Original article

Shilajit potentiates the effect of chemotherapeutic drugs and mitigates metastasis induced liver and kidney damages in osteosarcoma rats

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Abstract

Osteosarcoma is a primary malignant cancer of the bone identified by the direct formation of osteoid tissue or immature bone by cancer cells. The liver and kidneys represent two major secondary organs to which osteosarcoma metastasizes. In this study, we assessed Shilajit, a phytomineral diffusion traditionally used in Ayurvedic medicine, for its possible protective effects against metastasis induced liver and kidney damages in an osteosarcoma rat model. Osteosarcoma rats displayed typical dysregulation of serum levels of hepatic and renal functional markers (p < 0.05) including aspartate aminotransferase (AST)* and alanine aminotransferase (ALT), alkaline phosphatase (ALP), total proteins, albumin, bilirubin, creatinine, urea, and uric acid. Changes in functional markers were also positively correlated with marked histopathological alterations in liver and kidney tissues. Whereas Shilajit’s treatment of osteosarcoma rats in combination with CMF (cyclophosphamide, methotrexate, and 5-fluorouracil) chemotherapy drug cocktail significantly (p < 0.05) reversed the studied functional markers to their near-normal levels. Co-treatment of shilajit and drug cocktails also markedly alleviated histopathological changes in liver and kidney tissues. Correlation co-efficient analysis of hepatic and renal functional markers revealed a significant inter-association among these markers. Collectively, present data indicate that shilajit may potentiate the effects of chemotherapy drugs and mitigate the metastasis-induced liver and kidney damage in osteosarcoma. Thus, the findings of this study substantiate the beneficial health effects of shilajit and promote its regular consumption.

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1. Introduction

Osteosarcoma (OS), also named osteogenic sarcoma, is one of the commonest primary tumors of the skeleton recognized by osteogenic progenitor cells or mesenchymal cells producing osteoid and immature bone (Bielack et al., 2009). Worldwide, 2–4/million OS cases per year are reported to occur with the first increase in incidence between 15 and 19 years which is followed by a second minor peak in people above 60 years (Mirabello et al., 2009; Whelan et al., 2012). The OS most commonly originates in the metaphysis of the proximal tibia, distal femur, proximal humerus and proximal femur, while it is rarely found in the axial skeleton. In localized OS, 60 %–70 % survival rate is reported to reach a plateau in 5 years since the commencement of systematic chemotherapy (Stiller et al., 2001). However, in metastasis cases, a 20 % survival rate is still unexpected (Bielack et al., 2002; Kager et al., 2003; Mialou et al., 2005). Importantly, subclinically, most OS patients are likely to have micrometastatic lesions at the time of diagnosis. However dismal 15 %–20 % of OS cases which are newly diagnosed, are successfully diagnosed with metastasis (Kaste et al., 1999). The high propensity of OS to metastasize is the major cause of poor prognosis and failed treatments. OS is heterogeneous when it comes to its metastasis. For instance, tumors may grow concomitantly with the primary tumor while the treatment is on or soon after a long gap or after the end of the treatment regimen. Interestingly, metastasized tumors are
identical in histology to that of the primary tumor or display a variation in a different differentiation pathway (Kager et al., 2003; Odri et al., 2022). The metastatic osteosarcoma cells go through a series of crucial stages to populate and grow in the secondary site and eventually advance to clinically identifiable tumors. Additionally, the biology of metastasis differs from the primary tumor with regard to karyotype, cell cycle, metabolism, surrounding microenvironment, and differentiation. These variations are reported to be caused due to differences in the expression of relevant genes, changes in molecule profiles as well as interaction with tumor surroundings (Gorlick et al., 2003; PosthumaDeBoer et al., 2011). Several recent and previous studies reported that osteosarcoma could metastasize to extraskeletal organs such as the liver and kidney, rendering the disease treatment more complex and difficult (Dalinka et al., 1971; Daw et al., 2001; Hagay et al., 1986; Sigdel et al., 2021). Chemotherapy remained the primary choice for the treatment of metastasized OS. However, the outcome of chemotherapy in most cases is unsatisfactory with lower than expected recovery, relapse of tumor and the well-known side effects of chemotherapy. Chemotherapy-linked shortcomings led to renewed interest in research finding dietary intervention with minimal or no side effects and high potency in the treatment of OS (Leite et al., 2021; Rabelo et al., 2022).

