

Article

Avicequinone C Isolated from *Avicennia marina* Exhibits 5 α -Reductase-Type 1 Inhibitory Activity Using an Androgenic Alopecia Relevant Cell-Based Assay System

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Abstract: *Avicennia marina* (AM) exhibits various biological activities and has been traditionally used in Egypt to cure skin diseases. In this study, the methanolic heartwood extract of AM was evaluated for inhibitory activity against 5 α -reductase (5 α -R) [E.C.1.3.99.5], the enzyme responsible for the over-production of 5 α -dihydrotestosterone (5 α -DHT) causing androgenic alopecia (AGA). An AGA-relevant cell-based assay was developed using human hair dermal papilla cells (HHDPCs), the main regulator of hair growth and the only cells within the hair follicle that are the direct site of 5 α -DHT action, combined with a non-radioactive thin layer chromatography (TLC) detection technique. The results revealed that AM is a potent 5 α -R type 1 (5 α -R1) inhibitor, reducing the 5 α -DHT production by 52% at the final concentration of 10 μ g/mL. Activity-guided fractionation has led to the identification of avicequinone C, a furanonaphthaquinone, as a 5 α -R1 inhibitor with an IC₅₀ of 9.94 \pm 0.33 μ g/mL or 38.8 \pm 1.29 μ M. This paper is the first to report anti-androgenic activity through 5 α -R1 inhibition of AM and avicequinone C.

Keywords: avicequinone C; *Avicennia marina*; 5 α -R1 inhibitory activity; cell-based bioassay

1. Introduction

Avicennia marina (AM), commonly called the grey or white mangrove, is a species of mangrove trees belonging to the Acanthaceae family. Traditionally it is used in Egypt to cure skin diseases [1] against fish stings, ringworms, sores, boils, skin ulcers and scabies [2]. It has also been used as a contraceptive [2] and in treating rheumatism [3]. In the literature, AM has been reported to exhibit antifertility [4], anticancer [5], antimicrobial [6] and antitumor [7] activities. Phytochemically, AM has been found to contain a variety of natural product groups, including naphthalene derivatives, flavones, iridoid glucosides, prenylpropanoid glycosides, abietane ditriterpenoid glucosides, flavonoid terpenoids and steroids [1].

AM has been used as a contraceptive due to its effects on the body's endocrine system [2]. The exact mechanism through which AM causes contraception is not yet understood, but most oral contraceptives affect the steroidogenesis pathway through either increasing or decreasing hormones or their related receptor levels or affecting the activities of the enzymes involved [8,9]. One of the enzymes present in the steroidogenesis pathway is 5 α -reductase (5 α -R), which converts testosterone (T) to 5 α -DHT through the reduction of the $\Delta^{4,5}$ double bond [10]. Overproduction of 5 α -DHT, a much more potent androgen, causes androgen-dependent diseases such as benign prostate cancer, acne and androgenic alopecia (AGA) [10]. Of these diseases, AGA is the main focus of this research work.

AGA is the major type of scalp hair loss in humans, affecting some 60%–70% of the worldwide population [11,12]. It affects 50% of males by the age of 50 and up to 70% of all males in their later life, while it affects only 25% of women by the age of 49% and 41% by the age of 69 years [13]. It is characterized by the miniaturization of the large, thick pigmented terminal hairs with diameters of greater than 0.03 mm into small, fine, non-pigmented vellus hairs with a diameter of 0.03 mm or less [11,14]. The miniaturization, due to the overproduction of 5 α -DHT, results in the premature entry of the hair follicle into the catagen (transition) phase and the delay in the transition from the telogen (resting) to anagen (growth) phase, resulting in the shortening of the growth phase [15]. Therefore, one potential target for treating AGA is to inhibit this enzymatic reaction within the hair follicle. Two isoforms of this enzyme has been identified in different parts of the hair follicle, namely 5 α -R1 and 5 α -R type 2 (5 α -R2) [10]. 5 α -R1 is present in the dermal papilla cells, epidermal and follicular keratinocytes, while 5 α -R2 is present in the inner layer of the outer root sheath, inner root sheath, interfollicular keratinocytes and might be present in the dermal papilla cells [16,17]. Different isoforms that catalyze the same reduction reaction are thus present in different parts of the hair follicle, however, only the dermal papilla cells are the site of action of 5 α -DHT. In addition, they are the main regulator of hair growth as it plays an essential role in induction of new hair follicles and maintaining hair growth [18,19].

Therefore, in this study a cell-based assay system using dermal papilla cells commercially available as HHDPCs was used in order to evaluate the potential of AM as an 5 α -R inhibitor, specifically for treating AGA. The assay system was coupled with a non-radioactive TLC detection technique.

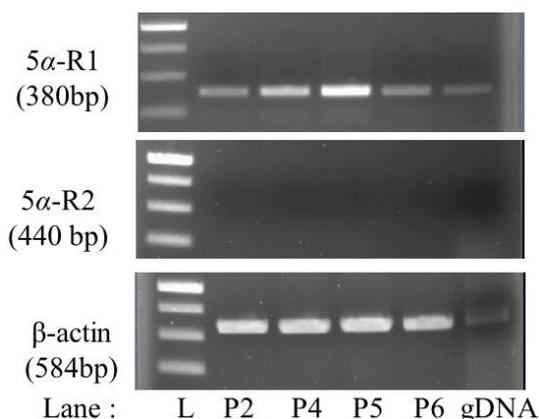
In addition, activity-guided fractionation through a preparative TLC technique was carried out in order to obtain pure bioactive compound(s) from the AM extract.

