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IN-VITRO EVALUATION OF ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF *BUCHANANIA LANZAN SPRENG.* BARK

C. Pramod*[1a], Dr. Ratheesh M.[2a], Svenia P. Jose[2b] and Feena Paul[1b]

[1a]Associate Professor DPS, CPAS, Kottayam.
[2b]Assist. Professor, Svenia P Jose Research Scholar St Thomas College Pala Kottayam.
[1b]Research Scholar M.G. University Kottayam.

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***Corresponding Author**
C. Pramod
Associate Professor DPS, CPAS, Kottayam.

ABSTRACT

Methods: The bark was shade dried and coarsely powdered and then subjected to hot continuous percolation by Soxhlet apparatus using ethanol as solvent. Preliminary phytochemical analysis was carried out on extract. The antioxidant, anti-inflammatory and antiarthritic potential was evaluated by in vitro. In vitro methods include Nitric oxide scavenging assay, DPPH assay, Protein denaturation and proteinase inhibition methods. In vivo antiinflammatory activity was established by using carrageenan induced paw edema and cotton wool granuloma methods. In vivo antiarthritic potential was evaluated by collagen induced arthritis model. After 20 days of collagen administration, EEBL 200mg/Kg was administered to experimental Sprague dawley rats for next 20 days. Changes in body weight, paw volume, arthritic score were assessed weekly. In addition, on 41st day, radiological analysis, histopathological evaluation, TNF-α expression by RT PCR analysis were performed. **Results and Discussion**: In vitro studies showed that EEBL had got good nitric oxide and DPPH scavenging activity and also possess significant antiinflammatory activity. In in vivo studies, extract treatment in experimental rats at the dose of 200mg/Kg showed a decrease in the paw volume and granuloma weight indicating the possibility of EEBL be a promising antiinflammatory agent. Studies on EEBL treated collagen induced arthritic animals, showed an increase in body weight, significant reduction in paw volume, arthritic score and TNF-α expression. Radiological and histopathological analysis also strengthens the evidence of resolution of arthritis. **Conclusion:** The study confirmed the antiinflammatory and antiarthritic potential of *Buchanania lanzan Spreng.* bark belongs to the family *Anacardaceae.*

KEYWORDS: EEBL (ethanolic extract of *Buchanania lanzan bark*); TNF-α (Tumor necrosis factor-α); DPPH (Diphenyl picryl Hydrazyl).

1. INTRODUCTION

Inflammation is defined as the 'reaction of the living microcirculation and its contents to injury'. The term microcirculation describes the system of small vessels (arterioles, venules and capillaries) supplying the tissues with blood, within which are the various classes of leucocytes important in the inflammatory process. The injury can be any sort of damage to tissues, i.e. traumatic, heat, radiation, immunological or infectious. The unique feature of the inflammatory process is the reaction of blood vessels, leading to the accumulation of fluid and leukocytes in extravascular tissues. The inflammatory response is closely intertwined with the process of repair. Inflammation serves to destroy, dilute, or wall off the injurious agent, and it sets into motion a series of events that try to heal and reconstitute the damaged tissue. Kumar et al.

An uncontrolled and persistent inflammation may act as an etiologic factor for many of the chronic illnesses. Although it is a defense mechanism, the complex events and mediators involved the inflammatory reaction can induce, maintain or aggravate many diseases. (Patsnaik et al., 2011). Prolonged inflammation leads to the rheumatoid arthritis, atherosclerosis, hay fever, ischemic heart diseases etc and inflammation is a common manifestation of infectious diseases like leprosy, tuberculosis, syphilis, asthma, inflammatory bowel syndrome, nephritis, vascularitis, celiac diseases, auto-immune diseases etc. Arthritis, an autoimmune disorder, is a chronic inflammatory disease which manifests itself in multiple joints of the body. The inflammatory process primarily affects the lining of the joints (synovial membrane), but can also affect other organs. The inflamed synovium leads to erosions of the cartilage and bone, and sometimes to joint deformity. Pain, swelling, and redness are common joint manifestations.

2. MATERIALS AND METHODS

2.1. a. Collection and authentification of sample

Buchnania lanzan Spreng. was collected from Thrikaippetta area of Wynad District and authentified by Dr.Jomy Augustine, HOD, Department of Botany, St. Thomas College, Pala. A voucher specimen (Voucher No:2266) was preserved at University College of Pharmacy, Cheruvandoor Campus. M.G.University.

