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RESEARCH ARTICLE

# Effect of Ethanolic Extract of Leaf of *Piper Betle* Linn as Immunomodulatory Agent: A Unique Role of Phytochemicals.

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**ABSTRACT**

**Background**: Biochemical and immunological effect of leaf of *Piper betle* Linn in rat were studied. **Objective:** To evaluate the immunomodulatory effect of leaf of *Piper betle Linn*. **Methods:** Ethanolic EXTRACT OF LEAF OF *Piper betle* Linn were administered at doses of 150 and 250 mg/kg body weight for 30 days in wistar albino rats. Immunomodulatory changes and biochemical correlations were investigated. **Results:** Ethanolic extract of leaf of Piper betle I Linn showed increased antibody production in a dose dependent manner. It enhances the production of RBC, WBC and HB. Conclusion: Ethanolic extract of leaf of Piper betle Linn has positive immunomodulatory activities.

**INTRODUCTION:**

The medicinal plants are being used for prophylactic and therapeutic purposes from ancient ages, especially in south Asian countries. The leaf of Indian traditional plant *Piper betle* Linn of the Piperaceauae family (Flowering Plants) has been chosen for our study. The plant has long been used traditionally and various effects against different pathological conditions have been reported in ancient Ayurvedic literature. It includes effect against inflammation, bleeding, pus formation, gastrointestinal problems and digestive disorders. The stalk of the said plant leaf has been shown to possess antifertility effect on ovary and testis of albino rats.. Action against human pathogenic bacteria and phytopathogenic fungi has also been seen. Other established evidences of action include regulation of digestive amylase and lipase activities, antticarcinogenous effect in various cultures and action against gastric carcinoma and gastric tumors development. Various antioxidant and other active constituents has been isolated from the plant including chavicol , hydroxychavicol, flavonoids, anthocyanin's , piperol , highly specific PAF receptor antagonists like piperol A and piperol B, piperbetlol and methylpiperbetol. Antiplatelet and anti-inflammatory factors –triterpenes and β-sitosterol has been isolated from the plant.Inhibitory action of the leaf on the initiation of 7,12-dimethylbenz[a] anthracene –induced mammary carcinogenesis and inhibitory action towards timor promoter 12-O-hexadecanoylphorbol-13-acetate (HPA) –induced *Epstein –Barr* virus (EBV) activation in *RAJI* cells cells has been repoted , to mention some .

Headspace sampling and gas chromatographic-mass spectromeric determination of amphetamine and methamphetamine in betel has been reported. Biological activities of the essential oil from the leaves of *Piper betle* Linn has been found *in vitro* to be highly active against the growth of Keratinophilic fungi , *Arthroderma behamial , Microsporium gypsum , etenomyces serratus* etc.

The use of traditional plants in medicine has been mentioned in different Vedas. The writings indicate the medicinal uses of plants dates back to as old as 4000-5000BC (etc);Piper betle Linn has been used traditionally for treatment of different diseases , and the wide variation of its propertie include antiulcerogenic activity, antibacteriocidal activities , antioxidant activities etc. As antioxidant activities are very closely related to the immunomodulatory acxtivities, and the various natural antioxidants play a vital role in the modulation of immune system,the present study was undertaken to evaluate the immunomodulatory activities of Piper betle Linn.

**MATERIALS AND METHODS:**

The leaf of Piper betle Linn , in dried form was supplied by M/s Surendra Nath Das and Co., Kolkata and it was identified from the Botanical Survey of Indi a, Botanical Gardens , Hawrah , India as Piper betle Linn of PIPERACEAUE(Flowering Plants ) family. A voucher specimen has been preserved in our laboratory.

**METHOD OF EXTRACTION:**

**PLANT EXTRACT:**

Fresh plants of Piper betle Linn leaves were cleaned , dried ,*cut into fine pieces of 1-2mm thickness , pasted with 95% ethanol forming a* slime.Cold percolated for 7 days at27±1°C with 95% ethanol.Then *filtered through Whatmann No 1 filter paper .The process is repeated thrice . The residue is discarded and filtrate is subjected to partial concentration in a rotary evaporator at 30℃ and ~cm of*



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*mercury and lyophilized under vacuum* .Extract (2.2% w/w) was used as drug for further studies.

