Results
**Effect of *Cissus quadrangularis* on BMP-2 gene expression in rat femoral fracture callus**

BMP-2 plays a key role in fracture repair, especially at the early stage of fracture healing (Tsuji *et al.*, 2006). The present study was carried out in femur fractured rat treated with *C. quadrangularis*. Fracture callus from control and *C. quadrangularis* treated rats were removed at each time point and mRNA expression of BMP-2 in the fracture callus was quantified by real-time RT-PCR ([Fig. 1](#)). Ethanolic extract of *C. quadrangularis* significantly increased (3-4 fold) the BMP-2 mRNA expression when compared to control on post fracture days 7, 14 and 21. **Fig. 2** shows the protein expression of BMP-2 in control and *C. quadrangularis* treated groups. The BMP-2 protein expression was also significantly increased (*p*<0.05) in the callus of *C. quadrangularis* treated rats when compared to respective control at all-time points.

**Effect of *Cissus quadrangularis* on BMP-4 gene expression in rat femoral fracture callus**

BMP-4 plays an important role in maintaining chondrogenic phenotype by stimulating the synthesis of type II collagen (Steinert *et al.*, 2003). In this study, *C. quadrangularis* significantly increased (2 fold) BMP-4 mRNA expression when compared to control on post fracture days 7, 14 and 21 ([Fig. 3](#)). The levels of BMP-4 protein were also significantly increased (*p*<0.05) in *C. quadrangularis* treated rats when compared to control on post fracture days 7 and 14 ([Fig. 4](#)).
Effects of *Cissus quadrangularis* on BMP-5 and BMP-6 mRNA expression in rat femoral fracture callus

BMP-5 transcripts are expressed at the earliest stages of skeletal development in most bones (Solloway and Robertson, 1999). Fig. 5 shows the mRNA expression of BMP-5 on post-fracture days 7, 14 and 21 in the control and experimental groups. *C. quadrangularis* treatment registered a remarkable increase (3 fold) in BMP-5 mRNA expression in the femoral fracture callus compared to control.

BMP-6 is reported to stimulate cartilage and bone formation *in vitro* as well as *in vivo* (Yamaguchi *et al.*, 1996). *C. quadrangularis* treatment induced a 2.5 fold increase in BMP-6 mRNA expression in the femoral fracture callus at all-time points studied (Fig. 6).

Effects of *Cissus quadrangularis* on BMP-7 and BMP-14 mRNA expression in rat femoral fracture callus

BMP-7 stimulated the proliferation, osteogenic differentiation and mineralization in human osteoporotic tissues and in estrogen-deficient rat fracture callus explant cultures (Wei *et al.*, 2010). Fig. 7 represents the mRNA expression of BMP-7 on post-fracture days 7, 14 and 21 in the control and experimental groups. *C. quadrangularis* treatment increased (2 fold) the BMP-7 mRNA expression in the femoral fracture callus on day 14 when compared with respective control.

BMP-14 is also known as growth differentiation factor-5 and cartilage-derived morphogenetic protein-1 (GDF-5, CDMP-1). BMP-14-deficient mouse fractures exhibited a delay in peak area cell density, callus organization and bone formation compared with controls (Chhabra *et al.*, 2005). *C. quadrangularis* treatment induced a
significant increase (1.5-2 fold) in BMP-14 mRNA expression in the femoral fracture callus compared to control at all-time points studied (Fig. 8).

**Effect of *Cissus quadrangularis* on IGF-I and IGF-II mRNA expression in rat femoral fracture callus**

IGFs promote cell proliferation and matrix synthesis by chondrocytes and osteoblasts (McCarthy *et al.*, 1989). These two cell types are largely responsible for the formation of fracture callus. *C. quadrangularis* significantly increased (3-4 fold) the IGF-I (Fig. 9) and IGF-II (Fig. 10) mRNA expression when compared to controls on post fracture days 7, 14 and 21.

**Effect of *Cissus quadrangularis* on IGF-IR gene expression in rat femoral fracture callus**

IGF-1R-deficient mice showed organ hypoplasia, delayed skeletal calcification, severe growth retardation, and invariably died postnatally as a result of respiratory dysfunction (Liu *et al.*, 1993). In this study, *C. quadrangularis* significantly increased (2 fold) the IGF-IR mRNA expression when compared to controls on post fracture days 7, 14 and 21 (Fig. 11). The levels of IGF-IR protein were also significantly increased (*p*<0.05) in *C. quadrangularis* treated rats when compared to controls at all the 3 time points studied (Fig. 12).

**Effect of *Cissus quadrangularis* on IGFBP-3 gene expression in rat femoral fracture callus**

IGFBP-3 is the third most abundant IGFBP expressed in human osteoblasts (Miyakoshi *et al.*, 1999). *C. quadrangularis* significantly increased (2 fold) the IGFBP-3 mRNA expression when compared to controls on post fracture days 7, 14...
and 21 (Fig. 13). IGFBP-3 protein expression was also significantly increased ($p<0.05$) in *C. quadrangularis* treated rats when compared to controls on post fracture days 7, 14 and 21 (Fig. 14).