2.1. b Preparation of Buchnania lanzan bark extract

Fresh bark was collected and shade dried at room temperature to remove the moisture, then coarsely powdered by using electric grinder. The powdered materials stored in an air tight container and used for further extraction procedure.

Extraction procedure

Extraction of coarsely powdered bark of Buchnania lanzan was carried out using ethanol by hot continuous percolation method using soxhlet apparatus. 450g of shade dried, coarsely powdered bark were taken extracted with 2L of ethanol in the round bottom flask and extraction was continued for 10 hours. The extract was collected and concentrated by gentle heating and followed by using vacuum evaporator. The concentrated extract was then weighed, stored and calculated the percentage yield.

3 *IN-VITRO* STUDIES

3.1 Anti oxidant studies

Various antioxidant activity methods have been used to monitor and compare the antioxidant studies. In this study the following methods are used to evaluate the antioxidant activity of the plant Buchanania lanzan Spreng.

3.1. a Nitric Oxide Scavenging Assay

Nitric oxide free radical is generated from the sodium nitroprusside in aqueous solution at physiological pH. This nitric oxide is spontaneously interacts with the oxygen to produce stable products (nitrates, nitrites), which can be determined using Griess reagent. The antioxidant molecules or the free radical scavengers compete with the oxygen leading to the reduced production of nitrite (Balakrishnan, et al., 2009).

Estimation procedure

In this assay 0.5ml Sodium nitroprusside (5mmol-1) in Phosphate buffer saline solution pH7.4, was mixed with different concentration of the extract and incubated at 25°C for 180 minutes. A control was prepared without the test compound, but an equivalent amount of ethanol . After 3 hrs, 1.5ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-Naphthyl ethylene diamine dihydrochloride) was added and incubated for 30 minutes for colour development. Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1-naphthyl ethylene diamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was calculated with reference to the standard. The scavenging activity on the nitric oxide was expressed as inhibition percentage using the following equation. (Sharma, et al., 2012).

$$\% \text{ Nitric oxide Scavenging} = \frac{\text{Absorbance of control-Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

The tests were carried out in triplicates. The extract concentration providing 50% inhibition (IC50) was calculated from the graph of inhibition percentage plotted against extract concentration.

3.1.b DPPH Assay (2, 2-diphenyl -1-picrylhydrazyl)

The radical scavenging activity of extracts was determined by using DPPH assay according to Chang et al., (2001). The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10mg/ml DMSO) was used as reference.

1,1-diphenyl-2-picryl hydrazyl is a stable free radical with red colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

$$DPPH + [H\text{-}A] \rightarrow DPPH\text{-}H + (A)$$

3.2Anti inflammatory studies

3.2.a Protein denaturation method

Protein Denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an oraganic solvent or heat. Most biological proteins lose their biological function when denatured. For example, enzymes lose their activity, because the substrates can no longer bind to the active site. Protein denaturation is one of well documented causes of inflammation. Production of auto antigens in certain rheumatic diseases may be due to in vivo protein denaturation. Mizushima and others have used protein denaturation as an in vitro screening model for antiinflammatory compounds. Compound containing phenylstyrylketone inhibit bovine serum albumin (BSA) denaturation and possess antiinflammatory activity. It has been reported that one of the features of several non-steroidal antiinflammatory drugs is their ability to stabilize (prevent denaturation) heat treated albumin at physiological pH. Denaturation of protein is one of the causes of inflammation. The mechanism of denaturation probably involved in the alteration of electrostatic hydrogen, hydrophobic and disulphide bonding. The percentage inhibition of protein denaturation was compared with standard drug Diclofenac sodium at the same concentration of sample taken (Verma, et al., 2011; Bole, et al., 2011).

Procedure

Reaction mixtures were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 370C for 20 minutes

and the temperature was increased to keep the samples at 570C for 3 minutes. After cooling added 2.5ml of phosphate buffer to the above solutions. The absorbance was measured by using UV visible spectrophotometer at 416nm (Mizushima et al., 1968; Leelaprkash and Dass 2011).

Percentage inhibition = [100-(optical denity of test solution – optical density of product control) ÷ (optical density of test control)] ×100.

