PHYTOTHERAPEUTIC INVESTIGATION OF MAJOR HERBAL STEROIDS TO EXPLORE THEIR POTENTIAL AS AN ALTERNATIVE TO SYNTHETIC STEROIDS

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CERTIFICATE

*This is to certify that the thesis entitled "**Phytotherapeutic investigation of major herbal steroids to explore their potential as an alternative to synthetic steroids**" represents bonafide and genuine research work of Mr. Nilesh Kanaiyalal Patel carried out under my guidance and supervision. The work presented in this dissertation was carried out at Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India and is upto my satisfaction.*

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DECLARATION

I hereby declare that thesis entitled **"Phytotherapeutic investigation of major herbal steroids to explore their potential as an alternative to synthetic steroids"** is a bonafide and genuine research work carried out by me, under the guidance of **Prof. (Dr.) Navin R. Sheth**, Head, Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India. The results presented in this dissertation are original and has not been submitted in part or full for any degree/diploma to any University.

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"If you steal from one author its plagiarism; if you steal from many its research." - Wilson Mizner

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NILESH K. PATEL

Content

List of Abbreviation

Abbreviation	Full Name
AA	Adjuvant Arthritis
AA	Adjuvant arthritis
ACTH	Adrenocorticotropic Hormone
African ph	*African* Pharmacopoeia
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AR	Analytical reagent
AST	Aspartate aminotransferase
CAH	Congenital adrenal hyperplasia
CBG	Corticosteroid-binding globulin
CFA	Complete Freund's adjuvant
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
cumm	Cubic millimeter
DA	*Dioscorea alata*
EAA	Ethanolic extract of *Agave americana*
EDA	Ethanolic extract of *Dioscorea alata*
EDTA	Ethylene diamine tetraacetic acid
EHI	Ethanolic extract of *Hemidesmus indicus*
ELAM-1	Endothelial leukocyte adhesion molecule-1
ESX	Ethanolic extract of *Solanum xanthocarpum*
ETF	Ethanolic extract of *Trigonella foenum graecum*
European ph	*European* Pharmacopoeia
FLS	Fibroblast-like synoviocytes
GGT	Gamma-glutamyl transferase

GR	Glucocorticoid receptor
HC	Hydrocortisone
HI	*Hemidesmus indicus*
HPTLC	High performance thin layer chromatography
IAEC	Institutional Animal Ethical committee
ICAM-1	Intracellular adhesion molecule-1
IOP	Intraocular pressure
IU	International Unit
mMol	Milimoles
OVA	Antiovalbumin
PAS	Periodic acid-Schiff reagent
RBC	RBC red blood corpuscle
Rf	Retardation factor
SEM	Standard error of mean
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SX	*Solanum xanthocarpum*
TF	*Trigonella foenum-graecum*
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α
W/W	Weight per Weight
WBC	White blood cells
WHO	World health organization

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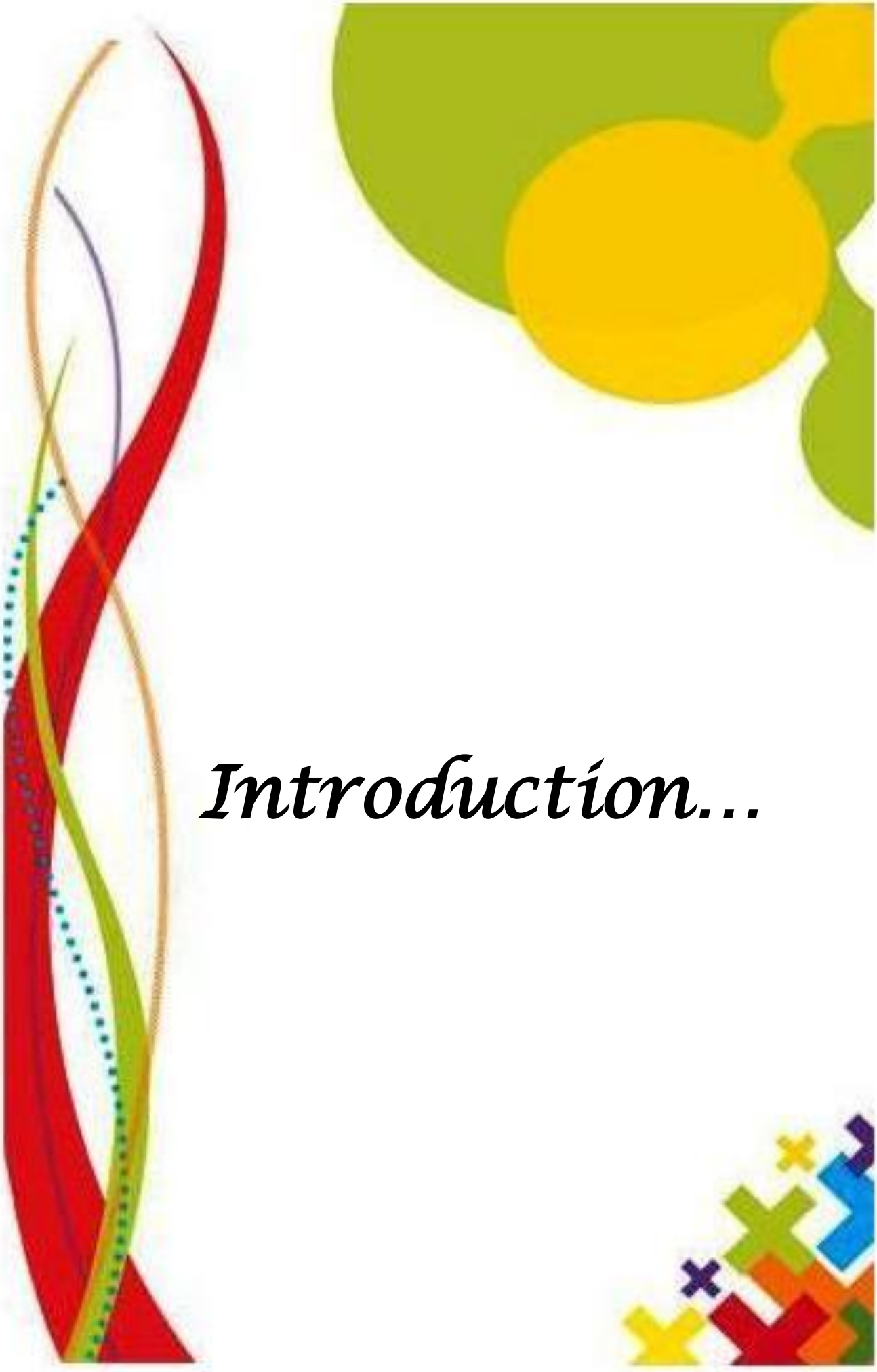


Introduction…

1. Introduction

Steroids are an unique class of chemical compounds that are found throughout the animal and plant kingdom and this class includes sterols such as cholesterol, ergosterol, bile acids, steroid hormones (Petrow *et al.*, 1996). The modern era of steroid research began with steroid chemistry in the early 1900s. Dr. Adolf Windaus, a Gottingen chemist, worked for over 20 years on the isolation of steroids, development of assays for detecting steroids, and the use of classical chemistry to elucidate steroid structures (Adolf *et al.*, 1909). Hundreds of distinct steroids are found in plants, animals, and fungi. All steroids are made in cells either from the sterol lanosterol (animals and fungi) or the sterol cycloartenol (plants). Both sterols are derived from the cyclization of the triterpene squalene Steroid molecules possess a common chemical skeleton of four fused rings, consisting of three six-membered rings and a five-membered ring. Chemically, this hydrocarbon scaffold is a cyclopentano perhydrophenanthrene, describing the three rings of phenanthrene and the cyclopentane ring (Gareth *et al.*, 2003).

Pregnenolone serves as the common precursor in the formation of the adrenocorticoids and other steroid hormones. This steroid is converted through enzymatic oxidations and isomerization of the double bond to a number of physiologically active steroids, including the adrenocorticoids cortisol (hydrocortisone), corticosterone, and aldosterone (Auchus *et al.*, 2003)

Since the introduction of cortisone and hydrocortisone anti-inflammatory steroids have remained an important and un-replaced drug class. Although not without adverse effects, these compounds have continued to be the "drug of choice "in the treatment of afflictions ranging from the moderate skin rash to severe acute inflammatory disorders (Baxter *et al.*, 1979). The generation of corticosteroids is intricately balanced when the production and regulatory process malfunctions, the result is either an excess (Cushing’s syndrome) or deficiency (Addison's disease) in glucocorticoid levels.

Glucocorticoids and their metabolites were recognized early as possessing powerful anti-inflammatory and immunomodulatory properties. Even before 1950,

reports of the anti-arthritic properties of cortisone by Hench and coworkers indicated the potential for these compounds to reduce the suffering of patients with inflammatory diseases (Wepierre *et al.*, 1979)

Prednisone is an inactive form of Prednisolone which is drug of choice in systemic anti-inflammatory, anti-asthmatic and for immunosuppressive effect. Dexamethasone and Betamethasone are long acting steroids used as anti-inflammatory and for immunosuppressive effect where water retention is undesirable. Beclometasone and budesonide are locally used anti-inflammatory and for immunosuppressive agents. Flucortisone is a drug of choice for mineralocorticoids effect. triamcinolone having only glucocorticoid activity and mineralocorticoids activity is absent while Dexamethasone and Betamethasone are having minimum mineralocorticoids activity compare to other steroids (Rang and Dale *et al.*, 2003).

Adrenocorticosteroids are also used in treatment of non-adrenal disorders like angioneurotic edema, asthma, contact dermatitis, allergic rhinitis, urticaria, lupus erythematosus, polymyositis, allergic conjunctivitis, optic neuritis, autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura, multiple myeloma, cerebral edema, multiple sclerosis, organ transplantation, prevention of infant respiratory distress, sarcoidosis, aspiration pneumonia, atopic dermatitis, seborrheic dermatitis and seborrheic dermatitis (Goldfien *et al.*, 1998).

Adverse effects of adrenocorticoids are always a big question mainly, for long term administration. Adrenocortical insufficiency results due to suppression of the pituitary-adrenal axis and inhibition of ACTH production. Musculoskeletal effects like muscle wasting, muscle pain and weakness, delayed wound healing, atrophy of protein matrix of bone are observed with use of steroid. Immune system also affected with increased susceptibility to infection, especially with high dose and systemic glucocorticoids. Fluid and electrolyte disturbances occur due to mineralocorticoid-like action by glucocorticoids results in sodium retention. Ocular effects include mainly increases in intraocular pressure (IOP) and the development of posterior subcapsular cataracts. Endocrine effects include varying effects including hypercorticism, amenoriea, hyperglycemia. GI disturbances are also observed like varying effects including nausea, anorexia, increased appetite, and the development and increased

severity of ulcers. Nervous system and psychological effects like headache, vertigo, insomnia, increased motor activity, euphoria, mood swings, depression, anxiety etc. also observed. Dermatologic effects include skin atrophy and skin thinning from a decrease in collagen synthesis, impaired wound healing, acne, increased perspiration. (Olin *et al.*, 1996)

Production of steroid drugs is a large scale industry, total world consumption of steroids precursors was one thousand tones, two third of which came from diosgenin and the remaining one third from the variety of miscellaneous sources. Till today, more than four thousand plant species have been investigated which has resulted in the identification of some thirty naturally occurring steroids sapogenins many of which could provide valuable source materials for steroids compounds (Applezweig *et al.*, 1962).

Major herbal steroids include diosgenin, solasodine, tigogenin, yoccagenin, ticogenin, barbourgenin, sarsepogenin, smilagenin, yamogenin, hecogenin. Diosgenin is mainly obtained from *Allium fuscoviolacenum, Allium narcissifolium, Aspidistra elatior, Balanites roxburghii, Convallaria keisukei, Costus speciosus, Dioscorea composita, Dioscorea floribunda, Dioscorea deltoidea, Dioscorea gracillima, Dioscorea polystachya, Dioscorea prazeri, Dioscorea sativa, Dioscorea septembola, Funkia ovata, Kallstroemia pubescens, Ophiopogon japonicus, Paris polyphyalla, Polygonatum latifolium, Polygonatum multiflorum, Smilax exfelsa, Solanum introsum, Solanum indicum, Tamas communis* and *Tribulus terrestris, Trigonella foenum* and *Trigonella tschonoskii.*

Plant material that used as source of Solasodine are *Solanum eleagnifolium, Solanum jubatum, Solanum incanum, Solanum khasianum, Solanum laciniatum, Solanum mammosum, Solanum marginatum, Solanum platanifolium, Solanum sodomaeum, Solanum tomentosum, Solanum trachysyphyum, Solanum trilobatum, Solanum verbascifolium* and *Solanum xanthocarpum (*Amrit *et al.*, 1999).

Yamogenin can be derived from plants like *Aspargus officinalis, Smilex officinalis* and *Trigonella foenum graceum*. Hecogenin is mainly found from agave

genus. In various species like *A. americana, A. cantala, A. sisalana, A. angustifolia,* leaves yield a valuable fiber (Marston *et al.*, 1985).

Herbal medicines are safer than allopathic medicine. There would be no serious side effects associated with longer use of herbal material. Herbal extract contains many chemical constitutes which eliminate the unwanted effects of active principle. In many cases it has been shown that potential harm is minimized through a selected method of preparation. Herbal material is being used in specific way particularly by addition of other material which may decrease the toxicity by countering unwanted pharmacological effects (Evans *et al.*, 2004).

Today 80 % of the world's populations are dependent on herbs for their health. Some herbal treatments are well established, and have undergone clinical testing. This approach is best called phytotherapy and uses one remedy for one condition based on proper scientific testing (Eric *et al.*, 1963)

A large number of academic, industrial and government institutes are conducting research on the steroid in India but there is few evidence of systemic study of steroids. Herbal steroids are mainly used for production of synthetic steroids. Herbal steroid are also used in phytotherapy. A few studies showed it to be anti-arthritic, anti-inflammatory, and Immunosuppressant. Benefits were also claimed in cases of antibacterial and anti-allergic. The collective scientific evidence, scarce as it is, shows that herbal steroid is more likely to build profit margins compare to available synthetic steroids (Swami *et al.*, 2005).

Steroid possesses glucocorticoid as well as mineralocorticoid activity. There is no credible research on the evaluation of herbal steroids. Apart from precursor for allopathic steroids, many activities have been reported for important herbal steroids.

Dioscorea Species can be used in preparation of medicaments for preventing mucositis caused by radiotherapy and chemotherapy (Faming *et al.*, 2005). The ethanolic extract of *Dioscorea membranacea* exhibited potent inhibitory activity against β hexosaminidase release as a marker of degranulation in RBL-2H3 cells, which shows their anti-allergic activities (Tewtrakul *et al.*, 2006). Diosgenin obtained

from *Dioscorea composita* shows antiphlogistic activity with dose of 1-500 mg daily (Roux *et al.*, 1964). Diosgenin modulates certain aspects of acquired immunity, including the enhancement of antigen-specific IgG2a and IFN-γ expression, which may be mediated through the up-regulation of Th1 differentiation (Jan *et al.*, 2007). *Dioscorea membranacea* contain Nitric oxide inhibitory substances and it is used for treatment of the inflammatory diseases (Tewtrakul *et al.*, 2007). Diosgenin, induces apoptosis, cell cycle arrest and COX activity in osteosarcoma cells and Diosgenin treatment caused an inhibition of 1547 cell growth with a cycle arrest in G1 phase and apoptosis induction (Moalic *et al.*, 2001) Plant steroid, diosgenin, causes an inhibition of the growth of fibroblast-like synoviocytes (FLS) from human rheumatoid arthritis (RA), with apoptosis induction associated with cyclooxygenase-2 (COX-2) up-regulation (Liagre *et al.*, 2004). Tribulus terrestris extracts for antibacterial, antiviral, anti-herpes cream. This product was also found to be very successful in suppository form for the treatment of vulvo-vaginal, vulvo-hemorrhoidal and colonic conditions (Alexis *et al.*, 2002).

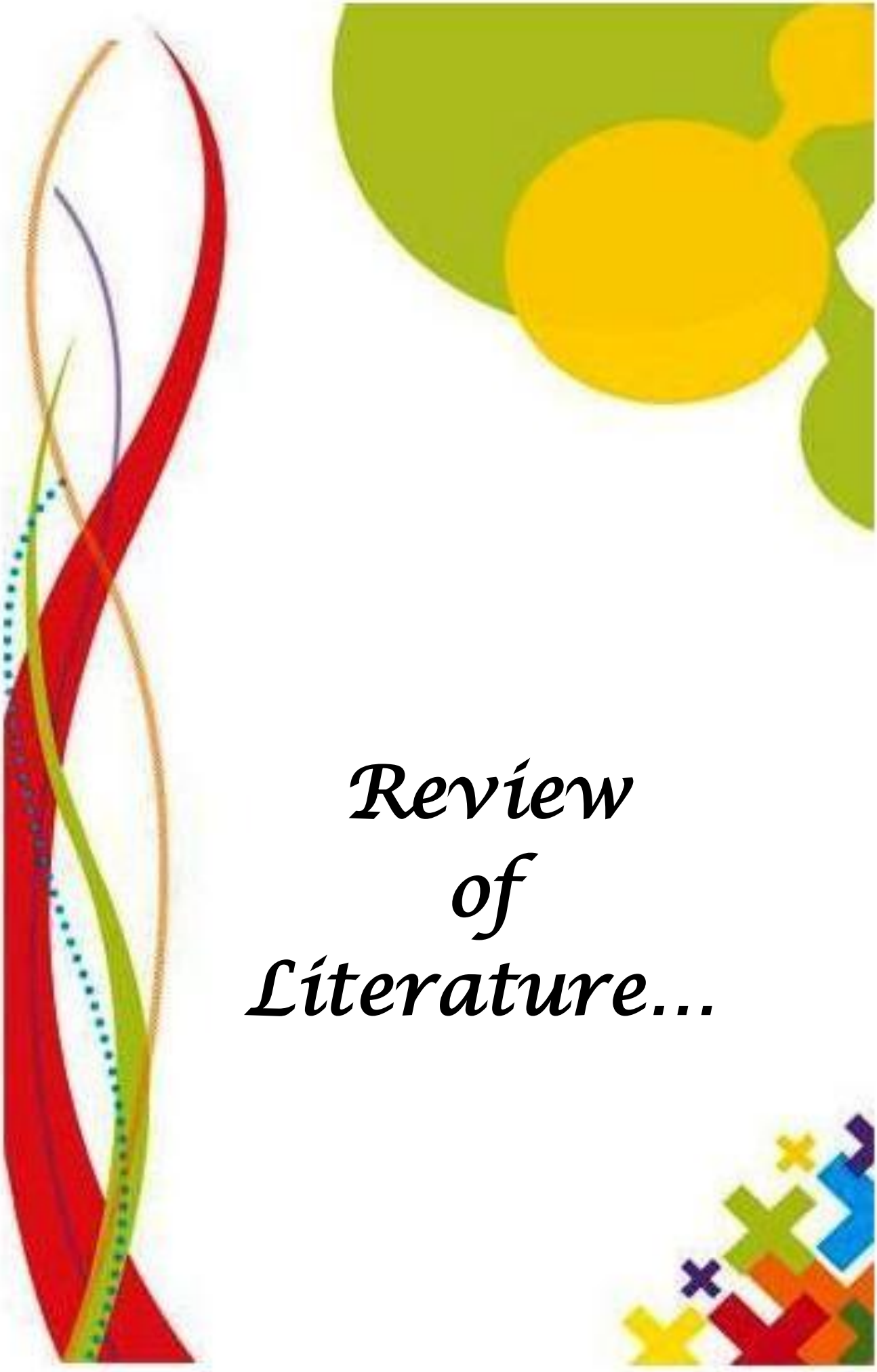
Many experiments have been reported on evaluation of therapeutic activity of Solanum Species. The anti-inflammatory effect of solasodine and of methanol extract of *Solanum trilobatum* was evaluated and it showed significant anti-inflammatory activity (Emmanuel *et al.*, 2006). Solasodine and preparation of solasodine organic acid salts useful as antitumor, anti-asthmatic or anti-inflammatory agent (Liu *et al.*, 2004). Solasodine hydrochloride from the fruit juice of *Solanum nigrum* or *Solanum aviculare* can be prepared and it can be used for treatment of neoplasm, asthma, and inflammation as oral or injection medication (Liu *et al.*, 2002).

Yamogenin is mainly found along with diosgenin containing plant. The nomenclature of yamogenin its self suggest its origin from wild yam and other varity of yam. Fenugreek seeds have been widely studied for their reputed antidiabetic, hypocholesterolemic and antifertility effects. The consumption of defatted fenugreek can help in the management of diabetes, hypercholesterolemia, prevention of atherosclerosis and coronary heart disease (Al-Habori *et al.*, 2002). A formulation of Smilax china which considered as good source for Yamogenin has been reported for treatment of gynecological inflammation (Han *et al.*, 2007).

Agave genus is the only reported source for hecogenin and many agave plants have been identified as source for hecogenin (Marston *et al.*, 1985). The methanolic extract of *Agave intermixta* may be mediated by its constituent hecogenin and might contribute to the anti-inflammatory activity shown by this species (Quilez *et al.*, 2004). Anti-inflammatory activity also reported for Lyophilized aqueous extract obtained from *Agave americana* (Peana *et al.*, 1997) Hecogenin is shown to inhibit 5α-reductase type 1 activity in I-C cells and its preparations suitable for treating acne, seborrhea. These preparations may contain anti-inflammatory or anti-irritant agents such as ursolic acid (L'oreal *et al.*, 2002).

The roots of *Hemidesmus indicus* are woody and have a sweet taste, with cooling effect, and used in various ailment of diseases, a well-known drug in the Ayurveda system of medicine (Kirtikar and Basu *et al.*, 1991). Inflammation induced by Viper venom and Propiono bacterium acne are reported to be treated by root extract (Jain *et al.*, 2003). A lep of root powder applied topically is used to treat swellings, inflammation and chronic rheumatism (Alam *et al.*, 1994, 1998). Many other reports suggest the activity against inflammation induced due to pathogenesis for example, asthma, leprosy, swelling of joints, inflamed eyes and in chronic cough, etc.

Many pharmacologic activities of steroid containing plants and herbal steroids viz. diosgenine, solasodine, yamogenin, sarsepogenin and hecogenin have been reported (Applezweig *et al.*, 1962).



Review of Literature...

2. Review of Literature

2.1 Steroids

2.1.1 Introduction to steroids

Corticosteroids are a class of steroid hormones that are produced in the adrenal cortex. Corticosteroids are involved in a wide range of physiologic systems such as stress response, immune response and regulation of inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels, and behavior. Glucocorticoids such as cortisol control carbohydrate, fat and protein metabolism and are anti-inflammatory by preventing phospholipid release, decreasing eosinophil action and a number of other mechanisms. Mineralocorticoids such as aldosterone control electrolyte and water levels, mainly by promoting sodium retention in the kidney.

2.1.2 Classification

Some of the common categories of steroids (Facts about gonane, 2010):

1) Animal steroids
    a) Insect steroids
        i) Ecdysterone
    b) Vertebrate steroids
        i) Steroid hormones
            (1) Androgens, estrogens, and progestagens.
            (2) Corticosteroids include glucocorticoids and mineralocorticoids.
            (3) Anabolic steroids
        ii) Cholesterol
2) Plant steroids
    a) Phytosterols
    b) Brassinosteroids
3) Fungus steroids
    a) Ergosterols

In general, corticosteroids are grouped into four classes, based on chemical structure. Allergic reactions to one member of a class typically indicate an intolerance of all members of the class. This is known as the "Coopman classification" after S. Coopman, who defined this classification in 1989. (Coopman . *et al.* 1989)

The highlighted steroids are often used in the screening of allergies to topical steroids (Wolverton *et al.* 2001)

Group A

(Short- to medium-acting glucocorticoids)

Hydrocortisone, Hydrocortisone acetate, Cortisone acetate, Tixocortol pivalate, Prednisolone, Methylprednisolone, and Prednisone.

Group B

Triamcinolone acetonide, Triamcinolone alcohol, Mometasone, Amcinonide, Budesonide, Desonide, Fluocinonide, Fluocinolone acetonide, and Halcinonide.

Group C

Betamethasone, Betamethasone sodium phosphate, Dexamethasone, Dexamethasone sodium phosphate, and Fluocortolone.

Group D

Hydrocortisone-17-butyrate, Hydrocortisone-17-valerate, Aclometasone dipropionate, Betamethasone valerate, Betamethasone dipropionate, Prednicarbate, Clobetasone-17-butyrate, Clobetasol-17-propionate, Fluocortolone caproate, Fluocortolone pivalate, and Fluprednidene acetate.

2.1.3 Chemistry

Steroids are a unique class of chemical compounds that are found throughout the animal and plant kingdom, and this class includes sterols such as cholesterol and ergosterol, bile acids, and steroid hormones. Modern scientific research on steroid chemistry and biochemistry began in the early twentieth century, and several major treatises on the subject have been published (Nes *et al.* 1977).

The biological and medical significances of steroids have been observed since ancient times, even though the exact chemical nature and properties of steroids began to be understood only in the late 1920s and early 1930s

Egyptians and Romans used extracts of plants such as purple foxglove to treat dropsy. The physical effects of castration were well recognized in eunuchs and in medieval castrati choirs.

Scientific observations in more modern times began examining the biological consequences of hormones without the realization of nature of the chemicals involved. Berthold, a Gottingen physiologist, reported the effects of implanted testis in studies with cocks in 1849 (Berthold *et al.* 1849). In 1855, Addison discovered the relationship of the adrenal glands with a particular disease characterized by bronze skin color (ll), and this disease of chronic adrenal insufficiency is now referred to as Addison's disease.

The modern era of steroid research began with steroid chemistry in the early 1900s. Dr. Adolf Windaus, a Gottingen chemist, worked for over 20 years on the isolation of steroids, development of assays for detecting steroids, and the use of classical chemistry to elucidate steroid structures. Munich was engaged in natural products chemistry, including bile acids (Wieland *et al.*,1916), and was awarded the Nobel prize in 1928 for his research on this subject. The original chemical structures of cholesterol and other steroids proposed in 1928 were subsequently found to be incorrect, and correct structures were identified in 1932 (Rosenheim *et al.*, 1932).

Chemical studies on compounds involved in reproduction began in the 1920s, and in 1929 Adolf F. Butenandt and Edward A. Doisy independently reported the

isolation of an active steroid sex hormone estrone, from the urine of pregnant women (Doisy *et al.* 1930). Throughout the 1930s, many steroid hormones were isolated and structures determined, including progesterone by Butenandt and corticosteroids by Reichstein.

The synthesis of steroids followed shortly thereafter by research groups led by Bachmann, Woodward, Robinson, and Cornforth (Woodward *et al.* 1951)

Studies on the biochemical mechanism of action of steroids began in the late 1950s with the use of tritiated estrogens of high specific activity. Jensen and Jacobson reported the accumulation of physiological levels of estrogen in target organs and postulated the presence of a receptor. Research on the biochemistry and molecular biology of steroid hormone action has exploded in the past few decades and constitutes a major effort in the field today.

Two important discoveries in the late 1940s and early 1950s had a dramatic effect not only on steroid research but also on the pharmacological applications of steroids. The first was the clinical report by Hench from the Mayo Clinic on the significant improvement in patients with rheumatoid arthritis following cortisone treatment. The second was the application of estrogen and progestin preparations for contraception, demonstrated by Pincus and colleagues. These two series of studies showed for the first time that steroids could be considered as drugs. As a result, extensive research on the medicinal chemistry, pharmacology, and clinical studies of steroid agonists and antagonists has evolved and continues to provide new insights and new medicinal agents for therapies in many different diseases and chemoprevention strategies.

2.1.4 Structure and Physical Properties of Steroids

Steroid molecules possess a common chemical skeleton of four fused rings, consisting of three six-membered rings and a five-membered ring (Figure 2.1). Chemically, this hydrocarbon scaffold is a cyclopentanoperhydro- phenanthrene, describing the three rings of phenanthrene (rings A, B, and C) and the cyclopentane

ring (ring D). In steroids, the phenanthrene ring system is completely saturated (hydrogenated) and is thus referred to as a perhydrophenanthrene. This steroid scaffold contains 17 carbon atoms, and the numbering of the carbon atoms begins with the carbons of the phenanthrene and is then followed by numbering the remaining carbons of the cyclopentane ring (Figure 2.1). Additional carbon atoms on steroids include angular methyl groups attached to C13 and C10 and alkyl substituents on C17 (Figure 2.2). When the steroid nucleus is drawn in a two dimensional representation, the steroid scaffold appears planar and substituents on carbons of the steroid scaffold may be located either above or below the "plane" of the steroid.

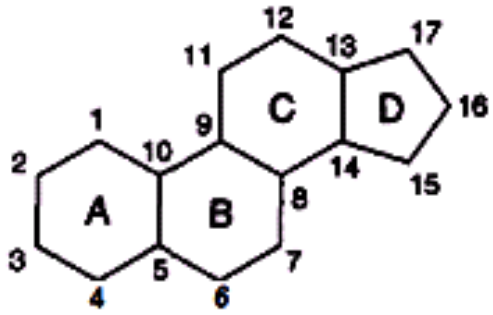


Figure 2.1 Basic steroid structure

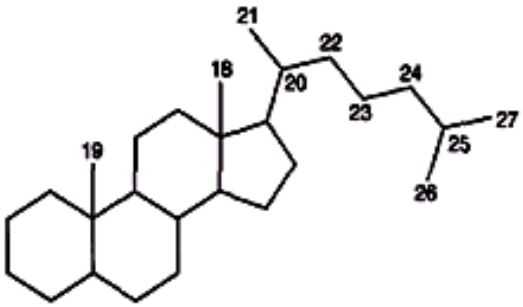


Figure 2.2 Side chain on steroid scaffold

Substituents located above the plane are drawn with solid lines or with solid wedges, and these moieties are referred to as being in the p-configuration. Substituents located below the plane are drawn with dashed lines and are referred to as having the a-configuration. The angular methyl groups numbered 18 and 19 are attached in the p-configuration (above the steroid plane) to C13 and C10, respectively.

The systematic names for steroids are based on the steroid hydrocarbon system, and the particular systematic name begins by selection of the stem name based on the hydrocarbon system (Figure 2.3). Cholestane is the term used for

steroids with 27 carbon atoms (i.e., the C_{27} steroid structure). Pregnanes are steroids with 21 carbon atoms, androstanes have 19 carbon atoms, estranes have 18 carbon atoms, and gonanes have 17 carbon atoms.

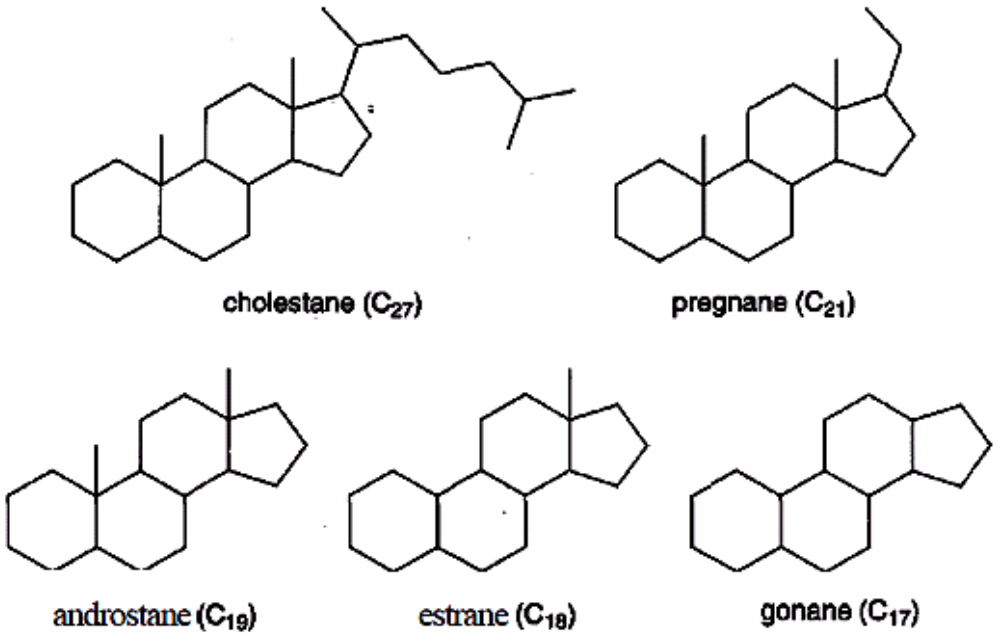


Figure 2.3 Structure of steroid stem names**.**

Examples of the trivial names, systematic names, and chemical structures for common steroids are illustrated in Figure 2.4. Cholesterol is the central steroid of the animal kingdom and functions as an essential component of cell membranes and as a biosynthetic precursor to other steroids in the body. Cholesterol has 27 carbon atoms, a hydroxyl group in the P-configuration at carbon 3, and contains a carbon-carbon double bond between carbons 5 and 6.

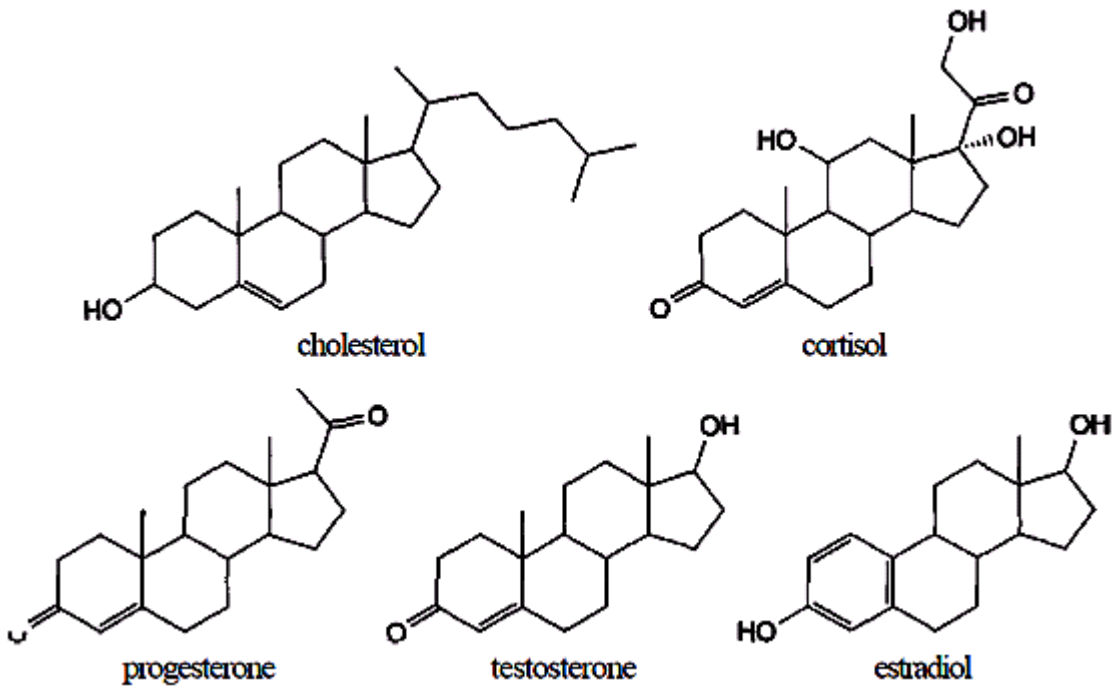


Figure 2.4 Structure of common steroids

2.1.5 Pharmacology of adrenal corticoids

The adrenal cortex synthesizes two classes of steroids: the *corticosteroids* (glucocorticoids and mineralocorticoids), which have 21 carbon atoms, and the *androgens*, which have 19 (Figure 2.5).

The actions of corticosteroids historically were described as glucocorticoid (carbohydrate metabolism-regulating) and mineralocorticoid (electrolyte balance-regulating), reflecting their preferential activities. In humans, *cortisol* (*hydrocortisone*) is the main glucocorticoid and aldosterone is the main mineralocorticoid (Figure 2.6). Although the adrenal cortex is an important source of androgen precursors in women, patients with adrenal insufficiency can be restored to normal life expectancy by replacement therapy with glucocorticoids and mineralocorticoids (Allolio *et al.*, 2002).

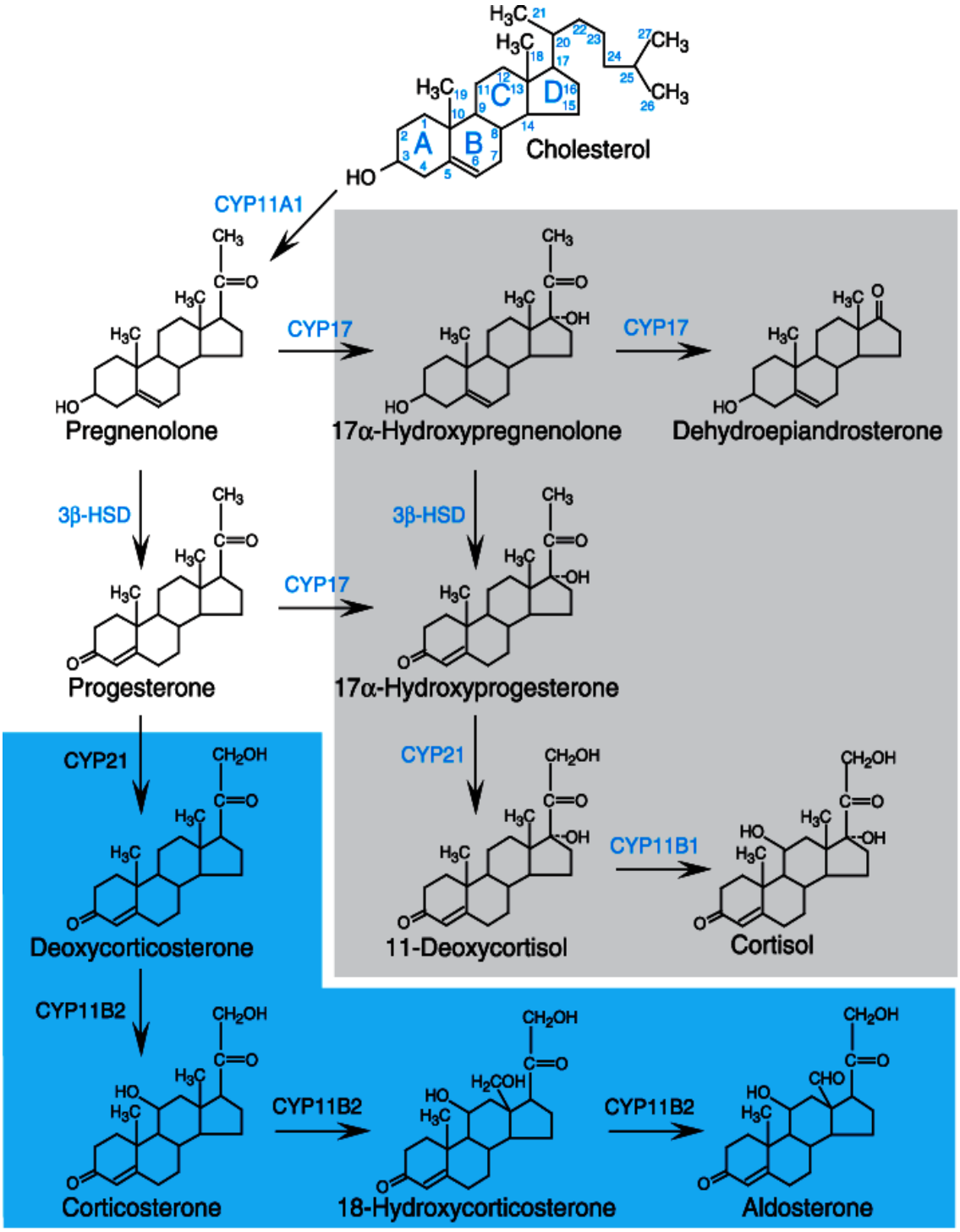


Figure 2.5 Pathways of corticosteroid biosynthesis

2.1.5.1 Physiological actions.

The effects of corticosteroids are numerous and widespread, and include alterations in carbohydrate, protein, and lipid metabolism; maintenance of fluid and electrolyte balance; and preservation of normal function of the cardiovascular system, the immune system, the kidney, skeletal muscle, the endocrine system, and the nervous system. In addition, corticosteroids endow the organism with the capacity to resist such stressful circumstances as noxious stimuli and environmental changes.

Until recently, corticosteroid effects were viewed as physiological (reflecting actions of corticosteroids at doses corresponding to normal daily production levels) or pharmacological (representing effects seen only at doses exceeding the normal daily production of corticosteroids). More recent concepts suggest that the anti-inflammatory and immunosuppressive actions of corticosteroid of the major "pharmacological" uses of this class of drugs also provide a protective mechanism in the physiological setting.

Many of the immune mediators associated with the inflammatory response decrease vascular tone and could lead to cardiovascular collapse if unopposed by the adrenal corticosteroids. This hypothesis is supported by the fact that the daily production rate of cortisol can rise at least tenfold in the setting of severe stress. In addition, as discussed below, the pharmacological actions of corticosteroids in different tissues and their physiological effects are mediated by the same receptor. Thus, the various glucocorticoid derivatives used as pharmacological agents generally have side effects on physiological processes that parallel their therapeutic effectiveness.

