



Influence of Extract Derived Cell Cultures of Broccoli against Osteoporosis in Ovariectomized Rats

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Abstract

This study was conducted to enhance broccoli secondary metabolites; using plant biotechnology techniques and tissue culture as well as to evaluate the therapeutic effect of broccoli extract against osteoporosis in ovariectomized (OVX) rats. Cell culture technique was implemented in order to achieve and produce polyphenols from leaf explants of broccoli. The high-performance liquid chromatography (HPLC) technique was used to evaluate the polyphenolic profiles of ethanolic extracts for cell cultures compared to the dried inflorescences of broccoli (cultivated plant). However, total phenol and flavonoid contents were determined by spectrophotometric technique. Induction of osteoporosis was performed via ovariectomy operation where rats were divided into 5 groups (10 rats each). Group 1: Sham operated control group and the remaining rats were ovariectomized for 12 weeks to produce osteoporosis model. Group 2: Ovariectomized osteoporotic positive control group. Groups 3-5: Ovariectomized rat daily received 17- β -estradiol (10 μ g/kg), broccoli cell suspension culture ethanol extracts (300 & 600 mg/kg) orally. The obtained results showed that Murashige and Skoog basal nutrient medium (MS) fortified with 2.25 μ M of 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.55 μ M of kinetin (kin.) showed the best medium for calli production. Addition of methionine enhanced polyphenolic production in cell suspension culture which was identified by HPLC. Administration of broccoli cell suspension extract elevated the uterine weight, high density lipoprotein-cholesterol and estradiol 2 (E2) serum levels and reduced serum levels of lipid profile, liver and kidney functions, osteocalcin and total procollagen type 1 N-terminal propeptide (TPINP). In conclusion, broccoli may be a promising source of secondary metabolites; polyphenol contents and flavonoids for treatment of bone loss and cell damage in osteoporosis.

Keywords: Broccoli; Secondary metabolites; Cell suspension culture; Polyphenols; Osteoporosis; Osteocalcin; Total procollagen type 1 N-terminal propeptide.

1. Introduction

Osteoporosis is a major health problem, which affects over 200 millions of people around the world and its frequency increases by age [1]. The prevalence of osteoporosis is affected by the economic state which is increased in industrialized countries more than others [2]. It is also affected by low socioeconomic levels and sociocultural attitudes where a high record was reported in Upper Egypt [3]. Awareness is very important, so earlier diagnosis and interventions prior to the first fracture are highly desirable [4].

Women are vulnerable to bone loss in which bones become more fragile, porous and susceptible to fracture after and during menopause [5]. Bone health maintenance is associated with sun exposure (maintaining vitamin D levels), diet, exercise and genetics. Meanwhile, osteoporosis is a multi-factorial degenerative disease and is affected by hormonal decrease; androgen in men and estrogen in women especially in menopausal period when bone leakage is initiated [6]. Normal integrity of bone density kept with daily intake of fruits /vegetables and herbs. They

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decline calcium loss and bone change because of their content of polyphenols [6]

Brassica oleracea L. var. italica (Broccoli) is one of cruciferous vegetables related to Brassicaceae family, have a high nutritional value, low caloric content and high dietary fiber contents. It is a source of important phytochemicals, such as phenolic compounds (flavonoids and phenolic acids), ascorbic acid, carotenoids (β -carotene) and glucosinolates [7]. These secondary metabolites have been widely considered to be anti-carcinogens and antioxidants [8]. The Intake of such phytochemicals may alleviate bone loss related to elderly [9].

Many strategies were attempted to synthesize the desired secondary metabolites in appreciable quantity and at competitive economic value but failed to gain commercial exploitation [10]. Current advances in plant biotechnology techniques provide opportunity for the production of useful secondary metabolites from cell or tissue instead of whole plant cultivation [11]. Plant tissue cultures have been considered over the last 50 years as an alternative of bioactive compounds production [12]. Plant cell suspension cultures are capable of producing particular medicinal compounds at a rate similar or superior to that of naturally grown whole plants [13]. The possibility of enhancing and/or altering the production of bioactive plants can be achieved through media modification and growth hormone variation [14]. What give us a lead, the malleable interconnection between bone health and dietary polyphenols and documented by the latest scientific literature where they can reduce the oxidative stress as well as its role in reduction of inflammation by proinflammatory signaling, modulation of osteoblastogenesis, osteoclastogenesis, and osteoimmunological action [15].

To the best of our knowledge, no previous studies have assessed the therapeutic potential of broccoli cell suspension culture ethanol extract (BCSE) against osteoporosis in ovariectomized rats. Accordingly, this study aimed to i) enhancement of broccoli secondary metabolites; polyphenol contents using plant biotechnology techniques, ii) Qualitative and quantitative estimation of polyphenolic contents for calli and cell suspension cultures extracts compared to the cultivated one using spectrophotometric and HPLC techniques, iii) Evaluating the therapeutic effect of broccoli cell suspension culture ethanol extract (BCSE) against osteoporosis in ovariectomized rats.

2. Material and Methods

2.1. Material

2.1.1 Plant material

Seeds of broccoli (*Brassica oleracea* L. var. *italica*), Family Brassicaceae (Cruciferae), were purchased from Agriculture Research Center, Cairo, Egypt

(ARC) and were used for the in-vitro plant tissue culture technique. While fresh inflorescences of Broccoli were collected from rural areas of villages (Giza Governorate) during December, 2018 and January, 2019. These Inflorescences were cleaned by washing in tap water to eliminate any surface contamination and sliced into small homogeneous pieces. All determinations were made on fresh products, immediately after washing. A voucher specimen (No. 14-01-2019) was deposited at the herbarium of National Research Centre, Cairo, Egypt.

2.1.2. Animals

Fifty female, Sprague Dawley rats (weight, 250 ± 10 g; age, eight-week-old) used for the main experiment and forty-eight Swiss albino mice (weighed 15–25 g) used for acute toxicity experiment were obtained from the animal house colony, National Research Centre, Cairo, Egypt. All animals were acclimated to a colony room with an ambient temperature of $22 \pm 1^\circ\text{C}$, humidity of $50 \pm 10\%$, and a 12 h light/dark cycle for at least 10 d before the start of the experiment in metal cages. Food and water were available to the rats. All experiments were carried out according to the ethical guidelines for care and use of experimental animals approved by the Ethical Committee of the National Research Centre, Cairo, Egypt.

2.1.3. Reagents, Chemical and kits

All reagents and solvents for phytochemical and HPLC analysis were of analytical grade and provided from Fisher Scientific (Fisher Scientific, Fair Lawn, New Jersey). Aluminum chloride, sodium carbonate (analytical grade), chlorogenic acid and rutin were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Cholesterol, triglyceride (TG), high density lipoprotein-cholesterol (HDL-cholesterol), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), alkaline phosphatase (ALP), creatinine and Blood urea nitrogen (BUN) were purchased from Biodiagnostic kit, Egypt. 17β -estradiol (folone) was purchased from Misr Pharmaceutical Company. RatEstradiol 2, osteocalcin and total procollagen type 1 N-terminal propeptide (TPINP) ELISA kits were purchased from Cusabio Technology Llc, Drg International, USA and MyBioSource, China. All other chemicals, used throughout the experiment, are of the highest analytical grade available.

2. Methods

2.1.1. Seeds surface sterilization

Seeds of broccoli were washed with sterile distilled water, immersed in ethanol (70%) for 1 min subsequently soaked in 20 % of commercial bleach solution for 15 min. The sterilized seeds were then rinsed three times with sterilized distilled water and exploited to obtain sterilized plantlets [16].

2.1.2. Preparation of leaf explants

Sterilized seeds were aseptically cultured into jars containing 50 ml of the half strength of MS basal nutrient medium to get stock sterilized leaf explants [17]. Then, they were used as sterilized starting plant materials for calli induction.

2.1.3. Callus induction.

Sterilized leaf explants of broccoli were excised from the aseptic seedlings and cultured on solidified MS medium. Cultures were gelled by 0.7% agar added prior autoclaving at 1.2 Kg / cm² for 15 min. The pH of the medium was adjusted to 5.8 by addition of 0.1 N HCl and /or 0.1 N KOH. The cultivation was done in 100 ml glass jars containing 25 ml of MS-medium supplemented with combinations from cytokinins; kinetin (Kin.) and benzyladenine (BA) and auxins; 2,4-Dichlorophenoxyacetic acid (2,4-D).at different concentrations. All cultures were incubated for 28 days (4 weeks) either under light conditions (Cool light fluorescent lamps 1400 lux for 16h / day) or under complete darkness. Cultures were incubated in a growth chamber at 26 ±1°C. Calli frequency formation was calculated and recorded. Each treatment was represented with five replicates. Percentage of calli formation under light or dark conditions was recorded [18].