3.2. b Poteinase inhibition method

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play important role in the development of tissue damage during in inflammatory reactions and significant level of protection was provided by proteinase inhibitors (Leelaprakash and Dass 2011). Recent studies have shown that many flavonoids and related polyphenols contributed significantly to the antioxidant and antiinflammatory activities of many plants. Hence, the presence of bioactive compounds in the ethanolic extract of different parts of plants may contribute to its, antimicrobial, antioxidant and antiinflammatory activity (Sakat, et al., 2009).

Procedure

The reaction mixture was incubated at 37^0C for 5min and then 1ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20min, 2ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210nm against buffer as blank. The experiment was performed in triplicate. The percentage of inhibition of proteinase inhibitory activity was calculated. (Chatterjee and Das, 1996)

Percentage Inhibition =

$$\frac{\text{(Absorbance of control – absorbance of test)} \times 100}{\text{Absorbance of control}}$$

4. RESULTS

4.1 Results of in vitro studies

4.1a Antioxidant studies

Nitric oxide scavenging assay

The percentage inhibition obtained in the different concentrations of sample extract were compared to that of standard and it is tabulated in Table 4.1 and the calculation of IC50 values of standard Gallic acid and extract were depicted in Figure 7.1 and 7.2.

Table 4.1: Percentage inhibition of Nitric oxide radical by ethanolic extract of Buchanania lanzan Spreng. bark and Gallic acid.

Sl.no	Concentration (µg/ml)	OD Gallic acid	%inhibition Gallic acid	OD EEBL	%inhibition EEBL
1	Control	0.712±0.003		0.712±0.003	
2	12.5	0.453±0.003	36.43	0.556±0.003	21.75
3	25	0.397±0.003	44.27	0.497±0.003	30.14
4	50	0.320±0.002	49.91	0.437±0.002	38.59
5	100	0.277±0.003	61.0	0.383±0.003	46.26
6	200	0.202±0.003	71.57	0.341±0.002	52.1
7	300	0.105±0.003	85.32	0.226±0.002	68.31

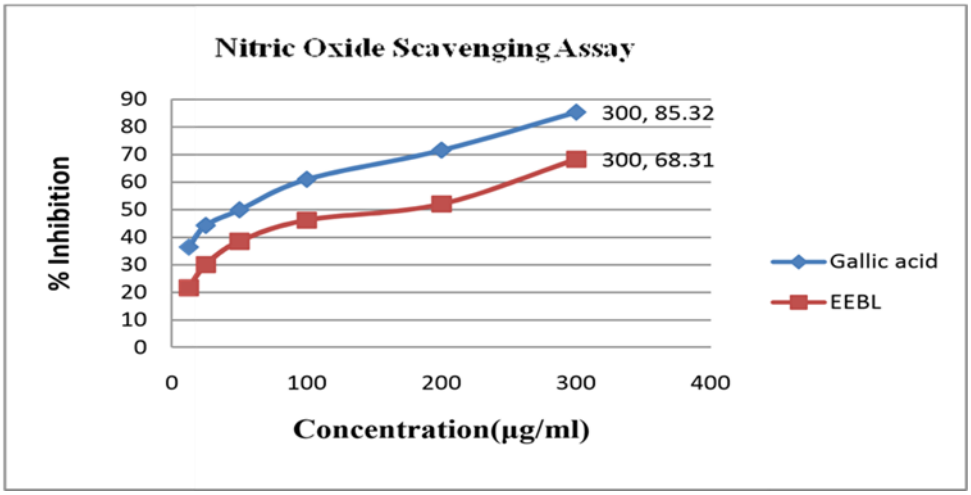

Figure 7.1: Nitric oxide radical Scavenging Assay

From the graphical method, IC50 values of Gallic acid and extract were found to be 65.26µg/ml and 165.57µg/ml respectively. Each concentrations of EEBL showed a comparable Nitric oxide scavenging activity with standard Gallic acid. i.e. EEBL possess Nitric oxide scavenging activity as compared with Gallic acid.

