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EVALUATION OF PHYTOCHEMICAL PROFILE AND ANTIOXIDANT ACTIVITY OF *EQUISETUM HYEMALE* L.

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ABSTRACT

Objective: *Equisetum hyemale* (EH) has been traditionally used for bone healing. This study focussed on chemical profile and antioxidant potential of aerial part of EH with a view to support its role in improving bone health. **Methods:** The phytochemicals were identified and quantified using HPTLC. The analysis of macro and micro elements was done using inductively coupled plasma mass spectrometer. The antioxidant activity was studied using DPPH free radical scavenging, β-Carotene-linoleic acid assay and reducing power assay. **Results:** The HPTLC studies of aqueous-ethanol (50%) extract of EH (AEEH) showed the presence of rutin (429.4 µg. g^{-1}), β-sitosterol (372.9 µg. g^{-1}), lupeol (259.4 µg. g^{-1}), diosgenin (163.1 µg. g^{-1}), and ellagic acid (27.5 µg. g^{-1}). The key mineral elements identified in the dried powder of EH were iron, zinc, manganese, copper and chromium. The total phenol and total flavonoid content in AEEH were 332.32 mg.g^{-1} and 38.46 mg.g^{-1} dry extracts, respectively. The AEEH showed significant ($p<0.05$) scavenging of DPPH free radicals, IC 50 was 0.081 mg.ml^{-1}.). The anti-lipid peroxidation activity was 48.06% and 60.82 % at a concentration of 1.20 mg/ml for AEEH and α-tocopherol respectively. The reducing power was recorded as 0.268 ± 0.045 for AEEH and 0.362 ± 0.043 for the standard BHA, at a concentration of 1.25 mg/ml. **Conclusion:** Our study justified a prospective role of the *E. hyemale* in bone healing, owing to the presence of various phytocompounds and significant antioxidant potential.

KEYWORDS: *Equisetum hyemale*, Rutin, Diosgenin, Antioxidant, Bone healing.

INTRODUCTION

Equisetum hyemale L. (EH) commonly known as scouring rush is one of the fifteen members of genus *Equisetum* which is the sole surviving representative of class *Sphenopsida.* Morphologically EH is deep green, erect, unbranched and bears hollow stem with reduced leaves at nodes. The cone is sharply pointed, about 7 mm-15 mm long and partly concealed by the teeth of the uppermost sheath. The plant typically grows in wet places, ponds, marshes, wet woodland and the banks of lakes and rivers (Notburga et al. 2005).

Phytochemical investigations suggest the presence of phenols including ferulic acid isomers, feruloyl and caffeoyl glucosides, flavonoids, flavonol glucosides, and alkaloids (Fonsa et al. 2013; Li et al. 2012; Park et al. 2011). Traditionally, the plant is used by people of Mexico for curing kidney disorders, edema, and urinary tract infections (Canales et al. 2005). It is also used for alopecia, tuberculosis, brittle fingernails, rheumatic diseases, gout, frostbite, profuse menstruation and nasal, pulmonary, wounds, burns and gastric hemorrhage (Santos et al. 2010). Moreover, the stems are boiled in water to make a solution which is used to wash sores on children's skin (Thie et al. 1999). The plant relieves symptoms of rheumatoid arthritis (iHerb.com) and osteoporosis owing to its high silica content which strengthens the connective tissues. In addition, the plant is rich in minerals and bioflavonoids which may further contribute to its medicinal properties (www.puralibre.com).

Bone is an organ with tremendous healing properties owing to its repair and regeneration potential. When an injury occurs in bone, the damaged tissues release free radicals, which impair the process of healing (Mohamad et al. 2012; Sheweita et al. 2007). The discrepancy between free radical generation and antioxidant mechanism in biological systems is termed as oxidative stress, and damages the cellular macromolecules and functions. However, when timely combated by the natural defense system of the body singly or in combination with antioxidants provided externally, it may hasten the process of healing. Antioxidants thus facilitate bone healing by scavenging free radicals. Calcium is another major component responsible for fracture healing, however, its uptake and availability in the biological systems becomes a limiting factor (Singh et al. 2011). Literature suggests the bone healing properties of many herbs like *Cissus quadrangularis*, *Cassia occidentalis*, *Horsetail species*, *Moringa oeliferia*, *Vitex negundo* etc. *Cissus quandrangularis* contains vitamins and steroids that markedly hasten the rate of fracture healing by influencing early regeneration of all connective tissues involved in the healing and quicker mineralization of callus. The leaves and stems of *Moringa olifera* are known to have a large amount of calcium bound in form of calcium oxalate crystals, more vitamin than carrot, more calcium than milk more iron than spinach more vitamin c than orange and more potassium than in banana (Singh et al. 2011).

