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Amelioration of postmenopausal osteoporosis and anticancer properties of an antioxidant enriched fraction from *Hygrophila spinosa* T. Anders

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ABSTRACT

In the current study, we have explored an antioxidant enriched fraction (AEF) from whole plant of *H. spinosa* for management of menopausal complications like osteoporosis and cancer. The AEF was identified by determining the total phenolic content, total flavonoid content and antioxidant potential (DPPH method and total antioxidant capacity assay). Ethyl acetate fraction of the crude ethanol extract comprised of the highest antioxidant potential and considered as AEF. HPTLC analysis of AEF confirmed the presence of apigenin and luteolin, which were further investigated for their estrogen receptors modulator effect by docking study. GC/MS analysis also demonstrated the presence of β-sitosterol, and lupeol in AEF. Antiosteoporotic activity was studied by ovariectomized animal model and in vitro anticancer potential by MTT assay. AEF significantly improved biomechanical parameters, body and uterine weight, phosphorous and calcium level in serum and urine. AEF also restored bone turnover markers like alkaline phosphatase, tartrate resistant acid phosphatase and hydroxyproline along with serum cholesterol and triglycerides (biochemical parameters). Histopathology showed restoration of normal bone architecture after AEF and raloxifene treatment. Docking study revealed the high affinity of apigenin and luteolin towards both estrogen receptors (α and β). AEF exerted dose dependent anticancer potential against breast and ovarian cancer cell lines with lowest IC_{50} (43 μg/ml) against SKOV-3 cell line. Our findings conclude that AEF from *H. spinosa* could be used as an alternative and economical botanical drug for treatment of menopausal osteoporosis and cancer.

1. Introduction

Postmenopausal osteoporosis (PMO) has become a major health hazard, afflicting about 50% of postmenopausal women worldwide, and is thought to be a disease with one of the highest incidences in senile people (Sambrook and Cooper, 2006). In India, the expected population by 2050 will be 1613 million out of which 315 million would be adults over 60 years indicating more prevalence of osteoporosis compared to 26 million in 2003 (Desai et al., 2017). Change of trabecular architecture of bone and crystalline properties of mineral deposits occurs during PMO leading to structural failure (fracture) of sites rich with cancellous bone (Zhang et al., 2006a). In women, PMO is predominantly due to reduction in estrogen, which leads to decline in bone formation and increase in bone resorption activity. Sedentary lifestyle, environmental hazards, ovarian disorder, early on-set of puberty, amenorrhea, stress and hormonal variation increases prevalence of menopausal osteoporosis (Jay and Stephen, 2004). Alteration in osteoblastic and osteoclastic functions leads to osteoporosis. Activated osteoclast generates reactive oxygen species (ROS) as superoxides and the level of malondialdehyde increases in blood in osteoporosis. These oxidative stresses also contribute to bone loss in osteoporosis (Desai et al., 2017).

Current treatment regimen of PMO mainly targets i) inhibition of bone resorption, ii) increasing bone formation, and iii) both inhibiting resorption and increasing formation of bone. The drugs used for these purpose are biphosphonates, raloxifene, calcitonin, hormone replacement therapy (HRT), vitamin D, teriparatide, strontium ranelate and selective estrogen receptor modulators (Kapur et al., 2008; Satpathy et al., 2015). However, these strategies have higher risk-to-benefit ratio as these are associated with side effects like breast and ovarian cancer, vaginal bleeding, hypercalcemia, breast tenderness, hot flushes, stroke and heart attack (Colditz et al., 1995; Tripathi, 2008). Therefore, search of new drugs with high benefit-to-risk ratio, low cost and side effects are preferable for management of osteoporosis. Phyto-estrogenic medicines sometimes called “dietary estrogens” are increasingly used

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as alternative therapy for management of PMO. Isoflavones, coumestans, lignins and their derivatives, flavonoids and stilbenoids may be considered phytoestrogens as they can interact with estrogen receptor, alter gene expression, and otherwise affect hormones (Occhiuto et al., 2007; Bedell et al., 2014). Phytochemicals like quercetin, berberine, rutin, luteolin, luteolin-7-O-β-D-glucopyranoside, apigenin-4′-O-β-D-glucoside, wedelolactone, 6′-O-trans-cinnamoyl-catalpol, epigallocatechin-3-gallate, genistein, afzelin, astragalin, pratensein, kaempferol, isorhamnetin and hyperoside (Yogesh et al., 2011; Jia et al., 2012) have shown antiosteoporotic activity. Various mechanisms for antiosteoporotic property of phytoconstituents include i) Inhibition of bone resorption and osteoclast formation, ii) Increased osteoblast mineralization, iii) Reduction of oxidative stress, bone resorbing cytokines and osteoclast differentiation factor, iv) Increasing bone formation parameters like calcium, phosphorus, parathyroid hormone and osteocalcin, v) Stimulation of osteoblastic proliferation and alkaline phosphatase (ALP) activity, vi) Inhibition of osteoclastic tartrate-resistant acid phosphatase (TRAP), vii) Enhancement of bone biomechanical features, and viii) Interaction with estrogen receptor α and/or β.

