



GC-MS analysis and *in vitro* anti-androgenic activity of

Kaempferia rotunda Linn extract

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Abstract

Testosterone is converted to a more potent androgen, dihydrotestosterone (DHT) by 5 α -reductase and causes various hormonal actions. Anti-androgen can be used for the treatment of several disorders related to male sex hormone. In this study, the *in vitro* anti-androgenic activity and chemical components of hexane, dichloromethane and ethanolic extracts of *Kaempferia rotunda* Linn. rhizome were investigated. The various concentrations of extracts were tested on anti-androgenic activity by acting against the conversion of testosterone. The hexane extract was the most potent ($IC_{50} = 0.43 \pm 0.10$ mg/mL) and showed the IC_{50} value in the same range to that of positive control; ethinylestradiol ($IC_{50} = 0.26 \pm 0.02$ mg/mL). The different chemical profiles of all extracts studied were observed by GC-MS. The results indicated that most of volatile components including mono- and sesquiterpenes were present in hexane extract. Further studies on isolation and identification of active components are needed. This plant might be a new source for anti-androgens.

Keywords: *Kaempferia rotunda*, Anti-androgen, 5 α -Reductase, GC-MS, Sesquiterpenes

Introduction

Androgens have been indicated to be factors inciting androgenic alopecia, benign prostatic hyperplasia (BPH), prostate cancer, acne, and hirsutism. Testosterone is the major circulating androgen acting on many androgen-responsive tissues. It is metabolized to a more potent androgen, dihydrotestosterone (DHT) by 5 α -reductase. These androgens can bind to androgen nuclear receptors and cause various hormonal actions (Bartsch, Rittmaster, & Klocker, 2002; Russell & Wilson, 1994). Hence, the anti-androgens which exhibit inhibitory activity on 5 α -reductase and/or block androgen receptor may be useful for treatment of these androgen-dependent disorders. A number of synthesized 5 α -reductase inhibitors have been reported including

finasteride (Proscar, Propecia) and more recently dutasteride (Avodart, Avolve); two steroidal 5 α -reductase inhibitors which already used for treatment of BPH and male pattern baldness (Andersen, Nickel, Marshall, Schulman, & Boyle, 1997). However, finasteride causes possible adverse effects; gynecomastia, muscle growth impairment and severe myopathy (Aggarwal, Thareja, Verma, Bhardwaj, & Kumar, 2010; Gormley, 1995). Therefore, the finding of the new more selective anti-androgenic compounds are needed and natural products may fulfill this requirement.

Kaempferia rotunda L. belongs to Zingiberaceae family. It is commonly known as Wan thip pha ya net (Thai), Wan hao non (Thai), bhuichampaka (Sanskrit), bhuchampa (Hindi) and blackhorn (English). It is a fragrant aromatic herb with a



tuberous rhizome. The rhizomes are used medicinally throughout Southeast Asia as stimulant, against gastric disorders, to relieve headache and eye diseases. Moreover, the leaves and rhizomes of *K. rotunda* are eaten fresh or cooked as a vegetable (Chayamarit, Pooma, & Pattharahirantricin, 2014; Larsen & Larsen, 2006). It has been reported to show anti-oxidation (Lotulung, Minarti, Kardono, & Kawanishi, 2008), anti-allergic effect (Madaka & Tewtrakul, 2011), antimutagenic effect (Atun, Arianingrum, Sulistyowati, & Aznam, 2013), insecticidal effects (Nugroho, Schwarz, Wray, & Proksch, 1996), antifeedant (Stevenson, Veitch, & Simmonds, 2007), antimicrobial effects (Pratiwi et al., 2015), antibacterial effects (Kabir & Reza, 2014) and anthelmintic effects (Agrawal et al., 2011). This plant also exerts various effects including reduce platelet-activation (Jantan et al., 2004; Jantan et al., 2008), wound healing effect (Imam, Rout, Sutar, Sharma, & Sutar, 2013), antihyperglycemic effect, antinociceptive effect (Sultana et al., 2012) and antiproliferative effect on Ehrlich ascites carcinoma and human breast cancer cells (Atun & Arianingrum, 2015; Kabir & Reza, 2014). The main constituent, crotepoxide, is useful for the inhibition of tumors (Kupchan, Hemingway, Coggon, McPhail, & Sim, 1968; Prasad et al., 2010). Although various biological effects of *K. rotunda* rhizome has been described, the anti-androgenic activity have not been reported yet. From our preliminary study, eighteen ethanolic crude rhizome extracts of Zingiberaceae plants were screened for inhibitory activity on testosterone conversion using *in vitro* enzymatic assay. *K. rotunda* was found as one of the active crude extracts (unpublished data). In this study, the *in vitro* anti-androgenic activities of hexane, dichloromethane and ethanolic rhizome extracts of *K. rotunda* were investigated for the first time and their volatile

components were also studied using gas chromatography-mass spectrometry (GC-MS).

