ANTIOXIDANT AND IMMUNOMODULATORY ACTIVITY OF *GLYCYRRHIZA GLABRA* AND *BAUHINIA VARIEGATA* IN RATS

THESIS

By

KANIKA

(V-2012-30-011)

Submitted to

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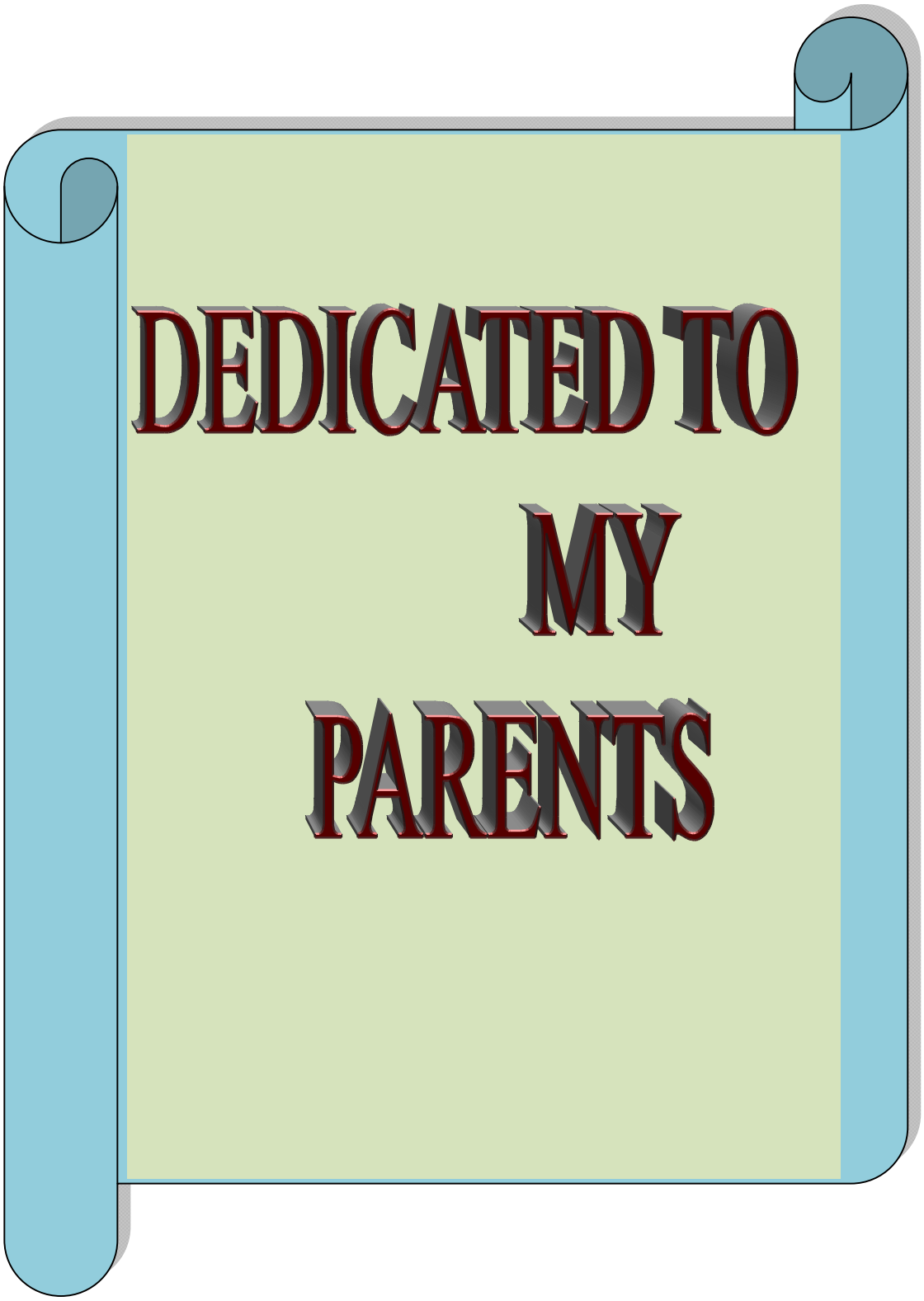
DEDICATED TO
MY
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CERTIFICATE - I

This is to certify that the thesis entitled ñ**Antioxidant and Immunomodulatory activity of *Glycyrrhiza glabra* and *Bauhinia variegata* in rats"** submitted in partial fulfillment of the requirements for the award of the degree of Master of Veterinary Science in the discipline of **Veterinary Pharmacology and Toxicology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Kanika (V-2012-30-011)** daughter of Shri Raj Kumar Kaistha under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been duly acknowledged.

(C.Varshneya)

(Major Advisor)

Place :

Dated :

CERTIFICATE - II

This is to certify that the thesis entitled ñ**Antioxidant and Immunomodulatory activity of *Glycyrrhiza glabra* and *Bauhinia variegata* in rats**ò submitted by **Kanika (V-2012-30-011)** daughter of Shri Raj Kumar Kaistha to the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur in partial fulfillment of the requirements for the degree of Master of Veterinary Science in the discipline of **Veterinary Pharmacology and Toxicology** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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Needless to mention, all errors and omissions are mine.

Place: Palampur **(KANIKA)**
Dated:

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LIST OF ABBREVIATIONS

Abbreviations	Meaning
ABTS	2,2ô-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt
A:G	Albumin: Globulin
dl	Decilitre
^0C	Degree Celsius
DLC	Differential Leucocyte Count
Fig.	Figure
g	Gram (s)
>	Greater than
HA	Haemagglutination
hrs	Hours
H_2O_2	Hydrogen peroxide
Kg	Kilogram
<	Less than
µl	Microlitre
mg	Milligram
ml	Millilitre
mm	Millimeter
min	Minute
m	Molar
No.	Number (s)

P	Page
%	per cent
S.E.	Standard Error
TLC	Total Leucocyte Count
DPPH	2,2-diphenyl-1-picrylhydrazyl
IC_{50}	Inhibitory concentration 50
$SRBC_s$	Sheep red blood cells
RBC	Red blood cell
DTH	Delayed type hypersensitivity
PBS	Phosphate buffered saline
$K_2Cr_2O_7$	Potassium dichromate
Cr	Chromium
NSS	Normal saline solution
Hb	Haemoglobin
PCV	Packed cell volume
TEC	Total Erythrocyte Count
FACS	Fluorescence-activated cell sorting (FACS)
FITC	Fluorescein isothiocyanate

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ABSTRACT

The present investigation was undertaken to study the antioxidant and immunomodulatory action of *Glycyrrhiza glabra* root and *Bauhinia variegata* stem bark extract. The fine powders of roots of *Glycyrrhiza glabra* and stem bark of *Bauhinia variegata* were subjected to methanolic, aqua-methanolic and aqueous extraction and recovery of the extracts was 15.5, 19 and 10.24%, respectively, in case of *Glycyrrhiza glabra* and 30, 9.65 and 16.66 %, respectively for *Bauhinia variegata*. ABTS quenching activity (expressed as IC_{50} values) was *0.114 mg* ml^{-1}, *0.193 mg* ml^{-1} and *0.709 mg* ml^{-1} for methanolic, aqua-methanolic and aqueous extracts of *Glycyrrhiza glabra*, respectively whereas it was *0.276 mg* ml^{-1}, *0.302 mg* ml^{-1} and *0.757 mg* ml^{-1} *for Bauhinia variegata extracts*, respectively. DPPH radical quenching activity (expressed as IC_{50} values) was *0.159 mg* ml^{-1}, *0.571 mg* ml^{-1} *and 0.552 mg* ml^{-1} for methanolic, aqua-methanolic and aqueous extracts of *Glycyrrhiza glabra* , respectively whereas it was *0.258 mg* ml^{-1}, *0.395 mg* ml^{-1} *and 0.410 mg* ml^{-1} *for Bauhinia variegata extracts,* respectively. These results suggested that methanolic extract had better quenching activity for reactive radicals. The *in vivo* antioxidant activity was studied in rats (administred extract of both plants at dose of 100 mg kg^{-1} b.w. p.o. for 30 days) by evaluating lipid peroxidation, reduced glutathione and catalase in liver, kidney and erythrocytes of male Wistar rats (130-190g). Potassium dichromate at dose rate of 30mg kg^{-1} b.w. orally for 30days was used to induce oxidative stress in rats. The studies revealed a significant ($P<0.05$) decrease in lipid peroxidation in liver, kidney and erythrocytes and a significant ($P<0.05$) increase in reduced glutathione level in the liver and kidney of *Bauhinia variegata* and *Glycyrrhiza glabra* treated rats in comparison to oxidative stress induced rats. Further immunomodulatory studies were conducted in the rats immunocompromised with dexamethasone (5mg kg^{-1} b.w. interaperitoneally for 5 days). Dexamethasone caused immunosuppression characterized by significant lymphocytopenia.The studies further revealed a significant increase in footpad thickness of *Glycyrrhiza glabra* treated rats at 24 and 48 hours of challenge with 10 % SRBCs administered interadermally on sub planter region of footpad. There was a significant increase in the absolute lymphocyte count and haemagglutination titre in *Glycyrrhiza glabra* treated rats. On differential leucocyte count a significant lymphocytopenia observed in deaxamethasone treated rats. The histopathological evaluation of lymphoid tissue exhibited the enhanced lymphocyte proliferation in lymphoid follicles in *Glycyrrhiza glabra* and *Bauhinia variegata* treated rats. There was significant ($P<0.05$) increase in CD4+ receptor cell count in lymphocytes in *Glycyrrhiza glabra* treated rats in comparison to rats immunocompromised with dexamethasone. Further *Glycyrrhhiza glabra* had significantly ($P<0.05$) higher CD4+ cell count as compared to *Bauhinia variegata* treated rats and CD4+ cell count was comparable to placebo treated animals.The present studies revealed that *Glycyrrhiza glabra* has got a better antioxidant as well as immunomodulatory activity as compared to *Bauhinia variegata*.

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(Signature of student with date) (Signature of Major Advisor)

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(Head of Department)

CHAPTER-1

INTRODUCTION

The herbs occupy an important place in various ancient system of medicine. These plants possess secondary metabolites which are pharmacologically and commercially important. These secondary metabolites are responsible for various bioactivities including antioxidant and immunomodulatory actions. The virtues of herbal medicine are fully and extensively utilised in the traditional health care system of India, Ayurveda. However, the traditional wisdom of herbal medicine has not been scientifically validated.

The free radicals are highly reactive substances formed in the body as a result of metabolic processes. Many of these molecular species are oxygen (and some time nitrogen) centered, oxygen free radicals and its non radical products are associated with reactive oxygen species (ROS).The increased production of ROS seems to accompany most forms of tissue injury. The involvement of ROS in aging and in many chronic diseases has been considered. The defense provided by antioxidant systems is crucial for the survival of organisms.

The detoxification of ROS in the cell is provided by both enzymatic and non-enzymatic systems which constitute the antioxidant defence systems. These antioxidants play a role in delaying, intercepting or preventing oxidative reaction catalysed by free radicals (Sharma *et al.* 2011a).

The immune system is a remarkably versatile defense system that has evolved to protect animals from invading pathogenic microorganisms and to eliminate disease. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders. Immunomodulation is required when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders.

Immunomodulation using medicinal plants can provide an alternative approach to conventional chemotherapy for a variety of diseases, especially when host defense

mechanisms are compromised in various infectious diseases, metabolic and autoimmune diseases (Mazumder *et al.* 2012).

Glycyrrhiza glabra (GG) (Licorice, Fabaceae/Papilionaceae) is a plant has a rich ethnobotanical history. The roots are used as a folk medicine both in Europe and eastern countries. The main components are the triterpene saponins, glycyrrhizin and glycyrrhetic acid, which are believes to be partly responsible for anti-ulcer, anti-inflammatory, anti-diuretic, anti-epileptic, anti- allergic and anti-oxidant properties of the plant as well as their ability to ófightô low blood pressure. Furthermore, *Glycyrrhiza glabra* extracts have been shown to possess antidepressant like: memory-enhancing activities and produce anti-thrombotic effects (Sharma and Agarwal 2013).

Bauhinia variegata (Kachnar) belonging to Family Caesalpiniaceae, is a deciduous tree found throughout India, at high altitude up to 1800m in Himalayas. The stem bark of *Bauhinia variegata* is used as astringent and tonic. The Kachnar is used as an antidote for snake poison. It has anthelmintic property. The plant is also used to treat tuberculosis, malaria and skin ailments (Patil *et al.* 2012).

The bark powder of the *Bauhinia variegata* is a major ingredient of the herbal tonic *Kanchanar guggul* and is used as an ayurvedic remedy prescribed to increase the white blood cells. Phytochemical characterization shows the presence of tannins, steroids, alkaloids, flavonoids and saponin in the stem bark of *Bauhinia variegata* Linn (Parekh *et al.* 2006). Phytochemical studies showed the presence of flavonoids, triterpenes and saponin in the stem bark of *Bauhinia variegata* Linn, which are the class of compounds known to possess immunostimulant properties (Ghaisas *et al*. 2009).

The present study aims at pharmacological validation of antioxidant and immunomodulatory activities with following objectives:

OBJECTIVES:

1. To investigate physical properties i.e. colour, texture of various extracts and *in vitro* validation of antioxidant properties of various extracts of *Glycyrrhiza glabra* and *Bauhinia variegata.*

2. To investigate immunomodulatory and antioxidant action of *Glycyrrhiza glabra* (root) and *Bauhinia variegata* (bark) in rats.

2.1 USES IN TRADITIONAL MEDICINE AND HISTORY

2.1.1 *Glycyrrhiza glabra* (Mulethi)

Glycyrrhiza glabra (Mulethi) was one of the major drugs in Ayurveda and Sushruta Samhita and used in Europe in the middle ages (Vispute and Khopade 2011). The plant grows in Europe, the Middle East and Western Asia (Nitalikar *et al*. 2010) and commonly known as liquorice and sweet wood belonging to Leguminosae family, Jothi-madh in Hindi, Yashti-madhuh, Madhuka in Sanskrit (Meena *et al*. 2010).

Glycyrrhiza glabra (Mulethi) used as carminative and expectorant (Zadeh *et al*. 2013). *Glycyrrhiza glabra* has pharmacological properties (including antitussive, antiulcer, antimicrobial, antiviral, antioxidant, antiinflammatory, hepatoprotective and anticancer activities and is used mainly for the treatment of peptic ulcer, hepatitis C, pulmonary and skin diseases (Saxena 2005, Maurya *et al*. 2009).

Glycyrrhiza glabra Linn., often used as a flavoring agent to mask bitter taste in various ayurvedic preparations (Kanimozhi and Karthikeya 2011).

2.1.2 *Bauhinia variegata* (Kachnar)

Bauhinia variegata (Kachnar) is a flowering plant belongs to the family leguminosae is native of southeastern Asia and southern China. It is commonly known as mountain ebony, orchid-tree, poor-manôs orchid, camel's foot and Napoleon's hat. Its leaves, flower buds, flower, stem, stem bark, seeds and roots are used in traditional medicine for treatment of bronchitis, leprosy, and tumors. *Bauhinia variegata* is a deciduous tree found throughout India, at high altitude up to 1800m in Himalayas. The stem bark of *Bauhinia variegata* is used as astringent and tonic agent. The Kachnar is used as antidote for snake poison. Its bark has anthelmintic property. The plant is also used to treat tuberculosis, malaria and skin ailments (Prasher and Venkataraman 2010, Al-Snafi 2013 and Patil *et al.* 2012).

Bauhinia variegata (Kachnar) has antimicrobial, antioxidant, antihyperlipidemic and other activities (Ahmed *et al.* 2012, Sharma *et al*. 2011c and Rajani and Ashok 2009). The stem bark of *B. variegata* is reported to possess hepatoprotective, anthelmintic, and antidiabetic activities (Mali and Dhake 2011).

2.2 CHEMICAL COMPOSITION

2.2.1 *Glycyrrhiza glabra*

Glycyrrhiza glabra contains glycyrrhizin a saponin glycoside 60 times sweeter than cane sugar; glycyrrhizin (glycyrrhizic acid and glycyrrhizinate) 10-25% of plant extract is major principle active constituent (Roshan *et al.* 2012).

The plant contains triterpenes, saponins, flavonoids, polysaccharides, pectins, simple sugars, amino acids, mineral salts, microelements and some other substances (Ammar *et al*. 2012). *Glycyrrhiza glabra* contains glucose up to 3.8%, sucrose up to 2.4-6.5%,bitter principles, resins, mannite, asparagines up to 2-4% and fat 0.8% (Vispute and Khopade 2011).

It has suggested that the yellow colour of the roots of *Glycyrrhiza glabra* is due to the flavoanoid contents. The major flavonoids are liquiritin, isoliquiritin (a chalcone) and other compounds. The isoflavones glabridin and hispaglabridins A and B have antioxidant activity whereas; glabridin and glabrene possess estrogen-like activity (Yamamura *et al.* 1992, Vaya *et al*. 1997, Tamir *et al*. 2001).

2.2.2 *Bauhinia variegata* (Kachnar)

There is presence of alkaloids, oil, fat glycoside, carbohydrates, Phenolics, Tannins, lignin, saponins, flavonoids and Terpinoids in stem bark of *Bauhinia variegata* (Dhale 2011). Flavonol glycoside 5,7,3',4'-tetrahydroxy-3-methoxy-7-O-alpha-L-rhamnopyranosyl(1-->3)-O-beta-galactopyranoside (1) are present in *Bauhinia variegata (*Yadava and Reddy 2003).

The stem bark is reported to contain 5,7 dihydroxy and 5,7 dimethoxy flavanone-4-O-L rhamnopyrosyl-ɓ-D-glycopyranosides, Kaempferol-3-glucoside, lupeol and betasitosterol (Reddy *et al*. 2003, Zhao *et al.* 2005 and Rajani and Ashok 2009).

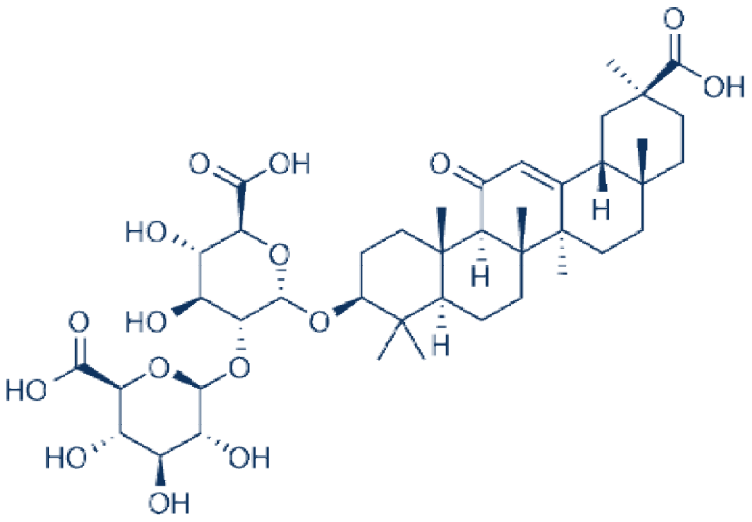


Fig 2.1 Chemical structure of glycyrrhizic acid (Vispute and Khopade 2011)

2.3 BIOLOGICAL AND PHARMACOLOGICAL EFFECTS OF *GLYCYRRHIZA GLABRA* AND *BAUHINIA VARIEGATA*

2.3.1 IMMUNOMODULATORY AND ANTIOXIDANT EFFECT OF MEDICINAL PLANTS

Immunomodulation is described as the ability of a nutrient, herb or other substance to promote healthy immune function. Immunomodulators include both immunomodulatory and immunosuppressive agents. Certain plant compounds have been shown in experimental studies to have immunostimulating properties, that is, they appear to help stimulate defense mechanisms by activating immune cells such as macrophages, lymphocytes (T cells, B cells and natural killer cells) and the cytokines (Lersch 1992).

Immunomodulation using medicinal plants can provide an alternative and adjective approach to conventional chemotherapy for a variety of diseases, especially when host defense mechanism has to be activated under the conditions impaired has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders (Mazumder *et al.* 2012).

Aloe vera triggers both specific and non-specific responses of immune system due to its alkaloids content (Chandu *et al* 2011). *Alstonia scholaris* has a significant immunomodulatory activity (Iwo *et al*. 2000). An acidic polysaccharide (ginsan) isolated from *Panax ginseng* has been reported to activate multiple effector pathways of the immune system and radioprotection (Kim *et al.* 1990).

An antioxidant can be defined as: ñany substance when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate (Halliwell and Gutteridge 1995).

There are two major groups of antioxidants in living cells: enzymatic antioxidants and non-enzymatic antioxidants. These groups are divided into several subgroups. The en- zymatic antioxidants are divided into primary and secondary enzymatic antioxidants (Carocho and Ferreira 2013).

2.3.2 *Glycyrrhiza glabra* (Mulethi)

i. IMMUNOMODULATORY EFFECT

Glycyrrhiza uralensis polyssacharides increase the pinocytic activity, the production of nitric oxide (NO), interleukin-1 (IL-1), IL-6 and IL-12 in fish. *Glycyrrhiza uralensis* polyssacharides have ability to modulate macrophage immune functions (Cheng *et al*. 2007).

ii. ANTIOXIDANT ACTIVITY

Lateef *et al.* (2012) reported that the crude methanolic extract of *Glycyrrhiza glabra* has antioxidant property studied by *in vitro* method**.**

The methanolic extract of powdered dry roots of *Glycyrrhiza glabra* showed good antioxidant activity (Morteza-Semnani et al. 2003) on the basis of in vitro antioxidant activity of *Glycyrrhiza glabra* substantiated by (Chopra et al. 2013) studied the in vitro free radical scavenging activity of methanolic root extract of *Glycyrrhiza glabra* by DPPH method and found that the extract had good antioxidant activity at 500µg/ml with per cent inhibition of 67.22 per cent and IC_{50} value was 359.45µg/ml.

