INVESTIGATION OF ANTI-ANDROGENIC COMPOUNDS FROM CURCUMA AERUGINOSA ROXB.

NUNGRUTHAI SUPHROM

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy Degree
in Pharmaceutical Sciences (International Program)
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This thesis entitled "Investigation of Anti-androgenic Compounds from *Curcuma aeruginosa* Roxb." submitted by Nungruthai Suphrom in partial fulfillment of the requirements for the Doctor of Philosophy Degree in Pharmaceutical Sciences (International Program) is hereby approved.

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Nungruthai Suphrom

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ABSTRACT

Androgens are known to involve with some symptoms and diseases such as androgenic alopecia, benign prostatic hyperplasia and prostate cancer. Testosterone is metabolized to a more potent androgen, dihydrotestosterone (DHT) by 5α-reductase and causes various hormonal actions. Anti-androgens which exhibit inhibitory activity on 5α-reductase and/or block androgen receptor may be useful for treatment of these androgen-dependent disorders. Our group recently discovered high anti-androgenic activity of a hexane extract from rhizomes of *Curcuma aeruginosa* Roxb. (Zingiberaceae) both *in vitro* and *in vivo*. Although the anti-androgenic effects of *C. aeruginosa* have been described, the active constituents are yet to be determined. Therefore, we aimed to isolate and identify the anti-androgenic compounds in this plant. Their anti-androgenic activity and potential mechanisms were also investigated. The study extended to quantitative and qualitative analyses of the active markers in the plant extract and stability studies which will give necessary information for the development of any product containing *C. aeruginosa*.

Six sesquiterpenes isolated from *C. aeruginosa* i.e. germacrone (**1**), zederone (**2**), dehydrocurdione (**3**), curcumenol (**4**), zedoarondiol (**5**) and isocurumenol (**6**) showed enzymatic inhibition on the conversion of testosterone. Germacrone (**1**) showed the highest inhibitory activity with IC_{50} of 0.42 ± 0.05 mg/mL. It also showed anti-androgenic effect on human prostate cancer cells (LNCaP) when proliferation was testosterone-induced. The growth of flank glands of male Syrian hamsters depends on

circulating androgen. It was found that **1** (3, 30, 100 μg) could inhibit testosterone-induced flank gland growth but was ineffective on that of DHT-induced. The similar activity profile was observed on the 5α–reductase inhibitor, finasteride (100 μg) treatment group. The receptor binding assay suggested that **1** did not bind to androgen receptor. Thus, anti-androgenic activity of **1** may not be associated with androgen receptor. Using all spectra of effects obtained, one of the possible mechanisms of **1** for anti-androgenic activity was inhibition of 5α-reductase.

The cytotoxic effect studies showed that *C. aeruginosa* extract together with isolated compounds (compounds **2-4**) had no cytotoxic effect on human foreskin fibroblast (HF) cell at the concentrations of 0.0001–10 μg/mL while compounds **1** and **5** tended to increase cell viability at the concentration of 10 and 1 μg/mL, respectively. The cytotoxic effect was also tested on LNCaP cells. The result showed that *C. aeruginosa* extract and compounds **3-6** (0.00001-10 μg/mL) showed no cytotoxic effect on LNCaP cell. It should be noted that **1** decreased cell viability at the highest concentration tested (10 μg/mL or 45 μM) while **2** dose-independently reduced LNCaP cell viability. The effect on the cells of **2** could perhaps be explained by growth inhibition rather than causing cell death as the increase of the concentration from 0.00001-10 μg/mL gave the same range of cell viability (70-80%).

Thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) were developed for qualitative and quantitative determination of chemical constituents in *C. aeruginosa* extract. The fingerprints of the extracts were made using these techniques. The HPLC gradient elution step of CH_3CN and 0.5 mM phosphate buffer pH 3.0 was applied to quantitatively determine **1** and **2** in the crude extract. Moreover, two GC-FID (system 1 and system 2) for determination of marker compounds (**1**, **2**, **4** and **6**) in crude extract and **1** which was studied as pure compound were optimized. The rest of the active sesquiterpenes could not be detected by GC due to their thermal instability. The calibration curves were linear between the concentration ranges of 18.75-250 μg/mL (system 1) and 15.625-250 μg/mL (system 2). The method was validated for limit of detection, limit of quantitation, linearity, precision and accuracy. These methods can be applied to determine marker compounds in *C. aeruginosa* for quality control purpose. The

amount of the most active compound, **1**, in *C. aeruginosa* rhizome dried powder was approximately 0.2-0.4 mg/g.

