RESEARCH ARTICLE Indian Journal of Animal Research, Volume 56 Issue 3: 323-329 (March 2022)

Comparative Evaluation of Nutraceuticals (*Curcuma longa* L., *Syzygium aromaticum* L. and *Olea europaea*) with Single-agent Carboplatin in the Management of Canine Appendicular Osteosarcoma

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10.18805/IJAR.B-4485

ABSTRACT

Background: Canine appendicular osteosarcoma (OSA) is the most common bone malignancy in dogs.

Methods: Spontaneous cases of canine OSA presented for three years were treated with different standard therapeutic protocols and compared with a novel plant formulations or nutraceuticals prepared from combination of turmeric, clove and olive oil. Different diagnostic modalities like survey and three view thoracic radiograph, abdominal ultrasonography (USG) with spectral Doppler USG, greyscale USG, strain elastography, fine needle aspiration cytology (FNAC), computed tomography and excisional biopsy were used to detect appendicular OSA and for staging of the primary bone tumor.

Result: Canine bone tumor of appendicular OSA primarily affects large breed dogs with median age 10.32 years (range, 2.6-13 years) and median body weight 32.95 kg (range, 14-41 kg). Among four treatment groups, disease-free interval (DFI) and overall survival times were longer in groups of amputation followed by carboplatin as adjuvant chemotherapy (9 no.) than in groups with neoadjuvant chemotherapy with carboplatin followed by amputation (2 no.) and amputation along with nutraceuticals treatment (6 no.) and were shorter in the control group with amputation alone (6 no.). Herbal nutraceuticals can be included in the therapeutic regimen of canine osteosarcoma for increasing the overall survival time.

Key words: Adjuvant chemotherapy, Carboplatin, Myelosuppression, Neoadjuvant chemotherapy, Osteosarcoma, Phytochemcials.

INTRODUCTION

Osteosarcoma (OS) is a malignant spindle cell primary bone tumor of mesenchymal origin characterized by the formation of osteoid or immature bone matrix or both by the tumor cells. Appendicular osteosarcoma (OSA) is the most common primary OSA with 75% occurrence, 24% involving axial skeleton and skull and 1% soft tissue (Boerman *et al.,* 2012). OS primarily affects large- and giant-breed dogs like Greyhounds, Rottweilers and Great Danes compared to mixed-breed dogs (Spodnick *et al.*, 1992; Mc Neill *et al.*, 2007). Mostly, at presentation, canine OSA is commonly found in stage IIB and usually, after detecting metastatic disease, the patient's survival period is very brief, ranging from 18 to 66 days (Saam *et al.,* 2011). In the majority of cases treated with amputation alone, death is due to the development of metastatic disease (Spodnick *et al.*, 1992; Liptak *et al.,* 2004). Clinical studies have shown that survival times in OSA dogs can be extended by administrating chemotherapy (Schimdt *et al.*, 2013). A multitude of factors have been associated with the prognosis of survival of a patient with OSA and those are tumor size, tumor metastasis (tumor grade and mitotic index), or anatomical locations such as humeral surface (Mc Neill, 2007), serum alkaline phosphatase (Liptak *et al.,* 2004) and lymph node metastasis (Ahuja *et al.*, 2008). Other than chemotherapeutic agents, nutraceuticals such as turmeric, clove and olive oil can be used for the treatment of solid tumors due to their potent anticancer and chemoprotective properties (Table 1). Curcumin has several therapeutic potentials such as anti-inflammatory, cytokine release, antioxidant, immunomodulatory, enhancing

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How to cite this article: Sahoo, A.K., Nath, I., Senapati, S.B., Panda, S.K., Das, M.R. and Patra, B.K. (2022). Comparative Evaluation of Nutraceuticals (*Curcuma longa* L., *Syzygium aromaticum* L. and *Olea europaea*) with Single-agent Carboplatin in the Management of Canine Appendicular Osteosarcoma. Indian Journal of Animal Research. 56(3): 323-329. DOI: 10.18805/IJAR.B-4485.

Submitted: 13-04-2021 **Accepted:** 31-05-2021 **Online:** 11-06-2021

Table 1: Phytoconstituents of nutraceuticals and their biological effects.