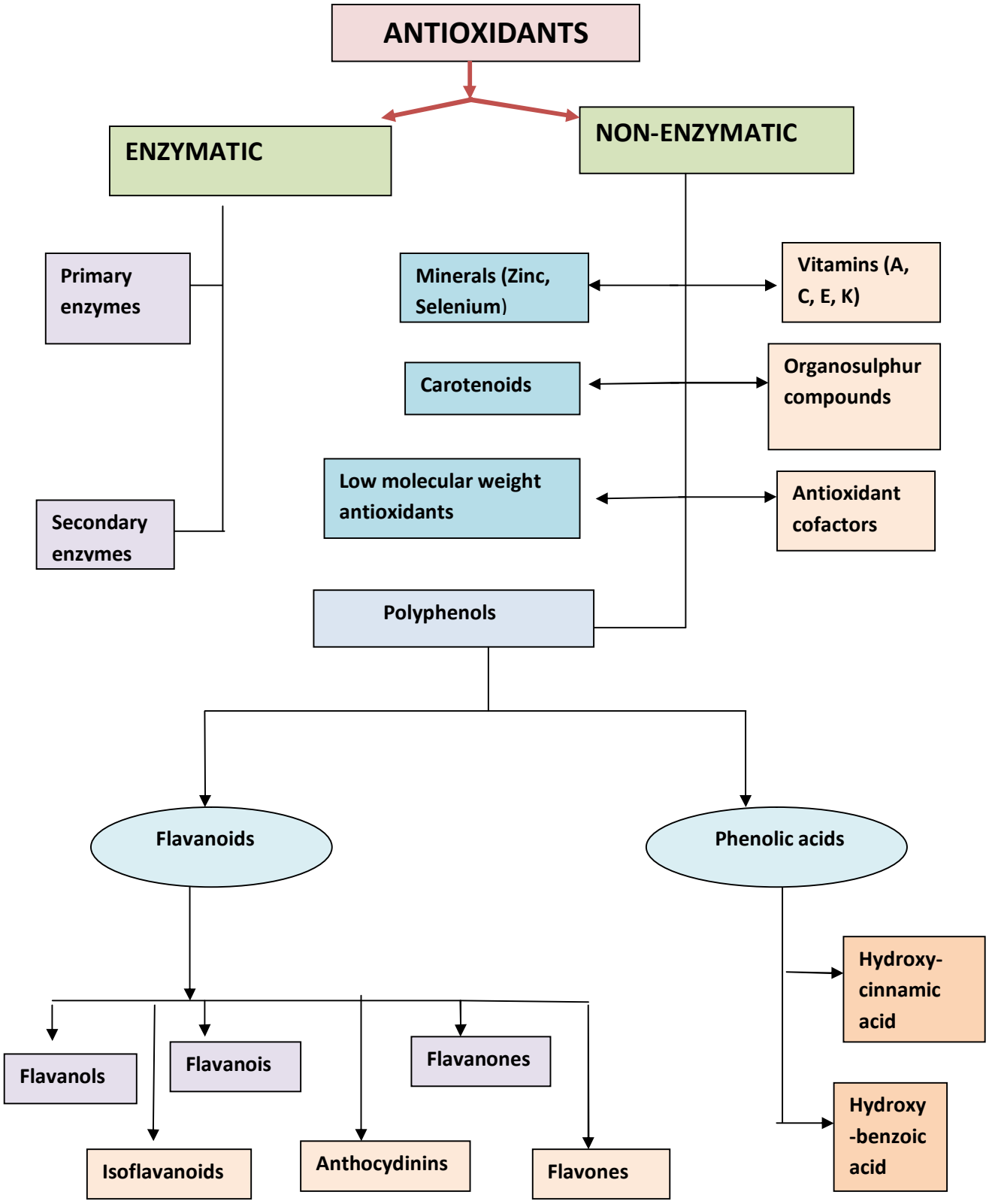


Fig 2.2 Schematic representation of classification of antioxidants Sanghera *et al.* (2013)

Tohma and Gulcin (2010) investigated antioxidant radical scavenging activity of lyophilized aqueous and ethanolic extract of roots of Turkish Liquorice (*Glycyrrhiza glabra*) by employing ABTS scavenging activity assay. A significant decrease ($P<0.01$) in the concentration of ABTS due to scavenging capacity of extracts at all concentrations was observed. Also, both the extracts were found effective ABTS radical scavengers in a concentration dependent manner.

Nand *et al.* (2012) determined the antioxidant activity of *Glycyrrhiza glabra* by ABTS assay. Results showed that the methanolic extract of *Glycyrrhiza glabra* exhibit potent antioxidant property with IC_{50} value of 21.37±1.422 µg/ml.

Sultana *et al.* (2010) examined the antioxidant properties (free radical scavenging activity) of *Glycyrrhiza glabra* by DPPH method and IC_{50} was calculated by using ascorbic acid, potential antioxidant as a positive control. The results denoted the presence of antioxidant principles in the extract.

Dayananda *et al.* (2010) studied the antioxidant property of *Glycyrrhiza glabra* root extract and the study showed that the effective antioxidative activity of methanolic extract in scavenging free radicals in comparison to other solvents. The antioxidant activities of the *Glycyrrhiza glabra* extracts were explained in terms of the total phenolics and flavanoid contents in the extract.

Al-Terehi *et al.* (2012) reported that extract of *Glycyrrhiza glabra* contains different polar compounds and are responsible for antioxidant activity. The extract inhibited the oxidation activity of anticancer drug; cyclophosphamide in rats.

iii. ANTI-BACTERIAL ACTIVITY

Glycyrrhiza glabra extract at high concentration has *in vitro* effect against *Salmonella typhi*, *S. paratyphi* B, *Shigella sonnei*, *S. flexneri* and enterotoxigenic *E.coli* studied by disc diffusion method (Shirazi *et al*. 2007).

The glycyrrhizin is commercially used as a vehicle in orally administered products inhibits the growth of some bacteria as well as dental plaque formation. *In vitro* studies also demonstrated that the aqueous and ethanolic extracts of *Glycyrrhiza gabra* have inhibitory effects on *Staphylococcus aureus* and *Staphylococcus pyogenes*. The plant also exhibited the antimicrobial activity against Gram-positive and Gram-negative bacteria (Zadeh *et al.* 2013).

iv. WOUND HEALING ACTIVITY

Ameri *et al*. (2013) evaluated the wound healing property of *Glycyrrhiza glabra* by application topical gel preaparation containing 2.5% of *Glycyrrhiza glabra* aqueous extract in MRSA-infected excision and incision wound models in mice. Mupirocin ointment was applied as a standard treatment antibiotic. The results indicated that gel formulation promoted wound healing in both models by influencing wound contraction and epithelization phases.

v. ANTI-HYPERGLYCEMIC ACTIVITY

Kalaiarasi and Pugalendi (2009) studied the hyperglycaemic effect of 18 beta-glycyrrhetinic acid, aglycone of glycyrrhizin, in streptozotocin induced diabetic rats.18 beta-glycyrrhetinic acid was shown to prevented the diabetic changes

vi EXPECTORANT ACTIVITY

Although the specific mechanism of action remains unknown, *Glycyrrhiza glabra* has been shown to be throat soothner and expectorant and is equivalent to codeine expectorant. It is proposed that carbenoxolone, a semisynthetic compound derived from *Glycyrrhiza glabra*, is able to stimulate gastric and tracheal mucus secretions and produces demulcent and expectorant effects (Murray 1998).

vii. SPASMOLYTIC ACTIVITY

Mills and Bone (2000) showed liquiritin present in the roots of *Glycyrrhiza glabra* is inactive as an anti- spasmodic. However, when hydrolysed by heat and converted to isoliquiritigenin, it showed strong spasmolytic activity.

viii. TOXICITY

The intake of higher doses of licorice over an extended period may cause sodium retention, hypertension and cardiac complaints (Khare 2004, Esmaeili *et al*. 2006). Yazdi *et al*. (2011) studied the anticonvulsant activity of *Glycyrrhiza glabra* leaves extract in mice and reported the LD_{50} value of extract was 3g/kg b.w.

2.3.3 *Bauhinia variegata* (Kachnar)

i. IMMUNOMODULATORY EFFECT

The ethanolic extract of stem bark of *Bauhinia variegata* showed the immunomodulatory activity on the primary and secondary antibody responses. It was also increased the phagocytic index and percentage neutrophil adhesion (Kirtikar and Basu 1991).

Immunomodulatory effect of ethanolic extract of stem bark of *Bauhinia variegata* in swiss albino mice was studied. Specific cell mediated immune response was studied by performing delayed type of hypersensitivity (DTH) in mice treated with ethanolic extract of stem bark of *Bauhinia variegata*. The non specific immune response was studied by performing the model of cecal ligation and puncture (CLP) model induced abdominal peritonitis in mice treated with ethanolic extract of *Bauhinia variegata*. In DTH model ethanolic extract of *Bauhinia variegata* at the oral dose of 250 and 500 mg kg^{-1} p.o. showed significant rise in the mean difference of footpad thickness in immunosuppresed group when compared with cyclosporine control. In the cecal ligation and puncture induced abdominal peritonitis model, ethanolic extract of *Bauhinia variegata* at the dose of 500 mg kg^{-1} p.o showed significant increase in survival of animals. The ethanolic extract of *Bauhinia variegata* shows the specific activation of cellular immune system in the immunosuppressed animal and also non specifically enhanced the immune system by activation of the monocyte macrophage system and natural killer cells. The ethanolic extract of *Bauhiniaa variegata* as immunomodulatory agent which acted by stimulating both specific and non-specific response of immunity (Shaikh *et al*. 2011).

ii. ANTIOXIDANT ACTIVITY

The crude extracts of *Bauhinia variegata* were studied for their antioxidant activity by DPPH radical scavenging assay. The lowest antioxidant activity was found in chloroform fraction. The ethyl acetate, methanol and n-hexane fractions showed moderate scavenging activity as compared to standard antioxidant querceitin (Uddin *et al.* 2012).

The ethanolic and aqueous extracts of stem bark and root of *Bauhinia variegata* L. were assessed for *in vitro* antioxidant activity by various methods. A significant antioxidant activity was observed (Rajani and Ashok 2009).

The total phenolic contents were estimated by Folin-Ciocalteu colorimetric method. The methanolic extract showed the highest amount of total phenolics. The results showed that the *Bauhinia variegata* is a rich source of phenolic compounds, the basis of its traditional use in different systems of medicines. Phenolics are important secondary metabolite responsible for

free scavenging activity that leads to the antioxidant activity of plant. Antioxidant property is directly correlated with the total phenolic contents in extracts (Negi *et al*. 2012).

iii. ANTI-INFLAMATORY EFFECTS

The non woody aerial parts of *Bauhinia variegata* yielded six flavonoids with triterpene caffeate. These compounds inhibited the lipopolysaccharides and interferon gamma induced nitric oxide (NO) and cytokines production for anti-inflammory effect (Koteswara *et al.* 2008).

iv. NEPHROPROTECTIVE EFFECT

The ethanolic and aqueous extracts of root of *Bauhinia variegata* Linn provided antioxidant and protective effect in gentamicin-induced nephrotoxity in rats (Sharma *et al*. 2011b).

v. ANTIULCER EFFECT

The ethanolic extract of *Bauhinia variegata* decreased the volume of gastric secretion, total free acidity and ulcer index in gastric ulcer induced rats (Rajkapoor *et al.* 2003).

As immune system is known to be involved in the etiology as well as pathologic mechanisms of many diseases it has tremendously increased the need of drugs which are effective on immune system. To overcome the drawbacks of the synthetic immunomodulators there is a need of development of herbal immunomodulators in future.

Both antioxidant protection and immunomodulation play a major role in adjunctive therapy in various pathological conditions. Therefore, to explore the antioxidant and immunomodulatory activities of roots of *Glycyrrhiza glabra* and stem bark of *Bauhinia variegata* the present study was undertaken.

CHAPTER-3

MATERIALS AND METHODS

The antioxidant and immunomodulatory activities of *Glycyrrhiza glabra* and *Bauhinia variegata* were studied using *in vitro* and *in vivo* methods. Male Wistar rats (130-190g) used as experimental animals for *in vivo* studies.

3.1 MEDICINAL PLANT

3.1.1 COLLECTION/PROCUREMENT OF PLANTS

The following plant materials were collected/procured:

Plant	**Faimily**	**Part**
Glycyrrhiza glabra (Mulethi)	Papilionaceae; Fabaceae	Root
Bauhinia variegata (Kachnar)	Caesalpiniaceae; Leguminosae	Stem bark

3.1.2. PREPARATION OF EXTRACTS

The roots of *Glycyrrhiza glabra* were procured from local market and stem bark of *Bauhinia variegata* was collected from nearby areas of Palampur. The identification of plant material was done at Department of Biodiversity (IHBT, CSIR Palampur H.P.). The plant materials were cleaned; shade dried and grounded to obtain fine powder. The powder was used for preparation of the extracts.

The mixture was filtered through filter paper. The filtrate was vacuum dried in rotary vacuum evaporator (BUCHI Rotavapor R-210, Vacuum Controller V-850) at temperature according to solvent used. The extract was evaporated to thick consistency by vacuum pump and lyophilized and stored at 4°C (Table 3.1).

3.2 *IN VITRO* ANTIOXIDANT ACTIVITY: *In vitro* antioxidant activities were determined according to following method:

3.2.1 MEASUREMENT OF TOTAL ANTIOXIDANT ACTIVITY BY ABTS

The total antioxidant activity of plant extracts was determined according to the method of Re *et al.* (1999). ABTS was dissolved in distilled water to form 2 mM concentration.70 mM solution of potassium persulphate formed and 50 ml of ABTS solution was mixed with 200µl of 70 mM potassium persulphate.The final concentration was allowed to stand in the dark at room temperature for 12-16 hours before use. ABTS solution reacted with potassium persulphate to form $ABTS^{+}$ free radicals. The resulting $ABTS^{+}$ solution was diluted with phosphate buffer, pH 7.4, to an absorbance of 0.70 ± 0.02 at 734 nm.

Table 3.1 Preparation of various types of extracts

Type of extract	**Amount of powder soaked in solvent**	**Amount of solvent used**
Methanolic (*Bauhinia variegata)*	100 g	400 ml of 95% methanol
Aqua-methanolic (*Bauhinia variegata)*	100 g	200 ml water and 200 ml of methanol
Aqueous (*Bauhinia variegata*)	60 g	400 ml of water
Methanolic (*Glycyrrhiza gabra)*	50 g	400 ml of methanol
Aqua-methanolic (*Glycyrrhiza glabra)*	50 g	200 ml of water and 200 ml of methanol
Aqueous (*Glycyrrhiza glabra*)	50 g	400 ml of water

The antioxidant activities of extracts of *Bauhinia variegata* and *Glycyrrhiza glabra* were evaluated in concentration ranging from 0.062 mg/ml to 1 mg/ml and compared with standard of trolox (0.015 mg/ml to 1 mg/ml).

Radical scavenging analysis was performed by mixing 20 εl of the sample solution into 2.0 ml of $ABTS^+$ solution and reading the absorbance at 734 nm after 1, 2, 3, 4 and 5 minutes. A blank solution of 20 εl 70% methanol in 2.0 ml of $ABTS^+$ solution was prepared and analyzed. The concentration activity curve was plotted and compared with standard antioxidant.

% $ABTS^+$ inhibition = [1 ï (A_{734}nm Sample/ A_{734}nm Blank)] x 100

3.2.2 DPPH RADICAL SCAVENGING ACTIVITY

The ability of extracts to scavenge DPPH radicals was determined according to the method of Hsu *et al.* (2006). A 100 εM solution of DPPH in methanol was prepared. The DPPH radical scavenging action of *Glycyrrhiza glabra* extract was evaluated in concentration between 1 to 0.06 mg/ml and that in *Bauhinia variegata* extract from 1 to 0.06 mg/ml. Butylated hydroxyl toluene (BHT) was taken as standard antioxidant. The free radical scavenging action of standard butylated hydroxyl toluene (BHT) was evaluated in the range of 1 to 0.03 εg/ml.

A 0.5 ml sample solution was added to 2.0 ml of DPPH in a 20 ml test tube. A control solution was prepared by adding 0.5 ml of methanol to 2 ml DPPH solution. The samples were vortexed for 10 to 15 seconds and held at room temperature (22 ± 3°C) in the dark for 30 minutes. The absorbance of the test and control solutions was determined at 517 nm and the per cent DPPH radical scavenging activity was calculated as follows:

% DPPH radical scavenging activity = [1ï (A_{517}nm test / A_{517}nm control)] x 100

3.3 PROCUREMENT AND MAINTENANCE OF EXPERIMENTAL ANIMALS

A total of 96 male Wistar rats (130-190 g) were procured from Disease Free Animal House, Hisar and were maintained in the Experimental Animal House of the Department of Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Palampur, Himachal Pradesh. All the experiments were performed in accordance with the permission of Institutional Animal Ethical Committee permission registeration number by 259, at CSKHPKV Palampur, dated 17-10-13)

3.3.1 HOUSING AND MAINTENANCE

On arrival, all the animals were examined for any abnormality and ill health and were weighed. The animals were acclimatized in new environment for 21 days. Rats were housed

in polypropylene cages (Tarson, India) and rice husk was provided as the bedding material. The animals were maintained under standard conditions (23 ± 1°C, 12 h light/dark cycles). The standard animal feed and clean water were provided *ad libitum*. All the animals were kept in hygienic conditions.

3.4. EXPERIMENTAL DESIGN

The experiment was designed for the evaluation of antioxidant and immunomodulatory potential of *Glycyrrhiza glabra* and *Bauhina variegata* in male Wistar rats.

3.4.1 EXPERIMENTAL STUDIES FOR ANTIOXIDANT ACTION

For the evaluation of antioxidant action, rats were divided into four groups of six rats each (Table 3.2). The oxidative stress was induced using potassium dichromate (chromiumVI).

Table: 3.2. Experimental design to evaluate the antioxidant effect of *Glycyrrhiza glabra* and *Bauhinia variegata.*

Group	Drug	No. of rats	Dose (mg/kg b.w.)		Route
			Extract	$K_2Cr_2O_7$ as Cr(VI)	
I	Control	6	-	-	oral
II	$K_2Cr_2O_7$	6	-	30	oral
III	$K_2Cr_2O_7$ + *Bauhinia variegata*	6	100	30	oral
IV	$K_2Cr_2O_7$ + *Glycyrrhiza glabra*	6	100	30	oral

3.4.2 EXPERIMENTAL STUDIES FOR IMMUNOMODULATORY ACTION

For the evaluation of immunomodulatory action, rats were divided into four groups of eighteen rats each (Table 3.3). The immunosuppression was induced using Dexamethasone.

Table: 3.3 Experimental designs to evaluate the Immunomodulatory effect of *Glycyrrhiza glabra* and *Bauhinia variegata*

Group	Drug	No. of rats	Dose (mg/kg)		Route
			Extract	Dexamethasone	
I	Control	18	-	-	-
II	Dexamethasone	18	-	5	Interaperitoneally
III	Dexamethasone + *Bauhinia variegata*	18	100	5	Interaperitoneally + oral
IV	Dexamethasone + *Glycyrrhiza glabra*	18	100	5	Interaperitoneally + oral

Table 3.4 EXPERIMENTAL PROTOCOL

The experimental studies were conducted in following chronological order:

DAY	ACTIVITY
-21	Animals procured and put in quarantine period for 20 days
0	Body weight of animals was recorded and animals were divided into groups according to the body weight. Oral gavaging of extracts and potassium dichromate started once a day in groups for antioxidant study and in immunomodulatory groups, dexamethasone immunosuppressive injected interaperitoneally twice a day started.

5	Dexamethasone injected interaperitoneally for 5 days. Blood sample collected for haematology.
6	Oral gavaging of extracts was started in immunomodulatory groups as per schedule.
7	Body weight of animals recorded.
14	Body weight of animals recorded.
16	Animals sensitized with SRBCs injected interaperitoneally (0.3 ml) on right hind footpad.
22	Rats were challenged with SRBCs for haemagglutination test and SRBCs injected on footpad for delayed type of hypersensitivity study.
28	Blood sample collected from rats for haemagglutination test, footpad thickness recorded at 0 time and DTH groups were challenged with SRBCs.Footpad thickness recorded as per schedule.
30	Footpad thickness recorded for DTH test as per schedule. Body weights of animals were recorded and animals were sacrificed for organs and blood collection. Organ weight (liver and kidney) of the representative groups was recorded.

3.6. GROWTH RESPONSE

3.6.1 BODY WEIGHT AND BODY WEIGHT GAIN

Rats from each group were weighed at weekly interval up to 4 weeks using calibrated weighing balance. Body weight and body weight gain per group was expressed in gram (g).

3.6.2 FEED CONVERSION RATIO (FCR)

FCR was calculated by dividing the feed intake during 4 week study period with the body weight gain.

3.7 ORGAN BODY WEIGHT RATIO

The rats were sacrificed by cervical dislocation after anaesthesia using chloroform and relative organ weight (g/100g) of liver, kidney, spleen was calculated.

3.8 *IN VIVO* ANTIOXIDANT ACTIVITY

3.8.1 OXIDATIVE STATUS IN LIVER AND KIDNEY

PREPARATION OF TISSUE HOMOGENATE: 0.5g of tissue sample was taken and mixed in 5 ml of ice cold saline. The tissue homogenates were prepared by trituration in mortar with pestle.

i LIPID PEROXIDATION (LPO)

The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was used for estimation of lipid peroxidation. The extent of lipid peroxidation was expressed in terms of MDA (malondialdehyde) production, determined by the thiobarbituric acid (TBA) method of Shafiq-ur-Rehman (1984). The procedure is detailed below:

Reagents:

1. 10 % Trichloroacetic acid (TCA) solution: 10 g of TCA dissolved in 100 ml of distilled water.
2. 0.67 % Thiobarbituric acid (TBA): 0.67 g of TBA in 100 ml of warmed distilled water.

Procedure:

1 ml of tissue homogenate was incubated at 37^{o}C for 2 h. To each sample, 1 ml of 10% TCA was added. This was mixed thoroughly and centrifuged at 2000 rpm for 10 min. To 1 ml of supernatant, an equal volume of 0.67 % TBA was added and kept in boiling water bath for 10 min. The reaction mixture was cooled and diluted with 1 ml of distilled water. The absorbance was read at 535 nm. The blank was prepared by using distilled water in place of tissue homogenate. Calculation was done using the molar extinction coefficient of MDA-TBA complex, 1.56 X 10^5 $M^{-1}cm^{-1}$. The amount of lipid peroxidation was expressed as nM malondialdehyde (MDA) formed per g of packed cells.

$$\text{LPO (nM MDA g}^{\square\square}) = \frac{\text{OD}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times 10^{\square} \times \text{DF} \times \text{IT}$$

Where, LPO- Lipid peroxidation

OD-Optical density

EC- Extinction coefficient

DF-Dilution factor (10)

I-Incubation time (in min)

iii. **REDUCED GLUTATHIONE (GSH)**

GSH was assessed by estimating free-SH groups, using DTNB method of Sedlak and Lindsay (1968).

Reagents:

1. (0.01 M) 5, 5, Dithiobis (2-nitro) benzoic acid (DTNB): 99 mg of DTNB reagent was dissolved in 25 ml of methanol.
2. 1 M tris buffer (pH 8.9): 121 g of tris base was dissolved in 950 ml of distilled water and final volume was made up to 1000 ml using distilled water.
3. TCA (50 %): 50 g of TCA in 100 ml of distilled water.

Procedure:

1 ml of tissue homogenate (as prepared previously) was mixed with 1 ml of 50% TCA and incubated at room temperature for 15 min. The mixture was centrifuged at 3000 rpm for 15 min. From this, 0.8 ml of supernatant was taken and to it 1.6 ml of 1 M tris buffer was added followed by 0.4 ml DTNB (0.01 M). The absorbance was recorded at 412 nm within 5 min. The blank was prepared by using distilled water in place of tissue homogenate.

Calculation was done by using the extinction co-efficient (EC = 13100 M^{-1} cm^{-1}) following formula and the results were expressed in mM of GSH g^{-1} of the wet tissue.

$$\text{GSH}(\text{mMg}^{-1}) = \frac{\text{OD}}{\text{EC}} \times \frac{\text{Volume of supernatant} \times \text{Volume of buffer}}{\text{Volume of supernatant} \times \text{Weight of tissue}} \times 1000 \times \text{DF}$$

Where, OD - Optical density

EC - Extinction coefficient

DF - Dilution factor (10)

iii. CATALASE

Catalase was estimated in tissues by spectrophotometric method as described by Aebi (1983). The procedure is detailed below:

Reagents:

1. Phosphate buffer (50 mM , pH 7.0)

2. Hydrogen peroxide (30 mM): 0.34 ml of 30% H_2O_2 was diluted with buffer. The optical density of diluted H_2O_2 at 240 nm should be around 1.5. The buffered H_2O_2 solution was prepared fresh.

