JGTPS, 2015, Vol. 6(3):2854-2859

ISSN- 2230 - 7346

Journal of Global Trends in Pharmaceutical Sciences

Journal home page: www. JGTPS. Com

ANTI-OSTEOPOROTIC ACTIVITY OF ETHANOLIC LEAVES EXTRACT OF *VITEX LEUCOXYLON* AGAINST CHRONIC ALCOHOL ABUSE MODEL INDUCED OSTEOPOROSIS IN RATS

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ABSTRACT

The present study was aimed to investigate the anti-osteoporotic activity of ethanolic leaves extract of *Vitex leukoxylon* (EEVL) on chronic alcohol abuse (CAA) model induced osteoporosis. Chronic alcohol abuse model was performed in male rats by giving 20% alcohol (3g/kg) through *i.p* thrice in a week up to 4weeks. Healthy albino rats were randomly divided into four groups of 6 animals each. First group served as normal group II served as disease control group III treated with Calcium and Vit-D3 (4.28mg/kg and 250 IU/kg, *p.o*), group IV treated with *Vitex leukoxylon* extract (500mg/kg,*p.o*) respectively for 4 weeks. Biochemical parameters (Serum Ca^{2+}, Phosphorus and Alkaline phosphatase) and Urinary parameters (Ca^{2+}, Phosphorus and urine creatinine) were examined. The animals were subjected to X-ray studies. Rats treated with EEVL and Vit-D_3 and calcium supplements showed significant ($p<0.01$) increase in serum calcium, phosphorous levels whereas alkaline phosphatase was significantly ($p<0.001$) reduced in rats treated with EEVL. Vit-D_3 and calcium supplements and EEVL treatment significantly ($p<0.001$) decreased urine creatinine, calcium and phosphorous levels compared to disease control group. X-ray data indicated that EEVL prevented bone loss supporting the biochemical and urine parameters. These findings suggest that the EEVL had shown significant anti osteoporotic activity when compared with disease control group. Further studies are required to determinethe active components that are responsible for its anti-osteoporotic activity.

Key words: *Vitex leukoxylon,* Anti-osteoporotic activity, X-ray of hind limbs, chronic alcohol abuse

INTRODUCTION

According to WHO osteoporosis is defined as a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue leading to enhanced bone fragility and consequent increase in fracture risk. Bone is important tissue to maintain various function in the body are: To form the frame work that support to the body, to provide protection to the brain and vital organs and blood cell formation (Hemopoiesis) in the bone marrow.

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This is the reason why some of the bone related problems like osteoporosis, rheumatoid arthritis, and Osteomalacia are the area of concern in drug development. Excessive use alcohol may cause depletion of bone density by enhancing of osteoclast activity due to elevation of PTH [1]. Medications used to treat osteoporosis are generally of two types: anti resorptive agents (The drugs which slow down the progression of bone loss) and bone-building agents (The drugs promote increasing bone mass). Vertebroplasty and kyphoplasty are minimally invasive spine procedures used for the treatment of painful osteoporotic vertebral compression fractures. Excessive use of Bisphosphonates may cause upper GI disorders such as Dysphagia, esophagitis and gastro esophageal ulcers. ERT slightly increases risk of endometrial cancer and stroke in women.

Hormone replacement and calcium supplement therapies have increased risk of heart diseases, breast cancer, blood clots and dementia [2]. To prevent all undue physical, mental and financial suffering by patients, there is an extreme importance for the better alternative therapeutic management especially from natural resources which are thought to be healthier and safer for the treatment of osteoporosis.

In ancient system of medicine, a several number of herbal plants have been used for osteoporosis, bone calcification and fracture. In Ayurveda (Dravyagunapranalika) VL have been reported to use bone disorders. *Vitex leukoxylon*was traditionally used to treat fever, joint pains, jaundice, anaemia, headache, asthma, cancer, wounds. *Vitex leukoxylon* was reported to have hepatoprotective [3], anti-cancer [4], anti-microbial [5], anti-inflammatory and anti-pyretic Property [6]. It contains flavonoids, anthraquinones, saponins, proteins, carbohydrates and terpenes. Calcium is present in the leaves all Vitex species [7]. β- Sitosterol, dimethyl terphthalate, vitexin,isovitexin, agnuside and aucubin were isolated fromthe leaves or barks of *V. leucoxylon* [8].