Methods and Materials

General experimental procedures

Testosterone remaining from the enzymatic activity was measured using high performance liquid chromatography (HPLC) (Agilent Technologies, Palo Alto, CA, USA). HPLC analysis was equipped with an Agilent 1100 series; G1311A Quaternary pump, G1322A degasser, G13158 diode array detector (DAD), G1316A column oven and an auto injector (Agilent 1200 series) with a 20 μ L loop. The phenomenex Luna 5 μ C-18(2) column (150 x 4.6 mm) was used for the HPLC separation. Gas chromatography-mass spectrometry (GC-MS) analysis was performed on a Hewlett Packard (Agilent Technologies, Palo Alto, CA, USA) model 6890 gas chromatograph equipped with a mass selective detector (MS). A fused silica capillary Hewlett Packard HP-5 (5% phenyl methyl siloxane) column (30 m x 0.25 mm i.d., 0.25 μ m film thickness) was used for the GC separation.

Chemicals

Testosterone, ethinylestradiol, prednisolone and tris-(hydroxy methyl)-aminomethane were purchased from Sigma-Aldrich (USA). Propylene glycol (PG) was obtained from Ajax Finechem (Australia), β -nicotinamide adenine dinucleotide phosphate (NADPH, tetrasodium salt) from Calbiochem (USA). Hexane, methanol (MeOH), ethanol (EtOH), dichloromethane (CH_2Cl_2), and dimethyl sulfoxide (DMSO) were bought from RCI Labscan Ltd (Thailand) and thiopental sodium from Abbott (Thailand). Alkane standard solutions (C_8 - C_{20} and C_{21} - C_{40}) were bought from Fluka analytical (Germany).



Plant material and Extraction

Fresh rhizomes of *K. rotunda* were collected from Amphur Khaokhor, Phetchabun province, Thailand (March, 2014). The plant material was identified by Dr. Pranee Nanggam. The voucher specimen (collection number: 003497) is kept at Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand.

The powdered rhizomes of *K. rotunda* (2.74 kg) were extracted with hexane (15 litres) for 3 days at room temperature and filtered. The maceration procedure was repeated 3 times. The filtrates were evaporated under reduced pressure and pooled to produce the hexane extract (16.50 g). The marc was further extracted using CH_2Cl_2 and 95% EtOH, respectively using the same procedure. The filtrates obtained were evaporated to produce 71.44 g of CH_2Cl_2 extract and 99.70 g of EtOH extract.

Preparation of rat liver enzyme

A crude microsomal fraction from the livers of male Sprague Dawley rats (6 weeks of age) was prepared by the method of Matsuda et al. (2001) with some modifications and used as a source of 5 α -reductase (Matsuda et al., 2001; Suphrom et al., 2012). The protocol was approved by Naresuan University Animal Ethics Committee (Approval no. 56 04 0062). Briefly, the rats were pre-anesthetized with sodium thiopental and then killed, the livers were removed, the blood washed out with Krebs-ringer phosphate buffer pH 7.4 and the livers were further homogenized in Tris-HCl buffer pH 7.4. This homogenate was centrifuged sequentially at 880, 4775 and 8595 \times g with collection of the supernatant and recentrifuging at the next speed. The final supernatant was stored at -80 °C.

Measurement of *in vitro* anti-androgenic activity by acting against the conversion of testosterone

The *in vitro* inhibitory activity against the conversion of testosterone to DHT of samples was conducted according to the method described in