Procedure:

To 2 ml of phosphate buffer in quartz cuvette, 20 µl of tissue homogenate was added and mixed well .The reaction was started by the addition of 1 ml of 30 mM H_2O_2 and the decrease in absorbance was recorded at every 10 sec interval for 1 min at 240 nm in a U.V. spectrophotometer (SHIMADZU UV-1800).

The results were expressed as µmol of H_2O_2 decomposed per min per mg protein using 36 as molar extinction coefficient of H_2O_2.

Calculation:

$$\text{Catalase} = \frac{\partial \text{OD}_{\square}}{\text{time}} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times \frac{1}{\text{mg of protein in 0.1ml}}$$

3.8.2 OXIDATIVE STATUS IN ERYTHROCYTES

i SEPARATION OF ERYTHROCYTES

The heparinized blood samples were centrifuged at 2000 rpm for 15 min. Plasma and buffy coat were removed and resulting erythrocyte pellets were washed thrice with NSS. The 33% dilution of packed RBC was made in PBS (pH 7.4). This 33% packed RBC suspension was used for estimation of lipid peroxidation..

ii LIPID PEROXIDATION (LPO)

Membrane peroxidative damage in erythrocytes was determined in terms of MDA (malondialdehyde) production, determined by TBA (thiobarbituric acid) method of Shafiq-ur-Rehman (1984).

Reagents:

1). Normal saline solution (NSS)

2). 10% Trichloroacetic acid (TCA) solution

3). 0.67% Thiobarbituric acid (TBA) in warm distilled water.

Procedure:

To 1 ml of 33% packed erythrocytes, 1 ml of 10% TCA was added. After thorough mixing, the mixture was centrifuged at 389 g for 10 min. To 1 ml of supernatant, 1 ml of 0.67% TBA was added and kept in boiling water bath for 10 min, cooled and diluted with 1 ml of distilled water. Blank was made by adding all the reagents except the packed erythrocytes which were substituted with equal volume of distilled water. The absorbance was read at 535 nm.

Calculation:

The amount of lipid per-oxidation was expressed as n mol MDA formed/ml packed cells. The molar extinction coefficient (EC) of MDA-TBA complex at 535 nm was 1.56 X 10^5/M/cm.

$$\text{LPO nM MDA gm}^{\square\square\square} = \frac{\text{OD}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times 10^{\square} \times \text{DF} \times \text{I}$$

3.8.2 ASSESSMENT OF IMMUNOMODULATORY ACTIVITY

i. HUMORAL IMMUNE RESPONSE (HAEMAGGLUTINATION TEST)

Six rats from each group were sensitized with sheep red blood cells (SRBCs) by intraperitoneal injection of 0.2 ml of 20% SRBCs suspended in PBS per animal on day 16 of the experiment. The animals were challenged with SRBCs on day 23. Serum was collected after 7 days of challenge. Haemagglutination (HA) was carried out by micro titration technique according to the procedure described by (Mazumdar *et al*. 2013) in V shaped 96 wells microtitre plate. 0.05ml of phosphate buffer saline was added to each well of the microtitre plate. Then 0.05ml serum samples were added to the first well and thereafter two fold serial dilutions were made up to last well. Then 0.05 ml of 0.5% SRBCs suspension was added to all the wells. Negative control contained 0.05 ml of phosphate buffered saline and 0.05ml of SRBCs. The plate was swirled gently for mixing and uniform distribution of erythrocytes and left overnight at room temperature. The HA pattern (a diffused sheet of agglutinating RBC covering the bottom of the wells) was read and the titre was recorded as

reciprocal of the highest dilution showing complete agglutination of the erythrocytes. The results were expressed as $\log_2$ values.

ii. CELL MEDIATED IMMUNE RESPONSE

DELAYED TYPE OF HYPERSENSITIVITY TEST

Delayed type of hypersensitivity test was done by method as described by Manjuladevi *et al.* (2013). On the 23rd day, 0.3ml of 10% SRBCs was injected interaperitoneally, after 7 days the 0.3 ml of 10% SRBCs was injected in sub plantar region of left hind paw and normal saline in the right hind paw in same volume. Foot pad oedema in rats was used for the detection of cellular immune response. The foot pad thickness was measured using vernier calliper at 0, 12, 24, 48 and 72 hours (which indicates that the oedema formed is due to hypersensitivity reaction). The foot pad reaction was expressed as the difference in thickness in mm between the right and left hind paw.

3.9. CD4+ and CD8+ COUNT BY FLOW CYTOMETERIC METHOD

Estimation of CD4+ and CD8+ cell count was done by Flowcytometery (BD FACS Calibur) in Department of Veterinary Microbiology, COVAS, CSKHPKV, Palmpur (H.P.)

COLLECTION OF BLOOD SAMPLES

The blood was collected directly from retro-orbital plexus in sterilized ependorff vials with EDTA as anticoagulant.

PREPARATION OF BLOOD SAMPLES

100µl of whole blood was transferred to 5ml polystyrene tube and incubated for 30 minutes at 4°C with anti CD4+ and anti mAbs labeled with FITC. After incubation, RBCs were lysed by FACS lysed solution. 2 ml of 1× FACS lysing solution was added to tubes. Samples were agitated and incubated for 10 minutes. Samples were washed with PBS twice and washed cells were suspended in 0.3 ml and analysed in FASC. The per cent (%) total of CD4+ and CD8+ cell count were noted.

3.10. BIOCHEMICAL PROFILE

The blood was collected directly from retro-orbital plexus in sterilized ependorff vials with heparin as an anticoagulant and plasma was used for biochemical estimation.

3.10.1 TOTAL PROTEIN

The total proteins were estimated by Biuret method (Henry and Winkleman 1974). The total protein values were expressed in gdl^{-1}.

3.10.2 ALBUMIN

The albumin was analyzed by using the BCG method (Doumas *et al*. 1971). The albumin values were expressed in gdl^{-1}.

3.10.3 GLOBULIN

Globulin values were calculated by subtracting the value of albumin from the corresponding total protein values. The globulin values were expressed in gdl^{-1}.

3.10.4 ALBUMIN: GLOBULIN (A: G) RATIO

Albumin: globulin ratio was calculated by dividing albumin values by corresponding globulin value.

3.11 HAEMATOLOGICAL PROFILE

3.11.1 BLOOD COLLECTION AND ESTIMATION OF HEMATOLOGICAL PARAMETERS

The blood was collected directly from retro-orbital plexus by using microcapillary tube in an ependorff vial containing EDTA (1-2 mg ml^{-1}).

Total leucocyte count (TLC), absolute lymphocyte count, absolute granulocyte count, differential leucocyte count (DLC), hemoglobin (Hb), packed cell volume (PCV) and total erythrocyte count (TEC) were estimated by Auto Hematology Analyzer (MINDRAY BC-2800 Vet) in Department of Veterinary Surgery and Radiology, COVAS, CSKHPKV, Palampur.

3.12 HISTOPATHOLOGY

The lymphoid tissues (spleen and mesenteric lymph node) were collected and fixed in 10 per cent (%) formalin. Histopathological examination of test tissues was made on haematoxylin-eosin stained section of 4 micron thickness following the routine processing.

3.13. STATISTICAL ANALYSIS

The data was analyzed by Analysis of variance test (ANOVA, one way) using the Graph Pad Instat version 3.00 for windows (Graph Pad Software, San Diego, California, USA, and www.Graphpad.com) and the significant differences between mean values was determined using Tukey-Kramer Multiple comparison test. The data was presented as mean ± standard error. The inter group comparisons were made at 5% level of significance.

CHAPTER-4

RESULTS AND DISCUSSION

The present study was aimed at studying the effects of extracts of *Bauhinia variegata* and *Glycyrrhiza glabra* on the basis of antioxidant and immunomodulatory activities. Besides growth performance, haematological profile, biochemical parameters and histopathological studies were also conducted.

4.1 COLOUR AND PERCENT RECOVERY OF THE EXTRACTS

Table.4.1 Colour and percent recovery of different extracts of *Glycyrrhiza glabra* and *Bauhinia*

S.No	Sample	Type of Extract	Colour	Percent recovery (%)	Dry Powder (mg) equivalence/100mg of extract
1	*Glycyrrhiza glabra*	Methanolic	Crystalline black	15.5	645.16
		Aqua- methanolic	Dark black	19	526.31
		Aqueous	Dark greenish black	10.24	976.56
2	*Bauhinia variegata*	Methanolic	Dark crystalline brown	30	333.33
		Aqua- methanolic	Dark crystalline brown	9.65	1036.26
		Aqueous	Dark brown	16.66	600

Concentration (mg/ml)	% Inhibition		
	Methanolic	Aqua-methanolic	Aqueous

variegata

The colour of *Glycyrrhiza glabra* roots was light brownish yellow whereas, that of *Bauhinia variegata* bark was light brown. The colour and percent recovery of different extracts is presented in table 4.1 and plate 4.1.

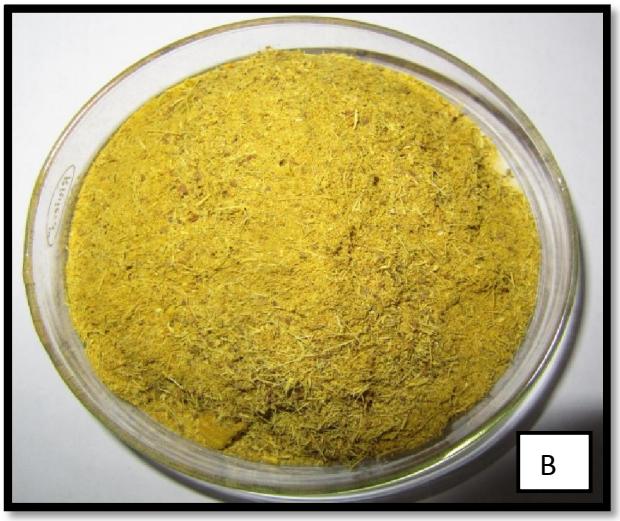
The percent recovery of different type of extracts of *Glycyrrhiza glabra* ranged between 10.24 to 19 percent and that of *Bauhinia variegata ranged* between 9.65 to 30 percent. The results showed that the aqua-methanolic extract of *Glycyrrhiza glabra* and methanolic extract of *Bauhinia variegata* has better extraction recovery of phytoconstituents.

Previously, extractive yield of stem bark of *Bauhinia variegata* was evaluated by Patil et al. (2012) in aqueous extract and was found to be 9 per cent which was comparable to present study in aqua-methanolic extract.

The per cent recovery of *Glycyrrhiza glabra* roots following methanolic extraction, studied by Mishra *et al*. (2011) was 16.07 per cent which was also almost identical to the present study. A slight variation in the recovery of extracts of the tested plant materials might be due to the different availability of extractable components in the plants and nature of solvents.


A


D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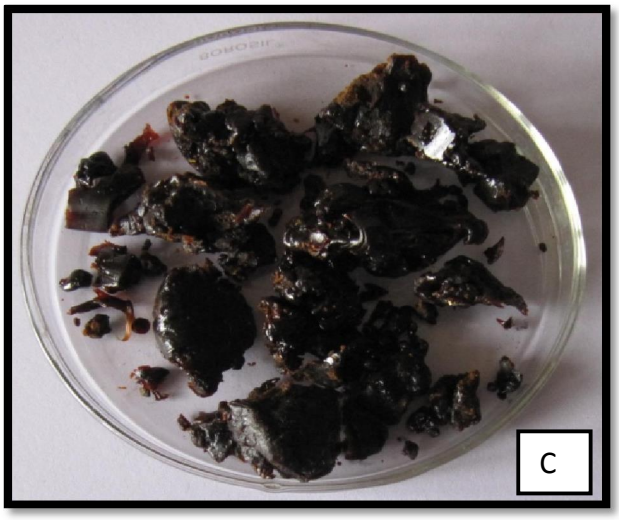
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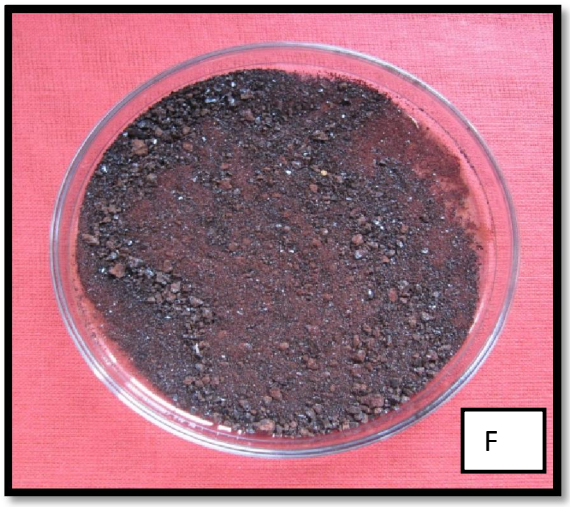
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4.2 STUDY OF ANTIOXIDANT ACTIVITY IN VITRO

4.2.1 MEASUREMENT OF TOTAL ANTIOXIDANT ACTIVITY

ABTS reacts with potassium persulphate to produce $ABTS^+$ *radical cations, a blue green chromogen with absorption maximum at 734 nm. The* extent of decolourization is an indicator of antioxidant activity of the sample. The antioxidants convert the $ABTS^+$ radical cation to a colourless ABTS (Bhatia *et al.* 2011).

The IC_{50} values of both plants (*Glycyrrhiza glabra and Bauhinia variegata)* were compared with trolox (standard). The IC_{50} value of trolox was found to be 0.173 mg ml^{-1}. The per cent inhibition at different concentrations and IC_{50} value of standard is shown in table 4.2 and fig 4.1.

The IC_{50} value of ABTS radical scavenging activity of methanolic, aqua-methanolic and aqueous extracts of *Glycyrrhiza glabra* was found to be 0.114 mg ml^{-1}, 0.193mg ml^{-1} and 0.709mg ml^{-1}, respectively (Table 4.3 and fig. 4.2).

Table 4.2 Total antioxidant activity of trolox by ABTS method

Concentration (mg/ml)	% Inhibition
0.015	6.48 ± 1.293[a]
0.03	10.93 ± 1.993[a]
0.06	26.89 ± 2.645[b]
0.12	81.97 ± 2.654[c]
0.25	89.76 ± 0.58[d]
0.5	100 ± 0.00[e]
IC_{50} value	**0.173 mg/ml**

Mean values ± standard error (n=3), the means with same superscripts in between rows do not differ significantly at 5% level

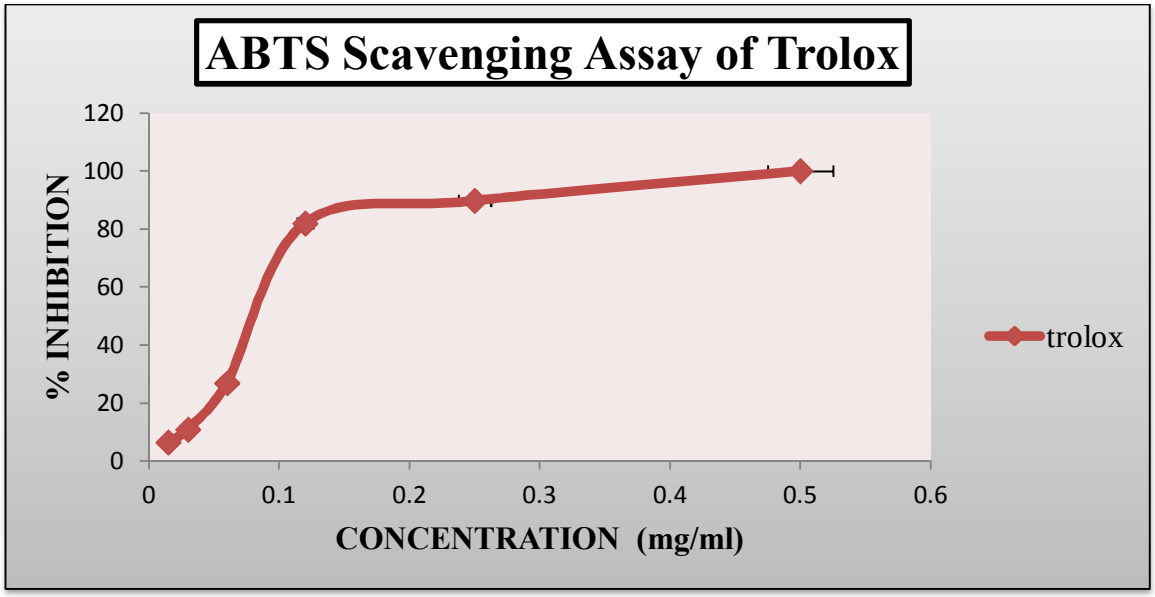


Fig. 4.1 Total antioxidant activity of trolox standard by ABTS method

Table 4.3 Total antioxidant activity of Glycyrrhiza glabra by ABTS method

Concentration (mg/ml)	% Inhibition		
	Methanolic	Aqua-methanolic	Aqueous
0.06	31.02 ± 0.381[a]	46.95 ± 1.405[a]	22.00 ± 0.425[a]
0.12	58.59 ± 0.552[b]	50.51 ± 0.706[a]	25.95 ± 1.428[b]
0.25	63.15 ± 0.643[c]	51.69 ± 0.075[b]	35.47 ± 0.281[c]
0.5	79.37 ± 0.372[d]	52.44 ± 0.254[c]	44.65 ± 0.370[d]
1	96.13 ± 0.731[e]	64.96 ± 1.613[d]	59.38 ± 0.322[e]
IC_{50} Value	0.114 mg/ml	0.193 mg/ml	0.709 mg/ml

Mean values ± standard error (n=3), the means with same superscripts in between rows do not differ significantly at 5% level

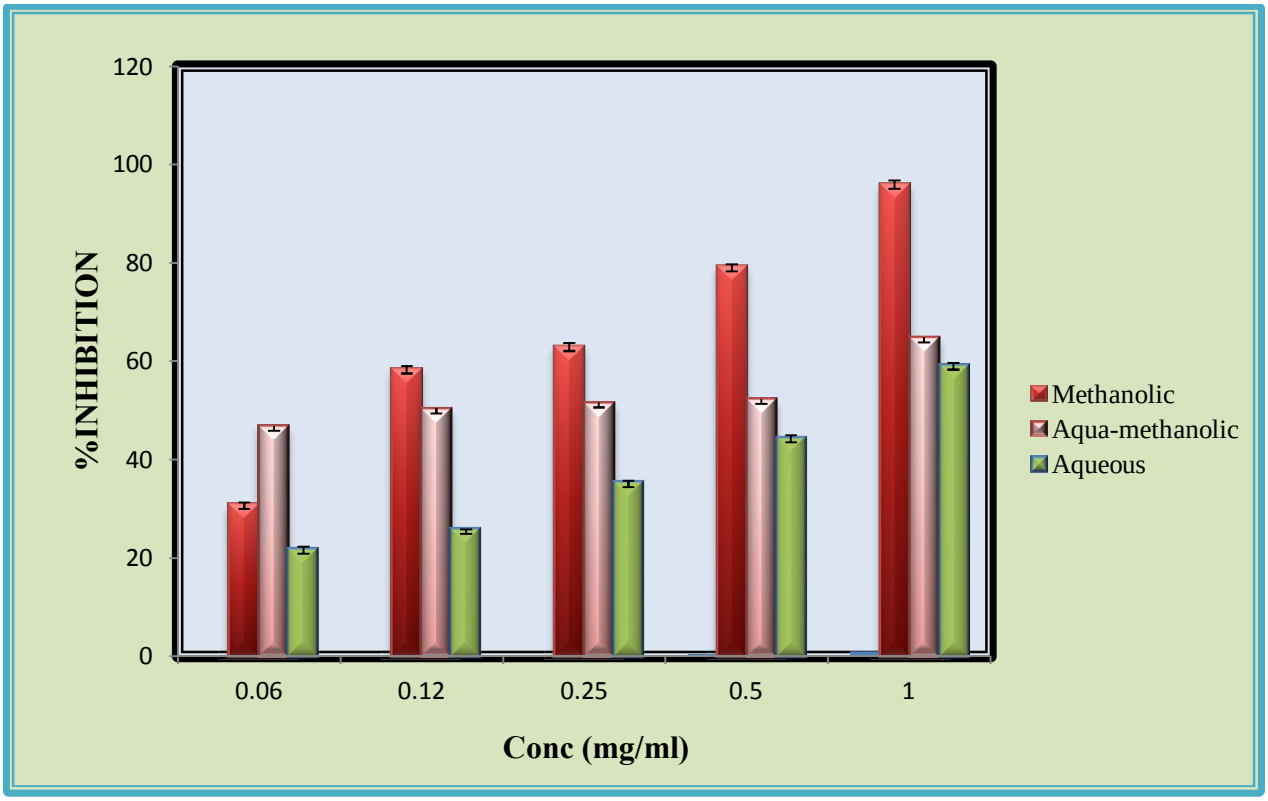


Fig. 4.2 Total antioxidant activity of Glycyrrhiza glabra by ABTS method

As shown in table 4.4 and fig. 4.3 the IC_{50} value of the methanolic, aqua-methanolic and aqueous extract of *Bauhinia variegata* was found to be 0.276 $mg\ ml^{-1}$, 0.302 $mg\ ml^{-1}$ and 0.757 $mg\ ml^{-1}$ respectively.

Tohma and Gulcin (2010) investigated antioxidant radical scavenging activity of lyophilized aqueous and ethanolic extract of roots of Turkish Liquorice (*Glycyrrhiza glabra*) by employing ABTS scavenging activity assay. A significant decrease ($P<0.01$) in the concentration of ABTS due to scavenging capacity of extracts at all concentrations was observed. Also, both the extracts were found effective ABTS radical scavengers in a concentration dependent manner.

Nand *et al.* (2012) determined the antioxidant activity of *Glycyrrhiza glabra* by ABTS assay. Results showed that the methanolic extract of *Glycyrrhiza glabra* exhibit potent antioxidant property with IC_{50} value of 21.37±1.422 µg ml^{-1}. The antioxidant activities in present study with respect to *Glycyrrhiza glabra* were, however comparitively lower.

