# The Possible Therapeutic Effects of Propolis on Osteoporosis in Diabetic Male Rats

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**Abstract: Objectives**: the objectives of the present study were to confirm the anti-osteopathic effects of propolis as well as to clarify the possible mechanisms of these effects. **Methods:** Thirty six adult male albino rats, weighing 180–200 gm, were used for this study. Ten rats were served as healthy control group (group I), while diabetes mellitus was induced in the rest of the rats by intravenous injection of a single dose of alloxan (40 mg/kg). Rats with blood glucose level less than 300 mg/dL were excluded. The diabetic rats were divided into two equal groups; group II: diabetic non- treated group and group III: diabetic rats treated with a single daily dose of propolis (0.3g/kg) that was supplemented orally in aqueous solution for six weeks. At the end of experimental period, blood samples were obtained and sera were separated for estimation of a bone formation marker (osteocalcin) and urine samples were collected for estimation of a bone resorption marker (hydroxyproline). Bone samples were also collected and osteoprotegerin gene expression was determined with real time-polymerase chain reaction (RT-PCR). **Results:** In the present study alloxan-induced diabetes was characterized by significant decrease in serum levels of osteocalcin (OC) and bone minerals (calcium and phosphorus) concentration in ash of right femurs as well as osteoprotegerin (OPG) gene expression in left femurs. Treatment of diabetic rats with propolis induced significant decrease of fasting blood glucose levels. On the other hand rats treated with propolis showed significant increase in serum levels of OC; together with increased calcium (Ca) and phosphorus (P) bone concentration as well as OPG gene expression; however, all these results were significantly lower than the control group. The hydroxyproline (HYP) levels in urine, showed non-significant changes between different groups. **Conclusion**, propolis may have therapeutic value for osteoporotic bone diseases as it improves bone mineralization and potentiates bone formation. However further researches are needed before its clinical application in humans.
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**Key words:** Osteoporosis, diabetes, propolis, osteocalcin, hydroxyproline and osteoprotegerin.

## 1. Introduction:

The Greek word "propolis" means to glue and it describes also the role of propolis to cement openings of the bee hive. Thus propolis is also named "bee glue" **(Bogdanov, 2012).** Propolis is composed mainly by the plant resins and exudates that bees gather. Bees add wax, and also some secretions and pollen to it. The typical components of popular propolis are the phenolics; flavonoid aglycones, (flavones and flavanones), phenolic acids and their esters **(Qian *et al.*, 2008).** However, the constituents of propolis vary widely due to climate, season, location and year **(Bogdanov, 2012).**

Propolis has several biological and pharmacological properties, as antimicrobial, anti-inflammatory and antioxidant effects (**Kanbur *et al.*, 2009) ,** that is why it is currently used in formulations for treatment of cold syndrome (upper respiratory tract infections, common cold, and flu-like infections), wound healing, treatment of burns, acne, and neurodermatitis (**Wagh, 2013).**

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both **(Brown and Sharpless, 2004).** The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs **(Wongdee and Charoenphandhu, 2011).** In addition to neurovascular, ocular and renal complications, osteopenia and osteoporosis are important debilitating problems in DM patients **(Jackuliak and Payer, 2014).**

Several studies reported that propolis could decrease levels of blood glucose as well as modulate blood lipid and reduce atherosclerosis in patients with diabetes mellitus **(Matsui *et al.*, 2004; Fuliang *et al.*, 2005 and El-Sayed *et al.*, 2009).** Moreover some researchers claim that propolis may play an important role in prevention of bone loss associated with the hyperglycemic state in diabetic rats **(Ang *et al.*, 2009 and Al-Hariri *et al.*, 2011)**. Hence the objectives of the present study were to confirm the anti-osteopathic effects of propolis as well as to clarify the possible mechanisms of these effects through investigating the osteoprotegerin gene expression and bone turnover markers in diabetic male rats exposed to propolis.