Shilajit, which is known by different names such as salajeet, mumie, mumijo, mummiyo, and moominjo, is a phytomineral diffusion found in sedimentary rocks and appears blackish-brown colored semi-solid matter with a sharp odor and pungent tang, formed due to the extended humification process of many plants (Agarwal et al., 2007; Carrasco-Gallardo et al., 2012a, 2012b). The chemical composition of shilajit is well characterized and found to contain 60–70 % humus (Agarwal et al., 2007; Aldakheel et al., 2021; Ghosal et al., 1976; Schepetkin et al., 2003). Shilajit has been used extensively in Indian traditional medicine Ayurveda to treat chronic diseases and a number of ailments due to its medicinal properties (Jafari et al., 2019; Schepetkin et al., 2003). Shilajit is shown to act as a chemotherapeutic agent against urinary bladder cancer and to induce apoptosis in human breast cancer cells (Kloskowski et al., 2021; Rahmani Barouji et al., 2020b). Wound-induced inflammation and aspirin-induced gastric lesions were found to be abrogated in rats by shilajit treatment (Ghasemkhani et al., 2021; Kim et al., 2021). Shilajit activated peritoneal macrophages and splenocytes in tumor bearing murine during different tumor growth stages (Rahmani Barouji et al., 2020a). Shilajit is also attributed to a number of beneficial effects against neurological disorders including Alzheimer’s disease (Bhattarai et al., 2016; Calfo et al., 2020; Carrasco-Gallardo et al., 2012a, 2012b). Importantly, shilajit has been shown to prevent liver damage in high-fat diet-induced non-alcoholic fatty liver disease and induce apoptosis in hepatic cancer cells (Ghezelbash et al., 2020; Pant et al., 2016). The prognosis of metastatic OS cases remains off the mark (Agarwal et al., 2007; Carrasco-Gallardo et al., 2012a, 2012b). The CMF cocktail was intravenously administered on days 1 and 8 and days 17, 18, and 19 at a dose of shilajit (150 mg/kg body weight) and OS + CMF + HDS was served as osteosarcoma positive rats, OS group rats received 4 T1 cancer cells and served as osteosarcoma positive rats, OS + CMF group rats received a CMF cocktail of drugs, OS + CMF + LDS group rats received a low dose of shilajit (150 mg/kg body weight) and OS + CMF + HDS were purchased locally. All other used reagents and chemicals were of standard grade.

2.2. Animals

Thirty-five male adult Sprague –Dawley rats weighing about 220 g were sourced from the College of Pharmacy, King Abdulaziz University, Jeddah and kept in an animal care facility to acclimatize to the environment. Animals had free access to water and a standard diet. The ethics committee approved guidelines were followed to carry out animal experiments. The study was approved by the ethical committee at the College of Science, King Abdulaziz University, Jeddah, Saudi Arabia (No. 845–19/2019).

2.3. Preparation of extract

Shilajit extract was prepared by dissolving it in boiling water at 100° C and placed in a 70° C incubator for 30 h to allow it to dry completely. After drying, shilajit extract was dissolved in HBSS and filtrated through a 0.2-mm filter before administering to animals. (Das et al., 2016).

2.4. Cell line

The 4 T1 cells (ATCC, USA) were cultured in RPMI medium supplemented with 1% penicillin/streptomycin and10% FBS.