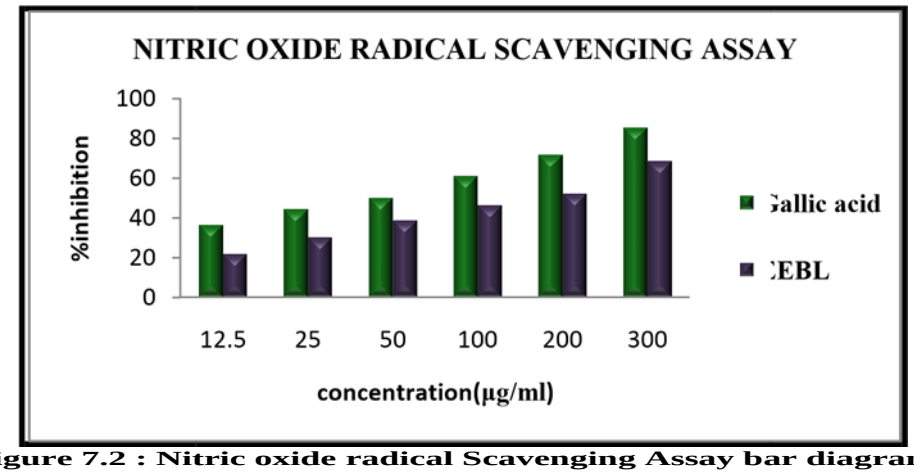

Figure 7.2 : Nitric oxide radical Scavenging Assay bar diagram

`DPPH ASSAY

The percentage inhibition obtained in the different concentrations of sample extract were compared to that of standard and it is tabulated in Table 7.3 and the calculation of IC_{50} values of standard Ascorbic acid and extract were depicted in Figure 7.3 and 7.4.

Table 7.3: Percentage inhibition of DPPH radical by ethanolic extract of *Buchanania lanzan* Spreng. bark and Ascorbic acid.

Sl.no	Concentration (µg/ml)	OD Ascorbic acid	%inhibition Ascorbic acid	OD EEBL	%inhibition EEBL
1	Control	1.46±0.02		1.46±0.02	
2	12.5	0.926±0.001	36.56	1.029±0.001	29.50
3	25	0.868±0.003	40.56	0.942±0.003	35.46
4	50	0.770±0.002	47.23	0.873±0.002	40.23
5	100	0.607±0.002	58.45	0.741±0.003	49..24
6	200	0.469±0.002	67.89	0.648±0.002	55.65
7	250	0.351±0.002	75.87	0.537±0.002	63.20

Values are mean±S.D of triplicate.

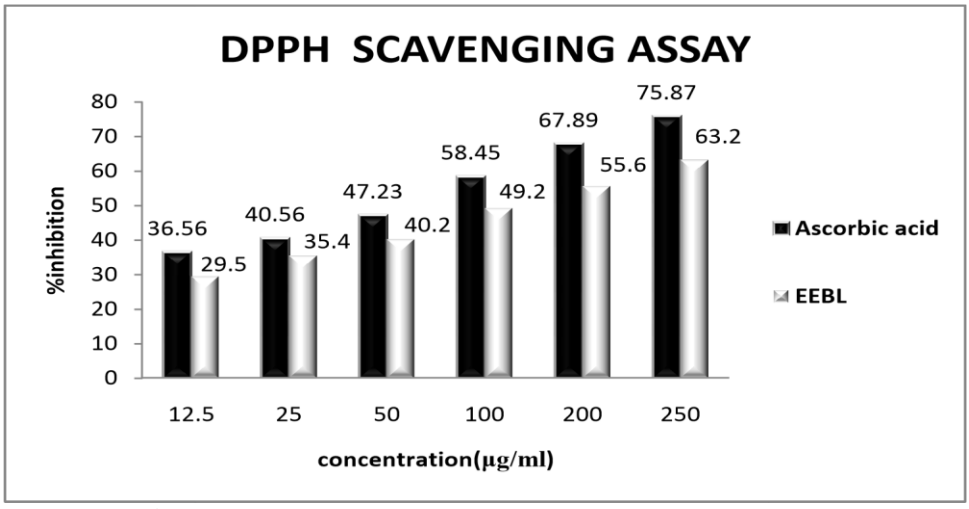

Figure 7.3: DPPH SCAVENGING ASSAY BAR DIAGRAM.

Figure 7.3 Illustrated the dose dependent DPPH radical scavenging activity of EEBL and standard Ascorbic acid.