Parts of nutraceuticals used in clinical study	Botanical Name (family)	Name of the phytoconstituents	Significant use and effect on neoplastic cells	Reference
Rhizome of turmeric	*Curcuma longa* L. (Zingiberaceae)	Curcumin (diferuloylmethane), (77%) demetossicurcumin (17%) bisdemetossicurcumina (3%),	Antitumour effect through downregulation of intracellular transcription factor such as NF-KB, activator protein1 (AP-1), cyclooxygenase II (COX2), Nitric oxide synthase, matrix metalloproteinase-9 (MMP-9) and STAT3 on various carcinomas such as ovarian, uterine, cervical, prostate and breast.	Hay *et al.,* 2019
Dried buds of clove	*Syzygium aromaticum* L. (Myrtaceae)	Eugenol (81.1%), β-caryophyllene, humulene, chavicol, methyl salicylate, α-ylangene and eugenone; the flavonoids eugenin, rhamnetin, kaempferol and eugenitin; triterpenoids like oleanolic acid, stigmasterol and campesterol	Interferes with cell signaling pathway nuclear factor kappa-B (NF-KB) and down regulates MMP-9 pathway Used in ovarian, liver, breast, colon and lung cancer.	Liu *et al*., 2014 Banerjee *et al*., 2006
Drupes of olive seeds available as virgin olive oil	*Olea europaea* L. (Oleaceae)	Olive oil polyphenol such as ole, oleuropein aglycone (Ole aglycone), oleacin or Oc	Human osteosarcoma cells Brest, colon, prostate, melanoma, promyelocytic leukemia cells.	Przychodzen *et al*., 2019 Hua *et al*., 2011

the apoptotic process and antiangiogenic properties (Levine *et al*., 2017). Cloves (*Syzygium aromaticum* L.) are the spices with the highest antioxidant properties along with anti-inflammatory effect and cytotoxicity to cancer cells (Yang *et al*., 2014) among natural products. Olive oil and its derivatives also have good chemoprotective and antioxidant activity in treating canine OSA cells (Hua *et al*., 2011).

The therapeutic potential of herbal ingredients or nutraceuticals such as the combination of turmeric, clove and olive oil has not been assessed before in solid tumors of canines. However, its proven anticancer effect has been well documented. Twenty-three dogs diagnosed with OSA were categorized into four different treatment groups and a comparison was made in terms of disease-free intervals and overall survival times as an outcome of the therapeutic approach.

MATERIALS AND METHODS

This study reported twenty-three (23) canine clinical cases of spontaneous OSA to the Departments of Teaching Veterinary Clinical Complex and Veterinary Surgery and Radiology from 2018-2021. On physical examination, persistent lameness and localized swelling were the most common findings with common symptoms such as non-weight bearing of the affected limb, anorexia and occasional acute pyrexia (102-104°C). Three view thoracic radiography, grey scale ultrasonography with power Doppler and strain elastography (LogiQ F series, GE healthcare) were performed for detecting probable thoracic metastasis, assessment of regional lymph node and spread of primary lesion. Surgical staging was done according to the TNM system into stage-I, II and III (Withrow *et al.,* 2013). Serum alkaline phosphatase was estimated in 20 dogs (20/23). FNAC was performed in five dogs with distal radius ulna OSA and humeral OSA and routinely stained with Wright-Giemsa. Computed tomography (CT) was performed in dogs affected with acetabular osteosarcoma under general anesthesia (Fig 2) in group II. Bone biopsies were performed by both closed technique (closed Core needle biopsy with a Michele Trephine as a minor invasive procedure without surgery) or open technique (excisional biopsy during surgical procedure). The tissues from the primary bone tumor were routinely stained with Haematoxylin and Eosin. With owner's consent, a biopsy of the prescapular lymph node was

Fig 1: Scapulectomy in a Rotweiller treated with carboplatin as adjuvant chemotherapy. *Inset*- Mediolateral view of scapula with cortical destruction of dorsal scapular spine and metastatic nodules in the lungs.

collected; the animal was euthanized with Thiopental sodium (25 mg/kg b wt, Injection Thiosol 1 gm vial) to collect mediastinal lymph node and then processed for routine H&E staining.