Corticosteroids are grouped according to their relative potencies in Na^+ retention, effects on carbohydrate metabolism (*i.e.*, hepatic deposition of glycogen and gluconeogenesis), and antiinflammatory effects. In general, potencies of steroids as judged by their ability to sustain life in adrenalectomized animals closely parallel those determined for Na^+ retention, while potencies based on effects on glucose metabolism closely parallel those for antiinflammatory effects. The effects on Na^+

retention and the carbohydrate/antiinflammatory actions are not closely related and reflect selective actions at distinct receptors, as noted above.

Some steroids that are classified predominantly as glucocorticoids (*e.g.*, cortisol) also possess modest but significant mineralocorticoid activity and thus may affect fluid and electrolyte handling in the clinical setting. In contrast, aldosterone is exceedingly potent with respect to Na^+ retention, but has only modest potency for effects on carbohydrate metabolism.

2.1.5.2 General Mechanisms for Corticosteroid Effects.

Corticosteroids interact with specific receptor proteins in target tissues to regulate the expression of corticosteroid-responsive genes, thereby changing the levels and array of proteins synthesized by the various target tissues (Figure 2.7). Although corticosteroids predominantly act to increase expression of target genes, there are well-documented examples in which glucocorticoids decrease transcription of target genes (De Bosscher *et al.*, 2003), as discussed below. In addition to these genomic effects, some immediate actions of corticosteroids may be mediated by membrane-bound receptors (Norman *et al.*, 2004).

The receptors for corticosteroids are members of the nuclear receptor family of transcription factors that transduce the effects of a diverse array of small, hydrophobic ligands, including the steroid hormones, thyroid hormone, vitamin D, and retinoids.

These receptors share two highly conserved domains: a region of approximately 70 amino acids forming two zinc-binding domains, called *zinc fingers*, that are essential for the interaction of the receptor with specific DNA sequences, and a region at the carboxyl terminus that interacts with ligand (the ligand-binding domain).

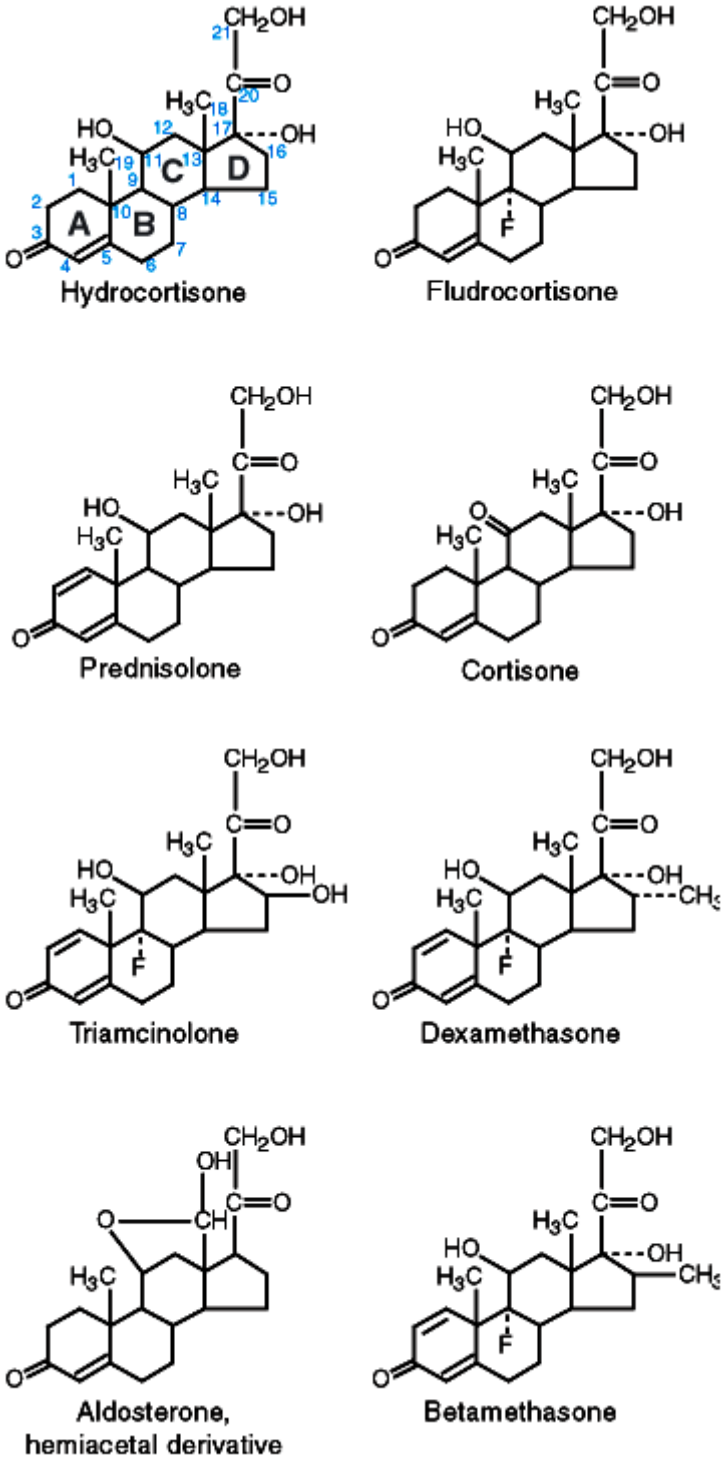


Figure 2.6 Structure and nomenclature of corticosteroid products and selected synthetic derivatives

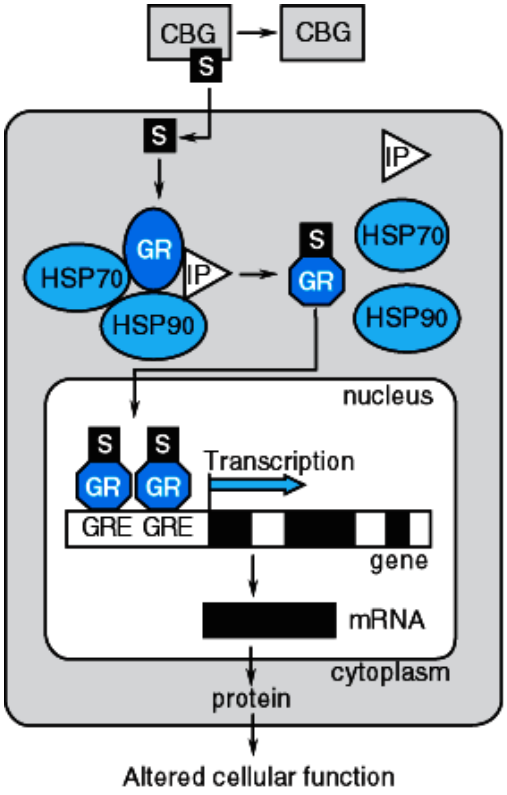


Figure 2.7 Intracellular mechanism of action of the glucocorticoid receptor

Although complete loss of glucocorticoid receptor (GR) function apparently is lethal, mutations leading to partial loss of GR function have been identified in rare patients with generalized glucocorticoid resistance (Bray *et al.*, 2003).

2.1.5.3 Glucocorticoid Receptor

The GR resides predominantly in the cytoplasm in an inactive form until it binds glucocorticoids (Figure 2.6). Steroid binding results in receptor activation and translocation to the nucleus.

The recognition that the metabolic effects of glucocorticoids generally are mediated by transcriptional activation, while the antiinflammatory effects largely are mediated by transrepression, suggests that selective GR ligands may maintain the

antiinflammatory actions while lessening the metabolic side effects (Coghlan *et al.*, 2003).

2.1.5.4 Carbohydrate and Protein Metabolism

Corticosteroids profoundly affect carbohydrate and protein metabolism. Teleologically, these effects of glucocorticoids on intermediary metabolism can be viewed as protecting glucose-dependent tissues (*e.g.*, the brain and heart) from starvation. They stimulate the liver to form glucose from amino acids and glycerol and to store glucose as liver glycogen.

The mechanisms by which glucocorticoids inhibit glucose utilization in peripheral tissues are not fully understood. Glucocorticoids decrease glucose uptake in adipose tissue, skin, fibroblasts, thymocytes, and polymorphonuclear leukocytes.

Similarly, the mechanisms by which the glucocorticoids promote gluconeogenesis are not fully defined. Amino acids mobilized from a number of tissues in response to glucocorticoids reach the liver and provide substrate for the production of glucose and glycogen. In the liver, glucocorticoids induce the transcription of a number of enzymes.

2.1.5..6 Lipid Metabolism

Two effects of corticosteroids on lipid metabolism are firmly established. The first is the dramatic redistribution of body fat that occurs in settings of endogenous or pharmacologically induced hypercorticism, such as Cushing's syndrome. The other is the permissive facilitation of the lipolytic effect of other agents, such as growth hormone and adrenergic receptor agonists, resulting in an increase in free fatty acids after glucocorticoid administration. With respect to fat distribution, there is increased fat in the back of the neck ("buffalo hump"), face ("moon facies"), and supraclavicular area, coupled with a loss of fat in the extremities.

2.1.5..7 Electrolyte and Water Balance

Aldosterone is by far the most potent endogenous corticosteroid with respect to fluid and electrolyte balance. Thus, electrolyte balance is relatively normal in patients with adrenal insufficiency due to pituitary disease, despite the loss of glucocorticoid production by the inner cortical zones. Mineralocorticoids act on the distal tubules and collecting ducts of the kidney to enhance reabsorption of Na^+ from the tubular fluid; they also increase the urinary excretion of K^+ and H^+. Conceptually, it is useful to think of aldosterone as stimulating a renal exchange between Na^+ and K^+ or H^+, although this does not involve a simple 1:1 exchange of cations in the renal tubule.

Glucocorticoids also exert effects on fluid and electrolyte balance, largely due to permissive effects on tubular function and actions that maintain glomerular filtration rate. Glucocorticoids play a permissive role in the renal excretion of free water.

In addition to their effects on monovalent cations and water, glucocorticoids also exert multiple effects on Ca^{2+} metabolism. Steroids interfere with Ca^{2+} uptake in the gut and increase Ca^{2+} excretion by the kidney. These effects collectively lead to decreased total body Ca^{2+} stores.

2.1.5.7 Cardiovascular System

The most striking effects of corticosteroids on the cardiovascular system result from mineralocorticoid-induced changes in renal Na^+ excretion, as is evident in primary aldosteronism. The resultant hypertension can lead to a diverse group of adverse effects on the cardiovascular system, including increased atherosclerosis, cerebral hemorrhage, stroke, and hypertensive cardiomyopathy.

2.1.5.8 Skeletal Muscle

Permissive concentrations of corticosteroids are required for the normal function of skeletal muscle, and diminished work capacity is a prominent sign of

adrenocortical insufficiency. In patients with Addison's disease, weakness and fatigue are frequent symptoms that may reflect an inadequacy of the circulatory system. Excessive amounts of either glucocorticoids or mineralocorticoids also impair muscle function. In primary aldosteronism, muscle weakness results primarily from hypokalemia rather than from direct effects of mineralocorticoids on skeletal muscle.

2.1.5.9 Central Nervous System

Corticosteroids exert a number of indirect effects on the CNS, through maintenance of blood pressure, plasma glucose concentrations, and electrolyte concentrations. Increasingly, direct effects of corticosteroids on the CNS have been recognized, including effects on mood, behavior, and brain excitability.

2.1.5.10 Formed Elements of Blood

Glucocorticoids exert minor effects on hemoglobin and erythrocyte content of blood, as evidenced by the frequent occurrence of polycythemia in Cushing's syndrome and of normochromic, normocytic anemia in adrenal insufficiency. More profound effects are seen in the setting of autoimmune hemolytic anemia, in which the immunosuppressive effects of glucocorticoids can diminish the self-destruction of erythrocytes.

2.1.5.11 Anti-inflammatory and Immunosuppressive Actions

In addition to their effects on lymphocyte number, corticosteroids profoundly alter the immune responses of lymphocytes. These effects are an important facet of the antiinflammatory and immunosuppressive actions of the glucocorticoids. Glucocorticoids can prevent or suppress inflammation in response to multiple inciting events, including radiant, mechanical, chemical, infectious, and immunological stimuli. Although the use of glucocorticoids as antiinflammatory agents does not address the underlying cause of the disease, the suppression of inflammation is of enormous clinical utility and has made these drugs among the most frequently prescribed agents. Similarly, glucocorticoids are of immense value in treating diseases

that result from undesirable immune reactions. These diseases range from conditions that predominantly result from humoral immunity, such as urticaria, to those that are mediated by cellular immune mechanisms, such as transplantation rejection. The immunosuppressive and antiinflammatory actions of glucocorticoids are inextricably linked, perhaps because they both involve inhibition of leukocyte functions.

Multiple mechanisms are involved in the suppression of inflammation by glucocorticoids. It is now clear that glucocorticoids inhibit the production by multiple cells of factors that are critical in generating the inflammatory response. As a result, there is decreased release of vasoactive and chemoattractive factors, diminished secretion of lipolytic and proteolytic enzymes, decreased extravasation of leukocytes to areas of injury, and ultimately, decreased fibrosis. Glucocorticoids can also reduce expression of proinflammatory cytokines, such as COX-2 and NOS2. Some of the cell types and mediators that are inhibited by glucocorticoids are summarized in Table 2.1 The net effect of these actions on various cell types is to diminish markedly the inflammatory response.

Table 2.1 Effects of Glucocorticoids on Components of Inflammatory / Immune Responses

CELL TYPE	FACTOR	COMMENTS
Macrophages and monocytes	Arachidonic acid and its metabolites (prostaglandins and leukotrienes)	Mediated by glucocorticoid inhibition of cyclooxygenase-2 and phospholipase A_2.
	Cytokines, including: interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α)	Production and release are blocked. The cytokines exert multiple effects on inflammation (*e.g.*, activation of T cells, stimulation of fibroblast proliferation).
	Acute phase reactants	These include the third component of complement.
Endothelial cells	Endothelial leukocyte adhesion molecule-1 (ELAM-1) and	ELAM-1 and ICAM-1 are intracellular adhesion molecules that are critical for leukocyte localization.

	intracellular adhesion molecule-1 (ICAM-1)	
	Acute phase reactants	Same as above, for macrophages and monocytes.
	Cytokines (*e.g.*, IL-1)	Same as above, for macrophages and monocytes.
	Arachidonic acid derivatives	Same as above, for macrophages and monocytes.
Basophils	Histamine, leukotriene C4	IgE-dependent release inhibited by glucocorticoids.
Fibroblasts	Arachidonic acid metabolites	Same as above for macrophages and monocytes. Glucocorticoids also suppress growth factor-induced DNA synthesis and fibroblast proliferation.
Lymphocytes	Cytokines (IL-1, IL-2, IL-3, IL-6, TNF-α, GM-CSF, interferon-γ)	Same as above for macrophages and monocytes.

The influence of stressful conditions on immune defense mechanisms is well documented, as is the contribution of the HPA axis to the stress response (Sapolsky *et al.*, 2000). This has led to a growing appreciation of the importance of glucocorticoids as physiological modulators of the immune system, where glucocorticoids appear to protect the organism against life-threatening consequences of a full-blown inflammatory response (Chrousos, 1995).

2.1.6 Pharmacokinetics of corticoids

2.1.6.1 Absorption

Hydrocortisone and numerous congeners, including the synthetic analogs, are orally effective. Certain water-soluble esters of hydrocortisone and its synthetic congeners are administered intravenously to achieve high concentrations of drug rapidly in body fluids. More prolonged effects are obtained by intramuscular injection of suspensions of hydrocortisone, its esters, and congeners. Minor changes in

chemical structure may markedly alter the rate of absorption, time of onset of effect, and duration of action.

Glucocorticoids also are absorbed systemically from sites of local administration, such as synovial spaces, the conjunctival sac, skin, and respiratory tract. When administration is prolonged, when the site of application is covered with an occlusive dressing, or when large areas of skin are involved, the absorption may be sufficient to cause systemic effects, including suppression of the HPA axis.

2.1.6.2 Transport, Metabolism, and Excretion.

After absorption, 90% or more of cortisol in plasma is reversibly bound to protein under normal circumstances. Only the fraction of corticosteroid that is unbound can enter cells to mediate corticosteroid effects. Two plasma proteins account for almost all of the steroid-binding capacity: corticosteroid-binding globulin (CBG; also called transcortin), and albumin. At normal or low concentrations of corticosteroids, most of the hormone is protein-bound. At higher steroid concentrations, the capacity of protein binding is exceeded, and a greater fraction of the steroid exists in the free state. Corticosteroids compete with each other for binding sites on CBG. CBG has relatively high affinity for cortisol and most of its synthetic congeners and low affinity for aldosterone and glucuronide-conjugated steroid metabolites; thus, greater percentages of these latter steroids are found in the free form.

All of the biologically active adrenocortical steroids and their synthetic congeners have a double bond in the 4,5 position and a ketone group at C 3 (Figure 2.6). As a general rule, the metabolism of steroid hormones involves sequential additions of oxygen or hydrogen atoms, followed by conjugation to form water-soluble derivatives. Reduction of the 4,5 double bond occurs at both hepatic and extrahepatic sites, yielding inactive compounds. Subsequent reduction of the 3-ketone substituent to the 3-hydroxyl derivative, forming tetrahydrocortisol, occurs only in the liver. Most of these A ring-reduced steroids are conjugated through the 3-hydroxyl group with sulfate or glucuronide by enzymatic reactions that take place in the liver,

and to a lesser extent in the kidney. The resultant sulfate esters and glucuronides are water-soluble and are the predominant forms excreted in urine. Neither biliary nor fecal excretion is of quantitative importance in humans.

Synthetic steroids with an 11-keto substituent, such as cortisone and *prednisone*, must be enzymatically reduced to the corresponding 11-hydroxy derivative before they are biologically active. The type 1 isozyme of 11-hydroxysteroid dehydrogenase catalyzes this reduction, predominantly in the liver, but also in specialized sites such as adipocytes, bone, eye, and skin. In settings in which this enzymatic activity is impaired, it is prudent to use steroids that do not require enzymatic activation (*e.g.*, hydrocortisone and prednisolone rather than cortisone or prednisone).

Such settings include severe hepatic failure and patients with the rare condition of cortisone reductase deficiency, who are unable to activate the 11-keto steroids because of a partial loss of 11 HSD1 activity and a relative deficiency in the enzyme hexose-6-phosphate dehydrogenase, which supplies reducing equivalents to the 11-hydroxysteroid dehydrogenase.

2.1.7 Structure-Activity Relationships

Chemical modifications to the cortisol molecule have generated derivatives with greater separations of glucocorticoid and mineralocorticoid activity; for a number of synthetic glucocorticoids, the effects on electrolytes are minimal even at the highest doses used (Table 2.2).

Table 2.2 Relative Potencies and Equivalent Doses of Representative Corticosteroids

Compound	**Anti inflammatory potency**	**Na^+-retaining potency**	**Duration of action***	**Equivalent dose, [a] (mg)**
Cortisol	1	1	S	20
Cortisone	0.8	0.8	S	25
Fludrocortisone	10	125	I	[b]
Prednisone	4	0.8	I	5

Prednisolone	4	0.8	I	5
6α-Methyl prednisolone	5	0.5	I	4
Triamcinolone	5	0	I	4
Betamethasone	25	0	L	0.75
Dexamethasone	25	0	L	0.75

* S, short (*i.e.*, 8-12 hour biological half-life); I, intermediate (*i.e.*, 12-36 hour biological half-life); L, long (*i.e.*, 36-72 hour biological half-life).

[a] These dose relationships apply only to oral or intravenous administration, as glucocorticoid potencies may differ greatly following intramuscular or intraarticular administration.

[b]This agent is not used for glucocorticoid effects.

Based on these differential potencies, the corticosteroids traditionally are divided into mineralocorticoids and glucocorticoids. Estimates of potencies of representative steroids in these actions are listed in Table 2.2.

In addition, these modifications have led to derivatives with greater potencies and with longer durations of action. A vast array of steroid preparations is available for oral, parenteral, and topical use. Some of these agents are summarized in Table 2.3.

Table 2.3. Available Preparations of Adrenocortical Steroids and Their Synthetic Analogs

Nonproprietary name (trade name)	**Types of preparations**	**Nonproprietary name (trade name)**	**Types of preparations**
Alclometasone dipropionate (ACLOVATE)	Topical	Cortisol (hydrocortisone) valerate (WESTCORT)	Topical
Amcinonide	Topical	Cortisone acetate	Oral,

(CYCLOCORT)		(CORTONE ACETATE)	injectable
Beclomethasone dipropionate (BECLOVENT, VANCERIL, others)	Inhalation	Desonide (DESOWEN, TRIDESILON)	Topical
Betamethasone (CELESTONE)	Oral	Desoximetasone (TOPICORT)	Topical
Betamethasone dipropionate (DIPROSONE, others)	Topical	Dexamethasone (DECADRON, others)	Oral, topical
Betamethasonesodium phosphate (CELESTONE PHOSPHATE, others)	Injectable	Dexamethasone acetate (DECADRON-LA, others)	Injectable
Betamethasonesodium phosphate and acetate (CELESTONE SOLUSPAN)	Injectable	Dexamethasonesodium phosphate (DECADRON PHOSPHATE, HEXADROL PHOSPHATE, others)	Topical, ophthalmic, otic, injectable
Betamethasone valerate (BETA-VAL, VALISONE, others)	Topical	Diflorasone diacetate (FLORONE, MAXIFLOR)	Topical
Budesonide (PULMICORT, RHINOCORT)	Inhalation	Fludrocortisone acetate* (FLORINEF)	Oral
Clobetasol propionate (TEMOVATE)	Topical	Flunisolide (AEROBID, NASALIDE)	Inhalation
Clocortolone pivalate (CLODERM)	Topical	Fluocinolone acetonide (FLUONID, SYNALAR, others)	Topical
Cortisol (hydrocortisone) (CORTEF, HYDROCORTONE,	Topical, enema, otic solutions, oral, injectable	Fluocinonide (LIDEX)	Topical

others)			
Cortisol (hydrocortisone) acetate (HYDRO CORTONE ACETATE others)	Topical, suppositories, rectal foam,	Fluorometholone acetate (FLAREX)	Ophthalmic
Cortisol (hydrocortisone) butyrate (LOCOID)	Topical	Flurandrenolide (CORDRAN)	Topical
Cortisol (hydrocortisone) cypionate (CORTEF)	Oral	Medrysone (HMS LIQUIFILM)	Ophthalmic
Cortisol (hydrocortisone) sodium phosphate	Injectable	Methylprednisolone acetate (DEPO-MEDROL, MEDROL ACETATE)	Topical, injectable
Cortisol (hydrocortisone) sodium succinate (A-HYDROCORT, SOLU-CORTEF)	Injectable	Methylprednisolone sodium succinate (A-METHAPRED, SOLU-MEDROL)	Injectable
Mometasone furoate (ELOCON)	Topical	Prednisone (DELTASONE, others)	Oral
Prednisolone (DELTA-CORTEF)	Oral	Triamcinolone (ARISTOCORT, KENACORT)	Oral
Prednisolone acetate (ECONOPRED, others)	Ophthalmic, injectable	Triamcinolone acetonide (KENALOG, others)	Topical, inhalation, injectable
Prednisolone sodium phosphate (PEDIAPRED, others)	Oral, ophthalmic, injectable	Triamcinolone diacetate (ARISTOCORT, KENACORT DIACETATE)	Oral, injectable
Prednisolone tebutate (HYDELTRA-T.B.A., others)	Injectable	Triamcinolone hexacetonide (ARISTOSPAN)	Injectable
* Fludrocortisone acetate is intended for use as a mineralocorticoid.			

Note: Topical preparations include agents for application to skin or mucous membranes in creams, solutions, ointments, gels, pastes (for oral lesions), and aerosols; *ophthalmic* preparations include solutions, suspensions, and ointments; *inhalation* preparations include agents for nasal or oral inhalation. (Bernard *et al.*, 2006)

2.2 Allopathic steroids and their therapeutic uses

2.2.1 Replacement Therapy

Adrenal insufficiency can result from structural or functional lesions of the adrenal cortex (primary adrenal insufficiency or Addison's disease) or from structural or functional lesions of the anterior pituitary or hypothalamus (secondary adrenal insufficiency). In developed countries, primary adrenal insufficiency most frequently is secondary to autoimmune adrenal disease, whereas tuberculous adrenalitis is the most frequent etiology in underdeveloped countries. Other causes include adrenalectomy, bilateral adrenal hemorrhage, neoplastic infiltration of the adrenal glands, acquired immunodeficiency syndrome, inherited disorders of the steroidogenic enzymes, and X-linked adrenoleukodystrophy (Carey *et al.*, 1997). Secondary adrenal insufficiency resulting from pituitary or hypothalamic dysfunction generally presents in a more insidious manner than does the primary disorder, probably because mineralocorticoid biosynthesis is preserved.

2.2.2 *Acute Adrenal Insufficiency*

This life-threatening disease is characterized by gastrointestinal symptoms (nausea, vomiting, and abdominal pain), dehydration, hyponatremia, hyperkalemia, weakness, lethargy, and hypotension. It usually is associated with disorders of the adrenal rather than the pituitary or hypothalamus, and sometimes follows abrupt withdrawal of glucocorticoids used at high doses or for prolonged periods.

2.2.3 *Chronic Adrenal Insufficiency*

Patients with chronic adrenal insufficiency present with many of the same manifestations seen in adrenal crisis, but with lesser severity. These patients require

daily treatment with corticosteroids (Coursin *et al.*, 2002). Traditional replacement regimens have used hydrocortisone in doses of 20 to 30 mg/day. *Cortisone acetate*, which is inactive until converted to cortisol by 11 HSD, also has been used in doses ranging from 25 to 37.5 mg/day.

2.2.4 *Congenital Adrenal Hyperplasia*

This term denotes a group of genetic disorders in which the activity of one of the several enzymes required for the biosynthesis of glucocorticoids is deficient. The impaired production of cortisol and the consequent lack of negative feedback inhibition lead to increased release of ACTH. As a result, other hormonally active steroids that are proximal to the enzymatic block in the steroidogenic pathway are produced in excess. Congenital adrenal hyperplasia (CAH) includes a spectrum of disorders whose precise clinical presentation, laboratory findings, and treatment depend on which of the steroidogenic enzymes is deficient.

2.2.5 Therapeutic Uses in Nonendocrine Diseases

Outlined below are important uses of glucocorticoids in diseases that do not directly involve the HPA axis. The disorders discussed are not inclusive; rather, they illustrate the principles governing glucocorticoid use in selected diseases for which glucocorticoids are more frequently employed. The dosage of glucocorticoids varies considerably depending on the nature and severity of the underlying disorder. For convenience, approximate doses of a representative glucocorticoid (generally prednisone) are provided.

2.2.6 *Rheumatic Disorders*

Glucocorticoids are used widely in the treatment of a variety of rheumatic disorders and are a mainstay in the treatment of the more serious inflammatory rheumatic diseases, such as systemic lupus erythematosus, and a variety of vasculitic disorders, such as polyarteritis nodosa, Wegener's granulomatosis, Churg-Strauss

syndrome, and giant cell arteritis. For these more serious disorders, the starting dose of glucocorticoids should be sufficient to suppress the disease rapidly and minimize resultant tissue damage. Initially, prednisone (1 mg/kg per day in divided doses) often is used, generally followed by consolidation to a single daily dose, with subsequent tapering to a minimal effective dose as determined by clinical variables.

While they are an important component of treatment of rheumatic diseases, glucocorticoids are often used in conjunction with other immunosuppressive agents such as *cyclophosphamide* and *methotrexate,* which offer better long-term control than steroids alone. The exception is giant cell arteritis, for which glucocorticoids remain superior to other agents. Caution should be exercised in the use of glucocorticoids in some forms of vasculitis (*e.g.,* polyarteritis nodosa), for which underlying infections with hepatitis viruses may play a pathogenetic role. Although glucocorticoids are indicated in these cases, there is at least a theoretical concern that glucocorticoids may complicate the course of the viral infection by suppressing the immune system. To facilitate drug tapering and/or conversion to alternate-day treatment regimens, the intermediate-acting glucocorticoids such as prednisone and methylprednisolone are generally preferred over longer-acting steroids such as dexamethasone.

2.2.7 *Renal Diseases*

Patients with nephrotic syndrome secondary to minimal change disease generally respond well to steroid therapy, and glucocorticoids clearly are the first-line treatment in both adults and children. Initial daily doses of prednisone are 1 to 2 mg/kg for 6 weeks, followed by a gradual tapering of the dose over 6 to 8 weeks, although some nephrologists advocate alternate-day therapy. Objective evidence of response, such as diminished proteinuria, is seen within 2 to 3 weeks in 85% of patients, and more than 95% of patients will have remission within 3 months. Cessation of steroid therapy frequently is complicated by disease relapse, as manifested by recurrent proteinuria. Patients who relapse repeatedly are termed *steroid-resistant* and often are treated with other immunosuppressive drugs such as

azathioprine or cyclophosphamide. Patients with renal disease secondary to systemic lupus erythematosus also are generally given a therapeutic trial of glucocorticoids.

2.2.8 *Allergic Disease*

The onset of action of glucocorticoids in allergic diseases is delayed, and patients with severe allergic reactions such as anaphylaxis require immediate therapy with epinephrine: for adults, 0.3 to 0.5 ml of a 1:1000 solution intramuscularly or subcutaneously (repeated as often as every 15 minutes for up to three additional doses if necessary). The manifestations of allergic diseases of limited duration such as hay fever, serum sickness, urticaria, contact dermatitis, drug reactions, bee stings, and angioneurotic edema can be suppressed by adequate doses of glucocorticoids given as supplements to the primary therapy

2.2.9 Bronchial Asthma and Other Pulmonary Conditions

Corticosteroids frequently are used in bronchial asthma. They sometimes are employed in chronic obstructive pulmonary disease (COPD), particularly when there is some evidence of reversible obstructive disease. Data supporting the efficacy of corticosteroids are much more convincing for bronchial asthma than for COPD. The increased use of corticosteroids in asthma reflects an increased appreciation of the role of inflammation in the immunopathogenesis of this disorder. In severe asthma attacks requiring hospitalization, aggressive treatment with parenteral glucocorticoids is considered essential, even though their onset of action is delayed for 6 to 12 hours. Intravenous administration of 60 to 120 mg of methylprednisolone (or equivalent) every 6 hours is used initially, followed by daily oral doses of prednisone (30 to 60 mg) as the acute attack resolves. The dose then is tapered gradually, with withdrawal planned for 10 days to 2 weeks after initiation of steroid therapy. In general, patients subsequently can be managed on their prior medical regimen.

2.2.10 Infectious Diseases

Although the use of immunosuppressive glucocorticoids in infectious diseases may seem paradoxical, there are a limited number of settings in which they are indicated in the therapy of specific infectious pathogens. One dramatic example of such beneficial effects is seen in AIDS patients with *Pneumocystis carinii* pneumonia and moderate to severe hypoxia; addition of glucocorticoids to the antibiotic regimen increases oxygenation and lowers the incidence of respiratory failure and mortality. Similarly, glucocorticoids clearly decrease the incidence of long-term neurological impairment associated with *Haemophilus influenzae* type b meningitis in infants and children 2 months of age or older.

A long-standing controversy in medicine is the use of glucocorticoids in septic shock (Annane and Cavaillon, 2003). Although studies initially suggested a benefit from the routine administration of glucocorticoids to subjects with septic shock associated with gram-negative bacteremia, subsequent studies showed that glucocorticoid therapy in supraphysiologic doses was actually associated with increased mortality.

2.2.11 *Ocular Diseases*

Ocular pharmacology, including some consideration of the use of glucocorticoids, is discussed in. Glucocorticoids frequently are used to suppress inflammation in the eye and can preserve sight when used properly. They are administered topically for diseases of the outer eye and anterior segment and attain therapeutic concentrations in the aqueous humor after instillation into the conjunctival cul-de-sac. For diseases of the posterior segment, systemic administration is required. Generally, ocular use of glucocorticoids should be supervised by an ophthalmologist.

2.2.12 *Skin Diseases*

Glucocorticoids are remarkably efficacious in the treatment of a wide variety of inflammatory dermatoses. As a result, a large number of different preparations and concentrations of topical glucocorticoids of varying potencies are available. A typical

regimen for an eczematous eruption is 1% hydrocortisone ointment applied locally twice daily. Effectiveness is enhanced by application of the topical steroid under an occlusive film, such as plastic wrap; unfortunately, the risk of systemic absorption also is increased by occlusive dressings, and this can be a significant problem when the more potent glucocorticoids are applied to inflamed skin.

2.2.13 *Gastrointestinal Diseases*

Glucocorticoid therapy is indicated in selected patients with inflammatory bowel disease (chronic ulcerative colitis and Crohn's disease; Patients who fail to respond to more conservative management (*i.e.,* rest, diet, and *sulfasalazine*) may benefit from glucocorticoids; steroids are most useful for acute exacerbations. In mild ulcerative colitis, hydrocortisone (100 mg) can be administered as a retention enema with beneficial effects. In more severe acute exacerbations, oral prednisone (10 to 30 mg/day) frequently is employed.

2.2.14 *Hepatic Diseases*

The use of corticosteroids in hepatic disease has been highly controversial. Glucocorticoids clearly are of benefit in autoimmune hepatitis, where as many as 80% of patients show histological remission when treated with prednisone (40 to 60 mg daily initially, with tapering to a maintenance dose of 7.5 to 10 mg daily after serum transaminase levels fall).

2.2.15 *Cerebral Edema*

Corticosteroids are of value in the reduction or prevention of cerebral edema associated with parasites and neoplasms, especially those that are metastatic. Although corticosteroids are frequently used for the treatment of cerebral edema caused by trauma or cerebrovascular accidents, controlled clinical trials do not support their use in these settings.

2.2.16 Diagnostic Applications of Adrenocortical Steroids

In addition to their therapeutic uses, glucocorticoids also are used for diagnostic purposes. To determine if patients with clinical manifestations suggestive of hypercortisolism have biochemical evidence of increased cortisol biosynthesis, an overnight dexamethasone test has been devised. Patients are given 1 mg of dexamethasone orally at 11 P.M., and cortisol is measured at 8 A.M. the following morning. Suppression of plasma cortisol to less than 1.8 µg/dl suggests strongly that the patient does not have Cushing's syndrome (Arnaldi *et al.*, 2003).

The formal dexamethasone suppression test is used in the differential diagnosis of biochemically documented Cushing's syndrome. Following determination of baseline cortisol levels for 48 hours, dexamethasone (0.5 mg every 6 hours) is administered orally for 48 hours. This dose markedly suppresses cortisol levels in normal subjects, including those who have nonspecific elevations of cortisol due to obesity or stress, but does not suppress levels in patients with Cushing's syndrome. In the high-dose phase of the test, dexamethasone is administered orally at 2 mg every 6 hours for 48 hours. Patients with pituitary-dependent Cushing's syndrome (*i.e.*, Cushing's disease) generally respond with decreased cortisol levels. In contrast, patients with ectopic production of ACTH or with adrenocortical tumors generally do not exhibit decreased cortisol levels. Despite these generalities, dexamethasone may suppress cortisol levels in some patients with ectopic ACTH production, particularly with tumors such as bronchial carcinoids, and many experts prefer to use inferior petrosal sinus sampling after CRH administration to make this distinction.

2.3 Toxicity of Adrenocortical Steroids

Two categories of toxic effects result from the therapeutic use of corticosteroids: those resulting from withdrawal of steroid therapy and those resulting from continued use at supraphysiological doses. The side effects from both categories

are potentially life-threatening and mandate a careful assessment of the risks and benefits in each patient. Following are list of issue related to toxicities of corticoids.

- Withdrawal of Therapy
- Fluid and Electrolyte Management
- Immune Responses
- Myopathy
- Cataracts
- Osteoporosis
- Osteonecrosis
- Regulation of Growth and Development

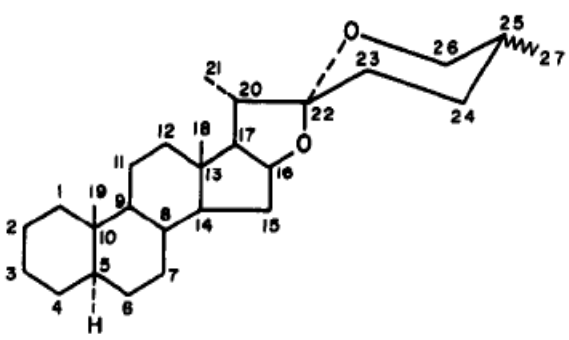
2.4 Herbal steroids

India has a rich heritage of plant based drugs both for use in preventive and curative medicines. Steroids and their related active metabolites are of great value in drug and pharmaceutical industry. They have numerous and diversified physiological functions and pharmacological effects such as influence on carbohydrate, protein, fat and purine metabolism; on electrolyte and water balance; on the functional capacities of the cardiovascular system viz., kidney, skeletal, muscle, nervous system and some organs and tissues. The term steroid (= sterol like) is derived from sterol (In Greek, Stereos= solid and ol=alcohol) as most of these compounds contain alcoholic group. All the steroids are structurally related and mostly saturated, colourless compounds found in plants and animals. Steroid includes a variety of compounds, among which sapogenins hold a very important position. Sapogenins when linked with sugar constitutes the saponins. Saponins are natural products, which have the property of forming soapy leather when shaken with water. Many plant contains sapogenins as principle chemical constituents (Table 2.4)

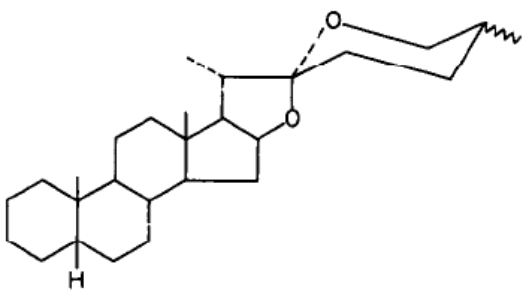
Table 2.4 Steroidal Sapogenin Yielding major Plants

S. No.	Name of Plant	Part used
1	*Agave species*	Leaves
2	*Allium fuscoviolacenum*	Bulbs
3	*Allium narcissifolium*	Bulbs
4	*Asparagus officinalis*	Roots
5	*Aspidistra elatior*	Underground parts
6	*Balanites roxburghii*	Fruits and leaves
7	*Convallaria keisukei*	Underground parts
8	*Costus speciosus*	Rhizome
9	*Dioscorea composita*	Rhizome
10	*Dioscorea deltoidea*	Rhizome
11	*Dioscorea floribunda*	Rhizome
12	*Dioscorea gracillima*	Rhizome
13	*Funkia ovata*	Leaves
14	*Kallstroemia pubescens*	Whole plant
15	*Polygonatum latifolium*	Leaves
16	*Polygonatum multiflorum*	Leaves
17	*Smilax aspera*	Leaves
18	*Smilax exfelsa*	Leaves
19	*Solanum indicum*	Fruits
20	*Solanum introsum*	Fruits
21	*Solanum khasianum*	Fruit
22	*Solanum laciniatum*	Fruit, Leaves and stem
23	*Solanum sodomaeum*	Fruit, leaves and bud
24	*Solanum trilobatum*	Fruit
25	*Solanum verbascifolium*	Fruit
26	*Solanum xanthocarpum*	Fruit
27	*Tribulus terrestris*	Over ground parts
28	*Trigonella foenum*	Leaves and seeds
29	*Trigonella foenum graceum*	Roots and Leaves
30	*Trigonella tschonoskii*	Under ground parts

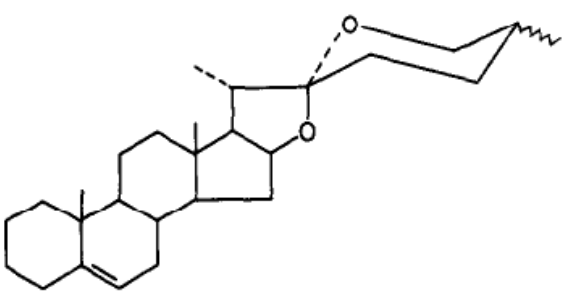
Production of steroid drugs is a large scale industry (Applezweig *et al.*, 1962). In 1967, total world consumption of steroids precursors was one thousand tones, two third of which came from diosgenin and the remaining one third from the variety of miscellaneous sources. Other steroidal alkaloids that could become available in large quantities are tomatidine, Solasodine and Neotigogenin. Till today, more than four thousand plant species have been investigated which has resulted in the identification of some thirty naturally occurring steroids sapogenins many of which could provide valuable source materials for steroids compounds (Figure 2.8).



