, 2011]. Palmitic acid isolated from *Schotia brachypetala* Sond., *Pelargonium* spp. And *Pentanisia prunelloides* (Klotzsch ex Eckl. & Zeyh.) Walp., were found antibacterial activity [Agoramoorthy, 2007].

Hexahydrofarnesyl acetone, present as one of the major volaile compounds, has also been suggested as a possible antimicrobial principle of volatile oils [Radulovic, 2011].

Isophytol is a Phytol isomer and isomerisation of Isophytol to Phytol in plant leaf waxes [Jerkovic, 2012].

Neophytadiene is presumably a chlorophyll metabolite. Chlorophyll chlorine rings can be constructed from several different side chains usually including the long diterpene alcohol phytol [Jerkovic, 2012]. Neophytadiene was identified as strong bactericidal compounds, an antifungal terpenoid identified in the red alga, *Centroceras clavulatum* (*C. agardh*) Montagn was also reported in several plants which were used as antipyretic, analgesic and vermifugic, including atopical application for sores and inflammation. In addition, this compound is showing significant antioxidant activity

($P<0.01$) in different plants. Even though several reports are available on antioxidant activity of *Eupatorium odoratum* L. major compounds responsible for antioxidant and its protective role through their anti-inflammatory response have not been reported [Venkata, 2012].

Tetradecanoic acid which is present in the leaf volatile oil has been reported to possess antimicrobial compound [Zito, 2010].

Phytol and Isophytol are acyclic terpenoids. Phytol is a diterpene that an important component of all plants used in cosmetics, shampoos, toilet soaps, household cleaners as it shows antimicrobial, anti-inflammatory, anticancer, antidiuretic activity. Interestingly, Phytol showed high antimicrobial against the food borne pathogens. Phytol is also important in the processing of glucose and can activate enzymes within the body that have strong positive effects on insulin level. This means that Phytol in the human diet could possibly help restore the metabolic functions of those with type-2 diabetes. And Phytol shows adjuvant and immune stimulatory activity [Venkata, 2012].

In this study, we screened for antibacterial activity for the volatile compounds of *C. latifolium* Bl. and *C. quadrangulare* Kurz. They showed potential activity against the microorganisms tested, the volatile compounds of these plants can be used as potential antibacterial agents.

4.5 Structure determination of isolated secondary metabolites

The methanolic extract of four *Combretum* species leaves showed antibacterial activities against gram-positive and gram-negative bacteria with MIC values of 1.25-2.50 mg/mL (*S. aureus*) and 0.16 mg/mL (*P. aeruginosa*), and showed anticancer activity against KB cancer cell line with IC_{50} of 34.34, 26.76 µg/mL (*C. deciduum* Coll. & Hemsl. and *C. quadrangulare* Kurz), MCF7 cancer cell line with IC_{50} of 28.84 and 26.63 µg/mL (*C. deciduum* Coll. & Hemsl. and *C. latifolium* Bl.), and NCI-H187 cancer cell lines with IC_{50} of 46.88 µg/mL (*C. quadrangulare* Kurz). All methanolic extracts of four *Combretum* species were non-cytotoxic against Vero cells. And methanolic extracts also showed potent antioxidant activities. For the ABTS method, the highest antioxidant activity was obtained with methanolic extracts of *C. latifolium* Bl. and *C. quadrangulare* Kurz (22.61, 19.85 mM Trolox/mg of extracts and 4.96, 4.35 mM

vitamin C/mg of extracts, respectively). For the DPPH method, the IC_{50} values of the methanolic extracts of *C. griffithii* Heur. & M.A., *C. latifolium* Bl. and *C. quadrangulare* Kurz were higher than the other extracts with 0.23, 0.13 and 0.13 µg/mL, respectively.

Therefore, from the above information, these three *Combretum* species extracts (*C. latifolium* Bl., *C. griffithi* Heur. & M.A., and *C. quadrangulare* Kurz) showed antibacterial activities, anticancer activities and antioxidant activities. These three species were selected for further study.

4.5.1 Structure determination of isolated secondary metabolites from the leaves of *C. griffithii* Heur. & M.A.

The obtained methanolic leaves extract of *C. griffithii* Heur. & M.A. (9.49 g), was then separated using column chromatography techniques to afford one pure compound. The structure determinations of all isolates were performed by interpretation of their IR, MS and NMR data, and then confirmed by comparison with literature values.