Among the *Equisetum species*, several phytochemical and pharmacological studies have been conducted on *E. arvense* which reveal its antiseptic, diuretic, anti-inflammatory, antioxidant, hepatoprotective, vasorelaxant and anti-nociceptive properties. Moreover, it has also been detected that the plant can affect bone metabolism, helping in the treatment of some bone

disorders, such as osteoporosis, and in the healing of osteocytic tissue (Costa-Rodrigues et al. 2012). However, reports stating the bone healing potential of *Equisetum hyemale* are meager. Despite the age-old use of EH among various tribes of the world, the published data on the validation of its medicinal properties is meager, moreover, the bone strengthening properties of the plant still need to be explored and understood in a systematic manner. The study focussed on the chemical profiling and antioxidant potential of aerial part of EH with a view to support its role in improving bone health.

MATERIALS AND METHODS

Plant material

The aerial part of EH was collected from Pantnagar, Uttarakhand, India, identified by Dr. AKS Rawat, of CSIR-National Botanical Research Institute (NBRI), Lucknow, and a voucher specimen (No. LWG 98570) was deposited in the department of Plant Systematics and Herbarium at CSIR-NBRI.

Chemicals and instruments

All chemicals and solvents used in the study were of analytical grade. DPPH was obtained from Sigma Chemicals (St. Louis, Mo, U.S.A.). Other chemicals were obtained from Ranbaxy Fine Chemicals Ltd., India. UV spectrophotometer (Shimadzu 160 IPC), homogenizer, centrifuge (Remi, India) and pH meter (Elico Ltd., India) were the instruments used for the study. HPTLC was performed on CAMAG system with winCATS 3.2.1 software and TLC silica gel plates were purchased from Merck.

Ethics Statement

For the collection of plants, no specific permits were required for the described field studies. For any locations/activities, no specific permissions were required. All locations where the plants were collected were not privately-owned or protected in any way and the field studies did not involve endangered or protected species.

Preparation of extract

The shade-dried aerial parts of *E. hyemale* (2 kgs) were ground and extracted with aqueous ethanol (50% v/v) for three times (3x10 L), for 6 h at room temperature. The extract was filtered using Qualigens 640de filter paper (12.5 cm diameter). The filtrate was evaporated using vacuum evaporator (BÜCHI Rotavapor® R-215) at reduced pressure and temperature

($55^0C \pm 2^0C$) and lyophilized (LABCONCO Free Zone Freeze Dry Systems). The lyophilized extract was stored at 4^0C until further use.

HPTLC analysis

Aqueous ethanolic extract (50%), reconstituted in the same solvent, and was used as test solution for HPTLC analysis. Standard solutions were prepared by dissolving 1mg of marker compound in methanol. The test solution was developed in different solvent systems for identification of markers. A known quantity of test and standard solutions was applied on a precoated silica gel GF254 plate of uniform thickness (0.2 mm). The plate was developed in the solvent system to a distance of 8 cm. Densitometric scanning was done by CAMAG Scanner with WinCats (3.1.1), and fingerprint profiles were recorded . The plate was observed under UV 254 and 366 nm and under visible light, after derivatization with 5% phosphomolybdic acid for diosgenin, and anisaldehyde for visualizing other compounds. The Rf values of the resolved components were recorded. The standard peak of all the reference marker compounds was scanned for their spectral analysis at the range of 200-700 nm wavelength and λ max was recorded. The identity of marker compounds was further verified by overlaying absorption spectra at three different levels, i.e. peak start, peak apex and peak end position of the spot of the respective marker compounds.