previous study (Suphrom et al., 2012) with some modification. Briefly, the reaction solution contained 50 μL of test compound dissolved in DMSO, 350 μL of Tris-HCl buffer pH 7.2, 100 μL of testosterone (0.5 mg/mL in the 1:1 v/v mixture of PG and Tris-HCl buffer pH 7.2) and 350 μL of the thawed rat liver enzyme solution (18.01 ± 0.10 mg/mL of total proteins). The reaction was started by adding 150 μL of NADPH (5.13 mg/mL in Tris-buffer pH 7.2). The mixture solution was incubated at 37 °C for 30 min and reaction was stopped by adding 3 mL of CH_2Cl_2 . Then 150 μL of prednisolone (0.1 mg/mL in MeOH) which was an internal standard was added to the reaction solution tube and shaken. The tube was kept in an ice bath and centrifuged at 2325 \times g for 10 min. The CH_2Cl_2 layer was transferred to another tube and evaporated to dryness. The residue was dissolved in 2 mL of MeOH. In addition, control samples were prepared with all the solutions including the DMSO but no test compound. The CH_2Cl_2 was added at time 0 and 30 min of incubation for the control (0 min) and the control (30 min), respectively. Ethinylestradiol, which has been reported as 5 α -reductase inhibitor, was used as a positive control (Nukui, 1997). The series of concentrations of three *K. rotunda* extracts (0.02–10 mg/mL) and ethinylestradiol (0.01–5 mg/mL) were tested for determination of IC_{50} values. The enzyme activity was determined from the remaining of testosterone after enzymatic reaction using HPLC. The phenomenex Luna 5 μm C-18 column was used as a stationary phase and 55% MeOH in water for the mobile phase. The flow rate was 0.8 mL/min and the injection volume was 20 μL . The separation was conducted at 40 °C for 30 min. The absorption detector was set at 254 nm. Percent enzymatic inhibition was determined using peak height ratios ($r = \text{peak height of testosterone} / \text{peak height of prednisolone}$) as shown in the equation below.



$$\text{Enzymatic inhibition (\%)} = \left(\frac{r \text{ of test sample} - r \text{ of control (30 min)}}{r \text{ of control (0 min)} - r \text{ of control (30 min)}} \right) \times 100$$

Gas Chromatography-mass spectrometry analysis of volatile components in *K. rotunda* extracts

The analysis for volatile components in three extracts were performed by GC-MS. High purity helium was used as carrier gas with constant flow rate 1.0 mL/min. The injector was set at 250°C and performed by split mode with a split ratio of 10:1 (in 1.0 µL). The column was as described above. The initial oven temperature was held at 70°C for 3 min, then programmed at 5°C/min to 280°C and finally held for 10 min. The temperature of transfer line heater was set at 280°C. The mass scanning range was set from 50–550 amu in full scan. Most volatile constituents were identified by computer matching the mass spectra with a standard library; wiley7n, and comparing obtained mass spectra of analytes with those of authentic standards from the National Institute of Standards and Technology (NIST) Chemistry WebBook (Babushok et al., 2007; Linstrom & Mallard, 2016), the retention indices (RIs) and with the mass spectra published previously. RIs were determined by analyzing a solution containing the homologous series of *n*-alkanes (C₈–C₃₂) and then calculated as described by van Den Dool and Dec. Kratz (1963).

Statistical analyses

The experiments of inhibitory activity test were performed in triplicate and the data were expressed as mean ± S.D. Statistical significance was determined by Student's *t*-test. A significance level of *p* < 0.05 denoted significance in all cases.

Results

In this study, the anti-androgenic activities of the hexane, CH₂Cl₂ and EtOH extracts from *K. rotunda* and ethinylestradiol (positive control) on the conversion of testosterone were determined using rat liver homogenate as a source of enzyme. Among three extracts tested, hexane expressed the most potent inhibitory activity. The concentration that could inhibit 50% of the enzymatic activity (IC₅₀) of hexane extract was 0.43 ± 0.10 mg/mL while that of CH₂Cl₂ extract was 1.17 ± 0.29 mg/mL (Table 1). For the EtOH extract, the highest concentrations tested (10 mg/mL) gave % enzymatic inhibition of only 22.52 ± 7.59. Therefore, its IC₅₀ value could not be calculated and reported as > 10 mg/mL.

Table 1 Inhibition effects of *K. rotunda* extracts on the conversion of testosterone to DHT. The data are expressed as means ± SD. Determinations were done in triplicate.

Samples	IC ₅₀ (mg/mL)
Hexane extract	0.43 ± 0.10
CH ₂ Cl ₂ extract	1.17 ± 0.29
EtOH extract	> 10
Ethinylestradiol (positive control)	0.26 ± 0.02

The analysis for volatile components in three extracts were performed by GC-MS instrument. Their total ion chromatograms were illustrated in

Figure 1. The different volatile compositions of each were observed. A total of 31 compounds in hexane extract were identified and listed in Table 2, where

the RIs of volatile compounds in sample were presented. As detailed in Table 2, a total of 16 and 9 compounds in CH_2Cl_2 and EtOH extracts were identified, respectively. The relative amount (%) of the compositions was calculated by peak-area normalization. The groups of volatile substances detected in *K. rotunda* were monoterpenes, sesquiterpenes, diterpenes, long chain hydrocarbons, ester of fatty acids, benzyl derivatives, cyclohexane

diepoxide and phytosterols. Most of monoterpenes and sesquiterpenes were present in hexane extract and some present in CH_2Cl_2 extract (see Figure 1 and Table 2). The main constituents found in hexane extract were benzyl benzoate (18.92%), pentadecane (10.90%) while that in CH_2Cl_2 and EtOH extracts were benzyl benzoate (9.39 and 5.46 %) and crotepoxide (33.11 and 42.92%).