Dogs in the present treatment protocol were categorized into four groups, group-I- Control with Amputation alone (6 no.), group-II -Amputation along with nutraceuticals treatment (6 no.), group-III- neoadjuvant chemotherapy with carboplatin followed by amputation (2 no.) and group-IV- amputation followed by carboplatin as adjuvant chemotherapy (9 no.). As amputation of the affected limb is the standard protocol for dogs with OSA (Seguin and Weigel, 2012), dogs that underwent amputation only without any additional therapy were included in control groups. Six dogs in group-I were treated with amputation only as palliative therapy for OSA in different stages. Scapulectomy was performed at the level of attachment to the body on the proximal portion for the tumors diagnosed in forelimb. In hind limbs, with hard palpable mass at distal one-third of the femur at the point above stifle joint in which amputation at the mid diaphysis of the femur was performed keeping

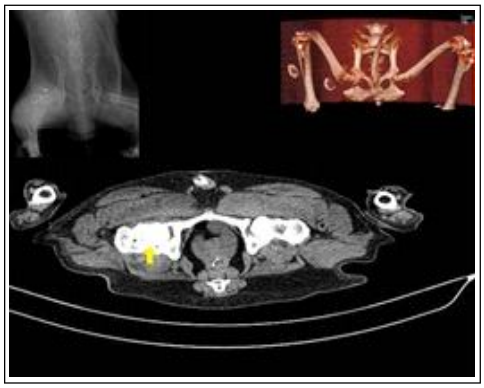


Fig 2: CT transverse image of right femur head in a Labrador with acetabular OSA (coxo-femoral joint) treated with amputation and nutraceuticals treatment. *Inset* (*left*). Radiography of hind limb showing sunburst appearance in right femur head and acetabulum on day of presentation. *Inset* (*upper right*). Three dimensional (3-D) reconstruction image of femur head showing neovascularisation.

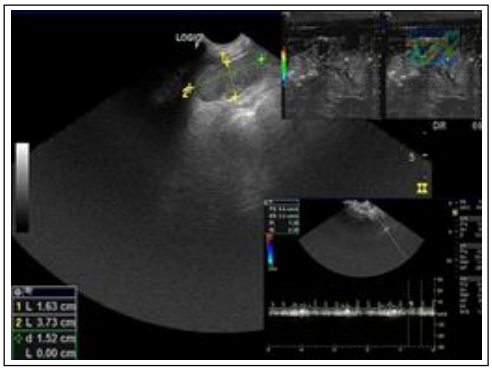


Fig 3: USG image of a typical metastatic lymph node (prescapular LN). *Inset* (*lower*)- Color Doppler and spectral waveform USG of prescapular LN with RI and PI >1. *Inset* (*upper*)- strain elastography of tumor mass showing hardness (blue).

3 cm soft tissue margin. In group-II, six patients affected with OSA were treated with amputation of the limb and subsequently fed with freshly prepared nutraceuticals pellets for a period of 4-6 weeks (28-42 days). Pellets were prepared by mixing 3gm turmeric, 2 gm clove with 5 ml of olive oil so that animal can swallow without any bitter taste. Healing effect, tumor regression and remission of clinical symptoms was assessed in different stages of the disease. The dog diagnosed with acetabular osteosarcoma was administered with Flunixin meglumine@2 mg/kg for 5 days and then with nutraceuticals orally once daily in empty stomach in the morning time for 36 days. The dog with an osteolytic lesion of distal tibia and thoracic metastasis (stage IIIA) was also administered with 5 gm mixture of nutraceuticals to assess the efficacy in critical conditions of patients with OSA. In group-III, neoadjuvant chemotherapy was administered in two patients with OSA in stage-IIB or IIA, with disease particularly confined to the foreleg. Injection Carboplatin (Kemocarb Inj 450 mg/45 ml Pfizer) as a single agent in neoadjuvant setting @ 300 mg/m^2 was administered seven days prior to surgical amputation followed by surgery and postoperative repetition of chemotherapy drug twice at 21-25 days interval. In group-IV, nine patients with OSA (stage-IIA or IIB) were followed with amputation along with standard carboplatin protocol (Fig 1). The drug was administered as single agent at three weeks interval in slow intravenous infusion with Normal saline (0.9% NaCl) for 30 minutes over 3 to 4 cycles depending on patient status, tumor regression and client's consent. The dose of carboplatin injection was fixed at 30 ml/m^2 or @ 300 mg/m^2 for all dogs above 30 kg or 1 m^2 in groups III and IV to reduce the cytotoxic effect. For this, exact body surface areas were calculated from body weights using a conversion table (Withrow *et al*., 2013). Statistical analysis was done through Kaplan Meier life table survival graph to compare disease free interval (DFI) and overall survival time (OST) among the dogs in four groups.