Estimation of mineral elements

For analysis of macro and micro elements in aerial part of *E. hyemale*, powdered samples were oven dried at 70^0C and wet digested in HNO_3 (69%, ACS, Germany) at 120^0C as described earlier by Dwivedi et al. (Dwivedi et al. 2010). The multielement calibration standard - 2A (MECS-2A, part No. 8500-6940) was used for standardization of macro and micro elements *viz.* Fe, Cu, Se, Ni, Zn and Co. During analysis, rhodium was used as internal standard. These elements were quantified by Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7500 cx). The recoveries of the standard reference material were Fe 92±6%, Cu 92±6%, Se 86±6%, Ni 84±6%, Zn 88±5% and Co 80±8%.

Total phenol and flavonoid content

The total phenol content (TPC) was determined using Folin-ciocalteau reagent and the total flavonoid content (TFC) was estimated using aluminum chloride method (Singh et al. 2009).

Scavenging effect on DPPH free radical

The free radical scavenging activity of the aqueous ethanolic extract of EH (AEEH) on stable radical 1, 1-diphenyl -2-picrylhydrazyl (DPPH) was evaluated according to Brand-Williams et al.(1995). Briefly, 2.0ml of extract at varying concentrations was mixed with 2.0 ml of DPPH solution in methanol (0.004% w/v). The mixture was allowed to stand at room temperature in dark for 20 min. The mixture was vortexed and absorbance was recorded at 517nm using a spectrophotometer. Ascorbic acid was used as a reference standard and control consisted of DPPH solution without extract. The test was performed in triplicate and percentage scavenging of DPPH free radical by extract was calculated using the equation: $(A_{control}- A_{test})/A_{control}$ X100, where $A_{control}$ is the absorbance of control and A_{test} is the absorbance in presence of extract or standard.

β-Carotene-linoleic acid assay

β-carotene bleaching assay was done according to Wettasinghe and Shahidi (1999). A 1ml of β-carotene solution ($0.2mg.ml^{-1}$ in chloroform) was pipetted into a round bottom flask containing 0.02ml of linoleic acid and 0.2 ml of 100% Tween-20. The mixture was evaporated in a rotary vacuum evaporator for 10 min to remove chloroform. After that, the mixture was immediately diluted with 100ml of distilled water with vigorous shaking to form an emulsion. Varying concentrations of extract and standard was added to 5ml of the emulsion in different test-tubes and the mixture was kept at 37^0C for 1h. The absorbance of sample and control was measured at time t=0 and t=60 min. Total antioxidant activity was calculated based on the following equation: $AA=[1-(A_0 - A_t) / (A_0^0 - A_t^0)] \times 100$

Where AA is antioxidant activity, A_0 and A_0^0 are the absorbance values measured at the initial incubation time for samples and control respectively while A_t and A_t^0 are the absorbance values measured in the samples or standards and control at t=60 min.

Reducing power assay

The reducing power was determined according to Oyaizu (1986). Different concentrations of AEEH were mixed with 2.5 ml of 200mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50^0C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 1000 rpm for 8 min. The upper layer (5ml) was mixed with 5ml of deionized water and 1 ml of 0.1% ferric chloride, and the absorbance was measured spectrophotometrically at 700nm. The assays were carried out in triplicate and the results expressed as a mean ± standard deviation. The

extract concentration providing absorbance value of 0.5 was calculated from the graph of absorbance at 700 nm against extract concentration. BHA (butylated hydroxy anisole) was used as a standard.

Statistical analysis

Correlation and regression analyses were performed using SPSS 10.0 software (SPSS Inc., USA). All data were reported as the mean ± standard deviation of three replications.

RESULTS

HPTLC analysis

The HPTLC fingerprint profile of AEEH displayed a vast array of peaks (Fig1). β sitosterol and lupeol spots were developed in solvent system toluene, ethyl acetate, formic acid (9:1:1), at Rf 0.40 and 0.52 respectively. Ellagic acid was observed in a solvent system, toluene, ethyl acetate, formic acid (5:4:1) at Rf 0.16. Rutin was observed in a solvent system, ethyl acetate, acetic acid, formic acid, water (10:1.1:1:2.6) at Rf 0.57, while diosgenin was observed in solvent system toluene, ethyl acetate (7:3) at Rf 0.58. Rutin content was highest (429.4 µg.g^{-1} extract) followed by β-sitosterol (372.9 µg. g^{-1} extract), lupeol (259.4 µg. g^{-1} extract), diosgenin (163.1 µg. g^{-1} extract), and ellagic acid (27.5 µg.g^{-1} extract).

