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5α-Reductase type 1 inhibition of *Oryza sativa* bran extract prepared by supercritical carbon dioxide fluid

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ABSTRACT

The three crude extracts including *Oryza sativa* (bran) from supercritical carbon dioxide fluid ($scCO_2$) process which gave the highest unsaturated fatty acid contents and biological activities including the antioxidative, tyrosinase inhibition, stimulation index on human normal skin fibroblast were selected from ten edible plants to prepare the semi-purified fractions. Fraction No. 3 of the *O. sativa* bran crude extract gave the highest content of unsaturated fatty acids and 5α-reductase (type 1) inhibition activity (5AR). Its linoleic acid (LN) and total unsaturated fatty acid (TUC) contents were significantly positive and linear correlated to 5AR on DU-145 cell line (at r of 1.00, $p < 0.01$). Its total phenolic contents and all biological activities also showed positive correlations to 5AR with $r > 0.9$ ($p < 0.05$). This study has demonstrated the potential of fraction No. 3 fractionated from the *O. sativa* bran crude extract prepared by $scCO_2$ to be developed as anti-androgenic alopecia products.

1. Introduction

In the US, an estimated of 40 million men and 20 million women suffer from baldness and have spent $1.5 billion annually on hair loss therapies [1]. Dihydrotestosterone (DHT), a potent male hormone, is the cause of genetic male pattern baldness since it has been shown that the DHT levels, numbers of the DHT receptors on the hair follicles and the 5α-reductase enzyme activity (which converts testosterone to DHT) increase in the balding scalp of androgenic alopecia patients [2–4]. DHT has three times greater affinity for androgen receptors than testosterone, which is the main cause of the androgenic alopecia leading to the miniaturization of hair follicle and hair shedding [5]. The 5α-reductase inhibitor type 2, finasteride has been approved by the US FDA to use in male pattern baldness, whereas dutasteride (the type 1 and 2 5α-reductase inhibitor) has been approved for the treatment of symptomatic benign prostatic hyperplasia (BPH), but still hold on phase III for the treatment of male pattern hair loss [6]. The type 1, 5α-reductase (SRD5A1) related to genetic male pattern baldness, is expressed predominantly in the skin, scalp, sebaceous gland, liver and brain, whereas the type 2, 5α-reductase (SRD5A2) is found predominantly in androgen target organs such as prostate, genital skin, and seminal vesicles [7]. Finasteride gives several side effects, such as the decrease of libido, erectile dysfunction, ejaculation disorder and gynecomastia, while dutasteride (5 weeks) has longer half life than finasteride (5–6 h) and is more difficult to reverse the side effects. Although finasteride can block the type 2, 5α-reductase in androgen target organs more than that in the hair follicle, it has been approved to use in male pattern baldness since it has lower side effects than dutasteride. Both drugs cannot be used in childbearing age females with the pregnancy category X which show the risks of fetal injury or birth defects.

Nowadays, natural extracts from several plants have been used for hair growth promotion such as *Asiasari radix* [8], *Eclipta alba* [9], essential oil of *Chamaecyparis obtuse* [10], *Zizyphus jujube* [11] *and Sophora flavescens* [12]. Most extracts have targeted on the induction of growth factors in hair follicle cells, such as insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF) and epidermal growth factor (EGF), but not on the production of 5α-reductase enzyme. The unsaturated fatty acids, such as γ-linolenic acid, linoleic acid and oleic acid, have been proved to have anti-hair loss activity by inhibiting 5α-reductase enzyme in the androgen responsive organs [13]. In fact, several edible plants contain these unsaturated fatty acids in variable amounts, such as *Carthamus tinctorius* L. (safflower), *Helianthus annuus* L. (sunflower), *Linum usitatissimum* L. (flaxseed), *Sorghum bicolor* (L.) Moench (sorghum) and bran of

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Oryza sativa L. (rice). As known, rice bran is discarded or used as livestock feed and oil production. Rice bran oil is edible and has been claimed for improving serum cholesterol levels and lipoprotein profiles similar to other vegetable oils, such as corn and safflower oil [14,15]. Rice bran oil has been extracted from rice bran using $scCO_2$ by both pilot [16] and lab scale [17]. The health promotion properties of rice bran are from its unsaturated fatty acid contents such as palmitic acid, oleic acid, linoleic acid and γ-linolenic acid, which are known as antioxidants and anti-cancer agents by stimulating the production of the substances which can protect cells from peroxides [18] and anti-hair loss agents [13]. The raw rice bran oil contains both unsaturated and saturated fatty acids, in which palmitic acid is a major acid (C18:0, 12–26%, w/w, typically 18%, w/w). The unsaturated fatty acids are mainly oleic acid (C18:1, 35–46%, w/w, typically 42%, w/w) and linoleic acid (C18:2, 25–38%, w/w, typically 37%, w/w) with the traces of C18:3 acid (0.4–3.8%, w/w) [19,20]. It has been reported that the quality of rice bran oil extracted by SFE was far superior to that produced by hexane extraction, especially at 80 °C [21]. The phenolic compounds and the unsaturated fatty acids containing in *C. tinctorius* have been shown to have antioxidant activity [22] which can recover or slow down the miniaturization of hair follicle. Their redox properties of many bioactive compounds can lead to hair shedding and act as reducing agents, hydrogen donators as well as the singlet oxygen quenchers in the hair follicle [23,24]. Also, there were several anti-hair loss medicinal plant recipes which have been traditionally used by the Chinese and Thai people for over 100 years. But, most of recipes and plants have no scientific evidences on anti-hair loss activity. The relationship between this activity and the antioxidant activity as well as the bioactive compounds in plants still never been explored. Recently, supercritical fluids have been introduced as an alternative one step at low temperature for the preparation of plant extracts. At the critical point, supercritical fluids have the density as liquid, but low viscosity with better flow property as gas. Carbon dioxide is a widely used gas to produce supercritical fluid because of its low critical temperature (Tc = 31.1 °C) and pressure (Pc = 73.8 bar). It has high solvating power at near critical point. Supercritical carbon dioxide ($scCO_2$) has been used for the substitution of organic solvent to extract many plant extracts containing thermal sensitive constituents with the advantages of not only being environmental friendly, non-toxic and nonflammable, but also inexpensive [25,26]. It has been used as an alternative to organic solvent for the extraction of many nuts and seeds, such as bran of *O. sativa* (rice) [27], *Arachis hypogaea* L. (peanuts) [28] and *Glycine max* (L.) Merr. (soybean) [29]. Although, the maceration method which is a simply and low cost method, it requires large amount and high purity of the organic solvents which are usually hazardous and flammable solvent wastes and are generally cumbersome [26]. Moreover, it is the non-selective, time consuming and toxic fume emission extraction in comparing to the supercritical carbon dioxide extraction procedure.