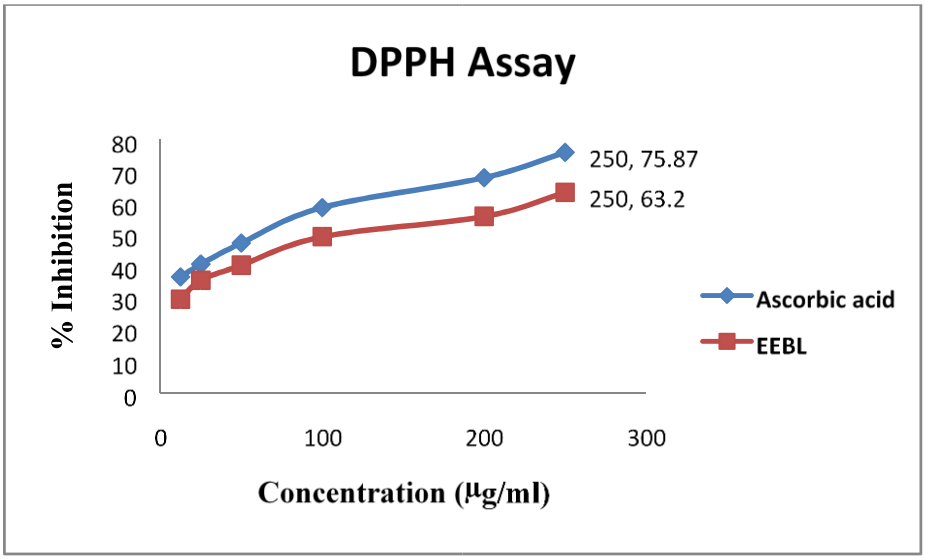

Figure 7.4: DPPH SCAVENGING ASSAY

IC_{50} values of Ascorbic acid and EEBL were found to be 78.3µg/ml and 142.14µg/ml respectively. EEBL exhibited a dose dependent DPPH radical scavenging activity as compared to standard Ascorbic acid.

7.2.2: Anti-inflammatory studies

Protein denaturation method

The percentage inhibition obtained in the different concentrations of sample extract were compared to that of standard and it is tabulated in Table 7.4

Table 7.4: Percentage inhibition of Protein denaturation by ethanolic extract of *Buchanania lanzan* Spreng. bark and Diclofenac.

SL NO.	Conc. (µg/ml)	OD of std	%inhibition of Diclofenac	OD of test	% inhibition of EEBL
1	control	0.612±0.002		0.612±0.002	
2	pc	0.208±0.002		0.163±0.003	
3	50	0.612±0.003	33.98	0.615±0.003	26.14
4	100	0.575±0.003	40.34	0.570±0.002	33.05
5	200	0.477±0.003	56.05	0.502±0.002	44.69
6	250	0.379±0.003	72.06	0.443±0.001	54.05
7	500	0.324±0.002	80.88	0.347±0.002	69.93

Values are mean±S.D of triplicate.

EEBL and Diclofenac showed a concentration dependent increase in the percentage inhibition in the protein denaturation which is depicted in Figure 7.5, with an IC50 values of Diclofenac and EEBL were found to be 157.7µg/ml 247.8 µg/ml.