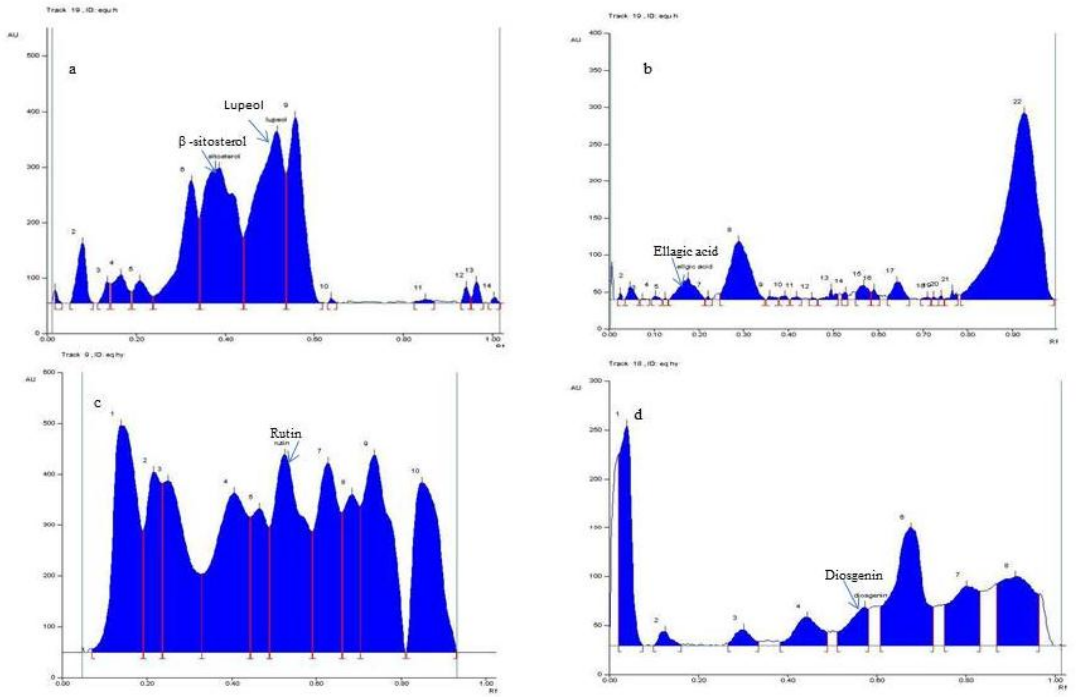

Fig 1: Densitometric scanning profile of aqueous-ethanol (50%) extract of *E. hyemale* (aerial part) showing phytochemical marker components (a) β-sitosterol and Lupeol, solvent system: toluene, ethyl acetate, formic acid (9:1:1); (b) Ellagic acid, solvent system: toluene, ethyl acetate, formic acid (5:4:1); (c) Rutin, solvent system : ethyl acetate, acetic acid, formic acid, water (10:1.1:1:2.6) and (d) Diosgenin, solvent system : toluene, ethyl acetate (7:3)

Estimation of mineral elements

The key mineral elements identified in the dried powder of EH aerial part were iron, zinc, manganese, copper and chromium (Fig 2). Certain minerals identified in trace amount were cobalt (0.513 ± 0.049 ppm), nickel (1.251 ± 0.112 ppm), selenium (1.512 ± 0.169 ppm), molybdenum (0.478 ± 0.025 ppm), cadmium (0.272 ± 0.045), lead (1.760 ± 0.324) and arsenic (0.314 ± 0.033). Interestingly, toxic elements like arsenic, molybdenum and lead were present within permissible limits.

Total phenol and flavonoid content

The total phenol and total flavonoid content in AEEH were 332.32 $mg.g^{-1}$ and 38.46 $mg.g^{-1}$ dry extracts, respectively.

Scavenging effect on DPPH free radical

The AEEH showed significantly high ($p<0.05$) scavenging of DPPH free radicals (Fig 3). A 50% of free radicals produced by DPPH were scavenged at a concentration of 0.081 $mg.ml^{-1}$ as compared to 0.047 $mg.ml^{-1}$, in case of ascorbic acid. The effective concentration (EC_{50}).