2.2.4 Cell suspensions production

Cell suspension culture of broccoli (BCS) was established from friable obtained leaflets calli cultures according to the described method by Torres [19]. A passage of calli (0.5 g fresh weight) were re-cultured into 125-ml Erlenmeyer flasks containing 25 mL of MS liquid culture medium supplemented with 2.25 µM /l 2,4-D + 0.55 µM/l Kin.in the presence of 150 mg/l of methionine. Cultures were incubated on a rotary shaker (125 rpm) at 25 ± 1°C under photoperiod (16-h light/8-h dark; cool white fluorescent tubes; 50 µE/m²/s). Cell suspension cultures were sub cultured at 15 days intervals and pH medium were optimized at 5.7. The following parameters were recorded after 4, 8, 12, 16, 20 and 24 days of cultivation as follow: -
1- Cell number was counted during the growth period of cultivation as a growth parameter [20].
2- Packed cell volume (PCV) was determined according to the method of Patrick [21].

2.2.5 Phytochemical analysis

2.2.5.1 Extraction of the plant material

Callus, BCS and dried inflorescence of broccoli were separately extracted by 70% ethanol (v/v) in a water bath at 70 °C for 5 minutes [22]. Each extract was sonicated using an ultrasound extraction method [23]. Then it was transferred into another flask for overnight maceration and filtered. The combined filtrate was evaporated under reduced pressure at 40 OC using a rotary evaporator. Finally, the dry extract was weighted.

2.2.5.2 Sample preparation for quantitative and qualitative estimation of total phenolic and flavonoid contents

Known weight of dried ethanol extract of each callus, cell suspension culture (BCSE) and the dried inflorescence was prepared as stock solution (1mg /ml) by dissolving it in 80% ethyl alcohol and completed to 100 ml with 80% ethyl alcohol.

2.2.5.3 Quantitative estimation of total phenolics and flavonoids contents

All assays were performed using UV spectrophotometer (UV/VIS, 2041 spectrophotometer) with reference to pre-established standard calibration curves. Total phenolic content (TPC) was determined using Folin –Ciocalteu colorimetric method [24], with some modification. Absorbance of the clear solution was measured at λ_{max} 760 nm. TPC was determined from a standard calibration curve, the mean of three readings were used and results were expressed as milligrams of chlorogenic acid equivalents per 100 milligrams of the extract (mgCAE/100 mg extract). The total flavonoid content (TFC) of each prepared extract was determined using colorimetric assay based on measuring the intensity of the color developed when flavonoids were complexed with aluminium chloride reagent. The absorbances of the color for each of standard and tested extracts were measured at λ_{max} 415 nm. TFC content was calculated from a calibration curve using rutin as standard and expressed as mg rutin /100 mg extract [24]. All measurements were done in triplicate and the mean of three readings were recorded.

2.2.5.4 HPLC analysis of phenolic contents

Chromatographic separation and identification of the polyphenolic constituents of each ethanol extract were performed using HPLC analysis (Agilent 1260 series). The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5–8 min (60% A); 8–12 min (60% A); 12–15 min (82% A) and 15–16 min (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 µl for each of the sample solutions. The column temperature was maintained at 35 °C. Extracts were injected in the

HPLC column after filtration through a 0.45 μm Acrodisc syringe filter (Gelman Laboratory, MI).

2.2.6 Biological design

2.2.6.1 Acute oral toxicity study for BCSE

The acute toxicity study was conducted as per the Organization for Economic Co-operation and Development (OECD) guidelines [25]. Eight groups (n=6) of Swiss albino mice represented as four groups in each sex, one group of each sex was orally given saline represented as control group while the other three groups were orally administered BCSE in single doses of 500, 1000 and 2000 mg/kg. Animals were closely monitored during the first 4 h, and then daily for a total of 14 days. Clinical signs and mortality were recorded.

2.2.6.2 Experimental design

Osteoporotic model induction: fifty rats subjected to bilateral ovariectomy according to the classic method [26]. After anesthetization by intraperitoneal injection of pentobarbital sodium (3.0 ml/kg), an abdominal incision was made around the midpoint between the lower margin of the free ribs and iliac crest, where the ovary is located. A suture was placed around the ovarian artery and vein prior to the removal of the ovary. To close the incision tightly, the muscles were repositioned in layers and sutured with resorbable sutures, and the skin was closed with nylon 4-0 sutures. The animals were given antibiotics postoperatively to prevent infection.

Animals were randomly classified into five groups, each of 10 animals. Group I: (Negative control): Rats subjected to sham operation and received daily only 5 ml/kg saline for 12 weeks, orally. Group II (OVX): Bilaterally ovariectomized rat and daily received 5 ml/kg saline for 12 weeks, orally [27]. Group III (OVX-E2): Bilaterally ovariectomized rat daily received 17- β -estradiol (10 $\mu\text{g}/\text{kg}$) orally [28]. Group IV (OVX-BCSEL): Bilaterally ovariectomized rat daily received broccoli cell suspension culture extract BCSE (300 mg/kg) for 12 weeks, orally as low dose [29]. Group V (OVX-BCSEH): Bilaterally ovariectomized rat daily received BCSE (600 mg/kg) for 12 weeks, orally as a high dose [30].

2.2.6.3 Determination of lipid profile, liver and kidney function

Blood samples were collected from the retro-orbital venous plexus of rats under light anesthesia in clean test tubes, allowed to clot, then centrifuged for 15 minutes at 3000 r.p.m [31]. Serum was separated and stored into eppendorf tubes at - 20 $^{\circ}\text{C}$ to be used for biochemical analysis of lipid profile; Cholesterol,

TG and HDL-chol, liver functions; GPT, GOT, and ALP as well as kidney functions; creatinine and urea.

2.2.6.4 Determination of estradiol 2, osteocalcin and TPINP

Serum levels of estradiol2, osteocalcin and TPINP were determined using ELISA (Enzyme-Linked Immunosorbent Assay) kit. The research team followed the manufacturer's instructions of CUSABIO TECHNOLOGY and My BioSource kits for calculating the results. Standards and samples were pipetted into wells with immobilized antibodies specific for rat estradiol2, osteocalcin and TPINP and then were incubated 30 min at 37 $^{\circ}\text{C}$. After incubation and washing, horseradish peroxidase conjugated streptavidin was pipetted into the wells and incubated 30 min at 37 $^{\circ}\text{C}$, which were washed once again. TMB (tetramethylbenzidine) substrate solution was added to the wells and incubated 15 min at 37 $^{\circ}\text{C}$; color developed proportionally to the amount of estradiol2, osteocalcin and TPINP bound. Color development was discontinued (Stop Solution) and after 10 min color intensity was measured at λ_{max} 450 nm.

2.2.6.5 Histopathological study

After collection of blood samples, rats were sacrificed by decapitation [32]; the bone and uterus were taken off for histopathological study. Autopsy samples were taken from the rat's bone as well as uterus in different groups and fixed in 10% formalin for twenty-four hours and decalcified in formic acid. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 $^{\circ}\text{C}$ in a hot air oven for twenty-four hours. Paraffin beeswax tissue blocks were prepared for sectioning at 4 microns thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by Hematoxylin & Eosin stain for examination through the light microscope [33]. All dead animals were collected, frozen until incineration.

2.2.6.6 Statistical analyses

Experiment of callus production was designed in a completely randomized pattern and the obtained data were expressed as mean \pm standard error of the mean and statistically analyzed using one-way analysis of variance (ANOVA) and followed by Duncan's.

Biological results were expressed as mean \pm standard error of the mean. Data were analyzed by one-way analysis of variance (ANOVA) and followed by Fisher's LSD. Multiple comparison test was used to assess the significance between-group differences at $P < 0.05$ using GraphPad Prism 6 (GraphPad Software Inc, San Diego, CA, USA).

3. Results

Stock sterilized germinated seedlings were formed on the half strength of MS basal nutrient medium which were implemented in the form of calli induction experiment.