Evaluation of Lethal Dose and Toxicological Investigation of leaf of Piper betle Linn:Toxicological evaluations were made according to standard procedures for development of new drug

**EXPERIMENTAL DESIGN:**

Animals were divided into three groups such as having 5 rats and treated accordingly.

**Group I:** Control

**Group II:** Animals treated with ethanolic extract of leaf of Piper betle I Linn (150 mg/kg bw)

**Group III:** Animals treated with ethanolic extract of leaf of Piper betle Linn (200mg/kg0

Antigen Preparation: Crystalline Bovine Serum Albumin fraction V (BSA) was used as non-cellular antigen for the present investigation.

**SOLUBLE BOVINE SERUM ALBUMIN (sBSA):**

S –BSA was prepared by overlaying the BSA poeder in isotonic saline .1.0 mg/ml of saline (0.15N) .It was allowed to dissolve without agitation as is used as antigen in the investigation.

Collection of Sheep Red Blood Cells

SRBC were collected in Alserver's solution from animal husbandry without contamination.To avaoid allogennic difference the sheep red blood was used throughout the study.

**IMMUNIZATION:**

After 3 days of exposure to the toxicant, rats were immunized with optimum dose of 0.5 ml of antigen. The antigen was injected through the intraperitonial route using 3 ml tuberculin syringe. Secondary immunization was also done with the same dose of antigen through the same route on the $15^{th}$ day after primary immunization. Antigen administration and serial bleeding of animals were always done between 2-4 pm to avoid circadian rhythmic variations on the immune response.

**BLOOD COLLECTION FROM EXPERIMENTAL ANIMALS**:

Blood samples were collected from a tailvein by snipping the tip of the tail. The tipps of the tail was cleaned with spirit and snipped with clean scissors .The blood was collected in EDTA, rinsed vials for haematological studies and antigen antibody titration.

**NORMAL SERUM AND ANTISERUM COLLECTION:**

The blood was collected from the control and test animals by snipping the caudal vein rinsed with 1% EDTA and kept at RT for 20 minutes. The serum was separated by snipping down the clot at 3000 rpm for 15-20 mins and then collected in sterilized storage vials.It was kept at 57 degree celcius temperature in a water bath for 30 mints to inactivate complement and stored at 20 degree celcius temperature until use.

**ANTIBODY TITRATION:**

**PASSIVE HAEMOAGGLUTINATION ASSAY:**

**CHROMIC CHORIDE METHOD:**

The assay was used to determine anti – BSA antibodies in the serum .Two fold dilutions of the antiserum (50 microlitre per well were made with saline in a U bottom microtitre plate. 50 microlitre of 2 % BSA coupled SRBC in saline was added to each well. For effective mixing, the microtitre plate was hand shaken and incubated for an overnight at 37 dgree celcius .The highest dilution of the serum samples showed detectable macroscopic agglutination was recorded and expressed as Log 2 antibody titre of the serum.

**COUPLING OF BSA TO SRBC:**

The chromic chloride method for immunological purposes was followed by Goding(1976).In this present study , $CrCl_3$ used as a coupling agent for the coupling of BSA to SRBC.Fresh sheep erythrocytes were washed thrice by using phosphate buffered saline and stored at 4 degree celcius . One volume of the chrmic chloride solution was added to an equal volume of the protein antigen in 0.15M saline and then added to one volume of packed red cells immediately.Then it was mixed well and kept at room temperature for 4 mins. The coupled red cells are then washed three times in 10-20 volumes of 0.11 M NaCl and resuspended in 0.15 M Nacl with 2 % BSA.

**HAEMATOLOGICAL ANALYSIS:**

The fresh whole blood samples were used for the estimation of leucocytes, erythrocytes counts and Hb, RBC, WBC.

**BIOCHEMICAL TESTS:**

Total plasma protein, albumin, globulin, alkaline phosphatase, SGOT, SGPT were analyzed by Semi Auto Analyzer (Chem 400).

**TOTAL PROTEIN:**

Total protein was assayed by modified Biuret, End Point Assay Method. The peptide bonds of protein react with cupric ions in alkaline solution's .The Biuret solution

contains sodium potassium tartarate, which helps in maintaining the solubility.

**ESTIMATION OF ALBUMIN AND GLOBULIN:**

Albimin and Globulin was assayed by Bromocresol Green End Point Assay Method .At pH 3.68, albumin acts as a cation and binds to anionic dye.

