American Journal of BioScience
2014; 2(6): 217-221
Published online December 02, 2014 (http://www.sciencepublishinggroup.com/j/ajbio)
doi: 10.11648/j.ajbio.20140206.15
ISSN: 2330-0159 (Print); ISSN: 2330-0167 (Online)

Bioactive propolis and bone loss reduction in an ovariectomized rat model of hypogonadal osteoporosis

Aiman Al-Qtaitat[1, *], Said Al-Dalaen[2], Samir Mahgoub[3], Mohammad Al-Rawashdeh[4], Jean E. Aaron[5]

[1]Department of Anatomy and Histology, Faculty of Medicine, Mutah University, Karak, Jordan
[2]Department of Pharmacology, Faculty of Medicine, Mutah University, Karak, Jordan
[3]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Mutah University, Karak, Jordan
[4]Department of Orthopaedic Surgery, Alkarak Governmental Hospital, Karak, Jordan
[5]School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds, UK

Email address:

aimanaq2000@yahoo.com (Al-Qtaitat A.), aldalaensm@yahoo.com (Al-Dalaen S.), samir_mhgb@yahoo.com (Mahgoub S.), mrawashdeh73@yahoo.com (Al-Rawashdeh M.), J.E.Aaron@leeds.ac.uk (Aaron J. E.)

To cite this article:

Aiman Al-Qtaitat, Said Al-Dalaen, Samir Mahgoub, Mohammad Al-Rawashdeh, Jean E. Aaron. Bioactive Propolis and Bone Loss Reduction in an Ovariectomized Rat Model of Hypogonadal Osteoporosis. *American Journal of BioScience.* Vol. 2, No. 6, 2014, pp. 217-221.
doi: 10.11648/j.ajbio.20140206.15

Abstract: Osteoporosis particularly affects post-menopausal women for which the ovariectomized (OVX) rat is an established model and validation tool for agents of bone loss prevention. Objective: The potency of the natural substance propolis was investigated with regard to bone loss in ovariectomized animals. Methods: The complex chemical nature of propolis extract was confirmed by HPLC. Adult female albino rats (n=70; weight 150 – 200 gm), all ovariectomized at 24 weeks were randomly divided into controls (Group I, n=35; OVX/ H_2O-supplement), for comparison with treatment (Group II, n=35; OVX/ propolis-supplement) administered as 400 mg of propolis/kg daily for 9 weeks. Blood biochemical analysis included serum calcium (Ca), phosphorus (P), magnesium (Mg), alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP), with serum osteocalcin (OC) determined by enzyme-linked immunosorbent assay (ELISA). Urine biochemical analysis measured Ca, P and creatinine (Cr). In addition, the corresponding densitometry of bone status comprised the bone mineral content (BMC) and density (BMD) at the proximal, distal and total femur by dual energy X-ray absorptiometry (DEXA; PIXImus), while the morphometry of the femoral shaft thickness was determined in longitudinal sections using an automated image analyzer. Results: A total of 27 compounds including flavonoids was identified in the propolis extract by HPLC. In comparison with OVX controls the skeleton was more substantial morphometrically in the animals receiving propolis, in terms of femoral shaft width ($p<0.01$) and BMD and BMC ($p< 0.01$). Simultaneously, the urine biochemical indices of Ca, P, Cr and Ca:Cr were significantly reduced ($p<0.01$) by propolis, while serum TRAP (an index of bone resorption) was also significantly lower ($p<0.05$), as were serum OC and ALP (indices of bone formation; $p<0.05$). Conclusion: Propolis is chemically complex and statistically bioactive in the oestrogen-deficient rodent, maintaining bone mass by reducing remodeling possibly by interaction with oestrogen receptors.

Keywords: Ovariectomy-Induced Osteoporosis, Rat Femoral Densitometry, Biochemical Remodelling Indices, Propolis Constituents, Bone Loss Reduction, Bone Turnover Reduction

1. Introduction

The metabolic bone disease osteoporosis has been defined by the WHO as "a systemic, skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility". It is global, costly and disabling and fractures of the wrist, spine and hip are diagnostic. At the same time, it is multifactorial and in particular includes age-related sex hormone diminution. In consequence, a gradual process of bone loss in both sexes is especially evident in women after the menopause due to decreased estrogen secretion [1]. For the prevention of hypogonadal bone loss, hormone replacement therapy (HRT) has been used widely [2] with the

acknowledgement of certain low risks with long-term usage (for example, breast, ovarian and endothelial cancer [3]). The bisphosphonates are a frequent alternative in the treatment of osteoporosis, although they too have unwanted side effects (for example, oesophageal cancer and jaw osteonecrosis [4]). Within the present pharmacological lexicon and at the periphery of current investigation are the polyphenolic compounds (flavonoids and phenolic acids [5]). Flavonoids have been characterized as selective estrogen receptor modulators (SERMs) with similar beneficial effects on bone to raloxifene [6]. They have been reported to inhibit bone loss in rats, not only by reducing osteoclastic resorption but also by increasing osteoblastic formation, a combination resulting in stronger bones [7].