Table 4.4 Total antioxidant activity of Bauhinia variegata by ABTS method

Mean values ± standard error (n=3), the means with same superscripts in between rows do not differ significantly at 5% level

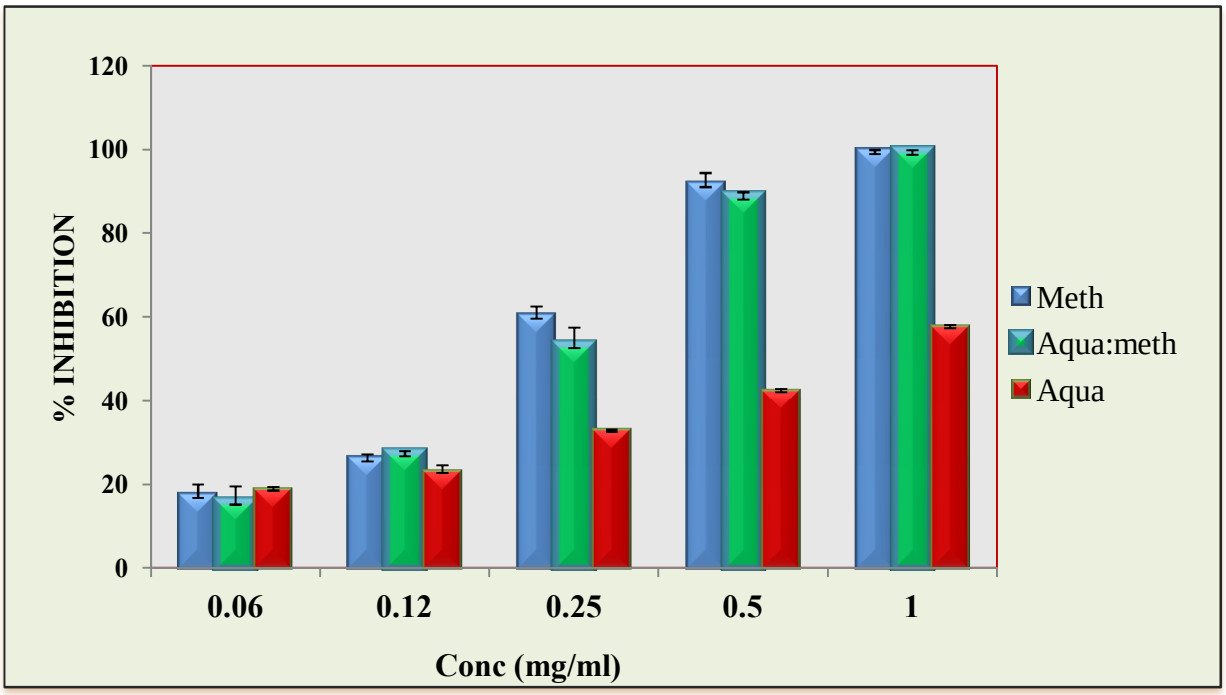


Fig. 4.3 Total antioxidant activity of Bauhinia variegata by ABTS method

Similarly, IC_{50} value of methanolic extract of stem bark of Bauhinia variegata evaluated by Pandey et al. (2012) was 19.5 μg^{-1}ml indicating a stronger antioxidant activity.

4.2.2 DPPH RADICAL SCAVENGING ASSAY

The DPPH is relatively stable nitrogen centered free radical that accepts electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with reducing agents as a result electrons become paired, forming the corresponding hydrazine (Sharma et al. 2011).

The IC_{50} values of different extracts of both plants were compared with trolox as standard and IC_{50} value of butylated hydroxyl toluene (BHT) was found to be 0.014 $mg\ ml^{-1}$. The per cent inhibition at different concentrations and IC_{50} value of standard is shown in table 4.5 and fig 4.4.

Plate 4.2 Clinical signs on skin (A- skin infection and B- alopecia) in rats after treatment with dexamethasone as immunosuppressive

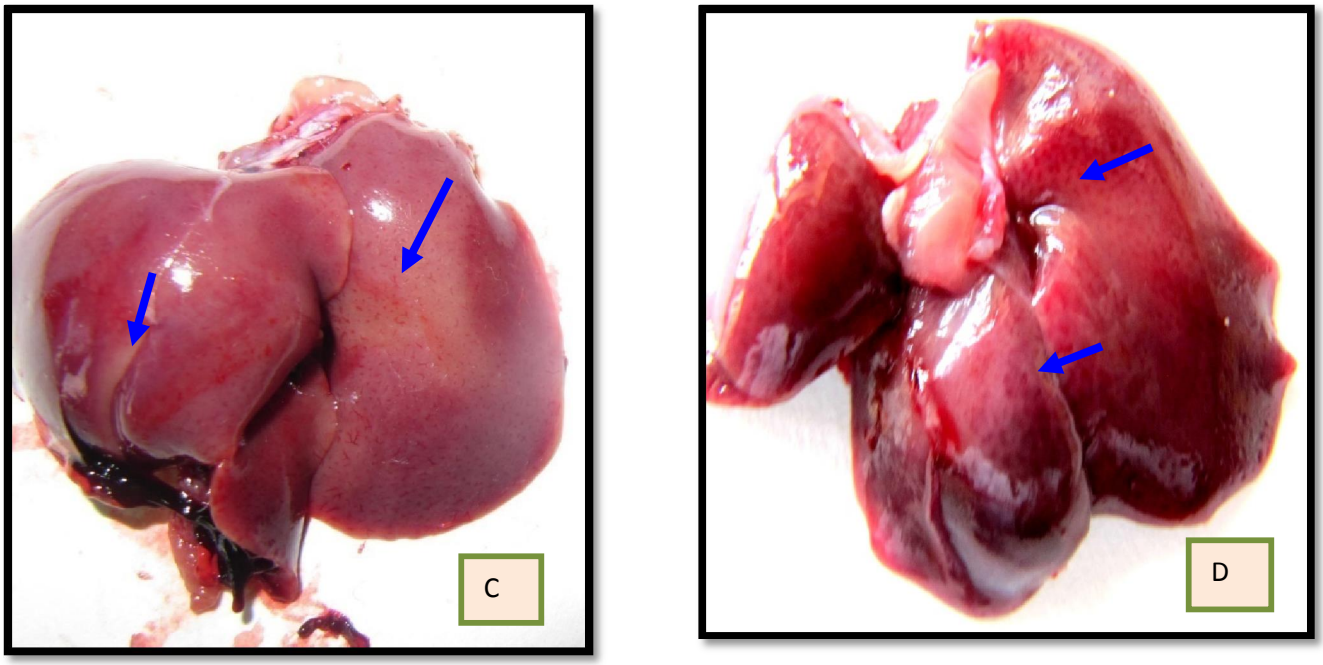


Plate 4.3 Gross lesions in liver of rats (C- yellowish discolouration, D – haemorrhages) after treatment with potassium dichromate as oxidative agent

Table 4.5 Free radical scavenging activity of BHT standard

Concentration (mg/ml)	% Inhibition
0.03	23.16 ± 0.083[a]
0.06	49.49 ± 1.294[b]
0.25	94.47 ± 0.047[c]
0.5	95.17 ± 0.107[d]
1	96.28± 0.158[e]
IC_{50} value	0.014 mg/ml

Mean values ± standard error (n=3), the means with same superscripts in between rows do not differ significantly at 5% level

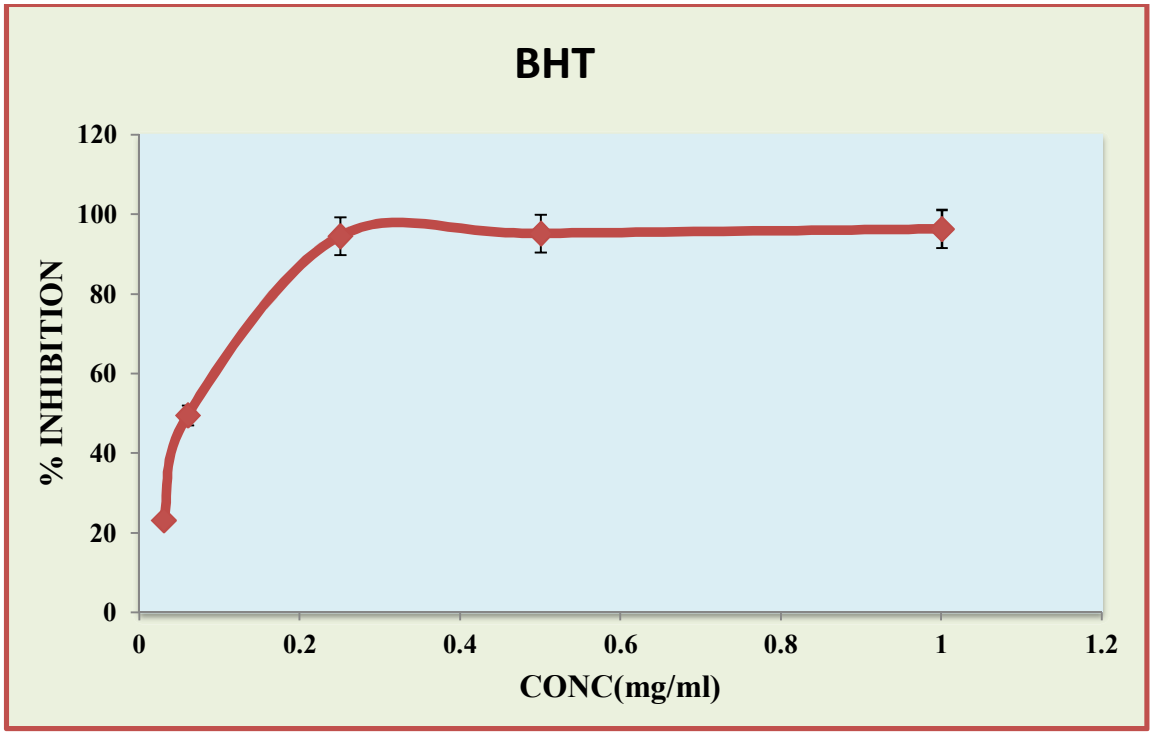


Fig. 4.4 Free radical scavenging activity of BHT by DPPH method

The IC_{50} value for free radical scavenging of methanolic, aqua-methanolic and aqueous extracts of *Glycyrrhiza glabra* was found to be 0.159 mg ml^{-1}, 0.571 mg ml^{-1} and 0.552 mg ml^{-1}, respectively (Table 4.5).

Chopra et al. (2013) studied the in vitro free radical scavenging activity of methanolic root extract of *Glycyrrhiza glabra* by DPPH method and found that the extract had good antioxidant activity at 500 μg^{-1}ml with per cent inhibition of 67.22 per cent and IC_{50} value was 359.45 $\mu g\ ml^{-1}$. In the present study the per cent inhibition at same concentration i.e.500 $\mu g\ ml^{-1}$ was higher and IC_{50} value was lower. In the present study a better DPPH quenching activity was followed by methanolic root extract.

Table 4.6 Free radical scavenging activity of *Glycyrrhiza glabra*

Concentration (mg/ml)	**% Inhibition**		
	Methanolic	**Aqua-methanolic**	**Aqueous**
0.06	24.75 ± 1.087[a]	4.85 ± 1.346[a]	6.32 ± 1.020[a]
0.12	50.29 ± 50.29[a]	12.74 ± 3.928[a]	11.91 ± 1.777[a]
0.25	70.71 ± 70.71[b]	33.16 ± 0.435[b]	40.28 ± 4.799[b]
0.5	75.47 ± 0.4716[c]	58.20 ± 2.330[c]	63.95 ± 1.282[c]
1	78.49 ± 1.898[d]	73.90 ± 0.218[d]	70.81 ± 0.903[d]
IC_{50} value	**0.159 mg/ml**	**0.571 mg/ml**	**0.552 mg/ml**

Mean values ± standard error (n=3), the means with same superscripts in between rows do not differ significantly at 5% level.

Table 4.7 and fig. 4.6 represents the free radical scavenging activity of *Bauhinia variegata* by DPPH method. The IC_{50} value for free radical scavenging activity of methanolic, aqua-methanolic and aqueous extracts of *Bauhinia variegata* was found to be 0.258 mg ml^{-1}, 0.395 mg ml^{-1} and 0.410 mg ml^{-1}, respectively.

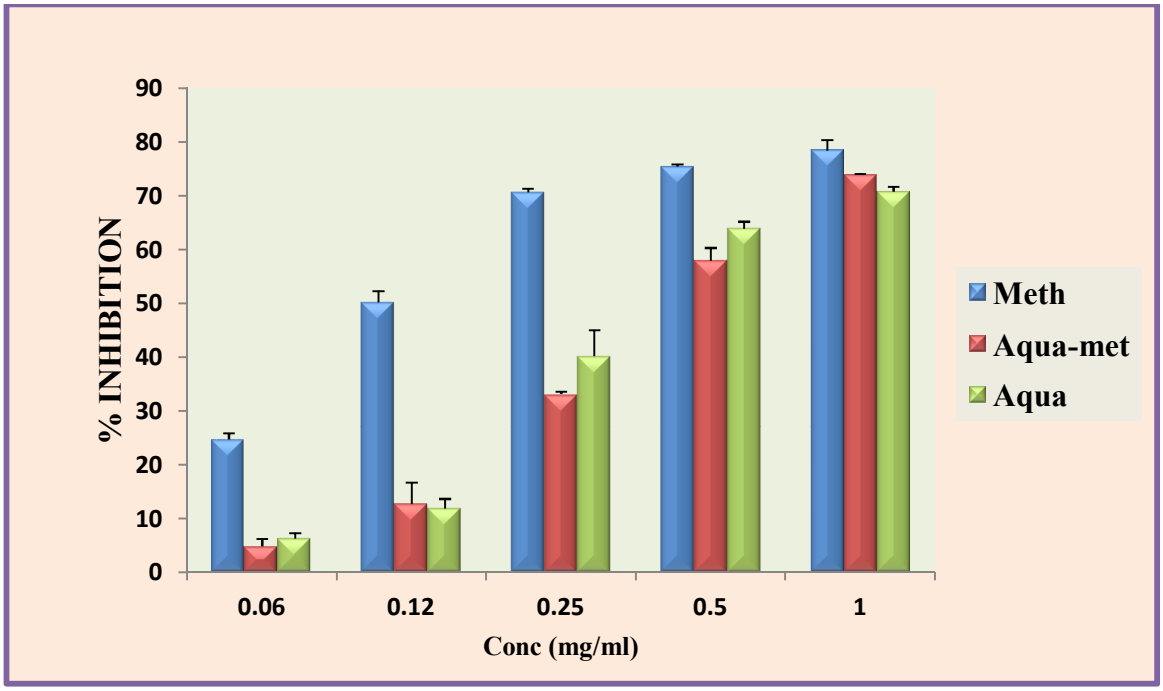


Fig. 4.5 Free radical scavenging activity of *Glycyrrhiza glabra* by DPPH method

Table 4.7 Free radical scavenging activity of *Bauhinia variegata*

Concentration (mg/ml)	% Inhibition		
	Methanolic	Aqua-methanolic	Aqueous
0.06	18.73 ± 0.570[a]	10.90 ± 2.065[a]	19.31 ± 0.720[a]
0.12	32.95 ± 0.620[b]	24.54 ± 1.844[b]	43.01 ± 1.327[b]
0.25	65.54 ± 1.082[c]	48.30 ± 1.147[c]	54.44 ± 1.065[c]
0.5	87.67 ± 2.068[d]	80.61 ± 0.165[d]	61.42 ± 0.483[d]
1	90.30 ± 0.347[e]	81.88 ± 0.663[e]	65.52 ± 0.275[e]
IC_{50} value	0.258 mg/ml	0.395 mg/ml	0.410 mg/ml

Mean values ± standard error (n=3), the means with same superscripts in between rows do not differ significantly at 5% level.

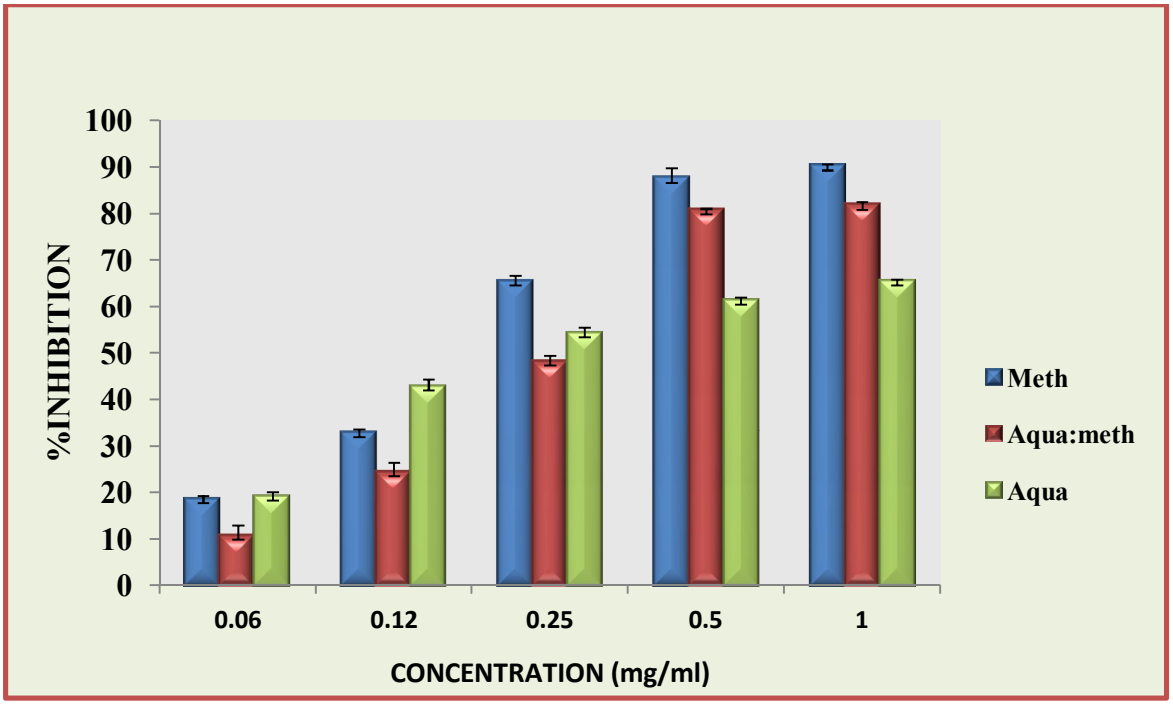


Fig. 4.6 Free radical scavenging activity of Bauhinia variegata by DPPH method

Kumar et al. (2005) evaluated the antioxidant activity of methanolic extract of Bauhinia racemosa L. stem bark by DPPH method and IC_{50} value was found to be 152.29 µg ml^{-1}, which is lower than the IC_{50} value of methanolic extract of Bauhinia variegata stem bark obtained in the present study. Rajani and Ashok (2009) studied the ethanolic and aqueous extracts of Bauhinia variegata Linn. for in vitro antioxidant activity by DPPH method. The percentage scavenging of various free radicals were compared with standard antioxidants such as ascorbic acid and butylated hydroxyl anisole (BHA). *Significant antioxidant activity was observed at concentration 10 µg ml^{-1} and IC_{50} values for alcoholic and aqueous extracts of stem of Bauhinia variegata were also evaluated (45.85 µg ml^{-1} and 30.50 µg ml^{-1}, respectively) which were quite less than IC_{50} values of alcoholic and aqueous extracts observed in present study.*

4.3 IN VIVO STUDIES

4.3.1 GROWTH PERFORMANCE

The effect of *Glycyrrhiza glabra* and *Bauhinia variegata* on body weight was studied at weekly intervals and the results are presented in Table 4.8.

Table 4.8 Effect of 4 weeks treatment of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts on body weight (g) in rats

S.NO.	GROUP	1ST Week	2ND Week	3rd Week	4th Week
1	CONTROL	181.00 ± 5.62[a]	231.50 ±14.2[a]	227.5±9.04[a]	258.83±10.90[a]
2	Cr	137.00 ± 8.12[a]	176.33 ± 9.00[a]	198.83±10.2[a]	180.0± 13.63[a]
3	Cr +G.G.	161.50 ± 9.62[a]	206.50 ± 8.00[a]	218.50 ±10.3[a]	243.50±11.5[a]
4	Cr+B.V.	137.50 ± 9.73[a]	178.83 ± 6.12[a]	183.33 ±16.28[a]	199.33±18.53[a]
5	Dex	188.16±10.35[a]	198.6 ±11.35[a]	217.5 ± 9.76[a]	227.50 ±0 .60[a]
6	Dex + G.G.	145.80± 7.36[a]	172.33 ±3.88[a]	186.1 ± 4.46[a]	206.16 ± 4.47[a]
7	Dex + B.V.	145.66± 9.07[a]	180.33 ± 9.17[a]	203.33± 6.00[a]	212.33±10.94[a]

Mean values ± standard error (n=6), the means with same superscripts in between column do not differ significantly at 5% level.

Cr- Potassium dichromate

B.V. - *Bauhinia variegata*

G.G.- *Glycyrrhiza glabra*

Dex- Dexamethasone

Tables 4.8 - 4.10 and figures 4.7 -4.9 represent the body weight, weight gain and FCR, respectively in rats. The treatment with *Glycyrrhiza glabra* and *Bauhinia variegata* extracts did not significantly ($P>0.05$) influence the weekly body weight and Feed Conversion Ratio (FCR) was observed in all the treated groups.

Table 4.9 Effect of 4 weeks treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* methanolic extracts on body weight gain (g) in rats

S.No.	Group	Initial Weight (g)	Final Weight (g)	Weight Gain (g)
1	CONTROL	181.00 ±5.62[a]	258.83±10.90[a]	77.83 ± 12.46[a]
2	Cr	137.00 ±8.12[a]	180 ± 13.63[a]	43.00 ± 6.19[a]
3	Cr + G.G	161.50 ±9.62[a]	243.50 ±11.51[a]	82.50 ± 14.94[a]
4	Cr + B.V.	137.50 ±9.73[a]	199.33±18.53[a]	61.83 ± 13.74[a]
5	Dex	188.16±10.35[a]	227.50 ± 0 .60[a]	39.17 ± 6.29[a]
6	Dex + G.G.	145.80 ± 7.36[a]	206.16 ± 4.47[a]	69.66 ± 13.78[a]
7	Dex+B.V.	145.66 ± 9.07[a]	212.33 ±10.94[a]	66.66 ± 5.00[a]

Mean values ± standard error (n=6) means with same superscripts in between column do not differ significantly at 5% level.

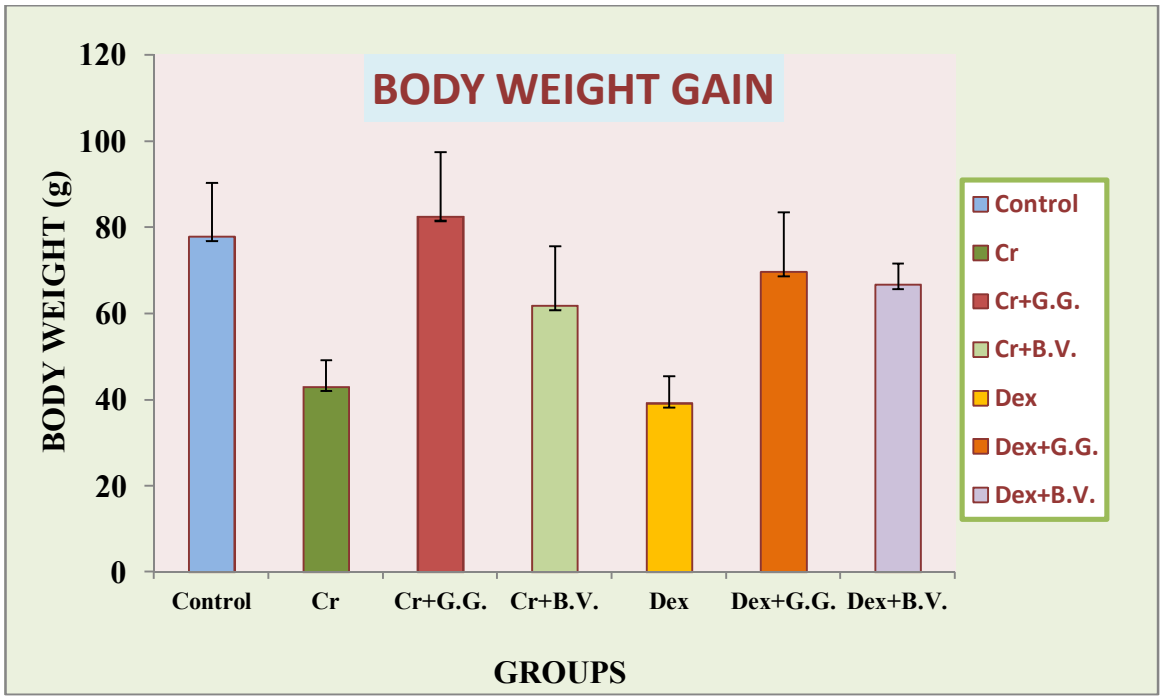


Fig 4.7 Effect of 4 weeks treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* methanolic extracts on body weight gain (g) in rats

Table 4.10 Effect of 4 weeks treatment of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts on feed conversion ratio in rats

S.No.	Group	FCR
1	CONTROL	22.70 ± 4.48[a]
2	Cr	38.62 ± 5.35[a]
3	Cr + B.V.	33.52 ± 9.44[a]
4	Cr + G.G	30.00 ± 14.03[a]
5	Dex	48.30 ± 13.62[a]
6	Dex+B.V.	23.08 ± 1.58[a]
7	Dex + G.G.	25.78 ± 4.62[a]

Mean values ± standard error (n=6), the means with same superscripts in between rows do not differ significantly at 5% level.