RESULTS AND DISCUSSION

Of the twenty-three dogs treated during this study, all are large breed dogs, including Labrador (11), German shepherd (5), Rottweiler (2), Bull Mastiff (1), Great Dane (1), Golden Retriever (1), excluding one Spitz and one nondescript < 15 kg. The median age of dogs at initial presentation was 10.15 years (range, 2.6-13 years) and the median body weight was 31.86 kg (range, 14-57 kg). Most of the dogs were > 10 years of age and sexually intact male or female. The primary site of occurrence of the tumor was the proximal or distal third of long bone starting from the scapula (2), humerus (3), radius-ulna (11), femur(3), tibio-fibula, synovium of the elbow joint (1), proximal femur involving acetabulum (1), synovium of stifle joint (1) and hock joint (1). The typical radiographic lesion found were cortical bone lysis and/or proliferative or palisading cortical bone (sunburst effect), periosteal lifting caused by subperiosteal hemorrhage (Codman's triangle), loss of the fine trabecular pattern in metaphyseal bone and calcification extending into

surrounding soft tissue. The site of metastasis was primarily the lungs with metastatic nodule throughout lung parenchyma and occasionally lymph nodes (n=2). Cytology of smears prepared from osteosarcoma lesion in the distal radius and ulna also showed metastatic changes such as the pleomorphic population of malignant mesenchymal cells with ovoid (osteoblast-like appearance) morphology and evident anisocytosis. Prominent eccentric polarized nuclei and clumped chromatin were observed in most cells (Fig 4). Histological criteria of malignancy were atypical osteoblasts characterized by hyperchromatism, increased nuclear volumes and high mitotic index. The sample was highly cellular with abundant production of partially mineralized osteoid matrix between the neoplastic cells. There was variation in cell size (anisocytosis) with concentric and abnormal cellular arrangement (Fig 5). On grey scale ultrasonography (Fig 3), size of the prescapular lymph node on short-axis (S or w=1.63 cm) and long axis (L-3.73 cm) were measured indicating larger size metastatic nodes. The lymph nodes had a sharp border and hypoechoic texture compared to adjacent musculature and the presence of coagulation necrosis within the node indicates pathologic or metastatic node. On power Doppler ultrasonography (Fig 3 *lower inset*) increase in peripheral vasculature was seen and on spectral Doppler ultrasound, the vascular resistance values in terms of Resistive Index (RI) and Pulsative Index (PI) were found as 0.59 cm and 1.39 cm. With strain elastography (Fig 3 *upper inset*), hard tumors tend to be stiffer and appear blue (grade 3 and 4) compared to soft tumors that appear red. In case of persistent lameness of hindquarter, survey radiography detected new bone formation with cortical destruction in pallisading manner which was confirmed through computed tomography (CT) of pelvis where increase in density of right acetabular cavity, measured as Hounsfield units (HU) was noticed (Fig 2). Total serum ALP activity was measured in 20 dogs before surgery and found within the normal reference range in 14 dogs (median range 130-210 U/L) and six above normal reference range, which had poor prognostic survival.

Scapulectomy was performed in case of primary tumor mass in proximal scapula, humerus, radius and ulna. In hind limb, coxo-femoral surgical amputation and amputation from mid diaphysis of femur was done for OSA affecting lower portion of rear limb. The treatment group with the longest survival time was the group that received adjuvant chemotherapy (amputation followed by chemotherapy) with a Disease-free interval (DFI) of 289 days. In these patients, metastatic changes were evidenced in stage-IIA or IIB with no signs of pulmonary metastasis. This was significantly higher than the groups treated with neoadjuvant chemotherapy, phytochemicals or nutraceuticals, or surgical amputation. The patients in the primary stage of appendicular OSA progression (IA or IB) were mostly treated with postoperative phytochemical therapy and the results were also very promising with remission from clinical symptoms and tumor size happening within 28-42 days. The sample