1) Structure determination of compound CG-3.2

Compound CG-3.2 (0.0685 g) was obtained as color needles crystals. The R_f value is 0.25 [silica gel/*n*-hexane : ethyl acetate (95:5)]. It was identified as 3*β*-Taraxerol.

This compound was previously isolated from leaves of *Alchornea latifolia* Sw. [Setzer, 2000], aerial parts of *Strobilanthes callosus* Nees. [Singh, 2002], fruits of *Rhizophora mucronata* Lam. [Laphookhieo, 2004], bark of *Bridelia micrantha* Baill. [Kouam, 2005], stem and twigs of *Rhizophora stylosa* Griff. [Li, 2008], leaves of *Combretum vendae* A.E. van Wyk [Eloff, 2008], bark of *Cupania dentata* [Hernandez-Chavez, 2012], leaves of *Mangifera indica* L. [Sangeetha, 2010 ; 2013], stem bark and wood of *Tridesmostemon omphalocarpoides* Engl. [Fru, 2013], leaves of *Heritiera littoralis* Dryand [Christopher, 2014].

The IR spectrum of CG-3.2 (Figure 6.1) indicated absorption bands at 3485.12 cm^{-1} (OH), 2918.83 cm^{-1} (CH_2), 2850.70 cm^{-1} (CH), 1472 cm^{-1} (C=C), and 1037.61 cm^{-1} (C-O).

The EIMS (Figure 6.2) afforded a molecular ion peak [M^+] at *m/z* 426, consistent with the molecular formula $C_{30}H_{50}O$ (D.B.E.=6), while other major fragments appeared at *m/z* 287, 148, 135, 133 and 69.

The ^{1}H- NMR spectrum of compound CG-3.2 (Figure 6.3 and Table 4.18) and *J*-resolved measurements helped in assigning each proton and their relative stereochemistries. Its spectrum, several signals for methyl groups, showed signals for oxymethine (δ 3.21 ppm, dd, *J*= 11.0, 4.7 Hz, H-3) and olefinic hydrogens (δ 5.55 ppm, dd, *J*= 8.2, 3.2 Hz, H-15) [de Oliveira, 2012]. H-3 proton appeared as typical of the axial proton associated with a 3*β*-hydroxy group [Corbett, 1972]. It showed to be *β*-from, and was determined by infinite zigzag O-H••O hydrogen bonded chains [Kouam, 2005]. Rest of proton appeared in the high field region in between 0.70-2.00 ppm.

The ^{13}C- NMR spectrum of compound CG-3.2 (Figure 6.4 and Table 4.19) showed, several signals for C-sp^3, signals for olefinic carbons, complatible with the presence of triterpenes type lup-20(29)-ene (δ 116.9 and 158.0 ppm). Also was observed signals at δ 79.1 ppm, characteristics of oxymethine carbon and carbonyl groups at C-3 [de Oliveira, 2012].

From these finding and by comparison with previous published spectral data [Hernandez-Chavez, 2012], led us to assign this structure to be 3*β*-Taraxerol. The chemical structure was shown in Figure 4.6. This compound was first separated from the leaves of *C. griffithii* Heur. & M.A.

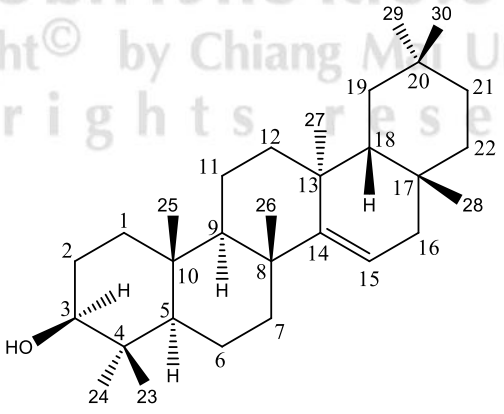

Figure 4.6 Structure of 3*β*-Taraxerol

Table 4.18 ^{1}H NMR Assignments of Compound CG-3.2 (in $CDCl_3$) and 3β-Taraxerol (in $CDCl_3$)