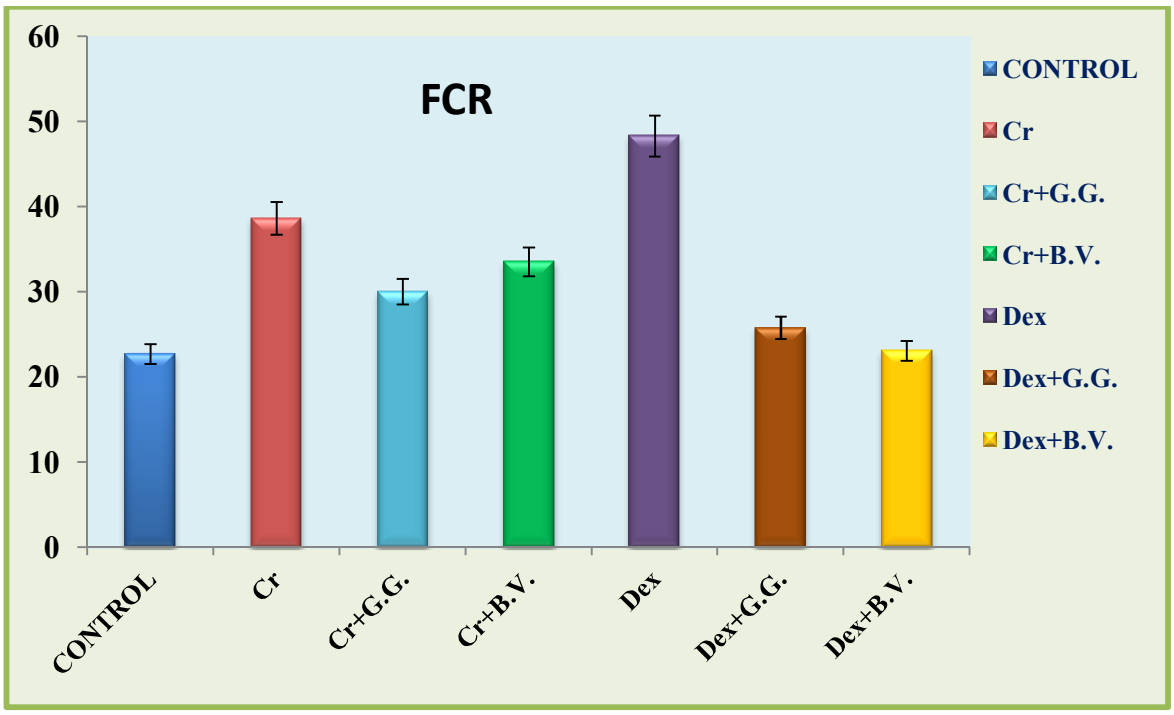


Fig. 4.8. Effect of 4 weeks treatment of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts on feed conversion ratio in rats

4.3.2 ORGAN BODY WEIGHT RATIO (g/100g)

The relative weight of liver and kidney in rats of all treatment groups were recorded on day 30 of the experiment and results are presented in table 4.11.

Table 4.11 The relative weight of liver and kidney in rats of all treatment groups were recorded on day 30 of the experiment

S.No.	Treatment group	Liver	Kidney
1	**Control**	4.36 ± 0.422^{a}	0.40 ± 0.098^{a}
2	**Cr**	3.99 ± 0.221^{a}	0.36 ± 0.045^{a}
4	**Cr+ G.G.**	3.86 ± 0.564^{a}	0.38 ± 0.05^{a}
5	**Cr +B.V.**	3.60 ± 1.122^{a}	0.40 ± 0.10^{a}
6	**Dex**	4.60 ± 0.427^{b}	0.42 ± 0.03^{bc}
7	**Dex + G.G.**	4.28 ± 0.39^{ad}	0.38 ± 0.03^{a}
8	**Dex + B.V.**	4.20 ± 0.527^{ac}	0.37 ± 0.04^{ad}

Mean values ± standard error (n=6), the means with same superscripts in between rows do not differ significantly at 5% level.

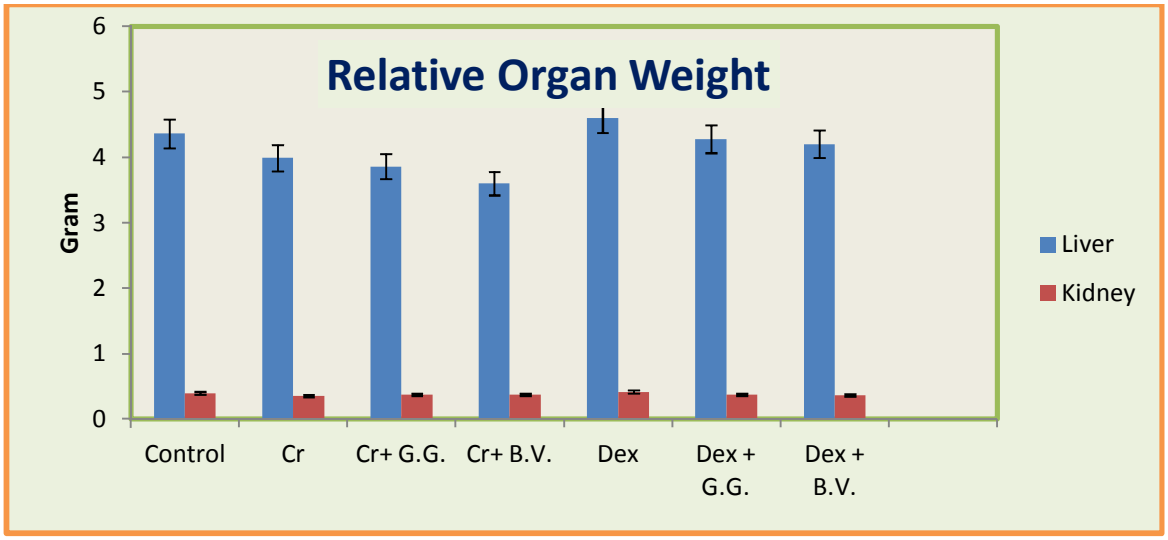


Fig. 4.9 The relative weight of liver and kidney in rats of all treatment groups were recorded on day 30 of the experiment

There was a non significant (P>0.05) change in relative weight of liver and kidney in rats treated with potassium dichromate (30mg kg^{-1}. b.w.) for 30 days. However, there was significant (P<0.05) increase in relative liver and kidney weight in dexamethasone treated rats. Dumas *et al*. (2003) studied the mitochondrial energy metabolism in dexamethasone induced under nutrition rat model and the findings revealed that five days treatment of dexamethasone (1-5mg kg^{-1}. b.w.) increased the liver weight to 23 per cent. Thus, these findings in consonance with the findings of Dumas *et al.* (2003).

4.3.3 *IN VIVO* ANTIOXIDANT ACTIVITY

To assess the *in vivo* antioxidant activity of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts, various parameters like lipid peroxidation, reduced glutathione and catalase were estimated in liver, kidney and erythrocytes of male Wistar rats.

i. LIPID PEROXIDATION (LPO)

The liver damage leads to cell necrosis and causes increase in lipid peroxidation and decrease in glutathione levels (Sowanya *et al*. 2013). The level of MDA gives indication of lipid peroxidation as a result of oxidative stress induced damage of cell membranes and also indicates the free radical scavenging property of plant extracts (Hari *et al*. 2012).

Table 4.12 Effect of 30 days treatment of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts on lipid peroxidation in liver, kidney (nM MDA g^{-1} of tissue) and in erythrocytes (nM MDA ml^{-1}) in rats

S. No.	Treatment group	Liver	Kidney	Erythrocytes
1	Control	8.82 ± 0.41^{a}	5.60 ± 1.20^{a}	0.91 ± 0.03^{a}
2	Cr	21.82 ± 1.80^{b}	11.35 ± 1.68^{b}	1.44 ± 0.04^{b}
3	Cr +G.G	10.84 ± 0.27^{ad}	6.45 ± 0.22^{ad}	0.92 ± 0.01^{ae}
4	Cr +B.V	14.49 ± 1.53^{cd}	6.87 ± 0.38^{ad}	1.04 ± 0.01^{cd}

Mean values ± standard error (n=6), the means with same superscripts in between rows do not differ significantly at 5% level.

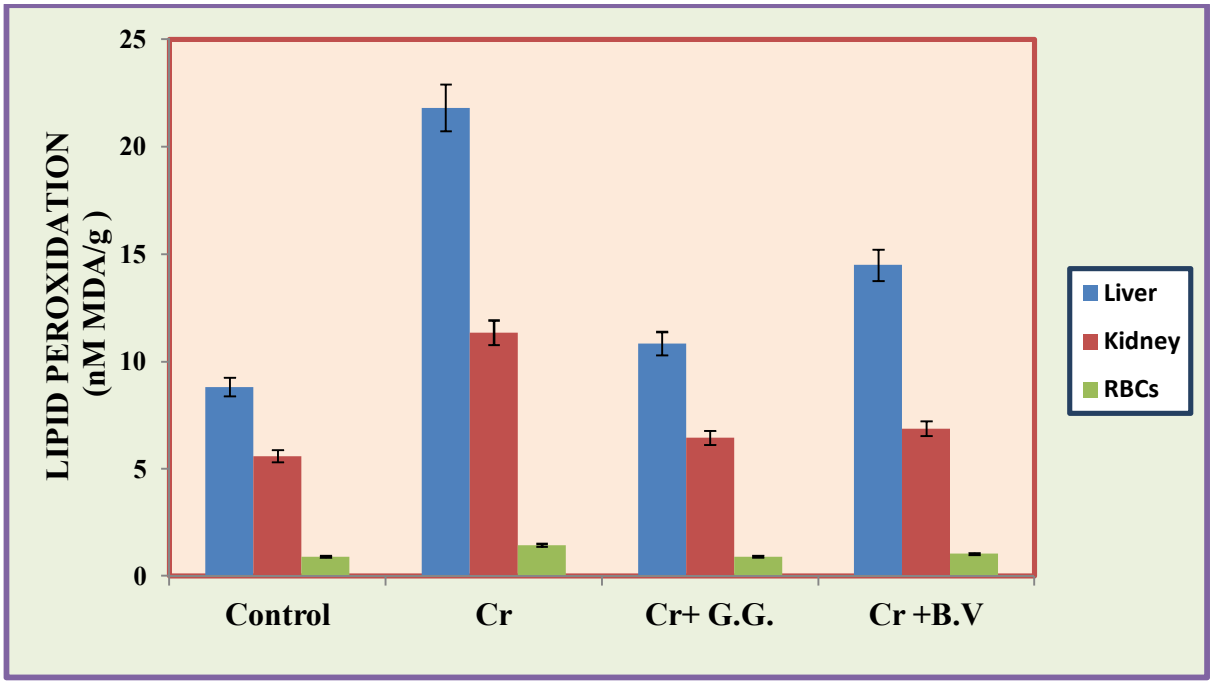


Fig. 4.10 Effect of 30 days treatment of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts on lipid peroxidation in liver, kidney (nM MDA g^{-1} of tissue) and in erythrocytes (nM MDA ml^{-1}) in rats

Liver, kidney and erythrocytes MDA levels significantly ($P<0.05$) increased in rats following 30 days treatment of chromium (30mg kg^{-1}b.w.) alone as compared to the control animals. The increase in MDA levels due to lipid peroxidation caused by chromium induced

oxidative stress in liver, kidney and erythrocytes was significantly ($P<0.05$) decreased by *Glycyrrhiza glabra* extract treatment when compared with chromium treated group.

Similarly, *Bauhinia variegata* significantly ($P<0.05$), decrease the MDA level in liver, kidney and erythrocytes of rats as compared to chromium alone treated rats.

Similar findings were reported by Muralidharan *et al.* (2009) in sodium nitrite induced hypoxic rats following treatment with the aqueous extract of *Glycyrrhiza glabra* roots extract (250 and 500 mg kg^{-1}b.w.).

Kanimozhi and Karthikeyan (2011) evaluated the effect of *Glycyrrhiza glabra* leaf extract in 1, 4 Dichlorobenzene (300mg kg^{-1}b.w.) induced stress in rats. The level of malondialdehyde (MDA), an end product of lipid peroxidation, markedly increased following treatment rats. However, after treatment with *Glycyrrhiza glabra* Linn. extract, MDA level returned to their original value. These findings were further substantiated by other workers with respect to their studies on *Glycyrrhiza glabra* in mice and rats (Visavadiya and Narasimcharya (2006), Yingkai *et al.* 2009 and Chowdhary *et al.* 2013).

Similarly, a decrease in lipid peroxidation (in terms of MDA levels) by ethanolic extract of *Bauhinia variegata* in different tissues of rats has been observed by previous workers (Hari *et al*. 2012 and Marasani *et al*. 2013).

ii. Reduced Glutathione

Reduced glutathione level was significantly reduced in liver and kidney of chromium treated rats as compared to control rats.

There was a significant ($P<0.05$) increase in reduced glutathione level in liver and kidney of *Glycyrrhiza glabra* and *Bauhinia variegata* treated rats as compared to chromium treated group.

Table 4.13 Effect of 30 days treatment of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts on reduced glutathione (mMg^{-1}) in tissue in rats

S.No.	Treatment group	Liver	Kidney
1	Control	0.83 ± 0.07^{a}	0.67 ± 0.04^{a}
2	Cr	0.32 ± 0.05^{b}	0.09 ± 0.07^{b}
3	Cr + G.G.	0.71 ± 0.22^{ac}	0.39 ± 0.14^{de}
4	Cr + B.V.	0.68 ± 0.03^{ac}	0.25 ± 0.03^{ce}

Mean values ± standard error (n=6), the means with same superscripts in between rows do not differ significantly at 5% level

Pandey and Agarwal (2009) was also observed enhanced reduced glutathione level due to *Bauhinia variegata* treatment in DMBA- induced stressed rats similar to present findings.

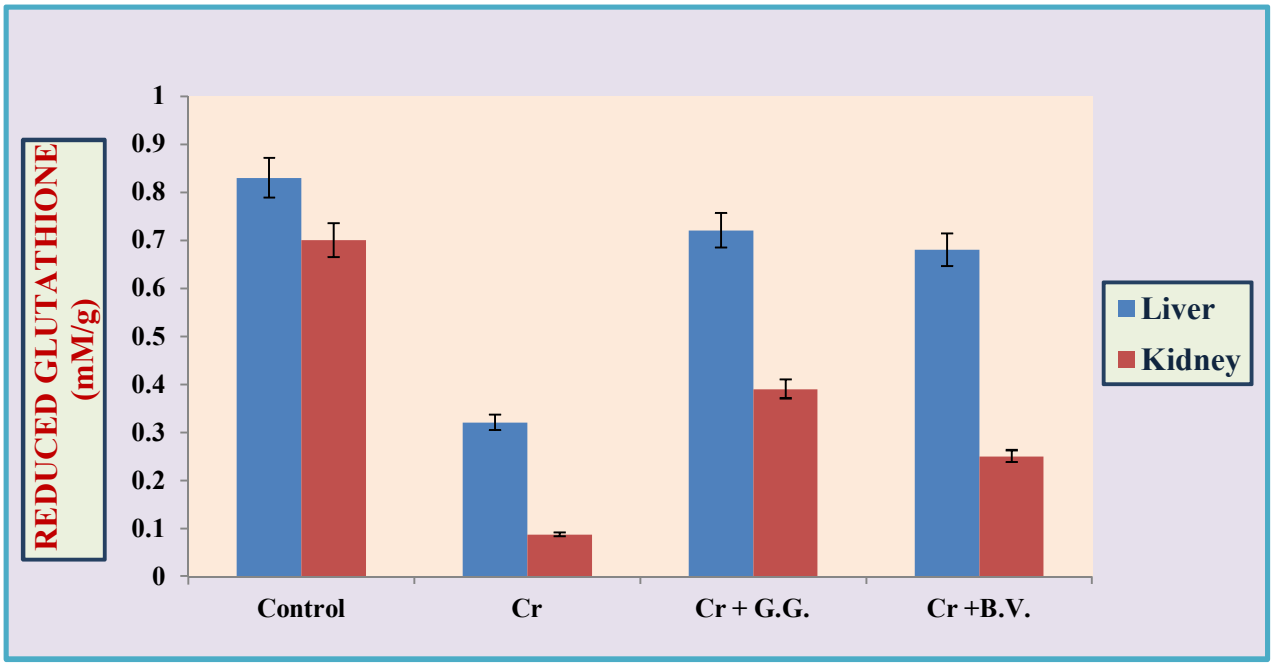


Fig 4.11 Effect of 30 days treatment of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts on reduced glutathione (mMg^{-1}) in tissue in rats

Similarly Marasani *et al.* (2013) evaluated the anti-stress activity of alcoholic extract of *Bauhinia variegata* bark (200-400 mg kg^{-1} p.o.) in rats for 7 days. Oxidative stress was induced

with iron overload (ferrous sulphate, 30 mg kg^{-1} i.p.) and reported that the alcoholic extract treated animals showed increased reduced glutathione enzyme level and decreased lipid peroxidation in brain homogenate.

Balaraman *et al.* (2004) studied the antioxidant activity of DHC-1(a herbal formulation (100-200 mg kg^{-1} p.o, once daily) having *Glycyrrhiza glabra* as one of the component for 30 days in rats and reported that the inhibition of lipid peroxidation and enhancement of antioxidant enzymes (SOD and CAT) activity along with reduced glutathione by *Glycyrrhiza glabra* in liver of rats.

iii. Catalase

The increase of catalase activity in *Bauhinia variegata* and *Glycyrrhiza glabra* treated rats not significantly different (P>0.05) as compared to chromium treated rats.

Table 4.14 Effect of 30 days treatment of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts on catalase activity (µmolH_2O_2utilized $min^{-1}mg^{-1}$ of protein) in liver tissue of rats

Organ	Control	Cr	Cr +G.G.	Cr +B.V.
Liver	53.60 ± 8.06^{a}	31.65 ± 2.18^{b}	39.00 ± 0.88^{a}	40.45 ± 4.03^{a}

Mean values ± standard error (n=6) means with same superscripts in between columns do not differ significantly at 5% level

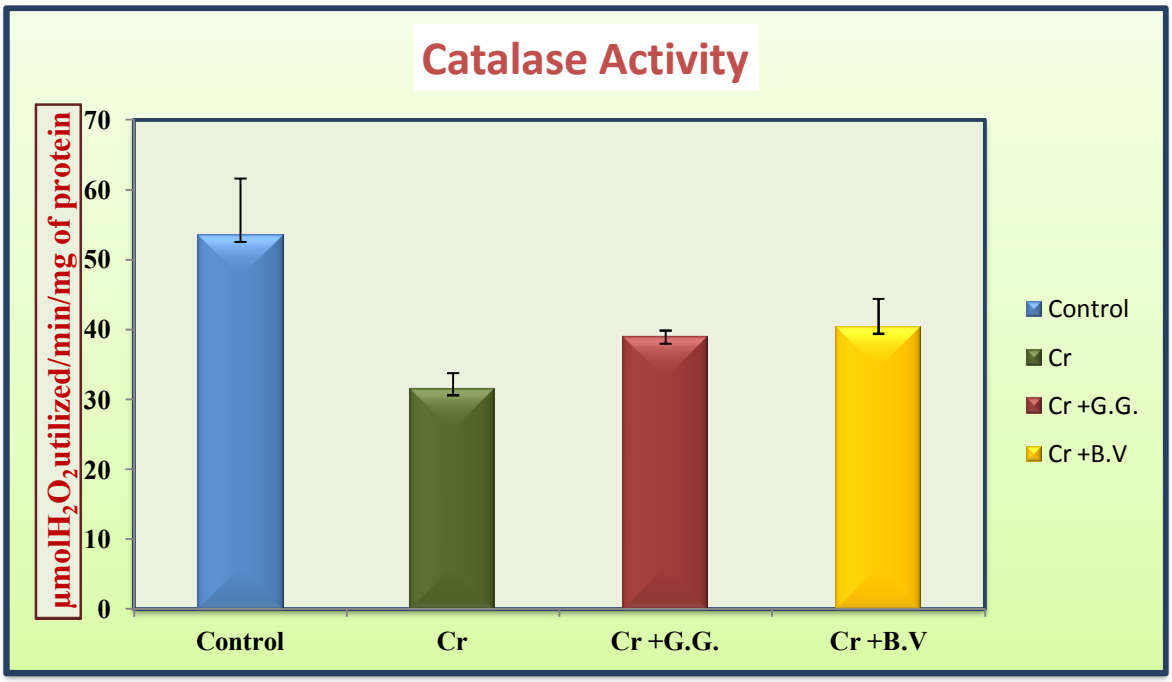


Fig 4.12 Effect of 30 days treatment of *Glycyrrhiza glabra* and *Bauhinia variegata* methanolic extracts on catalase activity (µmolH_2O_2utilized $min^{-1}mg^{-1}$ of protein) in liver tissue of rats

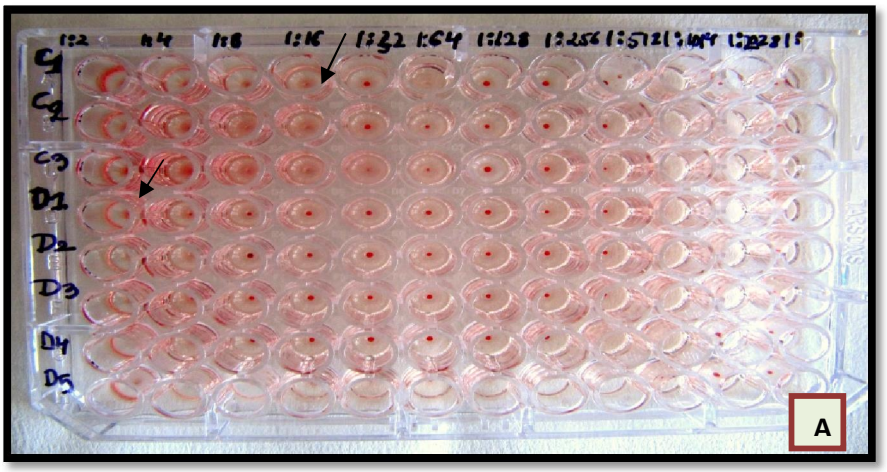
Thus, the findings of the present study are in consonance with the findings of Visavadiya and Narasimcharya (2006) and Sharma and Agarwal (2014) with respect to catalase activity following treatment mice and rats with *Glycyrrhiza glabra* extract**.**

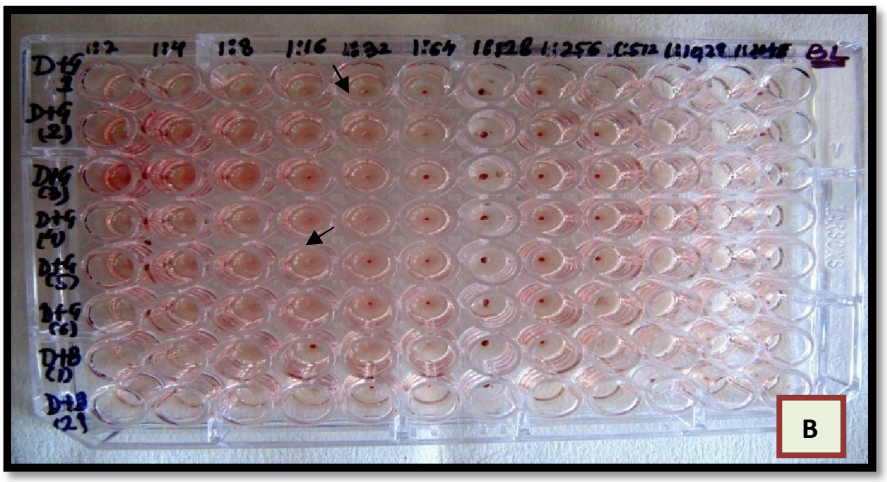
4.3.4 IMMUNOMODULATORY ACTIVITY

i. Humoral immune response

a. Haemaglutination Test

There was a significant ($P<0.05$) decrease in $\log_2$ value of haemagglutination titre following dexamethasone (5 mg kg^{-1} b.w.) treatment in mice. However, treatment with *Bauhinia variegata* and *Glycyrrhiza glabra* significantly increased the titres in rats immunocompromised with dexamethasone (Table 4.15, fig. 4.13 and plate 4.4).