## 2. Materials and Methods:

### Animal and experimental design

Thirty six adult male albino rats, weighing 180–200 gm, were used for this study. Rats were kept in plastic cages at room temperature (23-25°C) under a day/night rhythm in our institutional facility with free access to food and water.

Ten rats were served as healthy control group (group I), while diabetes mellitus was induced in the rest of the rats by intravenous injection of a single dose of alloxan (40 mg/kg) through the tail vein **(Fuliang *et al.*, 2005)**. After 7 days the blood glucose levels were measured with an Accu-chek Active strip test in a glucometer (Roche Diagnostic, Mannheim, Germany). Rats with blood glucose level less than 300 mg/dL were excluded. The diabetic rats were divided into two equal groups (ten rats each); group II: diabetic non- treated group and group III: diabetic rats treated with a single daily dose of propolis (0.3g/kg) that was supplemented orally in aqueous solution for six weeks **(Elwakkad *et al.*, 2008).** At the end of experimental period, urine samples, blood samples and bone samples were collected.

### Sample collection and biochemical assays:

At the end of the experiment, urine samples were collected from all rats, in metabolic cages for estimation of hydroxyproline (HYP). Hydroxyproline concentration was determined spectrophotometrically using the method of **Neuman and Logan (1950).** Fasting blood glucose levels (FBG) were measured with an Accu-chek Active strip test in a glucometer (Roche Diagnostic, Mannheim, Germany). Blood samples were obtained from the orbital sinus of overnight fasted rats under light ether anaesthesia. Blood was immediately centrifuged at 3000 (rpm) for 20 minute. Sera were separated and stored at -80 C until the day of analysis. In the blood specimens, biochemical bone formation marker; osteocalcin (OC) was measured using, enzyme-linked immunoassay kits (Formosa Biomedical kits).

The right femurs of all experimental rats were dissected out, and left at room temperature for 24 hours then dried in an oven at 100°C for 24 hours, and then ashed in a furnace at 800°C for 12 hours. The ash of each femur was dissolved in 3 mL of 70% nitric oxide and centrifuged; the supernatant was separated for the measurement of calcium (Ca) and phosphorus (P), and by the standard colorimetric method (**Norazlina *et al.*, 2002).** The left femurs were used for osteoprotegerin (OPG) gene expression measurement.

### Detection of osteoprotegerin gene expression with real time-polymerase chain reaction (RT-PCR):

The technique described by **Pfaffl MW (2001)** was used for RT-PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies.

1-**Ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis:**

Total RNA was isolated from bone tissue homogenates using trizol reagent (TM) (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically. The integrity of the RNA was studied by gel electrophoresis on a 1% agarose gel, containing ethidium bromide. First-strand cDNA synthesis was performed with the Super Script Choice System (Life Technologies, Breda, the Netherlands) by mixing 2 µg total RNA with 0.5 µg of oligo (dT)12-18 primer in a total volume of 12 µL. After the mixture was heated at 70°C for 10 min, a solution containing 50 mmol/L Tris HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, 10 mmol/L DTT, 0.5 mmol/L dNTPs, 0.5 µL RNase inhibitor, and 200 U Superscript Reverse Transcriptase was added, resulting in a total volume of 20.5 µL. This mixture was incubated at 42°C for 1 h and then stored at –80°C until further use.

### 2-real time quantitative PCR

For real time quantitative PCR, 1 µL of first-strand cDNA diluted 1:10 in RNase-free water was used in a total volume of 25 µL, containing 12.5 µL 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer. Primers, designed with the Primer Express software package (Applied Biosystems), the sequence for OPG gene (forward 5′- TTG TGT GAC AAA TGT GCT CC-3′, (Reverse 5′- GAC GTC TCA CCT GAG AAG -3′) and GAPDH primers (forward 5′-TTCGACAGTCAGCCGCATCTTCTT-3′, reverse 5′-CAGGCGCCCAATACGACCAAATC -3′). PCR reactions, consisting of incubation in 95°C for 10 min (1 cycle), 94°C for 15 s, and 60°C for 1 min (40 cycles), were performed on an ABI Prism 7900 HT Fast Real Time PCR system (Applied Biosystems). Data were analyzed with the ABI Prism 7900 sequence detection system software (genetic analyzer; version 2.2) and quantified with the comparative threshold cycle method with beta actin as a house keeping gene reference.