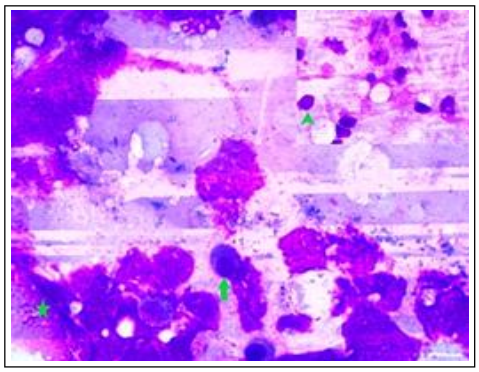


Fig 4: Cytology of smears from distal radius bone aspirate in a dog (Wright' stain, 100 x magnifications) with neoplastic ovoid and pleomorhic cells, anisokaryosis, eccentric nucleus (green arrow) and hyperchromasia surrounding a pink fibrillar extracellular osteoid matrix (asterix). *Inset.* Pleomorphic osteoblastic cells (anisokaryosis)

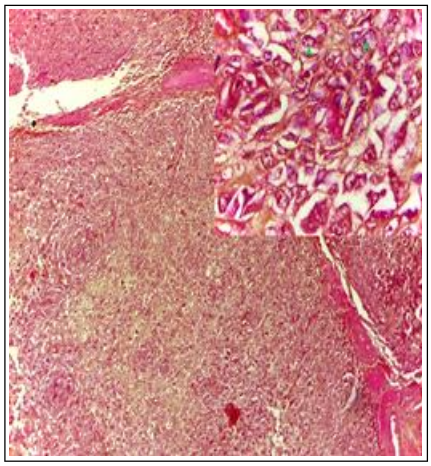


Fig 5: H&E staining (10X) of canine OSA lesion with concentric layer of osteoblasts was found nested within the eosinophilic matrix suggestive of osteoid and high mitotic figure with anisocytosis. *Inset.* Increase in nuclear volume (nuclear to cytoplasmic ratio) and multiple cytoplasmic vacule.

size in the neoadjuvant setting was very less (2) and one dog also died before second dose of carboplatin administration making the survival time insignificant. Thoracic radiography before death showed multiple pulmonary metastatic nodules indicative of stage-IIIB. Mild chemotherapeutic toxicosis (Myelosuppression like thrombocytopenia and neutropenia and gastrointestinal toxicosis) developed during carboplatin injection administration in both adjuvant and neoadjuvant settings. In case of severe toxicity, close monitoring of CBC (thrombocytopenia and neutropenia) were done and supportive medicine was prescribed (Inj. Perinorm with Metochlopromide Hcl 0.25 mg/kg body weight, Inj. Mikacin @ 10 mg/kg body weight as anti-diarrhoeal antibiotics and Ringer's lactate @ 40 ml/kg bwt). The photomicrograph of the tissue sample collected from the prescapular lymph node (Fig 6) and mediastinal lymph node (Fig 7) showed similar

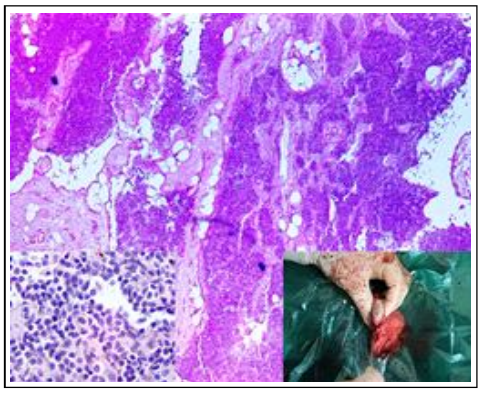


Fig 6: H&E staining (4X) of palpable prescapular lymphnode. Disorganized germinal centre with trabecular sinuses were clearly visible indicating lymphoid structure. *Inset left*. 100X magnification- anisocytosis, hyperchromasia and high mitotic figures 0-5 /HPF. *Inset*- Excisional biopsy of the prescapular lymph node.