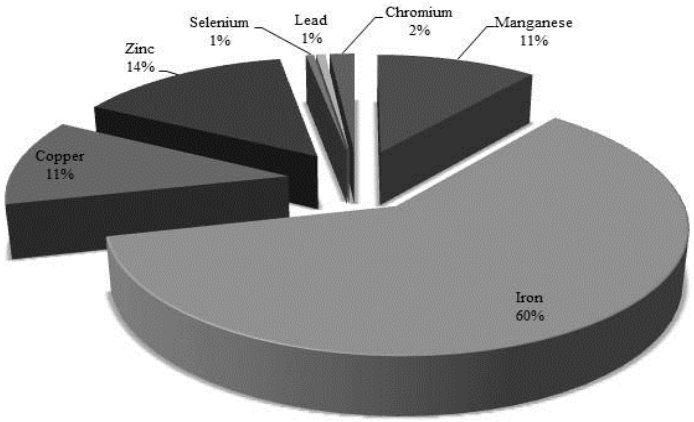

Fig 2: Analysis of mineral elements in aerial part of *E. hyemale* .These elements were quantified by Inductively Coupled Plasma Mass Spectrometer

was 3.52 mg mg^{-1} DPPH for AEEH and 2.05 mg mg^{-1} DPPH for ascorbic acid. The antiradical power 28.38 and 48.75 for AEEH and ascorbic acid, respectively (Table 1).

Table 1: IC50 (mg/ml) , EC50 (mg/mg DPPH) and Anti-radical power of E.hyemale and Ascorbic acid estimated by 1, 1-diphenyl -2-picrylhydrazyl (DPPH) free radical scavenging activity

	IC^{*}_{50} (mg/ml)	EC^{**}_{50} (mg/mg DPPH)	ARP^{***}
E. hyemale	0.081	3.52	28.38
Ascorbic acid	0.047	2.05	48.75

β-Carotene-linoleic acid assay

The antioxidant activity of AEEH was demonstrated by its scavenging effect on the linoleate free radical in a concentration-dependent trend (Fig 3). The antioxidant activity was 48.06% and 60.82 % at a concentration of 1.20 $mg.ml^{-1}$ for AEEH and α-tocopherol respectively. The AEEH and α-tocopherol showed 50% antioxidant activity at an effective concentration (EC_{50}) of 1.38 $mg.ml^{-1}$ and 0.073 $mg.ml^{-1}$ respectively.

Reducing power assay

The reducing power of AEEH increased with concentration. The absorbance recorded at 700 nm was 0.268 ± 0.045 for AEEH and 0.362 ± 0.043 for the standard BHA, at concentration of 1.25 $mg.ml^{-1}$. The effective concentration at which the absorbance would be 0.5 was recorded as 2.31 ± 0.04 and 1.76 ± 0.03 for AEEH and BHA respectively (Fig 4).

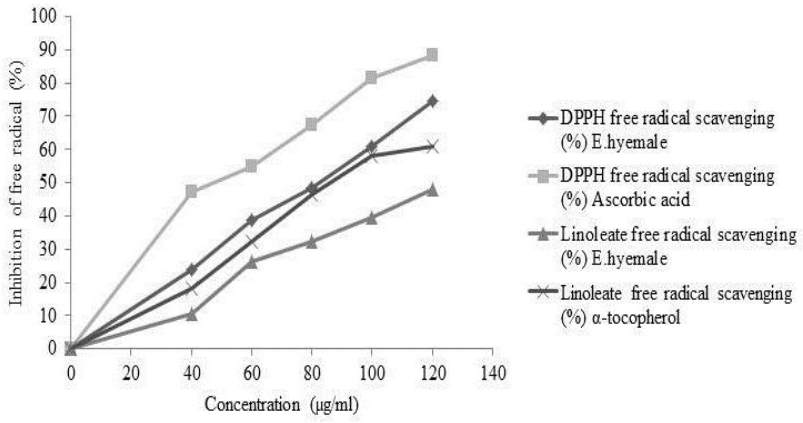

Fig 3: Antioxidant activity of aqueous-ethanol (50%) extract of aerial part of *E.hyemale* (a) DPPH free radical scavenging potential represented as bar graph, ascorbic acid was the standard; (b) β-carotene bleaching potential, showing inhibition of linoleate free radical, represented as line graph, α-tocopherol was the standard.
Results are presented as mean ± SD of three determinations