### Statistical analysis

Data are expressed as means ± standard error (SE). Statistical comparison between different groups were done using one way analysis of variance (ANOVA) followed by Tukey HSD multiple comparison test to judge the difference between various groups. All calculations were performed using the SPSS 16.0 software package. Significance was accepted at $P< 0.05$.

## 3. Results

In the present study alloxan-induced diabetes was characterized by significant decrease in serum levels of OC and bone minerals (Ca and P) concentration in ash of right femurs as well as OPG gene expression in left femurs **(Table 1)**.

At the end of the treatment period, diabetic rats treated with propolis showed significant decrease in fasting blood glucose levels. On the other hand serum levels of OC were increased significantly. In bone, Ca and P bone concentration as well as OPG gene expression were significantly increased; however, all these parameters were significantly lower than the control group.

On the other hand the HYP levels in urine, showed non-significant changes between different groups.

**Table (1): Bone minerals, bone turnover markers and OPG in different groups.**

| GROUPS / Parameters | Group I (Control) | Group II (diabetic non- treated) | Group II (diabetic treated with propolis) |
|---|---|---|---|
| **FBG (mg/dL)** | 98.52±0.60[a] | 320.52±0.30[b] | 120.52±0.32[c] |
| **Ca (%)** | 38.52±0.68[a] | 26.06±0.75[b] | 31.45±0.35[c] |
| **P (%)** | 17.31±0.34[a] | 14.77±0.31[b] | 15.63±0.28[c] |
| **OC (ng/ml)** | 693.28±0.39[a] | 102.96±0.41[b] | 500.11±0.29[c] |
| **HYP (µg/24h)** | 48.71±0.69[a] | 49.96±0.79[a] | 47.42±0.67[a] |
| **OPG (%)** | 0.99±0.31[a] | 0.17±0.45[b] | 0.58±0.85[c] |

Means followed by the same letter within the same row are not significantly different at $p<0.05$ level using Tukey HSD test.

## 4. Discussion:

In the present study alloxan-induced diabetes was characterized by significant decrease in serum levels of OC and bone minerals (Ca and P) concentration in ash of right femurs as well as OPG gene expression in left femurs. Similar results were found by **Martin and McCabe (2007**) who reported that diabetes was associated with a decrease in Ca and P of bone ash of streptozotocin induced diabetic rats. Also, continuous bone loss and deficient bone mineralization have been found in diabetic mice **(Demontiero *et al.*, 2012).**

Although several studies reported that bone health was compromised with diabetes, but the definitive causes were not known **(Wongdee and Charoenphandhu, 2011).** However previous data suggested that increased osmolarity associated with diabetes contributes to decreased activity of osteoblasts **(Coe *et al.*, 2013**). DM also induces lipid accumulation in the marrow of long bones, thereby leading to the expansion of marrow cavity and thinning of cortical envelope. The osteoblast-adipocyte shift might also reduce the number of differentiated osteoblasts available for bone formation (**Wang *et al.*, 2010**). In addition diabetic patients show low levels of insulin-like growth factor I, which is an anabolic hormone that maintains healthy bone formation (**van Dijk *et al.*, 2014).**

In consistent to the results of this study **Abuohashish *et al.* (2013**) noticed that osteoblast secretion of osteocalcin was decreased in streptozotocin induced diabetic rats, indicating that bone formation as assessed by osteocalcin was decreased. However HYP levels in urine, showed non-significant changes between different groups indicating that in hyperglycemic status, bone formation was probably more affected than bone resorption leading to osteopenia **(Sirasanagandla *et al.*, 2014).**

Osteoprotegerin (OPG) is a secreted member of the tumor necrosis factor (TNF) receptor superfamily, which has a strong anti-resorptive effect on bone. It exerts its effect through neutralization of the receptor activator for NFκB ligand (RANKL), a cytokine with strong osteoclast-inducing activity (**Rasmussen *et al.*, 2006**).