2.5. Induction of osteosarcoma

For bone cancer induction, rats were anesthetized with chloral hydrate and a superficial carving of nearly 1 cm size was made just over the knee that overlaid the patella. Gentle pressure was put to find condyles of the distal femur and using a 30-gauge needle a hole was made in the right femoral medullary cavity., which was replaced with a 10-μL syringe. Four microliters of PBS containing 1.0 × 10^8 4 T1 tumor cells were injected into the intramedullary space of the femur. The syringe was kept in place for one extra minute to avoid 4 T1 cells from backing up through the injection track, a syringe was left in its place for one more minute. The injection site was locked with a gelatin sponge and washed multiple times with normal saline. The skin was sutured with 5–0 Ethicon Vicryl rapid suture. Following the procedure, animals were left to recover for 3 days prior to using them for further experiments (Lelekakis et al., 1999; Rosol et al., 2003).

2.6. Treatment with CMF cocktail of chemotherapy drugs

The cyclophosphamide, methotrexate and 5-fluorouracil (CMF) cocktail of drugs was prepared by mixing 10 mg/kg body weight. of cyclophosphamide, 1 mg/kg body weight. Methotrexate, and 10 mg/kg body weight of 5-Fluorouracil dissolved in normal saline. The CMF cocktail was intravenously administered on days 1 and 8 for 6 rounds with a gap of 14 days between each round. The above mentioned dosages of drugs were selected from previously published studies on rats (Tomoda et al., 2005; Witzel et al., 1992).

2.7. Experimental design

The OS rats were randomly divided into 5 groups designated as control, OS, OS + CMF, OS + CMF + LDS (low dose shilajit) and OS + CMF + HDS (high dose shilajit) with each group consisting of 7 animals. Control group rats received 0.9 % normal saline and served as normal healthy rats, OS group rats received 4 T1 cancer cells and served as osteosarcoma positive rats, OS + CMF group rats received a CMF cocktail of drugs, OS + CMF + LDS group rats received a low dose of shilajit (150 mg/kg body weight) and OS + CMF + HDS...
group rats received a high dose of shilajit (250 mg/kg body weight). Osteosarcoma was induced in rats as described above. Osteosarcoma rats were treated with a CMF cocktail of chemotherapy drugs with or without shilajit as detailed above. Osteosarcoma rats were treated with either a low dose (150 mg/ml) or a high dose (250 mg/ml) of shilajit to assess dose dependent effects.

Control and all the treatment group rats had free access to food and water. Animals were examined daily for any change in their behavior and food and water intake. Throughout the experimental period, animals were maintained in ambient temperature and relative humidity and allowed to have free access to food and water.

2.8. Blood sampling

Following the end of treatment duration, rats were anesthetized by diethyl ether and sacrificed by cervical dislocation. Blood was collected without anticoagulants. Coagulated blood was centrifuged at 300 rpm for 10 min and serum was separated and stored until used. Liver and kidney were resected and immediately frozen in liquid nitrogen and part of the organs were also stored in formalin solution for histopathological examinations.

2.9. Determination of serum liver and kidney functional markers

Liver and kidney functional markers were measured using standard methods or commercial kits. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme activities were quantified according to the methods described by (Pappas, 1989; Reichling and Kaplan, 1988). Serum bilirubin (Cat#MAK126), total protein (Cat#TP0100), and albumin (Cat#MAK125) were quantified using commercially available kits following the instructions of the manufacturer (MERCK, Germany). Serum creatinine, uric acid and urea were enzymatically measured based on the procedure detailed earlier (Fossati et al., 1980; Taussky, 1956). Serum alkaline phosphatase (ALP) (Cat#MAK447) levels were measured by commercial kits (MERCK, Germany).

2.10. Histopathological study

Liver and kidney specimens were collected and immediately fixed with 10% formalin and dehydrated in increasing concentrations (70, 80 and 90%) of ethanol. Specimen were further processed in xylene and finally paraffin embedded. About 4-μm sections were cut and stained with hematoxylin and eosin (H&E) dye. The H&E stained slides were visualized under a light microscope.