Position	Compound CG-3.2 δ_H (ppm) (multiplicity, *J* in Hz)	3β-Taraxerol [Hernandez-Chavez, 2012] δ_H (ppm) (multiplicity, *J* in Hz)
3	3.21 (1H, dd)	3.24 (1H, dd)
7a	2.04 (1H, dt)	2.03 (1H, dt)
15	5.55 (1H, dd)	5.53 (1H, dd)
16a	1.93 (1H, dd)	1.92 (1H, dd)
23	1.01 (3H, s, CH_3)	0.98 (3H, s, CH_3)
24	0.80 (3H, s, CH_3)	0.80 (3H, s, CH_3)
25	0.93 (3H, s, CH_3)	0.93 (3H, s, CH_3)
26	1.05 (3H, s, CH_3)	1.09 (3H, s, CH_3)
27	0.91 (3H, s, CH_3)	0.91 (3H, s, CH_3)
28	0.82 (3H, s, CH_3)	0.82 (3H, s, CH_3)
29	0.95 (3H, s, CH_3)	0.95 (3H, s, CH_3)
30	0.90 (3H, s, CH_3)	0.90 (3H, s, CH_3)

Table 4.19 ^{13}C NMR Assignments of Compound CG-3.2 (in $CDCl_3$) and 3β-Taraxerol (in $CDCl_3$)

Position	Compound CG-3.2 δ_C (ppm)	3β-Taraxerol [Hernandez-Chavez, 2012] δ_C (ppm)
1	37.7	38.0
2	27.1	27.1
3	79.1	79.1
4	38.9	39.0
5	55.5	55.5
6	18.8	18.8
7	33.7	35.1
8	38.7	38.7
9	48.7	48.7
10	36.6	37.5
11	17.5	17.5
12	35.1	35.8
13	37.5	37.6
14	158.0	158.1
15	116.9	116.9
16	35.8	36.6
17	38.0	37.7
18	49.2	49.2
19	41.3	41.3
20	28.8	28.8
21	33.3	33.7
22	31.9	33.1
23	28.0	28.0
24	15.4	15.4
25	15.4	15.4
26	29.8	29.8

Table 4.19 (continued)

Position	Compound CG-3.2 δ_C (ppm)	3β-Taraxerol [Hernandez-Chavez, 2012] δ_C (ppm)
27	25.9	25.9
28	29.9	29.9
29	33.1	33.3
30	21.3	21.3

4.5.2 Structure determination of isolated secondary metabolites from the leaves of *C. latifolium* Bl.

The obtained methanolic leaves extract of *C. latifolium* Bl. (1 g), was then separated using preparative TLC techniques to afford two compounds. The structure determinations of all isolates were performed by interpretation of their MS and NMR data, and then confirmed by comparison with literature values.

1) Structure determination of compound CLP-2

Compound CLP-2 (0.0362 g) was obtained as white powder. The R_f value is 0.70 [silica gel/chloroform : methanol (9:1)]. It was identified as lipid (long chain fatty acid), based on their ^{1}H NMR data (Figure 6.5). Diagnostic signals representative of lipid appeared at δ 1.26 ppm (brd) for the methylene envelope δ 2.35 ppm (t, J = 7.5 Hz), and δ 4.29 ppm (m). This compound was continued to identify by using GC-MS. The qualitative and quantitative analysis of CLP-2 was listed in order of elution from a HP-5 capillary column (Table 4.20). The components of CLP-2 were identified by a comparison with mass spectra from libraries (Wiley7n.1 and NIST) and the data reported in the literature [Zito, 2010]. A typical GC chromatogram is presented in Figure 4.7.

In total, 5 compounds were identified, corresponding to 60.71% of the mixture of hydrocarbons, including *n*-Octacotane (28.48%), Docosane (14.53%) and Eicosane (8.30%) as shown in Table 4.20.