3.1 Callus production from leaf explants of Broccoli

The effect of supplementation MS-medium with 2,4-D, Kin and BA (μM) at different concentrations on frequency of callus formation from leaf explants of broccoli cultures were investigated and statically calculated. The data were presented in Table (1) for light and dark condition. High frequency of calli production 45.12(%) was recorded under light condition, while it was 65.12 (%) under complete darkness (Table 1 and Fig. 1). MS medium fortified with 2.25 μM of 2,4-D and 0.55 μM of kin showed the best medium for calli production compared with other supplementations.

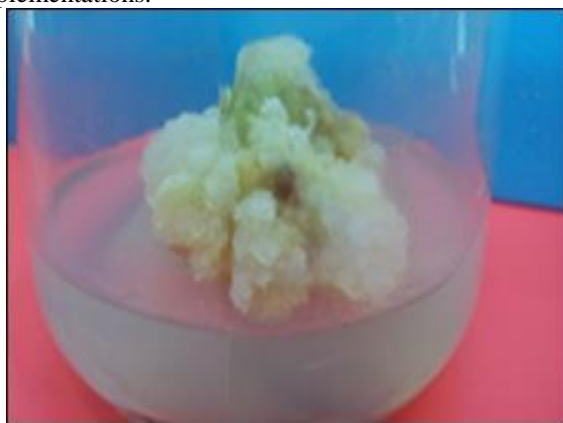


Fig.1 Leaf calli culture of broccoli incubated under completely darkness for 28 days.

3.2 Cell suspension production

Friable calli were saved further cultured on liquid MS medium augmented with 2.25 μM of 2,4-D and 0.55 μM of kin. in the presence of 150 mg/l methionine. After 24 days, the cell number and the packed cell volume were evaluated as the main parameters that affect the mass calli production. As shown in Fig. (2), the maximum cell number 4.37 ($\times 10^5$) was recorded with broccoli cell cultures after 16 days of cultivation. While, the highest packed cell volume 1.28 was reported after 24 days of cultivation. However, the significant and economic period of broccoli cell cultures production was recorded after 16 days of cultivation.

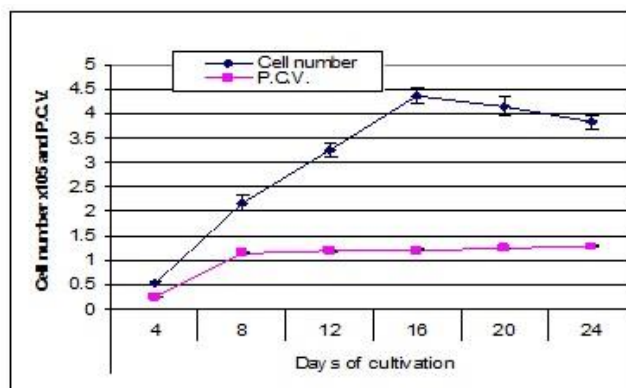


Fig.2 Cell number $\times 10^5$ and Packed Cell Volume (P.C.V.) of broccoli leaf cultures during 24 days of cultivation

3.3 Chemical analysis

3.3.1 Estimation of TPC and TFC using UV spectrophotometer

The total phenolics (TPC) and flavonoids (TFC) contents for ethanol extracts of broccoli calli, cell suspension culture and dried inflorescences (cultivated plant) were recorded (Table 2 and Fig. 3). From the pre-established standard calibration curves, TPC of calli and cell suspension culture extracts were 4.15 and 4.92 mg CAE/100mg, respectively. While, the TFC were found 0.243 mg RE/ 100mg extract for calli and 0.362 mg RE/ 100mg extract for cell suspension culture. On the other hand, TPC and TFC of the dried extract of the cultivated plant were 0.60 mg CAE/ 100mg extract and 0.33 mg RE/ 100mg extract, respectively.

3.3.2 HPLC analysis for ethanol extracts.

The obtained chromatograms from the analysis of the ethanol extracts of calli, cell suspension cultures and the dried plant, showed significant qualitative variations in the polyphenolic profiles and variation in the concentration of each individual compound (Fig. 4-6). Peaks were identified by congruent retention times (min.) and UV spectra in comparison with those of the standards. Phenolic acids and flavonoid compounds concentrations are expressed as ($\mu\text{g/g}$ dry weight) samples the concentrations of individual polyphenols were summarized in Table (3). The analysis resulted in identification of 12, 13 and 13 compounds in the tested extracts, respectively. Gallic acid was the major phenolic acid in the all extracts followed by ellagic and chlorogenic acids in calli and BCSE. Chlorogenic acid is major than ellagic acid in the dried inflorescences. Ellagic acid, methyl gallate, syringic, caffeic and cinnamic acids were detected in 4.65, 4.10, 3.48, 2.26 and 1.76 folds in BCSE with respect to dried inflorescences. Naringenin, catechin,

taxifolin, rutin and Kaempferol were the main detected flavonoids in all extracts. Naringenin was the major one in BCSE and calli extract with 3.83 and 3.24 folds, respectively. Kaempferol, rutin and taxifolin were recorded in BCSE with 2.79, 1.39 and 1.23 folds, respectively. Meanwhile, catechin was recorded as the main flavonoid in the cultivated plant.

3.4 In vivo study

3.4.1 Acute toxicity study

The obtained results demonstrated that BSCE showed no observable signs of toxicity or mortality up to a dose of 2000 mg/kg. Thus, indicating that the median lethal dose (LD50) could be higher than 2000 mg/kg in mice.

3.4.2 Effect of BCSE on osteoporosis

3.4.2.1 Effect of BCSE on uterine weight and lipid profile

The uterine weight was reduced in OVX group compared to the sham group, while oral

administration of 17- β -estradiol and BSCE (300 and 600 mg/kg) to ovariectomized rats for 12 weeks resulted in a significant increase in the uterine weight when compared with ovariectomized rats. Moreover, treatment with a high dose of broccoli increased the uterine weight by 6% when compared with the estradiol group and returned it to its normal value (**Table 4**).

Ovariectomy increased serum levels of cholesterol and TG and produced a decrease in HDL-chol levels compared with the sham operated group. Administration of 17- β -estradiol and broccoli (600 mg/kg) decreased serum cholesterol and TG levels as well as elevated HDL-chol, while administration of broccoli (300 mg/kg) decreased serum TG levels only and elevated HDL-chol compared with OVX group. Treatment with high dose of broccoli returned HDL-chol to its normal level and had the same potency as estradiol in reducing cholesterol and TG serum levels and elevated HDL-chol by 40% in comparison with estradiol group (**Table 4**).

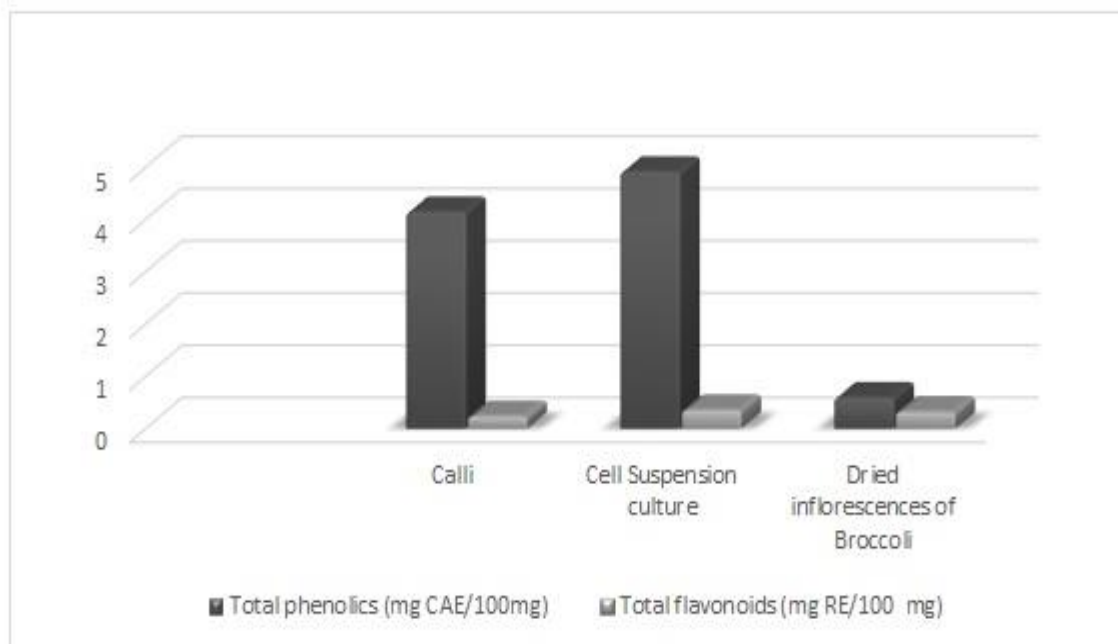


Fig.3 Total phenolic and flavonoids contents of Broccoli calli, cell suspension culture and the dried inflorescence extracts.