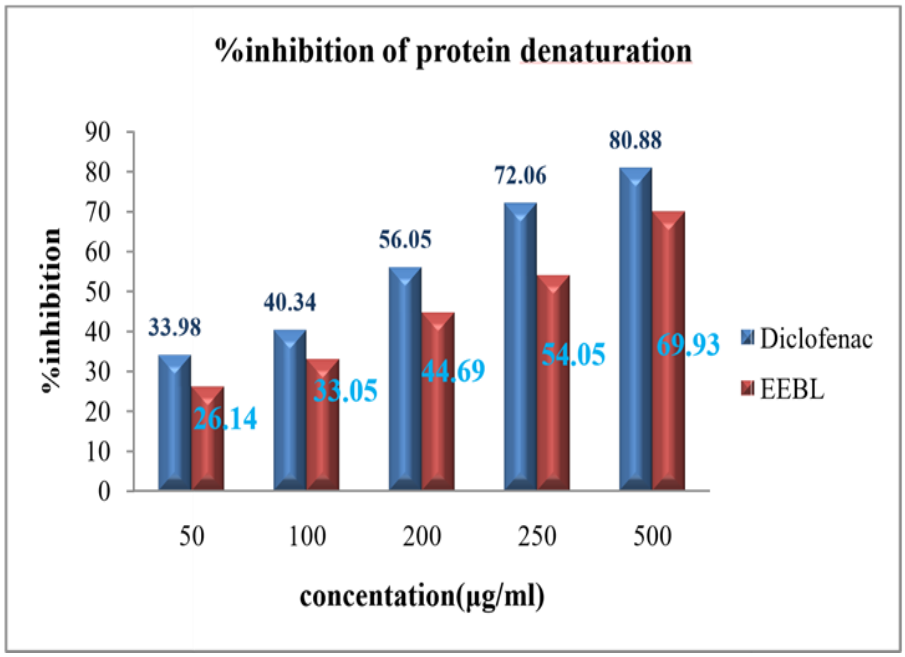

Figure 7.5: Percentage inhibition of protein denaturation.

Proteinase inhibition Assay

The percentage inhibition obtained in the different concentrations of sample extract were compared to that of standard and it is tabulated in Table 7.5.

Table 7.5: Percentage inhibition of Proteinase by ethanolic extract of *Buchanania lanzan* Spreng. bark and Diclofenac.

SL NO.	Conc (µg/ml)	OD of Diclofenac	%inhibition Diclofenac	OD of EEBL	%inhibition of EEBL
1	control	0.522±0.002		0.522±0.002	
2	50	0.321±0.002	38.54	0.350±0.005	32.95
3	100	0.273±0.002	47.73	0.320±0.003	38.69
4	200	0.239±0.002	54.24	0.281±0.002	46.25
5	250	0.187±0.003	64.16	0.243±0.002	53.84
6	500	0.106±0.003	79.78	0.187±0.001	64.17

Values are mean±S.D of triplicate

EEBL and Diclofenac showed a concentration dependent increase in the percentage inhibition in the proteinase which is depicted in Figure 7.6, with an IC_{50} values of Diclofenac and EEBL were found to be **143.3** µg/ml **262.8** µg/ml respectively.

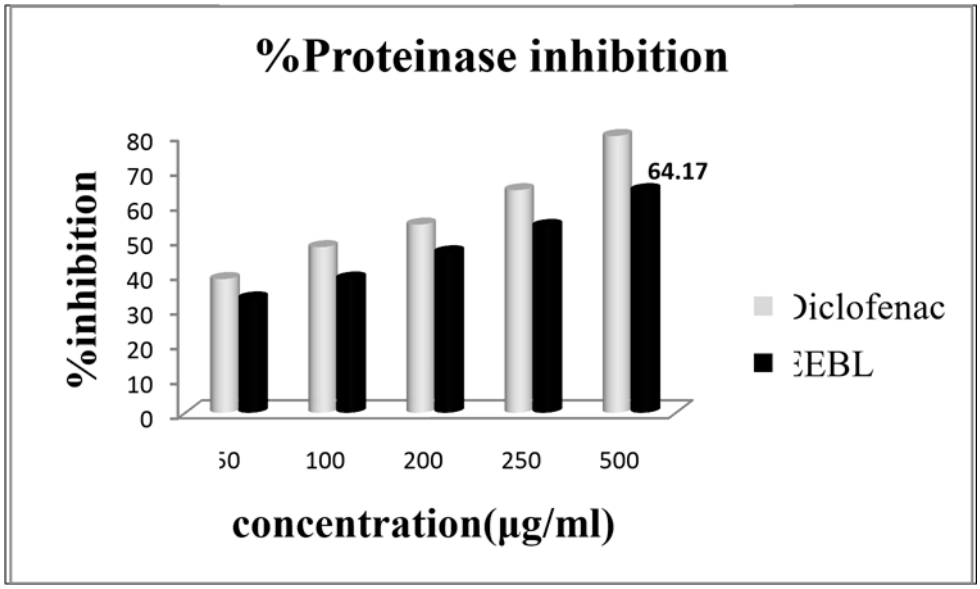

Figure 7.6: Percentage Proteinase inhibition.