RANKL and OPG are a key agonist/antagonist cytokine system that regulates differentiation, fusion, survival, activation and apoptosis of osteoclasts. RANKL increases the pool of active osteoclasts by activating its specific receptor RANK located on osteoclastic cells, thus increasing bone resorption, whereas OPG, which neutralizes RANKL, has the opposite effect (**Galluzzi *et al.*, 2005**).

In consistent to this work, **Rasmussen *et al.* (2006)** observed increased plasma OPG in diabetic patients with nephropathy and with signs of cardiovascular disease. In addition, they reported that plasma OPG was positively correlated to kidney function, glycated hemoglobin (HbA1c) and systolic blood pressure. This refers to the involvement of OPG in the development of diabetic vascular complications **(Kang *et al.*, 2009).**

Propolis contains large amounts of flavonoids which are benzopyrone derivatives found in all photosynthesizing cells. Flavonoids have many biological effects in animal systems and most probably, the beneficial effects of propolis and honey

are the result of their effects **(Grange and Davey, 1990).**

Previous studies have shown that propolis reduced fasting blood glucose (FBG) through improving oxidative stress and lipid metabolism in alloxan-induced diabetic rats as it reduces plasma levels of total cholesterol, malondialdehyde (MDA) and nitrous oxide synthase (NOS), while increases superoxide dismutase (SOD) levels **(Zhu *et al.,* 2011 and Usman, 2014).**

Moreover, water extracts of propolis have been studied to prevent the destruction of beta cells by inhibiting the activation of IL-1β and NO synthase activity in diabetic patients (**Rifa'i and Widodo, 2014).** On the other hand propolis is known to contain high-level of nutrient factors including vitamins, polyphenols, and amino acids that would be expected to improve insulin sensitivity. Thus, intake of propolis to decline the expression of inflammatory molecules is one of strategies to ameliorate hyperglycemia in diabetic patients **(Mahmoud and Al-Ozairi 2013; Aoi *et al.,* 2013).**

The antihyperglycemic effect of propolis may contribute to prevention of bone loss associated with diabetes **(Al-Hariri *et al.,* 2011).**

In agreement with the results of this study, **Elwakkad *et al.* (2008)** found that fish liver oil and propolis increase the bone formation markers (osteocalcin and of bone alkaline phosphatase) and decrease the bone resorption ones (N-telepeptide of type 1 collagen) in epileptic rats treated with valproate (which cause osteoporosis). They increase the OPG and decrease RANKL which inhibit the osteoclastogenesis.

In bone marrow- derived cell cultures, **Pileggi *et al.* (2009)** observed the direct inhibitory effect of propolis on late stages of osteoclast maturation. Moreover **Ang *et al.* (2009)** reported that propolis inhibits differentiation of osteoclasts through increasing OPG which binds RANKL that leads to bone resorption. Others found that caffeic acid phenethyl ester which is an active component of propolis attenuates osteoclastogenesis and bone resorption via the suppression of RANKL (**Ha *et al.,* 2009).**

**Thus,** the results of this work confirm that propolis can protect from bone loss associated with diabetes mellitus in alloxan-induced diabetic rats as mentioned in earlier reports **(Ang *et al.,* 2009 and Al-Hariri *et al.*,2011)**. In addition this study reports that this antiosteopathic effect could be through increasing osteoprotegerin gene expression.

**In conclusion**, propolis may have therapeutic value for osteoporotic bone diseases as it improves bone mineralization and potentiates bone formation. However further investigations are needed to confirm the direct effect of propolis on osteoprotegerin gene expression level and to identify the active component responsible for this effect. Also, further researches are needed before its clinical application in humans.

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