Tigogenin, 25*R*, 3β-OH
Gitogenin, 25*R*, 2α, 3β-OH
Karatavegenin, 25*R*, 2α-OBz, 3β, 5α, 6β-OH
Alliogenin, 25*R*, 2α, 3β, 5α, 6β-OH
Hecogenin, 25*R*, 3β-OH, 12-CO
Agigenin, 25*R*, 2α, 3β, 6β-OH
Digalogenin, 25*R*, 3β, 15β-OH
Rockogenin, 25*R*, 3β, 12β-OH
β-Chlorogenin, 25*R*, 3β, 6β-OH
Digitogenin, 25*R*, 2α, 3β, 15β-OH
Neotigogenin, 25*S*, 3β-OH
Neochlorogenin, 25*S*, 3β, 6α-OH
Paniculogenin, 25*S*, 3β, 6α, 23β-OH
Neoagigenin, 25*S*, 2α, 3β, 6β-OH
Neoalliogenin, 25*S*, 2α, 3β, 5α, 6β-OH
Solagenin, 25*S*, 3-CO, 6α-OH
Sisalagenin, 25*S*, 3β-OH, 12-CO



Yonogenin, 25*R*, 2β, 3α-OH
Tokorogenin, 25*R*, 1β, 2β, 3α-OH
Epimetagenin, 25*R*, 2β, 3α, 11α-OH
Metagenin, 25*R*, 2β, 3β, 11α-OH
Prometagenin, 25*R*, 2β, 3β, 11α-OH, 4-ene
Nogiragenin, 25*R*, 3β, 11α-OH
Isorhodeasapogenin, 25*R*, 1β, 3β-OH
Sarsasapogenenin, 25*S*, 3β-OH
Rhodeasapogenin, 25*S*, 1β, 3β-OH
Convallagenin A, 25*S*, 1β, 3β, 5β-OH
Convallagenin B, 25*S*, 1β, 3β, 4β, 5β-OH
Neotokorogenin, 25*S*, 1β, 2β, 3α-OH



Diosgenin, 25 *R*, 3β–OH
Ruscogenin, 25 *R*, 1β, 3β–OH
Yuccagenin, 25 *R*, 2α, 3β–OH
Kammogenin, 25 *R*, 2α, 3β–OH, 12–CO
Pennogenin, 25 *R*, 3β, 17α–OH
Prazerigenin A, 25 *R*, 3β, 14α–OH
Epidiosgenin, 25 *R*, 3α–OH
Epiruscogenin, 25 *R*, 1β, 3α–OH
Yamogenin, 25 *S*, 3β–OH

Figure 2.8 Common herbal Saponin molecules

2.5Lesser side effects of herbal drugs

Steroids can cause liver disorders, stunted growth in children and teenagers, personality changes, ulcerous acne and high cholesterol levels. Because steroids are made from synthetically-produced testosterone, which imitates human sex hormones, they can also cause wasting of the testicles, impotence and negative changes in sexuality. Sometimes, when steroids are used illegally, they are injected with needles. Sharing these needles can also pass on the Human Immunodeficiency Virus, HIV, that causes the deadly disease AIDS. (Brancazsio *et al.*, 1986)

2.6 Traditional uses of herbal steroids

Commercially source material for steroids is only few species belonging mainly to the genus *Dioscorea* and *Solanum*. Although diosgenin has been identified in other species such as *Costus speciosus, Trigonella foenum graceum* and *Kallstromia pubescens* but there is no evidence at present that they would be interesting commercially, and therefore, they have not been described in detail. Efforts have been directed towards the cultivation of several *Solanum* species as the source material for the production of steroids. Genus *Dioscorea*, with over 600 species is widely distributed in tropical world, except few species in temperate. Some

of the species like *Dioscorea alata* and *Dioscorea esculenta* have been cultivated for a long for their edible tubers. There are about 15 species of this genus, which are known to contain steroidal sapogenins chiefly diosgenin. In the world, Mexico, Guatemala, Costa Rico, India and China are the major diosgenin producing countries. Most of the world production of diosgenin today is met from Central American species mainly *Dioscorea floribunda* and *D. composita*. The total turnover of bulk steroids in the world is estimated to be about 500 million US dollars and estimated world usage to be somewhere between 550-650 tones of diosgenin. In India, *Dioscorea deltoidea* and *D. prazeri* occurring in North-West and North-East Himalayas respectively are the natural sources of diosgenin.

2.7 Current status and plant profile of selected herbal steroids

2.7.1 *Trigonella foenum*-graecum

2.7.1.1 Definition

Fenugreek consists of the dried ripe seeds of *Trigonella foenum-graecum* L. (Fabaceae)

2.7.1.2 Vernacular Name

Sanskrit : Methika
Bengali : Methi
English : Fenugreek
Gujrati : Methi
Hindi : Methi
Kannada : Menthya Soppu
Malayalam : Ulluva
Marathi : Methis

Figure 2.9 Plant of *Trigonella foenum*

Figure 2.10 Seeds of *Trigonella foenum*

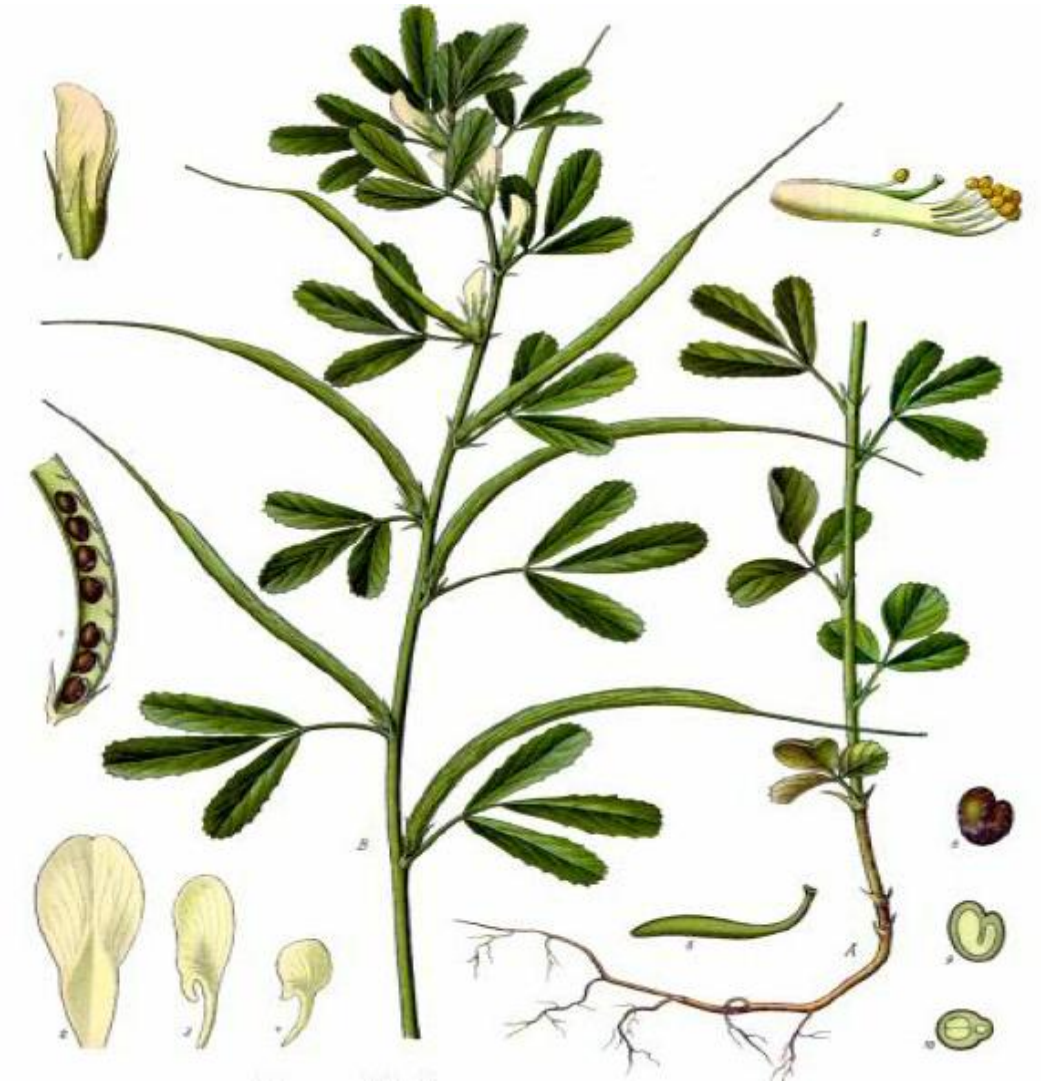


Figure 2.11 Morphological Character of *Trigonella foenum*

2.7.1.3 Description

Indigenous to the Mediterranean region, China, India and Indonesia and cultivated in these countries (*British Ph, 1996*). Annual aromatic herb, up to 60 cm high with a well developed taproot and much branched roots. Stem solitary or basally branched, terete, slightly pubescent, green to purple (Figure 2.9). Leaves petiolate, alternate, trifoliolate; stipules triangular, small, adnate to the petiole. Rachis short. Leaflets obviate or oblong, 1.5–4.0 cm long, 0.5–2.0 cm wide, upper part of margin denticulate. (The Ayurvedic Ph. India, 1999)

Flowers whitish, solitary, axillary, subsessile, 12–15 mm long. Calyx campanulate, finely pubescent, tube 4.5 mm long, with five lobes. Pistil with sessile ovary, glabrous style and capitate stigma. Fruits straight to occasionally sickle-shaped, linear pods, glabrous, with fine longitudinal veins, terminating in a beak 2–3 cm long. Seeds oblong-rhomboidal, 3– 5 mm long and 2–3 mm wide, with a deep furrow dividing each into two unequal lobes (Figure 2.10), with rounded corners, rather smooth, brownish (De Guzman *et al.*, 1999).

a) Macroscopic Characters

Odor: characteristic, aromatic; taste: slightly bitter (*European ph. 2000*).

Seeds oblong-rhomboidal, 3.0–5.0 mm long, 2.0–3.0 mm wide, 1.5–2.0 mm thick, with rounded corners, rather smooth. (*African ph. 1985)* Yellowish-brown to reddish brown, with a deep furrow dividing each seed into two unequal lobes, and a deep hilum at the intersection of the two furrows. Texture hard, not easily broken (Figure 2.11). Testa thin, endosperm translucent and viscous; cotyledons two, pale yellow, radical curved, plump and long (*European ph. 2000*).

b) Microscopic Characters

Transverse section shows an epidermis of palisade cells, one layer, with thick cuticle and thick lamellated walls, and a relatively large lumen at the lower part. Longitudinal pit-canals fine and close. Sub-epidermal layer of basket-like cells, with bar-like thickening on the radial walls, followed by a parenchymatous layer.

Endosperm of several layers of polyhedral cells with stratified mucilaginous contents and thickened walls. Cotyledons of parenchymatous cells containing fixed oil and aleurone grains up to 15 μm in diameter (*African ph. 1985).*

C) Powdered plant material

Yellowish-brown showing fragments of the testa in sectional view with thick cuticle covering epidermal cells, with an underlying hypodermis of large cells, narrower at the upper end and constricted in the middle, with bar-like thickenings of the radial walls. Yellowish-brown fragments of the epidermis in surface view, composed of small polygonal cells with thickened and pitted walls, frequently associated with the hypodermal cells, circular in outline with thickened walls. Fragments of the hypodermis viewed from below, composed of polygonal cells with bar-like thickenings extending to the upper and lower walls. Parenchyma of the testa with elongated, rectangular cells with slightly thickened walls. Fragments of endosperm with irregularly thickened, sometimes elongated cells, containing mucilage (*African ph. 1985).*

2.7.14 Identity, purity and strength (*African ph. 1985, British Ph, 1996)*

Foreign matter	:	Not more than 2%
Total Ash	:	Not more than 5%
Acid-insoluble ash	:	Not more than 2%
Alcohol-soluble extractive	:	Not less than 5%
Water-soluble extractive	:	Not less than 20%

Microbiological Purity tests

Tests for specific microorganisms and microbial contamination limits are as described in the WHO guidelines on quality control methods for medicinal plants (*QC Methods, WHO, 1998*).

Pesticide residues

The recommended maximum limit of aldrin and dieldrin is not more than 0.05 mg/kg. For other pesticides, see the *European pharmacopoeia* and the WHO guidelines on quality control methods for medicinal plants and pesticide residues (*QC Methods, WHO, 1998*).

2.7.1.5 Substitutes and adulterants (Henriette's herbal., 1999)

The taste fenugreek seeds are often substituted with maple syrup and the leaf substituted with celery leaf. The Fenugreek seed power is mainly adulterated with starchy material and such adulteration can be detected by Iodine test because fenugreek powder does not contain starchy material

2.7.1.6 Phyto chemistry (Hansel *et al.*1994, Bisset *et al.*, 1994)

It contains Alkaloides like Trimethylamine, Neurin, Trigonelline (up to 0.37%), Choline, Gentianine, Carpaine and Betain. Amino acids found are Isoleucine, 4-Hydroxyisoleucine, Histidine, Leucine, lysine, L-tryptophan, Argenine. Major Saponins (0.6–1.7%) are Graecunins, fenugrin B, fenugreekine, trigofoenosides A-G. It also contains Steroidal sapinogens like Yamogenin, diosgenin, smilagenin, sarsasapogenin, tigogenin, neotigogenin, gitogenin, neogitogenin, yuccagenin. Other chemical constituents like Coumarin, lipids, vitamins, minerals also reported (Figure 2.12).

The structure of trigonelline is presented below (Raghunathan *et al.*, 1982).

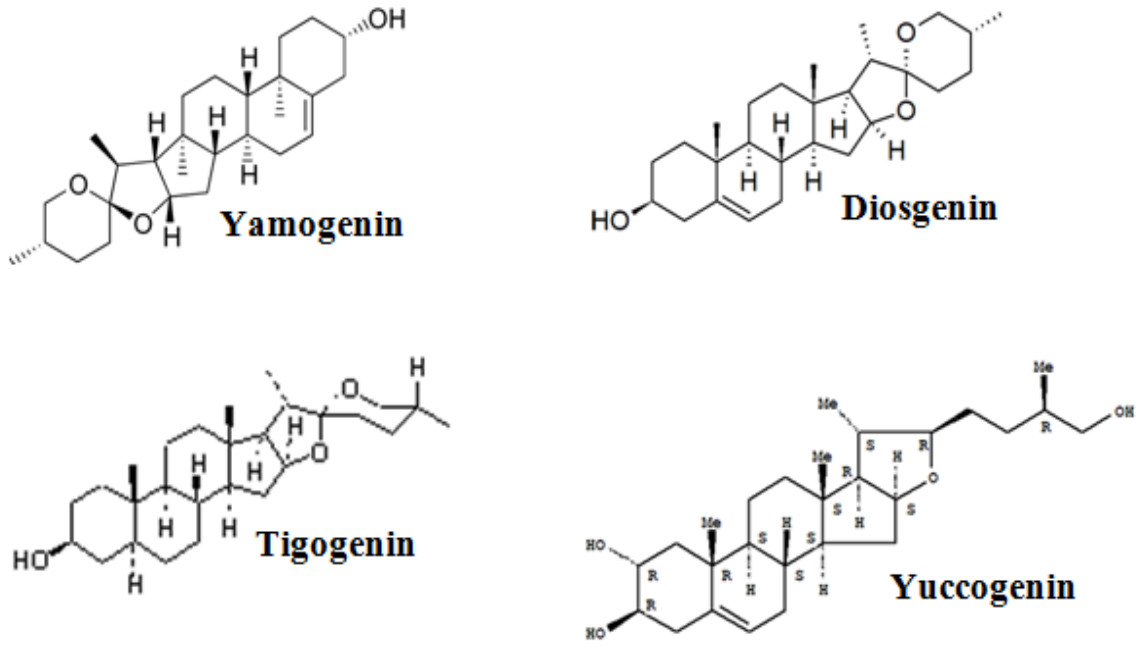


Figure 2.12 Major Saponins from *Trigonella foenum*

2.7.1.7 Traditional uses

As an adjunct for the management of hypercholesterolaemia, and hyperglycaemia in cases of diabetes mellitus (*Sharma et al., 1990*). Prevention and treatment of mountain sickness (*Bensky et al., 1993*). Internally for loss of appetite, and externally as a poultice for local inflammations (Blumenthal *et al., 2000*). Treatment of pain, and weakness and oedema of the legs (*Chinese Ph. 2000*). As an aphrodisiac, carminative, diuretic, emmenagogue, emollient, galactagogue and tonic (*Farnsworth et al., 2001*). Treatment of abdominal colic, bronchitis, diarrhoea, eczema, gout, indigestion, dropsy, fever, impotence, chronic cough, liver disorders, wounds and the common cold. Fenugreek restores nitrogen in the soil as it comes from the pea family, hence used in Organic farming as a soil renovator. Fenugreek plants are also used as an oriental cattle fodder (Farnsworth *et al., 2001*).

2.7.1.8 Experimental and Clinical Pharmacology

Owing to its effect on blood glucose levels in diabetic patients, *Trigonella foenum* should only be used in conjunction with oral antihyperglycaemic agents or insulin under the supervision of a health-care professional (*Mahmoud et al., 1992*).

An aqueous and a chloroform/methanol extract of the seeds were not mutagenic in the *Salmonella*/microsome assay using *S. typhimurium* strains TA98 and TA100. The extracts were also not mutagenic in pig kidney cells or in trophoblastic placental cells (Mahmoud *et al., 1992*). Dried seeds, extracts, fl uidextracts and tinctures. Store in a tightly sealed container away from heat and light (Bluementhal *et al.*, 2000).

Numerous clinical studies have assessed the effects of the seeds on serum cholesterol and glucose levels in patients with mild to moderate hypercholesterolaemia or diabetes (Al- Habbori *et al.*, 1998).

In a crossover trial, the effects of a powder of the seeds of *Momordica charantia* (MC) or *Trigonella foenum-graecum* (TF), or a combination of the two investigated in 20 hypercholesterolaemic non-insulin dependent diabetes mellitus patients. Mean serum total cholesterol was significantly ($P < 0.001$) decreased after treatment (Awal *et al.*, 1999).

In a placebo-controlled clinical trial, the effect of ginger and Trigonella on blood lipids, blood sugar, platelet aggregation, and fibrinogen and fibrinolytic activity was investigated. (Bordia *et al.,1997*).

A prescribed diet with or without the seeds was given to 60 patients with non-insulin dependent diabetes for a 7-day preliminary period and then for a 24-week trial. The 24-hour urinary sugar excretion was significantly reduced (Sharma *et al., 1996*).

The effect of the seeds on blood glucose and the serum lipid profile was assessed in patients with insulin-dependent (type I) diabetes patients. The high density-lipoprotein cholesterol concentrations remained unchanged (Raghuram *et al., 1990*).

In a long-term study, patients with diabetes ingested seeds per day for 24 weeks. No changes in body weight or levels of liver enzymes, bilirubin or creatinine were observed, (Sharma *et al., 1996*).

Antihypercholesterolaemic activity

Intragastric administration of an ethanol extract of Trigonella daily for 4 weeks to hypercholesterolaemic rats reduced plasma cholesterol levels by 18% and 25%, respectively (Stark *et al., 1993*).

Antihyperglycaemic activity

Oral administration of 250.0 mg of an aqueous or methanol extract of seeds daily to normal and diabetic rats significantly reduced blood glucose levels after eating or the administration of glucose ($P < 0.05$) (Ali *et al., 1995*).

Intragastric administration seeds to rats with or without alloxan-induced diabetes produced a significant decrease ($P < 0.05$) in blood glucose (Khosla *et al., 1995*). Intragastric administration of a single dose of an ethanol extract of the seeds to mice with or without alloxan-induced diabetes reduced serum glucose levels. (Ajabnoor *et al., 1988*).

Anti-implantation activity

Extracts of the seeds (undefined) exhibited anti-implantation effects (approximately 30%) in rats when administered orally in a single dose of pregnancy. The average number of fetal implants was significantly decreased ($P < 0.05$) (Rastogi *et al., 1993*).

Antioxidant activity

Administration of 2 g/kg bw of the seeds in the diet of rats with alloxaninduced diabetes lowered lipid peroxidation, increased the glutathione and β-carotene concentrations and reduced the α-tocopherol content in the blood (Ravikumar *et al., 1999*).

Gastrointestinal effects

Administration of a steroid-enriched extract of the seeds per day in the diet to rats with or without streptozotocin-induced diabetes significantly ($P < 0.01$) increased food intake and the motivation to eat (Petit *et al.*, 1995).

Toxicology

Intragastric administration of a debitterized powder of the seeds to mice and rats, did not produce any signs of acute toxicity or mortality. Pathological changes observed included fatty cytoplasmic vacuolation in the liver, necrosis of hepatocytes with lymphocytic infiltration, epithelial degeneration of the renal tubules, catarrhal enteritis, myositis and peritonitis (Dhawan *et al., 1977*).

Intragastric administration of an aqueous or 95% ethanol extract of the seeds (dose not specified) stimulated uterine contractions in healthy and pregnant rats, mice and guinea-pigs. (Setty *et al.*, 1976).

2.7.2 *Dioscorea alata*

2.7.2.1 Definition: *It consist of* a tuberous root *of Dioscorea alata var. purpurea*, known as purple yam belonging to family Dioscoreaceae.

2.7.2.2 Vernacular Name:

English : Greateryam, Asiatic yam, Guyana arrowroot, water yam

Hindi : Khamalu, Chupri alu

Gujarati :Ratalu

Marathi : kondfal

Tamil : rasa valli kilangu

Bengali : Bengo nari, Chupri alu, Kham alu

Kannada : Tuna genasu

Telugu : Dukka pendalam, Gunapendalamu, Niluvapendalamu

Oriya : Kambo alu

Malayalam : Kachil, Kavuttu

2.7.2.3 Description:

Normally grows for 8-10 months, then goes dormant for 3-4 months, with aerial stems dying back during dormancy. Fertile seeds rarely produced; spread by aerial tubers and fragments of underground tuber (Figure 2.13).

Depending upon the yam variety, of which there are about 200, its flesh may be of varying colors including white, ivory, yellow or purple while its thick skin may either be white, pink or brownish-black. Their shape is long and cylindrical (oftentimes having offshoots referred to as "toes") while their exterior texture is rough and scaly. Yams have a very starchy and slippery texture and when cooked, will either be creamy or firm, depending upon the variety. Their taste is earthy and hardy, with most varieties having minimal, if any, sweetness. (George *et al.*, 2007)

a) Macroscopic

Vigorously twining herbaceous vine, from massive underground tuber (Figure 2.14). Stems to 10 m (30 ft) or more in length, freely branching above; internodes square in cross section, with corners compressed into “wings,” these often red-purple tinged. Aerial tubers (bulbils) formed in leaf axils (not as freely as in D. bulbifera), elongate, to 10 cm (4 in) x 3 cm (1.2 in), with rough, bumpy surfaces. Leaves long petioled, opposite (often with only 1 leaf persistent); blades to 20 cm (8 in) or more long, narrowly heart shaped, with basal lobes often angular. Flowers small, occasional, male and female arising from leaf axils on separate plants (i.e., a

dioecious species), male flowers in panicles to 30cm (1 ft) long, female flowers in smaller spikes. Fruit a 3-parted capsule; seeds winged **(**Langeland *et al., 2008)*

Figure 2.13 *Dioscorea alata* plant

Figure 2.14 *Dioscorea alata* tuber root

b) Microscopic

The outer part of the yam tuber forms several layers of cork and the inner part is formed by a tissue of parenchyma cells, which are interwoven with vascular channels. Starch is contained within the tissue in thin walled parenchymatous cells and the non-carbohydrate components are mainly present in the cell wall. The thin cell wall imparts stability to individual cells while the cell walls of the major cells provide rigidity, strength and shape to the plant cell (Degras, 1986). It is assumed that starches with a range of granular structures behave uniquely. Starch granules were predominantly oval, round, elliptical or triangular with a few being irregular (Baah *et al.,*2009*)*

2.7.2.4 Identity, Purity and Strength (Horng *et al.*, 1999)

Foreign matter	:	Not more than 2%
Total Ash	:	Not more than 5%

Acid-insoluble ash	:	Not more than 0.05%
Alcohol-soluble extractive	:	Not less than 10%
Water-soluble extractive	:	Not less than 10%

2.7.2.5 Substitutes and adulterants (Zinash *et al.*, 2008)

Dioscorea alata- water yam, winged yam, purple yam

Dioscorea opposita- Chinese yam (in China)

Dioscorea bulbifera- air potato (found in both Africa and Asia)

Dioscorea esculenta- lesser yam

2.7.2.6 Phyto chemistry

Diascorea alata contains alkaloids (58.73%), terpenoids (92.06%), flavonoids (90.48%), saponins (50.79) and tannins (31.74%) (Maridass *et al.*, 2010) . The most predominant phytochemical characteristic of yam is the presence of dioscorine alkaloid and diosgenin, Dioscin saponin (Figure 2.15). Although dioscorine and diosgenin are traditionally considered as toxic, such toxicity is removed by washing, boiling and cooking (Eka *et al.*, 1998)

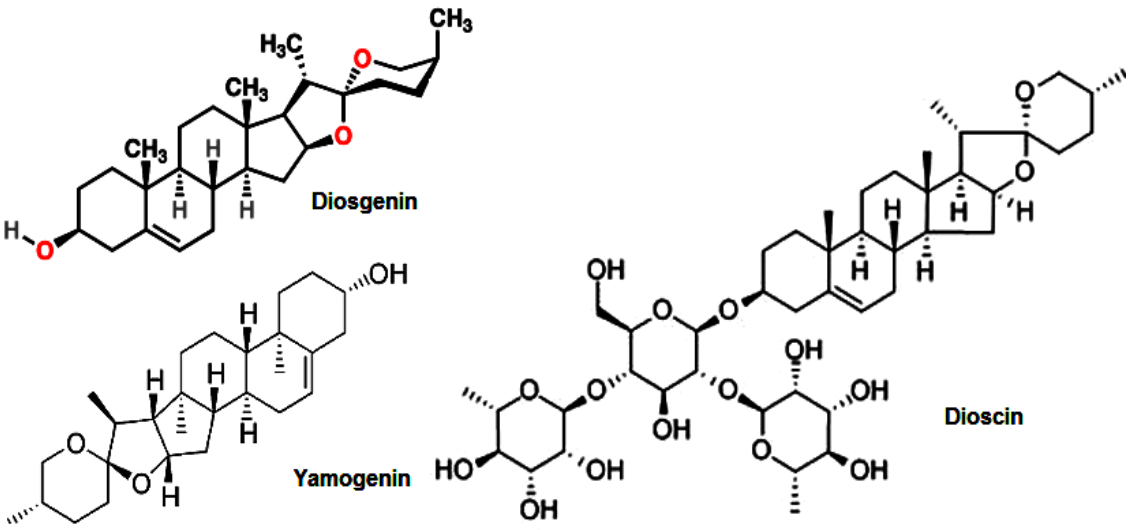


Figure 2.15 Major Saponins of *Dioscorea alata*

2.7.2.7 Traditional uses

Yams are a good source of vitamin B6. High intakes of vitamin B6 have also been shown to reduce the risk of heart disease. Yams are a good source of potassium, a mineral that helps to control blood pressure. Dioscorin, a storage protein contained in yam, may also be of benefit to certain individuals with hypertension. Preliminary research suggests that dioscorin can inhibit angiotensin converting enzyme, which would therefore lead to increased kidney blood flow and reduced blood pressure.

Wild yam also has some history of traditional use in herbal medicine, especially Chinese herbal medicine, as a botanical that can affect organ system function. While the focus here has been on kidney function, wild yam (or Chinese yam) has also been used to support the female endocrine system. For example, there has been traditional use of this root in conjunction with lactation. We've only seen one high-quality, peer-reviewed research study in which women were actually given wild yam (in the form of a topical cream) to determine the impact of this plant on menopausal symptoms. Although this research showed some very limited benefits from the wild yam cream--and no side effects--none of the symptom changes were

statistically significant. In summary, we'd say that there's no research evidence to support the claim that yam has special benefits when it comes to menopause, but that more research is needed in this area because there is a clear connection between yam, diosgenin, and endocrine function that is not yet understood. (George *et al.*, 2007)

2.7.2.8 Experimental and Clinical Pharmacology

Antidiabetic activity

In alloxan induced diabetic rats, the body wt. of the D alata ext. treated animals had shown a significant increase ($P < 0.001$) after 21 days treatment. The blood glucose level was reduced significantly by 47.48% and 52.09% after 21 days treatment at dose levels 100 and 200 mg/kg, respectively. Serum lipid levels, total protein, albumin, and creatinine were reversed toward near normal in treated rats as compared to diabetic control. The results indicate that ethanol ext. of DA tubers possesses significant antidiabetic activity (Maithili et al 2011)

Antioxidant activity

The radical scavenging activity of DAME was comparable with that of BHT (85%) at 100 mg/mL concn. DAME also showed higher reducing power than DAAE. A significant ($p<0.01$) correlation was observed between the phenolic content and the radical scavenging activity of the exts. indicating the contribution of phenolic compounds for the observed antioxidant effect. (Ahmed *et al.* 2009)

Immunomodulatory Activity

Lin *et al.*, (2009) shown that Tainong yam dioscorins have a higher ability to stimulate the phagocytic activity of the lymphoid cells than Japanese yam dioscorins, whereas Japanese yam dioscorins possess more abilities than Tainong yam dioscorins to enhance the proliferation of the lymphoid cells.

Increased splenic cytotoxic activity following the administration of mucilages from MJ yam was observed, Furthermore, the production of specific antiovalbumin

(OVA) antibody and OVA-stimulated splenic cell proliferation were also enhanced by all mucilage groups. It is suggested that the tuber mucilage may function as an immunomodulatory substance (Shang *et al.*, 2007).

Anti-fenton reaction activity

Wang *et al.* 2007, shown that a common, major ansialdehyde-sulfuric acid stained spot (possibly a polysaccharide mucilage) with an R_f of 0.09 may be the most likely contributor to the anti-Fenton reaction activities of the yam tuber exts. investigated. The present study identifies the mechanism of the health benefit of the Dioscorea family. The copper-chelating and absorbing capability of yam tuber pulp exts. may be useful in functional screening.

Mucosal hydrolase activity

The 25% yam diet was sufficient to modulate intestinal enzyme activities, but not the blood plasma and hepatic cholesterol levels. The blood plasma and liver cholesterol lowering effects of the 50% yam diet could be due to increased fecal fat and steroid excretion (chen *et al.*, 2003)

Angiotensin Converting Enzyme Inhibitory Activity

Hsu et al (2002) found that the ACE inhibitory activity was increased from 51.32% to about 75% during 32 h hydrolysis. The smaller peptides were increased with increasing pepsin hydrolytic times. Dioscorin and its hydrolyzates might be a potential for hypertension control when people consume yam tuber.

Many other activites like a-Glucosidase inhibitory action (Mitsui *et al.*, 2001), carbonic anhydrase and trypsin inhibitor activities (Hou e al., 2000), Antifungal activity (Aderiye *et al.*, 1996), Antiosteoporotic Activity (Peng *et al.*, 2011)

2.7.3 Hemidesmus indicus

2.7.3.1 Definition

Hemidesmus indicus commonly known as Indian sarsaparilla, belonging to the family Asclepiadaceae, is a slender laticiferous, twing, sometimes prostate or semi erect shrub, occurring over the greater part of India.

2.7.3.2 Vernacular Name:

Sanskrit	:	Ananti, Gopasuti, Sirivi
Bengali	:	Anantamul, Shvetashariva
English	:	Indian Sarasa Parilla
Gujrati	:	Upalsari, Kabri
Hindi	:	Anantamul
Kannada	:	Namada veru, Bili Namadaberu, Sogadeberu
Malayalam	:	Nannari, Nannar, Naruneendi
Marathi	:	Upalsari, Anantamula
Telugu	:	Sugandhi Pala, Tella Sugandhi

2.7.3.3 Description:

Upper Gangetic plain, eastwards to Bengal and the Sundribans, and from the Central provinces to South India and Ceylon. It is a slender, laticiferous, twining, sometimes prostrate or semi-erect shrub as shown in figure 2.16.

a) Macroscopic characters

Roots occur in pieces, about 30 cm long and 3-8 mm in diameter, cylindrical, thick, hard, somewhat tortuous, sparcely branched, provided with few thick rootlets

and secondary roots, external appearance dark brown, sometimes with violet grey tinge, centre yellow, woody, surrounded by a mealy white cortical layer, bark brownish, corky, marked with transverse cracks and longitudinal fissures and easily detachable from the hard central core, odor, characteristic, taste, sweetish, slightly acrid and aromatic (Figure 2.17).

Figure 2.16 *Hemidesmus indicus* Plant

Figure 2.17 *Hemidesmus indicus* root

b) Microscopic characters

Transverse section of root shows periderm consisting of three layers of tissues, cork, cork cambium and secondary cortex, cork cells radially flattened and rectangular in appearance filled with dark brown contents giving reactions of tannins, cork cambium, 2 or 3 layered, compressed, and filled with deep brown contents, secondary cortex, 3-4 layers of cells, similar to cork cells, with very little or no dark brown contents, secondary phloem consists of sieve elements, parenchyma, phloem ray cells along with several laticiferous ducts, parenchyma cells filled with starch grains, diameter 7-10 µ, occasional prismatic crystals of calcium oxalate, laticiferous ducts scattered in parenchymatous tissue, cambium very narrow: xylem traversed by narrow medullary rays, vessels and tracheids characterized by the presence of pitted markings, pith absent and centra region occupied by woody tissues.

2.7.3.4 Identity, Purity and Strength

Foreign matter : Not more than 2 %.

Total Ash : Not more than 4.3 %.

Acid insoluble ash : Not more than 0.3 %.

Alcohol soluble extractive : Not more than 6.5 %.

Water soluble extractive : Not more than 8.5 %.

TLC Identity Test

The TLC identity test for the drug has been reported using two solvent systems.

I. Toluene : Ethyl acetate : Methanol (8 : 2 : 0.5) for Vanillin.

II. Toluene : Ethyl acetate (9 : 1) for Lupeol.

The plate developed in I shows a band (R_f 0.56) corresponding to vanillin in both reference and test solution tracks. Other bands appearing in the test sample have R_f values 0.27 and 0.48. The plate developed in II shows a band (R_f 0.60) corresponding to lupeol in both reference and test solution tracks.

2.7.3.5 Substitutes and Adulterants

Ichnocarpus frutescens (L.) R.Br.

Decalepis hamiltonii Wight & Arn.

Cryptolepis buchanani Roem. & Schult.

2.7.3.6 Phyto chemistry

It has some potential as raw material for the semi synthesis of medicinal steroids, being a source of sarsasapogenin and smilagenin. The root contain 1.8-2.4% steroidal saponin (Figure 2.18).

Mukherjee et al., (1980) reported that roots of *H. indicus* contain steroids, terpenoids, flavanoids and saponins, but alkaloids is absent. Glycosides like indicine and hemidine were isolated from stem (*Prakash et al.,* 1991). The flavanoid glycosides identified in the flowers of H. indicus were hyperoside, isoquercitin and rutin (*Subramanian et al.,* 1968).

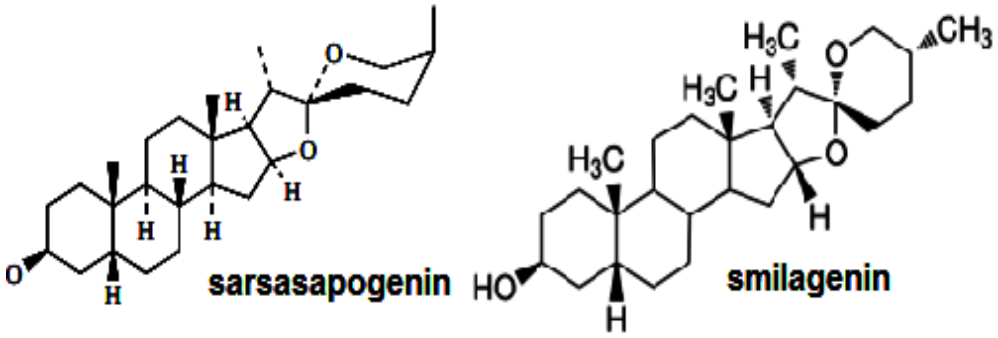


Figure 2.18 Major Saponins of *Hemidesmus indicus*

2.7.3.7 Traditional uses

Traditional Ayurveda medicine practitioners have used Sariva for hundreds and hundreds of years; it was used as a healing herb as well as a magical-spiritual dream herb. They used it to treat stomach problems, cure rashes, ease the mind, quell the symptoms of syphilis, to help induce trance states and deep meditation, and to clarify and prepare the mind for the dream world. Ayurveda tradition holds that the roots of the *Hemidesmus indicus* plant will transport the user to deeper states of sleep and through the four gates of dreaming, as written about by Carlos Castaneda, in The Art of Dreaming. It is used to help the experienced conscious dreamer achieve lucidity during the dream or REM phase of sleep.

Ayurveda healers also prescribed it to men suffering from low libido and sexual impotence, it is believed that one of active compounds produced by roots improves male testosterone levels and therefore improves sexual desire, sperm count and overall sexual performance. In traditional Hindi folk wisdom, the healer or sages used the roots to cleanse the blood of toxins, soothe skin irritations and rashes, to reduce the burning sensations caused by urinary tract infections, to reduce fevers, as well as to heal moderate cases of acne. Women use Sugandi roots to promote a healthy pregnancy and to reduce the possibility of a miscarriage.

2.7.3.8 Experimental and Clinical Pharmacology:

Antinociception

Verma et al. (2005) revealed that alcoholic extract of *H. indicus* possesses a dose dependent antinociceptive effect. It blocked both the neurogenic and inflammatory pain and its activity is due to the presence of triterpenes, flavanoids and sterols.

Anti-inflammatory

Dutta et al. (1982) found that the ethyl acetate extract of roots of *H. indicus* exhibited significant anti inflammatory activity in both acute and subacute inflammation.

Antiulcer

Anoop et al., (2003) established the antiulcer activity of *H. indicus var. indicus and var. pubescens.* It acts through mucoprotective action selectively inhibiting prostaglandin.

Hepatotonic and hepatotoxic

Prabakan et al. (2000) found out that oral administration of ethanolic (70%) extract of *H. indicus* significantly prevented Rifampicin and Isoniazid-induced hepatotoxicity in rats. *Baheti et al.* (2006) established the hepatoprotective effect of *H. indicus* by oral route.

Diuretic

Satoskar et al. (1962) found out that alcoholic and steam distilled extracts of roots of *H. indicus* had no significant diuretic activity, whereas aqueous extract caused a slight increase in urinary flow in rats, but not in dogs.

Antidiarrhoeal

Das et al. (2003) proved that methanolic extract elicited antidiarrhoeal activity which was effective than the standard antidiarrhoeal drug, Lomotil and the activity was due to inhibition of intestinal motility and its bactericidal activity.

Antivenom

The methanolic extract of *H. indicus* significantly neutralized by viper-venom induced lethality and haemorrhagic activity in albino rat and mouse. It could significantly neutralize lethality, haemorrhage, defibrinogenation, edema, PLA(2) activity induced by *D. russellii* venom. Alam *et al.* (1998) found out that 2-hydroxy-4-methoxy benzoic acid, isolated and purified from the methanolic extract possessed potent anti-inflammatory, antipyretic and antioxidant properties.

Antileprotic

Ethanolic (95%) extract was carried out for its delayed type cutaneous hypersensitivity stimulation effect by *Atal et al.* (1986). The aqueous extract of *H. indicus* was given orally at a concentration of 2% of diet in mice was active against *Mycobacterium leprae (Gupta,*1986). The mice were infected with the test organism taken from the leprosy patients. It delayed the cutaneous hypersensitivity stimulation at a dose of 100 mg/kg. It also possessed immune-modulator as well as immune-suppressant activities at 100 mg/kg. Phagocytosis was also decreased at 100 mg/kg.

Anticancer

Topical application of *H. indicus* resulted in significant protection against cutaneous tumourogenesis. The level of lipis peroxidation was significantly reduced. In addition, depleted levels of glutathione and reduced activities of antioxidant enzymes were restored, respectively which indicates its potent chemopreventive nature in skin carcinogenesis.

Chemopreventive

Sultana et al.(2003a) found that *H. indicus* is an effective chemopreventive agent in skin and capable of ameliorating cumene hydroperoxide induced cutaneous oxidative stress and tumor promotion. *Shetty et al.* (2005) found that the radioprotective effect on lipid peroxidation in rat liver microsomes and plasmid DNA protected microsomal membranes by minimizing lipid peroxidation, which could protect DNA from radiation.

Antioxidant and free radical scavenger

Methanolic (50%) extract demonstrated antioxidant properties by several *in vitro* and *ex vivo* models (Ravishankara *et al.*, 2002, Mary *et al.* 2003a) found out that methanolic extract of *H. indicus* roots inhibited lipid peroxidation and scavenges hydroxyl and superoxide radicals *in vitro*.