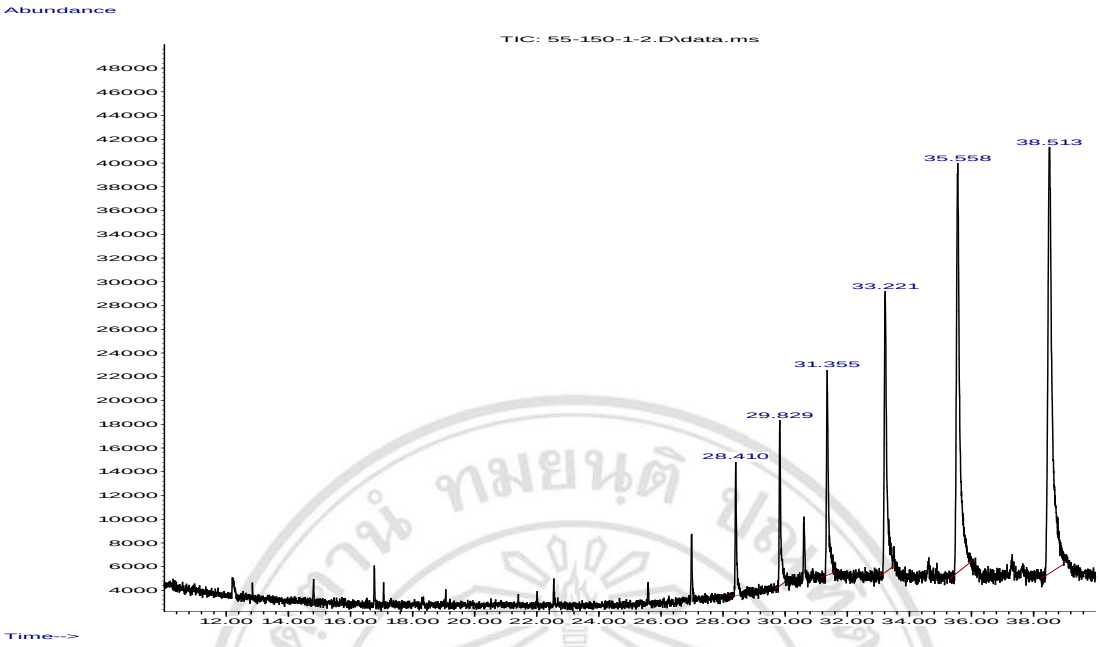

Figure 4.7 GC chromatogram of CLP-2

Table 4.20 Chemical compositions of CLP-2 compound

Peak no.	Compounds	RA[a]	RT[b]	RI[c]	RI[d]	References
1	Unknown	4.02	28.41	2224	-	-
2	Unknown	5.38	29.83	2257	-	-
3	Eicosane	8.30	31.36	2292	2014	[Zito, 2010]
4	Docosane	14.53	33.22	2410	2200	[Zito, 2010]
5	*n*-Octacotane	28.48	35.56	2595	2800	[Zito, 2010]
Group components						
Hydrocarbons		51.31				
Unidentified (3 in total, area > 0.5%)		9.40				
Total		**60.71**				

Note : [a]Relative area in % (peak area relative to total peak area) ; [b]Retention Time (min) ; [c]Retention Indices determined in this study ; [d]Retention Indices of Kovat index or references.

2) Structure determination of compound CLP-4

Compound CLP-4 (0.0361 g) was obtained as white powder. The R_f value is 0.67 [silica gel/chloroform : methanol (9:1)]. It was identified as lipid (long

chain fatty acid), based on their 1H NMR data (Figure 6.6). Diagnostic signals representative of lipid appeared at δ 1.28 ppm (brd) for the methylene envelope δ 2.35 ppm (t, J = 7.5 Hz), and δ 4.05 ppm (m). This compound was continued to identify by using GC-MS. The qualitative and quantitative analysis of CLP-4 was listed in order of elution from a HP-5 capillary column (Table 4.21). The components of CLP-4 were identified by a comparison of mass spectra from libraries (Wiley7n.1 and NIST) and the data reported in the literature [Zito, 2010]. A typical GC chromatogram is presented in Figure 4.8.

In total, 5 compounds were identified, corresponding to 63.60% of the mixture of hydrocarbons, including *n*-Nonacosane (39.21%), *n*-Heptacosane (14.71%), Eicosane (4.89%) and *n*-Heneicosane (3.25%) as shown in Table 4.21.