**Effect of *Cissus quadrangularis* on IGFBP-5 gene expression in rat femoral fracture callus**

IGFBP-5 is the most abundant IGFBP stored in bone and plays an important role in the regulation of bone formation (Amaar *et al.*, 2002). Fig. 15 shows the mRNA expression of IGFBP-5 on post-fracture days 7, 14 and 21 in the control and experimental groups. *C. quadrangularis* treatment registered a remarkable increase (2 fold) in IGFBP-5 mRNA expression in the femoral fracture callus compared to control. The levels of IGFBP-5 protein were also significantly increased ($p<0.05$) in *C. quadrangularis* treated rats when compared to controls on post fracture days 7, 14 and 21 (Fig. 16).

**Effect of *Cissus quadrangularis* on VEGF gene expression in rat femoral fracture callus**

VEGF plays an essential role in every step of the fracture repair cascade from being concentrated in fracture hematoma, to the final remodeling stages of fracture repair (Beamer *et al.*, 2010). This present study, *C. quadrangularis* significantly increased (2 fold) the VEGF mRNA expression when compared to controls on post fracture days 7 and 14 (Fig. 17). VEGF protein expression was significantly increased ($p<0.05$) in *C. quadrangularis* treated rats when compared to controls on post fracture days 7 and 14 (Fig. 18).
**Effect of Cissus quadrangularis on the specific activity of ALP in the rat femoral fracture callus**

ALP is involved in the bone mineralization processes. It is found enriched in matrix vesicles, which probably play a role in extracellular matrix processing and calcification of bone (Dean et al., 1994). The specific activity of ALP was significantly increased in the femoral fracture callus of *C. quadrangularis* treated rats when compared with control at all-time points studied (Fig. 19).

**Effect of Cissus quadrangularis on the specific activity of TRAP in the femoral fracture callus of rats**

TRAP is secreted from the ruffled border, dephosphorylates osteopontin and allows osteoclast migration. TRAP generates ROS that are targeted for degradation of the organic matrix components. The matrix degradation products are released from the osteoclast into the blood circulation together with TRAP, and the Secreted TRAP has been considered to be a marker of osteoclast activity (Halleen et al., 2001). The specific activity of TRAP was significantly decreased in the femoral fracture callus of *C. quadrangularis* treated rats when compared with control at day 7 and 14. No significant change was observed at day 21 in *C. quadrangularis* treated rats (Fig. 20).

**Effects of Cissus quadrangularis on the antioxidant enzymes (SOD, GPx and GST) in the femoral fracture callus of rats**

Fig. 21 shows the activity of SOD in the control and experimental groups. The SOD activity was significantly decreased (*p*<0.05) in the femoral fracture callus on post fracture day 7 and 14. However, *C. quadrangularis* treatment significantly
increased \((p<0.05)\) the SOD activity on post fracture day 7 and 14 in femoral fracture callus.

The GPx activity was decreased significantly \((p<0.05)\) on post fracture day 7, 14 and 21 in the femoral fracture callus. However, \textit{C. quadrangularis} treatment showed a significant increase in GPx activity \((p<0.05)\) on post fracture day 7, 14 and 21 in the femoral fracture callus when compared to respective control (Fig. 22).

The activity of GST in control and experimental groups was shown in Fig. 23. The GST activity was significantly reduced \((p<0.05)\) on post fracture day 7, 14 and 21 in the femoral fracture callus. However, the GST activity was significantly increased \((p<0.05)\) by \textit{C. quadrangularis} treatment on post fracture day 7, 14 and 21 in the femoral fracture callus.

Effects of \textit{Cissus quadrangularis} on the level of lipid peroxidation and hydrogen peroxide in the femoral fracture callus of rats

Femoral fracture registered an increased level of lipid peroxidation and hydrogen peroxide. However, interestingly, administration of \textit{C. quadrangularis} significantly reduced \((p<0.05)\) the levels of lipid peroxidation (Fig. 24) and hydrogen peroxide (Fig. 25) in the femoral fractured rats when compared to control at all- time points.
Unilateral fractures were produced in the left femur by anesthetizing 225-250 g of female Sprague-Dawley rats with Ketamine 80 mg/kg/bw/ip and xylazine 10 mg/kg/bw/im and using a scaled-down version apparatus originally described by Bonnarens and Einhorn (1984).

A midline anterior knee incision was made and a 23G-gauge needle (1.63 mm in diameter) was inserted into the left femur.

After closing the knee joint, radiographs were obtained to confirm pin placement.

The mid-diaphysis of the pinned femur was fractured by a force generated by dropping a 500 g weight from a height of 35 cm to produce fracture on the left femur.