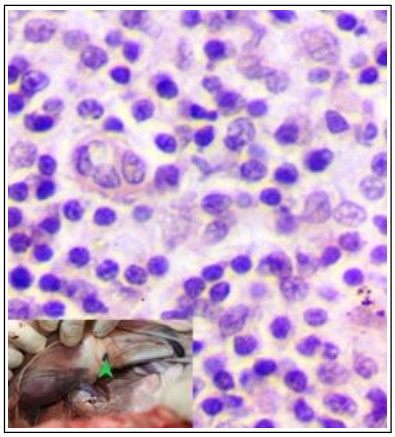
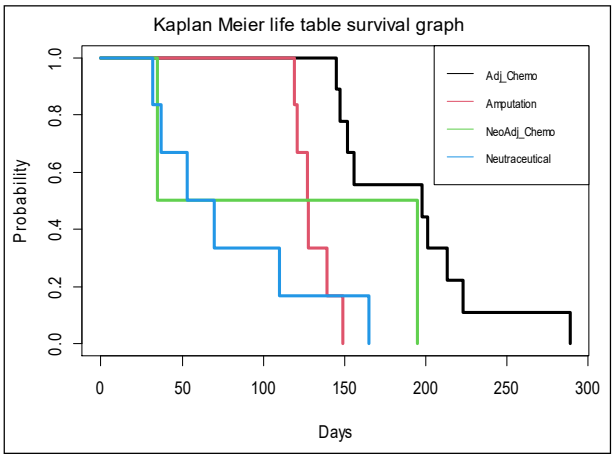


Fig 7: H&E stain-(100X) of mediastinal lymph node at necropsy, anisokaryosis, anisokaryosis, hyperchromasia, high mitotic figure (0-4/HPF), *inset* green arrow head- Necropsy of mediastinal lymph node after euthanasia of the patient.



Graph 1: Kaplan-Meier survival analysis for 23 dogs with OSA treated with amputation along with nutraceuticals (6 dogs in Group-I 1), 6 dogs with OSA treated with amputation alone (group 1), 2 dogs with OSA treated with preoperative carboplatin injection (group 3), amputation and postoperative adjuvant carboplatin chemotherapy (group 4).

malignancy characteristics as evident in the cytologic and histopathology collected from primary bone lesion indicating regional lymph node metastasis. Those are high mitotic index (0-5/HPF), anisocytosis, eccentric nucleus, increase in nuclear volume and high cell density,

The outcome of treatment in four groups were recorded and analyzed Kaplan-Meier life-table analysis (Graph-1). The DFI and OST for the groups under nutraceuticals combination therapy were 165 days and 185 days, whereas with amputation only the values were 149 and 179 days respectively. Dogs under group IV were having highest DFI and OST as 289 and 342 days respectively. Dogs which are under treatment with neoadjuvant chemotherapy have better survival rate with values 195 and 221 than group II.

The predominant bone cancer diagnosed both in human and canine patient is OSA sharing common attributes like tumor location, presence of micrometastatic disease at diagnosis, altered expression of several proteins along with p53 mutations (Fenger *et al*., 2014). Hence, understanding the disease in canine models and development of new therapeutic approach will eventually lead to numerous drug formulations in humans. The present study aimed to evaluate various treatment modalities such as surgical amputation, nutraceuticals or constituent phytochemicals postoperative to amputation, amputation along with chemotherapy (Carboplatin), neoadjuvant carboplatin therapy for increasing disease-free interval (DFI) and overall survival time in twenty-three cases of OSA in canines. The primary tumor location was more common in the front limb involving radius and ulna or both (n=11) in our population, also in accordance to previous reports indicating forelimb locations to be most common (Spodnick *et al*., 1992). Dogs with proximal humerus osteosarcoma and elevated serum alkaline phosphatase (ALP) have a shorter lifespan than dogs with the tumor in other appendicular skeletons which are negative prognostic factor (Boerman *et al*., 2012). Age is a prognostic factor for early mortality, not metastasis in canine OSA (Selmic *et al*., 2014), as happened with most of the dogs in the present population which were of more than ten years old. The survival time in these dogs was very less despite treatments such as amputation and amputation and chemotherapy that would result in a greater overall survival time in a younger population. In the present study, prescapular lymph node examined with power Doppler ultrasonography shows active areas of mixed or peripheral vascularisation (both hilar and peripheral vascularity), indicating malignancy which was later on confirmed with H&E staining of the concerned lymph node. The greyscale evaluation of nodes concerning size, border, echogenic hilus, intranodal necrosis was consistent with the criteria of metastatic nodes, whereas the shape (S/L ratio 0.46 <0.5) was marginally less indicative of a reactive node, but the