2. Results and Discussion

2.1. Expression of 5 α -R1 in HHDPCs

5 α -R is responsible for the conversion of T into 5 α -DHT causing AGA. Therefore, the presence of this enzyme within the HHDPCs was evaluated. The RT-PCR analysis revealed that the genes of 5 α -R1 were expressed in passages 2, 4, 5 and 6 of HHDPCs, while 5 α -R2 was not expressed in any of the passages (Figure 1). β -Actin, used as an internal control, was constantly expressed in all passages.

Figure 1. RT-PCR showing the expression of 5 α -Rs and β -actin within HHDPCs. A 1% agarose gel showing, from the top, the expression of 5 α -R1 (5 α -reductase type 1, 380 bp), 5 α -R2 (5 α -reductase type 2, 440 bp) and β -actin (584 bp) within passages 2, 4, 5 and 6 of HHDPCs. The 1-kb DNA ladder (L) shows the band sizes of 1 kb and 750, 500 and 250 bp from the top down.



These results suggested that the type 1 enzyme, not type 2, is likely to have a direct role in hair growth regulation. This corresponds with previous works showing that the predominant form of 5 α -R in HHDPC is 5 α -R1 [10,16,17]. The two isoforms have 47% sequence similarity [10] and are known to be specifically distributed in different organs within the human body [10,16]. However, finding a number of reports in the literature that use 5 α -R2 as the target enzyme for screening anti-AGA compounds is interesting [20–22]. This might be due to the previous clinical studies results showing that finasteride, a selective 5 α -R2 inhibitor, decreases the 5 α -DHT concentration and promotes hair growth in men with AGA [16,23]. This effect has been explained based on the results of immunohistochemical localization of 5 α -R2 within the inner root sheath keratinocytes in the hair follicle rather than its direct effect on 5 α -R1 within the HHDPCs which are known to be the main regulators of hair growth [10,16,17,24].

2.2. AM as 5 α -R1 Inhibitor

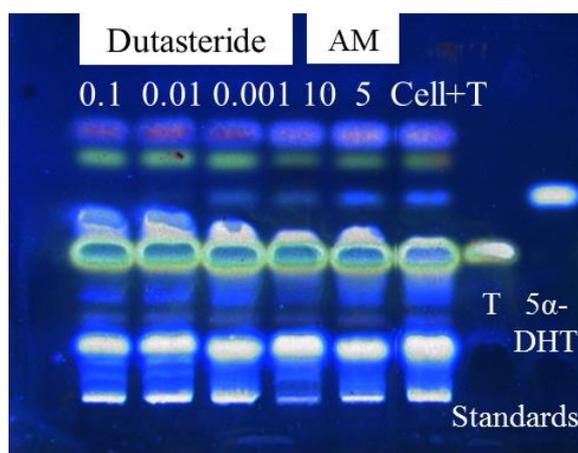
Overproduction of 5 α -DHT is caused by 5 α -R, therefore one potential target to treat AGA is to inhibit this enzymatic reaction. A lot of research has been conducted using different cell types (e.g.,

transfected rat [25] or insect [21] cell lines) for a cell-based assay, or 5α -R enzymes isolated from unrelated organs (e.g., rat liver [26–28], prostate [29,30], epididymis [20] or human prostate [31]) for a cell-free assay. The use of these indirect cells or 5α -R enzymes has raised questions on the reliability of the available assays, therefore, we have developed a new cell-based assay using the appropriate target cells of the hair follicle, HHDPCs which are involved in hair growth and AGA.

The developed HHDPC-based assay system was used to evaluate the 5α -R1 inhibitory activity potential of AM, using dutasteride, a well-known potent inhibitor, as a positive control. The inhibitory activity was detected at 366 nm on the TLC plates based on the amount of 5α -DHT produced relative to the internal control (Cell+T), through a simple method of dipping the developed silica gel 60 F₂₅₄ aluminum TLC plate into 42.5% phosphoric acid and then heating it at 120 °C for 20 min. As non-radiolabelled T was used as the substrate, the visual detection of 5α -DHT produced was recordable at 366nm using a TLC imager. There was no need to use the complicated detection techniques reported so far, which include radioactive image analyzers [27,30], TLC radioactive scanners [25], and HPLC radioactive detectors [21], or measuring the decrease in the radiolabelled T concentration using HPLC [20,28,29,31].

The results revealed that AM extract exhibited 5α -R1 inhibitory activity at the highest final non-toxic concentration of 10 $\mu\text{g/mL}$ as it significantly ($p < 0.05$) reduced 5α -DHT production by 52% compared to a non-significant reduction of 6.65% at 5 $\mu\text{g/mL}$ (Figure 2). Dutasteride exhibited complete inhibition at 0.1 and 0.01 $\mu\text{g/mL}$, while showing 16.5% reduction in 5α -DHT production at 0.001 $\mu\text{g/mL}$. The viability of the attached cells in the 96-well plate was $100.5\% \pm 2.02\%$ ($n = 3$), confirming the non-toxic effect of the AM extract.

Figure 2. 5α -R1 inhibitory activity of AM at 5 and 10 $\mu\text{g/mL}$ (6.65% and 52% inhibition, respectively) and dutasteride at 1, 0.01 and 0.001 $\mu\text{g/mL}$ (0%, 0% and 16.5% inhibition, respectively) using HHDPC-based assay system coupled with non-radioactive TLC detection technique. Cell+T is the internal control.