Materials and Methods:

Animals

Three months old female Albino rats of wistar strain (150-200gm) were selected. The animals were obtained from Raghavendra enterprises, Pvt. Ltd, Banglore, India. All the experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) of Sri Padmavathi School of Pharmacy (SPSP/CPCSEA/IAEC-1016/a/2014/006), Tiruchanoor, Tirupati, India.

Chemicals: Calcium and Vitamin-D_3 (Dr.Reddy's Laboratories Pvt. Ltd. Hyderabad, India), ketamine (Neon Laboratories Ltd. Thane, India), xylazine (Indian immunological Ltd. Andhra Pradesh, India). Calcium, Phosphorus, Creatinine and Alkaline phosphatase assay kits (AUTOSPAN).

Instruments

Electronic balance (Shimadzu, model no: DS-852J), Cooling centrifuge (Remi, model no: C-24 BL), Autoanalyzer (Mispa Excel, Version: 1.4e, Agappe diagnostics) were used for estimation of biochemical and urinary parameters.

Preparation of plant extract

The leaves of *Vitex leukoxylon* were collected from medicinal garden in Sri Padmavathi School Of Pharmacy, Tirupathi and authenticated by Professor Dr B. Sitaram, S.V. Ayurvedic College, Tirupati. The collected plant material was shade dried and milled to coarse powder by mechanical grinder. 250g coarse powder of leaves of *Vitex leucoxylon* soaked with 750 ml of 90% ethanol for 3 days. Then filtered, filtrate was collected and kept for distillation. Weight per ml calculation was done by loss on drying.

Acute toxicity studies

Acute toxicity studies were performed as per OECD-423 guidelines. The rats were fasted overnight, allowed water ad libitum, after which the alcoholic leaves extract of *Vitex leukoxylon* was administered orally at doses of 5, 50, 300, 2000 mg/kg body weight. Then the animals were observed for 14 days for the appearance of any of the toxicity manifestations like behavioral, neurological effects and mortality as described by Irwin *et al* (1968) [9].

INDUCTION OF OSTEOPOROSIS BY CHRONIC ALCOHOL ABUSE:

Alcohol administration was by a single daily i.p. injection of a 20% (vol/vol.) ethanol/saline solution at a dose of 3 g/kg. Alcohol injections were given 3 days/week [10].

Experimental design:

Rats were randomly divided into four groups each containing 6 animals. Group I served as normal control and group II served as a disease control were treated with 20% Ethanol (3g/kg) i.p thrice in a week up to 4weeks. Group III received 20% Ethanol (3g/kg) *i.p* Calcium and Vit-D_3 at doses of 4.28mg/kg and 250 IU/kg, p.o respectively thrice in a week up to 4weeks. Group IV received 20% Ethanol (3g/kg) *i.p* and ethanolic extract of Leaves of *Vitex leucoxylon* (EEVL) 500 mg/kg orally thrice in a week up to 4 weeks.

Estimation of biochemical and urinary parameters

At the end of treatment, blood samples was collected from the retro orbital venous plexus for estimating serum parameters namely calcium, phosphorus and alkaline phosphatase. Urine samples were collected after 4weeks treatment by housing the rats in individual metabolic cages after administration of distilled water (5 ml/animal) orally. The urinary parameters including Ca^{2+}, Phosphorus and creatinine were measured.

X-ray of hind limbs

Radiographs of the hind limbs were taken for the chronic alcohol abuse model animals on day 40. The animals were anaesthetized by intraperitoneal injection with chloroform. Radiographs were taken with an X-

ray apparatus in Veterinary University, Tirupathi.