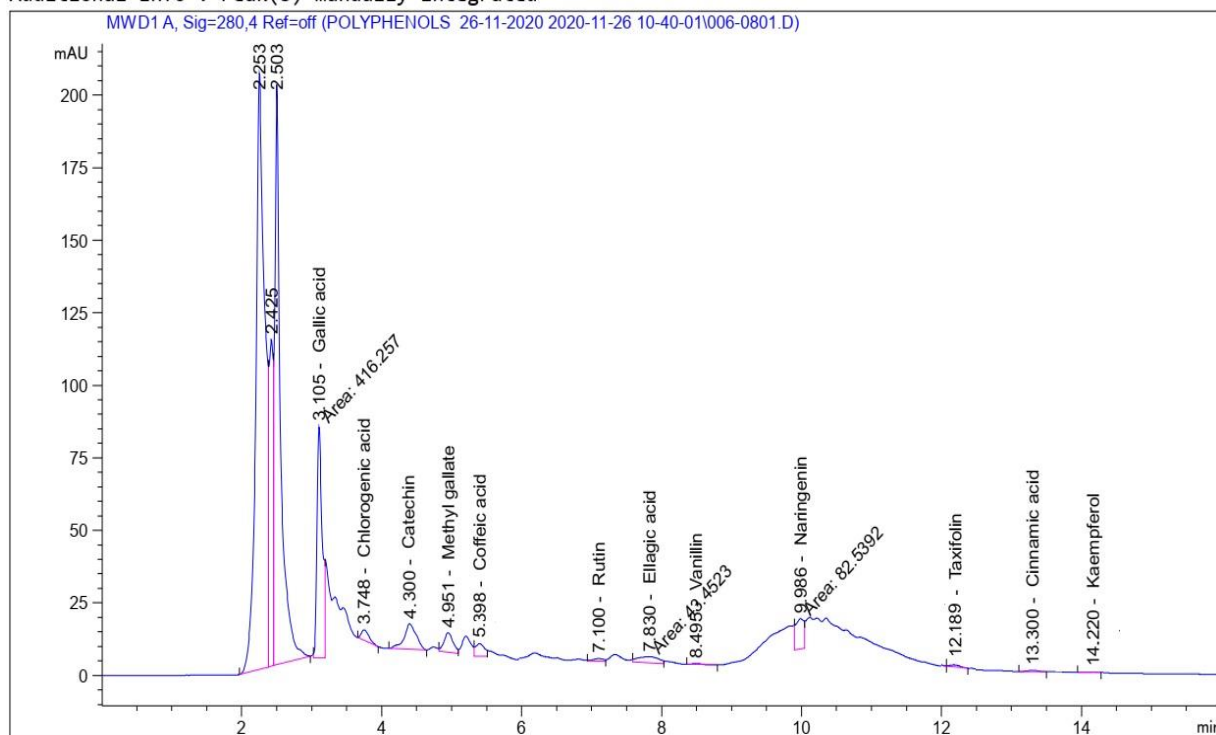


Fig.4 HPLC chromatogram of the phenolic compounds detected from Broccoli calli ethanol extract.

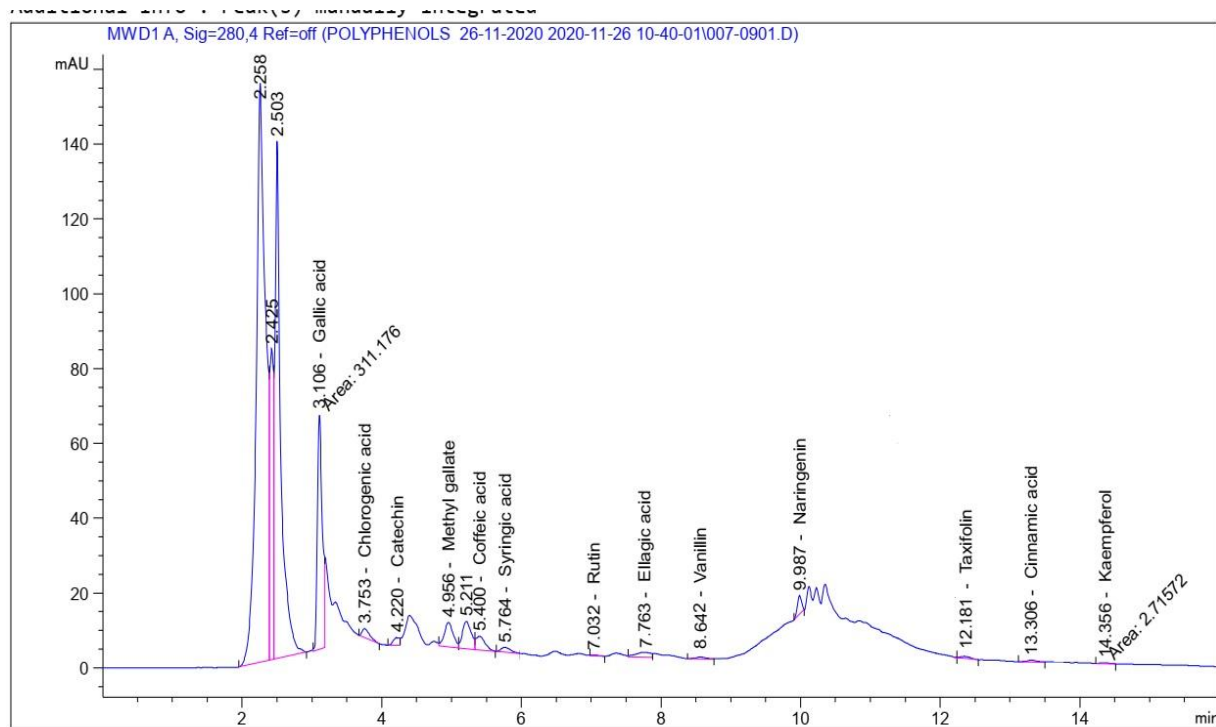


Fig.5 HPLC chromatogram of the phenolic compounds detected from Broccoli cell suspension culture ethanol extract (BCSE).

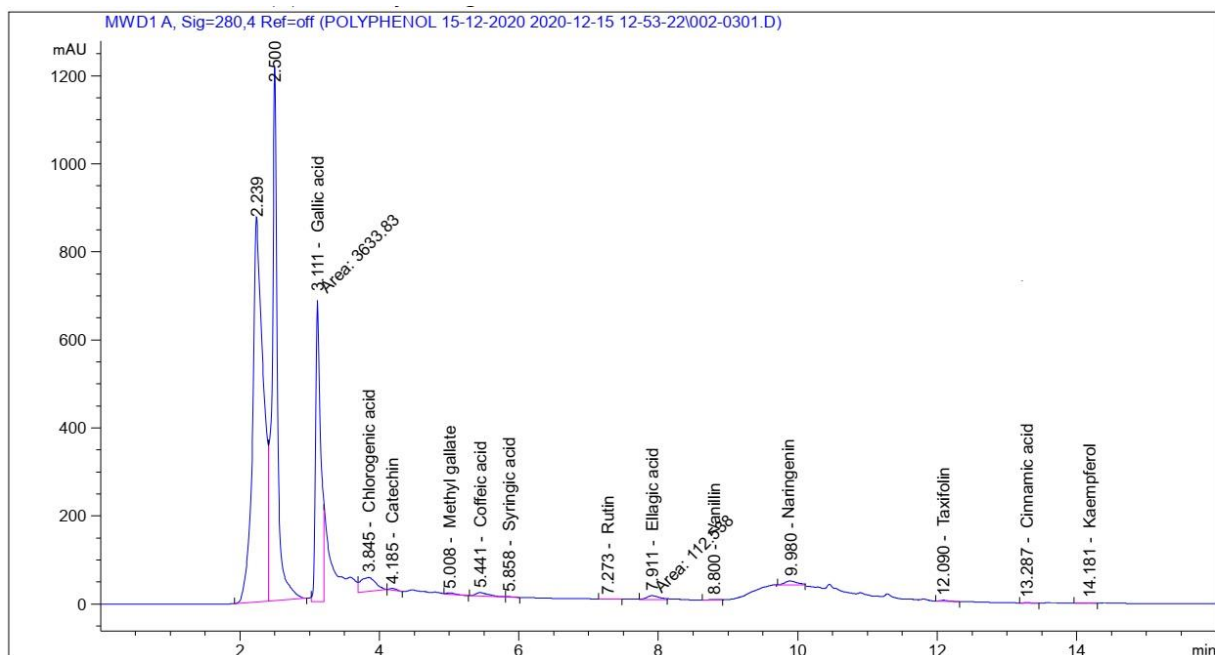


Fig.6 HPLC chromatogram of the phenolic compounds detected from the dried inflorescence ethanol extract of Broccoli.