This present study has compared the 5α-reductase type 1 inhibition in DU-145 cell line of the $scCO_2$ crude extract of *O. sativa* bran, *C. tinctorius* flowers and *S. bicolor* seeds and their semi-purified fractions. The relationship between the 5α-reductase type 1 inhibition activity and other biological activities as well as the bioactive contents in the crude and semi-purified extracts were evaluated.

2. Materials and methods

2.1. Materials

Gallic acid (99.0%), vitamin C (L-(+)-ascorbic acid, 99.5%), 2,2-diphenyl-1-picryhydrazyl radical (DPPH), EDTA, sulforhodamine B (SRB), dimethyl sulfoxide (DMSO), kojic acid (99.0%), ferrozine, finasteride (99.5%), Folin–Ciocalteu reagent and ferric chloride ($FeCl_2$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mushroom tyrosinase (4187 U/mg) and L-tyrosine were purchased from Fluka (Buchs, Switzerland). Linoleic acid (99.0%), oleic acid (98.5%) and 1,6 diphenyl-1,3,5-hexatriene were from Wako Pure Chemical Industrial Ltd. (Osaka, Japan). γ-Linolenic acid (99.5%) was purchased from Tokyo Chemical Industrial Ltd. (Tokyo, Japan). The standard dutasteride (99.5%) was purchased from Ka-Shing Business Macau Co., Ltd. (Macau, China). Siliga gel 60 was purchased from Merck (Damstadt, Germany). Dulbecco's modified Eagle's culture medium, antibiotics penicillin and streptomycin, fetal bovine serum and trypsin were purchased from HyClone (Logan, UT, USA). All other reagents and solvents were of analytical grade.

2.2. Plant crude extract

2.2.1. Plant sample

Parts of the ten edible plants which have been searched from the literature reviews to contain high amount of unsaturated fatty acids (γ-linolenic acid, linoleic acid and oleic acid) were collected from Chiang Mai Province in Thailand during October to November in 2008 (Table 1). The plant seeds used in this study were packed in vacuum plastic bags and purchased from Thai Cereals World Co., Ltd., Bangkok, Thailand. The % moisture contents in the seeds were in the range of 6–10%. The specimen samples were authenticated by a botanist at the Natural Products Research Development Center (NPRDC), Science and Technology Research Institute (STRI) at Chiang Mai University, Chiang Mai in Thailand.

2.2.2. Plant preparation

All plant parts were seeds, except *O. sativa* and *C. tinctorius* were bran and flower, respectively. The bran of *O. sativa* was passed through sieve No. 25 (0.707 mm). Other plant parts were ground into small pieces by a blender (Twist HR 1701, Philips, Indonesia) and passed through sieve No. 20 (0.841 mm). The plant powder was kept in a tight container at 4 °C until use.

2.2.3. Maceration method

Briefly, 200 g of the plant powder were macerated with 1 l of 95% (v/v) ethanol at room temperature (27 ± 2 °C) for 8 h and stirred every 2 h. The extract was filtered through the paper filter Whatman No. 1, connected with a vacuum pump. The residues were re-extracted more by the same process twice. All filtrates were collected, pooled and dried by a rotary evaporator (Rotavapor R210, Buchi, Switzerland) at 40 °C. The crude extracts were kept at −80 °C until use.

2.2.4. Supercritical carbon dioxide fluid extraction

Briefly, 200 g of the plant powder were put in the supercritical carbon dioxide fluid apparatus ($scCO_2$) (SFE-500MR-2-C50 System, Thar Instruments, Inc., Pittsburgh, USA) together with 25% (w/v) of 95% (v/v) ethanol as a co-solvent, in the chamber at 40 °C and 200 bar [30]. After 2 h, the pressure was released and the extract was collected. The plant extract residues were re-extracted more by the same procedure 3 times. All extracts were collected, pooled, mixed and dried by a rotary evaporator at 40 °C. The crude extracts were kept at −80 °C until use.

2.2.5. Determination of bioactive compounds and biological activities of the crude extract

The resulting extracts were determined for unsaturated fatty acid and total phenolic contents. The phytochemical tests were also investigated. Briefly, 20 mg of the crude extracts were dissolved in 80% (v/v) methanol and used for detecting the presence of alkaloids, anthraquinones, flavonoids, glycosides, carotenoids,