histopathology reports were confirmative of the metastatic node. This result is consistent with the earlier finding that though lymph node metastasis are rare in dogs with OSA as it spread through hematogenous route, but dogs with positive lymph node metastasis have a poorer prognosis than dogs with normal echotexture of lymph node (Ahuja *et al*., 2008). Strain elastography of the tumor mass was also done and stiffness may be due to calcium deposition or hard tumor mass. Most of the lesions in OSA were centered towards the distal metaphysis of humerus and radius-ulna because of the haematogenous route of spread of the disease and nutrient artery being located in the distal portion of the long bone. Deaths due to carboplatin administration are generally rare and mostly occur due to gastroenteritis with doses greater than 300 mg/m^2 (Schimdt *et al*., 2013). The dose-limiting toxicity of carboplatin in this study was myelosuppression, specifically neutropenia which was treated with supportive therapy. Disease-free interval (DFI) was defined as the interval between the date of amputation of the affected appendages and the date when first metastasis was diagnosed or tumor recurrence. Overall survival time (OST) was defined as the interval between date of amputation and date of death or euthanasia. Dogs that were censored in DFI analysis had either tumor recurrence; metastasis had not occurred before the end of the study period, lost to follow-up, died before relapse, or was alive at the end of the study period (Brenda *et al*., 2009). The group treated with nutraceuticals (turmeric, clove and olive oil) recovered from persistent lameness and clinical illness within a short span of 28-42 days may be due to primary stage of progression of solid tumor (IA) and tumor may be non-aggressive or have not disseminated to nearby regional lymph nodes and other organs of the body or thorax. Eugenol, the active component of clove, has selective antioxidant activity on human metastatic diseases through inhibition of MMP-9 (matrix metalloproteinase activity) pathway (Liu *et al*., 2014) and so when combined with Curcumin (active components of turmeric) showed the highest antioxidant potential against cancerous cells as reported by Pandey *et al*., 2014. The tumor regression phenomena and remission of clinical symptoms within 6-8 weeks were all due to synergistic effect of eugenol and curcumin which is consistent with earlier reports. The bioavailability of curcumin is enhanced in the presence of Clove. Phenolics (oleic acid and squalene), the active components of olive oil added to the mixture of turmeric and clove synergistically increases the antioxidant activity of all the three components against the cancerous cell (Waterman and Lockwood, 2007). Hence, we affirm that this study assessing the potential therapeutic efficacy of herbal ingredients or nutraceuticals in relation to carboplatin adjuvant therapy (a standard protocol for the treatment of appendicular Osteosarcoma) both in humans and animals opens possibilities of new herbal therapeutic regimen for curing patients in primary stages (stage- IA or IB) of OSA. This will in turn free the cancer patient from the unnecessary burden of chemotherapeutic toxicity.

CONCLUSION

In the present study, four types of treatment modalities for cure of appendicular osteosarcoma in dogs were assessed and treatment methods involving amputations along with nutraceuticals were found comparable to the standard carboplatin protocol of adjuvant chemotherapy. The therapeutic use of nutraceuticals or constituent phytochemicals of (turmeric, clove and olive oil) as a chemoprotective agent in the treatment of canine OSA patients are probably reported first in the present study. The sample size in group-II, amputation along with nutraceuticals treatment was minimum (6 no.), hence further elaborate study with larger sample size to determine proven therapeutic efficacy of these nutraceuticals (turmeric, clove and olive oil) in treatment of solid tumors (osteosarcoma) in canines is required.

ACKNOWLEDGEMENT

The author is thankful to the Central Instrumentation Facility (CIF), Odisha University of Agriculture and Technology (OUAT), Bhubaneswar for technical assistance on microscopy and Dr. D.K. Karna, Associate professor, Department of Animal Breeding and Genetics, CVSc and AH for statistical analysis.

Conflict of interest statement

There is no conflict of interest among authors to disclose.

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