2.11. Statistical methods

SPSS Statistical software was used for statistical analysis (SPSS, Inc, Chicago, IL, USA). Data collected from different experiments were expressed as the mean ± SD. Spearman correlation was carried out to examine the correlation among liver and kidney functional markers. Significance of variance between the means was obtained using the Student’s t-test or by one-way analysis of variance (ANOVA). Significance was set at p < 0.05.

3. Results

3.1. Body weight change

Changes in body weights of control and treatment groups are presented in Table 1. Body weights of osteosarcoma rats were found to be significantly lower than those of control. Body weights of osteosarcoma rats treated with CMF cocktail showed significant improvement and further increased in response to co-treatment of rats with CMF cocktail and shilajit. Shilajit at 250 mg/kg dose exhibited a better effect on body weight than 150 mg/kg dose.

3.2. Liver markers

Levels of liver functional markers in serum are presented in Table 2. Serum levels of ALT, ALP and AST were significantly augmented in osteosarcoma rats as compared to those in control. Whereas albumin total protein and bilirubin were significantly declined in osteosarcoma rats as against in control. Osteosarcoma rats treated with CMF cocktail displayed a significant decrease in ALT, AST and ALP levels and a significant spike in albumin, total protein and bilirubin as compared to those in untreated osteosarcoma rats. Co-treatment of CMF cocktail and shilajit at 150 mg/kg or 250 mg/kg concentrations led to a further significant decline in ALT, AST and ALP and a significant increase in albumin, total protein and bilirubin compared to those in osteosarcoma rats treated only with CMF cocktail. Importantly, Shilajit at 250 mg/kg concentration had significantly more positive effects on the studied functional markers as matched to that with 150 mg/kg concentration of shilajit.

3.3. Kidney functional markers

Kidney functional markers in the serum of control and treated rats are provided in Table 3. Compared to control, osteosarcoma rats exhibited a significant increase in creatinine, uric acid (UA) and urea. Treatment of osteosarcoma rats with CMF cocktail resulted in a significant reduction in all the studied markers matched to those in untreated osteosarcoma rats. On the other hand, combined treatment of CMF cocktail and shilajit at 150 or 250 mg/kg concentration further lowered creatinine, urea, and uric acid as compared to CMF cocktail alone treatment. Shilajit at 250 mg/kg dose exerted a superior effect in reducing these kidney markers than 150 mg/kg dose as shown in Table 4.

3.4. Correlations among the liver and kidney functional markers

Correlation coefficient values among the serum liver and kidney functional indices are shown in Table 2. It was found that liver and kidney functional markers included ALT, AST, and ALP. Uric acid and creatinine were significantly and positively correlated with each other. Whereas no significant correlations were found among other studied functional indices of liver and kidney.

3.5. Histopathology of liver tissue

Histopathological changes in liver tissue of control and treated groups are presented in Fig. 1. Hepatocytes, central vein, and sinusoids demonstrated different morphological changes in liver tissue of osteosarcoma than those in the control negative group. The dis-

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**Table 1**

| Groups                  | Initial weight (g) (N = 7) | Final weight (g) (N = 7) |
|-------------------------|----------------------------|--------------------------|
| Control                 | 220.34 ± 8.11*             | 284.45 ± 7.23*           |
| OS                      | 221.15 ± 8.09*             | 96.74 ± 5.36*            |
| OS + CMF                | 221.61 ± 9.27*             | 106.91 ± 6.74*           |
| OS + CMF_LDS           | 224.24 ± 4.12*             | 126.22 ± 5.28*           |
| OS + CMF + HDS          | 221.58 ± 5.92*             | 161.05 ± 5.86*           |

OS, osteosarcoma; CMF, cocktail of chemotherapy drugs; LDS, low dose shilajit; HDS, high dose shilajit. Data are expressed as mean ± SD, *p < 0.05 as compared to control and #p < 0.05 compared to OS group.
Correlation coefficient (r) among the studied liver and kidney functional markers.

Kidney functional parameters in serum of control and different groups of rats.