Stability of **1** in *C. aeruginosa* extract and in pure form both in solid and solution were investigated in various pHs and temperatures. The results showed that the remaining amount of **1** was still higher than 75% at all of pH conditions when the samples were kept for 14 days. For temperature effect studies, our results revealed that **1** was thermo-sensitive. At 4 and 25°C, the slightly decrease of **1** in crude extract semi-solid form was found after 1 month while the dramatic decrease to undetectable level of **1** after kept at 45°C for only 3 days was found. Moreover, **1** in crude extract was more stable than that in pure form. Oxygen might be one of factors causing the degradation of **1**. Surprisingly, the stability of **1** was improved when kept in the solution form. Our studies demonstrated that **1** was also slightly sensitive to light. Therefore, compound **1** in *C. aeruginosa* extract would be well protected when kept at low temperature and avoid oxygen and light exposure.

In conclusion, the anti-androgenic components of *C. aeruginosa* extract have been identified. The tentative mechanism of the extract as well as the most active compound, **1**, was inhibition of 5α–reductase. The methods of analyses of **1** and some sesquiterpenes in the extract were developed. The stability of **1** was also studied. Further development of **1** or *C. aeruginosa* extract for therapeutic applications related to their anti-androgenic activity is possible.

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ABBREVIATIONS

AGA	=	androgenic alopecia
ANOVA	=	analysis of variance
BPH	=	benign prostatic hyperplasia
br	=	broad (for NMR signals)
$CDCl_3$	=	deuterated chloroform
$CHCl_3$	=	chloroform
CH_2Cl_2	=	dichloromethane
CH_3CN	=	acetonitrile
cm	=	centimetre
CO_2	=	carbondioxide
COSY	=	correlation spectroscopy
COX-2	=	cyclooxygenase-2
d	=	doublet (for NMR signals)
D.B.E.	=	double bond equivalent
DEPT	=	distortioless enhancement by polarization transfer
DHT	=	dihydrotestosterone
DMEM	=	dulbecco's modified eagle's medium
DMSO	=	dimethylsulfoxide
EI-MS	=	electron impact ionization-mass spectrometry
ESI-MS	=	electro spray ionization- mass spectrometry
EtOAc	=	ethyl acetate
EtOH	=	ethanol
F	=	finasteride
FBS	=	fetal bovine serum
FM	=	flutamide
FP	=	fluorescent polarization
g	=	gram
GC-FID	=	gas chromatography-flame ionization detector
GC-MS	=	gas chromatography-mass spectrometry
HCl	=	hydrochloric acid

ABBREVIATIONS (CONT.)

HF	=	human foreskin fibroblast
HMBC	=	heteronuclear multiple-bond correlation spectroscopy
HMQC	=	heteronuclear multiple quantum correlation spectroscopy
HPLC	=	high performane liquid chromatography
hr	=	hour
HRP	=	horseradish peroxidase
IC_{50}	=	concentration that could inhibit 50% of the enzymatic activity
i.d.	=	internal diameter
iNOS	=	inducible nitric oxide synthase
J	=	coupling constant
KCl	=	potassium chloride
kg	=	kilogram
L	=	litre
LNCaP	=	androgen-sensitive human prostate cancer
m	=	multiplet (for NMR signals)
m/z	=	mass-over-charge ratio
MeOH	=	methanol
min	=	minute
MHz	=	megahertz
µg/mL	=	microgram per millilitre
µL	=	microlitre
µM	=	micromolar
mM	=	millimolar
mL	=	millilitre
MTT	=	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	=	β-nicotinamide adenine dinucleotide phosphate
nm	=	nanometre
NMR	=	nuclear magnetic resonance spectroscopy
NOSEY	=	nuclear overhauser effect spectroscopy
PDA	=	photodiode array

ABBREVIATIONS (CONT.)

PEG	=	polyethylene glycol
PG	=	propylene glycol
pH	=	power of hydrogen ion concentration
RPMI-1640	=	roswell park memorial institute formulation 1640
RSD	=	relative standard deviation
r	=	ratio
s	=	singlet (for NMR signals)
SD	=	standard deviation
SEM	=	standard error of mean
t	=	triplet (for NMR signals)
T	=	testosterone
TLC	=	thin layer chromatography
TMS	=	tetramethylsilane
U	=	unit
UV/Vis	=	ultraviolet-visible
5αR	=	5α-reductase