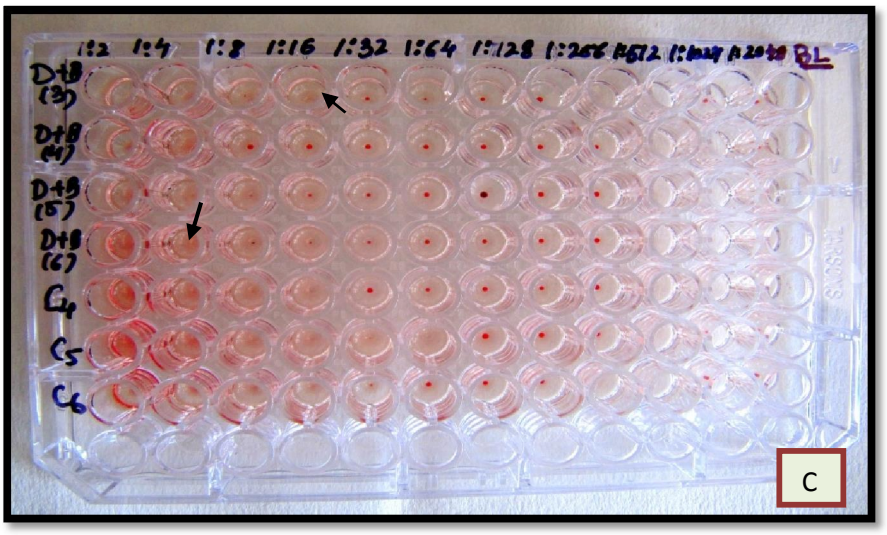


Plate 4.4 Microtiter plates (A- Control and Dex groups, B- G.G +Dex, C-B.V. +Dex) showed the haemagglutination titre against Sheep RBCs in different groups of rat

Table 4.15 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* methanolic extracts on haemagglutination titre (log_2 values) against sheep RBCs in rats

Group	Control	Dexa	Dexa +G.G.	Dexa +B.V.
Titre (log_2 values)	4.70 ± 0.34^{a}	1.51 ± 0.22^{b}	3.90 ± 0.31^{ad}	3.18 ± 0.31^{cd}

Mean values ± standard error (n=6) means with same superscripts in between columns do not differ significantly at 5% level

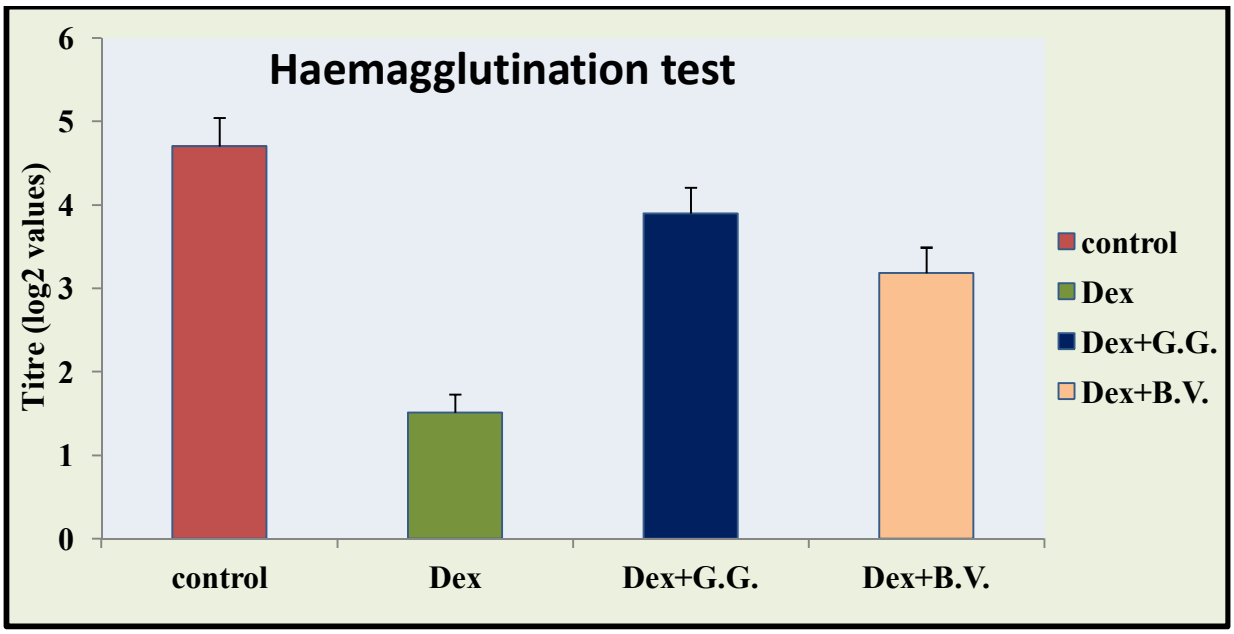


Fig. 4.13 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* methanolic extracts on haemagglutination titre (log_2 values) against sheep RBCs in rats

There was augmentation of the humoral immune response against sheep RBCs, as evidenced by increase in haemagglutination titre in *Glycyrrhiza glabra* and *Bauhinia variegata* treated rats due to increased response of T and B lymphocytes involved in the antibody synthesis. Thus, the findings of present studies indicated significant immunomodulation.

The findings with respect immunomodulation in the present studies are consistent with the findings of Sharma and Ray (1997) and Yingkai *et al.* (2009) with respect of *Glycyrrhiza glabra* and Ghaisas *et al.* (2009) with respect to *Bauhinia variegata* in mice.

ii. CELL MEDIATED IMMUNE RESPONSE

Cell mediated immune response was evaluated by injecting SRBCs in rats for delayed type hypersensitivity reaction on day 30 of the experiment. The increase in thickness of left footpad at 12, 24, 48 and 72 hours post challenge in different groups was measured using Vernier Calliper. The results are presented in table 4.16 and fig.4.14.

a. GROSS LESIONS

The gross lesions were characterized by redness, hot and painful focal swelling on foot pad at 12 and 24 hour of post challenge with SRBCs after 24 hours, swelling subsided.

b. DELAYED TYPE HYPERSENSITIVITY TEST

The delayed type of hypersensitivity test was characterized by the non- specific large influx of inflammatory cells at the sensitized site. The macrophages are the major prominent inflammatory cells in DTH. It is a type IV hypersensitivity reaction (Quadry 2004).

There was a non- significant ($P>0.05$) change in foot pad thickness at 12 hour of post challenge in all the groups. However, there was a significant ($P<0.05$) increase in foot pad thickness of rats at 24 and 48 hour of post challenge in *Glycyrrhiza glabra* treated group as compared to the control and dexamethasone treated groups. From 72 hours onwards in challenged rats the delayed response started subsided as shown in table 4.17 and fig. 4.1.

However, in case of *Bauhinia variegata* treated group the foot pad thickness was significantly ($P<0.05$) increased only at 48 hour of post challenge as compared to the control and dexamethasone treated groups. Intrestingly, *Glycyrrhiza glabra* group also revealed a significant increase from pretreatment level (0 hr) at 24 hours.

Table 4.16. Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* methanolic extracts on mean difference in foot pad thickness (mm) in delayed type of hypersensitivity reaction in rats

Hours Post Challenge	Control	Dexa	Dexa + G.G.	Dexa + B.V.
0	$0.30 \pm 0.05^{a\#}$	$0.57 \pm 0.17^{a\#}$	$0.68 \pm 0.25^{a\#}$	$0.60 \pm 0.0.07^{a\#}$
12	$0.33 \pm 0.07^{a\#}$	$0.68 \pm 0.13^{a\#}$	$0.73 \pm 0.12^{a\#}$	$0.72 \pm 0.22^{a\#}$
24	$0.60 \pm 0.0.15^{a\#}$	$0.63 \pm 0.14^{a\#}$	$1.97 \pm 0.21^{bc*}$	$0.95 \pm 0.16^{a\#}$
48	$0.40 \pm 0.097^{a\#}$	$0.62 \pm 0.22^{a\#}$	$1.07 \pm 0.16^{c\#}$	$1.10 \pm 0.19^{b\#}$
72	$0.35 \pm 0.11^{a\#}$	$0.67 \pm 0.06^{a\#}$	$0.93 \pm 0.12^{b\#}$	$0.72 \pm 0.19^{a\#}$

Mean values ± standard error (n=6), the means with same superscripts with in rows (alphabets) and between rows (#,*) do not differ significantly at 5% level.

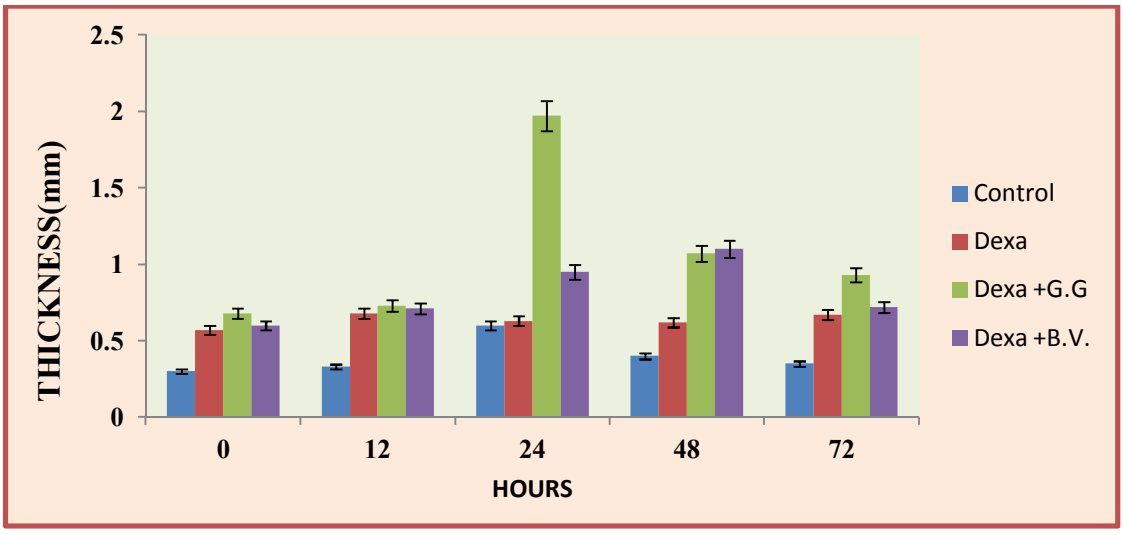


Fig. 4.14 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* methanolic extracts on foot pad thickness (mm) in Sheep RBCs induced delayed type hypersensitivity reaction in rats

A similar enhancement of cellular immune response characterized by enhancement of foot pad thickness following SRBC challenge has been observed by earlier workers following the treatment *Bauhinia variegata* extract (Shaikh *et al.* 2011) and *Glycyrrhiza glabra* (Manjuladevi *et al.* 2013). Thus, it is clearly evident from the present study of earlier studies that both the plants have triggering effect on cell mediated immunity.

iii. BIOCHEMICAL PARAMETERS

Table 4.17 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on total proteins, total albumin, and total globulin and albumin globulin ratio

Parameter	Control	Dexa	Dexa +G.G	Dexa +B.V.
Total Protein	6.83 ± 0.83^{a}	6.37 ± 1.45^{a}	6.40 ± 0.07^{a}	6.64 ± 0.62^{a}
Total Albumin	2.67 ± 0.79^{a}	2.04 ± 0.58^{a}	3.00 ± 0.67^{a}	3.89 ± 1.17^{ab}
Total Globulin	4.17 ± 0.77^{a}	4.32 ± 1.16^{a}	3.40 ± 0.65^{a}	2.74 ± 0.62^{a}
Albumin: Globulin	0.69 ± 0.33^{a}	0.49 ± 0.16^{a}	1.00 ± 0.58^{a}	2.02 ± 1.44^{bc}

Mean values ± standard error (n=6), the means with same superscripts in between columns do not differ significantly at 5% level.

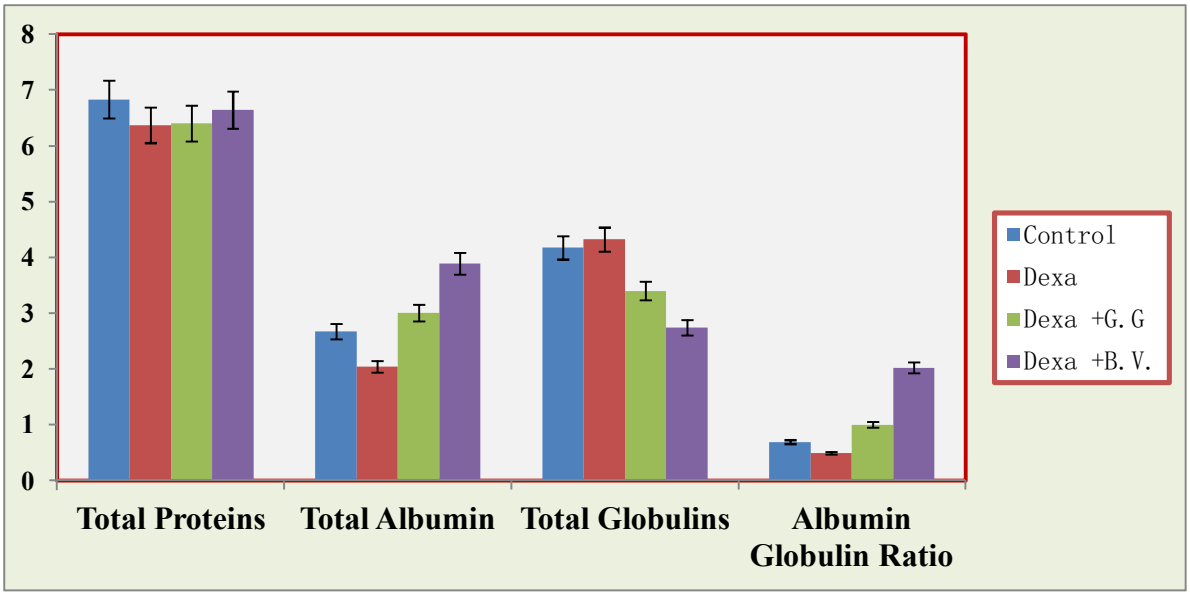


Fig. 4.15 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on total protein, albumin, globulin and albumin globulin ratio

The total protein and globulin level was not significantly (P>0.05) different in all the treated groups. However, there was a significant (P<0.05) increase in albumin globulin ratio in *Bauhinia variegata* treatment.

Short term effects of dexamethasone at different dosages (0.1-10.0 mg $kg.^{-1}$b.w.) were determined by Ulutas *et al.* (2007) on some acute phase proteins in Wistar rats. Hepatoglobin and ceruloplasmin concentrations tended to increase whereas; albumin concentration tended to decrease in dexamethasone treated rats. The results suggested that the dexamethasone treatment at different doses directly affects the synthesis of positive acute phase proteins.

In variance to observations of Sulaiman *et al.* (2010) and Marasani *et al*. (2013) in the present studies the non significant changes were observed in the protein levels following the dexamethasone treatment and also treatment with the twin extracts. The slight changes in protein levels, however, could be explained on the basis of dexamethasone induced changes in the alteration of acute phase proteins.

iv. HAEMATOLOGICAL PARAMETERS

Table 4.17 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on haematological parameters

Parameters		Control	Dexa	Dexa + G.G	Dexa + B.V.
Total Leucocytes Count (10^9/L)		6.67± 0.82^{a}	5.75± 0.46^{a}	5.05 ± 0.33^{a}	5.75 ± 0.456^{a}
Lymphocyte Count (10^9/L)		5.03± 0.64^{a}	0.87±0.09^{b}	2.43± 0.25^{cd}	1.98± 0.29^{bd}
Total Erythrocyte Count (10^{12}/L)		5.67±0.63^{a}	5.32± 0.24^{a}	5.81 ± 0.45^{a}	5.04 ± 0.34^{a}
Hb (g/dL)		10.27± 1.40^{a}	9.55± 0.64^{a}	10.85 ± 0.83^{a}	9.15 ± 0.67^{a}
PCV (%)		30.77±3.95^{a}	26.7± 1.72^{a}	29.96 ± 2.52^{a}	25.63± 1.77^{a}
DLC (%)	**Lymphocytes**	68.53±1.36^{a}	14.50±1.02^{b}	64.78±1.67^{ad}	53.86±5.85^{cd}
	Monocytes	2.51± 0.14^{a}	5.37± 0.43^{b}	3.18± 0.24^{ac}	3.32± 0.34^{ac}
	Granulocytes	31.02± 1.69^{a}	78.81±1.33^{b}	32.41±1.75^{ac}	40.57± 4.74^{ac}

Mean values ± standard error (n=6), the means with same superscripts in between columns not differ significantly at 5% level

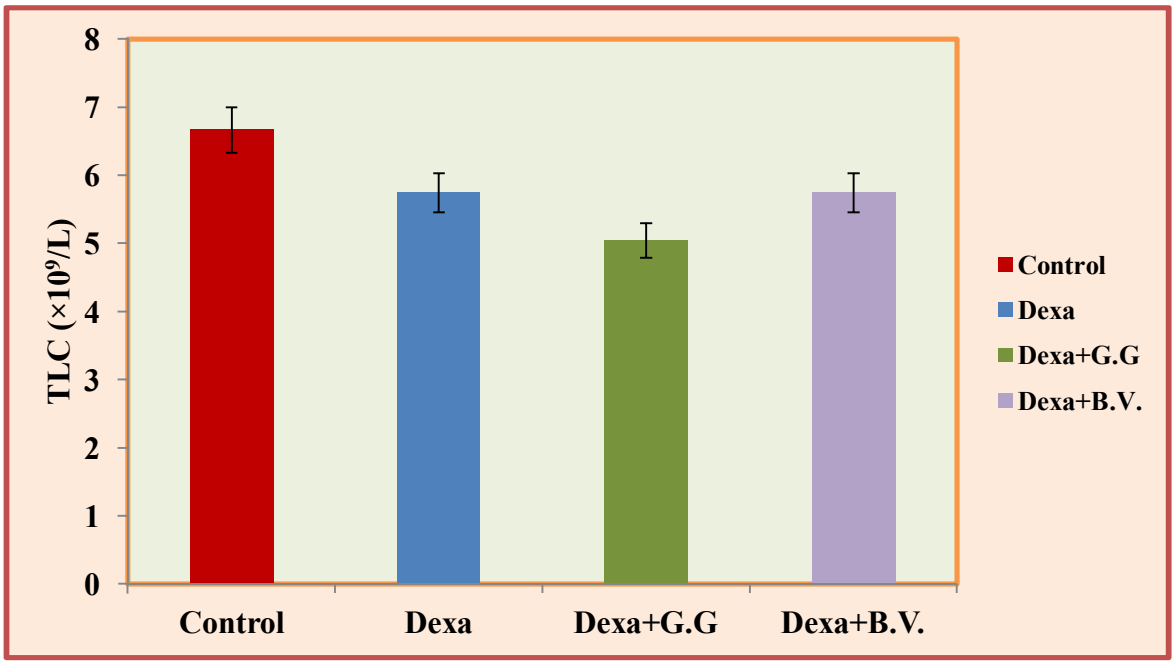


Fig 4.16 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on total leucocyte count

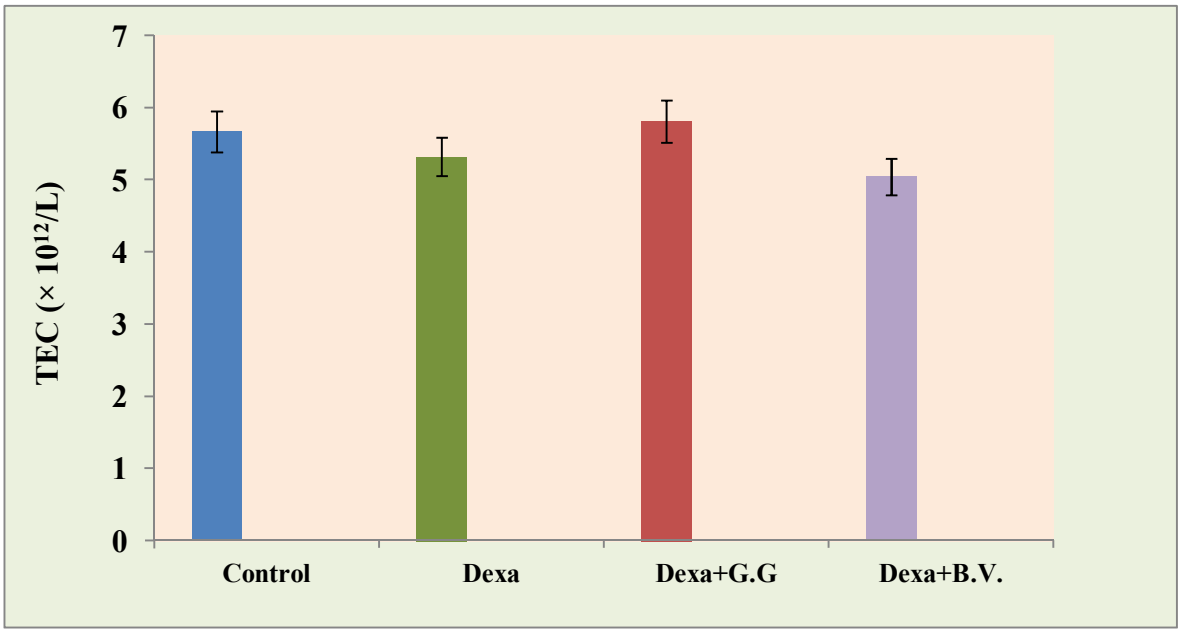


Fig 4.17 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on Total Erythrocyte Count