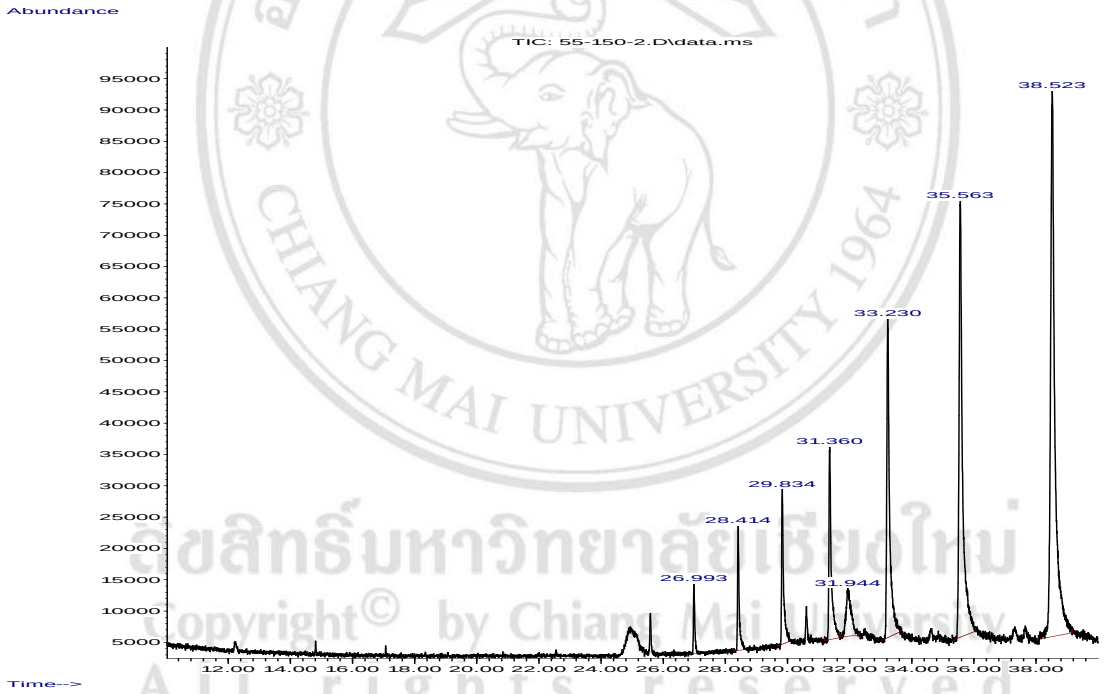

Figure 4.8 GC chromatogram of CLP-4

Table 4.21 Chemical compositions of CLP-4 compound

Peak no.	Compounds	RA[a]	RT[b]	RI[c]	RI[d]	References
1	Unknown	1.54	26.99	2178	-	-
2	*n*-Heneicosane	3.25	28.41	2224	2100	[Zito, 2010]
3	Eicosane	4.89	29.83	2257	2014	[Zito, 2010]
4	*n*-Heptacosane	14.71	33.23	2411	2700	[Zito, 2010]
5	*n*-Nonacosane	39.21	38.52	2851	2900	[Zito, 2010]
Group components						
Hydrocarbons		62.06				
Others		1.54				
Total		**63.60**				

Note : [a]Relative area in % (peak area relative to total peak area) ; [b]Retention Time (min) ; [c]Retention Indices determined in this study ; [d]Retention Indices of Kovat index or references.

4.5.3 Structure determination of isolated secondary metabolites from the leaves of *C. quadrangulare* Kurz

The obtained methanolic leaves extract of *C. quadrangulare* Kurz (10.41 g), was then separated using column chromatography techniques to afford two pure compounds. The structure determinations of all isolates were performed by interpretation of their MS and NMR data, and then confirmed by comparison with literature values.

1) Structure determination of compound CQ-3.2

Compound CQ-3.2 (0.0043 g) was obtained as white crystal. The R_f value is 0.45 [silica gel/*n*-hexane : ethyl acetate (9:1)]. It was identified as Lupeol.

This compound was previously isolated from leaves of *Phyllanthus reticulates* Poir. [Jamal, 2008], stem of *Diospyros rubra* Lec. [Prachayasittikul, 2010 ; Suryati, 2011], leaves of *Ficus deltoidea* Jack [Suryati, 2011], rhizomes of *Polypodium vulgare* [Prakash, 2012] and leaves of *C. micranthum* G. Don [Dawe, 2013].

The EIMS (Figure 6.7) afforded a molecular ion peak [M^+] at *m/z* 426, consistent with the molecular formula $C_{30}H_{50}O$ (D.B.E.=6), while other major fragments appeared at *m/z* 359, 331, 316, 287 and 246.

The ^{1}H- NMR spectrum of compound CQ-3.2 (Figure 6.8 and Table 4.22) showed a characteristic pattern to triterpenoid. H-29 showed two olefinic protons gives two doublets at δ 4.68 and δ 4.56 representing the exocyclic double bond protons. A singlet for 3 protons at δ 1.68 which was shown to be coupled to two vinyl protons (δ 4.68 and δ 4.56) was indicating for the presence of isopropenyl group of a triterpene. Seven tertiary methyl singlets at δ 0.77, 0.78, 0.85, 0.96, 0.98, 1.03, 1.68, and one secondary hydroxyl group as doublet of doublets at δ 3.18. The remaining protons appeared as complex multiplets in between 1.00-2.50 ppm.

The ^{13}C- NMR spectrum of compound CQ-3.2 (Figure 6.9 and Table 4.23) showed 30 signals for the terpenoid of lupine skeleton which was represented by seven methyl groups. The carbon bonded to the hydroxyl group C-3 appeared at 79.0, while the alkinic carbons appeared at 151.0 and 109.3 [Jamal, 2008].