2.3. TLC Profile and Activity-Guided Fraction of AM

The chemical complexity of AM extract was first observed by TLC. By using a silica gel plate and a mobile phase of toluene–acetonitrile–ethyl acetate–acetic acid in the ratio of 7:1:3:0.03, the constituents of AM appeared to be well separated as observed under the wavelengths of 254

and 366 nm (Figure 3a). Therefore, the preparative TLC technique was used to fractionate each observed band as fractions. Eight fractions were obtained from the preparative TLC, each of which showed clearly the presence of main constituent(s), especially at 366 nm (Figure 3b). Each fraction was then tested for 5 α -R1 inhibitory activity at the final concentration of 10 μ g/mL, and the results are shown in Figure 4. It can be clearly seen that fraction “4”, which is a mixture of at least three compounds, exhibited similar 5 α -R1 inhibitory activity to that of the methanolic crude extract of AM. Therefore, further purification of this fraction was carried out using a double developing TLC system of toluene–acetonitrile in the ratio of 8:2 as the mobile phase, which lead to the isolation of two blue compounds and one green compound labeled as B1, B2 and G1 (Figure 3c).

Figure 3. (a) TLC profile of the methanolic heartwood extract of AM visualized at 254 and 366 nm; (b) TLC plate visualized at 366 nm showing the isolated fractions; (c) Separation of B1, B2 and G1 from fraction “4” through double development of the TLC plate in 8:2 toluene–acetonitrile.

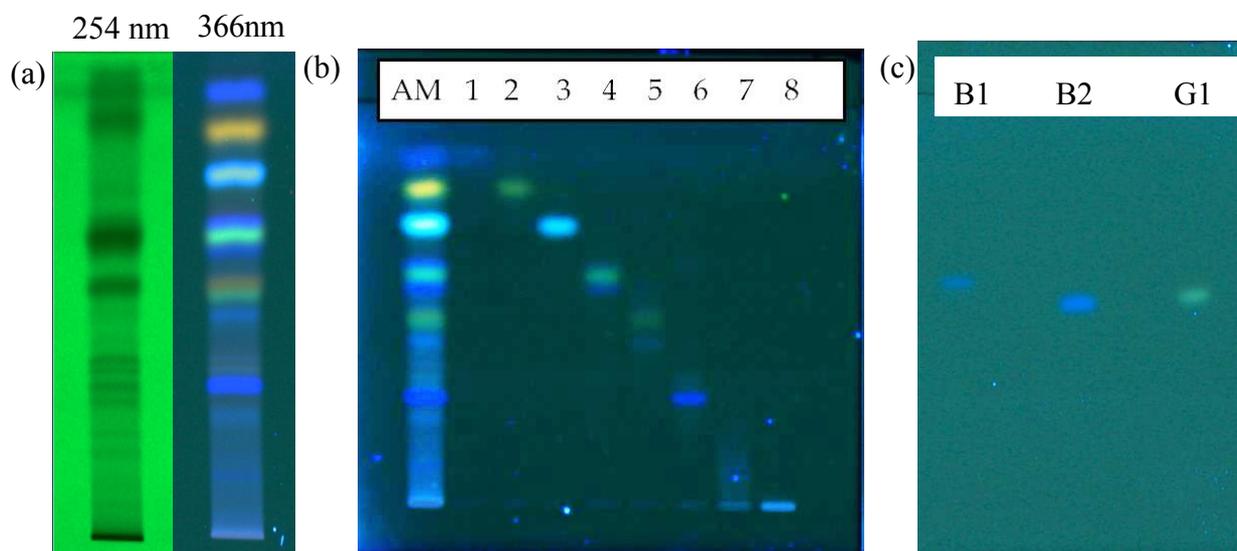
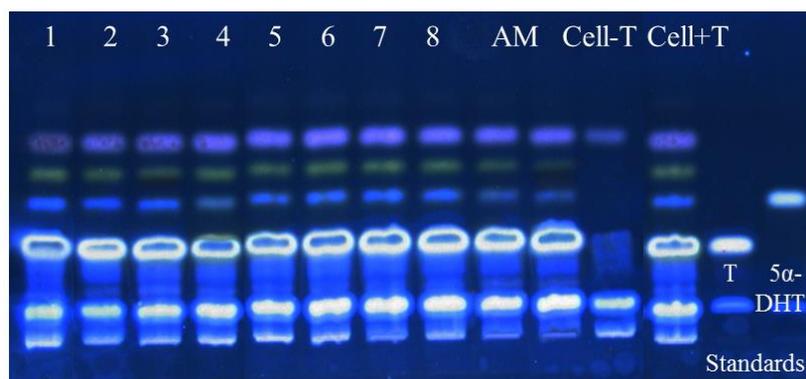
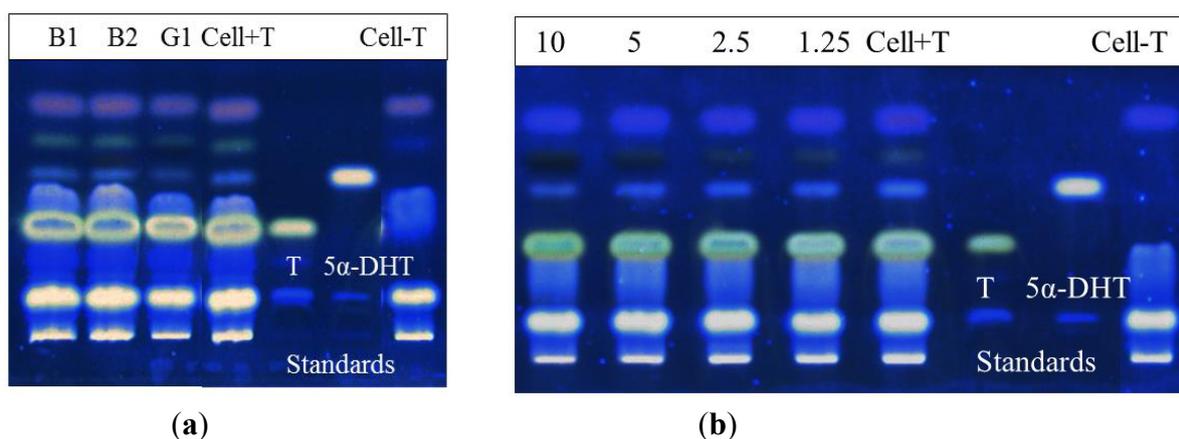