There was significant (P<0.05) decrease in lymphocyte count in all dexamethasone treated rats (i.e. ii, iii and iv) compared to control. A significant (P<0.05) increase in lymphocyte count was observed in *Glycyrrhiza glabra* treated rats compared to control however, *Bauhinia variegata* treatment did not significantly alter the lymphocyte count. Intrestingly, lymphocyte count was significantly increase by *Glycyrrhiza glabra* extract in immunocompromised animals. None the less both the extracts increased lymphocyte counts when studied in terms relative count (Table 4.17 and fig. 4.18)

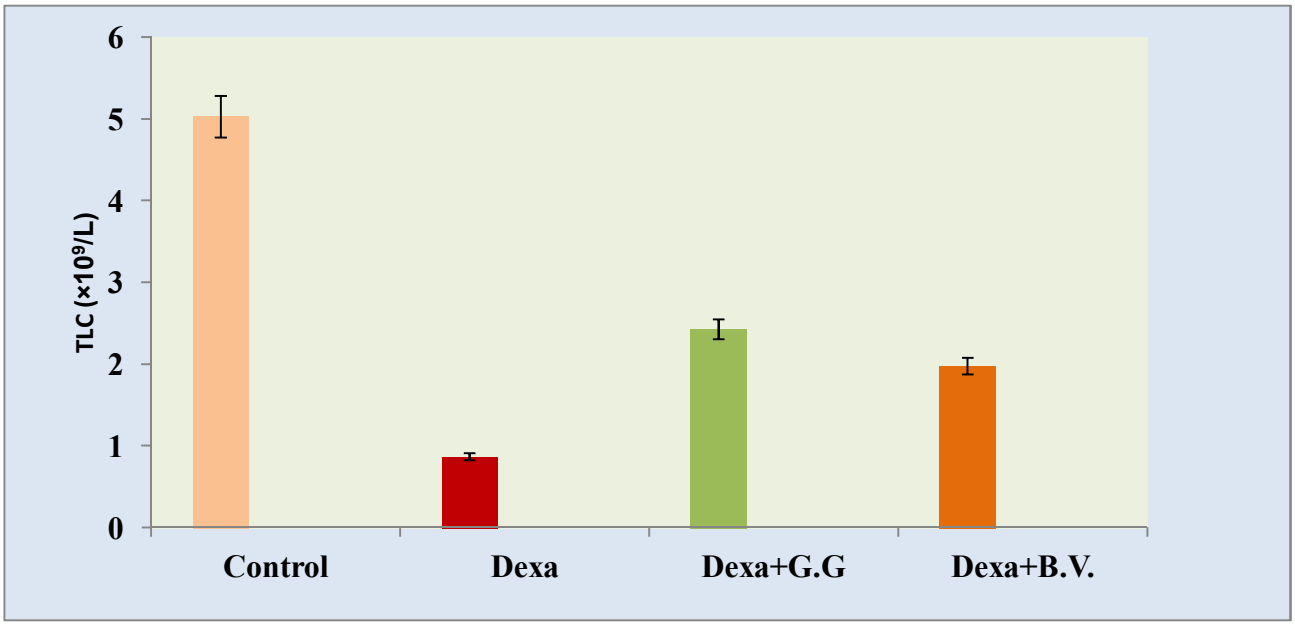


Fig 4.18 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on Total Lymphcyte Count

There was non significant (P>0.05) change in haemoglobin (Hb) of all treated groups as compared to the control group.

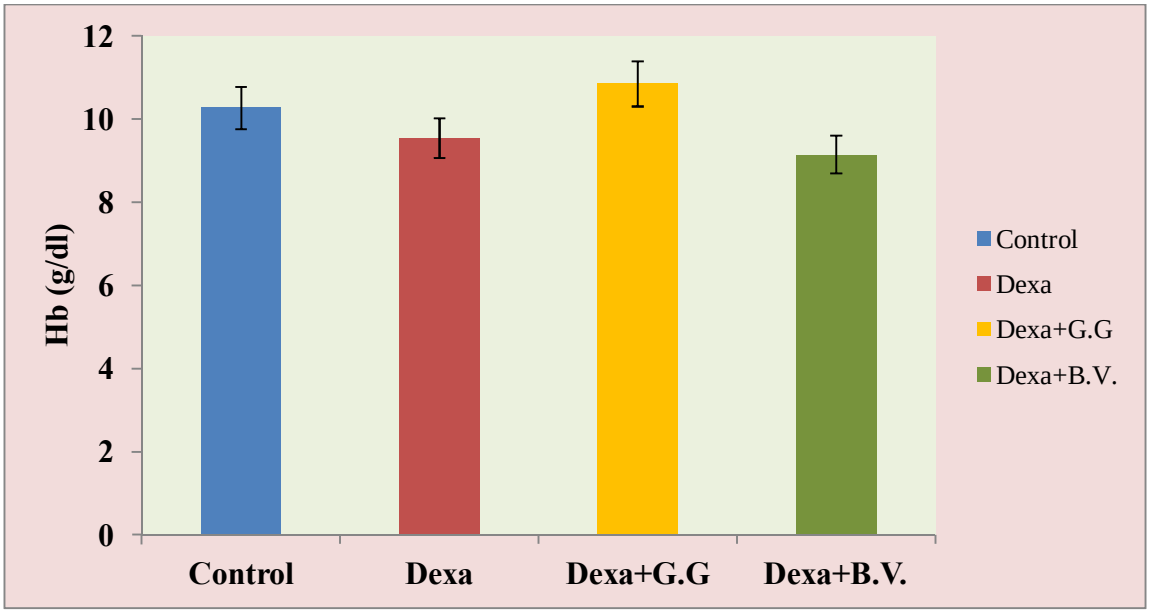


Fig 4.19 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on Haemoglobin

There was significant (P<0.05) decrease in per cent lymphocyte count in dexamethasone treated rats compared to control animals and significant increase (P<0.05) in per cent lymphocytes in *Bauhinia variegata* treated and *Glycyrrhiza glabra* treated rats compared to dexamethasone treated animals.

There was a significant (P<0.05) increase in per cent monocyte count in dexamethasone treated rats compared to control animals. The per cent monocyte count significantly (P<0.05) decreased compared to dexamethasone treated group in *Bauhinia variegata* and *Glycyrrhiza glabra* treated group.

The per cent granulocyte count was significantly (P<0.05) higher in dexamethasone treated group compared to control group. There was significant (P<0.05) decrease of per cent granulocyte count in *Bauhinia variegata* and *Glycyrrhiza glabra* treated groups compared to dexamethasone treated group.

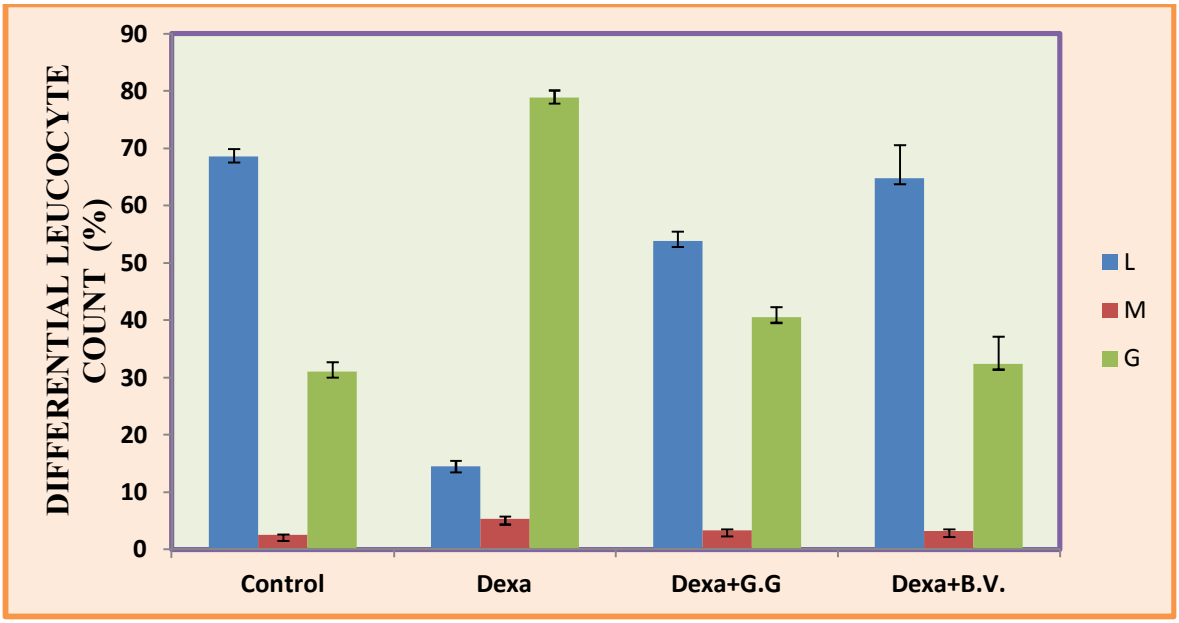


Fig 4.20 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on Differential Leucocyte Count

There was no change (P>0.05) in packed cell volume (PCV) of all treated groups as compared to the control group.

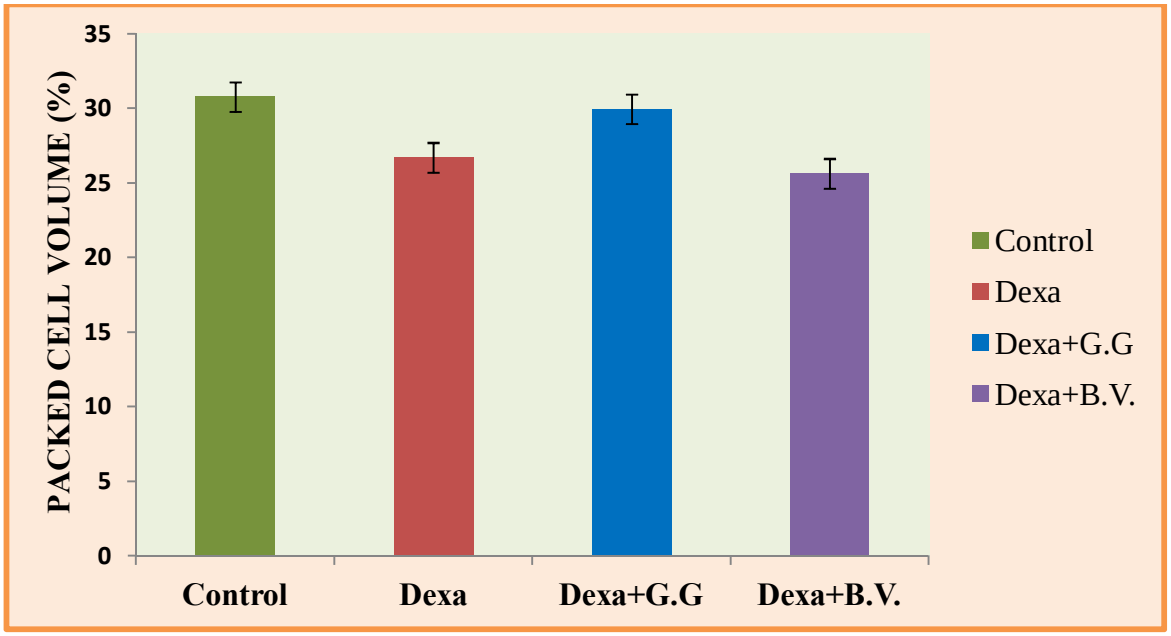


Fig 4.21 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on Packed Cell Volume

A lymphocytopenic response of dexamethasone in the present study could be explained on the basis of inhibition of lymphocyte adhesion molecules (Black 2002).

Dorohi *et al*. (2005) studied the immunomodulatory activity of various standaridized ethanolic extracts of various plants involving *Glycyrrhiza galbra* in laying hens. Blood samples were taken to study specific and non-specific response of extracts and concluded that the *Glycyrrhiza glabra* (20 μg^{-1}ml) had the co-mitogenic potential for both T and B avian lymphocytes ($P<0.05$).Thus the studies of Dorohi *et al*. (2005) confirm the findings of present study.

v. PER CENT TOTAL CD4+and CD8+ COUNT IN BLOOD

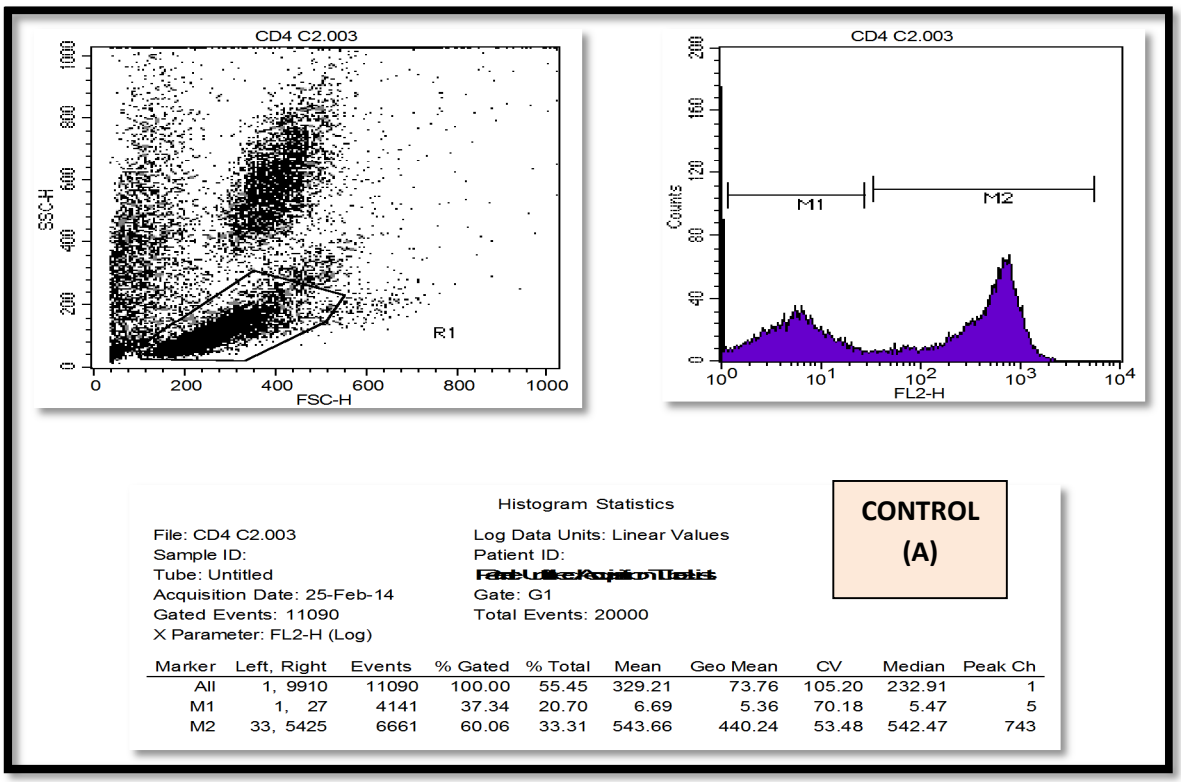
The CD4+ and CD8+ count was done in whole blood of rats following challenge with sheep RBCs. The results are presented in table 4.18, fig. 4.22 and plate 4.5, 4.6. There was significant ($P<0.05$) decrease in CD4+ count in all the rats immunosuppressed with dexamethasone including immunocompromised and treated with *Bauhinia variegata.* None of the treatment influenced CD8+ cell count. Interestingly the treatments with *Glycyrrhiza glabra* in immunocompromized rats had CD4+ count comparable to control animals and significant ($P<0.05$) increased as compared to dexamethasone treated group of animals. The increase in CD4+ cell count in *Glycyrrhiza glabra* treated animals was significant ($P<0.05$) as compared to *Bauhinia variegata* treated animals. These findings are in agreement with the observations with respect immunostimulatory effect of plant in the present studies.

Table 4.18 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on CD4+and CD8+count

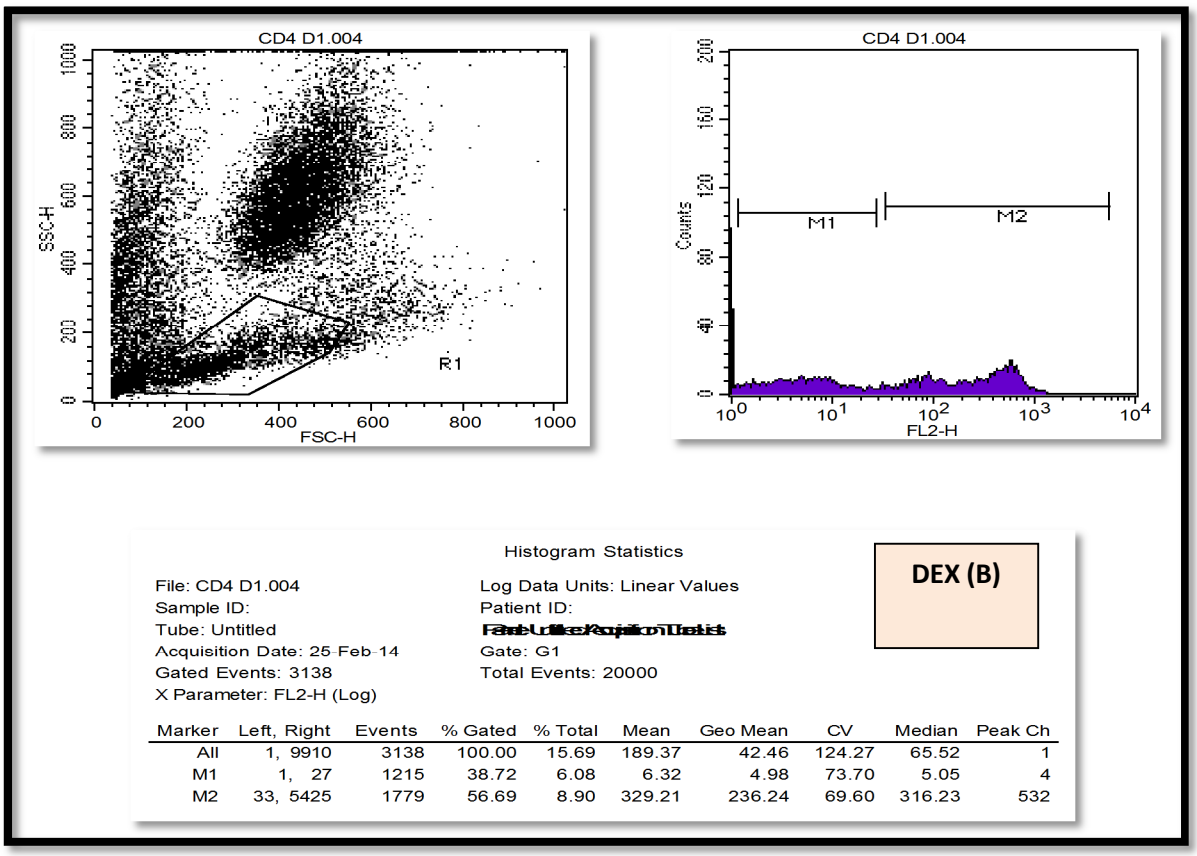
Receptor	Control	Dexa	Dexa + G.G.	Dexa + B.V.
CD4+	25.48 ± 7.83^{a}	8.89 ± 0.01^{b}	19.53 ± 0.27^{ad}	9.76 ± 0.31^{be}
CD8+	7.89 ± 2.03^{a}	2.83 ± 0.47^{a}	8.37 ± 2.74^{a}	6.09 ± 0.55^{a}

Mean values ± standard error (n=6) means with same superscripts in between columns do not differ significantly at 5% level

The findings of the present studies were in agreement with the findings of Ehiaghe *et al*. (2013) who observed no change in CD4+ and CD8+ count following oral administration of fresh aqueous extract of *Acalypha wilkesiana* supplements.

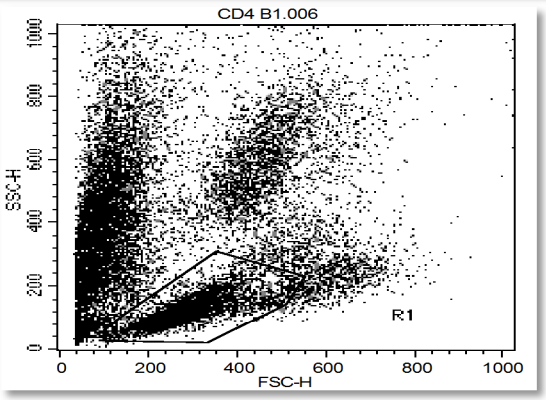


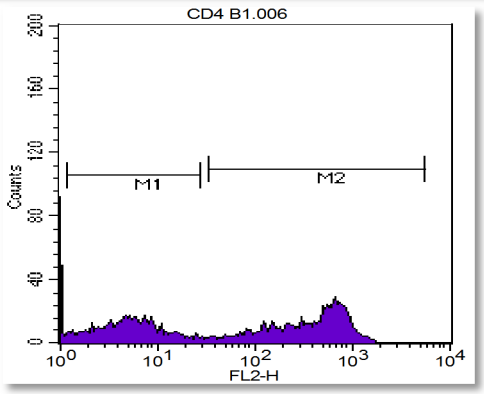
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	11090	100.00	55.45	329.21	73.76	105.20	232.91	1
M1	1, 27	4141	37.34	20.70	6.69	5.36	70.18	5.47	5
M2	33, 5425	6661	60.06	33.31	543.66	440.24	53.48	542.47	743



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	3138	100.00	15.69	189.37	42.46	124.27	65.52	1
M1	1, 27	1215	38.72	6.08	6.32	4.98	73.70	5.05	4
M2	33, 5425	1779	56.69	8.90	329.21	236.24	69.60	316.23	532

Plate 4.5 Histogram showed the per cent total of CD4+ receptor cell count in control (A) and dexamethasone (B) treated group of animals.





Histogram Statistics

Dex + B.V.

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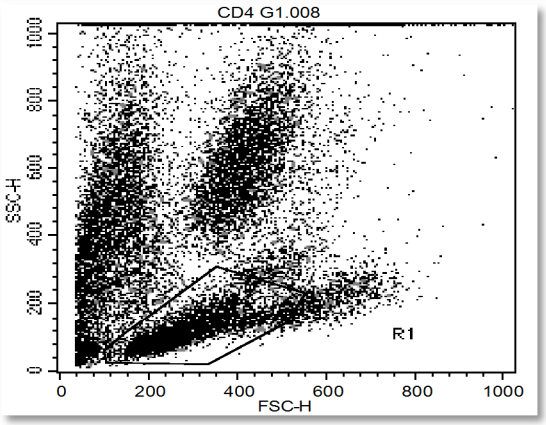
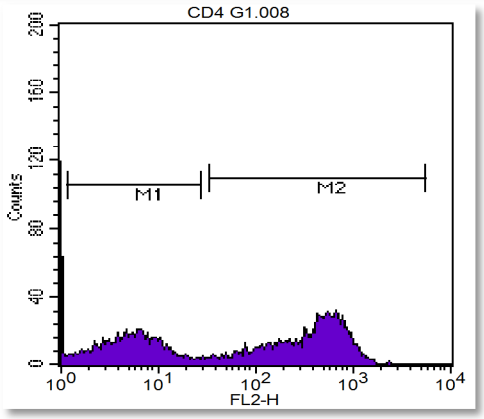
Acquisition Date: 25-Feb-14
Gated Events: 4929
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
[illegible]
Gate: G1
Total Events: 20000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	4929	100.00	24.65	278.84	59.45	113.12	132.16	1
M1	1, 27	1891	38.36	9.45	6.24	4.98	72.65	5.05	4
M2	33, 5425	2887	58.57	14.43	471.74	364.80	59.97	482.61	637





Histogram Statistics

Dex + G.G.