Propolis is a resinous natural substance collected by bees from tree bud exudates the species of which determine its extensive chemical composition. Like honey, propolis traditionally has anecdotal health benefits and in ancient cultures it was used to treat several diseases. Today it has the reputation of a natural alternative remedy available at health food stores for topical use including cosmetic purposes. Its recorded antimicrobial, antiviral and antioxidant properties have contributed to its consideration in veterinary practice and it has become commercially available as capsules, mouthwash solutions, creams, powders and throat lozenges, as purified, wax-free products (for a recent review see Wagh, 2013[8]). In the last decade or so the ancient putative biological activity of propolis has attracted sufficient evidence-based affirmation to warrant further investigation of its alleged immuno-modulatory, anti-inflammatory and antioxidant properties. This has resulted in the identification of more than 300 components [9], amongst which steroids, flavonoids, aromatic acids, terpenoids and diterpenic acids and polyphenolic compounds appear to be the principal constituents essential for its possible biological action [8,10]. As well as the plant source of the material, the geographical origin of propolis and ambient climatic conditions influence the relative proportions of its remarkable complexity of constituents.

Of special interest arising from the chemical analysis has been the potential estrogenic activity of propolis as examined both *in vitro* and *in vivo*. It was studied *in vitro* in relation to MCF-7 human breast cancer cell proliferation [11], in human estrogen receptor binding and in yeast-based steroid receptor transcription, while *in vivo* it was used to produce the immature rat uterotrophic effect [12]. Expanding upon this potential plant oestrogenic theme the aim of the present study was to evaluate propolis skeletally in the prevention of osteoporosis as induced by ovariectomy in rats (an established model for postmenopausal osteoporosis [13]). To our knowledge, this would seem to be the first investigation into the potential bone-related effects of this apparently bioactive substance in relation to hypogonadal bone turnover and imbalance induced by estrogen deficiency in an OVX animal model.

2. Materials and Methods

Propolis extract. The intact substance was collected during the spring (April to May) of 2013 from a honey farm in central Jordan. One gram, cut into small pieces, was extracted twice in 24 hours at room temperature with 50 ml of 70% ethanol. The alcoholic extract was evaporated to dryness under vacuum at 50^{o}C and the residue dissolved in methanol and passed through a 0.45 μm filter for HPLC analysis of the polyphenolics (Agilent 1100 Series spectrophotometer with UV detector and auto-sampler [14]). The identified polyphenolic constituents were quantified by chromatographic comparison with validated standards and response factors and concentration of these and the polyphenolics in the propolis sample was calculated [15].

Animals. The ethical regulations of the local Medical Research Ethics Committee (Faculty of Medicine, Mu'tah University, Jordan) were followed in the welfare and treatment of seventy female albino rats (Sprague Dawley strain), average weight 150-200g. They were fed according to the AIN-93M casein-based diet for 24 weeks (Table 1; [16]). Their weight range was 275-330gm. The environmental conditions (temperature, humidity and light) were standardized for acclimatization of the rats. The animals were anaesthetized with sodium pentobarbitone (35 mg/kg, I.P.) for aseptic bilateral ovariectomy which was followed by prophylactic ampicillin (4000 IU/kg I.P.) for three days and antiseptic paste (Coloplast; Humlebaek, Denmark) applied locally. The OVX animals were divided into Group I (n=35) which subsequently received food and water and no added propolis, and Group II (n=35) which was similarly fed and to which 400 mg propolis/kg daily was added to their food for the following 9 weeks during which their body weight was monitored weekly throughout. Their weight range was 290-335gm.

Table 1. *Composition of casein-based AIN-93M diet fed to OVX rats (Groups I and II).*

Ingredients	(g/kg diet)	Ingredients	(g/kg diet)
Corn starch	620.7	Mineral mixture (AIN-93M-MX)	35
Casein (85% protein)	140	Mineral mixture (AIN-93M-VX)	10
Sucrose	100	L-cystine	1.8
Corn oil[#]	40	Choline chloride	2.5
Fiber	50	Ter-butylhydroquinone	0.008

[#] Corn oil was used instead of soybean oil to eliminate any possible interference with isoflavones in soybean oil.