3.4.2.2 Effect of BSCE on liver and kidney functions

Elevated serum levels of GPT, GOT and ALP were observed in OVX group compared with the sham operated group. Administration of estradiol and broccoli (600 mg/kg) reduced serum levels of GPT, GOT, and ALP, while administration of BCSE (300 mg/kg) decreased serum GOT, and ALP levels only, compared with OVX group. Treatment with a high dose of broccoli had the same potency as estradiol in reducing GPT while it reduced the serum levels of GOT and ALP by 8% and 6% respectively, in comparison with estradiol group (**Table 5**).

Ovariectomy elevated serum levels of creatinine and BUN compared with the sham operated group. Administration of 17- β -estradiol and both doses of broccoli reduced serum levels of creatinine and BUN, compared with OVX group. Treatment with a high dose of broccoli had the same potency as estradiol in reducing creatinine serum level (**Table 5**).

3.4.2.3 Effect of BCSE on estradiol 2, osteocalcin and TPINP

Ovariectomy induced a reduction in serum estradiol 2 level by 75% with elevations in serum contents of osteocalcin by 3-fold and TPINP by 2.6-fold, compared to sham operated groups. Administration of estradiol and BCSE (300 and 600 mg/kg) increased serum estradiol 2 level by 2.7-fold, 2.5-fold and 3-fold respectively and decreased serum contents of osteocalcin by 60%, 55% and 65% respectively and TPINP by 59%, 46% and 70%

respectively, compared to OVX group. Moreover, treatment with a high dose of BCSE returned estradiol 2, and TPINP to their normal values. Furthermore, broccoli extract high dose treatment elevated estradiol 2 serum level by 8%, and decreased osteocalcin and TPINP serum levels by 14% and 28% as compared to 17- β -estradiol (Fig. 7-9).

3.4.3 Histopathological study

3.4.3.1 Histopathological study of bone

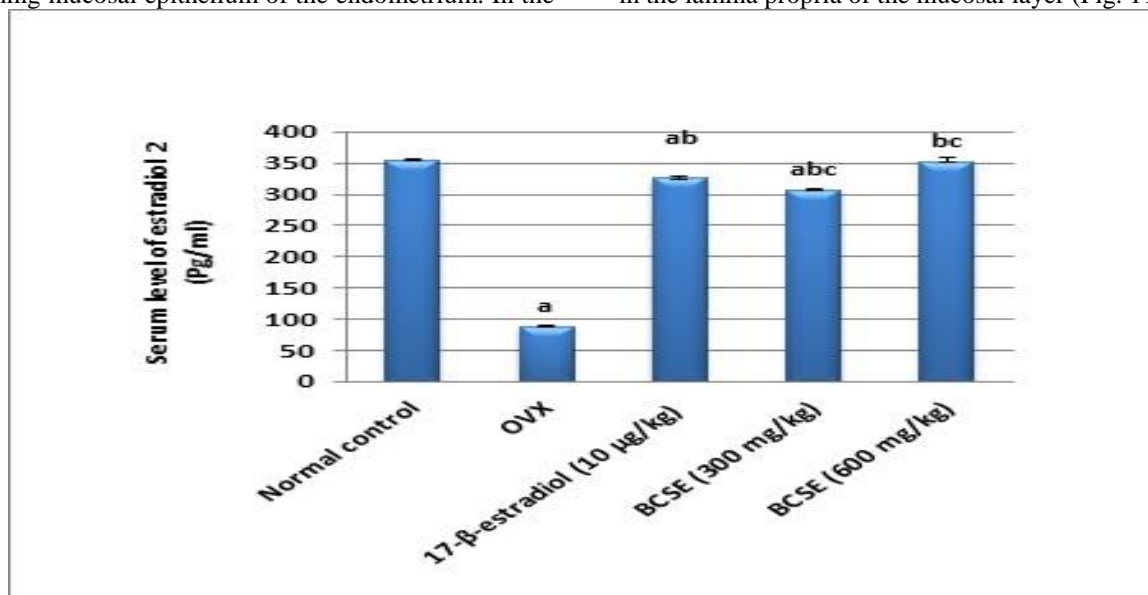
Control group showed no histopathological alteration (normal histological structure of the articular cartilaginous surface and the bone trabeculae of the epiphysis and metaphysis). OVX group showed osteoporosis with thinning in the bone trabeculae. Estradiol group showed no histopathological alteration. In the low dose BCSE group, there was osteoporosis in the bone trabeculae of the epiphysis and metaphysis. In the high dose BCSE group, there was no histopathological alteration (Fig. 10).

3.4.2.1. Histopathological study of uterus

Control group showed no histopathological alteration and the normal histological structure of the mucosal lining epithelium, lamina propria with glandular structure in the endometrium then the underlying myometrium and perimetrium of the serosa were recorded. OVX group showed edema, cellular proliferation and glandular hyperplasia in the lamina propria of the mucosal layer. In estradiol group, the endometrium showed oedema in the lamina propria of the mucosa. In the low dose BCSE group, epithelial

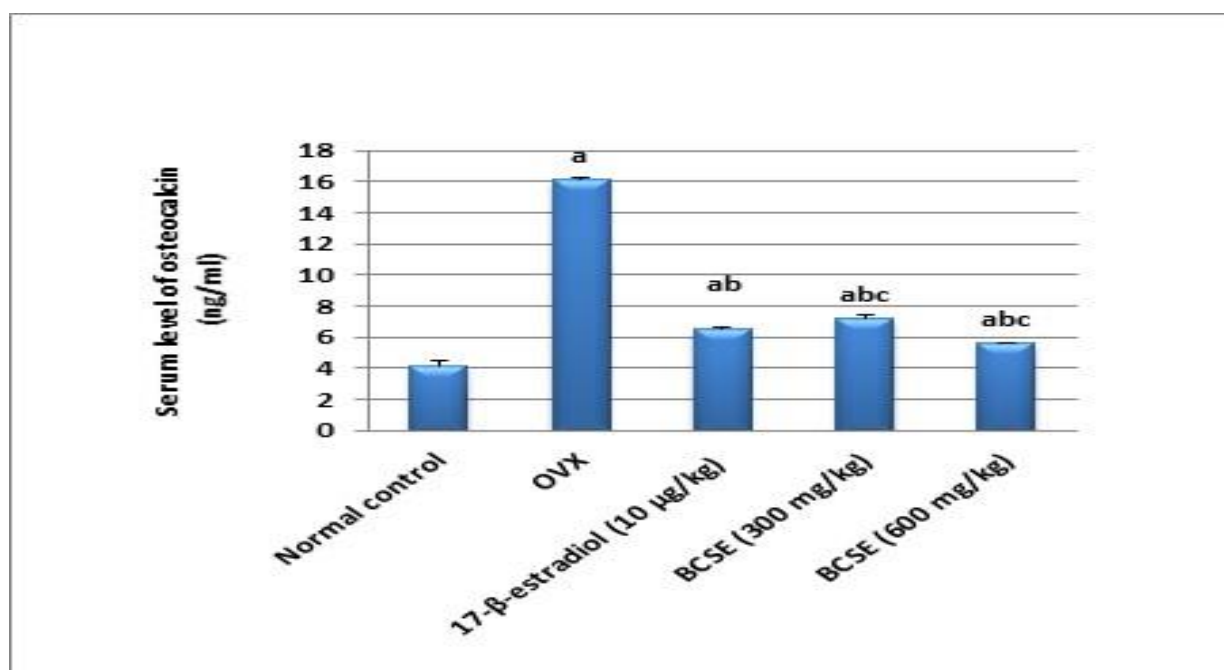
hyperplasia with polyps formation were detected in the lining mucosal epithelium of the endometrium. In the

high dose of BCSE group, oedema only was detected in the lamina propria of the mucosal layer (Fig. 11).