Figure 4. 5 α -R1 inhibitory activity assay of the eight AM fractions numbering 1–8 with 0, 19.2%, 13.3%, 50%, 18.9% 10.7%, 5.7% and 17.1% inhibition, respectively, comparing with the original AM extract (52% inhibition) using HHDPC-based assay system coupled with non-radioactive TLC detection technique. Cell+T is the internal control and Cell–T is the negative control.



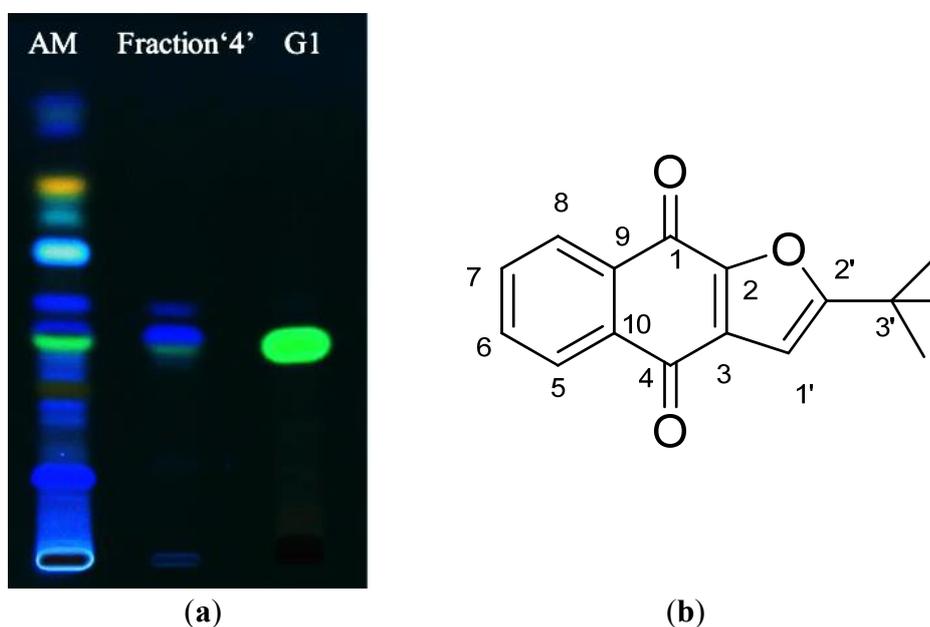
Each compound at the final concentration of 10 $\mu\text{g/mL}$ was again tested for $5\alpha\text{-R1}$ inhibitory activity. As shown in Figure 5a, only G1 alone showed $5\alpha\text{-R1}$ inhibitory activity similar to that of AM with a 50% reduction in $5\alpha\text{-DHT}$ production, giving an IC_{50} of $9.94 \pm 0.33 \mu\text{g/mL}$ (Figure 5b) while B1 and B2 showed only 15% and 12% inhibitory activity, respectively.

Figure 5. (a) $5\alpha\text{-R1}$ inhibitory activity of B1, B2 and G1 at the final concentration of 10 $\mu\text{g/mL}$; and (b) Dose-dependent response of G1 at 1.25, 2.5, 5 and 10 $\mu\text{g/mL}$ (25.5%, 33.2%, 36.3% and 52% inhibition, respectively). Cell+T is the internal control and Cell-T is the negative control.



Subsequently, G1 obtained from the preparative TLC was further purified by semi-preparative HPLC. This was achieved through the application of 45 mg of G1 in 450 μL of DMSO to a TSK gel ODS ($2 \times 25 \text{ cm}$, 5 μm) column using 30% acetonitrile as a mobile phase with a flow rate of 9 mL/min. G1 was eluted as a major pure compound peak at a retention time of 78 min (Figure 6a).

Figure 6. (a) Purity check of G1 compared with AM extract and fraction 4 by a silica gel TLC plate developed by toluene: acetonitrile in the ratio of 8:2 and visualized under 366 nm; (b) Structure of G1, identified as avicequinone C.



NMR analysis of G1 was based on the spectral data of both ^1H -NMR and ^{13}C -NMR. By comparison with previously reported ^1H -NMR and ^{13}C -NMR data [32,33] (Table 1), G1 was identified as naphtho 2'-(1-hydroxy-1-methylethyl)[2,3- β]furan-1,4-dione, or avicequinone C (Figure 6b) with a molecular formula of $\text{C}_{15}\text{H}_{12}\text{O}_4$ and a molecular weight of 256.

Table 1. NMR spectra data of G1 and avicequinone C in CDCl_3 .