The structure of this isolated compound CQ−3.2 was confirmed by comparison with the reported data [Prachayasittikul, 2009; Suryati, 2011] which were corresponding to those described for lup-20(29)-en-3b-ol, generally known as Lupeol. The chemical structure was shown in Figure 4.9. This is the first time it has been found Lupeol occurring in the leaves of *C. quadrangulare* Kurz.

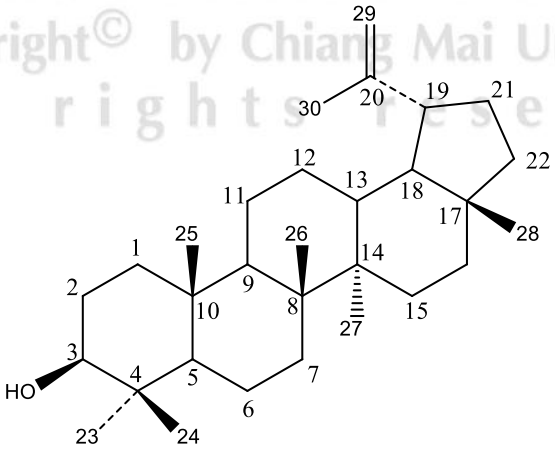

Figure 4.9 Structure of Lupeol

Table 4.22 ^{1}H NMR Assignments of Compound CQ-3.2 (in $CDCl_3$) and Lupeol (in $CDCl_3$)

Position	Compound CQ-3.2 δ_H (ppm) (multiplicity, *J* in Hz)	Lupeol [Suryati, 2011] δ_H (ppm) (multiplicity, *J* in Hz)
2	1.89 (2H, m)	1.62 (2H, m)
3	3.18 (1H, dd)	3.18 (1H, t)
5	0.67 (1H, t)	0.67 (1H, t)
6	1.39 (2H, m)	1.38 (2H, m)
9	1.24 (1H, t)	1.25 (1H, t)
18	1.29 (1H, dd)	1.35 (1H, dd)
19	2.37 (1H, m)	2.36 (1H, m)
23	0.98 (3H, s)	0.96 (3H, s)
24	0.77 (3H, s)	0.75 (3H, s)
25	0.85 (3H, s)	0.82 (3H, s)
26	1.03(3H, s)	1.02 (3H, s)
27	0.96 (3H, s)	0.94 (3H, s)
28	0.78 (3H, s)	0.78 (3H, s)
29	a.4.68 (1H, d) ; b. 4.56 (1H, d)	a. 4.68 (1H, d) ; b. 4.56 (1H, d)
30	1.68 (3H, s)	1.67 (3H, s)

Table 4.23 ^{13}C NMR Assignments of Compound CQ-3.2 (in $CDCl_3$) and Lupeol (in $CDCl_3$)

Position	Compound CQ-3.2 δ_C (ppm)	Lupeol [Prachayasittikul, 2010] δ_C (ppm)
1	38.7	38.7
2	27.4	27.4
3	79.0	79.0
4	38.8	38.8
5	55.3	55.3
6	18.3	18.3
7	34.3	34.3
8	40.8	40.8
9	50.4	50.4
10	37.1	37.1
11	20.9	20.9
12	25.1	25.1
13	38.0	38.0
14	42.8	42.8
15	27.4	27.4
16	35.6	35.6
17	43.0	43.0
18	48.0	48.0
19	48.3	48.3
20	151.0	150.9
21	29.8	29.7
22	40.0	40.0
23	28.0	28.0
24	15.4	15.3
25	16.0	15.9
26	16.1	16.1

Table 4.23 (continued)

Position	Compound CQ-3.2 δ_C (ppm)	Lupeol [Prachayasittikul, 2009] δ_C (ppm)
27	14.5	14.5
28	18.0	18.0
29	109.3	109.3
30	19.3	19.3

2) Structure determination of compound CQ-4.2

Compound CQ-4.2 (0.0065 g) was obtained as color needles crystals. The R_f value is 0.42 [silica gel/*n*-hexane : ethyl acetate (9:1)]. It was identified as 3*β*-Taraxerol similar compound CG-3.2.

The EIMS (Figure 6.10) afforded a molecular ion peak [M^+] at *m/z* 426, consistent with the molecular formula $C_{30}H_{50}O$ (D.B.E.=6), while other major fragments appeared at *m/z* 302, 287, 148, 135, 133 and 69.