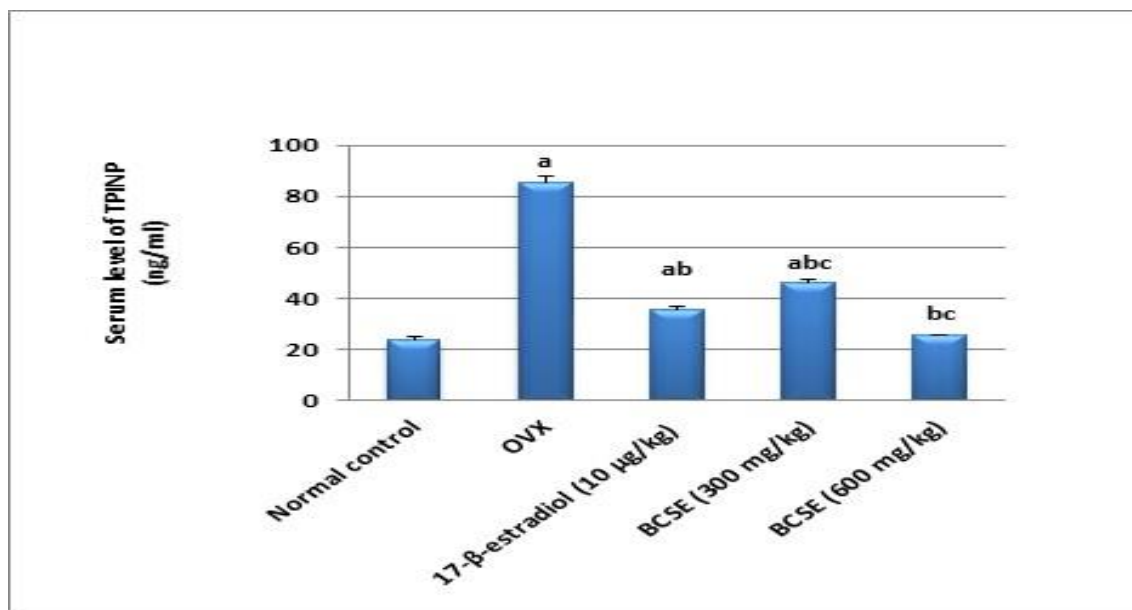
Data are presented as the mean \pm S.E. of (n=10) for each group. Statistical analysis was carried out by one-way analysis of variance followed by Fisher's LSD test. ^a Statistically significant from control group. ^b Statistically significant from OVX group. ^c Statistically significant from 17- β -estradiol group at P < 0.05.

Fig.7 Effect of Broccolicell suspension culture ethanol extracton serum level of Estradiol 2



Data are presented as the mean \pm S.E. of (n=10) for each group. Statistical analysis was carried out by one-way analysis of variance followed by Fisher's LSD test. ^a Statistically significant from control group. ^b Statistically significant from OVX group. ^c Statistically significant from 17- β -estradiol group at P < 0.05.

Fig.8 Effect of Broccolicell suspension culture ethanol extracton serum level of osteocalcin



Data are presented as the mean \pm S.E. of (n=10) for each group. Statistical analysis was carried out by one-way analysis of variance followed by Fisher's LSD test. ^a Statistically significant from control group. ^b Statistically significant from OVX group. ^c Statistically significant from 17- β -estradiol group at $P < 0.05$.

Fig.9 Effect of Broccolicell suspension culture ethanol extract on serum level of TPINP

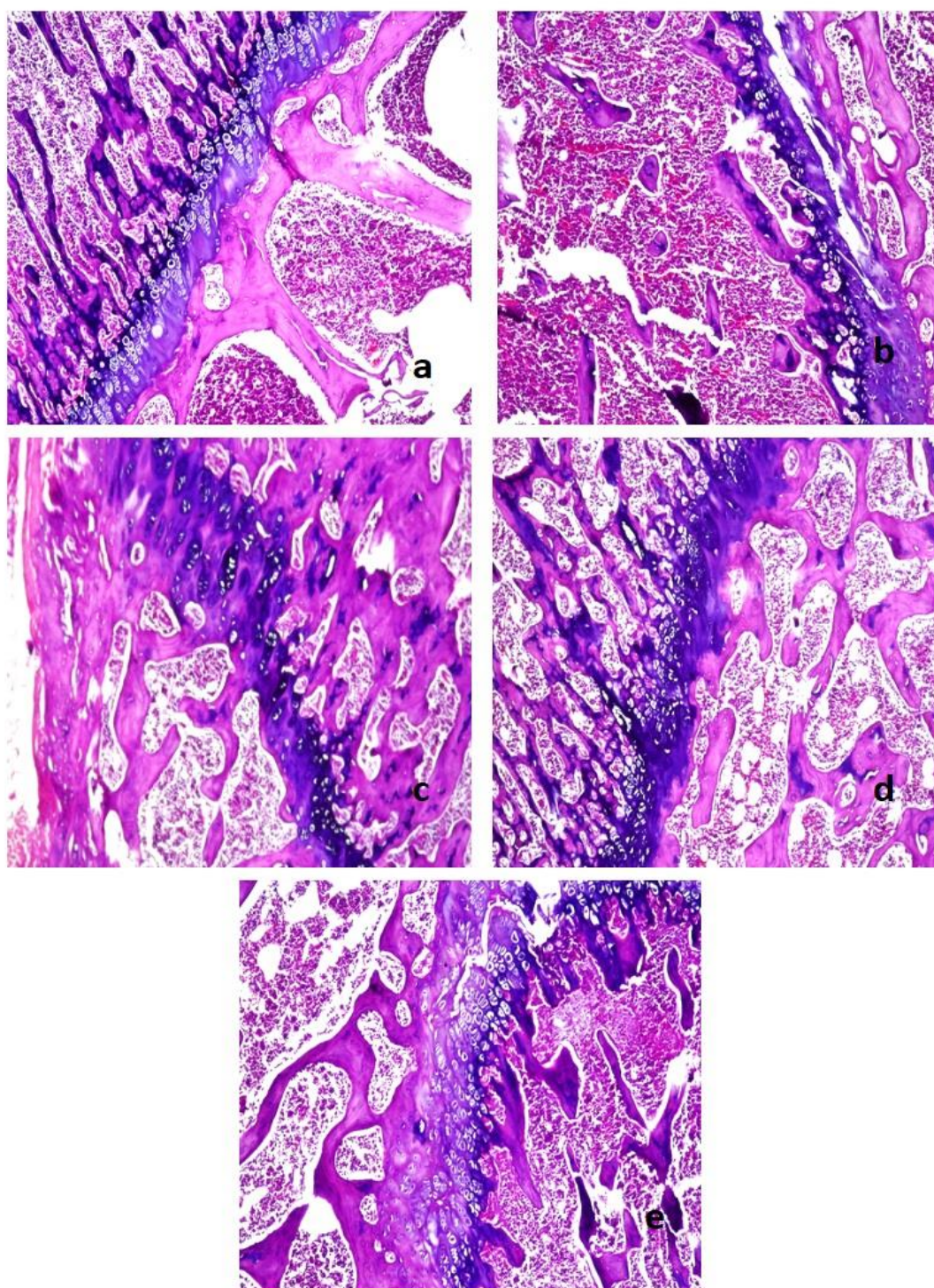
Table 1: Effect of MS-medium* supplemented with 2,4-D, Kin and BA (μ M) at different concentrations on frequency of calli formation from leaf explants of *Brassica oleracea* L. *Var italic* (Broccoli) cultured and incubated under light or dark conditions at $26 \text{ }^{\circ}\text{C} \pm 1$ for 28 days.