2.7.4 *Agave americana*

2.7.4.1 Definition

Agave consists of the dried leaves of *Agave americana* var. marginata family Amaryllidaceae (Agavecea) (Figure 2.19)

2.7.4.2 Vernacular Name

Sanskrit : Kantala, Vanketaki

English : Agave, Century Plant, maguey, American aloe

Gujrati : Ketaki

Hindi : Kamal cactus, Bharakhawar, Rakshpattah

Kannada : Kantala

Malayalam : Ageve, Anakaita

Marathi : Ketaki, Ghaipaat

Telugu : Kalabanda

2.7.4.3 Description

Century plant typically lives only 10 to 30 years. It has a spreading rosette (about 4 m/13 ft wide) of gray-green leaves up to 2 m (6.6 ft) long, each with a spiny margin and a heavy spike at the tip that can pierce to the bone. Large growing species. Which can be used as hedge plants. Agave leaves give good quality fibre. These plants flower only once in their life time. Century plant prefers full sun exposures and well drained soils, but is adaptable to a wide range of conditions, including coastal climates. It is cold hardy to 15° F. (perry *et al.*, 1982)

a) Macroscopic Characters

Century plant has no stem. Its thick and massive gray-green leaves originate from a basal rosette. The leaves get up to 6' long and 10" wide, and have sharp spines on the margins and tips. Solitary or slowly clumping succulent rosette that grows to a height of 90-120 cm. The arching leaves are characteristically variegated and have a broad creamy white band down the centre of each leaf, with grey-blue sharp-spine edges. The margin spines are recurved like fishhooks and the tip spines can be more than an inch long (Figure 2.20). The flower stalk is branched, 20-40' tall, and bears large (3-4") yellow-green flowers. Popular cultivars are 'Marginata' with yellow margins on the leaves,. (Floridata, *Plant Encyclopedia).* Fruits are flat disk seed enclosed in capsule

b) Microscopic Characters

Microcopy of plant leaf shoos that ultimate fibers are held together by sticky and waxy substances to finally form a technical fiber with section forms which are difficult to define. The ultimate fibers show oval and irregular sections with a large lumen Compared to other fibers from the same family, the *Agave americana L.* fibers are light. the *Agave americana L.* fibers are more hydrophilic than cotton, flax, and other vegetable fibers. (Slah *et al.* 2006)

Figure 2.19 Plant of *Agave americana*

Figure 2.20 Leaves of *Agave americana*

The fibers are yellow white to white and consist of fiber cell, large spiral vessels, and parenchyma cells containing single oxalate crystals up to 0.5 mm long. The cells of parenchyma are mostly destroyed and glittering crystals or pieces of crystals are left deposited on longitudinal rows on the fiber. The sclerenchymatious fibers cells are very uniform in structure, iodine and sulphuric acid gives yellowish brown color with wall. (Andrew *et al.*, 1907)

2.7.4.4 Identity, purity and strength

Foreign matter	:	Not more than 3%
Total Ash	:	Not more than 5%
Acid-insoluble ash	:	Not more than 2%
Alcohol-soluble extractive	:	Not less than 5%
Water-soluble extractive	:	Not less than 35%

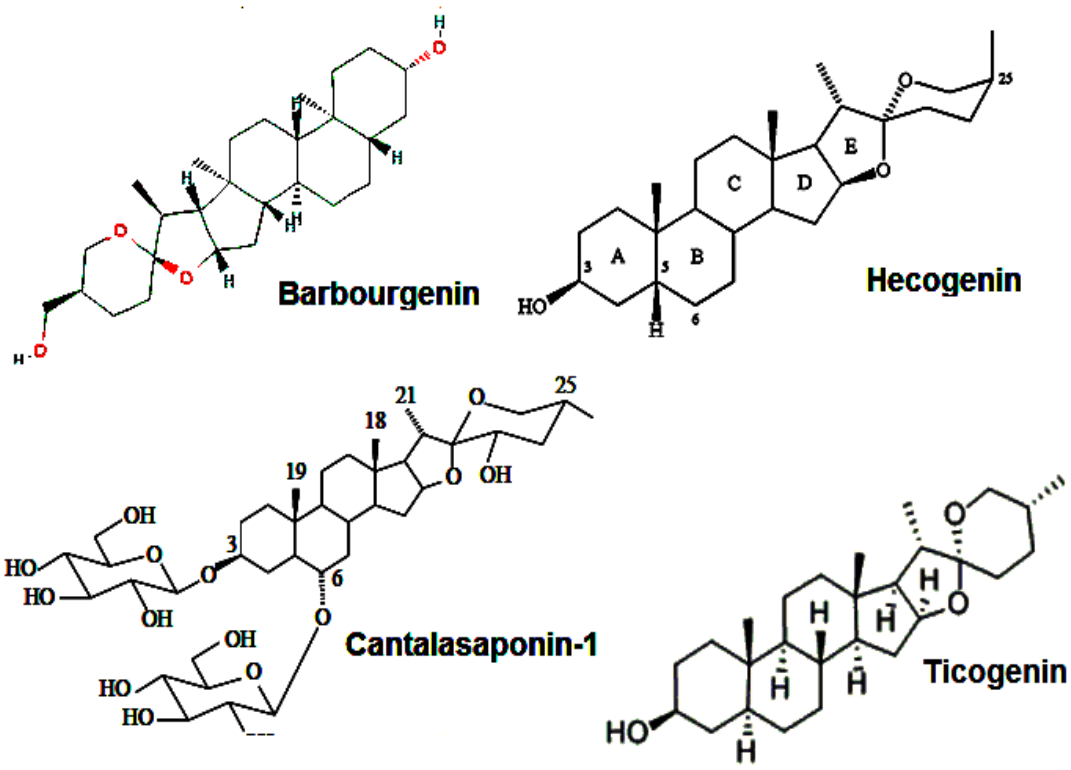


Figure 2.21 Major Saponins from *Agave americana*

2.7.4.5 Substitutes and adulterants

Agave americana var. *expansa*

Agave americana var. *latifolia*

Agave americana var. *oaxacensis*

Agave americana ssp. *Protamericana*

2.7.4.6 Phytochemistry

Several pregnane glycosides were discovered from Agavaceae plants. The species of the genus *Agave* constitute an important source of steroidal sapogenins (Bedour *et al.*, 1979; Gupta, 1995). In the pharmaceutical industry, these natural

compounds are used for the semisynthesis of medicinal steroids as corticosteroids, sexual hormones and steroid diuretics (Tyler *et al.*, 1988).

Previously isolated constituents are steroidal sapogenins and saponins (Blunden *et al.*, 1978), chromones, alcohols, and esters (Parmar *et al.*, 1992). *Agave americana* contains principle saponins hecogenin, cantalasaponin-1, Ticogenin and barbourgenin as shown in figure 2.21. (Wang *et al.*, 2006)

2.7.4.7 Traditional uses

Century plants are often used for fencing in Mexico and Central America. A dense hedge of these spiny succulents is impermeable to cattle and people. As an ornamental, century plant usually is grown in rock gardens, in cactus and succulent gardens, in Mediterranean-style landscapes, in borders, or as a specimen. It tends to dominate the landscape wherever it is grown. Century plant also is grown in containers where it stays much smaller than its outdoor brethren. Keep it in a cool, frost-free area in winter and put it out on the balcony or patio in summer. Water century plant only in the summer. The sap of century plant is used as a diuretic and a laxative. The juice of the leaves is applied to bruises and taken internally for indigestion, flatulence, constipation, jaundice and dysentery. If the flower stem is cut without flowering, a sweet liquid called agua miel ("honey water") gathers in the heart of the plant. This may be fermented to produce a beer-like drink called pulque, which may then be distilled to produce mezcal.

2.7.4.8 Experimental and Clinical Pharmacology

Concerning toxic effects, in a preliminary toxicological evaluation of *Aga6e sp.* 'Amole', doses of 1 g:kg administered to rats during 10 days did not manifest any lethal action (Segura, 1991), although adverse reactions such as gastritis and diarrhea were produced with doses higher than the therapeutic dose (Gupta *et al.*, 1995).

Anticancer activity

The ethanol extract of *A. americana* has a potential cytotoxic and antitumor activity. Antitumor activity of ethanolic extract of *A. americana* leaves demonstrated

by using PA-1 human cell line of ovarian teratocarcinoma. Brine shrimp lethality bioassay (LC50= 923.10 µg/ml), *Allium cepa* root meristem model and MTT assay (IC50 =0.01 µg/ml) showed potent cytotoxic and anticancer activity of ethanolic extract of *A. americana* leaves. The ethanolic extract of *A. americana* leaves has a cytotoxic and antitumor activity. (Ketan *et al.*, 2011)

Antifungal activity

Leaf ext. of *Agave americana* was evaluated for antifungal activity against Alternaria brassicae, the causal agent of Alternaria blight of Indian mustard [Brassica juncea (L.) Czern. & Coss]. Methanolic leaf ext. (crude ext.) of A. americana showed antifungal activity against A. brassicae. (Guleria et al ., 2009)

Anti-inflammatory activity

Doses of genins (total steroidal sapogenins, hecogenin and tigogenin) equiv. to the amt. in the lyophilized exts. produced an antiedeniatous effect which was much stronger and more efficacious than that obtained with an i.p. administration of 5 mg /kg of indomethacin or dexamethasone 21-phosphate at a dose equiv. to the molar content of hecogenin administered. At the doses used to evaluate the anti-inflammatory activity, the genins did not have any harmful effect on the gastric mucous membranes. (Peana *et al.*, 1997)

Three biogenetically related compounds have been isolated from *Agave americana*, namely 5-hydroxy-7-methoxy-2-tritriacontyl-4H-1- benzopyran-4-one, tetratriacontyl hexadecanoate and tetratriacontanol. The first two compounds exhibited significant antibacterial activity. (Parmar *et al.*, 1992)

Antiallergics and antihistaminics

An ethanolic ext. of A. americana showed dose-dependent inhibition on compound 48/80-induced histamine release from rat peritoneal mast cells. Emulsions, packs, shampoos, hair tonics, bath preparations, etc. containing extract were also formulated and their efficacies for skin troubles were examined. (Kawai *et al.*, 2000)

Other Activities

Agave american . has shown antibacterial and anti-inflammatory activities (Feng *et al.*, 1964; Peana *et al.*, 1997). Antimicotic, antiviral and antituberculosis are different effects described for species of this genus (Gupta *et al.*, 1995).

2.7.5 *Solanum xanthocarpum*

2.7.5.1 Definition

Kantakari consists of mature, dried whole plant of *Solanum surattense* Burm. f.,*Syn. Solanum xanthocarpum* Schrad. & Wendl, (Fam. *Solanaceae*), perennial (Figure 2.22), very prickly diffused herb of waste land, found throughout India. (Indian Herbal Ph. 1998)

2.7.5.2 Vernacular Name (Indian Herbal Ph. 1998)

Sanskrit : Vyaghri, Nidigdhika, Ksudra, Kantakarika

Bengali : Kantakari

English : Febrifuge plant

Gujrati : Bharingani

Hindi : Katai, Katali, Ringani, Bhatakataiya, Chhotikateri

Kannada : Nelagulla, Kiragulla

Malayalam : Kantakari chunda

Marathi : Bhauringani, Kataringani

Tamil : Kandangatri, Kandankatri, Kandanghathiri

Telugu : Nelamulaka, Pinnamulaka, Mulaka, Chinnamulaka, Vakudu

2.7.5.3 Description

a) Macroscopic characters

Fruit -Berry globular, measuring 0.8-1 cm in diameter, surrounded by persistent calyx at base unripe fruits variegated with green and white strips, ripe fruit shows different yellow and white shades (Figure 2.23). Root -10-45 cm long, few mm to two cm in diameter, almost cylindrical and tapering, transversely smoothened surface shows a thin bark and wide compact cylinder of wood, fracture, short, taste, bitter.

Figure 2.22 *Solanum xanthocarpum* Herb

Figure 2.23 *Solanum xanthocarpum* Fruits

Stem-herbaceous, prickly with prominent nodes and internodes, green when fresh, young branches, covered with numerous hairs, Leaves-petiolate, exstipulate, ovate--oblong or elliptic, sinuate or sub-pinnatifid, subacute hairy, Flower- bracteate, pedicellate, bisexual, pentamerous, regular, complete, bright blue or bluish purple. Seeds-circular, flat, numerous, embedded in a fleshy mesocarp about 0. 2 cm in diameter, glabrous taste, bitter and acrid. (Indian Herbal Ph. 1998)

b) Microscopic characters

Transverse section of mature fruit shows single layered epidermis, covered externally by a thin cuticle, 1-2 layers of collanchyma present below epidermis, narrow endosperm with embryo, some cells of endosperm contain oil globules. Petiole-transverse section of petiole shows circular to wavy outlines, epidermis single

layered, covered externally by a thick cuticle, hypodermis consists of 3-4 layers of collenchymatous, cells, , epidermis shows mostly stellate and rarely urn to tricellular hairs. Midrib-transverse section of midrib shows a biconvex structure, , some stellate hair present on epidermis. Lamina-transverse section shows dorsiventral structure, epidermis on either side, palisade ratio 1.7-4 (mean 2 .85). (Indian Herbal Ph. 1998)

2.7.5.4 Identity, Purity and Strength (Indian Herbal Ph. 1998)

Foreign matter	:	Not more than 2 %
Total Ash	:	Not more than 9 %
Acid-insoluble ash	:	Not more than 3 %
Alcohol-soluble extractive	:	Not less than 6 %
Water-soluble extractive	:	Not less than 16 %

2.7.5..5 Substitutes and adulterants

The commercial drug *of S. xanthocarpum* is often found adulterated with the allied species *S. indicum.* (Indian Herbal Ph. 1998)

2.7.5..6 Phytochemistry

Steroidal alkaloid solasodine is the principal alkaloid. Alcoholic extracts of the plant contain fatty and resinous substances. Solasonine is present in fruits (Figure 2.24). The glycoalkaloid content of fruits collected from plants growing in Jammu & Kashmir is reported to be 3.5 per cent (total alkaloids: 1.1%). The plant samples collected from Calcutta contained solasodine of 0.0287%. The presence of diosgenin in the plant has been reported (Pendse *et al.*, 1932). Seeds yield 19.3 % of a greenish yellow, semi-drying oil with a characteristic odour. The unsaponifiable matter of fruits contains two sterols, one of which is carpesterol (Gupta *et al.*, 1936). The dried plants give 10.8% ash and 7.6% soluble ash consisting mainly of potassium nitrate,

carbonate and sulphate. It contains 1.6% total sugar with 0.3 % glucose as reducing sugar. (Saiyed *et al.*, 1936) It contains alkaloids, tannin, sugar, starch, fat and oil. Protein, mucilage, lignin, cutin and calcium oxalate which react positively with different concentrations of acids, alkalis, salts and dyes. (Johansen *et al.*,1940)

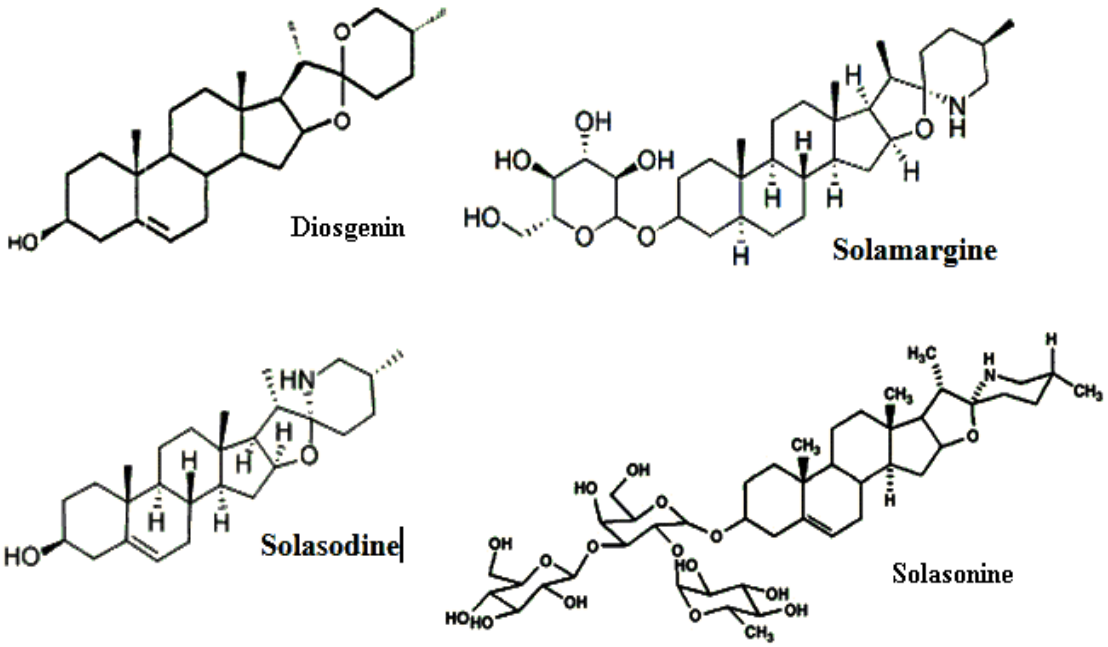


Figure 2.24 Major Saponins and Glycoalkaloids of Solanum Xanthocarpum

Plant shown presence of Solasodine in fruits; the glycoalkaloid content of fruits collected from plants growing in Jammu and Kashmir is reported to be 3.5 %. The presence of diosgenin in the plant has been reported. (Sato *et al.*,1953). It showen that the minor constituents are solamargine, solasonine (Kusano *et al.*, 1975). Its reported that cycloartenol, norcarpesterol, cholesterol and their derivatives. The yield of pure solasodine, a steroidal alkaloid is reported to be 5 gm/ kg. Which was double in quantity as compared to other previously described methods. Banik *et al.*, (1990) reported 0.028 % solasodine contents in *S. xanthocarpum*. Modified acid-dye method for detection of solasodine was found suitable.

2.7.5.7 Traditional uses

The roots, fruits and the whole plant are used for medicinal purpose. Roots are one of the constituents of Dasamulasava. The fumigation of kantakari is helpful in piles. The paste applied on swollen and painful joints in arthritis, reduces the pain and swelling effectively. Internally, kantakari is useful in vast range of diseases. In respiratory ailments like colds, asthma, sore throat, hoarseness of voice etc, it is valuable drug. The decoction of the roots works well in cough, when given with honey. The fruit is useful as an aphrodisiac in males and the seeds, in women for irregular menstruation and dysmenorrheal. The white flowered variety of kantakari (Laksmana) helps to promote conception in females. As the herb is stimulant to the heart and is a blood purifier, it is extremely beneficial in the treatment of cardiac diseases associated with edema.

2.7.5.8 Experimental and Clinical Pharmacology

Adaptogenic effects

Gupta *et al.,* (2009) demonstrated the adaptogenic effects of *Solanum xanthocarpum* and *Solanum nigrum* in forced swimming test and cold restraint stress models in swiss albino mice. They concluded that adaptogenic effects of steroidal saponins were found to be better than those of the total extracts.

Anthelmintic Activity

Priya *et al.,* (2010) showed antimicrobial, antioxidant and anthelmintic potential of extracts of *Solanum xanthocarpum* whole herb. Gunaslevi G *et al.*, (2010) demonstrated methanolic and aqueous extracts of *Solanum xanthocarpum* fruits have amazing anthelmintic activity of parasite.

Antiandrogenic potency

Dixit *et al.,* (1989) administered Solasodine obtained from the *Solanum xanthocarpum* berries to rhesus monkeys to evaluate their effect on testicular cell and

found antiandrogenic nature of the compound. Gupta RS *et al.*, (2002) showed that oral administration of Solasodine suggest antiandorgenic potency of Solasodine.

Anti asthmatic and respiratory activity

Govindan *et al.*, (1999) shown, *Solanum xanthocarpum* and *Solanum trilobatum* herbs significantly improved the various parameters of pulmonary function in asthmatic subjects. Govindan S *et al.*, (2004) proved clinical efficacy of *Solanum xanthocarpum* in the ventilator function of asthmatic individuals.

Mast cell stabilizing activity

Parmar *et al.*, (2008) revealed antihistaminic and mast cell stabilizing activity of ethanolic extract of *Solanum xanthocarpum* in experimental animal. Anti-infalmmatory activity was also revealed using acute (carrageenan-induced paw edema) and chronic (cotton pellet granuloma) models of inflammation. Antiasthamatic effect also reported by Navin *et al.* (2010).

Anti-urolithiactic and natriuretic activity

Patel *et al.*, (2010) evaluated the isolated compounds, solasonine and Solasodine, from methanolic extracts of *Solanum xanthocarpum* berries for antiurolithiatic activity on rodent and concluded that solasonine showed good natriuretic activity as compared to Solasodine.

Wound healing activity

Kumar *et al.*, (2010) verified the effects of fruits of *Solanum xanthocarpum* on experimentally induced excision and incision wound models in Sprague dowlay rats and suggested that methanolic extract of fruits possess significant wound healing potential.

Hypoglycaemic activity

Kar *et al.*, (2006) showed that aqueous extract of fruits of *Solanum xanthocarpum* found to possess significant hypoglycaemic activity compared to

standard glibenclamide in rats and mice. The *in vitro* study on glucose utilization by isolated rat hemidiaphragm suggested that the aqueous extract may have direct insulin like activity which enhances the peripheral utilization of glucose and have extra pancreatic effect.

Other pharmacological activities like Antibacterial activity (Saini V *et al.*, 2006), Antifungal activities (Singh *et al.*, 2007), Antimicrobial activity (Thenmozi *et al.*, 2008) Antinociceptive activity (Salar *et al.*, 2009)

Aim
&
Objectives…

3. Aim and Objectives

A steroid is a type of organic compound that contains a characteristic arrangement of four cycloalkane rings that are joined to each other. Examples of steroids include the dietary fat cholesterol, the sex hormones estradiol and testosterone, and the anti-inflammatory drug dexamethasone.

Steroids can be organ saving and even life saving when the autoimmune disease is very active. It can also help control the inflammation in rheumatoid arthritis and especially the acute flares of arthritis.

Steroids are considered as harmful drugs by many scientist and it may cause weight gain, raised blood pressure, worsening of diabetes, water retention, stomach irritation, acidity, cataract, osteoporosis, muscle weakness, increased susceptibility to infections etc.

Till today, more than four thousand plant species have been investigated which has resulted in the identification of some thirty naturally occurring steroids which could provide valuable source materials for steroids compounds (Atal *et al.*, 1982).

Many well established medicines originate from plants. For example, the painkiller morphine comes from poppies, aspirin comes from the bark of willow trees and digoxin (a drug used to treat heart failure) derives from foxglove. Many pharmacologic activities of steroid containing plants like Wildyam, kanthkari, Agave spices, Indian Sarsaparilla and Fenugreek have been reported. This may be a clue for investigating use of herbal steroids as therapeutic agents. **Hence, in order to find a safer alternate steroid treatment for management of various ailments, we have resolved to investigate therapeutic potential of herbal steroids.**

The objective of the present investigation was

1. To carry out phytochemical investigation of major steroid containing medicinal plants.
2. To screen various herbal steroids for corticoid potential by using *in-vivo* tests.
3. To screen selected herbal steroids for their steroidal activities and prepare a comparative index of their glucocorticoid activities by using *in-vivo* tests.
4. To investigate effect of selected herbal steroids on hepatic and hematological parameter.
5. To screen various selected herbal steroids for their steroidal activities and prepare a comparative index of their mineralocorticoid activities by using *in-vivo* tests.
6. To investigate quantitative effect of selected herbal steroids on urine content.
7. To design quality control parameter for steroidal herbal formulation.

Materials & Method…

4. Materials and Methods

4.1 Plant procurement, identification and authentification

Plant selected as major source of herbal steroid were Fenugreek (*Trigonella foenum graecum*) seed, Wild Yam (*Dioscorea alata*) underground tuber, Indian Sarsaparilla (*Hemidesmus indicus*) root, *Agave americana* leaf and Kantakari (*Solanum xanthocarpum*) fruits. *Agave americana* and Kantakari (*Solanum xanthocarpum*) collected from Saurashtra university campus while Fenugreek (*Trigonella foenum graecum*) seed and Indian Sarsaparilla (*Hemidesmus indicus*) purchased from local market, Rajkot. The wildyam was procured from modasa, district sabarkantha, Gujarat.

The study protocol was approved by Institutional animal ethics committee and the protocol no. is SU/DPS/IAEC/1001 date 11/02/2010 as attached in annexure.

The plant materials herbariums (No. SU/DPS/407-411, dated 14/10/2011) were prepared as per the guideline given by centre for advanced studies in plant biotechnology and genetic engineering, Saurashtra University, Rajkot and sent for the identification and authentification. The voucher specimens of the collected plant sample were deposited at department of pharmaceutical sciences, Saurashtra University, Rajkot (Figure 4.1-4.5)

4.2 Material and Reagents (AR Grade)

- Cyclohexane
- Acetone
- Chloroform
- Ethylacetate
- Methanol
- Water (Distilled)
- Toluene
- Propanol
- Formic acid
- Sulphuric acid
- Glacial acetic acid
- Antimony (III) Chloride
- Liebermann- Burchard reagent
- Anthrone
- Periodic acid-Schiff reagent

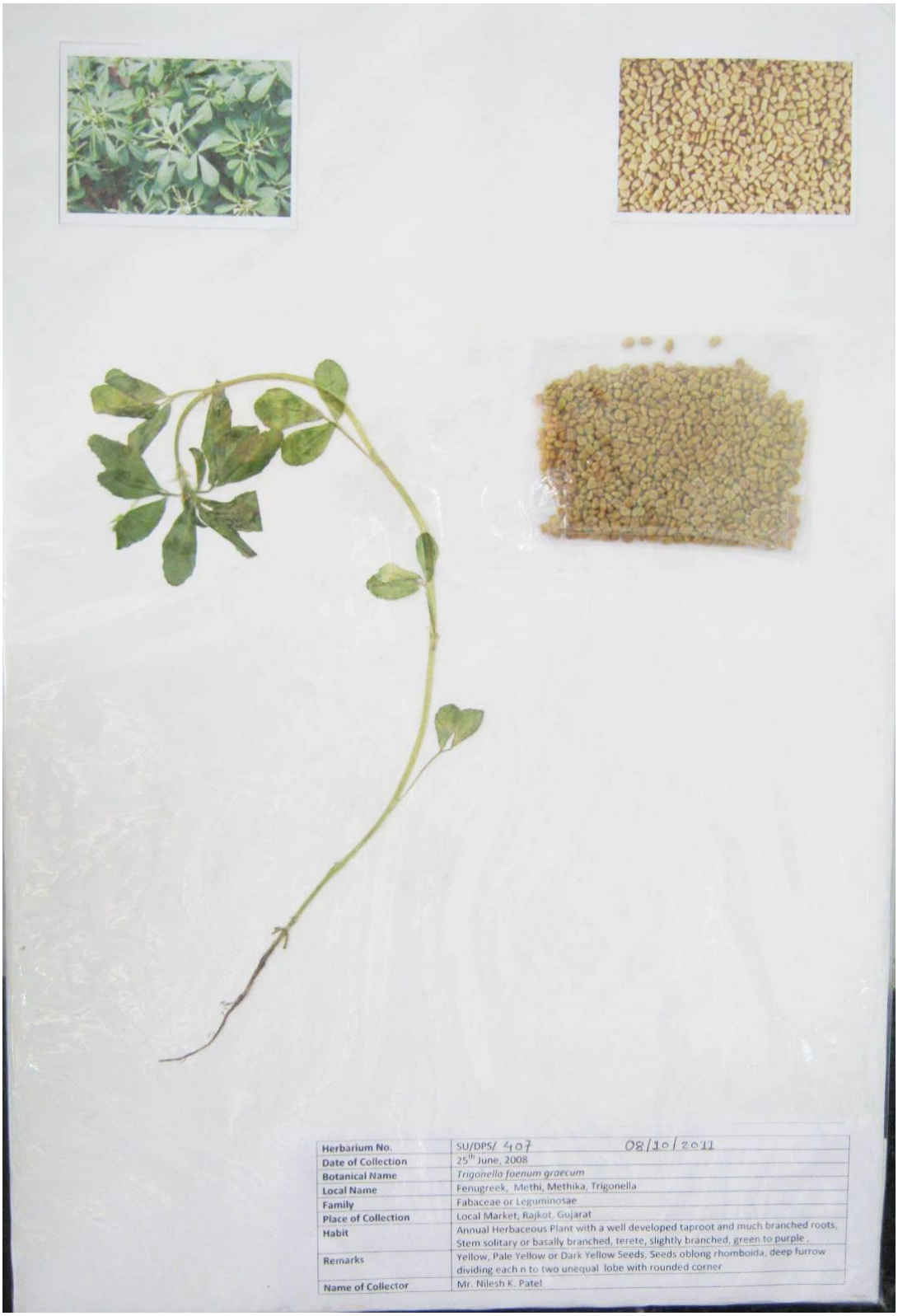


Figure 4.1 The authenticated herbarium specimen of *Trigonella foenum graecum* (Fenufreek)

Figure 4.2 The authenticated herbarium specimen of *Dioscorea alata* (Wild Yam)

Figure 4.3 The authenticated herbarium specimen of *Hemidesmus indicus* (Indian Sarsaparilla)

Figure 4.4 The authenticated herbarium specimen of *Agave americana (Century Plant)*

Figure 4.5 The authenticated herbarium specimen of *Solanum xanthocarpum (Kantakari)*

4.3 Macroscopic observations

The plant material was subjected to macroscopic studies which comprise of characters like shape, size, surface characteristics, texture, fracture, appearance of cut surface, color, odor, taste, smell etc. These characters were examined for primary identification and purity of material.

4.4 Plant extraction

All the chemicals used for experimental purpose were of analytical grade. The plant materials were washed with distilled water to remove dirt and shade dried. Routine pharmacognostic studies including organoleptic examination, macroscopic examination and microscopic examination were carried out to confirm the identity of material. The collected samples were subjected to grinding and passed through moderately coarse sieve and packed in wide mouth air tight jar.

Figure 4.6 soxhlet apparatus for Extraction

The powdered plant materials (100 gm) were soaked with 20 ml distilled water for 12 hrs before extraction with ethanol. The drug was defatted with petroleum ether (60-80°C) and extracted with ethanol (95%) using soxhlet apparatus continuously for 48 hrs (Fig. 4.6).

All ethanolic extracts were individually filtered, through Whatmann filter paper # 42 and evaporated to dryness at 50°C in oven. The ratio of powder to solvent was 1:5. The extracts were weighed for and yield were calculated and stored in desiccators till further use (Kotadia K, M.Pharm Thesis, 2005)

4.5 Phytochemical Evaluation (Hostettamann *et al.* 1995)

4.5.1 Chemical test

Selected chemical test were performed for presence of saponins and steroidal content in plant. Following chemical test were performed

4.5.1.1. Foam test: To 1 gm of drug add 5 ml of water; shake for few minutes, formation frothing which persists for 60-120 seconds in presence of saponins.

4.5.1.2. Haemolysis test: A drop blood on slide was mixed with few drops of aq. Saponin solution, RBCs becomes ruptured in presence of saponins.

4.5.1.3. Libermann Bruchard test: Plant extract of drug was extracted with $CHCl_3$, add few drops of acetic anhydride followed by conc. H_2SO_4 from side wall of test tube to the $CHCl_3$ extract. Formation of violet to blue colored ring at the junction of two liquid, indicate the presence of steroid moiety.

4.5.1.4. Antimony trichloride test: Plant extract of drug was extracted with $CHCl_3$, add saturated solution of $SbCl_3$ (Antimony trichloride**)** in $CHCl_3$ containing 20% acetic anhydride. Formation of pink color on heating indicates presence of steroids and triterpenoids.

4.5.2 Thin Layer Chromatography

4.5.2.1 Preparation of Test Solution

Accurately weighed 1 gram quantity of all five alcoholic extract were transferred to 10 ml volumetric flask and dissolved separately in 10 ml methanol. All five test sample were sonicated for 15 minutes in sonicator.

4.5.2.1Chromatographic conditions

- Stationary phase : Methanol pre-washed TLC Aluminum sheets of silica gel 60 F_{254}, layer thickness 0.2 mm 10x10 cm
- Mobile phase : Toluene: Ethyl acetate: Methanol (6:1:0.8 v/v/v)
- Chamber saturation Time : 45 min
- Distance run : 80 mm
- Scanning wavelength : 254 nm, 366 nm
- Temperature : 25± 2^{o} C

Thin Layer Chromatography was performed to ensure presence of saponins / Steroids in plant extracts. The prepared TLC plates were used to separate saponins/steroids from mixture and it's confirmed by spraying reagents. Twin trough chambers (10X10 cm) were used to provide saturate environment and developed TLC plates. All samples were applied with glass capillary.

4.5.2.2. Preparation of Lieberman Burchard reagent

Concentrated sulphuric acid (1 ml) is mixed with acetic anhydride (20 ml) and chloroform (50 ml). Heating at 80-90 ^{O}C gives required coloration.

4.5.2.3 Preparation of 20% H_2SO_4 reagent

Prepare 20% H_2SO_4 solution in water and spray on TLC plate. Heating at 80-90 ^{O}C gives required coloration.

4.6 Animals

Male albino wistar rats weighing 150–210 gm were used in this study. Animals were housed in groups of six rats on 12-hour light and 12-hour dark cycle; and were maintained in an air-conditioned animal quarter at a temperature of 22±2 °C and a relative humidity of 60±10 %. They were offered water and food *ad libitum*. The animals were acclimatized to the facilities for 5 days. Experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigation of experimental pain in conscious animals. The Institutional Animal Ethical committee (IAEC) has approved the protocol of the study. All efforts were made to minimize the number of animals used and their suffering.

4.7 Adjuvant arthritis in rats

Albino Wistar Male rats with an initial body weight of 150 to 200 g was used. On day 1, they were injected with ketamine (40mg/kg) intraperitoneally (Fahim *et al.* 1973) and anesthetized. Each animal injected into the sub plantar region of the left hind paw with 0.1 ml of heat-killed Mycobacterium tuberculosis (H37Ra) suspended in incomplete freund's adjuvant (4mg/ml). Vehicle administered orally and treated as control group, All animal were grouped (n=6) according to following,

Group-I Control Group

Group-II Standard Drug- Hydrocortisone

Group-III Ethanolic extract of *Trigonella foenum graecum* (ETF)

Group-IV Ethanolic extract of *Dioscorea alata* (EDA)

Group-V Ethanolic extract of *Hemidesmus indicus* (EHI)

Group-VI Ethanolic extract of *Agave americana* (EAA)

Group-VII Ethanolic extract of *Solanum xanthocarpum* (ESX)

All test drugs ETF (Asmena *et al.* 2009), EDA (Dhanabal *et al.* 2008), EHI (Anoop *et al.* 2008), EAA (Dana *et al.* 2006), ESX (Rahman *et al.*2003) *300mg*/kg per rat and the standard (Hydrocortisone-Primacort200) 10 mg per rat administered orally for 12 days. Purposely, from day 13 to 21, the animals received neither test compound nor the standard. Paw volumes of both sides and body weight were recorded on the day of injection, day 5, day 12, day 17 and day 21 whereby paw volume is measured plethysmographically (Vogel *et al.* 2002). The severity of the secondary lesions on day 12 is evaluated visually and graded according the following scheme

Table No. 4.1 Grading for Evaluation of Secondary lesions

Body Part	Effect	Score
Ears	Absence of nodules and redness	0
	Presence of nodules and redness	1
Nose	No swelling of connective tissue	0
	Intensive swelling of connective tissue	1
Tail	Absence of nodules	0
	Presence of nodules	1
Forepaws	Absence of inflammation	0
	Inflammation of at least 1 joint	1
Hind paws	Absence of inflammation	0
	Slight inflammation	1
	Moderate inflammation	2
	Marked inflammation	3

4.8 Liver glycogen deposition test in rats

4.8.1 Adrenalectomy

Albino Wistar male rats weighing 150–210 g are used. The dorsal fur is shaved; the rat is anesthetized with ether and placed on a block (8 × 3 × 2 cm) in order to elevate the viscera. A transverse incision about 5 mm long is made in the midline at the costovertebral angle. To remove the left adrenal gland, the skin is retracted to the ventral side and the lumbar muscles incised just superior and anterior to the splenic shadow. In this way, the adrenal gland appears directly beneath the incision and no exteriorization of the kidney is necessary (Figure 4.7).

The periadrenal tissue is grasped between the kidney and the adrenal by small curved forceps and the intact gland together with the periadrenal fat and the mesenteric attachments is removed in toto. The adrenal gland and its capsule are not touched and any remnants of the capsule to which cortical tissue may adhere are removed (Vogel *et al.* 2002). The bleeding is negligible in young animals so that no vessels need be tied off (Figure 4.8).

After ablation of the left adrenal, the animal is turned around and the right gland is removed through the original skin incision. A small incision through the lumbar muscles is made just above and anterior to the prominent lumbocostal artery which is seen near the costal margin. The curved forceps are inserted over the kidney and by elevating the liver, which covers the adrenal on this side; the gland is brought into view and grasped by the forceps removing again the intact gland with the periadrenal fat and the mesenteric attachments.

The incisions made in the lumbar muscles need not exceed 3 mm in length and may be made by spreading the blades of a pair of scissors, hemostasis or closure by sutures is not necessary. The incision is closed by a skin clip. The entire procedure is done in a time sufficiently short to avoid long-acting anesthetics. The animals appear normal in every aspect within a few min following the operation.

Figure 4.7 The kidney and adrenal gland beneath the incision

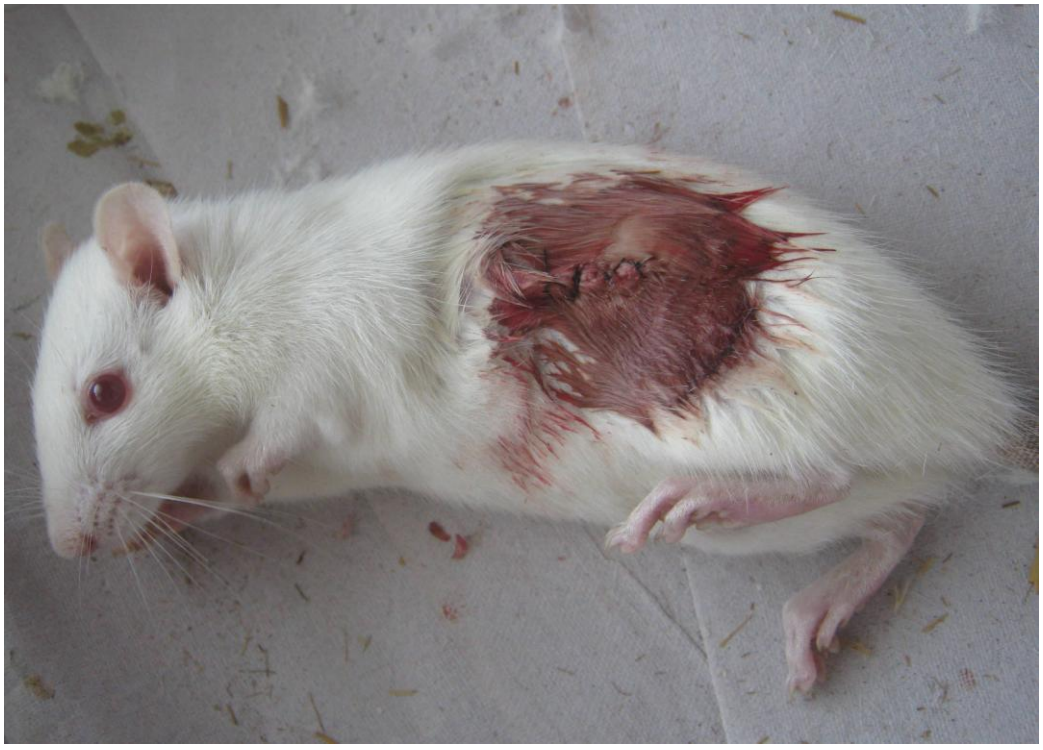


Figure 4.8 Post operative condition after adrenalectomy

4.8.2 Animal Experiment

Albino Wistar (Chen *et al.* 1952) Male rats (n=6) weighing 150–210 g are adrenalectomized. They are fed stock laboratory diet and 1% sodium chloride solution. On the morning of the fourth postoperative day, food is withdrawn. On the morning of the fifth day, the drinking fluid is withdrawn and the rats are grouped in following manner.

Group-I Control Group

Group-II Standard Drug- Hydrocortisone

Group-III Ethanolic extract of *Hemidesmus indicus* (EHI)

Group-IV Ethanolic extract of *Agave americana* (EAA)

Group-V Ethanolic extract of *Solanum xanthocarpum* (ESX)

Control group received only 1% w/v tween-80 solution in distilled water. Hydrocortisone- (Primacort-200), 10 mg/rat orally in 1% tween 80, given to group-II. Test drug Ethanolic extract of *Hemidesmus indicus* (EHI), Ethanolic extract of *Agave americana* (EAA) and Ethanolic extract of *Solanum xanthocarpum* (ESX) by 300 mg/kg per rat, oral dose given to group III, IV and V respectively. Seven hours later, the rats are sacrificed (Vogel *et al.* 2002).

The livers are removed and blotted on filter paper to remove blood, weighed, dropped into flasks containing 10 ml hot 30% potassium hydroxide and digested on a hot plate. The digest is diluted to 100 ml and appropriate dilution used for colorimetric estimation.

4.8.3 Estimation of Liver Glycogen

Ten ml 0.2% anthrone in 95% sulfuric acid is slowly added to 5 ml of liver digest dilution with cooling. The mixture is heated in a boiling water bath for 10 min and then placed into cold water. Optical density (Absorbance) is measured in a

spectrophotometer UV 1800 SHIMADZU at 620 nm using the anthrone-reagent as blank. Quantization of Liver glycogen carried out from Glucose calibration curves in range 20µg-100µg (Vogel *et al.* 2002).