The ^{1}H- NMR spectrum of compound CQ-4.2 (Figure 6.11 and Table 4.24) and *J*-resolved measurements helped in assigning each proton and their relative stereochemistries. Its spectrum, several signals for methyl groups, showed signals for oxymethine (δ 3.21 ppm, dd, *J*= 11.1, 4.7 Hz, H-3) and olefinic hydrogens (δ 5.55 ppm, dd, *J*= 8.2, 3.2 Hz, H-15) [de Oliveira, 2012]. H-3 proton appeared as typical of the axial proton associated with a 3*β*-hydroxy group [Corbett, 1972]. It showed to be *β*-from, and was determined by infinite zigzag O-H••O hydrogen bonded chains [Kouam, 2005]. Rest of proton appeared in the high field region in between 0.70-2.00 ppm.

The ^{13}C- NMR spectrum of compound CQ-4.2 (Figure 6.12 and Table 4.25) showed, several signals for C-sp^3, signals for olefinic carbons, complatible with the presence of triterpenes type lup-20(29)-ene (δ 116.9 and 158.1 ppm). Also was observed signals at δ 79.1 ppm, characteristics of oxymethine carbon and carbonyl groups at C-3 [de Oliveira, 2012].

From these finding and by comparison with previous published spectral data [Hernandez-Chavez, 2012], led us to assign this structure to be 3*β*-Taraxerol. The chemical structure was shown in Figure 4.10. This compound was first separated from the leaves of *C. quadrangulare* Kurz.

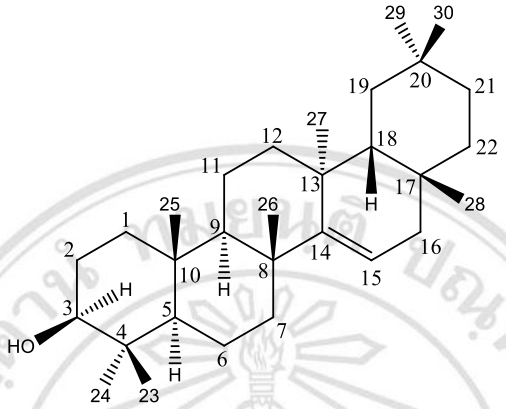

Figure 4.10 Structure of 3*β*-Taraxerol

Table 4.24 ^{1}H NMR Assignments of Compound CQ-4.2 (in $CDCl_3$) and 3β-Taraxerol (in $CDCl_3$)

Position	Compound CQ-4.2 δ_H (ppm) (multiplicity, *J* in Hz)	3β-Taraxerol [Hernandez-Chavez, 2012] δ_H (ppm) (multiplicity, *J* in Hz)
3	3.21 (1H, dd)	3.24 (1H, dd)
7a	2.05 (1H, dt)	2.03 (1H, dt)
15	5.55 (1H, dd)	5.53 (1H, dd)
16a	1.93 (1H, dd)	1.92 (1H, dd)
23	1.00 (3H, s, CH_3)	0.98 (3H, s, CH_3)
24	0.80 (3H, s, CH_3)	0.80 (3H, s, CH_3)
25	0.94 (3H, s, CH_3)	0.93 (3H, s, CH_3)
26	1.11 (3H, s, CH_3)	1.09 (3H, s, CH_3)
27	0.92 (3H, s, CH_3)	0.91 (3H, s, CH_3)
28	0.82 (3H, s, CH_3)	0.82 (3H, s, CH_3)
29	0.97 (3H, s, CH_3)	0.95 (3H, s, CH_3)
30	0.90 (3H, s, CH_3)	0.90 (3H, s, CH_3)

Table 4.25 ^{13}C NMR Assignments of Compound CQ-4.2 (in $CDCl_3$) and 3β-Taraxerol (in $CDCl_3$)

Position	Compound CQ-4.2 δ_C (ppm)	3β-Taraxerol [Hernandez-Chavez, 2012] δ_C (ppm)
1	37.7	38.0
2	27.1	27.1
3	79.1	79.1
4	39.0	39.0
5	55.5	55.5
6	18.8	18.8
7	33.7	35.1
8	38.8	38.7
9	48.7	48.7
10	36.7	37.5
11	17.5	17.5
12	35.1	35.8
13	37.6	37.6
14	158.1	158.1
15	116.9	116.9
16	35.8	36.6
17	38.0	37.7
18	49.3	49.2
19	41.3	41.3
20	28.8	28.8
21	33.3	33.7
22	29.9	33.1
23	28.0	28.0
24	15.4	15.4
25	15.4	15.4
26	29.7	29.8

Table 4.25 (continued)