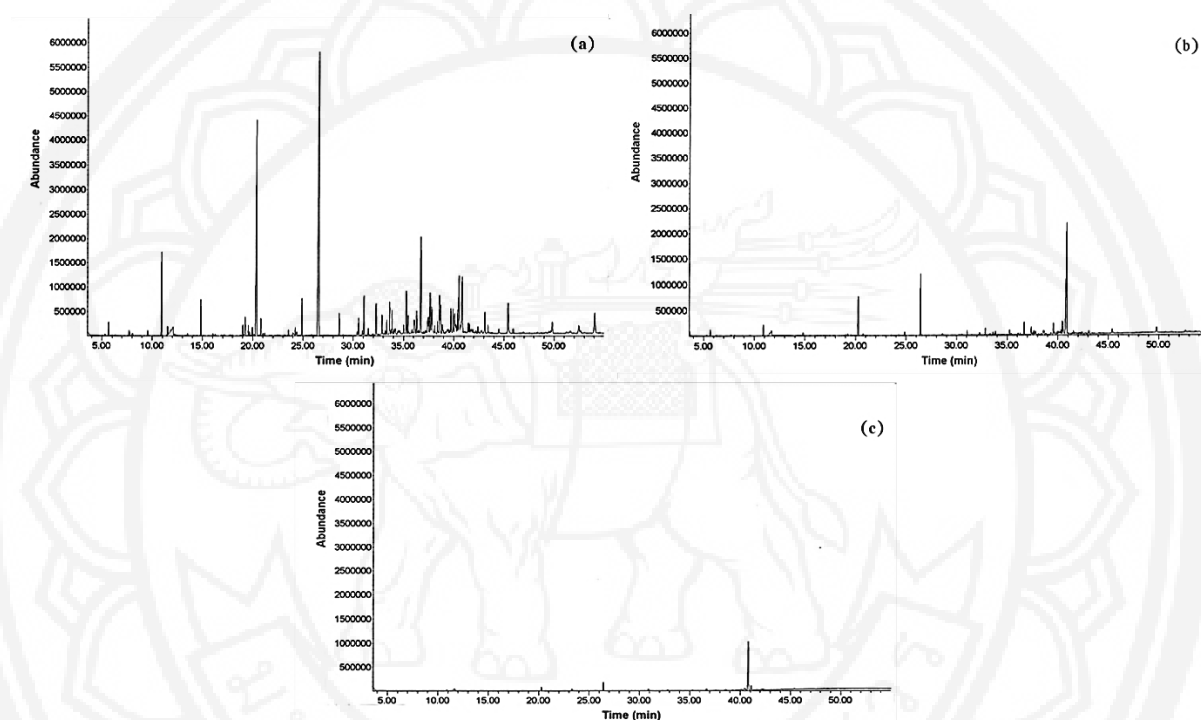


Figure 1 GC-MS total ion chromatograms of (a) hexane extract, (b) CH_2Cl_2 extract, and (c) EtOH extract

Table 2 Volatile compounds of *K. rotunda* extract identified by GC-MS

RT (min)	RI ^a	Identified compounds	Classification of compounds	Relative amount (%) ^b		
				hexane extract	CH_2Cl_2 extract	EtOH extract
5.32	932	α -pinene	monoterpene	0.05	0.14	NA ^c
5.67	947	camphene	monoterpene	0.33	0.69	NA
7.71	1030	1,8-cineole	monoterpene	0.15	0.11	NA
7.77	1032	benzyl alcohol	benzyl derivative	0.08	0.17	NA
8.05	1042	salicylaldehyde	benzyl derivative	0.08	0.08	NA
9.57	1097	linalool	monoterpene	0.16	0.12	NA
10.92	1145	camphor	monoterpene	2.43	1.49	0.79
11.52	1166	borneol	monoterpene	0.28	NA	NA



Table 2 (Cont.)