4.8.4 Preparation of calibration glucose curve

Weigh accurately 30 mg of Dextrose and transfer to 100 ml volumetric flask and dissolve in distilled water (300 µg/ml). Transfer 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml of above solution to 5 ml volumetric flask separately and dilute up to the mark with 30% KOH (stock solution). The final concentration of 5ml volumetric flask was 60 µg/ml, 120 µg/ml, 180 µg/ml, 240 µg/ml and 300 µg/ml. Ten ml 0.2% anthrone in 95% sulfuric acid is slowly added to 5 ml of glucose stock solution with cooling separately. The resultant solution will have concentration 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml. The mixture is heated in a boiling water bath for 10 min and then placed into cold water. Optical density (Absorbance) is measured in a spectrophotometer UV 1800 SHIMADZU at 620 nm using the anthrone-reagent as blank. Calibration curves are established using glucose as standard in range 20 µg-100 µg.

4.8.5 Hematological Evaluation

Blood collected from rat through retro orbital plexus and transfer 3 ml to EDTA coated blood collecting disposable tube and 2 ml to non-EDTA coated blood collecting disposable tube. Hematological evaluation carried out for quantitative estimation White blood cell, Red blood Corpuscle, Platelets, of 3 ml for all animals carried out for heamatological parameter, Serum glutamic pyruvic transaminase (SGPT), Serum glutamic oxaloacetic transaminase (SGOT), Serum Calcium and Serum potassium. All samples were analysed at Dr. Bhatt Pathology Laboratory, Rajkot.

4.8.5.1 Determination of Blood cells

The determination WBC, RBC and Platelets for all sample were performed on Automated 3 part blood cell counter BC 3000 plus with two counting modes, whole

blood and prediluted. Instrument works on the principle of electrical impedance method. Coulter's electrical impedance principle in which cells pulled through an aperture break an electric circuit, indicating both the presence of a cell and the size of the cell. Increased resistance between electrodes results in an electrical pulse while RBCs and Platelets counted together, separated by pulse heights. Sample size for whole blood and prediluted were 13 µl and 20 µl respectively. Automatic diluting, lyzing, mixing, rinsing and unclogging performed by BC 3000 plus.

4.8.5.1 Determination of SGPT and SGOT

The liver function test, SGPT and SGOT were performed on MISPA NANO fully automatic biochemistry analyzer by UV method without pyridoxal phosphate. Alanine aminotransferase (ALT) reagent and aspartate aminotransferase (AST) reagents were used for quantitative determination of SGPT and SGOT respectively. Enzyme reagent and subtract reagents were used for formation of color complex and absorbance was determine at 340nm. At 340nm, with the one-reagent procedure and the two reagent procedure, Activity (U/L) =ΔOD/min. x 1746 (Henderson *et al.* 2001).

Principle for SGPT

Kinetic determination of the alanine aminotransferase (ALT) activity :

L-Alanine + α- Ketoglutarate $\xrightarrow{ALT}$ Pyruvate + L-Glutamate

Pyruvate + NADH + H^+ $\xrightarrow{LDH}$ L-Lactate + NAD^+

Principle for SGOT

Kinetic determination of the aspartate aminotransferase (AST) activity :

L-Aspartate + α - Ketoglutarate $\xrightarrow{AST}$ Oxaloacetate + L-Glutamate

Oxaloacetate + NADH + H^+ $\xrightarrow{MDH}$ L-Malate + NAD^+

4.8.5.1 Determination of Serum Calcium and Serum potassium

Estimation of serum potassium and serum calcium was performed on Roche 9180 electrolyte analyzer by ion selective electrode measurement. Roche 9180 electrolyte analyzer can perform electrolytic analysis from whole blood as well as plasma. Electrolyte results can be obtained from just 95 µl of whole blood, serum, plasma, acetate or bicarbonate dialysate, or pre-diluted urine. Linearity in serum was established with the analysis of two specimen set in non clinical test; commercially prepared serum linearity standard for potassium and calcium and group of random patient serum sample.

4.8.6 Histopathology of Liver

Clean liver with distilled water and accurately weigh one of liver. Transfer the liver lobe to 10% formaline solution in wide mouth disposable plastic sample jar. Histopathology of liver performed at B.T. Savani Kidney Hospital, Rajkot. Wax blocks were made and section was made by microtome, stained by Periodic acid-Schiff (PAS) and photographed (Ulusoy *et al.* 2006).

4.9 Mineralocorticoid activity in rat

4.9.1 Animal Experiment

Albino Wistar (Pierre *et al.* 1980) Male rats weighing 150–210 gm are adrenalectomized as per 4.8.1. They are maintained on 1% NaCl solution as drinking fluid. On the morning of the fourth postoperative day, food and drinking fluid are withdrawn.

On the following day, each rat is given 5 ml water by stomach tube; one hour later 5 ml 0.9% NaCl orally and the rats are grouped (n=6) in following manner.

Group-I	Control Group
Group-II	Standard Drug- Hydrocortisone
Group-III	Ethanolic extract of *Hemidesmus indicus* (EHI)
Group-IV	Ethanolic extract of *Agave americana* (EAA)
Group-V	Ethanolic extract of *Solanum xanthocarpum* (ESX)

Control group received only 1% w/v tween-80 solution in distilled water. Test drug, Ethanolic extract of *Hemidesmus indicus* (EHI), Ethanolic extract of *Agave americana* (EAA) and Ethanolic extract of *Solanum xanthocarpum* (ESX) by 300 mg/kg per rat, oral dose given to group III, IV and V respectively. Hydrocortisone-(Primacort-200) 10 mg/rat given orally in 1% w/v tween-80 solution. The rats were lightly anesthetized with ether to induce emptying of the bladder and placed in metabolic cages for 4 h, again anesthetized with ether and removed from the cages. Urine volume is recorded and cages rinsed over the collection cylinders with a distilled water spray. Collections are diluted to 100 ml and appropriate dilutions analyzed for sodium ion (Vogel *et al.* 2002).

4.9.2 Urine Analysis

Sodium is expressed as percent of excretion of control animals. Collected urine sample stored in disposable urine collection bottle. Urine analysis performed for quantitative evaluation of Sodium, Potassium, Creatinine, and Chloride. All urine samples were analyzed at Dr. Bhatt Pathology Laboratory, Rajkot.

Determination of Sodium, Potassium, Chloride and Creatinine were performed on Dade Behring Dimension RxL automated clinical chemistry analyzer using respective reagents.

4.10 Statistical Analysis

The data are presented as mean±SEM. Statistical significance was tested between more than two groups using one-way ANOVA followed by the Dunnett's control comparisons test using a computer-based fitting program (Prism, Graphpad 5). Dunnett's Test is used as a *post hoc* test. In statistical significance testing, the p-value is the probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis is true. P value expressed as "Michelin Guide" scale, *: $P < 0.05$ (significant), **: $P < 0.01$ (highly significant) and ***: $P < 0.001$ (extremely significant).

4.11 Quality control of selected steroidal herbs

4.11.1 Instrument

(A) winCATS planner chromatography system manager
(B) Camag linomat 5 semi automatic spot / band applicator
(C) Twin trough chromatogram development glass chambers
(D) Photo recording UV cabinet
(E) Camag scanner 3 winCATS with quantitative evaluation
(F) Linomat syringe, 100 micro liters for TLC visualiser
(G) 0.45 micron syringe filters
(H) Sonicator

4.11.2 Preparation of Sample solution

Weigh accurately 30 mg finely powdered EHI, EAA and ESX, transfer to 100 ml volumetric flask and dilute up to 100 ml with methanol (AR Grade). Sonicate all three solutions for 15 minutes in sonicator and ensure all drugs remain in dissolved form. Transfer the solution with Whatman filter paper and store solution in 100 ml volumetric flask covered with aluminum foil (Stock solution).

The common extract solution prepared by adding equal volume (10 ml) of all thee plant extract stock solution and mixed for 15 minutes. Label the solution and use for HPTLC fingerprinting.

4.11.3 Prewashing and activation of precoated TLC plate

TLC Aluminum sheets of silica gel 60 F_{254} (Precoated) 10.0 x 10.0 cm sheet was placed in twin trough glass chamber containing methanol as mobile phase. Methanol was allowed to travel up to upper edge of plate (ascending method). Plate was removed, allowed to dry at 50^0 C for 10 min.

4.11.4 Mobile Phase Optimization

Different mobile phase were prepared and checked for resolution of methanolic solution of Ethanolic extract of *Trigonella foenum graecum* (ETF), Ethanolic extract of *Dioscorea alata* (EDA), Ethanolic extract of *Hemidesmus indicus* (EHI), Ethanolic extract of *Agave americana* (EAA) and Ethanolic extract of *Solanum xanthocarpum* (ESX). Out of all, primarily four different mobile phases were selected for HPTLC fingerprinting.

(1)	Cyclohexane / Ethyl acetate / Chloroform	1 : 1 : 1 v/v/v
(2)	Chloroform / Methanol	7.5 : 2.5 v/v
(3)	Toluene / Ethyl acetate / Formic acid	5 : 5 : 1 v/v/v
(4)	Toluene / Ethyl acetate / Methanol	6 : 1 : 0.8 v/v/v

Drug sample were applied 9000ng/spot and developed in different four mobile phase and evaluated for resolution.

4.11.3 HPTLC fingerprinting

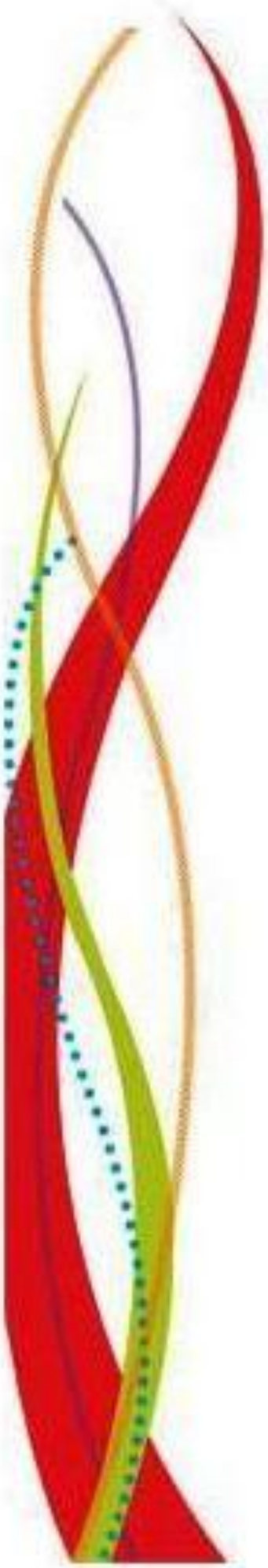
4.11.4.1 Chromatographic conditions

- Stationary phase : Methanol pre-washed TLC Aluminum sheets of silica gel 60 F_{254}, layer thickness 0.2 mm 10x10 cm
- Mobile phase : Toluene: Ethyl acetate: Methanol (6:1:0.8 v/v/v)
- Chamber saturation Time : 45 min
- Distance run : 80 mm
- Scanning wavelength : 254 nm, 366 nm

- Temperature : 25± 2^{o} C
- Band length : 6 mm
- Syringe size : 100 µl
- Distance between tracks : 20 mm
- Slit dimention : 4.0 X 0.45 mm Micro
- Scanning speed : 20 mm / second

4.11.4.2 Procedure for fingerprinting

Methanolic solution of Ethanolic extract of *Hemidesmus indicus* (EHI), Ethanolic extract of *Agave americana* (EAA) and Ethanolic extract of *Solanum xanthocarpum* (ESX) applied. On pre-washed, pre-coated TLC Aluminum sheets of silica gel 60 F_{254} 30µl (9000 ng per spot) of three drug solution and mixture were spotted with the help of spotting device using 100µl syringe. The plate was dried for 5 min at 50^{0} C and developed in previously saturated twin trough chamber at constant temperature 25±2^{0} C using Toluene / Ethyl acetate / Methanol 6 : 1 : 0.8 v/v/v, as mobile phase. After development the plate was dried at 50^{0} C for 10 min. Densitometric scanning was performed in the absorbance/reflectance as per prescribed chromatographic parameter.



Results…

5. Results

5.1 Plant extraction

The ethanolic extracts (95%) of Fenugreek (*Trigonella foenum graecum*) seed and Wild Yam (*Dioscorea alata*) underground tuber were dark brown sticky mass and remaining plant material extract were in solid after evaporation of solvent. The yield of plant ethanolic extract in % W/W for given procedure laid down in table 5.1.

Table 5.1 Ethanolic (95% v/v) plant extracts and their respective yields

Sr. No	Extract	Color and Consistency	Yield (%W/W)
1	ETF	Sticky, Dark brown mass	16.2
2	EDA	Sticky, Dark brown mass	8.0
3	EHI	Solid, Dark yellowish brown	7.5
4	EAA	Solid, Dark greenish	7.8
5	ESX	Solid, Dark yellowish	6.8

5.2 Phytochemical Evaluation

5.2.1 Chemical test

Steroidal saponins are chemical compounds abundant in different types of plant species. Their structure is composed of one or more hydrophilic glycoside moieties held by a lipophilic triterpene derivative.

Foam Test, Haemolysis test, Libermann Bruchard test and Antimony trichloride chemical test were performed and their observations were as per table 5.2.

In Foam test, ethanolic plant extract of all plant material shows characteristic foaming when mixed and shake with water. The comparative evaluation of all five extracts shown in figure 5.1.

Saponin containing plants are among the secondary metabolites and produce soap-like foam when they are shaken in water solutions. Soap like compound produces heamolysis in RBC. The aqueous solution of all plant extracts shows clear RBC rupture under microscope.

Libermann Bruchard test commonly used to check presence of steroidal moiety in plant material. All plant extracts clearly showed violet to blue colored ring at the junction between two liquids which supported presence of steroids.

The reaction of steroidal moiety with antimony trichloride also used to evaluate presence of steroids. All plant material extracts produced formation of pink color on heating which indicate presence of steroids and triterpenoids

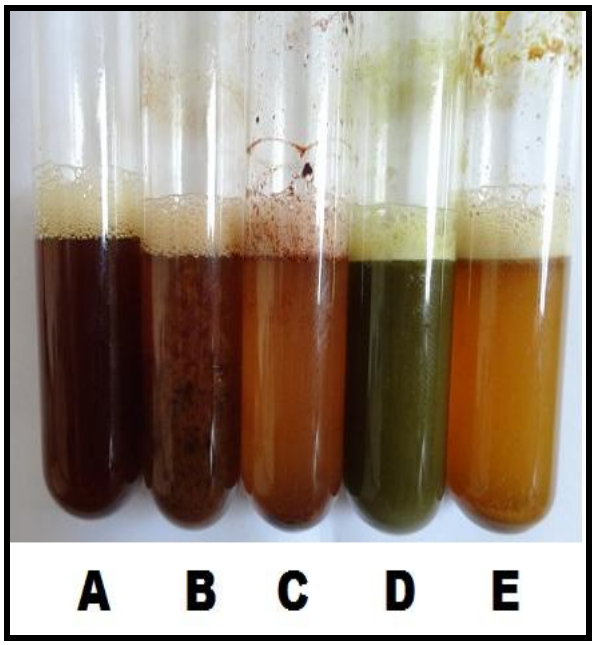


Figure 5.1 Foam test of all ethanolic plant extracts

A- Ethanolic extract of Trigonella foenum graecum

B- Ethanolic extract of *Dioscorea alata*

C- Ethanolic extract of *Hemidesmus indicus*

D- Ethanolic extract of *Agave americana*

E- Ethanolic extract of *Solanum xanthocarpum*

Table 5.2 Chemical test for all extracts

Chemical Test	**ETF**	**EDA**	**EHI**	**EAA**	**ESX**
Foam test	++	+	++	+	++
Haemolysis test	++	+	++	+	+
Libermann Bruchard test	++	++	+	++	++
Antimony trichloride test	+	+	+	+	+

[- Absence, + Present, ++ Abundance]

5.2.2 Thin Layer Chromatography

Ethanolic solution of all plant extracts were used to performe HPTLC analysis using TLC aluminum sheets of silica gel 60 F_{254}. Development of TLC plates performed on previously developed and optimized mobile phase, Toluene: Ethyl acetate: Methanol (6:1:0.8 v/v/v).

The TLC plate were sprayed with Libermann Bruchard reagent showed clear indication for presence of steroidal molecule (table 5.3) in all plant extracts. After development TLC, plates were visualized in white light (figure 5.2). In TLC profile of ETF spot, major steroidal spot were visualized at R_f 0.23 (dark purple), 0.34 (dark purple), 0.58 (dark purple) and 0.86(pink). In TLC profile of EDA spot, major steroidal spot were found at R_f 0.20 (light purple), 0.28 (light purple), 0.58 (light purple), 0.69 (light purple), 0.79 (light purple) and 0.98 (dark purple). EHI spot showed presence of steroidal spot at 0.19 (dark purple), 0.70 (dark pink), 0.79(dark pink) and 0.95(dark pink). In EAA spot, major steroidal compounds were separated at 0.21 (dark purple), 0.28(dark purple), 0.34(green), 0.74 (dark green) and 0.84 (dark green). ESX spot showed major steroidal band at R_f 0.23 (dark purple), 0.34 (light purple) and 0.38(light purple).

Another TLC plate was developed in 20% H_2SO_4 solution which gives red to pink color for triterpenoids and saponins (figure 5.3). All observation for TLC derivatized with 20% H_2SO_4 solution shown in table 5.4.

TLC developed with spraying reagent Libermann Bruchard and observed in UV_{366} (figure 5.4) and UV_{254} (figure 5.6) as well as TLC developed with spraying reagent 20% H_2SO_4 solution and observed in UV_{366} (figure 5.5) and UV_{254} (figure 5.7) serve additional basis for qualitative evalution by TLC method.

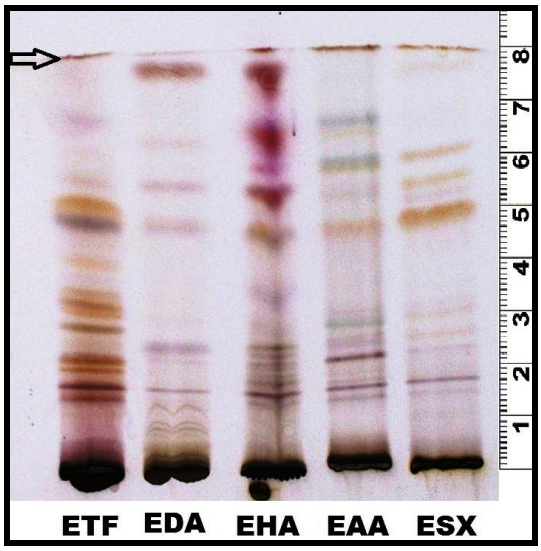


Figure 5.2 TLC developed with spraying reagent Libermann Bruchard and observed in white light

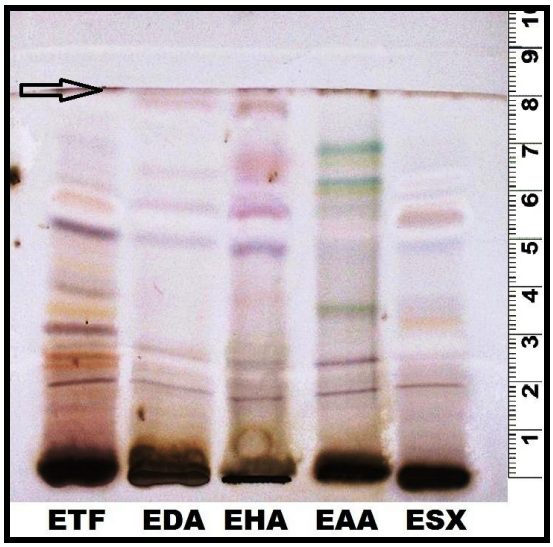


Figure 5.3 TLC developed with spraying reagent- 20% H2SO4 and observed in white light

Mobile Phase - Toluene: Ethyl acetate: Methanol (6:1:0.8 v/v/v)
ETF - Band of ethanolic extract of Trigonella foenum graecum
EDA- Band of ethanolic extract of *Dioscorea alata*
EHA- Band of ethanolic extract of *Hemidesmus indicus*
EAA- Band of ethanolic extract of *Agave americana*
ESX- Band of ethanolic extract of *Solanum xanthocarpum*
⇨- Indicate Solvent front

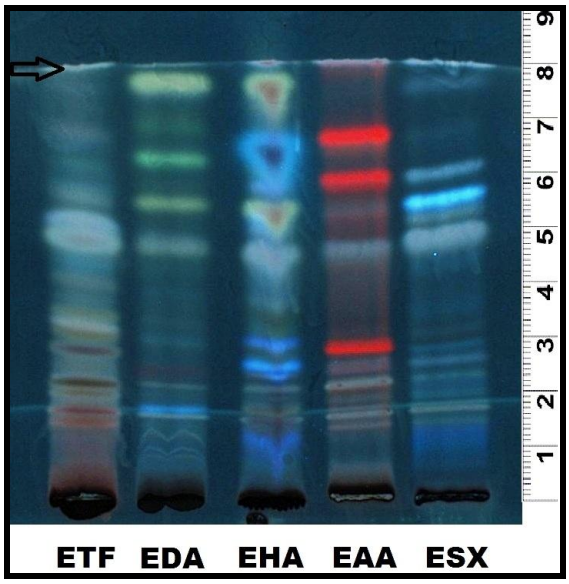


Figure 5.4 TLC developed with spraying reagent Libermann Bruchard and observed in **UV_{366}**

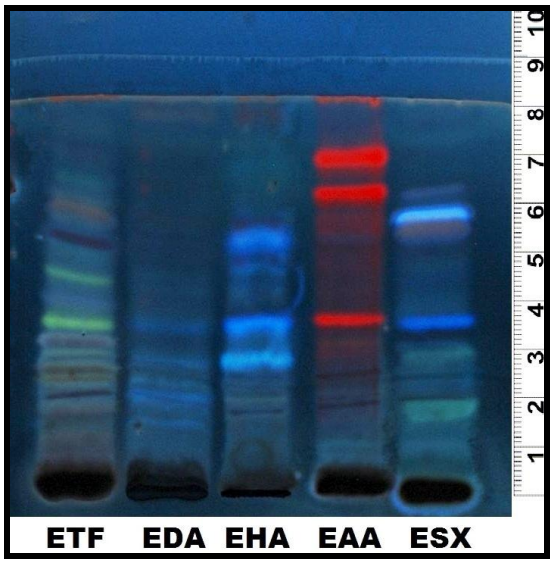


Figure 5.5 TLC developed with spraying reagent- 20% H2SO4 and observed in **UV_{366}**

Mobile Phase - Toluene: Ethyl acetate: Methanol (6:1:0.8 v/v/v)
ETF - Band of ethanolic extract of Trigonella foenum graecum
EDA- Band of ethanolic extract of *Dioscorea alata*
EHA- Band of ethanolic extract of *Hemidesmus indicus*
EAA- Band of ethanolic extract of *Agave americana*
ESX- Band of ethanolic extract of *Solanum xanthocarpum*
⇨- Indicate Solvent front

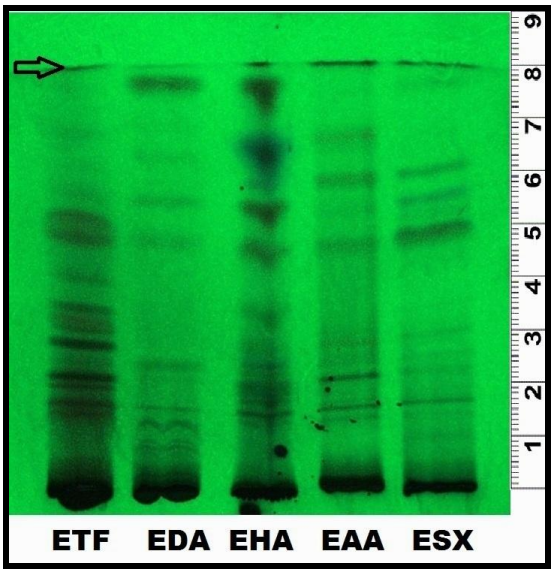


Figure 5.6 TLC developed with spraying reagent Libermann Bruchard and observed in **UV_{254}**

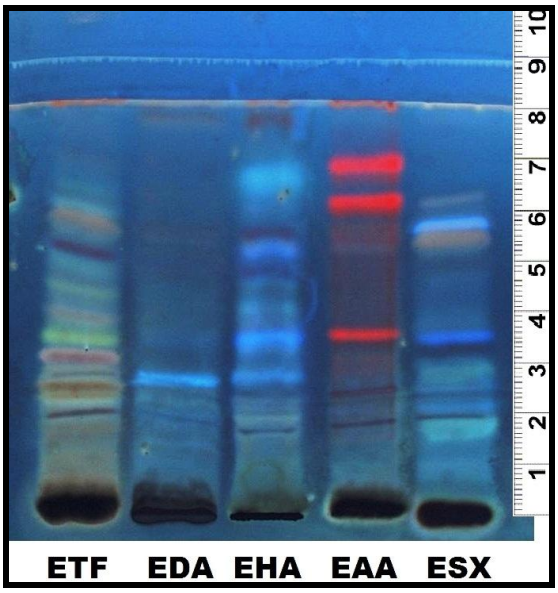


Figure 5.7 TLC developed with spraying reagent- 20% H2SO4 and observed in **UV_{254}**

Mobile Phase - Toluene: Ethyl acetate: Methanol (6:1:0.8 v/v/v)
ETF - Band of ethanolic extract of Trigonella foenum graecum
EDA- Band of ethanolic extract of *Dioscorea alata*
EHA- Band of ethanolic extract of *Hemidesmus indicus*
EAA- Band of ethanolic extract of *Agave americana*
ESX- Band of ethanolic extract of *Solanum xanthocarpum*
⇨- Indicate Solvent front

Table 5.3 TLC profile of all plant extracts derivatized with Libermann Bruchard reagent

TLC Band Test	Observation in White Light		Observation in UV_{366} Light		Observation in UV_{254} Light	
	R_f Value	Color of Band	R_f Value	Color of Band	R_f Value	Color of Band
ETF	0.23	Dark purple	0.23	Brownish purple	0.20	Dark black
	0.28	Light brown	0.28	Dark brown	0.25	Dark black
	0.34	Dark purple	0.35	Dark brown	0.35	Dark black
	0.39	Light brown	0.41	Light green	0.44	Dark black
	0.58	Dark purple	0.44	Green	0.60	Dark black
	0.63	Light brown	0.64	Light blue	0.63	Dark black
	0.86	Pink	0.69	Light blue		
EDA	0.20	Light purple	0.21	Light blue	0.15	Dark black
	0.28	Light purple	0.23	Blue	0.29	Dark black
	0.58	Light purple	0.58	Bluish green	0.59	Dark black
	0.69	Light purple	0.69	Light green	0.69	Dark black
	0.79	Light purple	0.78	Light green	0.98	Dark black
	0.98	Dark purple	0.98	Light green		
EHI	0.19	Dark purple	0.31	Light blue	0.31	Dark black
	0.29	Dark purple	0.35	Light blue	0.35	Dark black
	0.54	Dark purple	0.58	Light blue	0.58	Dark black
	0.64	Dark pink	0.68	Brownish green	0.68	Dark black
	0.70	Dark pink	0.81	Light blue	0.81	Dark black
	0.79	Dark pink	0.93	Brownish green	0.93	Dark black
	0.95	Dark pink				
EAA	0.21	Dark purple	0.19	Bluish green	0.19	Dark black
	0.28	Dark purple	0.31	Reddish purple	0.31	Dark black
	0.34	Green	0.36	Red	0.36	Dark black
	0.59	Light brown	0.65	Reddish purple	0.65	Dark black
	0.74	Dark green	0.75	Red	0.75	Dark black
	0.84	Dark green	0.86	Red	0.86	Dark black
ESX	0.23	Dark purple	0.23	Greenish blue	0.23	Dark black
	0.34	Light purple	0.34	Greenish blue	0.34	Dark black
	0.38	Light purple	0.60	Greenish blue	0.60	Dark black
	0.61	Light brown	0.66	Greenish blue	0.66	Dark black
	0.69	Light brown	0.71	Light blue	0.71	Dark black
	0.75	Light brown	0.78	Greenish blue	0.78	Dark black

Table 5.4 TLC profile of all plant extracts derivatized with 20 % H_2SO_4

TLC Band Test	Observation in White Light		Observation in UV_{366} Light		Observation in UV_{254} Light	
	R_f Value	Color of Band	R_f Value	Color of Band	R_f Value	Color of Band
ETF	0.25	Dark purple	0.28	Dark purple	0.28	Dark purple
	0.30	Light brown	0.30	Brownish blue	0.30	Brownish blue
	0.38	Dark purple	0.44	Light green	0.44	Light green
	0.40	Light brown	0.56	Light green	0.56	Light green
	0.48	Light purple	0.65	Dark purple	0.65	Dark purple
	0.63	Dark purple				
	0.70	Light orange				
EDA	0.24	Dark purple	0.20	Light blue	0.31	Light blue
	0.31	Dark purple	0.25	Light blue	0.44	Light blue
	0.63	Light purple	0.44	Light blue		
	0.73	Light purple				
	0.78	Light purple				
EHI	0.20	Dark purple	0.35	Bright blue	0.35	Bright blue
	0.31	Dark purple	0.45	Bright blue	0.45	Bright blue
	0.48	Light orange	0.68	Bright blue	0.68	Bright blue
	0.61	Dark purple			0.88	Bright blue
	0.69	Dark pink				
	0.81	Pinkish orange				
EAA	0.24	Dark purple	0.25	Dark purple	0.25	Dark purple
	0.31	Dark purple	0.31	Dark purple	0.31	Dark purple
	0.45	Dark green	0.39	Dark orange	0.39	Dark orange
	0.65	Light green	0.48	Bright red	0.48	Bright red
	0.75	Light green	0.78	Bright red	0.78	Bright red
	0.78	Dark green	0.88	Bright red	0.88	Bright red
	0.85	Light green				
	0.88	Dark green				
ESX	0.25	Dark purple	0.24	Greenish blue	0.24	Greenish blue
	0.40	Light orange	0.38	Greenish blue	0.38	Greenish blue
	0.48	Light purple	0.44	Light blue	0.44	Light blue
	0.61	Bluish purple	0.74	Bright blue	0.74	Bright blue
	0.69	Dark purple	0.78	Dark blue	0.78	Dark blue

5.3 Effect of treatment of plant extract on adjuvant arthritis in rats

In present protocol albino Wistar rat were treated with complete Freund's adjuvant to evaluate steroidal potential of ETF, EDA, EHI, EAA and ESX. The result of this study gives important direction for preparation of glucocorticoid and mineralocorticoid comparative index of selected herbs.

All animal were grouped (n=6) as control, hydrocortisone treated, ETF treated, EDA treated, EHI treated, EAA treated and ESX treated. Hydrocortisone 10 mg per rat administered orally and 300 mg/kg per rat dose kept common for all other treatment group for comparative evaluation of relative potencies.

Paw volumes of both sides (figure 5.8 (A) and figure 5.8 (B)) and body weight were recorded on the day of injection, day 5, day 12, day 17 and day 21 whereby paw volume is measured plethysmographically. The severity of the secondary lesions on day 12 is evaluated visually and graded according scheme given in materials and methods.

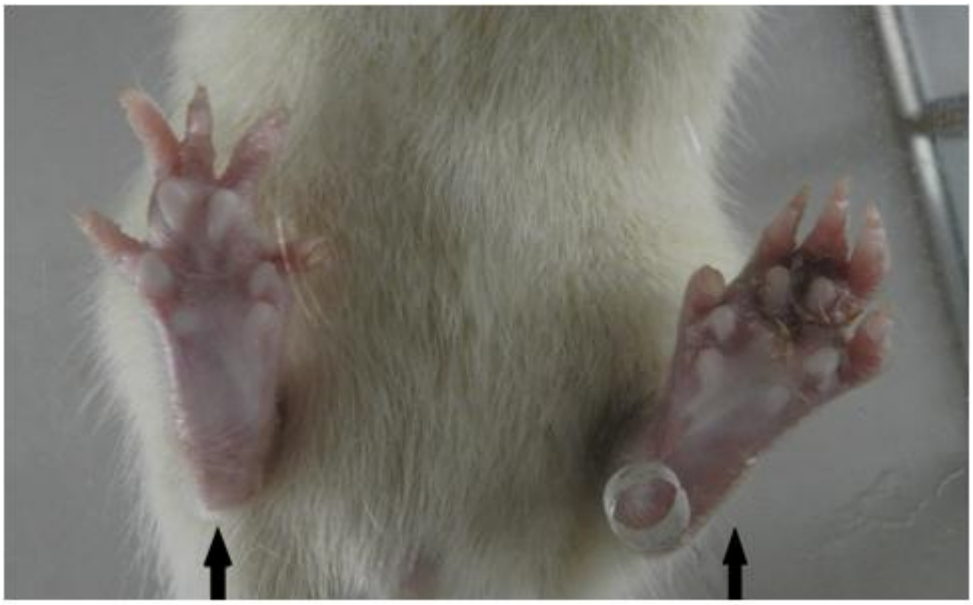


Figure 5.8 (A) Effect of complete Freund's adjuvant Injected left paw and Non-injected right paw of rat

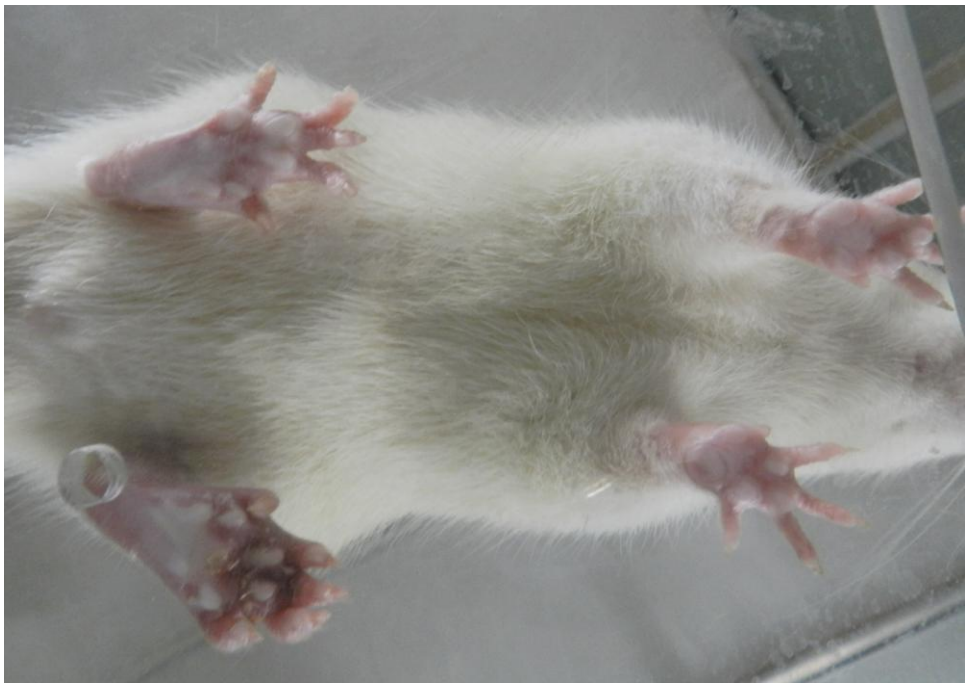


Figure 5.8 (B) Comparision of paw volume for both fore paw and both hind paw after complete Freund's adjuvant Injection

Decrease of weight of normally observed with catabolic steroids in rat. After the completion of study, on day 21 the weight were measure and change in weight compare with initial weight (day 1, before injection of CFA). The result for change in weight shown in table 5.5 while its graphical representation for day 5, day 12, day 17 and day 21 given in figure 5.9 (A) and Figure 5.10 (B)

On day 21, the increase in body weight in gram for control, HC, ETF, EDA, EHI, EAA and ESX were 36.33±1.358, 2.0 ±0.894***, 31.0 ±2.955, 28.83±2.428, 25.83±3.270*, 23.67±3.721* and 22.17±2.868** when compared with weight on day 1 (*p<0.05, **p<0.01 and ***p<0.001)

Table 5.5 Effect of HC, ETF, EDA, EHI, EAA and ESX on body weight during Adjuvant arthritis study

Group	Increase in Body Weight (gram)			
	At Day 5	**At day 12**	**At day 17**	**At day 21**
Control	12.00± 0.516	22.50±0.991	28.17±1.424	36.33±1.358
HC	8.50 ±0.341*	16.67±0.666*	9.66 ±0.802***	2.0 ±0.894***
ETF	9.83 ±0.601	18.17±1.249	22.83±2.040	31.0 ±2.955
EDA	10.50±0.763	18.33±1.542	23.00±2.324	28.83±2.428
EHI	10.00 ±0.816	18.50±1.522	22.33±2.431	25.83±3.270*
EAA	10.00 ±0.632	17.33±1.820	20.50±3.233	23.67±3.721*
ESX	11.17 ±1.641	17.00±1.983	19.33±2.171*	22.17±2.868**

Six rats were used in each group. The increase in body weight in gram was calculated by subtracting weight at day 1 from that after FCA injection. The value expressed mean±S.E.M. *p<0.05, **p<0.01 and ***p<0.001 vs control group.

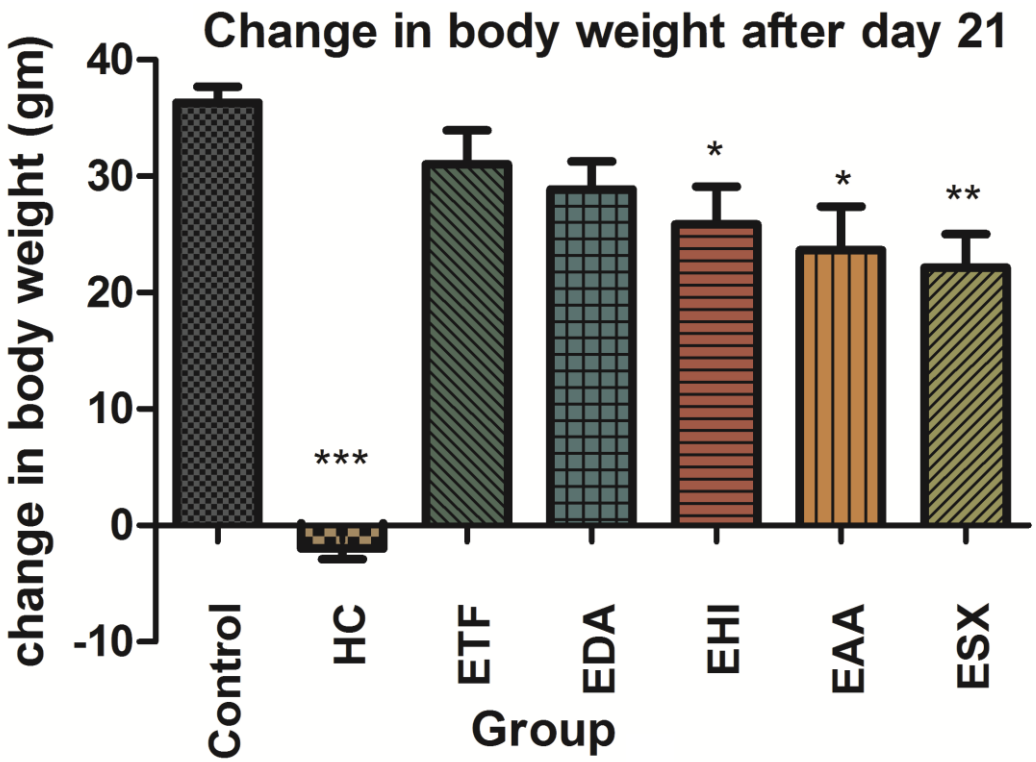


Figure 5.9 (A) Effect of HC, ETF, EDA, EHI, EAA and ESX on body weight on day 21 during adjuvant arthritis study. Each column represents the mean±S.E.M. of six rats. *p<0.05, **p<0.01 and ***p<0.001 vs. control group.