Position	Compound CQ-4.2 δ_C (ppm)	3β-Taraxerol [Hernandez-Chavez, 2012] δ_C (ppm)
27	25.9	25.9
28	29.8	29.9
29	33.1	33.3
30	21.3	21.3

4.6 Antibacterial activity of the isolated compounds from *Combretum* species

The isolated compounds from three *Combretum* species (*C. griffithii* Heur. & M.A., *C. latifolium* Bl. and *C. quadrangulare* Kurz) were subjected to antibacterial activity evaluation. The antibacterial activity against three bacterial strains (*S. aureus*, *E. coli* and *P. aeruginosa*) using the agar disc diffusion method was assessed by determination of the inhibition zone. Three conventional medicines (ampicillin, gentamicin and ceftriaxone) were used as positive controls. While, methanol and sterile water were used as negative controls. The results are shown in Table 4.26. The isolated compounds were investigated at the concentration of 1 mg/mL. The five isolated compounds (CLP2, CLP4, CG-3.2, CQ-3.2 and CQ-4.2) possessed the highest antibacterial efficacy against gram-negative strain *E. coli* with inhibition zones of 9.33-10.33 mm followed by gram-positive strain *S. aureus* with inhibition zones of 817-9.33 mm. The isolated compounds exhibited the lowest or no antibacterial efficacy against gram-negative strain *P. aeruginosa* with inhibition zones of 6.33-6.67 mm.

Table 4.26 Antibacterial activity of isolated compounds from leaves of four *Combretum* species

Samples	Zone of inhibition in (mm)[a]		
	Pathogen		
	S. aureus	*E. coli*	*P. aeruginosa*
CLP2 (Lipid)	9.17 ± 0.08	9.33 ± 0.08	6.33 ± 0.03
CLP4 (Lipid)	8.17 ± 0.08	9.67 ± 0.06	6.33 ± 0.03
CG-3.2 (3*β*-Taraxerol)	9.33 ± 0.03	10.33 ± 0.06	6.33 ± 0.03
CQ-3.2 (Lupeol)	8.83 ± 0.10	10.00 ± 0.10	6.67 ± 0.06
CQ-4.2 (3*β*-Taraxerol)	9.17 ± 0.08	10.33 ± 0.06	6.67 ± 0.03
Ampicillin[b]	56.67 ± 0.15	25.50 ± 0.05	6.50 ± 0.00
Gentamicin[c]	30.50 ± 0.05	37.00 ± 0.10	27.83 ± 0.08
Ceftriaxone[d]	34.50 ± 0.05	35.50 ± 0.05	26.00 ± 0.00

Not : [a]Diameter of inhibition zones (mm) including the diameter of disc (6 mm) ; value are given as mean ± SD of triplicate experiment ; tested volumn = 5 µg/disc ; [b,c,d]Antibiotic used as positive controls.

In this studied, we reported 3*β*-Taraxerol and Lupeol showed antibacterial activity against *S. aureus* and *E. coli*. Corresponding with the previous studied, Sangeetha *et al.* [2010] reported that 3*β*-Taraxerol is having higher antibacterial activity against *E. coli* followed by *S. aureus*, *P. aeruginosa* and *K. pneumonia*. And 3*β*-Taraxerol has been reported to exhibit anti-microbial activity against *K. pneumonia*, *E. coli* and *S. aureus*, respectively [Singh, 2002 ; Kabir, 2013]. And Amir *et al.* [2013] found 3*β*-Taraxerol showed strong activity against *S. aureus*. Suryati *et al.* [2011] reported that Lupeol showed more sensitive antibacterial activity against *S. aureus* and *E. coli*. In addition, Gallo *et al.* [2009] reported that Lupeol showed significant zone of inhibition in the cultures of 18 hospital strains of the gram-negative bacteria *P. aeruginosa*, *K. pneumoniae*, *S. typhi* and *E. coli*. Among Lupeol, which is a common constituent of grape, hazelnut, olive oils coconut butter, mango pulp, white cabbage and a variety of therapeutic plants. Lupeol exhibits a broad spectrum of biological activities and can be used as chemopreventive to avoid several diseases. This may be due to the synergistic effects of the bioactive agents in the crude extract. Although 3*β*-Taraxerol and Lupeol

are well known compounds, but this is the first report of these compounds from *C. griffithii* Heur. & M.A. and *C. quadrangulare* Kurz.

In the previous reported, triterpenoids being the major constituents of genus *Combretum*. Further study is needed to determine its mechanisms of action and structure-activity relationship.