Position	G1		Avicequinone C [32,33]	
	^1H (mult., J in Hz)	^{13}C	^1H (mult., J in Hz)	^{13}C
1	-	173.4	-	173.3
2	-	151.8	-	151.6
3	-	131.3	-	131.2
4	-	180.8	-	180.7
5	8.16 (m)	126.9	8.14 (m)	126.8
6	7.75 (m)	133.9	7.73 (m)	133.9
7	7.75 (m)	133.7	7.73 (m)	133.7
8	8.21 (m)	126.8	8.18 (m)	126.8
9	-	132.5	-	132.4
10	-	133.1	-	-
1'	6.82 (s)	102.6	6.80 (s)	102.6
2'	-	167.9	-	168.1
3'	-	69.4	-	69.3
4'	1.69 (s)	28.8	1.67 (s)	28.7
5'	1.69 (s)	28.8	1.67 (s)	28.7

Avicequinone C has a furanonaphthaquinone structure. It was first isolated from AM in 2000 by Ito. *et al.* [32]. The compound has been shown to have antimicrobial and antiproliferative activities [34]. In this study, avicequinone C showed its 5α -R1 inhibitory activity, with an IC_{50} value of $38.8 \pm 1.29 \mu\text{M}$. Although this value indicates moderate potency, it is still better than or equivalent to the reported IC_{50} values of many natural products isolated from plants, for example, the IC_{50} values of $112 \mu\text{M}$ for soyasaponin1 from *Pueraria thomsonii* [20], $85 \mu\text{M}$ for artocarpin from *Artocarpus incises* [35], $40 \mu\text{M}$ for emodin from *Polygonum multiflorum* Thunb [36], $31.7 \mu\text{M}$ for triolin from *Torillia japonica* [37], $390 \mu\text{M}$, $230 \mu\text{M}$, $220 \mu\text{M}$ and $220 \mu\text{M}$ for 1,7-diphenylhept-4-en-3-one, dihydroyashabushiketol, 5-hydroxy-7-(4"-hydroxy-3"-methoxyphenyl)-1-phenyl-3-heptanone and 5-hydroxy-7-(4"-hydroxyphenyl)-1-phenyl-3-heptanone from *Alpinia officinarum* [38] and $44 \mu\text{M}$, $103 \mu\text{M}$ and $48 \mu\text{M}$ for (-)-cubebin, (-)-3,4-dimethoxy-3,4-desmethylenedioxcubebin and piperine from *Piper nigrum*, respectively [39].

The presence of the 1,4-naphthoquinone nucleus of avicequinone C might be important for the activity, as it is in alizarin, which exhibited 5α -R1 inhibitory activity in both cell-free and cell-based assays with IC_{50} values of 3 and $6 \mu\text{M}$, respectively [25]. Purpurin, an anthraquinone, also exhibited 5α -R1 inhibitory activity in a cell-free assay with an IC_{50} of $2 \mu\text{M}$ [25]. However, to date only these two naphthoquinones have been identified as 5α -R inhibitors and no structure-activity relationships have been conducted on this group of compounds.

3. Experimental

3.1. Chemicals, Enzymes and Reagents

All of the organic solvents used were analytical grade and purchased from RCI Labscan (Bangkok, Thailand). Ultrapure grade dimethyl sulfoxide (DMSO) was purchased from Ameresco[®] (Framingham, MA, USA). T and 5 α -DHT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dutasteride was purchased from BDG Synthesis (Wellington, New Zealand). Agarose-LE was purchased from Affymetrix (Santa Clara, CA, USA). Mesenchymal stem cell medium and its supplements were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). Fetal bovine serum, 100 \times antibiotic-antimycotic solution, 10 \times PrestoBlue[®] (Life Technologies, Carlsbad, CA, USA), RPMI medium, 50 \times Tris-acetate-EDTA (TAE) buffer, 0.25% trypsin-EDTA and Platinum[®] *Taq* polymerase kit were purchased from Invitrogen (Grand Island, New York, NY, USA). A GeneRuler 1-kb DNA ladder was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). RNeasy[®] mini kit was purchased from Qiagen (Valencia, CA, USA). DNase I enzyme, EDTA, first-strand cDNA synthesis kit, dATP, dTTP, dCTP and dGTP were purchased from Fermentas (Waltham, MA, USA).

3.2. Plant Material and Extraction

The heartwood of AM was obtained from a local Thai-Chinese medicinal store and was first ground into powder and further subjected to maceration using 100% methanol at room temperature for two days. The methanolic extract was then evaporated to dryness at 45 °C using a rotary evaporator (Rotavapor R-210, Buchi, Flawil, Switzerland) and kept at −20 °C until used.

3.3. Culturing of HHDPCs

HHDPCs, obtained from Sciencell Research Laboratories, were grown in mesenchymal stem cell medium containing 5% fetal bovine serum (FBS), mesenchymal stem cell medium supplement, and 1 \times antibiotic-antimycotic solution at 37 °C in 5% CO₂. The cells between passages 2 to 6 were used in this study.

3.4. Checking for the Presence of 5 α -R in the HHDPCs

Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to identify the isoforms of 5 α -R (*i.e.*, 5 α -R1 and/or 5 α -R2), expressed in HHDPCs from passages 2, 4, 5 and 6. The forward and reverse primers for the two isoforms of 5 α -R and β -actin, shown in the Table 2, were designed from the protein region of the full length sequence obtain from the NCBI GenBank using Clone Manager (Scientific & Educational Software, Cary, NC, USA) and made to order at 1st Base Laboratories (Selangor, Malaysia). The PCR products were analyzed using 1% agarose gel electrophoresis.

Table 2. Forward and reverse primers and expected sizes of 5 α -Rs and β -actin.