MS medium supplemented with : (μ M)			Leaf explants <i>Brassica oleracea</i> L. <i>Var italic</i>	
2,4-D	Kin	BA	Incubation conditions	
			Light (16/8 h)	Dark
	0	0	---	---
0	0.55	0	1.05 \pm 0.13 ^a	3.56 \pm 1.09 ^a
	1.10	0	1.52 \pm 0.23 ^a	4.33 \pm 0.95 ^a
	0	0	4.2 \pm 0.15 ^a	12.58 \pm 1.84 ^a
1.125	0.55	0	23.15 \pm 2.7 ^c	35.18 \pm 4.24 ^b
	1.10	0	14.35 \pm 2.58 ^b	28.19 \pm 5.22 ^b
	0	0	11.25 \pm 2.17 ^{ab}	32.89 \pm 4.15 ^b
2.25	0.55	0	45.12 \pm 3.6 ^d	65.12 \pm 2.17 ^d
	1.10	0	37.12 \pm 2.12 ^d	60.12 \pm 5.16 ^{cd}
	0	0	12.17 \pm 1.48 ^b	15.24 \pm 2.25 ^a
4.5	0.55	0	25.22 \pm 2.95 ^c	52.15 \pm 4.95 ^c
	1.10	0	10.12 \pm 1.17 ^{ab}	49.97 \pm 5.12 ^c
	0	0.55	1.45 \pm 0.18 ^a	1.65 \pm 0.54 ^a
0	0	1.10	1.52 \pm 0.23 ^a	2.19 \pm 0.88 ^a
	0	0.55	5.4 \pm 0.17 ^a	24.17 \pm 2.45 ^b
	0	1.10	7.12 \pm 1.22 ^a	16.95 \pm 2.85 ^a
1.125	0	0.55	14.26 \pm 1.12 ^{ab}	29.85 \pm 2.53 ^b
	0	1.10	11.25 \pm 1.17 ^b	26.58 \pm 3.87 ^b
	0	0.55	7.12 \pm 0.65 ^a	21.12 \pm 2.98 ^b
2.25	0	1.10	8.48 \pm 0.95 ^a	14.89 \pm 2.25 ^a
	0	0.55		
	0	1.10		

*All treatments were modified with 1 g/l Casein hydrolysate.

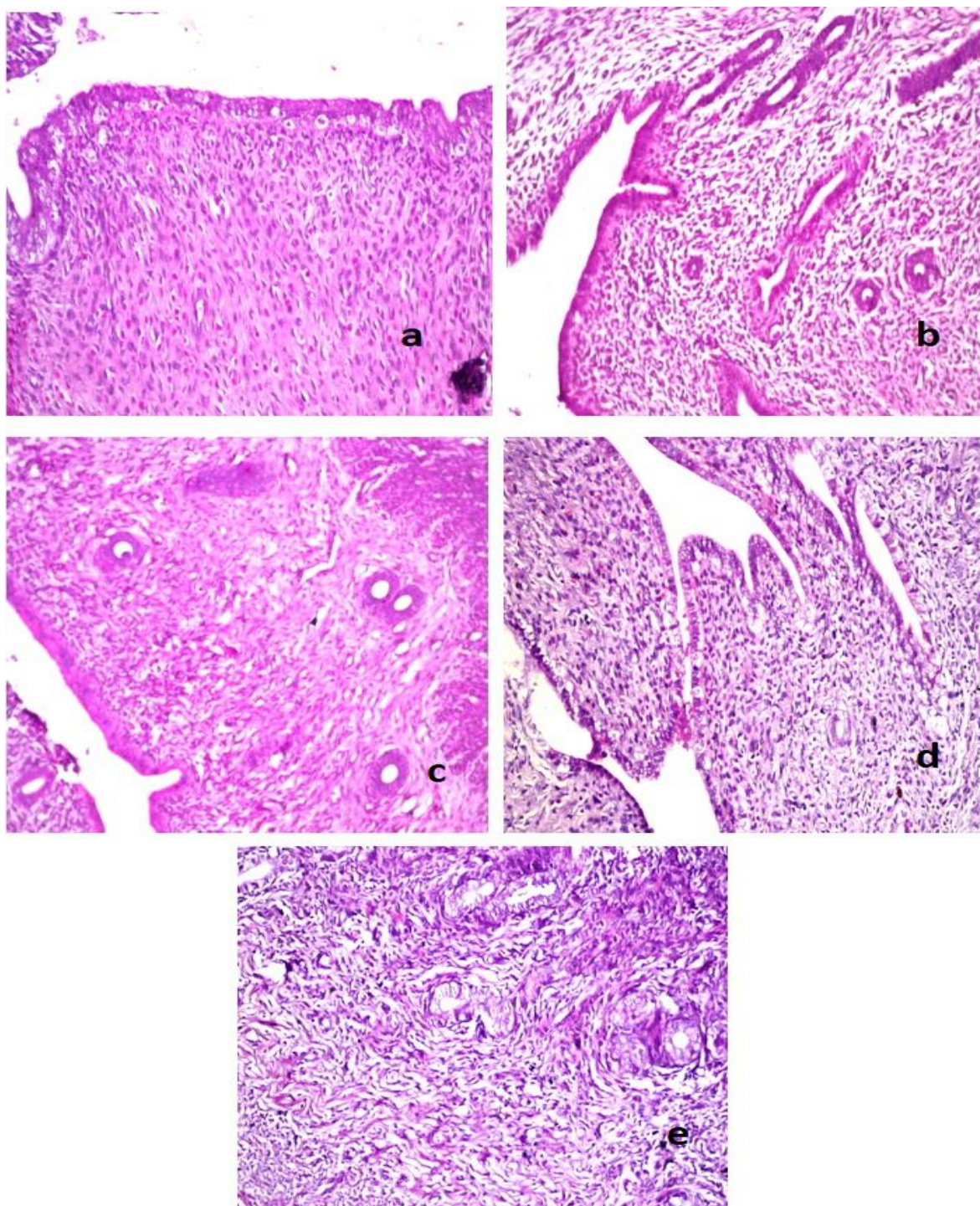
Each treatment is the average of 5 replicates (n = 5) \pm SE,

Means followed by the same letter were not significantly different according to Duncan,s multiple range test $P \leq 0.05$.



a) Control group: showing no histopathological alteration (normal histological structure of the articular cartilaginous surface and the bone trabeculae of the epiphysis and metaphysis). b) OVX group: showing osteoporosis with thinning in the bone trabeculae. c) 17- β -estradiol group: showing no histopathological alteration. d) Low dose of BCSE: There was osteoporosis in the bone trabeculae of the epiphysis and metaphysis. e) High dose of BCSE: There was no histopathological alteration.

Fig.10 Histopathological study of bone



- a) Control group: There was no histopathological alteration and the normal histological structure of the mucosal lining epithelium, lamina propria with glandular structure in the endometrium then the underlying myometrium and perimetrium of the serosa were recorded. b) OVX group: The lamina propria of the mucosal layer showed oedema cellular proliferation and glandular hyperplasia. c) $17\text{-}\beta\text{-estradiol}$ group: The endometrium showed oedema in the lamina propria of the mucosa. d) Low dose of BCSE: Epithelial hyperplasia with polyps formation were detected in the lining mucosal epithelium of the endometrium. e) High dose of BCSE group: oedema was detected in the lamina propria of the mucosal layer.

Fig.11 Histopathological study of uterus

Table (5): Effect of Broccoli cell suspension culture extract (BCSE) on liver and kidney functions

	Normal control	OVX	17- β -estradiol (10 μ g/kg)	BCSE (300 mg/kg)	BCSE (600mg/kg)
GPT (U/L)	13.10 \pm 0.33	41.34 \pm 2.61 ^a	18.11 \pm 0.23 ^{ab}	38.13 \pm 2.15 ^{ac}	19.20 \pm 0.37 ^{ab}
GOT (U/L)	91.04 \pm 2.09	244.60 \pm 1.42 ^a	109.28 \pm 2.75 ^{ab}	112.35 \pm 1.41 ^{ab}	100.75 \pm 2.94 ^{abc}
ALP (U/L)	145.00 \pm 3.77	406.80 \pm 2.62 ^a	168.00 \pm 2.61 ^{ab}	238.50 \pm 3.17 ^{abc}	157.50 \pm 2.22 ^{abc}
Creatinine (mg/dl)	0.66 \pm 0.01	1.42 \pm 0.01 ^a	0.71 \pm 0.03 ^b	0.93 \pm 0.01 ^{abc}	0.73 \pm 0.03 ^{ab}
BUN (mg/dl)	36.40 \pm 0.83	80.70 \pm 2.30 ^a	38.30 \pm 0.58 ^b	59.55 \pm 1.16 ^{abc}	46.72 \pm 0.92 ^{abc}

Data are presented as the mean \pm S.E. of (n=10) for each group. Statistical analysis was carried out by one-way analysis of variance followed by Fisher's LSD test. a Statistically significant from control group. b Statistically significant from OVX group. c Statistically significant from 17- β -estradiol group at P < 0.05.