Statistical analysis

All the data were expressed by Mean ± S.E.M and statistical analysis was performed by one way ANOVA followed by Tukeytest. The statistical significance was set at $p<0.01$and $P<0.001$.

RESULTS

Effect of EEVL on serum and urine calcium level in CAA model

There was significant ($p<0.001$) decrease in serum calcium levels on 30th day in disease control (G-II) group when compared to the normal group (G-I). The group (G-III) treated with calcium and Vit-D_3 showed significant ($p<0.001$) increase serum calcium levels on 30th day, when compared to the disease control group (G-II).Test group (G-IV) receiving EEVL showed significant ($p<0.01$) increase in serum calcium levels on 30th day, when compared to the disease control (G-II) group (Table 1). There was significant ($p<0.001$) increase in urine calcium levels on 30th day in CAA control (G-II) group when compared to the normal (G-I) group. The group treated with calcium and Vit-D_3 showed significant ($p<0.001$) decrease in urine calcium levels on 30th day, when compared to the disease control group.Administration of EEVL (G-IV) revealed that there was significant decrease ($p<0.01$) in urine calcium levels on 30th day, when compared to disease control (G-II) group (Table 1).

Effect of EEVL on serum and urine phosphorus level in CAA model

There was significant ($p<0.001$) reduction in the serum phosphorus levels on 30th day disease control group (G-II) when compared to the normal (G-I) group. The group (G-III) treated with calcium and Vit-D_3 showed significant ($p<0.001$) increase in serum phosphorus levels on 30th day, when compared to the disease control (G-II) group.The group (G-IV) treated EEVL showed marked($p<0.001$) enhancement of serum phosphorus on 30th day, when compared to the disease control (G-II) group (Table 1). However, the urine phosphorus level, which significantly ($p<0.001$) increased in CAA control (G-II) group when compared to the normal (G-I) group. The group (G-IV) receiving EEVL showed significant ($p<0.01$) reduction in urine phosphorus level, when compared to the disease control (G-II) group. Calcium and Vit-D_3 (G-III) treated group had shown significant ($p<0.001$) decrease in urine phosphorus level, when compared to the disease control (G-II) group (Table 1).

Effect of EEVL on serum Alkaline Phosphatase levels in CAA model

Increase in alkaline Phosphatase levels was markedly ($p<0.001$) increased on 30th day in disease control (G-II) group, when compared to the normal (G-I) group. The group (G-IV) receiving EEVL showed significant ($p<0.001$) elevation in serum ALP levels on 30th day, when compared to the disease control (G-II) group. Standard (Calcium and Vit-D_3) treated group had shown significant ($p<0.001$) reduction in serum ALP levels on 30th day, when compared to the disease control (G-II) group (Table 1).

Effect of EEVL on urine Creatinine levels in CAA model

CAA control (G-II) group showed significant ($p<0.001$) raise in urine Creatinine levels on 30th day, when compared to the normal group (G-I). The group (G-III) receiving Calcium and Vit-D_3 showed a significant ($p<0.001$) decrease in urine Creatinine on 30th day, when compared to the Disease control (G-II) group. Significant ($p<0.001$) reduction in urine Creatinine levels was observed on 30th day in group (G-IV) receiving EEVL when compared to the disease control (G-II) group (Table 1).

X-ray studies

Administration of alcohol (20%) has caused significant bone resorption, thinning of cortex in femur and tibia in disease control group when compared to normal animals.