Name	Primer pair		Expected size (bp)
	F	R	
5 α -reductase type 1 (5 α -R1) GenBank: NM_001047.2	F:5' ACTGCATCCTCCTGGCCATGTTC 3'	R:5' GGCATAGCCACACCACTCCATGA 3'	380
5 α -reductase type 2 (5 α -R2) GenBank: NM_000348.3	F:5' AAGCACACGGAGAGCCTGAA 3'	R:5' GCCACCTTGTGGAATCCTGTAGC 3'	450
β -actin (internal control) GenBank: NM_001101.3	F:5' ATGATGATATCGCCGCGCTC 3'	R:5' GCGCTCGGTGAGGATCTTCA 3'	584

3.5. Cytotoxicity of AM on HHDPC

In order to obtain a suitable starting concentration for testing the inhibitory activity, the highest non-toxic concentration of AM was determined. HHDPCs were seeded at a cell density of 1×10^5 cells/mL onto 96-well plates (100 μ L of 10,000 cells/well). After 24 h, the cells were separately treated with 100 μ L of AM or 1% DMSO (control). The concentration of AM ranges from 40, 20, 10 and 5 μ g/mL with the final concentrations of 20, 10, 5 and 2.5 μ g/mL, respectively. Cell viability was measured 24 h after the treatment using $1 \times$ PrestoBlue[®] (Life Technologies) reagent in RPMI medium. In the presence of viable cells, PrestoBlue[®] changes from a non-fluorescent blue color to a fluorescent purple-pink color, which is detected using the Multimode Detector DTX 880 (Beckman Coulter[®], Indianapolis, IN, USA), a bottom-read fluorospectrophotometer with an excitation/emission of 535/615 nm. The results showed that AM was not toxic to HHDPCs, (*i.e.*, cell viability more than 85%) up to the final concentration of 10 μ g/mL of AM. Therefore, this concentration was used as the starting concentration for the 5 α -R1 inhibitory activity test.

3.6. 5 α -R inhibitory Activity Test

HHDPCs were seeded at a cell density of 1×10^5 cells/mL onto 96-well plates (100 μ L of 10,000 cells/well). After 24 h, the cells were separately treated with 50 μ L of 4×10^{-4} M T and 50 μ L of 2% DMSO (internal control); 50 μ L of 4×10^{-4} M T and 50 μ L of 40 μ g/mL AM; and 100 μ L of 2% DMSO (negative control). The cells were treated for 48 h, before the cell culture medium was collected in Eppendorf tubes, and the attached cells were tested for cell viability using the $1 \times$ PrestoBlue[®] (Life Technologies) reagent in RPMI medium in order to avoid false positive result. T and its product, 5 α -DHT, were extracted from the cell culture medium using an equal volume of ethyl acetate. The ethyl acetate fraction was then dried, reconstituted with 20 μ L of methanol and spotted on a TLC Silica gel 60 F₂₅₄ aluminum plate (Merck, Darmstadt, Germany). The TLC plate was developed using toluene–acetone at a ratio of 8:2 as the mobile phase [30]. The developed TLC plate was dipped in a solution of 42.5% phosphoric acid and heated at 120 $^{\circ}$ C for 20 min, for the visual detection of 5 α -DHT at 366 nm using a TLC reprostar imager (Camag, Muttenz, Switzerland), and the amount was quantified using an image analyzing program, Quantity One (Bio-Rad, Hercules, CA, USA). The inhibitory activity was determined through the decrease in 5 α -DHT production relative to the internal control.

3.7. TLC Profile of AM

TLC was used to observe the complexity of the methanolic heartwood extract of AM. The TLC silica gel 60 F₂₅₄ aluminum plate was spotted with 10 µL of AM at the concentration of 3.5 mg/mL and was developed in toluene–acetonitrile–ethyl acetate–acetic acid in the ratio of 7:1:3:0.03 as the mobile phase. The developed plate was then visualized under the wavelengths of 254 and 366 nm.

3.8. Isolation and Structural Analysis of Bioactive Compound(s) within AM

Preparative TLC was used to separate and isolate the compounds in AM. The extract was developed, using the same system as mentioned above, on a preparative TLC Silica gel 60 F₂₅₄ glass plate and each band/fraction was isolated through scraping. The bands/fractions were then tested for 5 α -R inhibitory activity using the developed assay system. The purity of active compound(s) were checked using HPLC before structure elucidations were conducted using ¹H-NMR and ¹³C-NMR: ¹H-NMR (400 MHz, CDCl₃) δ_H (mult (*J* in Hz); H): 8.16 (*m*; 1H, H-5), 8.21 (*m*; 1H, H-8), 7.75 (*m*; 2H, H-6, H-7), 6.82 (*s*; 1H, H-1'), 1.69 (*s*; 2H, H-4', H-5'); ¹³C-NMR (100 MHz, CDCl₃) δ_C : 173.4 (C-1), 151.8 (C-2), 131.3 (C-3), 180.8 (C-4), 126.9 (C-5), 133.9 (C-6), 133.7 (C-7), 126.8 (C-8), 132.5 (C-9), 133.1 (C-10), 102.6 (C-1'), 167.9 (C-2'), 69.4 (C-3'), 28.8 (C-4', C-5').

3.9. Statistical Analysis

All of the experiments were performed in triplicate, and the data are presented as the means \pm SD. One-way single factor ANOVA was used and a *p*-value < 0.05 was taken to be statistically significant.