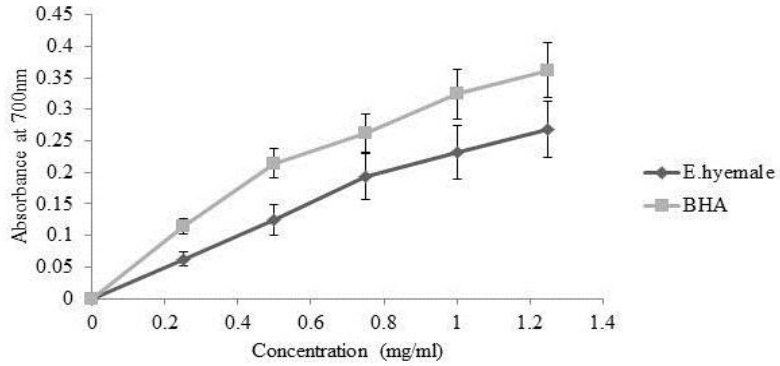

Fig 4: Reducing power of *E.hyemale* aqueous-ethanol (50%) extract and butylated hydroxy anisole (BHA). Higher absorbance indicates higher reducing power. Each value is expressed as mean ± standard deviation (n=3).

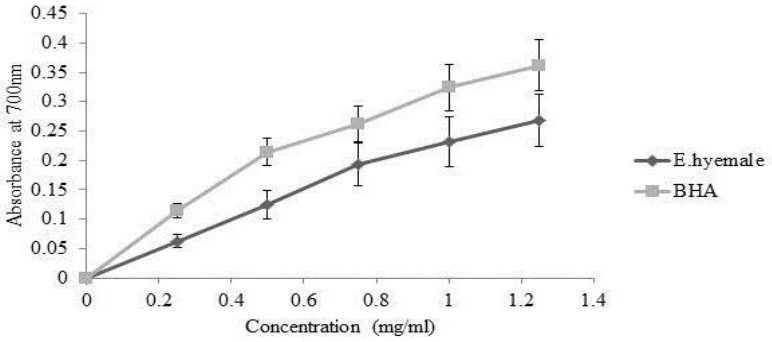

Fig 4: Reducing power of *E.hyemale* aqueous-ethanol (50%) extract and butylated hydroxy anisole (BHA). Higher absorbance indicates higher reducing power. Each value is expressed as mean ± standard deviation (n=3).

DISCUSSION

The HPTLC studies of AEEH reveal the presence of rutin (flavonoid), diosgenin (steroidal saponins), ellagic acid, lupeol, and β-sitosterol. Rutin was isolated for the first from *Ruta graveolens*, a plant known as an excellent remedy for pain and soreness in the bones, joints, tendons, and cartridge. The plant is used as a traditional remedy for treating rheumatism, arthritis and sore bones in northern Peru (Bussmann and Glenn 2011). Rutin is the dominant flavonoid glycoside in common buckwheat and was reported to exhibit antioxidative, anti-hemorrhagic and blood vessel protecting properties (Couch et al. 1946). A study by Horcajada-Molteni (2000) showed that rutin inhibits ovariectomy-induced trabecular bone loss in rats both by slowing down resorption and increasing osteoblastic activity. Rutin is also one of the active molecules responsible for the anti -osteoporotic activity of *Cissus quadrangularis* (Kumar et al. 2010). Another phyto-compound, diosgenin is also reported to

effect bone healing, while its anti -oxidant property was reported by Hisham et al. (2011). Vascular endothelial growth factor – A (VEGF – A) plays an important role in bone related angiogenesis; a critical process occurring during bone formation and fracture healing. In a study conducted by Yen et al. (2005), it was observed that in murine MC 3T3 – F 1 proteoblast like cells, VEGF – A, m– RNA and protein expression was significantly elevated in response to diosgenin, in a concentration-dependent fashion. Diosgenin treatment was found to induce strong angiogenic activity in *in vivo* angiogenesis assay. Diosgenin is also known for its anti-inflammatory activity, inhibiting macrophage-derived inflammatory mediators through downregulation of CK2, JNK, NF-kappa B and AP-1 activation (Jung et al. 2010). Oral treatment with triterpenes lupeol, lupeol palmitate or lupeol linoleate reduced synovial cavity thereby decreasing the destruction of articular cartilage and subchondral bone in arthritic adult male Winstar rats (Kweifio-Okai et al. 1995). Rats treated with lupeol esters showed reduced periosteal bone erosion with bone repair in the form of the periosteal new bone formation.