**ESTIMATION OF AST/SGOT:**

Estimation of SGOT is done by 2,4 DNPH method by Reitmann and Frankel .

**ESTIMATION OF ALT/SGPT:**

Estimation of SGPT wasdone by REitman and Frankel method which is an end product photometric method for the estimation of enzymatic activity .To obtain accurate results , method has been standardized with kinetic method (Standaed Karmen Unit Assay) This product is a single point calibration version of original medhod to maximize ease of use and convenience.

**ALKALINE PHOSPHATASE:**

Alkaline Phosphatase wasestimated by Kind and King's method, where alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0

**RESULTS:**

Administration of ethanolic extract of leaf of *Piper betle* Linn (150 mg/kg bw ) and 250 mg/kg bw produced dose dependent significant increase in antibody titre compared to control. The results are given in Table I.

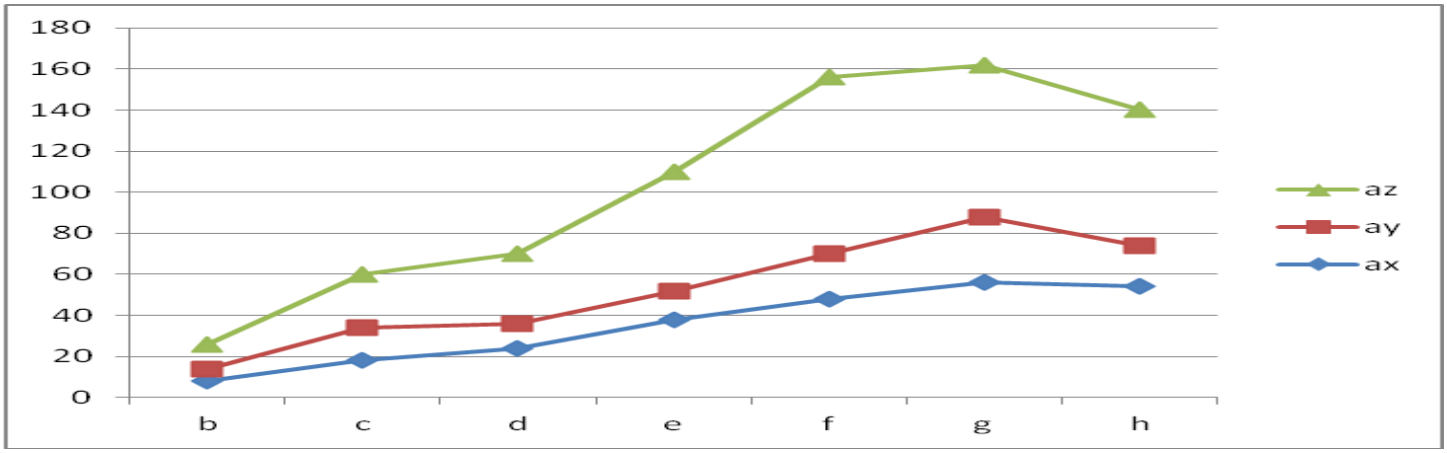


**Figure1: Effects of Piper betle extracts on humoral response to S-BSA exposed for 30 days**

Y Axis = Logarithmic Value; Axis= Exposure Time; b=2 days, c=4 days, d= 6 days, d= 8 days, e= 10 days, f= 12 days, g= 14 days, h = 16 days; Az= High Dose; Ay= Low dose; Ax= Normal Control