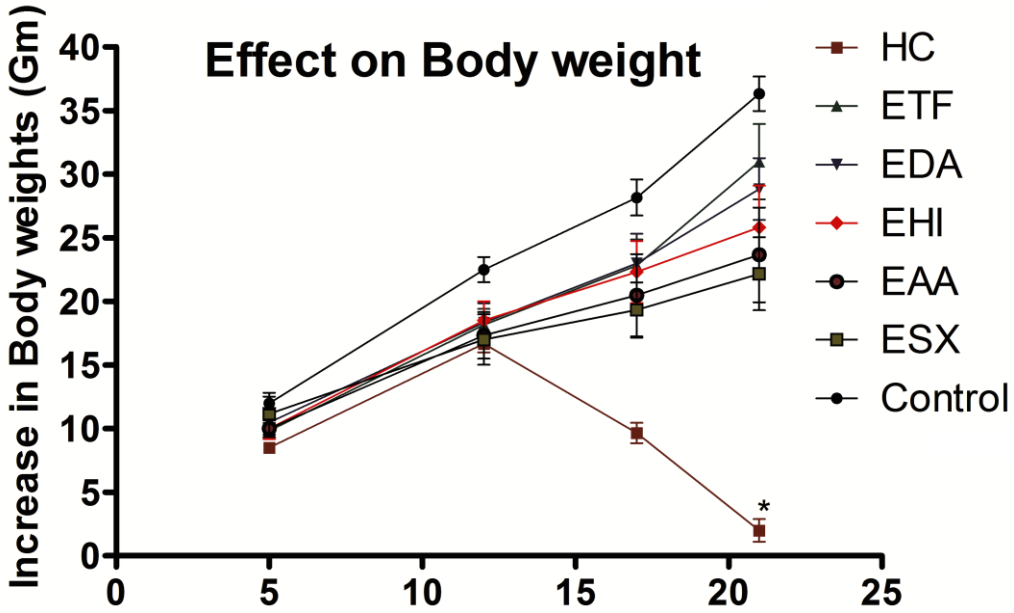


Figure 5.9 (B) Effect of HC, ETF, EDA, EHI, EAA and ESX on body weight during Adjuvant arthritis study. Each line represents the mean±S.E.M. of six rats. *p<0.05, **p<0.01 and ***p<0.001 vs. control group.

The injection of CFA into left hind paw of rat provoked inflammatory reaction marked by edema in left paw (Figure 5.8 (A)) edema in right paw as secondary infection (figure 5.8 (B)).

The change in paw volume in ml measure for both paw and compare with control. Change in paw volume expressed as change in ml and % inhibition by treatment group.

$$\% \text{ Inhibition of paw volume} = \frac{V_{Control} - V_{Test}}{V_{Control}} \times 100$$

Where,

$V_{Control}$ = Difference in Volume of paw of control (ml) compare to day 1

V_{Test} = Difference in Volume of paw of test group (ml) compare to day 1

The change in left (injected) paw volume compare to day 1 given in table 5.6 while percentage inhibition of paw volume (figure 5.26) compare to control group given in table 5.8. The change in right (non-injected) paw volume compare to day 1 given in table 5.7 while percentage inhibition of paw volume (figure 5.27) compare to control group given in table 5.9.

Paw volume measured on day 5 for left and right paw in ml. On day 5, the change in left (injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were 0.54±0.041, 0.35±0.042, 0.46±0.075, 0.44±0.038, 0.48±0.079, 0.42±0.073 and 0.44±0.085 ml respectively (figure 5.10). The percentage inhibition (left paw) on day 5 for HC, ETF, EDA, EHI, EAA and ESX were 14.51±5.166, 9.07±5.254, 1.41±5.855, 5.72±7.874, and 7.96±7.041 and 9.39±6.193 respectively (figure 5.11).

On day 5, the change in right (non-injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were 0.19±0.017, 0.09±0.0211, 0.13±0.046, 0.08±0.033, 0.09±0.042, 0.09±0.025 and 0.08±0.026 ml respectively (figure 5.12).

The percentage inhibition (right paw) on day 5 for HC, ETF, EDA, EHI, EAA and ESX were 16.31±4.380, 6.86±6.619, 5.67±8.293, 11.58±4.882, 12.06±3.513 and 13.24±6.934 respectively (figure 5.13).

On day 12, the change in left (injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were 0.76±0.031, 0.35±0.025***, 0.55±0.059, 0.55±0.049, 0.62±0.062, 0.53±0.081* and 0.58±0.067 ml respectively (figure 5.14). The percentage inhibition (left paw) on day 12 for HC, ETF, EDA, EHI, EAA and ESX were 29.25±2.960, 17.99±3.455, 10.31±4.767, 11.37±4.895, 14.41±6.348 and 14.02±4.071 respectively (figure 5.15) (***p<0.001). The reduction in edema for HC found extremely significant and EAA significant (*p<0.05, **p<0.01 and ***p<0.001).

The change in right (non-injected) paw volume (ml) on day 12 for control, HC, ETF, EDA, EHI, EAA and ESX were 0.26±0.025, 0.06±0.021***, 0.19±0.054, 0.09±0.019**, 0.11±0.036*, 0.12±0.032* and 0.11±0.025** ml respectively (figure 5.16). The percentage inhibition (right paw) on day 12 for HC, ETF, EDA, EHI, EAA and ESX were 29.18±4.354, 6.92±6.815, 13.70±5.452, 19.00±3.937, 17.51±3.649 and 19.85±5.793 respectively (figure 5.17) (*p<0.05, **p<0.01 and ***p<0.001). The reduction in edema for HC found extremely significant, EDA and ESX highly significantly reduced paw edema while EHI and EAA highly significantly reduced paw edema (*p<0.05, **p<0.01 and ***p<0.001).

Edema (Paw volume) measured on day 17 for left and right paw in ml. On day 17, the change in left (injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were 0.94±0.046, 0.12±0.042***, 0.63±0.062**, 0.66±0.054*, 0.65±0.060**, 0.57±0.076*** and 0.63±0.074** ml respectively (figure 5.18). The percentage inhibition (left paw) on day 17 for HC, ETF, EDA, EHI, EAA and ESX were 14.51±5.166, 9.07±5.254, 1.41±5.855, 5.72±7.874, and 7.96±7.041 and 9.39±6.193 respectively (figure 5.19). The reduction of edema for HC and EAA were extremely significant while ETF, EHI and ESX showed highly significant decrease in paw volume. EDA showed significant decline in paw volume (*p<0.05, **p<0.01 and ***p<0.001).

On day 17, the change in right (non-injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were 0.29±0.034, 0.09±0.038**, 0.22±0.054, 0.16±0.037, 0.18±0.038, 0.22±0.031 and 0.16±0.044 ml respectively (figure 5.20). The percentage inhibition (right paw) on day 17 for HC, ETF, EDA, EHI, EAA and ESX were 29.70±6.117, 8.89±6.340, 9.9±3.880, 14.34±4.835, 8.889±4.883 and 16.16±5.833respectively (figure 5.21). The reduction of edema for only HC found highly significant (**p<0.01).

Paw volume measured on day 21 for left and right paw in ml. On day 21, the change in left (injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were 1.13±0.065, 0.03±0.032***, 0.92±0.078, 0.79±0.067**, 0.66±0.054***, 0.61±0.077*** and 0.72±0.077*** ml respectively (figure 5.22). The percentage inhibition (left paw) on day 21 for HC, ETF, EDA, EHI, EAA and ESX were 65.54±2.482, 13.91±4.187, 15.34±1.997, 28.53±3.663, 29.14±4.300 and 25.26±3.939 respectively (figure 5.23). The reduction of edema for HC EAA, EHI and ESX were extremely significant and the reduction of edema for EDA was highly significant (**p<0.01 and ***p<0.001).

The change in right (non-injected) paw volume (ml) on day 21 for control, HC, ETF, EDA, EHI, EAA and ESX were 0.42±0.062, 0.05±0.023***, 0.15±0.031***, 0.12±0.046***, 0.15±0.049***, 0.21±0.043** and 0.13±0.041*** ml respectively (figure 5.24). The percentage inhibition (right paw) on day 21 for HC, ETF, EDA, EHI, EAA and ESX were 42.68±3.720, 27.34±4.022, 25.40±3.683, 28.22±5.343, 22.22±4.863 and 30.86±4.003 respectively (figure 5.25). The reduction in paw volume for HC, ETF, EDA, EHI and ESX found extremely significant while EAA highly significantly reduced paw edema (**p<0.01 and ***p<0.001).

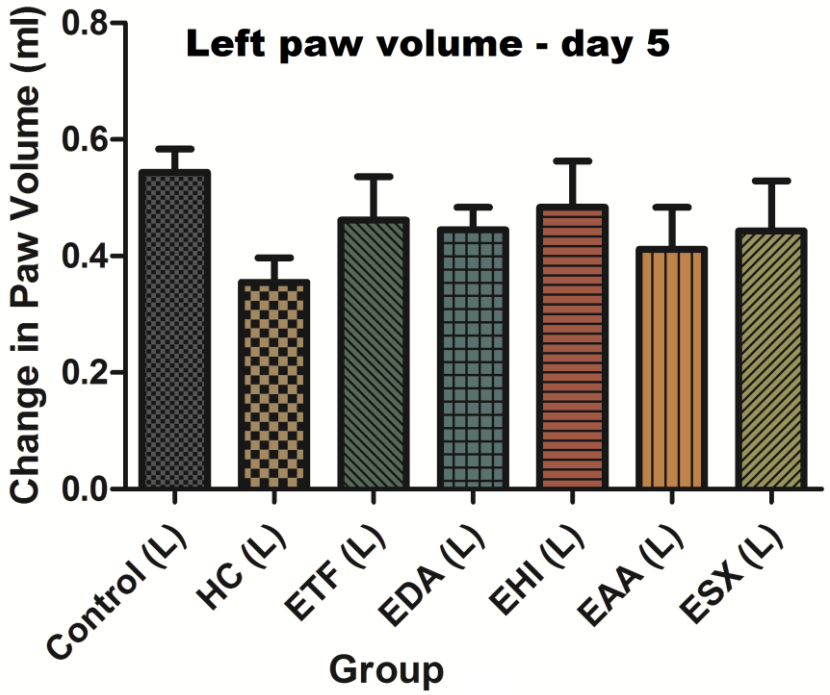


Figure 5.10 Effect of HC, ETF, EDA, EHI, EAA and ESX on left (injected) paw volume after 5 days during adjuvant arthritis study. Each column represents the mean±S.E.M. of six rats. *p<0.05 vs. control group.

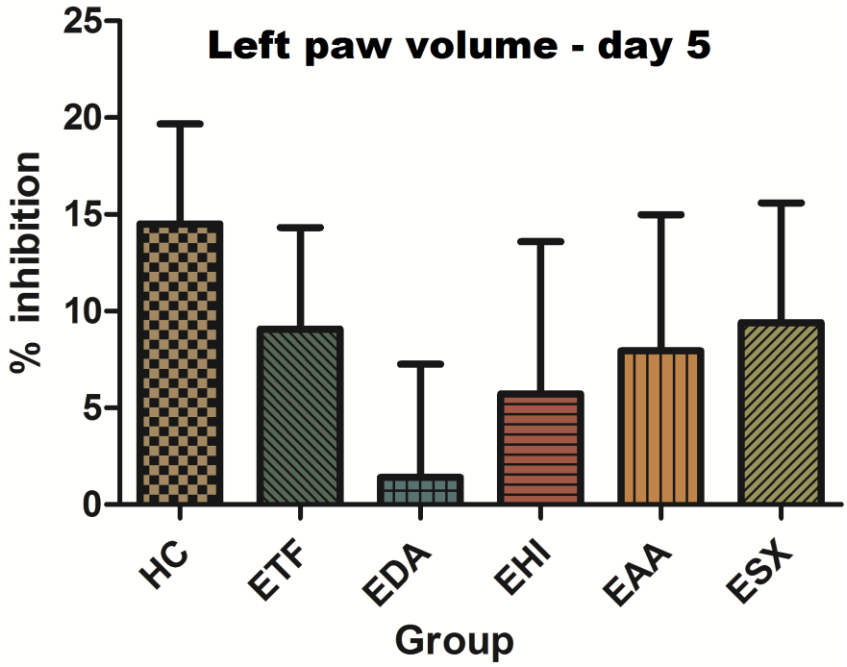


Figure 5.11 Percentage inhibition of left (injected) paw volume after 5 days during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

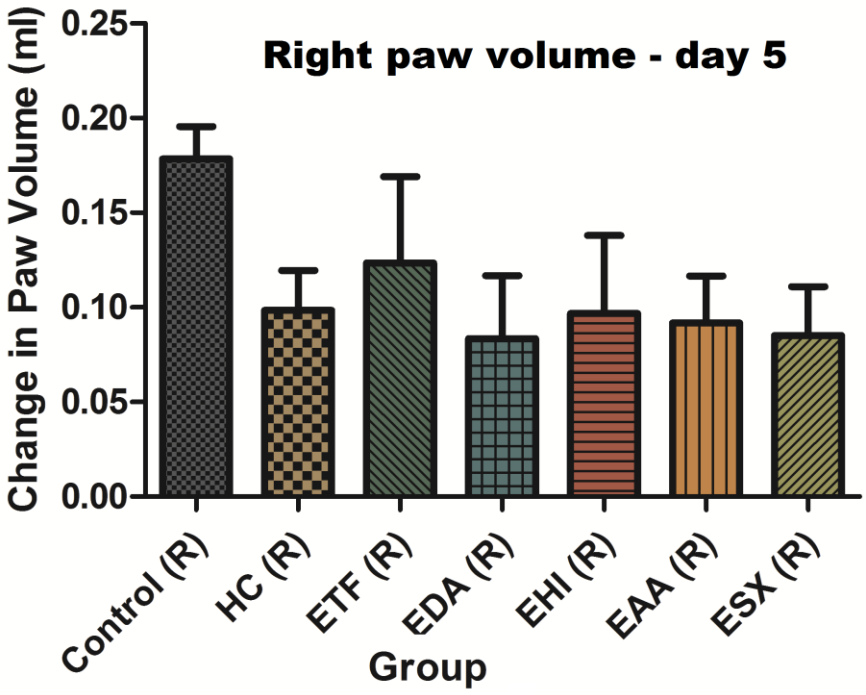


Figure 5.12 Effect of HC, ETF, EDA, EHI, EAA and ESX on right (non-injected) paw volume after 5 days during adjuvant arthritis study. Each column represents the mean±S.E.M. of six rats. *p<0.05 vs. control group.

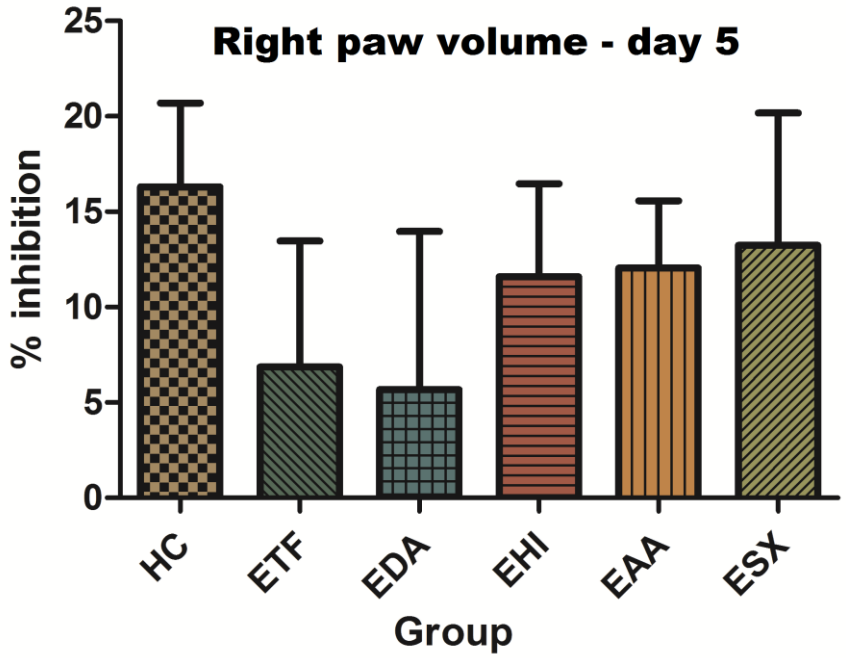


Figure 5.13 Percentage inhibition of right (non-injected) paw volume after 5 days during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

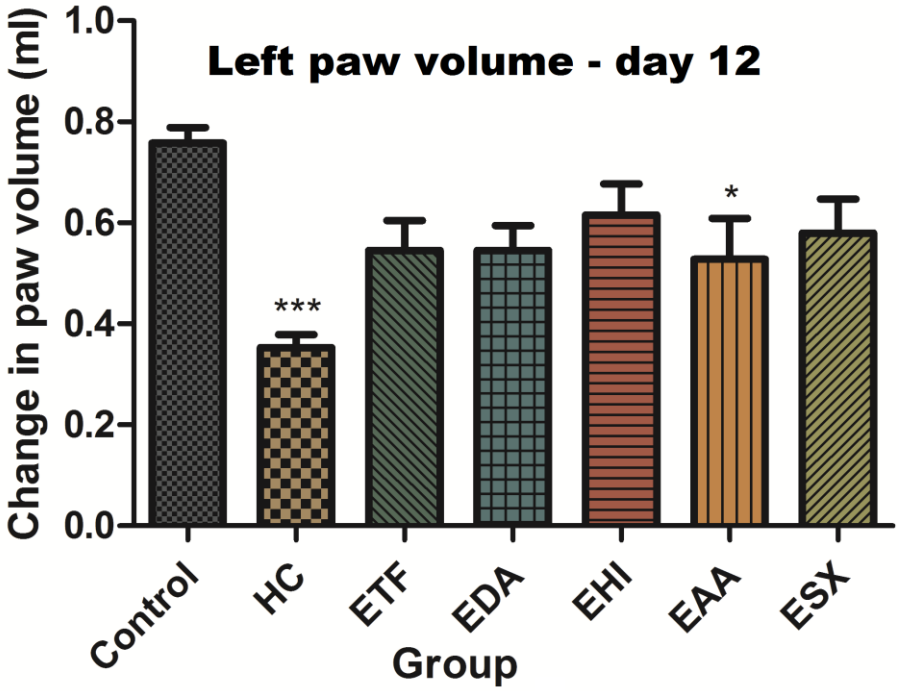


Figure 5.14 Effect of HC, ETF, EDA, EHI, EAA and ESX on left (injected) paw volume after 12 days during adjuvant arthritis study. Each column represents the mean±S.E.M. of six rats. *p<0.05 and ***p<0.001vs. control group.

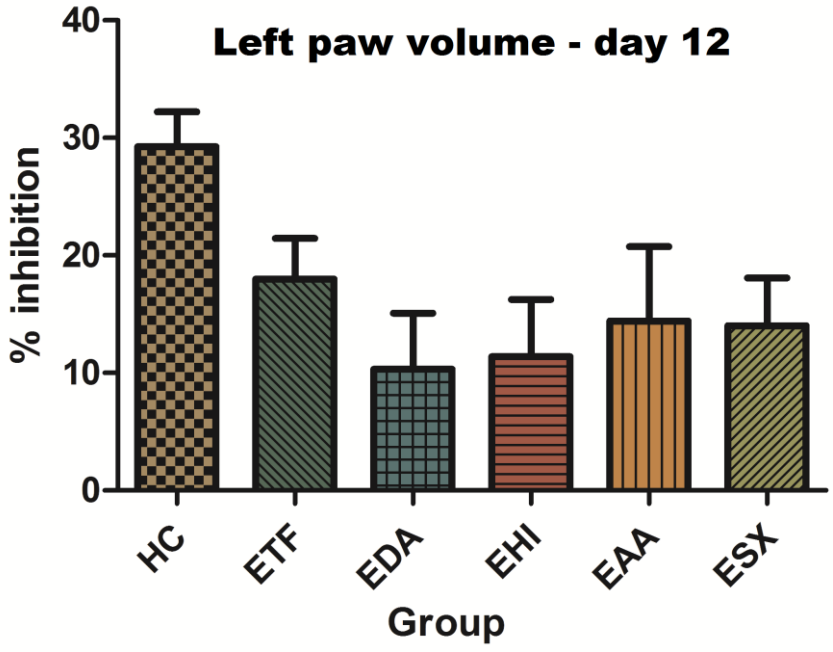


Figure 5.15 Percentage inhibition of left (injected) paw volume after 12 days during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

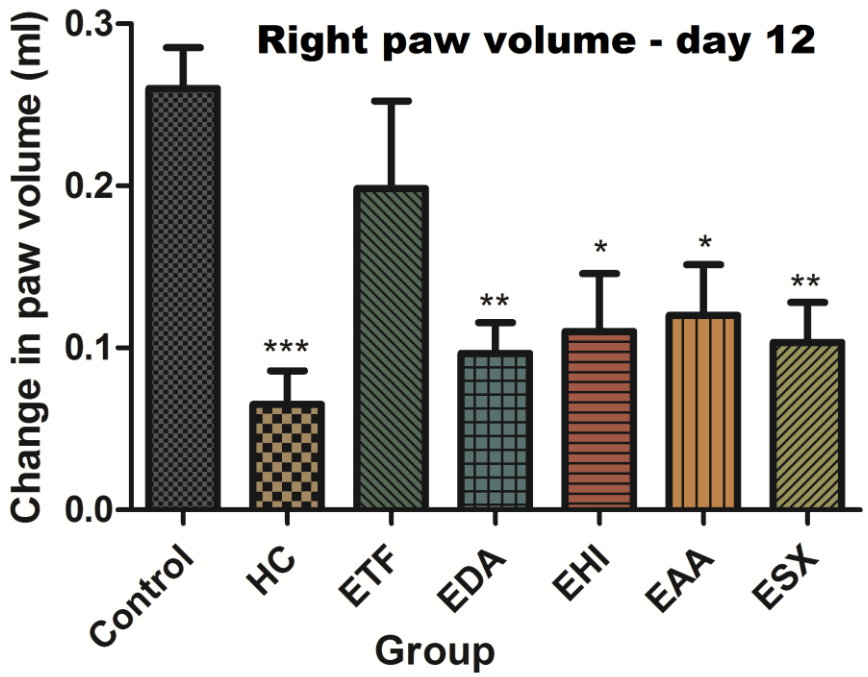


Figure 5.16 Effect of HC, ETF, EDA, EHI, EAA and ESX on right (non-injected) paw volume after 12 days during adjuvant arthritis study. Each column represents the mean±S.E.M. of six rats. *p<0.05, **p<0.01 and ***p<0.001 vs. control group.

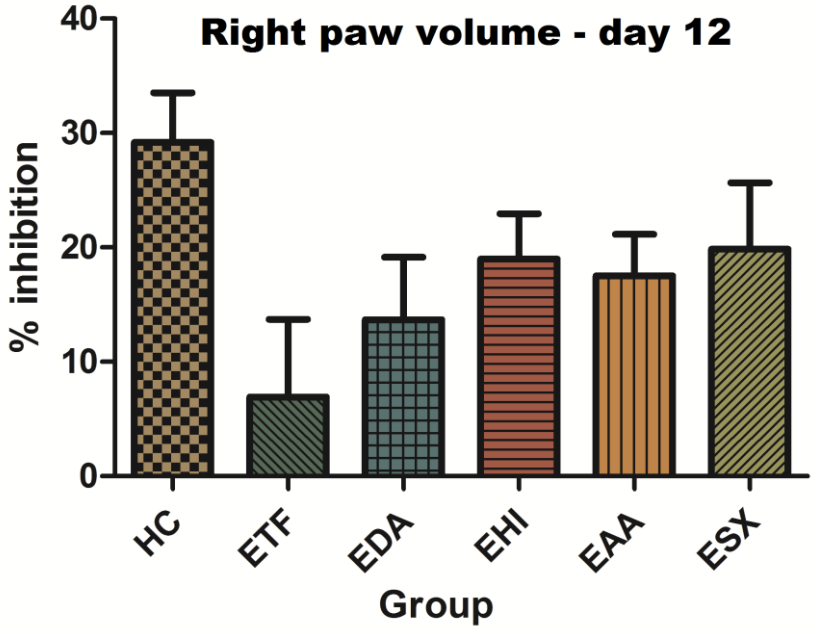


Figure 5.17 Percentage inhibition of right (non-injected) paw volume after 12 days during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

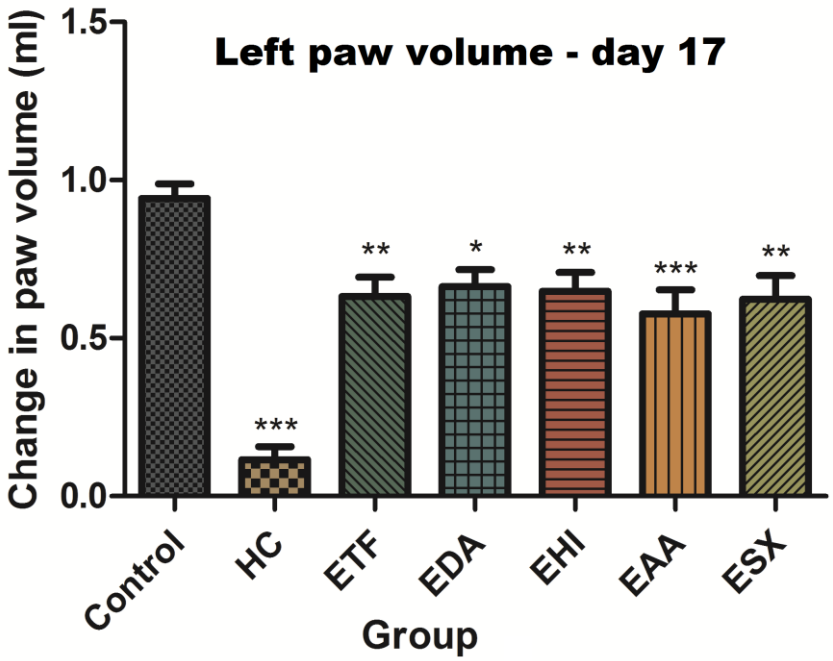


Figure 5.18 Effect of HC, ETF, EDA, EHI, EAA and ESX on left (injected) paw volume after 17 days during adjuvant arthritis study. Each column represents the mean±S.E.M. of six rats. *p<0.05, **p<0.01 and ***p<0.001 vs. control group.

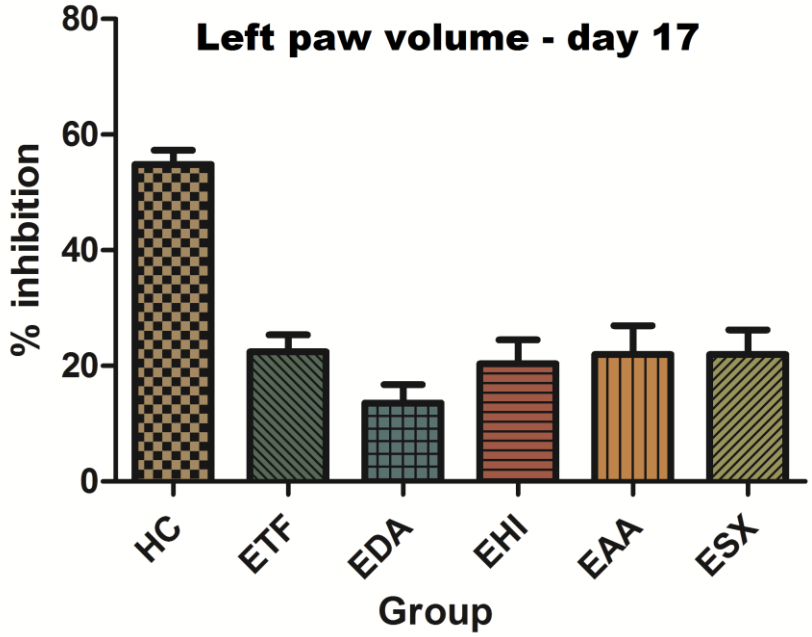


Figure 5.19 Percentage inhibition of left (injected) paw volume after 17 days during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

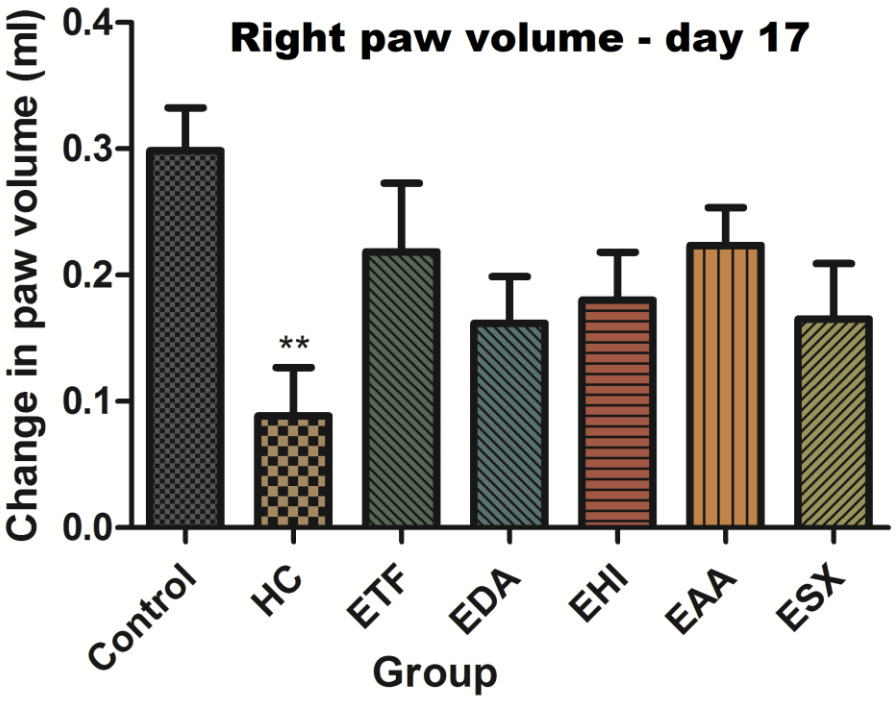


Figure 5.20 Effect of HC, ETF, EDA, EHI, EAA and ESX on right (non-injected) paw volume after 17 days during adjuvant arthritis study. Each column represents the mean±S.E.M. of six rats. **p<0.01 vs. control group.

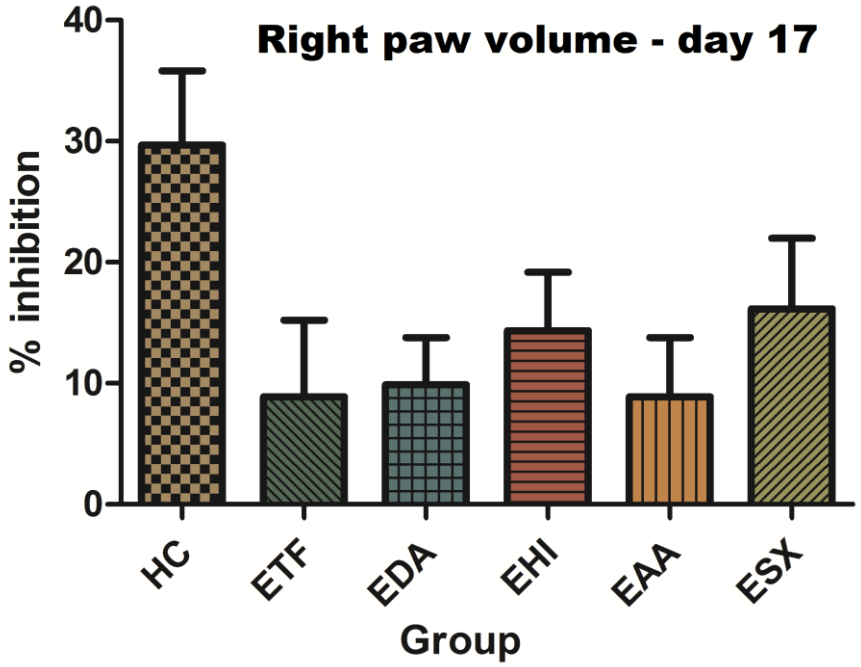


Figure 5.21 Percentage inhibition of right (non-injected) paw volume after 17 days during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

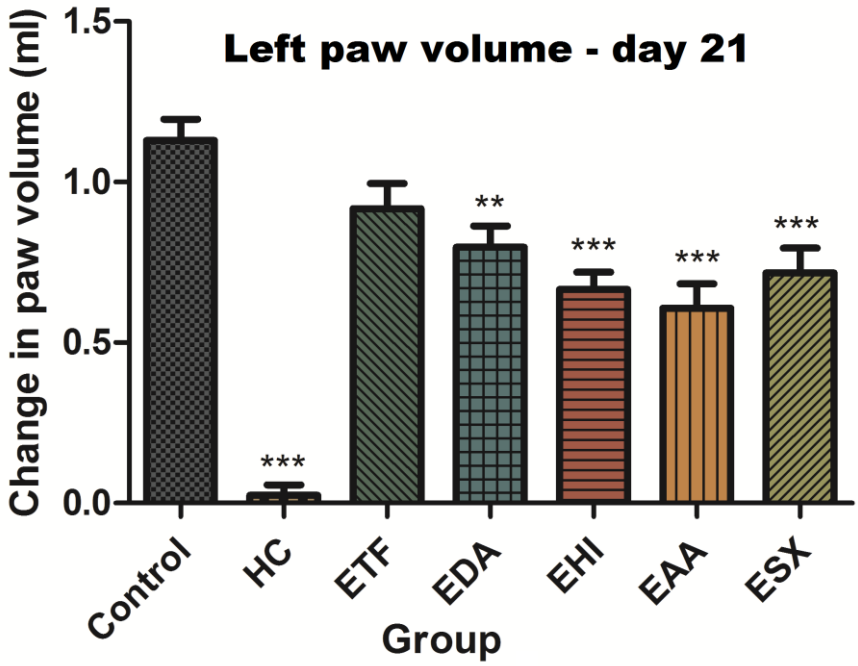


Figure 5.22 Effect of HC, ETF, EDA, EHI, EAA and ESX on left (injected) paw volume after 21 days during adjuvant arthritis study. Each column represents the mean±S.E.M. of six rats. **p<0.01 and ***p<0.001 vs. control group.

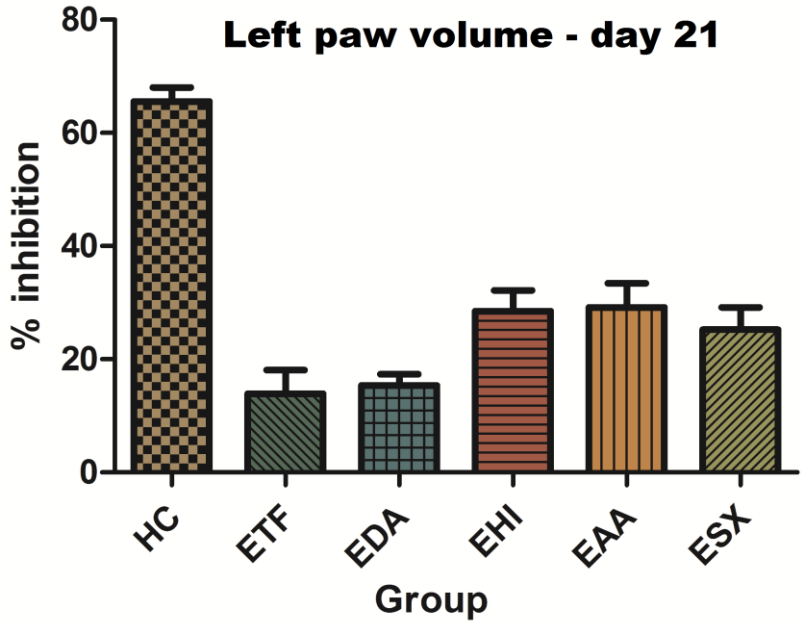


Figure 5.23 Percentage inhibition of left (injected) paw volume after 21 days during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

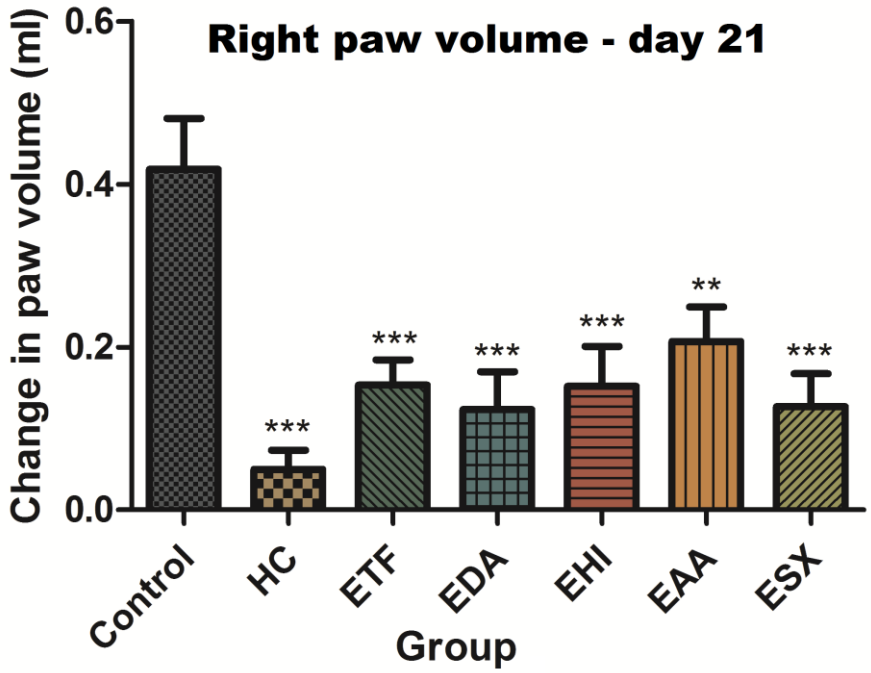


Figure 5.24 Effect of HC, ETF, EDA, EHI, EAA and ESX on right (non-injected) paw volume after 21 days during adjuvant arthritis study. Each column represents the mean±S.E.M. of six rats. **p<0.01 and ***p<0.001vs. control group.

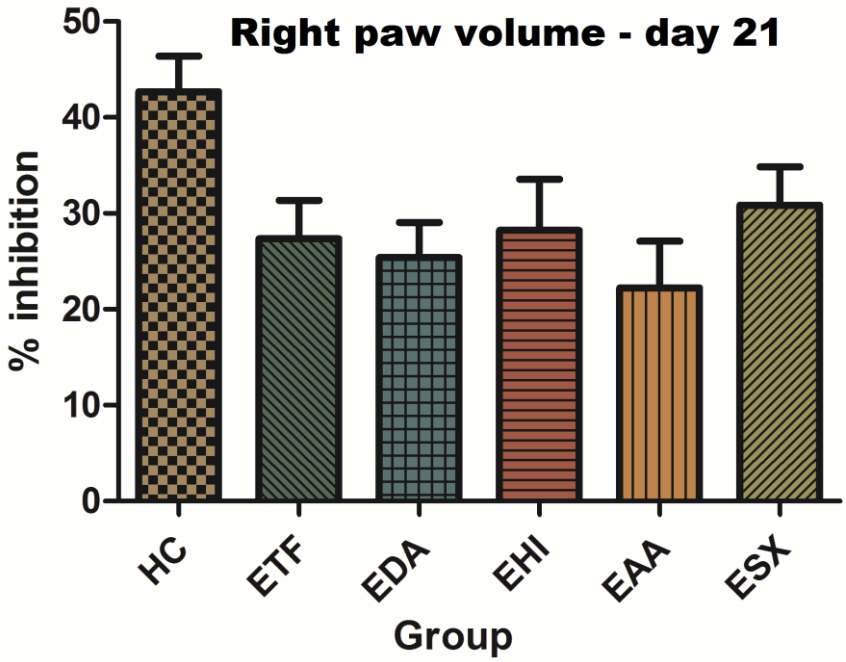


Figure 5.25 Percentage inhibition of right (non-injected) paw volume after 21 days during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

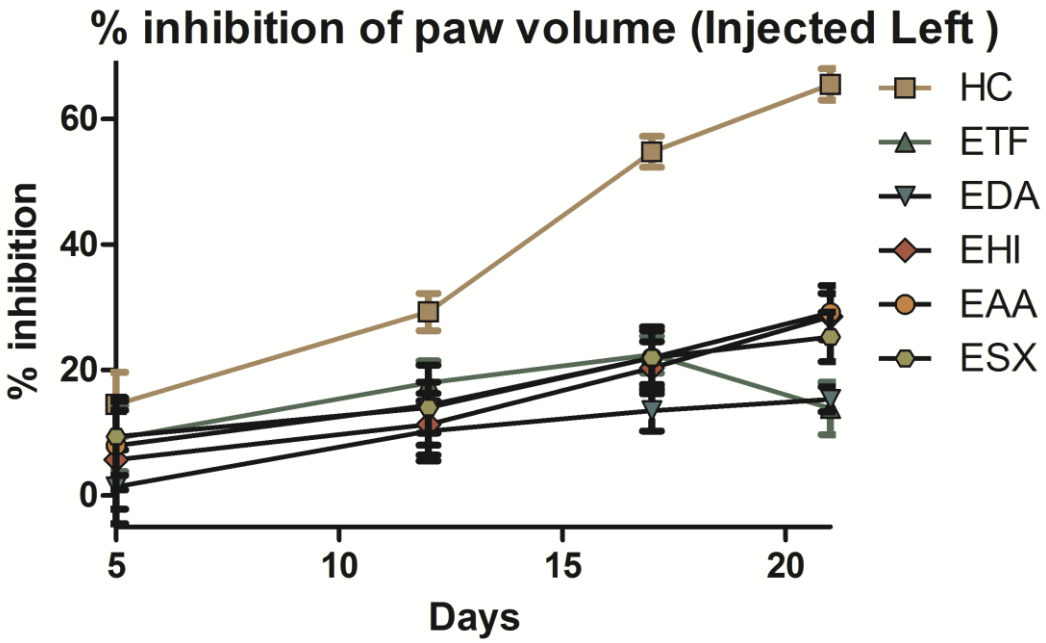


Figure 5.26 Percentage inhibition of left (injected) paw volume during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each value represents the mean±S.E.M. of six rats.