Various mineral elements play a key role in enhancing bone health. Iron acts as a cofactor in enzymes prolyl and lysyl hydroxylases, involved in collagen bone matrix synthesis. Iron is also a cofactor in 25-hydroxycholcalciferol hydroxylase, which is involved in transforming vitamin D to the active form, thereby affecting calcium absorption. Iron-deficient animals have lower bone mass and mechanical strength compared to iron-replete rats (Palacio, 2006). Zinc is an essential trace element, needed for osteoblastic activity, collagen synthesis, and alkaline phosphatase activity. It is involved in the differentiation of osteoblastic and osteoclastic cells and is required for growth, development and maintenance of bone health. Zinc increases alkaline phosphatase activity and stimulates osteocalcin production during fracture healing. In osteoporotic subjects, the level of skeletal zinc is lower than in controls. The combination of zinc and genistein has a synergistic effect on osteoblastic cells, it enhances bone mineralization and increases bone mass (Giganti et al. 2014). Copper influences bone formation, skeletal mineralization, and the integrity of the connective tissue. The copper-containing enzyme, lysyl oxidase, is essential for cross-linking of collagen fibrils, thereby increasing the mechanical strength of the protein and forming strong, flexible connective tissue. Copper deficiency decreased bone strength in animal studies. In humans, copper supplementation for 2 years was associated with a reduction in bone loss in premenopausal and postmenopausal women. Manganese is involved in the biosynthesis of mucopolysaccharides during bone matrix formation. It also acts as a cofactor for several

enzymes in bone tissue. Manganese supplementation, along with calcium, copper, and zinc showed a significant gain in bone health, as compared to calcium alone in postmenopausal women over a period of 2 years (Palacio, 2006).

The bone healing property of EH can also be correlated to its antioxidant potentials. Oxidative stress is a condition that can be characterized by an imbalance of pro-oxidants and antioxidants with the scale being tipped towards an excess of pro-oxidants creating an abnormally high concentration of ROS (reactive oxygen species) (Sharma et al. 2012). Recent studies have reported the impact of oxidative stress on osteoclast differentiation as well as on its function resulting in an increase in bone resorption (Callaway and Jiang, 2015; Cervellati et al. 2014). In addition to *in vitro* and animal models, there is also increasing clinical evidence that oxidative stress might be involved in the pathogenesis of osteoporosis (Melhus et al. 1999; Basu et al. 2001; Maggio et al. 2002). Flavonoids, (e.g. rutin), that act as anti-oxidants can help limit this damage by acting directly on reactive oxygen species or by stimulating endogenous defence system. Antioxidants are known to mitigate the damaging effects of oxidative stress on cells. Higher consumption of anti-oxidants has been correlated with a reduction in the risk for the development of osteoporosis (Prentice et al. 2006). Excellent free radical scavenging of the AEEH extract indicates the presence of antioxidants in EH. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid peroxidation. The antioxidant activity of carotenoids, in β-carotene bleaching assay, is based on the radical adducts of carotenoids with free radical from linoleic acid. The linoleic acid-free radical attacks the highly unsaturated β- carotene models. The presence of antioxidants neutralizes the linoleate free radical and other free radicals formed in the system, thus reducing the extent of β- carotene bleaching. Accordingly, the absorbance decreased rapidly in the control, without antioxidants, while color retention and thus absorbance was observed for a longer time in samples that contain antioxidants. It is probable that the antioxidants in AEEH reduced the extent of β- carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. AEEH also showed significant reducing power ($p<0.05$). The yellow color of the test solution changed to various shades of green and blue, depending on the reducing capacity of the extract. The presence of reducers, (antioxidants), converts the Fe^{3+}/ ferricyanide complex of the solution to Fe^{2+}/ ferrous form, which can be recorded at 700 nm. Thus higher absorbance indicates greater reducing power. The reducing properties are generally associated with the presence of reductones, which show antioxidant potential by breaking the free radical chain by donating a

hydrogen atom (Shimada et al. 1992).

CONCLUSION

The current study justifies a possible role of the *E. hyemale* in bone healing, owing to the presence of various phytocompounds and mineral elements, and significant antioxidant potential. However, a series of well-designed pharmacological studies followed by clinical trials are needed to take the study further.

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Conflict of Interest

The author declares no conflict of interest.

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