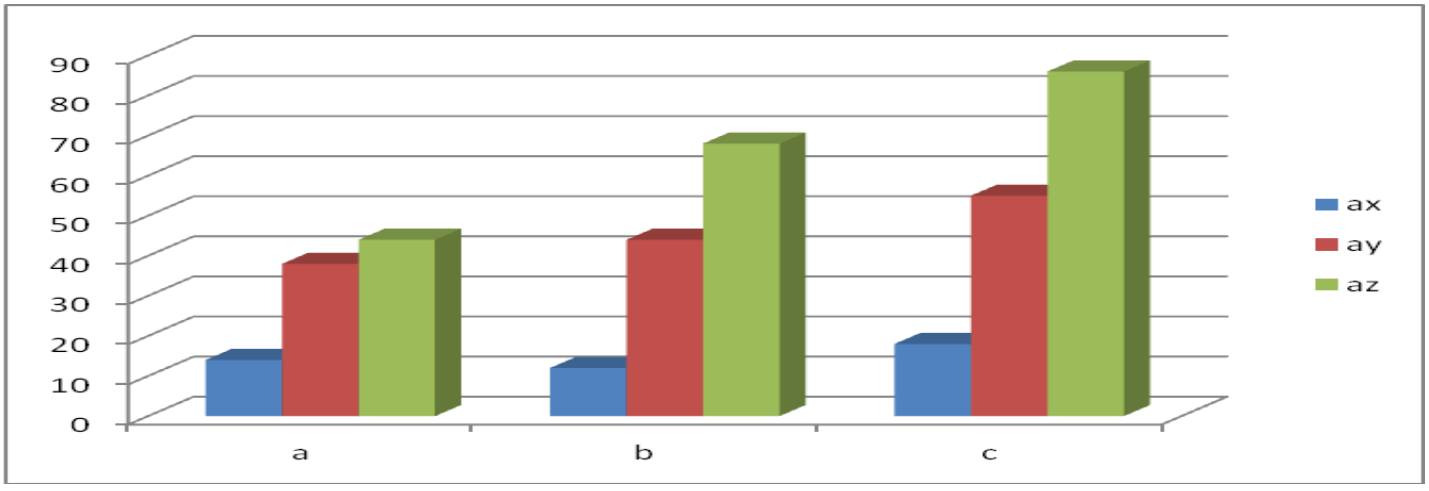


**Figure 2: Effect of Piper betle Linn on Erythrocyte Count of Wister Albino Rats**

Y Axis = Number of Erythrocyte (X10 $^{6}$);X Axis = Exposure Time (Days);Ax= Normal Control;Ay= Low Dose;Az= High dose

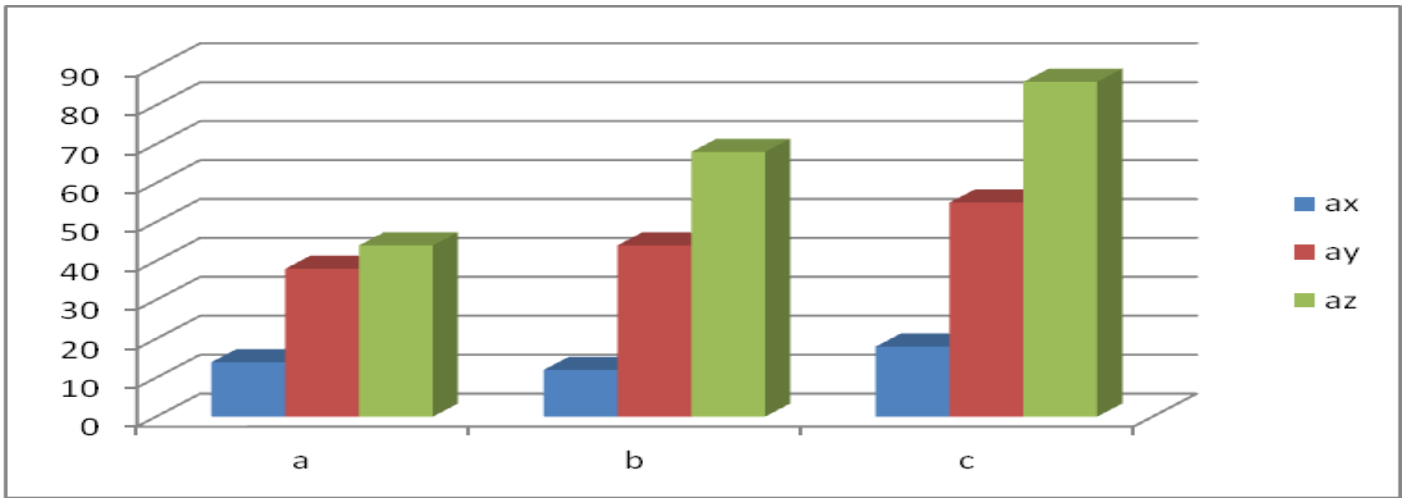


**Figure 3: Effect of Piper betle Extract on Total Leucocyte Count**

**HAEMATOLOGICAL CHANGES:**

WBC, RBC in ethanolic extract of leaf of *Piper betle* Linn groups was significantly higher compared with the control group during the experimental period. Fig II and III. Hb content also increased.