4. Conclusions

A natural furanonaphthaquinone exhibiting 5 α -R1 inhibitory activity (IC₅₀ of 38.8 \pm 1.29 µM) and identified as avicequinone C, a known compound, was successfully isolated from the heartwood of AM. This was accomplished by a simple activity-guided fractionation using TLC as a tool for both the cell-based assay detection and compound isolation. In addition, HHDPCs was used in this cell-based assay due to its properties of being the main regulator of hair growth as they are the only cells within the hair follicle that are the direct site of 5 α -DHT action, and therefore, considered to be the right target and used for the first time to screen for anti-AGA compounds. Further studies on the modification of avicequinone C might lead to more potent 5 α -R1 inhibitors with higher potential in treating AGA.

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Author Contributions

Ruchy Jain: Experimental design and conduct, data analysis and manuscript preparation; Orawan Monthakantirat: Preparation of plant crude extracts and structure elucidation; Parkpoom Tengamnuay:

Co-supervisor of Ruchy Jain, giving comments and suggestions; Wanchai De-Eknamkul: Main supervisor of Ruchy Jain, project leader, giving comments and suggestions and manuscript preparation.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Zhu, F.; Chen, X.; Yuan, Y.; Huang, M.; Sun, H.; Xiang, W. The chemical investigations of the mangrove plant *Avicennia marina* and its endophytes. *Open Nat. Prod. J.* **2009**, *2*, 24–32.
2. Liebezeit, G.; Rau, M.T. New Guinean mangroves—Traditional usage and chemistry of natural products. *Senckenb. Maritima* **2006**, *36*, 1–10.
3. Bandaranayake, W. Traditional and medicinal uses of mangroves. *Mangroves Salt Marshes* **1998**, *2*, 133–148.
4. Vinod Prabhu, V.; Guruvayoorappan, C. Phytochemical screening of methanolic extract of mangrove *Avicennia marina* (Forssk.) Vierh. *Pharm. Sin.* **2012**, *3*, 64–70.
5. Sukhramani, P.S.; Vidyasagar, G.; Patel, P.M. Biological screening of *Avicennia marina* for anticancer activity. *Pharm. Sin.* **2013**, *4*, 125–130.
6. Khafagi, I.; Gab-Alla, A.; Salama, W.; Fouda, M. Biological activities and phytochemical constituents of the gray mangrove *Avicennia marina* (Forssk.) Vierh. *Egypt. J. Biol.* **2003**, *5*, 62–69.
7. Patra, J.K.; Thatoi, H.N. Metabolic diversity and bioactivity screening of mangrove plants: A review. *Acta Physiol. Plant.* **2011**, *33*, 1051–1061.
8. Hsueh, A.; Wang, C.; Erickson, G. Direct inhibitory effect of gonadotropin-releasing hormone upon follicle-stimulating hormone induction of luteinizing hormone receptor and aromatase activity in rat granulosa cells. *Endocrinology* **1980**, *106*, 1697–1705.
9. Dimattina, M.; Albertson, B.; Seyler, D.E.; Loriaux, D.L.; Falk, R.J. Effect of the antiprogestin RU486 on progesterone production by cultured human granulosa cells: Inhibition of the ovarian 3 β -hydroxysteroid dehydrogenase. *Contraception* **1986**, *34*, 199–206.
10. Azzouni, F.; Godoy, A.; Li, Y.; Mohler, J. The 5 alpha-reductase isozyme family: A review of basic biology and their role in human diseases. *Adv. Urol.* **2011**, doi:10.1155/2012/530121.
11. Tobin, D.J. The biogenesis and growth of human hair. In *Hair Toxicology—An Important Bio-Monitor*; The Royal Society of Chemistry: Cambridge, UK, 2005.
12. Price, V.H. Androgenetic alopecia in women. In *Journal of Investigative Dermatology Symposium Proceedings*; Nature Publishing Group: London, UK, 2003; pp. 24–27.
13. Dinh, Q.Q.; Sinclair, R. Female pattern hair loss: Current treatment concepts. *Clin. Interv. Aging* **2007**, *2*, 189–199.
14. Stough, D.; Stenn, K.; Haber, R.; Parsley, W.M.; Vogel, J.E.; Whiting, D.A.; Washenik, K. Psychological effect, pathophysiology, and management of androgenetic alopecia in men. *Mayo Clin. Proc.* **2005**, *80*, 1316–1322.
15. Itami, S.; Inui, S. Role of androgen in mesenchymal epithelial interactions in human hair follicle. *J. Investig. Dermatol. Symp. Proc.* **2005**, *10*, 209–211.