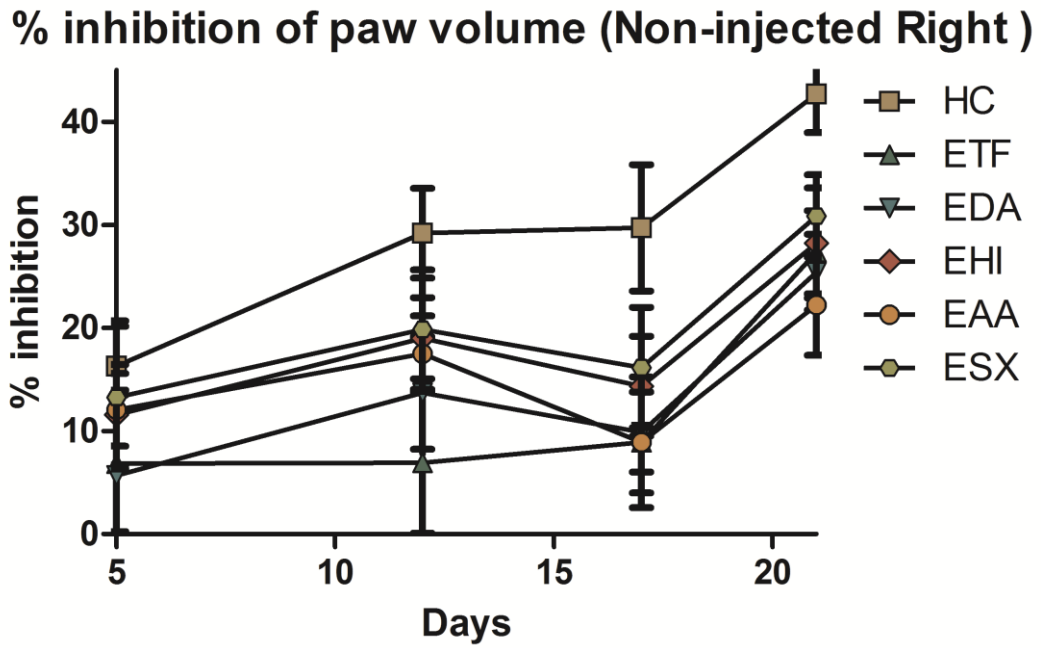


Figure 5.27 Percentage inhibition of right (non-injected) paw volume during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each value represents the mean±S.E.M. of six rats.

Table 5.6 Effect of HC, ETF, EDA, EHI, EAA and ESX on left (injected) paw volume during adjuvant arthritis study

Group	Change in Injected (Left) paw volume in ml.			
	At Day 5	**At day 12**	**At day 17**	**At day 21**
Control	0.54±0.041	0.76±0.031	0.94±0.046	1.13±0.065
HC	0.35±0.042	0.35±0.025***	0.12±0.042***	0.03±0.032***
ETF	0.46±0.075	0.55±0.059	0.63±0.062**	0.92±0.078
EDA	0.44±0.038	0.55±0.049	0.66±0.054*	0.79±0.067**
EHI	0.48±0.079	0.62±0.062	0.65±0.060**	0.66±0.054***
EAA	0.42±0.073	0.53±0.081*	0.57±0.076***	0.61±0.077***
ESX	0.44±0.085	0.58±0.067	0.63±0.074**	0.72±0.077***

Six rats were used in each group. The change in left paw volume in ml was calculated by subtracting weight at day 1 from that after FCA injection. The value expressed mean±S.E.M. *p<0.05, **p<0.01 and ***p<0.001 vs control group.

Table 5.7 Effect of HC, ETF, EDA, EHI, EAA and ESX on right (non-injected) paw volume during adjuvant arthritis study

Group	Change in Injected (Right) paw volume in ml.			
	At Day 5	**At day 12**	**At day 17**	**At day 21**
Control	0.19±0.017	0.26±0.025	0.29±0.034	0.42±0.062
HC	0.09±0.0211	0.06±0.021***	0.09±0.038**	0.05±0.023***
ETF	0.13±0.046	0.19±0.054	0.22±0.054	0.15±0.031***
EDA	0.08±0.033	0.09±0.019**	0.16±0.037	0.12±0.046***
EHI	0.09±0.042	0.11±0.036*	0.18±0.038	0.15±0.049***
EAA	0.09±0.025	0.12±0.032*	0.22±0.031	0.21±0.043**
ESX	0.08±0.026	0.11±0.025**	0.16±0.044	0.13±0.041***

Table 5.8 Percentage inhibition of left (injected) paw volume during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX

Group	% inhibition of paw volume of the injected left paw			
	At Day 5	At day 12	At day 17	At day 21
HC	14.51±5.166	29.25±2.960	54.81±2.484	65.54±2.482
ETF	9.07±5.254	17.99±3.455	22.45±2.934	13.91±4.187
EDA	1.41±5.855	10.31±4.767	13.55±3.246	15.34±1.997
EHI	5.72±7.874	11.37±4.895	20.37±4.135	28.53±3.663
EAA	7.96±7.041	14.41±6.348	21.98±4.952	29.14±4.300
ESX	9.39±6.193	14.02±4.071	21.98±4.274	25.26±3.939

Six rats were used in each group. The change in right paw volume in ml was calculated by subtracting weight at day 1 from that after FCA injection. The value expressed mean±S.E.M. *p<0.05, **p<0.01 and ***p<0.001 vs control group.

Table 5.9 Percentage inhibition of right (non-injected) paw volume during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX

Group	% inhibition of paw volume of the non-injected right paw			
	At Day 5	At day 12	At day 17	At day 21
HC	16.31±4.380	29.18±4.354	29.70±6.117	42.68±3.720
ETF	6.86±6.619	6.92±6.815	8.89±6.340	27.34±4.022
EDA	5.67±8.293	13.70±5.452	9.9±3.880	25.40±3.683
EHI	11.58±4.882	19.00±3.937	14.34±4.835	28.22±5.343
EAA	12.06±3.513	17.51±3.649	8.889±4.883	22.22±4.863
ESX	13.24±6.934	19.85±5.793	16.16±5.833	30.86±4.003

The immunosuppressive activity can also express by inhibition of lesion compare to control on day 21 (table 5.10). The arthritic lesion on day 21 for control, HC, ETF, EDA, EHI, EAA and ESX were 6.167±0.402, 1.167±0.167***, 4.667±0.422*, 3.167±0.477**, 2.333±0.333***, 2.167±0.307*** and 2.000±0.258*** respectively (figure 5.28 (A)). The percentage inhibition of arthritic lesion (figure 5.28 (B)) on day 21 for HC, ETF, EDA, EHI, EAA and ESX were 81.08±2.703, 29.72±3.419, 48.65±4.983, 62.16±5.405, 64.86±4.983 and 67.56±4.187 respectively. The suppression of arthritic lesion on day 21 for HC, EHI, EAA and ESX found extremely significant. ETF and EDA showed significant and highly significant inhibition of arthritic lesion (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).

The total percentage change (table 5.11) calculated by addition of percentage inhibition of left paw on day 5, percentage inhibition of right paw on day 12 and percentage inhibition of arthritic score on day 21 (figure 5.30). The total percentage change for HC, ETF, EDA, EHI, EAA and ESX were 124.77, 45.71, 63.76, 86.88, 90.33 and 96.8 respectively.

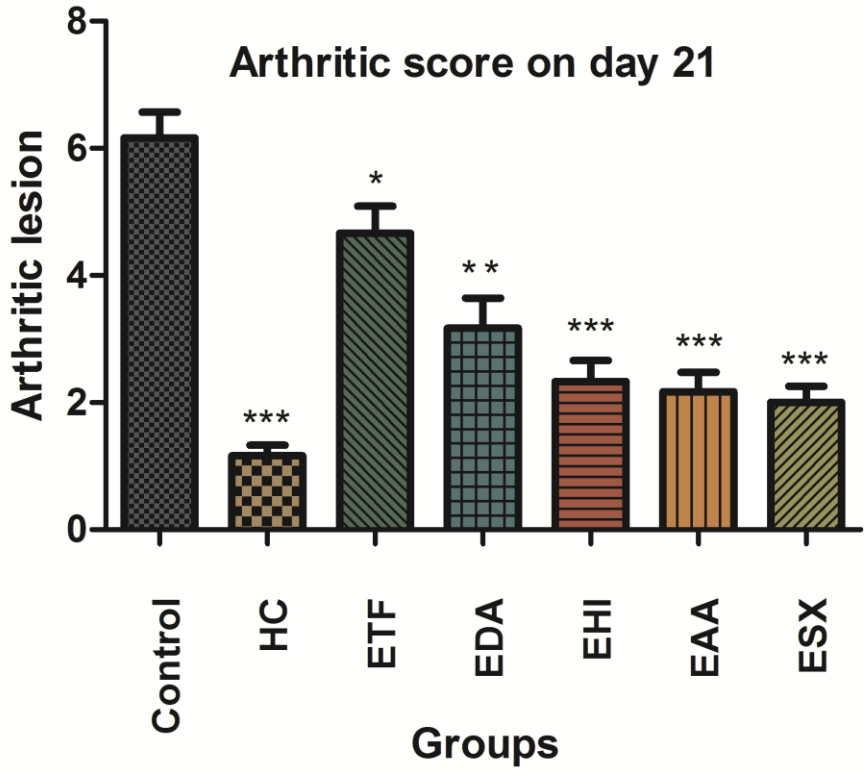


Figure 5.28 (A) Arthritic lesion on day 21 during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats. *p<0.05, **p<0.01 and ***p<0.001 vs. control group.

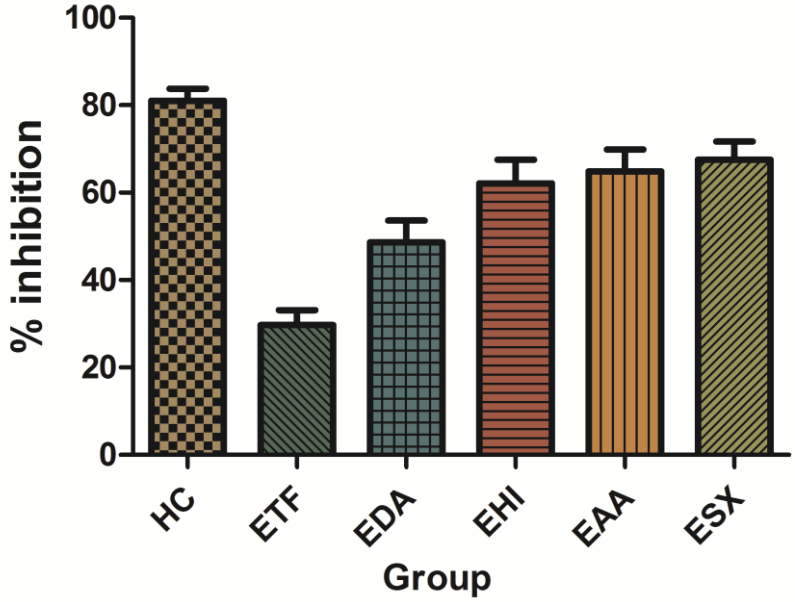


Figure 5.28 (B) Percentage inhibition of arthritic lesion during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

Table 5.10 Percentage inhibition of arthritic index during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX

Group	Arthritic index on day 21	% inhibition of arthritic lesion
Control	6.167±0.402	------
HC	1.167±0.167***	81.08±2.703
ETF	4.667±0.422*	29.72±3.419
EDA	3.167±0.477**	48.65±4.983
EHI	2.333±0.333***	62.16±5.405
EAA	2.167±0.307***	64.86±4.983
ESX	2.000±0.258***	67.56±4.187

Six rats were used in each group. The arthritic index was graded on the scale of 0-4. The value were expressed mean±S.E.M. *p<0.05, **p<0.01 and ***p<0.001 vs. control group

Table 5.11 Total percentage change during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX

Group	Total percentage change
HC	124.77
ETF	45.71
EDA	63.76
EHI	86.88
EAA	90.33
ESX	96.8

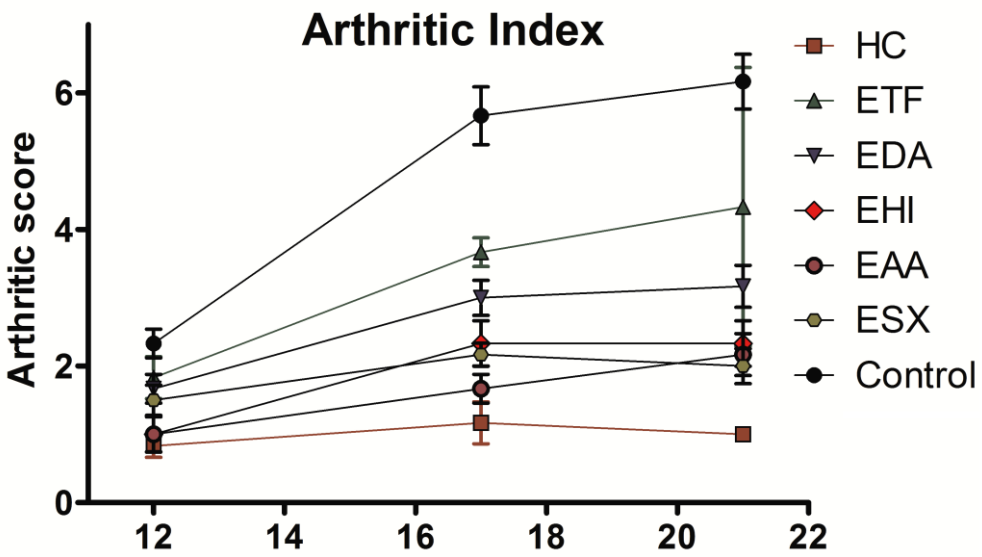


Figure 5.29 Arthritic index during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each value represents the mean±S.E.M. of six rats.

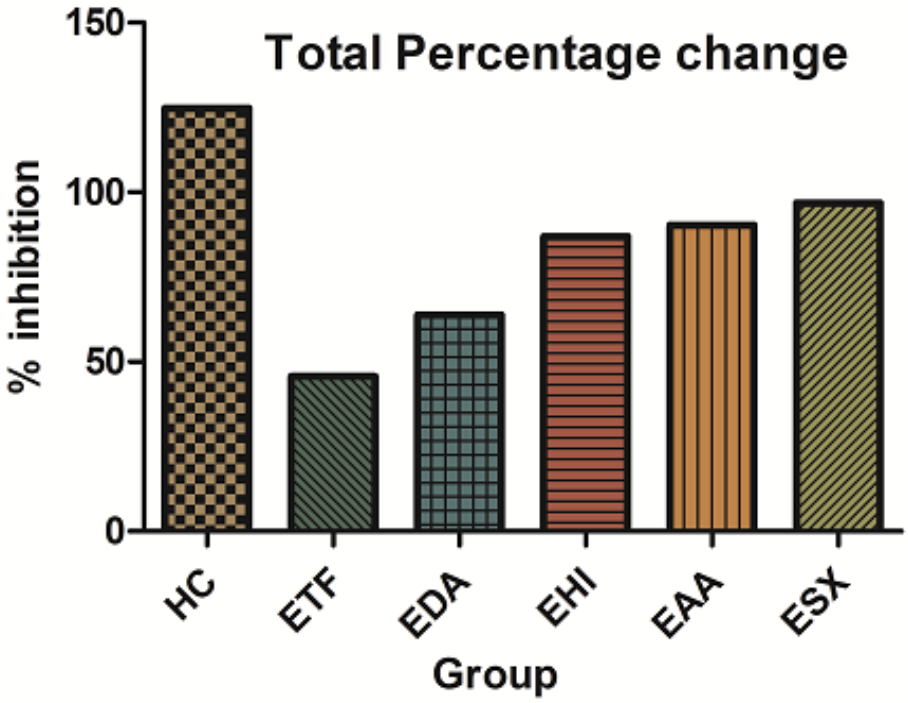


Figure 5.30 Total percentage change during adjuvant arthritis study by HC, ETF, EDA, EHI, EAA and ESX. Each column represents the mean±S.E.M. of six rats.

5.4 Effect of treatment of plant extract on liver glycogen deposition test in rats

The choice of the animal strain has been found to be very important for the performance of this test. Wistar rats have been proven to be very suitable in contrast to other sub strains. In present study albino Wistar rat were used as test animal and references (Nobuhiko *et al.*1975) available for liver glycogen deposition animal model (figure 5.31).

The Calibration curve *(*figure 5.32) of glucose for estimation of liver glycogen was prepared same as procedure explained in materials and methods. Linearity range of standard glucose calibration curve (n=3) was found in range of 20-100 µg/ml with co-relation coefficient (r^2) of 0.986 and the linearity equation was found to be Y = 0.006X + 0.103 (figure 5.33).

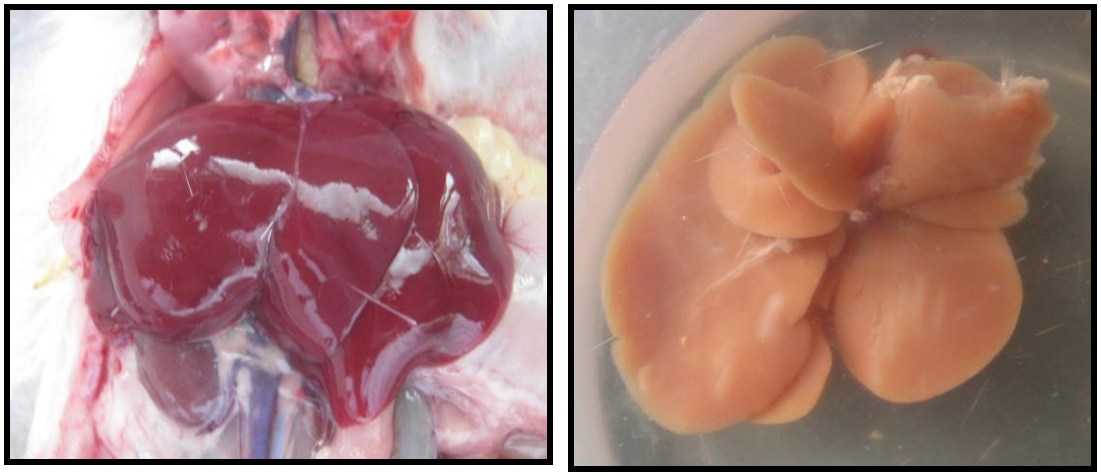


Image of Rat liver **Rat liver after removal of blood**

Figure 5.31 Rat liver during surgery and after removal of blood

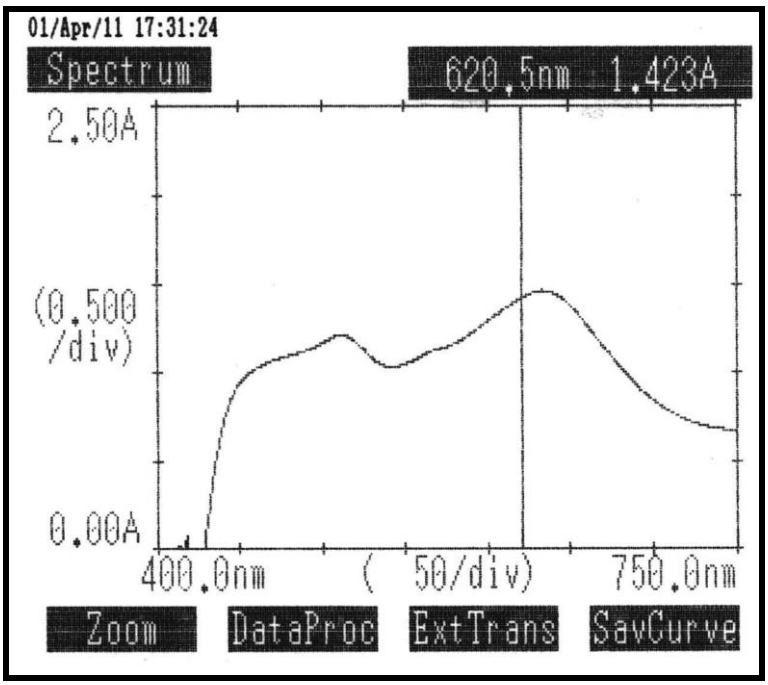


Figure 5.32 UV spectrum of glucose after interaction with anthrone reagent

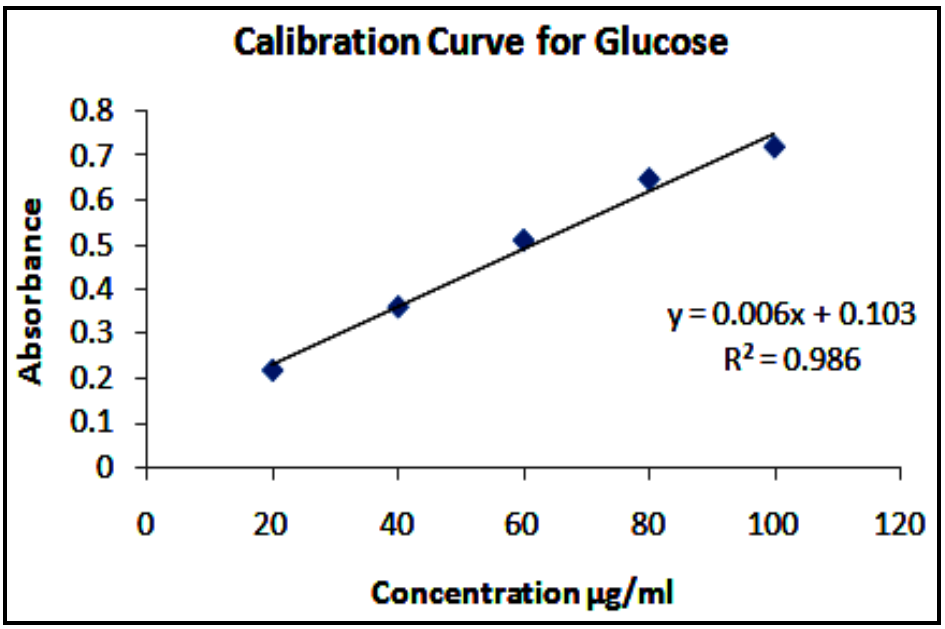


Figure 5.33 Calibration curve of glucose for estimation of liver glycogen

The glycogen deposition in liver was expressed as liver glycogen in mg per gm of liver (figure 5.34). The liver glycogen deposition was found for control, HC, EHI, EAA and ESX groups were 17.67 ± 1.279, 46.22 ± 2.279 ***, 27.90 ± 1.955 **, 27.17 ± 1.791** and 25.57 ± 1.731* respectively as shown in table 5.12. . The gain in liver glycogen for EHI and EAA was found highly significant while ESX was found significant when compared with control. The glycogen deposition in liver increase with extreme significance. (*p<0.05, **p<0.01 and ***p<0.001).

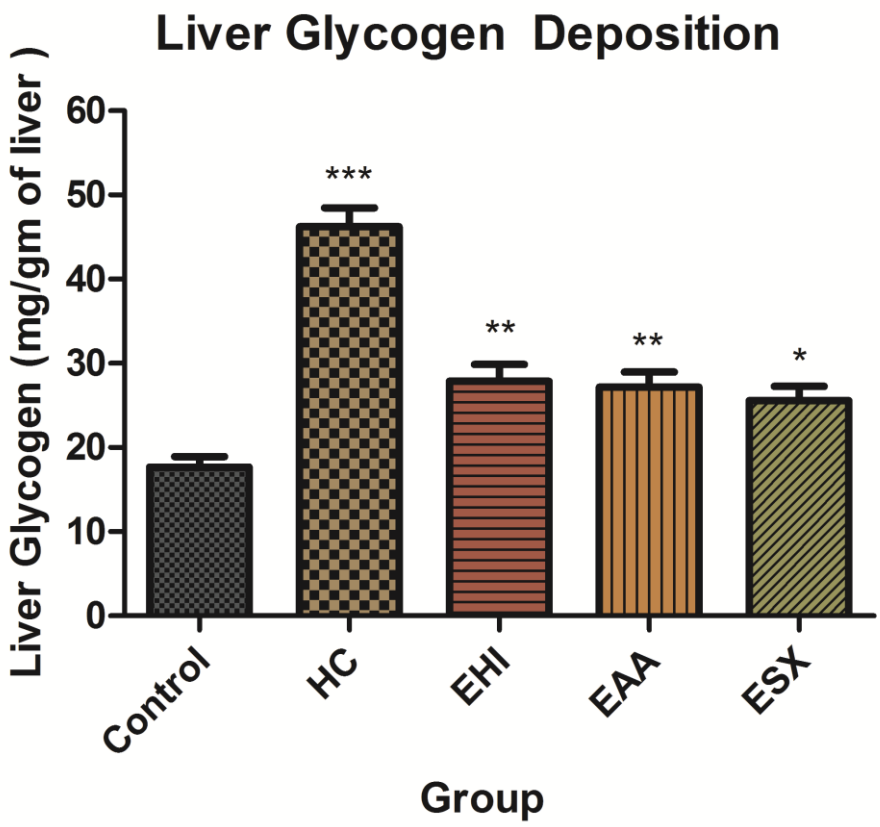


Figure 5.34 Effect of HC, EHI, EAA and ESX on liver glycogen during liver glycogen deposition study. Each column represents the mean±S.E.M. of six rats. *p<0.05, **p<0.01 and ***p<0.001 vs. control group.

Table 5.12 Effect of HC, EHI, EAA and ESX on liver glycogen deposition	
Group	**Liver Glycogen (mg/gm of liver)**
Control	17.67 ± 1.279
HC	46.22 ± 2.279 ***
EHI	27.90 ± 1.955 **
EAA	27.17 ± 1.791**
ESX	25.57 ± 1.731*

Six rats were used in each group. The liver glycogen deposition was calculated in mg per gram of liver. The value were expressed mean±S.E.M. *p<0.05, **p<0.01 and ***p<0.001 vs. control group

The hematological evaluation for blood cells, SGPT, SGOT, serum calcium and serum potassium was performed for control group and treatment groups. The results for hematological evaluation expressed in table 5.13.

The red blood corpuscle (RBC) count (10^6/cumm) for control, HC, EHI, EAA and ESX were found to be 7.39±0.519, 10.6±0.950**, 7.4 ± 0.705, 6.2 ± 0.568 and 7.4 ± 0.464 respectively (figure 5.35). The increase in RBC for HC was found to be highly significant as compare to control (**p<0.01).

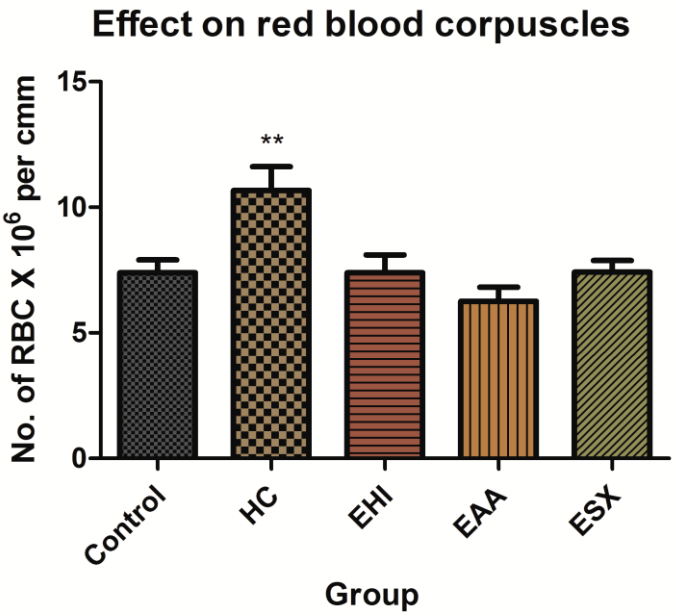


Figure 5.35 Effect of HC, EHI, EAA and ESX on red blood corpuscles during liver glycogen deposition study. Each column represents the mean±S.E.M. of six rats. **p<0.01 vs. control group.

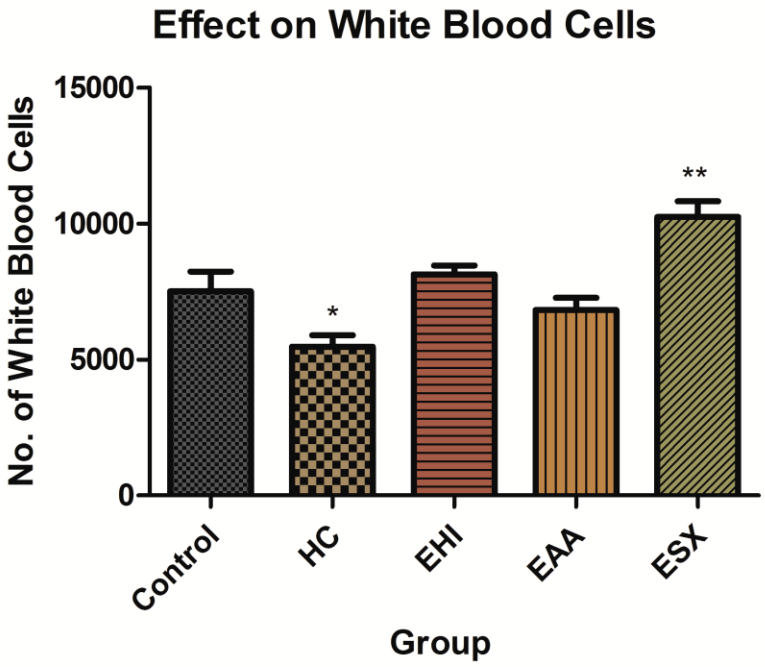


Figure 5.36 Effect of HC, EHI, EAA and ESX on white blood cell during liver glycogen deposition study. Each column represents the mean±S.E.M. of six rats. *p<0.05 and **p<0.01 vs. control group.

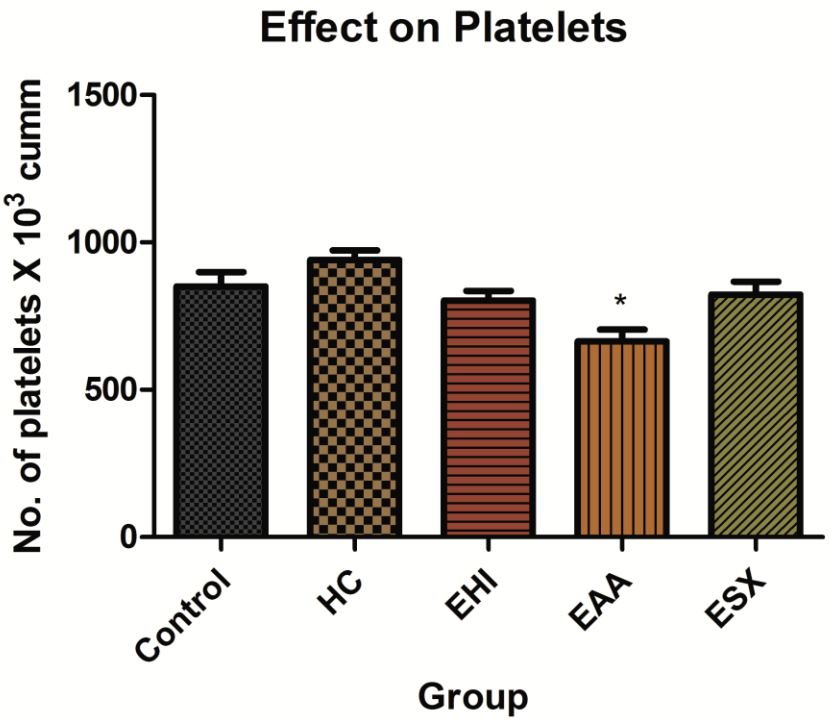


Figure 5.37 Effect of HC, EHI, EAA and ESX on platelets during liver glycogen deposition study. Each column represents the mean±S.E.M. of six rats. *p<0.05 vs. control group.

The white blood cells (WBC) count per cu mm for control, HC, EHI, EAA and ESX were found to be 7509 ± 721.7, 5469 ± 420.9 *, 8133 ± 323.0, 6819 ± 448.2 and 10239 ± 575.9 ** respectively (figure 5.36). The increase in WBC for HC was found to be significant as compare to control while ESX showed highly significant gain in WBC as compare to control (*p<0.05 and **p<0.01).

The platelets count per (10^3/cumm) for control, HC, EHI, EAA and ESX were found to be 851.0 ± 48.06, 940.0 ± 33.59, 803.7 ± 31.56, 664.0 ± 40.28 * and 823.3±43.47 (figure 5.37) only EAA showed significant decrease in platelet counts as compare to control (*p<0.05).

The serum calcium level (mg/dl) for control, HC, EHI, EAA and ESX were found to be 8.233 ± 0.567, 14.00 ± 1.378 **, 5.673 ± 0.943, 12.52 ± 0.953 * and 8.967±0.605 (figure 5.38). The HC shown highly significant increase in serum calcium level as compare to control while EAA shown significant gain in serum calcium level as compare (*p<0.05 and **p<0.01).

The serum potassium level (mMol/liter) for control, HC, EHI, EAA and ESX were found to be 5.100 ± 0.193, 2.667 ± 0.191***, 4.147 ± 0.353, 4.073 ± 0.582 and 4.300 ± 0.238 (figure 5.39). The HC showed extremely significant increase in serum potassium level as compare to control (***p<0.001).

Serum glutamate pyruvate transaminase, or SGPT (ALT) level (IU/L) for control, HC, EHI, EAA and ESX were found 62.30±4.594, 105.3±9.207**, 50.43±4.646, 56.73±10.67 and 48.30±4.838 (figure 5.40). The HC showed highly significant increase in SGPT as compare to control (**p<0.01).

SGOT or Serum glutamic-oxaloacetic transaminase (AST) level (IU/L) for control, HC, EHI, EAA and ESX were found 178.7 ± 11.98, 233.0 ± 8.71*, 174.0 ± 14.01, 193.3 ± 10.33 and 157.7 ± 10.09 (figure 5. 5.41). The HC showed significant increase in SGOT level as compare to control (*p<0.05).

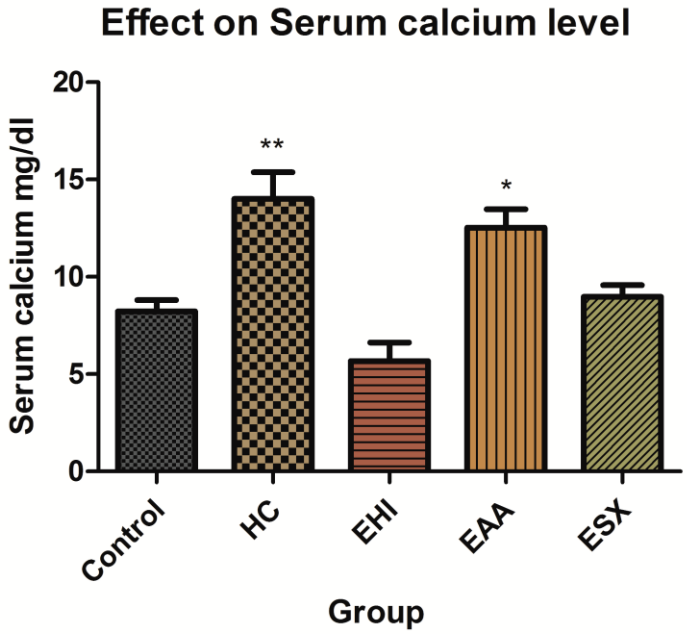


Figure 5.38 Effect of HC, EHI, EAA and ESX on serum calcium level during liver glycogen deposition study. Each column represents the mean±S.E.M. of six rats. *p<0.05 and **p<0.01 vs. control group.

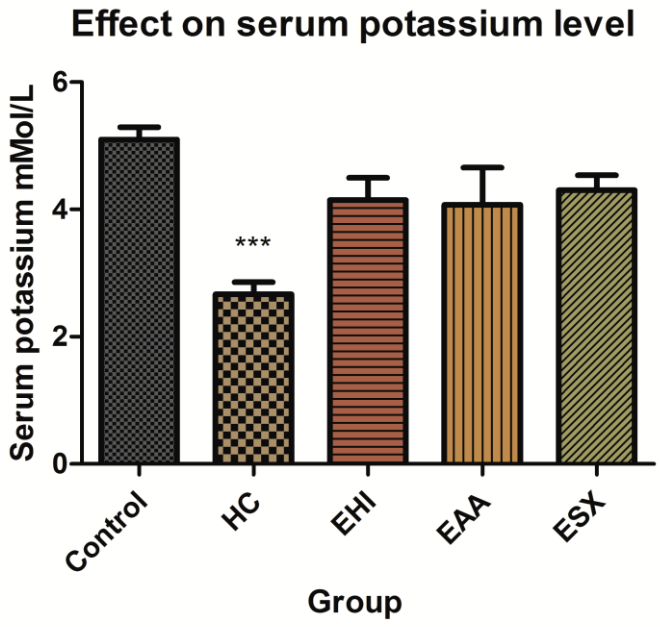


Figure 5.39 Effect of HC, EHI, EAA and ESX on serum potassium level during liver glycogen deposition study. Each column represents the mean±S.E.M. of six rats. ***p<0.001 vs. control group.

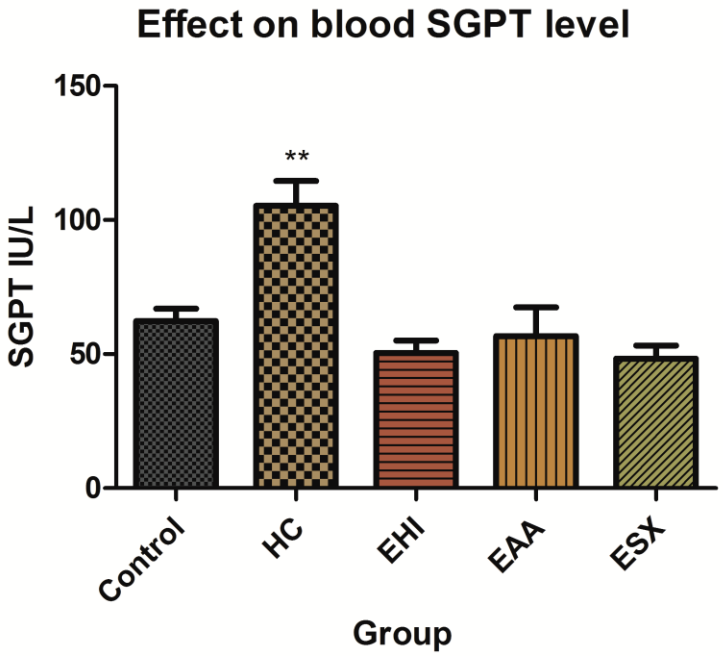


Figure 5.40 Effect of HC, EHI, EAA and ESX on SGPT (ALT) level during liver glycogen deposition study. Each column represents the mean±S.E.M. of six rats. **p<0.01 vs. control group.

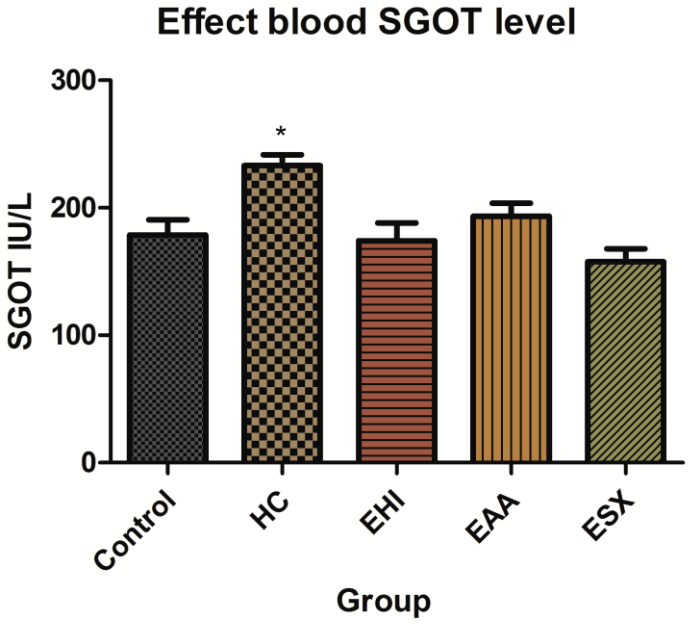


Figure 5.41 Effect of HC, EHI, EAA and ESX on SGOT (AST) level during liver glycogen deposition study. Each column represents the mean±S.E.M. of six rats. *p<0.05 vs. control group.