Blood and urine biochemistry. Both alkaline phosphatase (ALP) and osteocalcin (OC) are produced by osteoblasts and are often used as markers of bone formation [19], while tartrate-resistent acid phosphatase (TRAP) is released during osteoclastic bone resorption [20]. Similarly fasting urinary calcium excretion and the calcium:creatinine (Ca:Cr) ratio are used as indicators of net bone resorption [21] together with collagen degradation [22]. Serum analysis was performed to determine levels of calcium (Ca), phosphorous (P), magnesium (Mg), ALP and TRAP using standard colorimetric methods and BioDiagnostic kits (Amman, Jordan), and serum OC was measured by an ELISA kit specific for the rat (Biochemical Technology, Staughton, USA). Urine Ca, P and Cr were analyzed by the same methods.

Bone densitometry and morphometry. The bone mineral content (BMC) and related bone mineral density (BMD) of the two groups were measured proximally, distally and for the total femur using dual energy X-ray absorptiometry (PIXImus; GE Lunar Co, Wisconsin, USA). The corresponding femoral shaft thickness was measured in, 10μ thick longitudinal sections, 10 fields per section, using a computerized image analyzer (Leica Qwin 500) and the mean shaft thickness for each group was determined.

Statistical analysis. Data was compared by one way ANOVA followed by post hoc Sheffe's test using SPSS computer software Version 7.5. The paired t- test was used for the analysis of weekly weight gain, at 0.05 and 0.01 levels of significance.

3. Results

A total of 27 compounds was detected in the propolis extract including naringenin, quercetin-3, 3-dimethylether and pinocembrin. Also found were the three iso-flavones formonontin, genistein and prunetin (Table 2). The monitored weight gain of the OVX rats during the 9 week observation period showed no significant difference between those on the control diet and those receiving this complex propolis supplement (Table 3).

Table 2. *HPLC quantitative analysis of propolis polyphenolics*

	Substance	mg/g propolis		Substance	mg/g propolis		Substance	mg/g propolis
1	Hydrocinnamic acid	18.1	10	Quercetin-7-methylether	4.6	19	Hesperetin	3.9
2	Hydrocaffeic acid	15.7	11	Dimethylallyl caffeate	2.4	20	8-methoxy kaempferol	0.75
3	Coniferyl alcohol	2.8	12	Pinocembrin	39.4	21	Apigenin	1.02
4	Caffeic acid	1.6	13	Luteolin	3.7	22	Luteolin-3′-methylether	5.4
5	Eriordictyol	4.7	14	Quercetin	1.4	23	Prunetin	3.0
6	Liquiriteginin	2.0	15	Naringenin	14.5	24	Formononetin	1.8
7	Myricetin	5.6	16	Pinobankasin	0.83	25	Acacetin	7.0
8	Quercetin-3, 3-dimethylether	22.8	17	Quercetin-3-methylether	1.3	26	Biochanin A	10.2
9	Formonontin	10.1	18	Genistein	1.9	27	Pinostrobin	1.2
							Total	187.7

Table 3. *Weight (gm) comparison of the two groups of rats, at the start of the study, after 24 weeks and after 9 weeks of standard diet feeding or treatment.*

Group	Week 0	Week 24	Week 33
Group I (OVX-Control)	161.00±0.61a	305.78±0.27a	310.70±0.25a
Group II (OVX-Propolis treated)	160.95±0.71*	307.97±0.17*	309.05±0.16*

Values as mean±SE
*Insignificant difference compared to [a]

The serum biochemistry variables in the two groups are shown in Table 4 and there were significant differences. The propolis-supplemented Group II had lower ALP, TRAP and OC (remodelling variables) than Group I controls. No significant differences were found in serum Ca, P and Mg. The urine biochemistry is summarised in Table 5 and all the variables decreased significantly with propolis supplementation. Differences were also observed densitometrically with Group II propolis-treated having a significantly higher BMC and BMD than Group I controls at all the femoral locations examined (Table 6). These results were supported morphometrically in longitudinal slices where the mean femoral shaft thickness in the Group I controls was 203.08 μm ±0.73 compared to the significantly more substantial 501.24 μm±0.81 of the Group II propolis-augmented (P< 0.01).