RT (min)	RI ^a	Identified compounds	Classification of compounds	Relative amount (%) ^b		
				hexane extract	CH ₂ Cl ₂ extract	EtOH extract
12.20	1190	α -terpineol	monoterpene	0.06	NA	NA
14.84	1285	1-bornyl acetate	monoterpene	0.99	0.47	1.47
17.16	1372	ylangene	sesquiterpene	0.05	NA	NA
17.76	1395	tetradecane	hydrocarbon	0.04	NA	NA
19.02	1450	β -selinene	sesquiterpene	0.41	NA	NA
19.15	1445	α -gurjunene	sesquiterpene	0.04	NA	NA
19.68	1471	β -gurjunene	sesquiterpene	0.15	NA	NA
19.80	1476	γ -Selinene	sesquiterpene	0.03	NA	NA
19.94	1481	α -curcumene	sesquiterpene	0.26	0.11	NA
20.36	1498	pentadecane	hydrocarbon	10.90	5.30	2.39
20.78	1516	α -amorphene	sesquiterpene	0.56	0.29	NA
21.00	1525	γ -gurjunene	sesquiterpene	0.11	NA	NA
23.30	1623	propylparaben	benzyl derivative	NA	NA	1.41
23.54	1634	γ -eudesmol	sesquiterpene	0.18	NA	NA
24.24	1665	heptadeca-6,9-diene	hydrocarbon	0.26	0.13	NA
24.89	1694	heptadecane	hydrocarbon	1.03	0.46	0.29
26.57	1772	benzyl benzoate	benzyl derivative	18.92	9.39	5.46
30.54	1968	sandaracopimaradiene	diterpene	0.54	0.24	NA
30.91	1987	ethyl palmitate	ester of fatty acid	0.19	NA	1.24
34.00	2155	ethyl linoleate	ester of fatty acid	0.25	NA	NA
34.55	2186	ethyl stearate	ester of fatty acid	0.13	NA	0.46
40.88	2575	crotopoxide	cyclohexane diepoxide	2.68	33.11	42.92
52.46	3247	stigmasterol	sterol	0.57	NA	NA
54.06	-	β -sitosterol	sterol	1.61	NA	NA

^aRetention indices were calculated using a homologous series of *n*-alkanes (C₉-C₃₂)^bResults obtained by peak-area normalization^cNA: not available



Discussion

From our study, the hexane extract showed potent anti-androgenic activity. It is also the first time that anti-androgenic activity of *K. rotunda* was reported. The inhibitory activity of hexane extract was higher than that of CH₂Cl₂ extract about 3 folds, and EtOH extract did not show interesting activity. The positive control, ethinylestradiol showed the IC₅₀ value of 0.26 ± 0.20 mg/mL or 0.86 mM in our assay system which was in agreement to the studies of Matsuda et al. and Hirata et al. (IC₅₀ = 0.81 mM) (Hirata et al., 2007; Matsuda et al., 2001). Interestingly, hexane extract showed the same level of inhibitory effect to that of the positive control ($p < 0.05$). Since three extracts of *K. rotunda* showed different anti-androgenic activity, their chemical components were analyzed by GC-MS. The compounds detected in *K. rotunda* extracts were corresponded to that of the previous reports (Kumar, 2014; Sirata, Jamila, & Siew, 2005; Woerdenbag et al., 2004). The three extracts, contained different amounts of chemical components i.e. monoterpenes (α -pinene, camphene, 1,8-cineole, linalool, camphor, borneol, α -terpineol, 1-bornyl acetate), sesquiterpenes (ylangene, selinene, gurjunene, curcumene, amorphene, eudesmol), benzyl derivatives, ethyl ester of fatty acid (ethyl palmitate, ethyl linoleate, ethyl stearate) according to their different polarities.

Since hexane extract of *K. rotunda*, the most active extract, showed the higher terpenoids (mono- and sesquiterpenes) contents than the other two extracts, the correlation between anti-androgenic activity and terpenoids was observed. From the previous studies, sesquiterpenes i.e. germacrane- and guaiane-types sesquiterpenes isolated from *Curcuma aeruginosa* Roxb. were reported for anti-androgenic effect as they inhibited the enzymatic conversion of testosterone to DHT (Srivilai, Khorana, Waranuch, &

Ingkaninan, 2011; Suphrom et al., 2012). These terpenoids including borneol, camphene, camphor, 1,8-cineol, α -curumene have been previously reported for other bio-activities i.e. anti-inflammatory, antioxidant, antibacterial effects, antimicrobial effects before (Afzal, Oriqat, Akram Khan, Jose, & Afzal, 2013). The bio-assay guided fractionation might lead to the isolation and identification of anti-androgenic compounds from *K. rotunda*.

Conclusion and Suggestion

In conclusion, *K. rotunda* hexane extract was found to exhibit an *in vitro* anti-androgenic activity in the same potency as ethinylestradiol. The further isolation and structural elucidation of the anti-androgenic compounds in this extract is interesting.

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