6. DISCUSSION

Plants are playing a major role in the world for treating various ailments because of their safety, efficacy and cost effectiveness (**Agarwal, 2001).** A massive number of plants and formulations have been claimed to have antiinflammatory activity. Eventhough significant trendy of several herbal drugs in general, they are still unacceptable treatment modalities for inflammatory diseases. The limiting factors that contribute to this eventuality are, lack of standardization of herbal drugs, lack of identification of active principles and lack of toxicological evaluation. (Radha and Yogesh, 2005). The present study made an effort to rationalise the use of *Buchanania lanzan* Spreng. as antiinflammatory and antiarthritic agent.

The present study demonstrated the antiinflammatory and antiarthritic activity of ethanolic extract of *Buchanania lanzan* Spreng. bark in various *in vitro* and *in vivo* screening models.

Steroids, triterpenes and phenols are known to be antiinflammatory. Various studies on medicinal plants have reported promising antiinflammatory and antiarthritic activity of these phytoconstituents. Ethanolic extract of *Pistia stratiotes* produced a significant reduction in the joint swelling and showed only a mild chronic inflammatory change on histopathological analysis and the study suggested that these pharmacological activities could probably due to phytoconstituents like steroids, phenols and glycosides present in the plant. **(**Kyei, *et al*., 2012**).** The phytochemical screening results showed that EEBL contains steroids, triterpenoids, phenols, carbohydrate, tannins and saponins.

Nitric oxide (NO) is an important signalling molecule, produced as a part of the inflammatory response from activated cells and macrophages. NO plays a pivotal role as second messenger and an effecter molecule in a variety of tissues. NO also have been defined as an important molecule in inflammation and to the pathogenesis of arthritis. Nitric oxide or reactive nitrogen species formed during its reaction with oxygen or with superoxide such as NO2, N2O4, N3O4, nitrate and nitrite are very reactive. These compounds alter the architectucture and function of many cellular components. Therefore, compounds that inhibit excessive NO production may have beneficial therapeutic effects in inflammation and arthritis by blocking cartilage degradation **(Shukla *et al*., 2008).** With above literature support we assessed the antiinflammatory and antiarthritic acitivity The present study revealed that EEBL possess significant NO scavenging activity as compared with Standard Gallic acid with an IC50 of 165.57µg/ml.

The DPPH approach is simple and widely applied for the measurement of antioxidant activity. Discolouration of the sample which contains the DPPH and antioxidant agent indicated the hydrogen donating ability of the antioxidant compound. The present study showed that the EEBL possess Hydrogen donating ability with an IC50 of 142.14 µg/ml. Antioxidant capacity of the extract was found to be concentration dependent.

Hydrogen donating ability and radical scavenging activity makes the EEBL be a good anti oxidant. Antioxidant activity of EEBL provides a beneficial role in antiinflammatory property, because inflammatory processes are related to an increased production of free radicals.

Denaturation of proteins is a well documented cause of inflammation. Protein denaturation is a process in which proteins lose their tertiary and secondary structure by application of external stress. Most biological proteins lose their biological function when denatured

(Leelaprakash *et al.*, 2011). Denaturation of proteins is one of the causes of rheumatoid arthritis. Production of autoantigens in certain rheumatic diseases may be due to denaturation of proteins. The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding (Chattergee *et al*., 1996).

From the results of the present study it can be stated that EEBL is capable of inhibiting the denaturation of proteins in concentration dependent manner. EEBL showed 69.93% inhibition at 500µg/ml and standard Diclofenac showed 80.88% at the same concentration which supports the above literature.

Neutrophils are known to be inflammatory mediator, a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocytes proteinases play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors (Chattergee *et al*). EEBL exhibited significant antiproteinase activity at different concentrations. It showed maximum inhibition 64.17% at 500µg/ml and standard Diclofenac showed 79.78% at the same concentration. So, the EEBL showed a significant anti-inflammatory agent.

7. CONCLUSION

The present study highlights the antiinflammatory and antiarthritic potential of *Buchanania lanzan* Spreng. bark. The extract also seems to have potent antioxidant activity. The probable mechanisms behind the pharmacological properties include inhibition of prostaglandins (PGs)(via either negative regulation of COX-2 (cycloxygenase and lipoxygenase pathway)or induction of lipocortin) and negative regulation of genes of cytokines like TNF-α.

In vitro antioxidant studies revealed that EEBL had strong free radical scavenging activity. Thus, offered a potential effect of EEBL against free radical toxicity. Protein denaturation and proteinase inhibition methods established that EEBL possess significant inhibition effect.

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