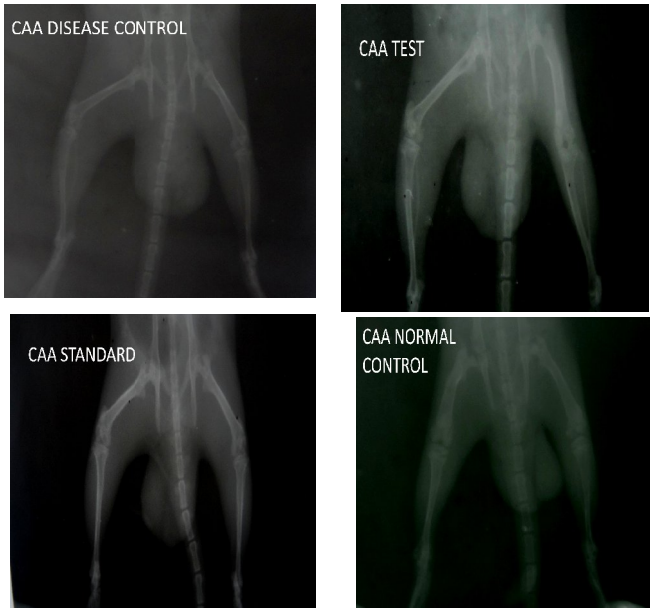
Treatment with Calcium and Vit-D_3(G-III)and EEVL (G-IV) showed significant protection against osteoporosis induced by chronic alcohol abuse, which is evident by the decrease in bone resorption and no thinning of cortex of bones when compared to disease control group as confirmed by figure 1.

TABLE: 1 Effect of EEVL on biochemical parameters and urinary parameters in chronic alcohol abuse rats

###p<0.001 when compared to normal control. ***p<0.001, **p<0.01 when compared to disease control

S. no	Group	SERUM			URINE		
		Calcium (mg/dL)	Phosphorus (mg/dL)	ALP (IU/L)	Calcium (mg/dL)	Phosphorus (mg/dL)	Creatinine (mg/dL)
I	Normal control	10.82 ± 0.254	5.625 ± 0.3630	123.9 ± 3.205	8.157 ± 0.45	4.48 ± 0.27	0.66 ± 0.0432
II	Disease control	5.768 ±0.413###	2.081 ± 0.05###	664 ± 34.70###	16.8 ± 1.2###	9.7± 0.42###	1.557± 0.072###
III	Standard(Calcium4.28mg/kg and vit-D_3 and 250 IU/kg *p.o*)	12.06 ± 0.290***	5.707 ± 0.40***	154.9 ± 7.018***	7.822 ± 0.64***	5.4 ±0.4***	0.83±0.037***
IV	Test (EEVL 500mg/kg *p.o*)	9.970 ± 0.396**	4.645 ± 0.22***	161.3 ± 6.501***	9.603 ± 0.480**	6.4± 0.33**	0.97 ± 0.07***

Figure: 1 Radiographic Analysis - X-Ray of hind limbs



DISCUSSION

The present study was carried out to evaluate the anti-osteoporotic effect of ethanolic extract of leaves of *Vitex leukoxylon* against CAA induced osteoporosis in male wistar rats. CAA induced osteoporosis in rats, a systemic skeletal disease characterized by destruction of bone by increasing bone resorption and inhibiting bone remodeling (Osteoid formation).Alcohol administered through intraperitoneal injection resulted in decreased cortical bone mass and drasticreductions in bone formation and mRNA levels for bone matrix proteins [1]. Calcium represents another important factor in the pathogenesis of osteoporosis. It has been postulated that Ca^{2+} metabolism plays a significant role in bone turnover, and deficiency of Ca^{2+} leads to impaired bone mineralization [11].Phosphorus is essential for development of bones. Most of the phosphorus present in combination with calcium in the bones. Alcohol shows alteration in PTH. PTH involved in absorption phosphorus from intestine. Serum ALP is an important biochemical marker of bone formation. The level of this enzyme is increased in osteoporosis and other bone metabolic disorders [12]. Creatinine is naturally occurring nitrogen compound synthesized by the liver,

kidney and pancreas. The majority of the creatinine pool is found in skeletal muscle (creatine phosphate) tissue (95%), with the remaining located in the brain, heart, retina and testes. Estimation of Creatinine is used to detect bone resorption.

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How to cite this article:

B. Pushpa Kumari*, K. Changappa, D. Ranganayakulu, M. Himasaila, K. Sundeep, Anti-osteoporotic activity of ethanolic leaves extract of *vitex leucoxylon* against chronic alcohol abuse model induced osteoporosis in rats , 6 (3): 2854 – 2859 (2015)