After awakening from the anesthesia, the rats were allowed for unrestricted full weight-bearing activity. Radiographs were obtained to confirm fracture induction.
After intramedullary pinning, induction of fracture is done by scale down method (500 g weight dropped from 35 cm height)

Adopted from Bonnarens and Einhorn, 1984
Female rat of 225-250 g weight

Anesthesia

Cleaning and shaving

Pin insertion

1 mm hole on condyles

Lateral incision

Closing the knee joint
Radiograph (X-ray)

X-Ray image of pin-inserted bone (Before fracture)

After fracture

Unilateral fracture induction

Fracture apparatus

Cont.....
Sample Collection

After fracture  \rightarrow  Cissus quadrangularis  \rightarrow  Callus

Day 14  \rightarrow  Day 21

14th day Control  C.Q.
21 days Callus
Control  C.Q.
Fig. 1: Effect of *Cissus quadrangularis* on BMP-2 mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of BMP-2 mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{-\Delta\Delta C_t}$ method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 2: Effect of *Cissus quadrangularis* on BMP-2 protein expression in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations

‘*’ denotes statistical significance at $p < 0.05$ when compared with control
Fig. 3: Effect of *Cissus quadrangularis* on BMP-4 mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of BMP-4 mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 4: Effect of *Cissus quadrangularis* on BMP-4 protein expression in the femoral fractured callus of rats

Each bar represents Mean ± SEM of 3 observations

‘*’ denotes statistical significance at $p < 0.05$ when compared with control
Fig. 5: Effect of *Cissus quadrangularis* on BMP-5 mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of BMP-5 mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The 2^{-ΔΔCt} method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
**Fig. 6**: Effect of *Cissus quadrangularis* on BMP-6 mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of BMP-6 mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 7: Effect of *Cissus quadrangularis* on BMP-7 mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of BMP-7 mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 8: Effect of *Cissus quadrangularis* on BMP-14 mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of BMP-14 mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{ΔΔCt}$ method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. “*” denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 9: Effect of *Cissus quadrangularis* on IGF-I mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of IGF-I mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 10: Effect of *Cissus quadrangularis* on IGF-II mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of IGF-II mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{-\Delta \Delta Ct}$ method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 11 Effect of *Cissus quadrangularis* on IGF-IR mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of IGF-IR mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{-\Delta\Delta}\text{Ct}$ method of relative quantification was used to determine the fold change in expression with $\beta$-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 12: Effect of *Cissus quadrangularis* on IGF-IR protein expression in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations

"*" denotes statistical significance at $p < 0.05$ when compared with control
Fig. 13: Effect of *Cissus quadrangularis* on IGFBP-3 mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of IGFBP-3 mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The 2^{ΔΔCt} method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 14: Effect of *Cissus quadrangularis* on IGFBP-3 protein expression in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations

"*" denotes statistical significance at $p < 0.05$ when compared with control
Fig. 15: Effect of *Cissus quadrangularis* on IGFBP-5 mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of IGFBP-5 mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{-\Delta\Delta C\text{t}}$ method of relative quantification was used to determine the fold change in expression with β-actin. Values are mean ± SEM of triplicate of 3 independent experiments. “*” denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 16: Effect of *Cissus quadrangularis* on IGFBP-5 protein expression in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations

‘*’ denotes statistical significance at $p < 0.05$ when compared with control
Fig. 17: Effect of *Cissus quadrangularis* on VEGF mRNA expression in the femoral fracture callus of rats

Representative graph showing real-time RT-PCR amplification of VEGF mRNA expression in the fracture callus of femur in CQ treated rat on post fracture days 7, 14 and 21. The $2^{\Delta\Delta C_t}$ method of relative quantification was used to determine the fold change in expression with $\beta$-actin. Values are mean ± SEM of triplicate of 3 independent experiments. ‘*’ denotes statistical significance at the level of $p \leq 0.001$ when compared with control.
Fig. 18: Effect of *Cissus quadrangularis* on VEGF protein expression in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations

‘*’ denotes statistical significance at $p < 0.05$ when compared with control
Fig. 19: Effect of *Cissus quadrangularis* on the specific activity of ALP in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations. ‘*’ denotes statistical significance at $p < 0.05$ level.
Fig. 20: Effect of *Cissus quadrangularis* on the specific activity of TRAP in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations. ‘*’ denotes statistical significance at $p < 0.05$ level.
Fig. 21: Effect of *Cissus quadrangularis* on the SOD activity in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations. ‘*’ denotes statistical significance at $p<0.05$ level.
Fig. 22: Effect of *Cissus quadrangularis* on the GPx activity in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations. ‘*’ denotes statistical significance at $p < 0.05$ level.
Fig. 23: Effect of *Cissus quadrangularis* on the GST activity in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations. ‘*’ denotes statistical significance at \( p < 0.05 \) level.
Fig. 24: Effect of *Cissus quadrangularis* on the lipid peroxidation in the femoral fracture callus of rats

![Graph showing the effect of *Cissus quadrangularis* on lipid peroxidation](image)

Each bar represents Mean ± SEM of 3 observations. ‘*’ denotes statistical significance at $p < 0.05$ level.
Fig. 25: Effect of *Cissus quadrangularis* on the level of hydrogen peroxide in the femoral fracture callus of rats

Each bar represents Mean ± SEM of 3 observations. ‘*’ denotes statistical significance at $p < 0.05$ level.