**BIOCHEMICAL ANALYSIS:**

The results showed that the increasing level of total protein in low and high dose. *Piper betle* Linn treated animals. When compared to control, albumin level was significantly increased for bothg low and high doses.SGOT was significantly altered for low and high doses. When compared to control, no significant change was observed.ALP was not changed for both low and high doses during the experimental period.

| Biochemical Parameters | Exposure | Control | 150mg/kg Body Weight | 250 mg/kg body weight |
|---|---|---|---|---|
| Protein(g/dl) | 0 | 6.71±0.32 | 6.51 ± 0.41 | 6.62 ±0.28 |
| | 15 | 6.92 ±0.27 | 6.71 ± 0.88 | 6.22 ±0.32 |
| | 30 | 6.96±0.11 | 6.21 ± 0.42 | 6.18 ± 0.54 |
| Albumin (g/dl) | 0 | 4.72±0.42 | 4.52 ±0.33 | 4.64 ±0.82 |
| | 15 | 4.70±0.46 | 4.80 ±0.52 | 4.72 ± 0.42 |
| | 30 | 4.82 ±0.51 | 4.64 ±0.26 | 4.54 ±0.56 |
| Globulin (g/dl) | 0 | 1.82±0.21 | 1.72 ±0.64 | 1.64±0.68 |
| | 15 | 1.96±0.71 | 1.85 ±0.65 | 1.78 ± 0.82 |
| | 30 | 1.32±0.42 | 1.42 ± 0.62 | 1.56 ±0.64 |
| SGOT(U/L) | 0 | 54.2± 6.12 | 53.76 ±7.20 | 55.82 ±6.82 |
| | 15 | 53.2± 6.30 | 54.22 ±6.62 | 55.72 ±7.54 |
| | 30 | 54.3 ±7.12 | 54.81 ± 6.81 | 56.82 ±6.42 |
| SGPT (U/L) | 0 | 21.42 ±4.72 | 22.52 ± 3.12 | 23.42 ± 2.84 |
| | 15 | 20.21 ±3.21 | 20.24 ±5.22 | 21.62 ± 1.82 |
| | 30 | 21.32 ±2.11 | 20.76 ±4.21 | 20.82 ± 3.72 |
| ALP(U/L) | 0 | 85.26 ± 10.32 | 84.42 ±9.76 | 85.72 ± 10.22 |
| | 15 | 84.21 ± 9.12 | 82.12 ±7.28 | 84.31 ±8.92 |
| | 30 | 86.15 ± 7.26 | 84.12 ± 6.56 | 84.28 ± 7.48 |

**Table 1: Effect of Piper betle Linn in Biochemical Parametersof Wistar Albino Rats**

**DISCUSSION:**

*Piper betle* Linn is found throughout the semitropical and tropical parts of South East Asia.This is used as medicinal plant in Ayurveda and Siddha systems of Medicine. In the present study, the immunomodulatory activity of *Piper betle* Linn was investigated in Wistar Albino Rat Model. The immune system is a complex system, to protect the host from invading and to eliminate diseases. Immunomodulators are being used as an adjuvant in conditions of immunodeficiency in cancer and other immunodeficiency sysndrome.(Mathew and Kuttan, 1999). In the present study Piper betle Linn showed increased antibody production .It may be the mediators of hypersensitivity rteactions and tissue responses to these mediators in the target organs by Piper betle Linn. Already there are many polyphenols and anthocyanin like compounds that have been proved to be very good antioxidants. The antioxidants are scavengers of free radicals and the natural antioxidants have been found to be very good immunomodulators.So , Piper betle Linn, being a natural antioxidant , the mechanism of action of the immunomodulation may be due to its antioxidant activities.Further studies will reveal the phytochemicals and the active components that are most prominent for the immunomodulatory activities. However the reverse pharmacology theory states that the active components are sometimes less active that the overall gross extract, due to the synergistic effects of the different phytochemicals that are present. Polymorphic leucocyte bursts, resulting into the production of free radicals, are being neutralized by the active components.As antioxidant activities and immunomodulatory activities are closely associated, it also reassures the theory. The herbal immunomodulators are very helpful in boosting the immune system and fighting against diseases. Further research is needed to extract and identify the active components, that is, phytochemicals that may prove to beextremely important as immunomodulating activities.

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