Hygrophila spinosa T. Anders (Acanthaceae) is described in Ayurvedic literature as Kokilasha "having eyes like Kokila or Indian cuckoo". Different parts of the plant viz. seeds, whole plant, leaves, and roots are used traditionally for treatment of various ailments, and phytomolecules such as lupeol, stigmasterol, β-sitosterol, gallic acid, quercetin, apigenin, luteolin, betulin, apigenin 7-O-glucuronide, apigenin 7-O-glucoside, and luteolin-7-O-rutinoside have been reported in the plant (Patra et al., 2009c; Hussain et al., 2010; Maji et al., 2014). *H. spinosa* has also been screened for antioxidant (Sridhar et al., 2013), antitumor (Nair et al., 2015), anti-inflammatory (Patra et al., 2009a) and antidiabetic (Thorve et al., 2012) activities.

Considering the wide pharmacological activities and presence of chemical constituents like apigenin, luteolin, lupeol, stigmasterol, β-sitosterol, apigenin 7-O-glucuronide, apigenin 7-O-glucoside, and luteolin-7-O-rutinoside in *H. spinosa*, we hypothesized to i) identify an antioxidant enriched fraction (AEF) from the plant, ii) recognize bioactive compounds in AEF by phytochemical analysis (HPTLC and GC/MS), iii) evaluate the antiosteporotic potential of AEF as oxidative stress is an important factor for bone loss, iii) examine the anticancer efficacy of AEF in breast and ovarian cancer cell line as these cancers are common in women after menopause and side effects of the available treatment strategies of PMO, iv) analyze the interaction of bioactive compounds with estrogen receptors by docking studies.

2. Materials and methods

2.1. Plant material and preparation of extracts

The whole plant of *H. spinosa* were collected from Guru Ghasidas University (GGU) campus, Bilaspur, Chhattisgarh, India and authenticated through ICAR-National Bureau of Plant Genetic Resources, Phagli, Shimla, India (No.: NBPGR-565-569). A voucher specimen has been preserved in the Institute of Pharmacy, GGU for future references. The fresh plant materials were cut into small pieces, dried under shade, and then coarsely powdered. Coarse powder material was completely extracted with 95% ethanol using soxhlet apparatus. The extract was dried with the help of rotary vacuum evaporator. The extract was suspended in distilled water and successively fractionated with different solvents as shown in Fig. 1. All the fractions were dried and stored in air tight container until further use.

2.2. Determination of total phenolic content (TPC) and total flavonoid content (TFC)

TPC and TFC were determined using Folin–Ciocalteu and aluminum chloride colorimetric methods, respectively (Madaan et al., 2011) by reconstituting the dried extracts in methanol (1 mg/ml). TPC of the samples was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg of GAE/g of DW) through the calibration curve of gallic acid. TFC was represented as mg of rutin equivalents per gram (mg RE/g) of the sample through a standard curve of rutin. All measurements were carried out in triplicate.

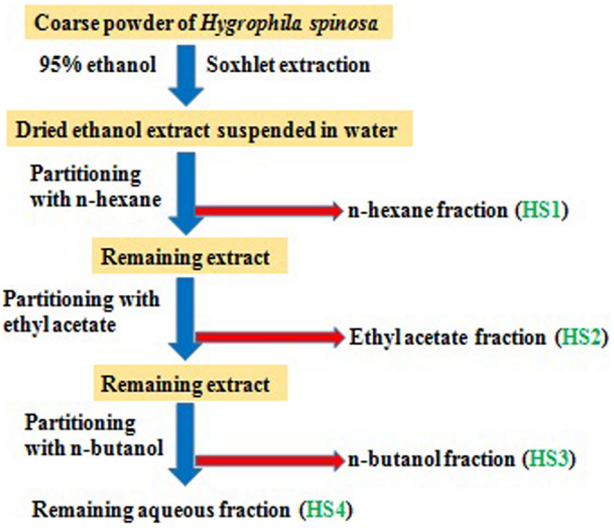

Fig. 1. Scheme for extraction and preparation of different fractions of *H. spinosa*.

2.3. Antioxidant activity

2.3.1. DPPH method

Antioxidant activity of the samples to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured spectrophotometrically (Patra et al., 2009b). Two (2.0) milliliters of various concentrations of the extracts (10–50 mg/ml in methanol) were added to 1.0 ml of methanolic solution of DPPH (0.2 mM). Methanol was used as control in place of the samples. The solutions were kept in the dark for 1 h at room temperature, and the absorbance at 517 nm was determined. The radical scavenging activity was represented as percentage inhibition of DPPH radical, and was calculated using the following formula. The concentration producing 50% inhibition (IC_{50}) was also determined.

$$\%\text{Inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

2.3.2. ABTS assay

Total antioxidant capacity (TAC) of extracts was determined based on their ability to interact with ABTS radicals (Spigno and Faveri, 2009). The assay procedure was performed following the instructions in the product information manual provided with the TAC assay kit from Sigma-Aldrich, MO, USA (Catalog Number MAK187). The components of the kit sufficient for 100 assays in 96 well plates were Cu^{+2} reagent (Catalog Number MAK187A), assay diluent (Catalog Number MAK187B), protein mask (Catalog Number MAK187C) and Trolox standard, 1 μmole (Catalog Number MAK187D). Briefly, 10 μl of sample, 90 μl of HPLC water and 100 μl of Cu^{+2} working solution were transferred to each well in a 96 well plate. The contents were mixed thoroughly using a horizontal shaker and incubated for 90 min in light protected condition at room temperature. Then, the absorbance was measured at 570 nm and Trolox equivalents in terms of μM/g of sample was calculated from the standard curve of Trolox.