Liver functional parameters in the serum of control and different groups of rats.

...tubular atrophy when compared with the normal renal tissue...

...osteosarcoma rats demonstrated shrinkage of glomerular tuft and...

...induced rats and other treated groups and the histological features...

...played further inhibition of the hepatic inflammation and hepato-

...control ans #p < 0.05 compared to OS group.

Table 2
Liver functional parameters in the serum of control and different groups of rats.

| Groups         | Parameters | Bilirubin | TP | Albumin | AST | ALT | ALP |
|----------------|------------|-----------|----|---------|-----|-----|-----|
| Control        | (mg/dl) n (7) | 0.43 ± 0.020* | 7.64 ± 0.15* | 4.92 ± 0.298* | 19.85 ± 2.91* | 19.28 ± 3.03* | 223.28 ± 26.47* |
| OS             | (g/dl) n (7) | 2.20 ± 0.22* | 3.35 ± 0.25* | 1.54 ± 0.222* | 100.28 ± 5.76* | 99.85 ± 5.87* | 698.00 ± 9.00* |
| OS + CMF       | (mg/dl) n (7) | 1.64 ± 0.198* | 4.88 ± 0.27* | 2.01 ± 0.307* | 81.42 ± 3.20* | 80.71 ± 4.71* | 593.00 ± 30.5* |
| OS + CMF, LDS  | (mg/dl) n (7) | 0.89 ± 0.028* | 5.71 ± 0.22* | 3.04 ± 0.171* | 66.42 ± 4.68* | 64.00 ± 1.82* | 432.28 ± 16.5* |
| OS + CMF, HDS  | (mg/dl) n (7) | 0.67 ± 0.026* | 6.45 ± 0.17* | 4.10 ± 0.230* | 56.71 ± 2.28* | 49.57 ± 1.71* | 350.71 ± 40.5* |

OS, osteosarcoma; CMF, cocktail of chemotherapy drugs; LDS, low dose shilajit; HDS, high dose shilajit; TP, total protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. Data are expressed as mean ± SD, *p < 0.05 as compared to control ans #p < 0.05 compared to OS group.

Table 3
Kidney functional parameters in serum of control and different groups of rats.

| Groups         | Parameters | Uric acid | Urea | Creatinine |
|----------------|------------|-----------|------|------------|
| Control        | (mg/dl)n    | 0.51 ± 0.035* | 20.85 ± 2.11* | 4.04 ± 0.097* |
| OS             | (mg/dl)n    | 2.40 ± 0.23* | 73.14 ± 2.26* | 7.41 ± 0.22* |
| OS + CMF       | (mg/dl)n    | 1.97 ± 0.13* | 61.71 ± 3.14* | 7.00 ± 0.31* |
| OS + CMF, LDS  | (mg/dl)n    | 1.62 ± 0.11* | 54.14 ± 3.1* | 6.17 ± 0.075* |
| OS + CMF, HDS  | (mg/dl)n    | 0.87 ± 0.067* | 38.42 ± 2.87* | 4.98 ± 0.106* |

OS, osteosarcoma; CMF, cocktail of chemotherapy drugs; LDS, low dose shilajit; HDS, high dose shilajit; Data are expressed as mean ± SD, *p < 0.05 as compared to control ans #p < 0.05 compared to OS group.

3.6. Histopathology of kidney tissue

Histopathological changes in kidney tissue of control and different treatment groups are presented in Fig. 2. Kidney tissue of osteosarcoma rats demonstrated shrinkage of glomerular tuft and tubular atrophy when compared with the normal renal tissue (Fig. 2 A &B). On other hand, mild tubular atrophy and slightly congested glomeruli were found in osteosarcoma rats treated with CMF cocktail as compared to untreated osteosarcoma rats (Fig. 2 C). A further improvement was noted in tubular atrophy and glomeruli of the kidney of rats treated with CMF cocktail and shilajit at 150 mg/kg dose (Fig. 2 D). Histology of kidney tissue of rats treated with CMF cocktail and shilajit at 250 mg/kg concentration displayed near normal features that were comparable to a normal control group (Fig. 2E).