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Acquisition Date: 25-Feb-14
Gated Events: 6584
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
[illegible]
Gate: G1
Total Events: 20000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	6584	100.00	32.92	269.32	61.56	113.11	140.11	1
M1	1, 27	2437	37.01	12.18	6.17	5.00	69.22	5.14	4
M2	33, 5425	3960	60.15	19.80	443.72	341.59	62.92	425.51	620

Plate 4.6 Histogram showed the per cent total of CD4+ receptor cell count in *Bauhinia variegata* (C) and *Glycyrrhiza glabra* (D) treated group of anima

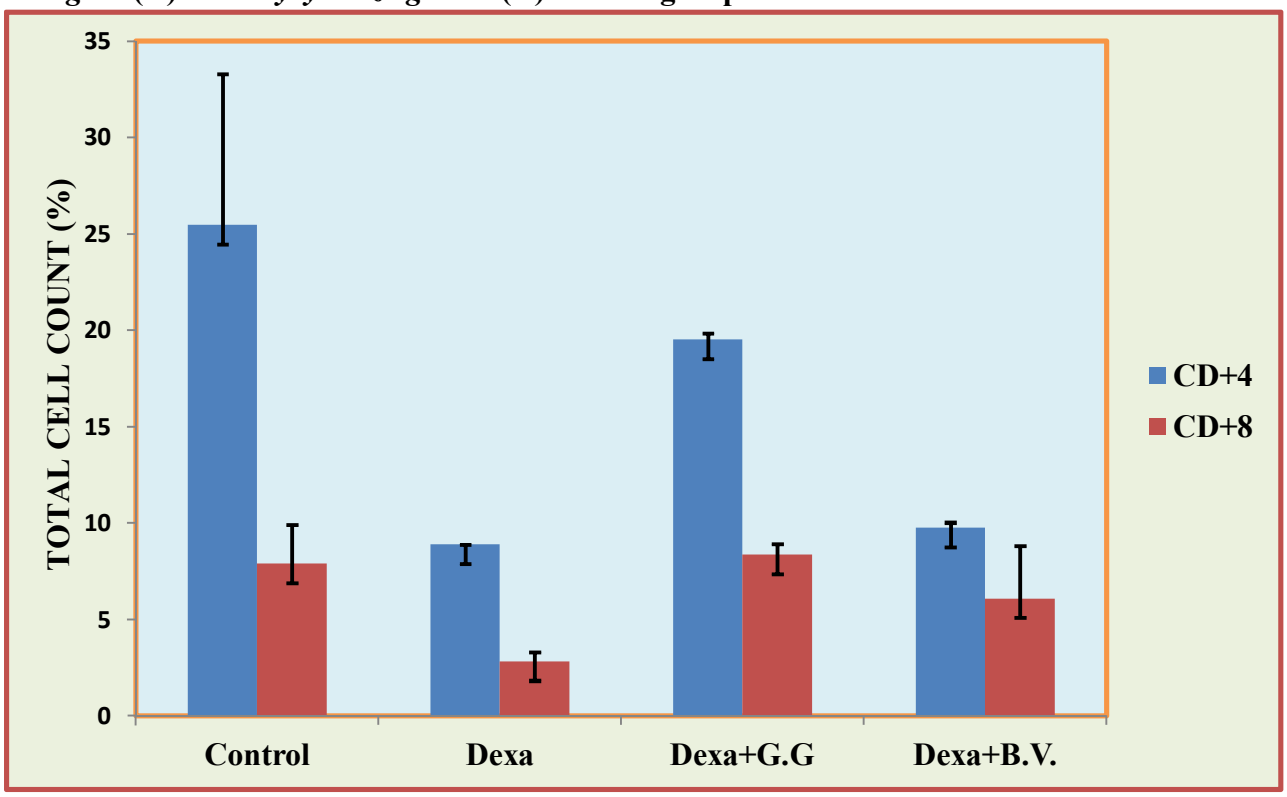


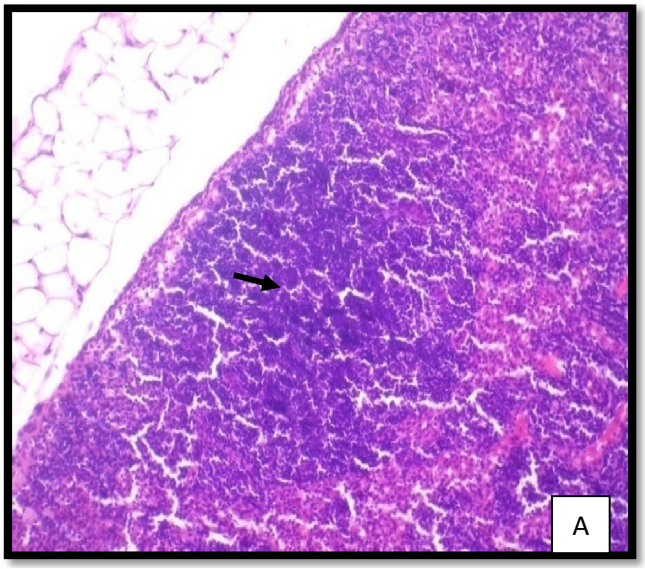
Fig 4.22 Effect of 30 days treatment of *Bauhinia variegata* and *Glycyrrhiza glabra* on CD4+ and CD8+ count

vi. HISTOPATHOLOGICAL STUDIES

As shown in plate 4.7 and 4.8 there was depletion of lymphocytes in mesenteric lymph node. In spleen there was decreased in size of lymphoid follicle in dexamethasone treated group as compared with control group. In case of *Glycyrrhiza glabra* and *Bauhinia variegata* treated groups there was an increased in number of reactive lymphoid follicles. Lymphoid follicles were intermingled with each other showing immunostimulatory response of the *Bauhinia variegata* and *Glycyrrhiza glabra* methanolic extracts.

Two weeks treatment of dexamethasone in male wistar rats at dose rate of 2.2, 6.25 and 12.5 mg kg^{-1} b.w. in distilled water orally resulted in toxico-pathological effects in different organs. There was depletion in lymphoid cells of spleen and decrease in relative weight of spleen (Rajwadi *et al.* 2011).

The depletion of lymphoid cells was consistent with the immunomodulatory response observed with respect to the humoral response against SRBC and cell mediated immunity in the present study.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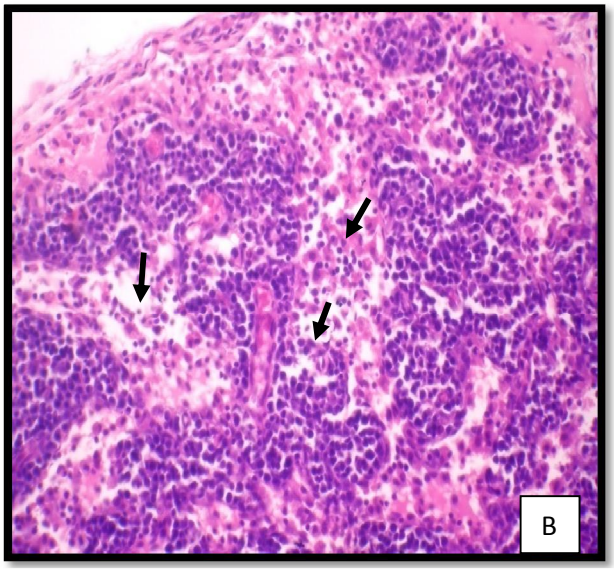
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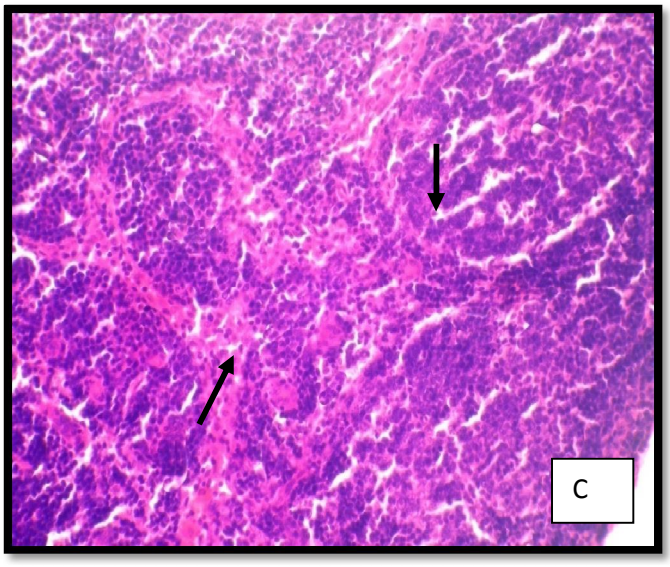
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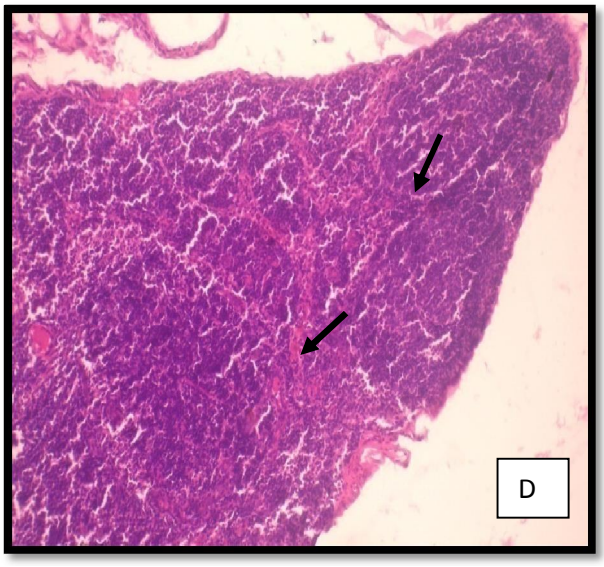
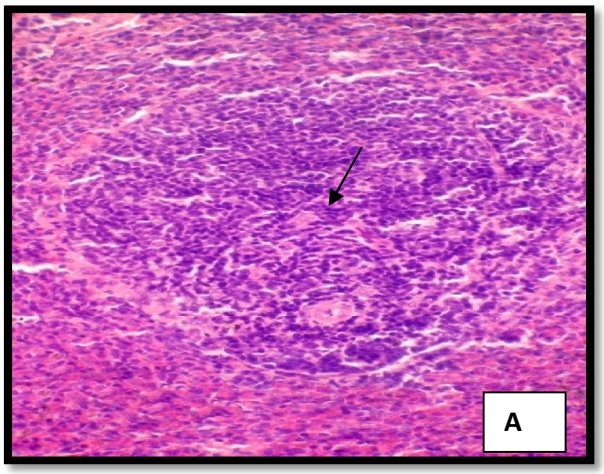
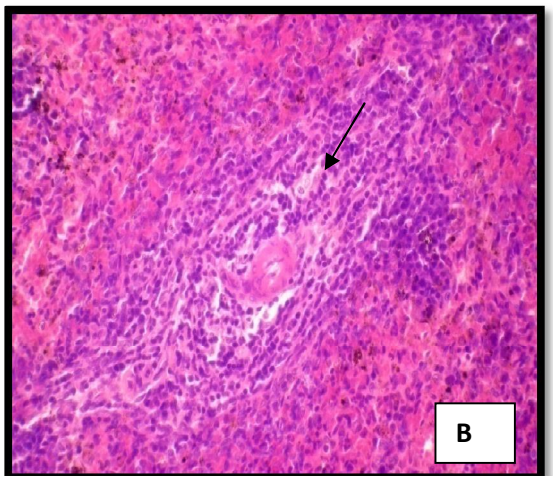
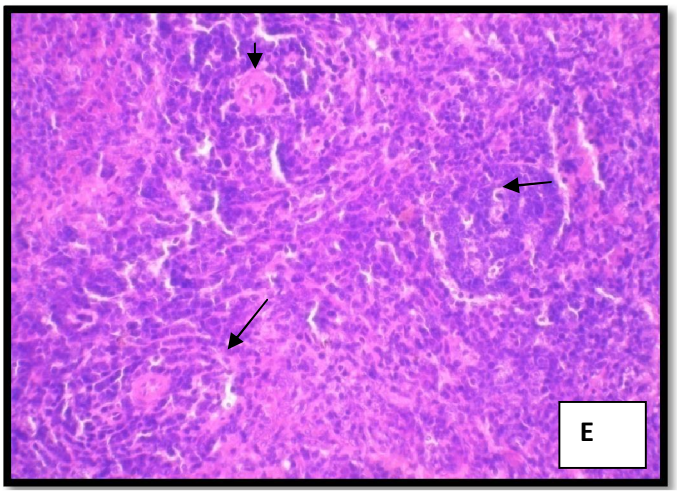
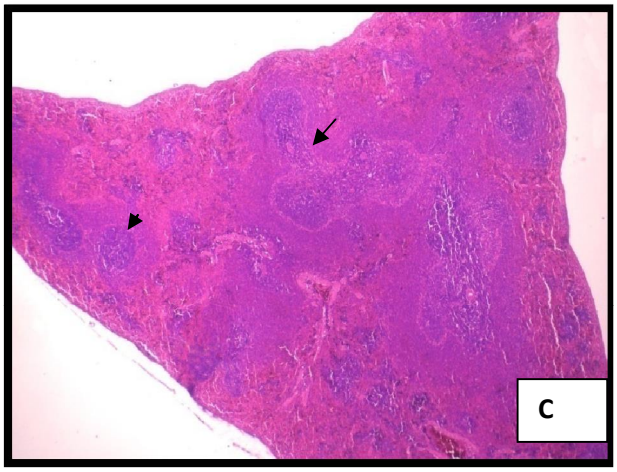

D

Plate 4.7 Photomicrographs of mesenteric lymph node of different treated groups rats









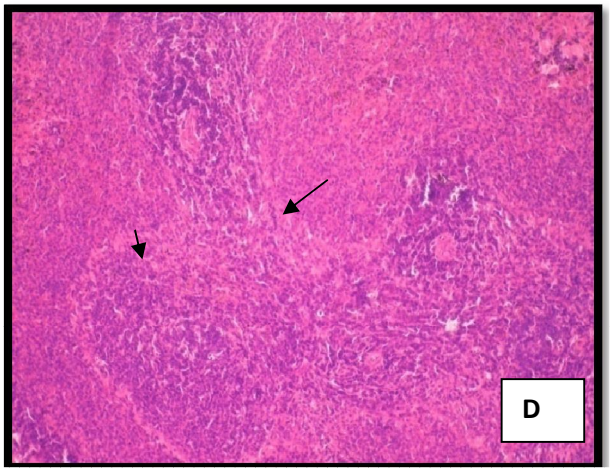


Plate 4.8 Photomicrographs of spleen tissue of different treated groups rats

A. Photomicrograph of mesenteric lymph node of control rats showed normal lymphocytes H&E × 33.

B. Photomicrograph of mesenteric lymph node of dexamethasone treated rats showed depletion of lymphocytes H&E × 132.

C. Photomicrograph of mesenteric lymph node of *Glycyrrhiza glabra* treated rats showed reactive and intermingled lymphoid follicles H&E × 132.

D. Photomicrograph of mesenteric lymph node of *Bauhinia variegata* treated rats showed reactive and intermingled lymphoid follicles H&E × 33

A. Photomicrograph of spleen of control rats showed normal area of lymphoid tissue H&E × 132

B. Photomicrograph of spleen of dexamethasone treated rats showed depletion of area of lymphoid tissue H&E × 132.

C. Photomicrograph of spleen of *Bauhinia variegata* treated rats showed reactive lymphoid follicles H&E × 132.

D. Photomicrograph of spleen of *Bauhinia variegata* treated rats showed reactive and intermingled lymphoid follicles H&E × 33.

E. Photomicrograph of spleen of *Glycyrrhiza glabra* treated rats showed reactive and intermingled lymphoid follicles H&E × 33.

CHAPTER-5

SUMMARY AND CONCLUSIONS

Free radicals are highly reactive substances formed in the body as a result of metabolic processes. The increased production of free radicals cause cell death and tissue injury due to damage on cell membrane. The involvement of free radicals in aging and in many chronic diseases and its effective protection has been a matter of great concern for the scientists world over. Detoxification of oxygen reactive species in the cell is provided by both enzymatic and non-enzymatic systems which constitute the antioxidant defense systems. These antioxidants play a role in delaying, intercepting or preventing oxidative reaction catalysed by free radicals. The plants and herbal medicaments are the major resources of exogenous antioxidants.

The immunomodulation means to immunopotentition, immunostimulation and immunosuppression (in autoimmune disorders) of immune system by an agent. The immunomodulation using medicinal plants can provide an alternative approach to conventional chemotherapy for a variety of diseases, especially when host defense mechanism has to be activated under the compromised immune response during various pathological conditions.

Both antioxidant protection and immunomodulation play a major role in adjunctive therapy in various pathological conditions. Therefore, to explore the antioxidant and immunomodulatory activities of roots of *Glycyrrhiza glabra* and stem bark of *Bauhinia variegata* the present study was undertaken.

The per cent recovery, physical properties and *in vitro and in vivo* antioxidant properties and immunomodulatory activity of both plants was studied. A total of 96 rats male Wistar rats (130-200g) were used. Potassium dichromate was used to induce oxidative stress while, dexamethasone was used as standard immunosuppressant. The effect of methanolic extracts of *Glycyrrhiza glabra* and *Bauhinia variegata* was studied in stressed as well as normal rats for 30 days.

In vivo antioxidant activity of methanolic extracts of *Glycyrrhiza glabra* and *Bauhinia variegata* was studied using various parameters like lipid peroxidation (liver, kidney and erythrocytes), reduced glutathione (liver and kidney) and catalase (liver) activity. The immunomodulatory action of extracts was assessed using immunological parameters like haemgglutination test (HA titer), delayed type of hypersensitivity test (DTH) and CD4+ and CD8+ cell count in blood and haemato-biochemical parameters like total protein, albumin, globulin, albumin: globulin , total leucocyte count, total lymphocyte count, haemoglobin, packed cell volume, differential leucocyte count and total erythrocyte count.

The colour of *Glycyrrhiza glabra* roots was light brownish yellow whereas, that of Bauhinia variegata bark was light brown. The percent recovery of different type of extracts of *Glycyrrhiza glabra* ranged between 10.24 to 19 percent and that of Bauhinia variegata ranged between 9.65 to 30 percent. Results showed that the methanolic and aqua methanolic extracts of *Glycyrrhiza glabra* and methanolic and aqueous extracts of Bauhinia variegata have more percent recovery of phytoconstituents.

The IC_{50} values of ABTS radical scavenging activity of methanolic, aqua-methanolic and aqueous extracts of *Glycyrrhiza glabra* were 0.114 mg/ml, 0.193mg/ml and 0.709mg/ml, respectively and of *Bauhinia variegata* extracts were 0.276 mg/ml, 0.302mg/ml and 0.757mg/ml, respectively. The IC_{50} value for DPPH radical scavenging of methanolic, aqua-methanolic and aqueous extracts of *Glycyrrhiza glabra* were 0.159 mg/ml, 0.571 mg/ml and 0.552 mg/ml, respectively whereas, in *Bauhinia variegata* extracts IC_{50} values were 0.258 mg/ml, 0.395 mg/ml and 0.410 mg/ml, respectively.

Glycyrrhiza glabra and *Bauhinia variegata* extracts treatment did not significantly ($P>0.05$) influence the weekly body weight, body weight gain and feed conversion ratio during four weeks period in rats. The relative organ weight (liver and kidney) in rats of all treated groups was recorded on day 30 of the experiment and no significant ($P>0.05$) change in relative organ weight in different groups except the rats treated with dexamethasone where a significant increase ($P<0.05$) was observed.

After 30 days treatment, a significant ($P<0.05$) increase in malondialdehyde (MDA) level (liver, kidney and erythrocytes) was observed in chromium treated rats as compared to the control animals. The treatment with *Glycyrrhiza glabra* extract and *Bauhinia variegata* provided significant decrease in lipid protection (in terms of MDA level) in liver, kidney and erythrocytes of chromium treated rats. Contrarily, the both plant extracts significantly

($P<0.05$) increased the reduced glutathione levels in liver and kidney of chromium treated rats.

There was a significant ($P<0.05$) decrease in $\log_2$ value of haemagglutination titre in dexamethasone (5 mg kg^{-1} b.w.) treated group as compared to the control whereas, significant ($P<0.05$) increase was seen in *Bauhinia variegata* and *Glycyrrhiza glabra* treated groups as compared to dexamethasone treated group.

A significant ($P<0.05$) increase in foot pad thickness at 24 and 48 hour of the challenge in *Glycyrrhiza glabra* treated group as compared to the rats compromised with dexamethasone. Further decrease in footpad thickness was observed at 72 hour of post challenge. *Bauhinia variegata* treated group exhibited significant ($P<0.05$) increase in foot pad thickness at 48 hour post challenge only.

A significant ($P<0.05$) decrease in per cent lymphocyte count was observed following dexamethasone treatment with *Bauhinia variegata* and *Glycyrrhiza glabra* provided significant increase ($P<0.05$) in per cent lymphocytes.

Histopathological studies showed intermingled and reactive lymphoid follicles showing immunostimulatory response of the *Bauhinia variegata* and *Glycyrrhiza glabra* methanolic extracts.

CONCLUSION:

- On the basis of in *vitro* as well as *in vivo* antioxidant studies and immunomodulatory activities of *Glycyrrhiza glabra* and *Bauhinia variegata* both the plants were found to possesss antioxidant activity. However, *Glycyrrhiza glabra* was found to be a better herbal antioxidant as well as immunomodulator.

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Brief Biodata of the Student

Name	:	Kanika
Fatherôs Name	:	Sh. Raj Kumar Kaistha
Motherôs Name	:	Smt. Aruna Kumari
Date of Birth	:	28-08-1988
Permanent Address	:	H.No. 67, W. No. 10, Deptot Bazaar, Dharamshala, Distt. Kangra, H.P.

Academic Qualifications:

S. No.	Degree/ examination passed	Year of passing	University/ board	Division	O.G.P.A/ Percentage	Medium of instruction
1	High school (10^{th})	2004	H.P.B.S.E	I^{st}	80.12%	English
2	Senior secondary (10+2)	2006	H.P.B.S.E	I^{st}	78.6 %	English
3	B.V.Sc. & A.H.	2012	CSKHPKV, Palampur	I^{st}	7.16 /10.0 (71.6%)	English
4	M.V.Sc. (Vety. Pharmacology and Toxicology)	2014	CSKHPKV, Palampur	I^{st}	7.75 /10.0 (7.75%)	English