16. Chen, W.; Orfanos, C. The 5 α -reductase system and its inhibitors. *Dermatology* **1996**, *193*, 177–184.
17. Bayne, E.; Flanagan, J.; Einstein, M.; Ayala, J.; Chang, B.; Azzolina, B.; Whiting, D.; Mumford, R.; Thiboutot, D.; Singer, I. Immunohistochemical localization of types 1 and 2 5-reductase in human scalp. *Br. J. Dermatol.* **1999**, *141*, 481–491.
18. Messenger, A.G. The control of hair growth: An overview. *J. Investig. Dermatol.* **1993**, *101*, 4S–9S.
19. Rho, S.-S.; Park, S.-J.; Hwang, S.-L.; Lee, M.-H.; Kim, C.D.; Lee, I.-H.; Chang, S.-Y.; Rang, M.-J. The hair growth promoting effect of *Asiasari radix* extract and its molecular regulation. *J. Dermatol. Sci.* **2005**, *38*, 89–97.
20. Murata, K.; Noguchi, K.; Kondo, M.; Onishi, M.; Watanabe, N.; Okamura, K.; Matsuda, H. Inhibitory activities of Puerariae Flos against testosterone 5 α -reductase and its hair growth promotion activities. *J. Nat. Med.* **2012**, *66*, 158–165.
21. Raynaud, J.-P.; Cousse, H.; Martin, P.-M. Inhibition of type 1 and type 2 5 α -reductase activity by free fatty acids, active ingredients of Permixon[®]. *J. Biochem. Mol. Biol.* **2002**, *82*, 233–239.
22. Pais, P. Potency of a novel saw palmetto ethanol extract, SPET-085, for inhibition of 5 α -reductase II. *Adv. Ther.* **2010**, *27*, 555–563.
23. Ellis, J.A.; Sinclair, R.; Harrap, S.B. Androgenetic alopecia: Pathogenesis and potential for therapy. *Expert Rev. Mol. Med.* **2002**, *2*, 1–11.
24. Eicheler, W.; Dreher, M.; Hoffmann, R.; Happle, R.; Aumüller, G. Immunohistochemical evidence for differential distribution of 5 α -reductase isoenzymes in human skin. *Br. J. Dermatol.* **1995**, *133*, 371–376.
25. Hiipakka, R.A.; Zhang, H.-Z.; Dai, W.; Dai, Q.; Liao, S. Structure-activity relationships for inhibition of human 5 α -reductases by polyphenols. *Biochem. Pharmacol.* **2002**, *63*, 1165–1176.
26. Matsuda, H.; Yamazaki, M.; Naruto, S.; Asanuma, Y.; Kubo, M. Anti-androgenic and hair growth promoting activities of *Lygodii Spora* (Spore of *Lygodium japonicum*) I. Active constituents inhibiting testosterone 5 α -reductase. *Biol. Pharm. Bull.* **2002**, *25*, 622–626.
27. Liu, J.; Kurashiki, K.; Shimizu, K.; Kondo, R. Structure-activity relationship for inhibition of 5 α -reductase by triterpenoids isolated from *Ganoderma lucidum*. *Bioorg. Med. Chem.* **2006**, *14*, 8654–8660.
28. Kumar, N.; Rungseevijitprapa, W.; Narkkhong, N.-A.; Suttajit, M.; Chaiyasut, C. 5 α -reductase inhibition and hair growth promotion of some Thai plants traditionally used for hair treatment. *J. Ethnopharmacol.* **2012**, *139*, 765–771.
29. Kumar, T.; Chaiyasut, C.; Rungseevijitprapa, W.; Suttajit, M. Screening of steroid 5 α -reductase inhibitory activity and total phenolic content of Thai plants. *J. Med. Plants Res.* **2011**, *5*, 1265–1271.
30. Roh, S.-S.; Kim, C.D.; Lee, M.-H.; Hwang, S.-L.; Rang, M.-J.; Yoon, Y.-K. The hair growth promoting effect of *Sophora flavescens* extract and its molecular regulation. *J. Dermatol. Sci.* **2002**, *30*, 43–49.
31. Pandit, S.; Chauhan, N.S.; Dixit, V. Effect of *Cuscuta reflexa* Roxb on androgen—Induced alopecia. *J. Cosmet. Dermatol.* **2008**, *7*, 199–204.
32. Ito, C.; Katsuno, S.; Kondo, Y.; Tan, H.; Furukawa, H. Chemical constituents of *avicennia alba*. isolation and structural elucidation of new naphthoquinones and their analogues. *Chem. Pharm. Bull.* **2000**, *48*, 339–343.

33. Rui, J.; Yuewei, G.; Huixin, H. Studies on the chemical constituents from leaves of *Avicennia marina*. *Chin. J. Nat. Med.* **2004**, *2*, 16–19.
34. Han, L.; Huang, X.; Dahse, H.-M.; Moellmann, U.; Fu, H.; Grabley, S.; Sattler, I.; Lin, W. Unusual naphthoquinone derivatives from the twigs of *Avicennia marina*. *J. Nat. Prod.* **2007**, *70*, 923–927.
35. Shimizu, K.; Fukuda, M.; Kondo, R.; Sakai, K. The 5 α -reductase inhibitory components from heartwood of *Artocarpus incisus*: Structure-activity investigations. *Planta Med.* **2000**, *66*, 16–19.
36. Cho, C.-H.; Bae, J.-S.; Kim, Y.-U. 5 α -reductase inhibitory components as antiandrogens from herbal medicine. *J. Acupunct. Meridian Stud.* **2010**, *3*, 116–118.
37. Park, W.S.; Son, E.D.; Nam, G.W.; Kim, S.H.; Noh, M.S.; Lee, B.G.; Jang, I.S.; Kim, S.E.; Lee, J.J.; Lee, C.H. Torilin from *Torilis japonica*, as a new inhibitor of testosterone 5 α -reductase. *Planta Med.* **2003**, *69*, 459–461.
38. Kim, Y.-U.; Son, H.K.; Song, H.K.; Ahn, M.-J.; Lee, S.S.; Lee, S.K. Inhibition of 5 α -reductase activity by diarylheptanoids from *Alpinia officinarum*. *Planta Med.* **2003**, *69*, 72–74.
39. Hirata, N.; Tokunaga, M.; Naruto, S.; Iinuma, M.; Matsuda, H. Testosterone 5 α -reductase inhibitory active constituents of *Piper nigrum* leaf. *Biol. Pharm. Bull.* **2007**, *30*, 2402–2405.

Sample Availability: Sample of the compound avicequinone C is available from the authors.

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