4. Discussion

Sterilized seedlings were induced from seeds cultured *in vitro* on MS medium free growth regulators [17]. The best augmentations of MS medium for calli production from leaf explants with addition of 2,4-D and kin. in different concentrations compared with other supplementations. It was observed, in current study, that incubation under complete darkness was better for mass calli production from leaf explants than incubation under light condition. Cell suspension cultures were produced from calli using MS medium fortified with the same supplementations in addition to 150 mg/l methionine. In this respect and in agreement of the obtained results, [34] obtained highest value of calli cultures from *Echinacea angustifolia* and *Echinacea pallida*, by culturing of leaf explants on MS medium supplemented with 3 mg/l 2,4-D. Karuppusamy [35] stated that, plant cell, tissue, and organ cultures have been established as routine work under sterile conditions from explants such as plant leaves, stems, roots, meristems, etc., for the production and extraction of hundreds of bioactive secondary metabolites. In this respect, the obtained results clearly showed high levels of total phenolics (TPC) contents in ethanol extracts of both calli and cell suspension cultures with 6.9 and 8.2-fold to the cultivated plant. Meanwhile, total flavonoid contents (TFC) in BCSE were found to be almost the same as the cultivated one. Whereas, the total phenolic and flavonoid content in broccoli Calabrese cultivar recorded 7.68 and 5.46mg/g DW (control), respectively [36]. Furthermore, Ovando-Domínguez et al.[37]stated that a higher content of total phenols was quantified in calli cultures of *Annona purpurea*(27.8 mg g⁻¹ dw) compared to *A.muricata*(23.2 mg g⁻¹ dw). The highest content of total flavonoids was observed in the callus of *A. purpurea* (8.0 μ g g⁻¹ dw). In addition, Misawa[38] stated that difference obtained in the concentration of the metabolites produced by *in vitro* culture is due to the fact that in plant cell cultures,

substances are not always produced qualitatively and quantitatively equal to those of mother plants.

HPLC analysis for BCSE revealed the presence of phenolic acids; ellagic acid, methyl gallate, synergic acid, caffeic acid, cinnamic acid and vanillin in 4.65, 4.1, 3.48, 2.26, 1.7 and 1.6 folds, respectively in comparison with their concentration in the original plant. Chlorogenic acid present in nearly the same concentration. While, the concentration of flavonoids; naringenin, kaempferol, rutin and taxifolin; were in 3.83, 2.79, 1.39 and 1.23 folds, respectively. Methyl gallate and kaempferol were reported to have antioxidant, anti-inflammatory, anticancer and antimicrobial activities [39][40]. Rutin also, has the same effects in addition to be a neuroprotective and on hormone therapy [41]. Qualitative and quantitative analysis using spectrophotometric Table (1) and HPLC Table (2) techniques for calli, BCS and dried inflorescences ethanol extract revealed the presence of TPC in 8 folds and nearly the same in TFC in BCSE with relative to cultivated plant. The elevation in the concentration of individual components was also recorded specially in BCSE in different folds, in addition to their biological activities. These observed data suggested and recommended the use of broccoli cell suspension culture ethanol extract (BCSE) for biological study against osteoporosis in ovariectomized rats.

Ovary secretes the sex steroids regulating fat profile [42] reducing bone resorption, stimulating bone formation [43] and regulating the liver function [44]. Ovariectomy, in this study, increased TG and LDL, decreased HDL and impaired ALP, creatinine, and BUN. Ovariectomy mimicked to post-menopausal state [45]. The main menopausal complaints are vasomotor symptoms[46], urogenital atrophy [47] and osteoporosis that is musculoskeletal alignment and tends to fractures with severe liver and kidney dysfunctions that evidenced by increased levels of AST, ALT[48]. Previously, ovariectomy developed

spontaneous hypercholesterolemia [49] and caused elevated levels of liver function [50], and kidney functions [51], these elevated levels of kidney functions mediated by absence of estrogen anabolic effect [52] or due to oxidative stress induced by ovariectomy that decreased glomerular filtration rate [53]

Treatment with BCSE, in current study, ameliorated lipid, renal and liver dysfunction and especially BCSE high dose had a potency in decreasing cholesterol, TG, GOT and ALP and elevating HDL-chol serum levels more than 17- β -estradiol, in addition, these effects is due to enhancement of flavonoids and phenolics contents in broccoli that have a great role in maintaining the normal hepatic and renal tissues structures [54][55].The flavonoid exhibited hepatoprotective against thioacetamide-induced liver injury mitigating the increment of liver function levels through its antioxidant activity [56]. In addition, Flavonoids and phenolic acids have lipid lowering effects [57]. Broccoli is rich with glucoraphanin compounds that lower LDL levels [58][59].Broccoli which contains phytochemicals such as kaempferol that inhibits osteoclastic cells reabsorption, has anti-inflammatory effect with the suppression of nuclear factor kappa B [60] and antioxidant effect that eliminate cellular responses to oxidative stress [61]. Broccoli active compound, indole-3-carbinol antagonized the aryl hydrocarbon receptor (AhR), which has a role in bone homeostasis maintenance [62].

The current results displayed that ovariectomy reduced uterine weight and decreased estradiol level and mimicked post-menopausal condition state. These results are in agreement with previous study [63][64]. BCSE increased uterine weight, elevated estrogen and returned estradiol 2 to its normal value controlling post-menopausal condition. Moreover, BCSE had more effect in elevating estradiol 2 serum level compared with 17 β -estradiol. This therapeutic effect has been attributed to phytochemicals in broccoli; phenolics and flavonoids. In a previous study, broccoli is very rich in ascorbic acid, quercetin, kaempferol, total phenolics and flavonoids [63]. Another study showed the effect of Kaempferol flavonoids of broccoli in elevating uterine weights and concentration of Estrogen receptors (ER- α) the in uterus [65].

Osteocalcin, non-collagenous protein in bone, is synthesized by osteoblasts and has a vital role in bone mineralization, metabolic regulation and calcium ion homeostasis [66]. Also, TPINP is a more sensitive bone biomarker for measurement of the bone formation rate in osteoporosis treatment. It is found in bone matrix (> 90%) and released during conversion of procollagen type 1 to collagen in the intracellular space. It is removed by proteases and exists in the blood stream [67]Ovariectomy, in this study, increased osteocalcin production and elevated serum

level of TPINP than the sham group, previously lower bone mineral density were showed in ovariectomized rat [68]. and increased serum level of TPINP [69]. It may be the first study on the effect of BCSE administration on serum levels of osteocalcin and TPINP. Its administration produced a decline in osteocalcin production and serum level of TPINP than ovariectomized group. High dose of BCSE decreased osteocalcin and TPINP serum levels more than 17 β -estradiol through enhanced flavonoids.

It is reported that flavonoids have bone anabolic action and therapeutic effect in ovariectomized rats *via* suppression of increased serum osteocalcin [70].kaempferol reduced bone loss and stimulated bone regeneration at fractured site [71][72]. Histopathological study confirmed the obtained results that showed that treatment with BCSE alleviated osteoporosis with thinning in the bone trabeculae, cellular proliferation and glandular hyperplasia in the lamina propria of uterus induced by ovariectomy. This study suggests that BCSE content of phenolic acids and flavonoids has anabolic activity and enhanced bone regeneration thus emphasizing broccoli's positive role on bone health.

Conclusion

BCSE contains high amounts of total phenolics and flavonoids which increased by plant biotechnology technique. BCSE flavonoids and total phenolics have osteomodulating and regenerating effects that might be helpful in the treatment of osteoporosis in this rat model via regulating liver and kidney function, ameliorating the increase in lipid profile, elevating estradiol 2, suppressing osteocalcin and TPINP, and alleviating the bone trabeculae thinning and glandular hyperplasia in the lamina propria of the uterus.

Conflicts of interest

The authors declare that there is no conflict of interest.

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