Table 4. *Serum biochemistry comparison of the two groups of OVX rats with and without propolis for nine weeks of treatment.*

Variable	Group I : OVX control	Group II : OVX propolis
ALP (U/l)	275.42±3.35[a]	156.14±11.27*
TRAP (U/l)	61.9 ±0.50[a]	40.8 ±1.43*
OC (ng/ml)	3.1 ±0.71[a]	0.45±0.08*
Ca (mg/dl)	8.72 ±1.43[b]	9.11±1.49#
P (mg/dl)	5.4 ±0.13[b]	6.01±0.07#
Mg (mg/dl)	2.38±0.38[b]	2.44±0.38#

Values as mean±SE
*Significant difference compared to [a] P< 0.05
#Insignificant difference compared to [b]

Table 5. *Urine biochemistry comparison of the two groups of OVX rats with and without propolis for nine weeks of treatment.*

Variable	Group I : OVX control	Group II : OVX propolis
Ca (mg/24 h)	0.470±0.105[a]	0.081±0.018*
P (mg/24 h)	0.931±1.070[a]	0.825±1.610*
Cr (mg/24 h)	0.532±2.041[a]	0.360±0.060*
Ca:Cr ratio	0.883±0.005[a]	0.225±0.380*

Values are mean±SE
*Significant difference compared to [a] P< 0.01

Table 6. *Bone densitometric BMC (g) and BMD (g/cm^2) femoral comparison (proximal, distal, total) of the two groups of OVX rats with and without propolis for nine weeks of treatment.*

Variable	Group I : OVX control	Group II : OVX propolis
BMD (Proximal)	0.104±0.015[a]	0.122±0.090[*]
BMC (Proximal)	0.072±0.036[a]	0.090±0.039[*]
BMD (Distal)	0.099±0.028[a]	0.115±0.037[*]
BMC (Distal)	0.056±0.032[a]	0.093±0.033[*]
BMD (Total)	0.105±0.005[a]	0.125±0.058[*]
BMC (Total)	0.188±0.092[a]	0.279±0.012[*]

Values as mean±SE
[*]Significant difference compared to [a] P< 0.01

4. Discussion

Two different skeletally-established methodologies have been applied to determine any bone response produced by propolis. Both the standard blood and urine biochemistry indices as measures of bone turnover [19, 20, 21, 22] and the regular morphometry/ densitometry indices as measures of bone mass suggested that over the 9 week treatment period propolis maintained a significant level of skeletal stability in an animal model of osteoporosis. The negative effect on human bone mass of oestrogen deficiency has been extensively recorded in the literature which also includes oophorectomized animal models ranging from primates to rodents (for example, Hodgkinson et al, 1978 [23]; Hordon et al, 2006 [24]). The results above illustrate some of the traits regularly reported with oestrogen decline including a high bone turnover, a negative calcium balance and weight gain [25, 26]. However, in the present investigation no difference was found between the weight gain of the OVX controls and their propolis-tested counterparts, even though a positive association between body weight and bone density is generally recognised [18]. This suggests that the propolis extract may not behave exactly like oestrogen in the regulation of body weight.

The phytoestrogens [27] are well known plant substances with a chemical structure resembling the endogenous oestrogen, estradiol, a similarity enabling them to bind to oestrogen receptors (ERs) producing oestrogenic or anti-oestrogenic effects [18]. It follows that the mechanism whereby propolis flavonoids effect bone turnover rate negatively as indicated by the blood and urine biochemistry may be due to a direct interaction with ER-β which is more abundant in the skeletal system than is ER-α which is prevalent in the reproductive system, especially mammary and uterine tissues. In this way the propolis flavonoids with a high affinity for ER-β may reduce bone loss without at the same time stimulating unwanted tissue proliferation elsewhere. Also contributing to the effect may be other propolis constituents such as steroids and terpenoids [10] some of which have a close structural relationship to estradiol, enabling their binding to ER-α as well as -β receptors [28], perhaps augmenting the propolis property.

At present little more than speculation surrounds the bone bioactivity of propolis and the evidence is limited as yet. At the same time, the results do support a biochemical basis for the possibility that the ancient remedy propolis does indeed contain active and potentially therapeutic ingredients within its complex chemical make-up, and these apparently include oestrogen-related skeletal modulators of bone mass and remodelling rate. It remains to be established whether matrix turnover stays above the threshold critical for daily microfissure control and chronic repair (for example, as fluoride apparently does not (Aaron et al, 1991[29]; 1992 [30]), and also whether propolis extract may offer pharmacological advantages in terms of fewer side-effects compared with current SERMS and other frontline established formulations. In particular, complementary bone histomorphometry is essential before propolis can be more seriously considered as a potential positive agent for the ageing human skeleton.

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