4. Discussion

In the present study, we assessed the possible beneficial effects of shilajit in combination with chemotherapeutic drugs in mitigating the detrimental effects of metastasis of osteosarcoma on the liver and kidney tissue in a rat model. Effects of shilajit were measured at two different doses; one at a higher concentration and the other at a lower concentration. The effect of shilajit on these tissues was evaluated by measuring their key functional components as well as by examining histopathological changes. Shilajit is a well-known medicinal plant based product that has been used in traditional medicine such as Ayurveda (Jafari et al., 2019; Schepetkin et al., 2003). The medicinal properties of shilajit are mainly attributed to its antioxidant properties (Schepetkin et al., 2003).

Osteosarcoma is the commonest primary bone tumor found in adolescents and children. OS is characterized by osteogenic progenitor cells or mesenchymal cells producing immature bone and osteoid (Biellack et al., 2009). Recent investigations have shown that osteosarcoma can metastasize to the liver and kidney (Dalinka et al., 1971). Rats in which osteosarcoma was induced displayed decreased body weight, and altered levels of liver and kidney functional markers. Besides these unfavorable changes to functional markers, osteosarcoma rats also demonstrated marked histological changes in the liver and kidney tissues. These findings clearly indicate the pathological features of metastasis of osteosarcoma to the liver and kidney. Treatment of these osteosarcoma rats with shilajit at low and high doses in combination with CMF cocktail drug significantly increased the body weight, lowered the liver

Table 4
Correlation co-efficient (r) among the studied liver and kidney functional markers.

|                | ALT     | AST     | UA      | Creatinine | Alp    |
|----------------|---------|---------|---------|------------|--------|
| ALT            | 0.985** | 0.972** | 0.961*  | 0.967*     | 0.996**|
| AST            | 0.985** | 0.955** | 0.939*  | 0.973**    | 0.948* |
| UA             | 0.972** | 0.955** | 0.939*  | 0.973**    | 0.948* |
| Creatinine     | 0.961** | 0.939*  | 0.973** | 0.964**    | 0.948* |
| Alp            | 0.967** | 0.953*  | 0.973** | 0.964**    | 0.948* |

TP, total protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. Data are expressed as mean ± SD, *p < 0.05 as compared to control ans #p < 0.05 compared to OS group. **p < 0.01.
Fig. 1. H&E stained liver tissue sections from control (A), OS (B), OS + CMF (C), OS + CMF + LDS (D), OS + CMF + HDS (E) rats. Histology of osteosarcoma rats (OS group) displaying ballooning degeneration of hepatocytes (indicated by triangle), sinusoid congestion (indicated by hallow arrow) and inflammation (arrow) as against normal histology seen in the control group. Tissue sections from OS + CMF groups showed regeneration of hepatocytes but lesser sinusoid congestion and moderate inflammation, Rats from OS + CMF + LDS group demonstrated reduced degeneration, no sinusoid congestion and mild inflammation in liver tissues, whereas near normal histology was observed in tissue sections from OS + CMF + HDS group rats.

Fig. 2. H&E stained kidney tissue sections from control (A), OS (B), OS + CMF (C), OS + CMF + LDS (D), OS + CMF + HDS (E) rats. Histology of osteosarcoma rats (OS group) displaying shrinking of glomerular tuft (arrow) and tubular atrophy (hallow arrow) as compared to normal histology seen in the control group. Tissue sections from OS + CMF groups showed slightly congested glomeruli and moderate tubular atrophy. Rats from OS + CMF + LDS group demonstrated no glomeruli congestion and mild tubular atrophy, whereas no apparent histological changes were noted in kidney tissue sections from OS + CMF + HDS group rats.
Contribution

FAA-Conceptualized the study, data analysis and review of the manuscript. EJJ- carried out the experiments and manuscript preparation.