Table 5.13 Effect of HC, EHI, EAA and ESX on Hematological parameter during liver glycogen deposition study

Group	RBC 10^6/cumm	WBC per cumm	Pletlet 10^3/cumm	Serum Ca^{++} Mg/dl	Serum K^+ mMol/L	SGPT (ALT) IU/L	SGOT (AST) IU/L
Control	7.39 ± 0.519	7509 ± 721.7	851.0 ± 48.06	8.233 ± 0.567	5.100 ± 0.193	62.30 ± 4.594	178.7 ± 11.98
HC	10.6 ± 0.950 **	5469 ± 420.9 *	940.0 ± 33.59	14.00 ± 1.378 **	2.667 ± 0.191***	105.3 ± 9.207 **	233.0 ± 8.71 *
EHI	7.4 ± 0.705	8133 ± 323.0	803.7 ± 31.56	5.673 ± 0.943	4.147 ± 0.353	50.43 ± 4.646	174.0 ± 14.01
EAA	6.2 ± 0.568	6819 ± 448.2	664.0 ± 40.28 *	12.52 ± 0.953 *	4.073 ± 0.582	56.73 ± 10.67	193.3 ± 10.33
ESX	7.4 ± 0.464	10239 ± 575.9 **	823.3 ± 43.47	8.967 ± 0.605	4.300 ± 0.238	48.30 ± 4.838	157.7 ± 10.09

Six rats were used in each group. The hematological parameters were calculated in blood. The value were expressed mean±S.E.M. *p<0.05, **p<0.01 and ***p<0.001 vs. control group

Histopathology of Liver

Liver glycogen was determined under standard conditions on intact and adrenalectomized rats. The liver uses glucose as a fuel and also has the ability to store it as glycogen and synthesize it from noncarbohydrate precursors (gluconeogenesis). Periodic acid-Schiff (PAS) is a staining method used to detect glycogen and other polysaccharides in tissues. The reaction of periodic acid oxidizes the diol functional groups in glucose and other sugars, creating aldehydes that react with the Schiff reagent to give a purple-magenta color. A suitable basic stain is often used as a counterstain. Wax blocks were made and section was made by microtome, stained by Periodic acid-Schiff (PAS) and photographed.

The effect of HC, EHI, EAA and ESX on histopathology of liver during liver glycogen deposition study shown in figure 5.42. The Histopathology of liver treated with HC clearly showed more deposition of glycogen in hepatocytes. The Glycogen deposition was comparatively observed more in HC, EHI and EAA while ESX showed least signs for glycogen deposition.

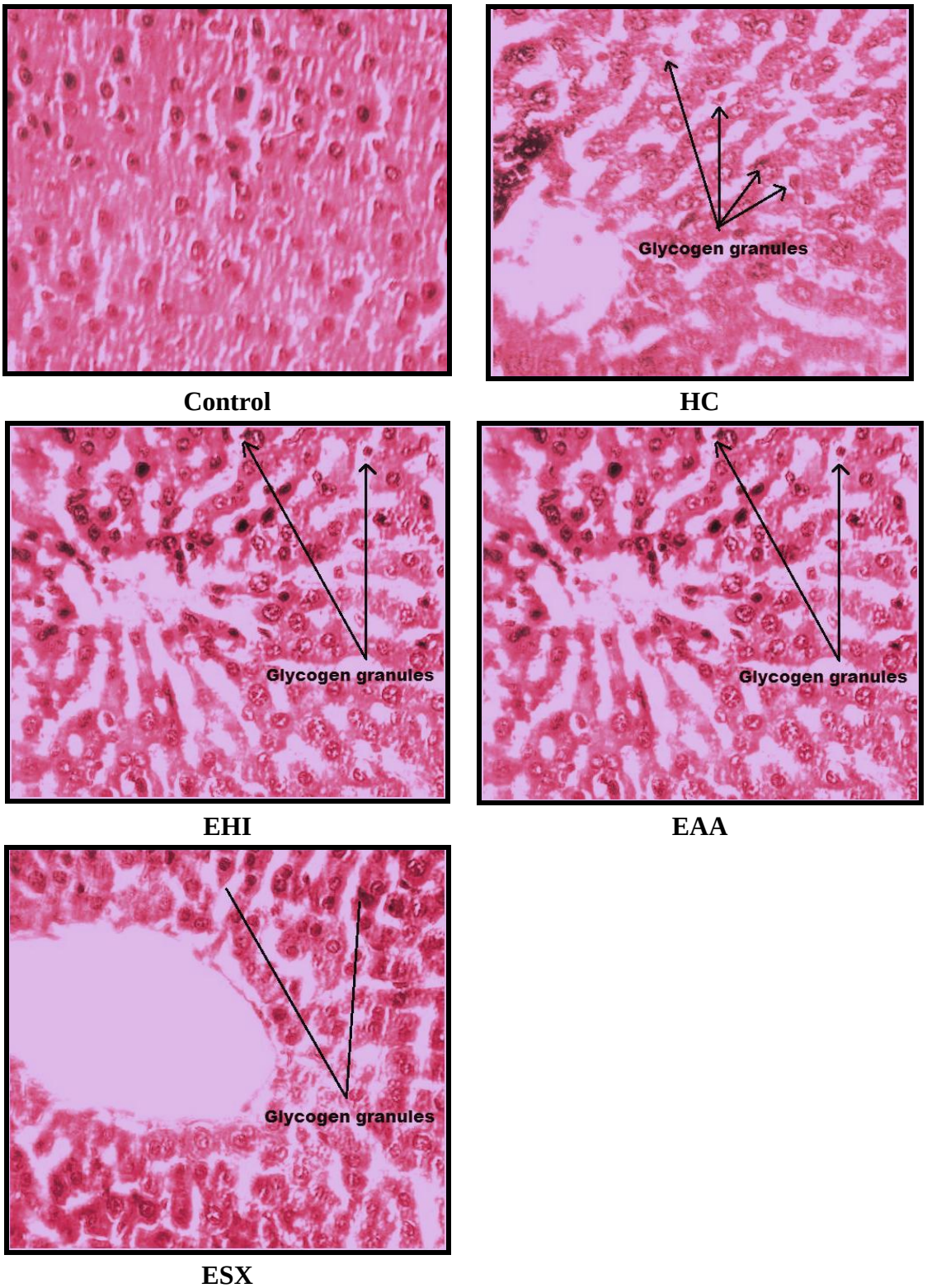


Figure 5.42 Effect of HC, EHI, EAA and ESX on histopathology of liver during liver glycogen deposition study

5.5 Effect of treatment of plant extract on sodium excretion in rat

The mineralocorticoid activity by measurement of sodium excretion carried out only for Hydrocortisone, Ethanolic extract of *Hemidesmus indicus* (EHI), Ethanolic extract of *Agave americana* (EAA) and Ethanolic extract of *Solanum xanthocarpum* (ESX). Along with sodium excretion, urine alalysis also performed for urine chlorides, urine potassium and urine Creatinine as shown in table 5.14.

The urine chloride (Cl^-) level (mMol/L) for control, HC, EHI, EAA and ESX were found to be 102.3 ± 5.453, 44.20 ± 6.583 ***, 104.3 ± 4.902, 91.33 ± 10.85 and 107.3 ± 9.404 (figure 5.43). HC showed extremely significant decrease in urine chloride level as compare to control (***p<0.001).

The urine Creatinine level (mg/dl) for control, HC, EHI, EAA and ESX were found to be 0.546 ± 0.049, 1.005 ± 0.129 **, 0.646 ± 0.069, 0.566 ± 0.083 and 0.600 ± 0.074 (figure 5.44). The HC showed highly significant gain in urine Creatinine level as compare to control (**p<0.01).

The urine sodium level (mMol/L) for control, HC, EHI, EAA and ESX were found to be 76.40 ± 3.530, 30.20 ± 3.878 ***, 59.78 ± 5.207, 48.80 ± 8.496* and 74.60 ± 7.871 (figure 5.45). The HC showed extremely significant fall in urine sodium level as compare to control while EAA showed significant reduction in urine sodium level as compare to control (*p<0.05 and ***p<0.001).

The urine potassium level (mMol/L) for control, HC, EHI, EAA and ESX were found to be 11.52 ± 0.8133, 19.06 ± 1.364 **, 12.62 ± 2.209, 13.34 ± 1.515 and 12.84 ± 1.547 (figure 5.46). HC showed highly significant increase in potassium excretion level as compare to control (**p<0.01).

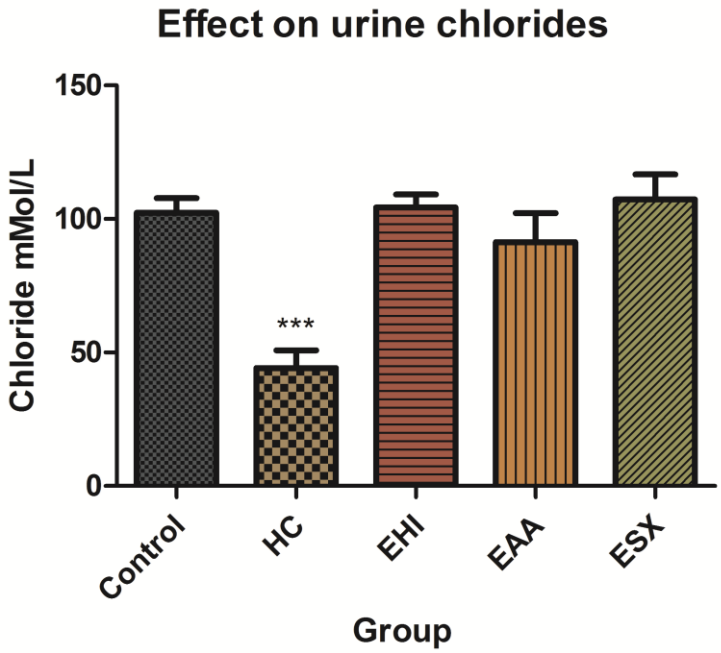


Figure 5.43 Effect of HC, EHI, EAA and ESX on urine chlorides (Cl^-) during evaluation of mineralocorticoid activity. Each column represents the mean±S.E.M. of six rats. ***p<0.001 vs. control group.

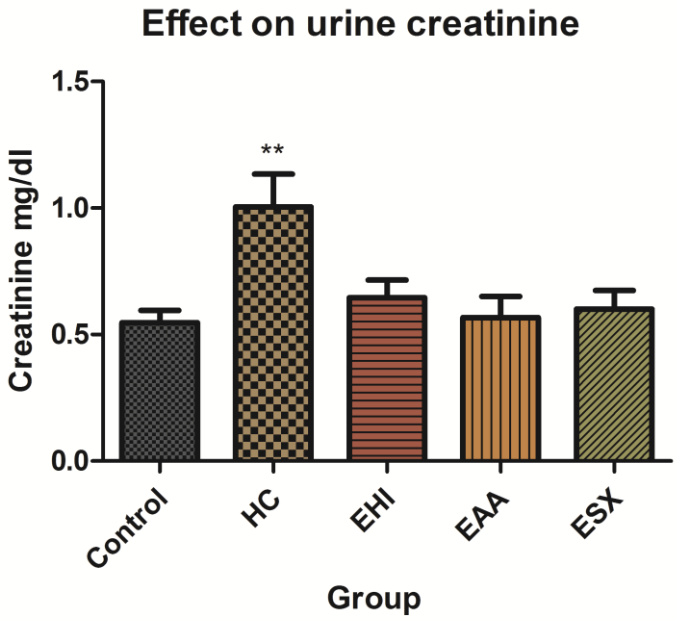


Figure 5.44 Effect of HC, EHI, EAA and ESX on urine Creatinine level during evaluation of mineralocorticoid activity. Each column represents the mean±S.E.M. of six rats. **p<0.01 vs. control group.

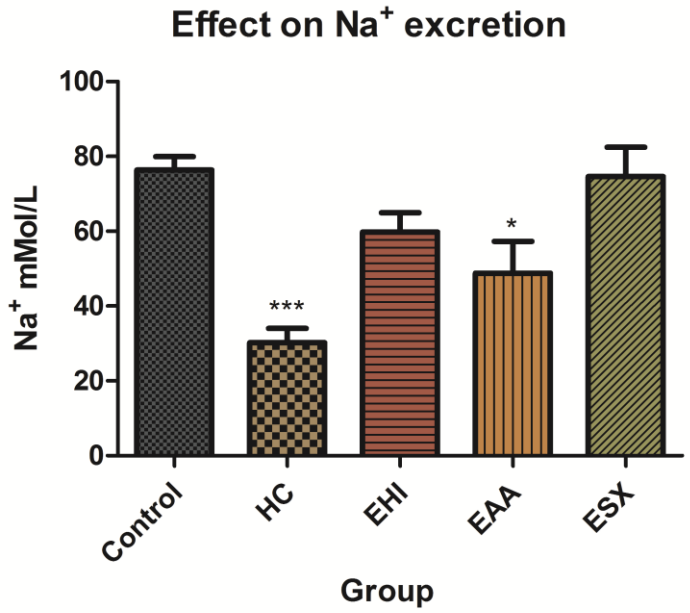


Figure 5.45 Effect of HC, EHI, EAA and ESX on Sodium (Na^+) excretion during evaluation of mineralocorticoid activity. Each column represents the mean±S.E.M. of six rats. *p<0.05 and ***p<0.001 vs. control group.

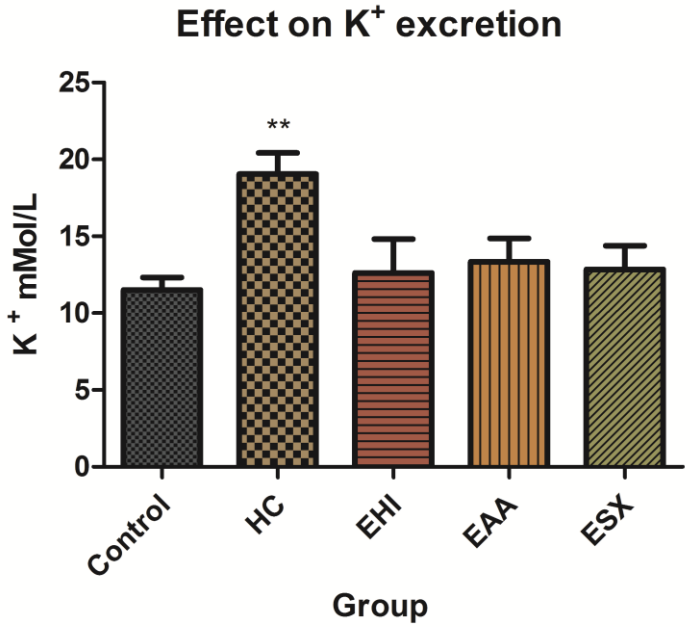


Figure 5.46 Effect of HC, EHI, EAA and ESX on Potassium (K^+) excretion during evaluation of mineralocorticoid activity. Each column represents the mean±S.E.M. of six rats. **p<0.01 vs. control group.

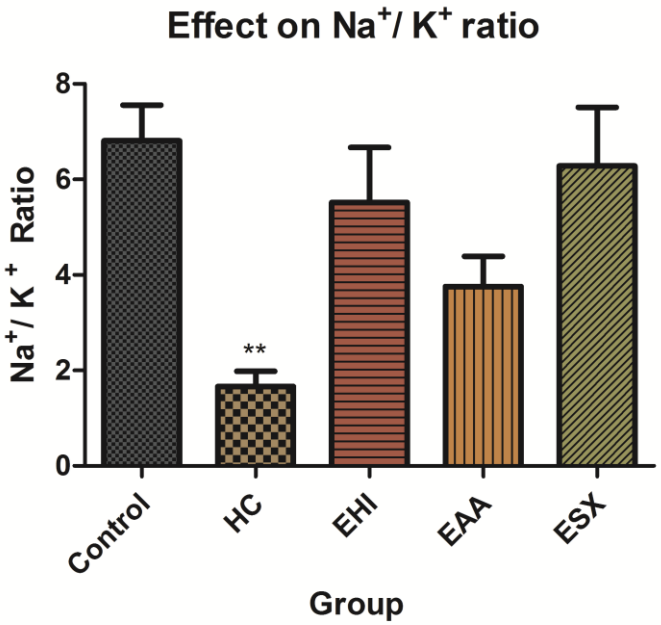


Figure 5.47 Effect of HC, EHI, EAA and ESX on Sodium /Potassium (Na^+ / K^+) Ratio during evaluation of mineralocorticoid activity. Each column represents the mean±S.E.M. of six rats. **p<0.01 vs. control group.

Table 5.14 Effect of HC, EHI, EAA and ESX on urine parameter during evaluation of Mineralocorticoid activity

Group	Chloride mMol / L	Creatinine Mg / dl	Sodium mMol / L	Potassium mMol / L	Na^+ / K^+ Ratio
Control	102.3 ± 5.453	0.546 ± 0.049	76.40 ± 3.530	11.52 ± 0.8133	6.81 ± 0.748
HC	44.20 ± 6.583 ***	1.005 ± 0.129 **	30.20 ± 3.878 ***	19.06 ± 1.364 **	1.66 ± 0.320 **
EHI	104.3 ± 4.902	0.646 ± 0.069	59.78 ± 5.207	12.62 ± 2.209	5.52 ± 1.148
EAA	91.33 ± 10.85	0.566 ± 0.083	48.80 ± 8.496 *	13.34 ± 1.515	3.75 ± 0.636
ESX	107.3 ± 9.404	0.600 ± 0.074	74.60 ± 7.871	12.84 ± 1.547	6.28 ± 1.229

Six rats were used in each group. The urine parameters were calculated and the value were expressed mean±S.E.M. *p<0.05, **p<0.01 and ***p<0.001 vs. control group

Table 5.15 Relative Potencies of Hydrocortisone, EHI, EAA and ESX

Sr. No.	Steroidal Compound	Glucocorticoid activity *(Liver glycogen deposition)*	Mineralocorticoid Activity *(Na^+ excretion)*
1	Hydrocortisone	1	1
2	EHI	0.61	0.57
3	EAA	0.58	0.73
4	ESX	0.55	0.36

The Na^+/K^+ ratio for control, HC, EHI, EAA and ESX were found to be 6.81±0.748, 1.66 ± 0.320 **, 5.52 ± 1.148, 3.75 ± 0.636 and 6.28 ± 1.229 (figure 5.47). The HC showed highly significant fall in Na^+/K^+ ratio as compare to control (**$p<0.01$)

Relative Potencies of Hydrocortisone, EHI, EAA and ESX were described in table 5.15. Glucocorticoid potential of (Liver glycogen deposition) HC, EHI, EAA and ESX were found to be 1, 0.61, 0.58 and 0.55 while Mineralocorticoid potential (Na+ excretion) of HC, EHI, EAA and ESX were found to be 1, 0.57, 0.73 and 0.36.

5.10 Statistical Analysis

In statistics, one-way analysis of variance (abbreviated one-way ANOVA) is a technique used to compare means of two or more samples (using the F distribution). This technique can be used only for numerical data. The ANOVA tests the null hypothesis that samples in two or more groups are drawn from the same population. When ANOVA shows a significant difference between groups, if one of the groups is a control (reference) group, Dunnett's Test is used as a *post hoc* test. In statistical significance testing, the p-value is the probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis is true. P value expressed as "Michelin Guide" scale, *: $P < 0.05$ (significant), **: $P < 0.01$ (highly significant) and ***: $P < 0.001$ (extremely significant). This multiple comparison test can be used to determine the significant differences between a single control group mean and the remaining treatment group means in an analysis of variance setting. It is one of the least conservative *post hoc* tests (Anscombe *et al.*, 1948).

5.11 Quality control of selected steroidal herbs

Various parameter like mobile phase, HPTLC band, saturation time, activation time and operational parameters were developed based on trial and error method. The mobile phase for optimization carried out with more than 10 different combination of mobile phase and selected 4 mobile phases which were again evaluated with optimized parameter. The development of four screened mobile phase showed in figure below. The figure 5.48 indicates visualization of HPTLC plate in UV_{254} using four mobile phase while figure 5.49 indicates visualization of HPTLC plate in UV_{366} using four mobile phase.

Ethanolic extract of *Trigonella foenum graecum* (ETF), Ethanolic extract of *Dioscorea alata* (EDA), Ethanolic extract of *Hemidesmus indicus* (EHI), Ethanolic extract of *Agave americana* (EAA) *and* Ethanolic extract of *Solanum xanthocarpum* (ESX) evaluated for presence of steroids by TLC while HPTLC performed only for EHI, EAA and ESX, those herbs which showed significant activity as steroids in adjuvant arthritis activity.

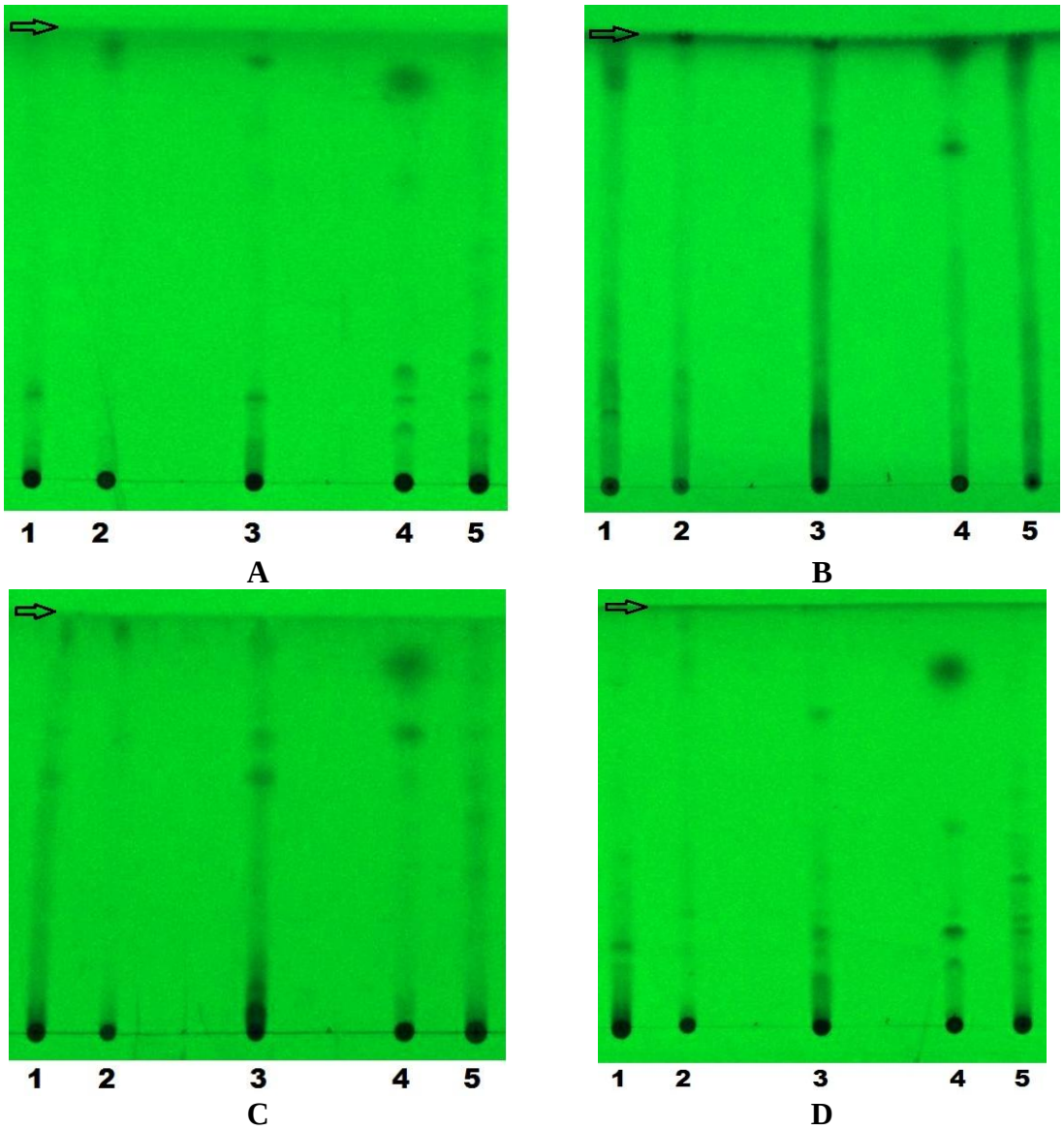


Figure 5.48 Thin layer chromatography for selected herbal steroids developed and visualized in UV_{254}

1-EDA, 2-EAA, 3-ESX, 4-EHI, 5-ETF

Mobile Phase

A. Cyclohexane/Ethyl acetate/ Chloroform 1:1:1 v/v/v
B. Chloroform/Methanol 75:25 v/v
C. Toluene/Ethyl acetate/Formic acid 5:5:1 v/v/v
D. Toluene/Ethyl acetate/Methanol 6:1:0.8 v/v/v

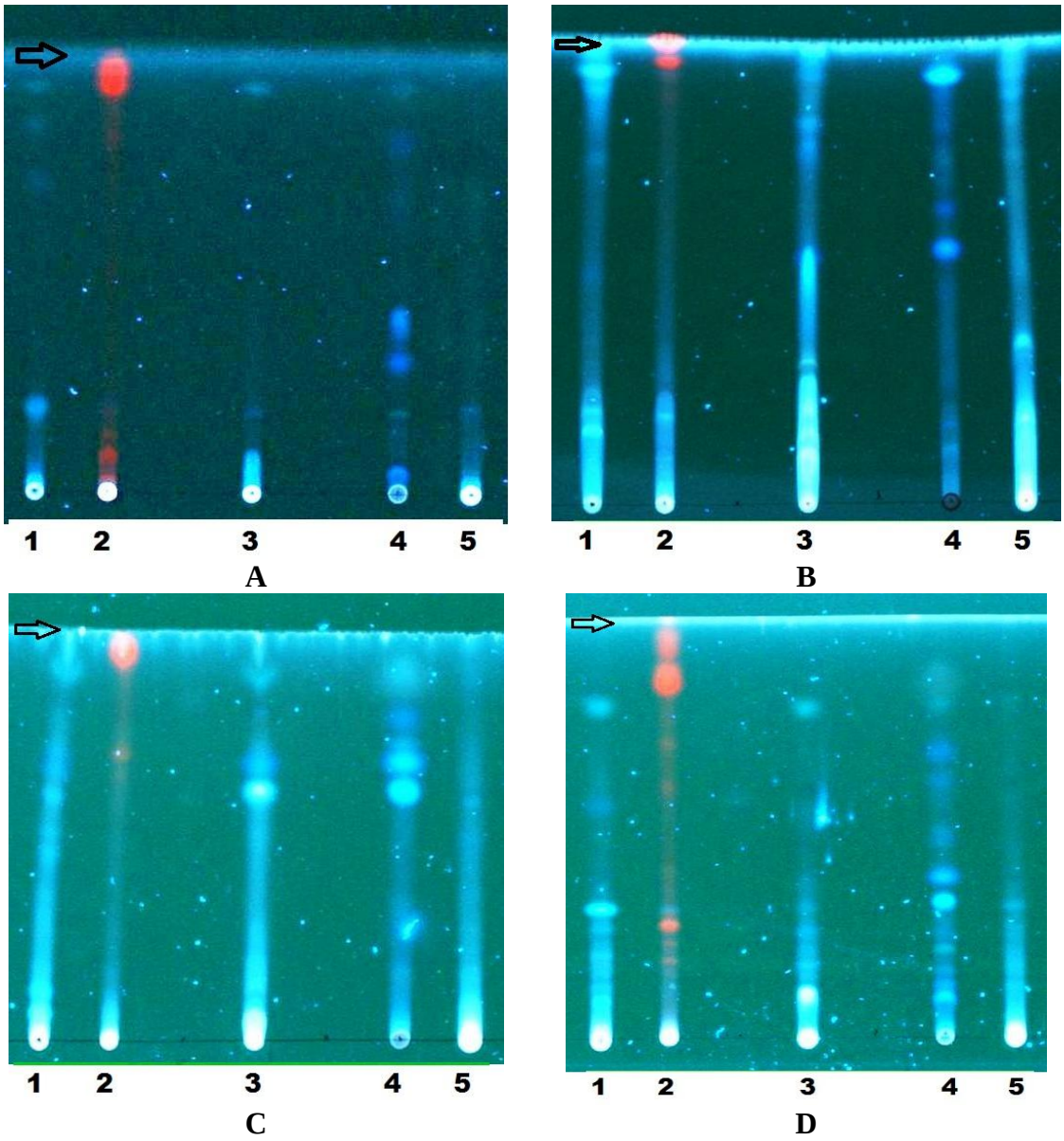


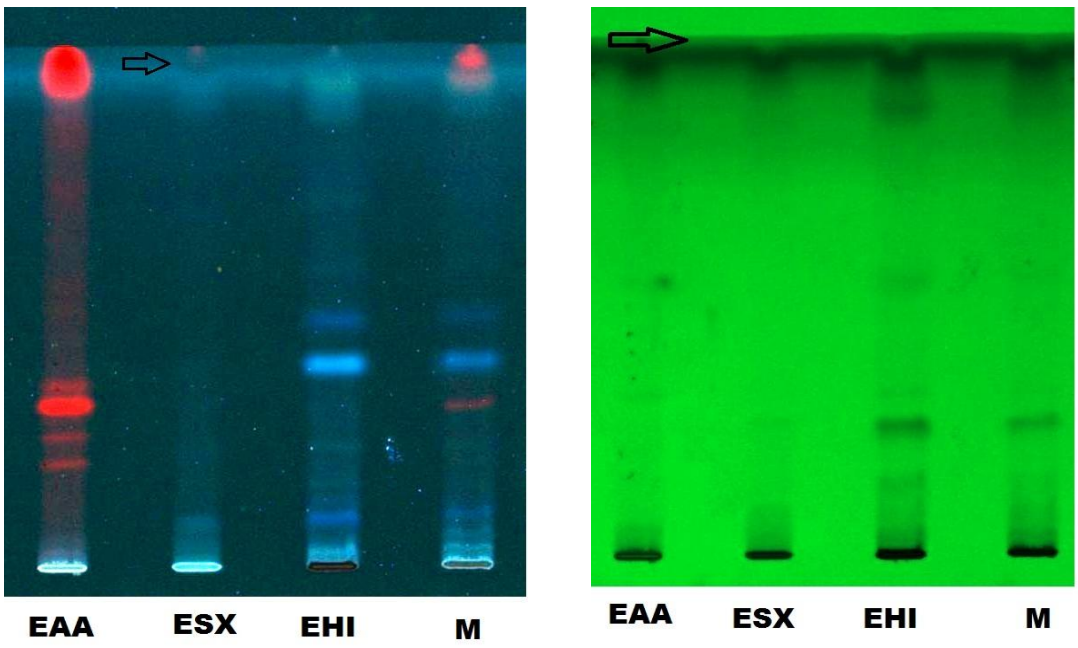
Figure 5.49 Thin layer chromatography for selected herbal steroids developed and visualized in UV_{366}

1-EDA, 2-EAA, 3-ESX, 4-EHI, 5-ETF

Mobile Phase

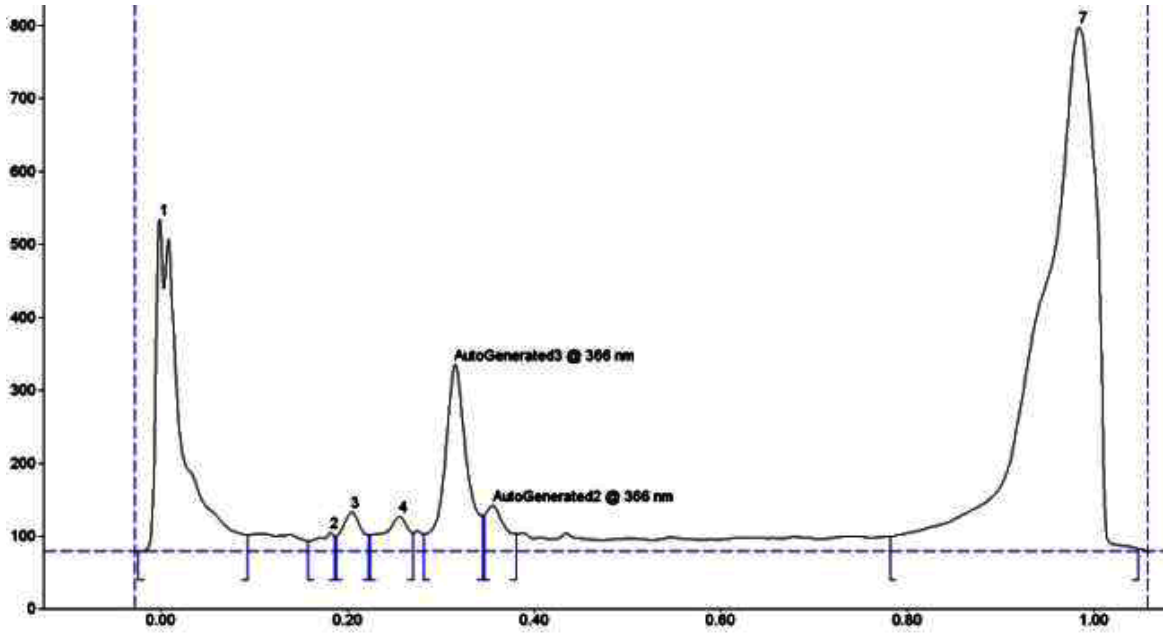
E. Cyclohexane/Ethyl acetate/ Chloroform 1:1:1 v/v/v
F. Chloroform/Methanol 75:25 v/v
G. Toluene/Ethyl acetate/Formic acid 5:5:1 v/v/v
H. Toluene/Ethyl acetate/Methanol 6:1:0.8 v/v/v

HPTLC fingerprinting for EAA, ESX, EHI and mixture of all three extract developed, dried and visualized. Figure 5.50 (A) showed visualization in UV_{366} and figure 5.50 (B) showed visualization in UV_{254}



A **B**

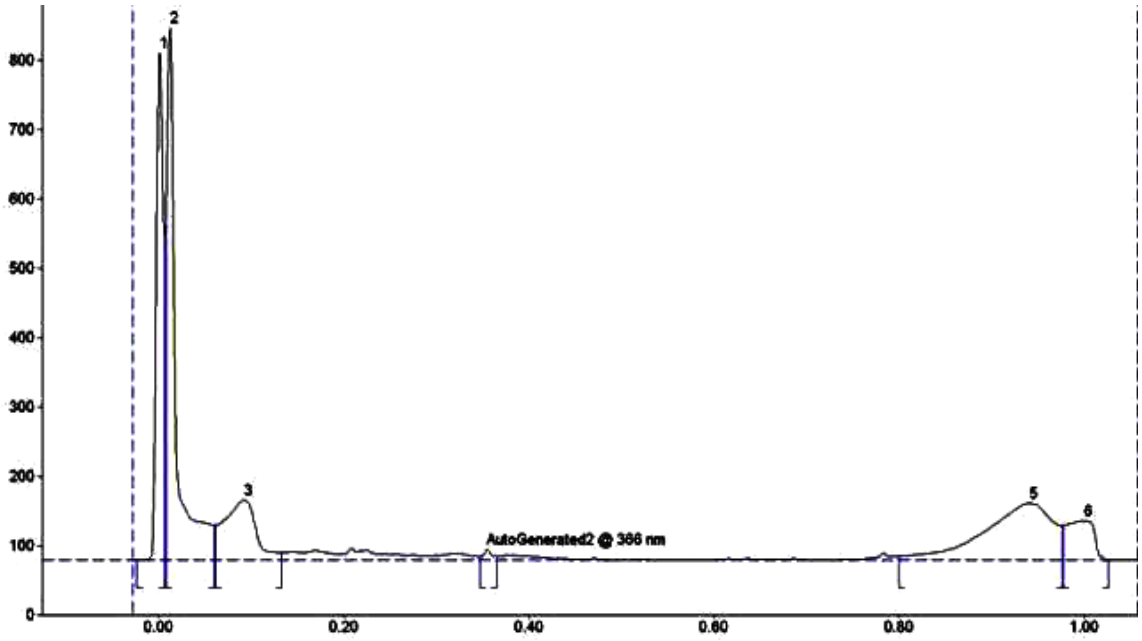
Figure 5.50 HPTLC fingerprinting for EAA, ESX, EHI and mixture of all three extract visualized in (A) UV_{366} and (B) UV_{254}



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	0.0	0.00	454.8	28.10	0.09	22.6	11222.1	18.62
2	0.16	14.0	0.18	25.3	1.56	0.19	19.7	446.3	0.74
3	0.19	20.2	0.20	53.6	3.31	0.22	22.3	1036.6	1.72
4	0.22	22.4	0.26	47.7	2.95	0.27	25.2	1198.8	1.99
5	0.28	23.2	0.32	255.9	15.81	0.35	48.6	5631.0	9.34
6	0.35	49.3	0.36	62.3	3.85	0.38	24.2	1222.4	2.03
7	0.78	20.6	0.99	718.8	44.41	1.05	3.8	39511.6	65.56

Figure 5.51 Chromatogram and HPTLC fingerprinting details of EAA

The Developed and scanned band of EAA showed good resolution of 7 bands when scanned ay UV_{366}. As showed in figure 5.51, the chromatogram of EAA produced major peak at R^f 0.07, 0.32 and 0.85. Figure 5.50 (A) clearly produced red colored peak in developed HPTLC plate.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	0.1	0.00	731.1	42.04	0.01	464.3	5212.9	22.91
2	0.01	488.5	0.01	767.9	44.15	0.06	50.6	7413.5	32.59
3	0.06	50.8	0.09	86.1	4.95	0.13	10.9	2789.7	12.26
4	0.35	5.2	0.36	15.4	0.89	0.37	5.3	131.8	0.58
5	0.80	5.6	0.94	82.3	4.73	0.98	50.0	5625.0	24.73
6	0.98	50.2	1.00	56.3	3.24	1.03	0.3	1577.0	6.93

Figure 5.52 Chromatogram and HPTLC fingerprinting details of ESX

The Developed and scanned band of ESX showed good resolution of 6 bands when scanned ay UV_{366}. As showed in figure 5.52, the chromatogram of ESX produced major peak at R^f 0.04, 0.09 and 0.88. Figure 5.50 (A) clearly produced light blue colored peak in developed HPTLC plate.

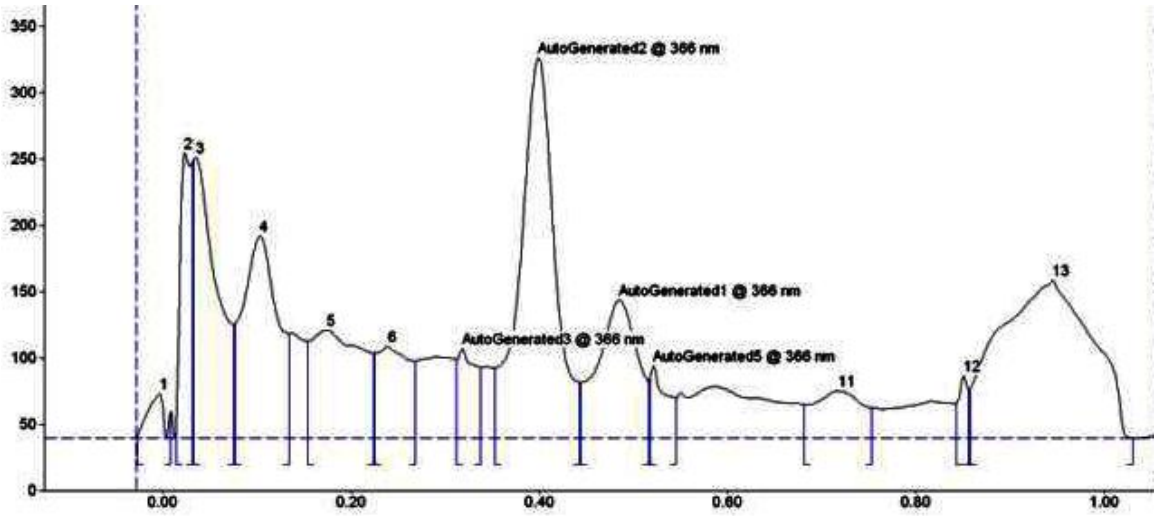


Figure Chromatogram of EHI

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.03	1.9	-0.00	33.6	2.28	0.01	19.5	522.9	1.08
2	0.01	5.7	0.02	214.8	14.56	0.03	207.6	2429.3	5.03
3	0.03	209.6	0.04	211.2	14.31	0.08	85.9	4846.9	10.03
4	0.08	86.3	0.10	152.3	10.32	0.14	78.9	5252.6	10.87
5	0.16	73.2	0.17	81.6	5.53	0.22	64.4	4154.9	8.60
6	0.23	64.4	0.24	68.9	4.67	0.27	57.9	2225.6	4.61
7	0.31	59.5	0.32	67.2	4.55	0.34	53.4	1290.1	2.67
8	0.35	53.1	0.40	286.5	19.41	0.44	42.3	9656.0	19.99
9	0.44	42.3	0.49	103.7	7.03	0.52	43.2	4077.9	8.44
10	0.52	45.2	0.52	54.7	3.70	0.55	30.5	872.5	1.81
11	0.68	25.4	0.72	35.6	2.41	0.75	22.9	1709.9	3.54
12	0.84	26.1	0.85	46.9	3.18	0.86	36.0	445.3	0.92
13	0.86	37.0	0.95	118.8	8.05	1.03	0.0	10820.2	22.40

Figure 5.53 Chromatogram and HPTLC fingerprinting details of EHI

The Developed and scanned band of EHI showed good resolution of 13 bands when scanned ay UV_{366}. As showed in figure 5.53, the chromatogram of EHI produced major peak at R^f 0.12, 0.40, 0.48 and 0.90. Figure 5.50 (A) clearly produced blue colored peak in developed HPTLC plate.

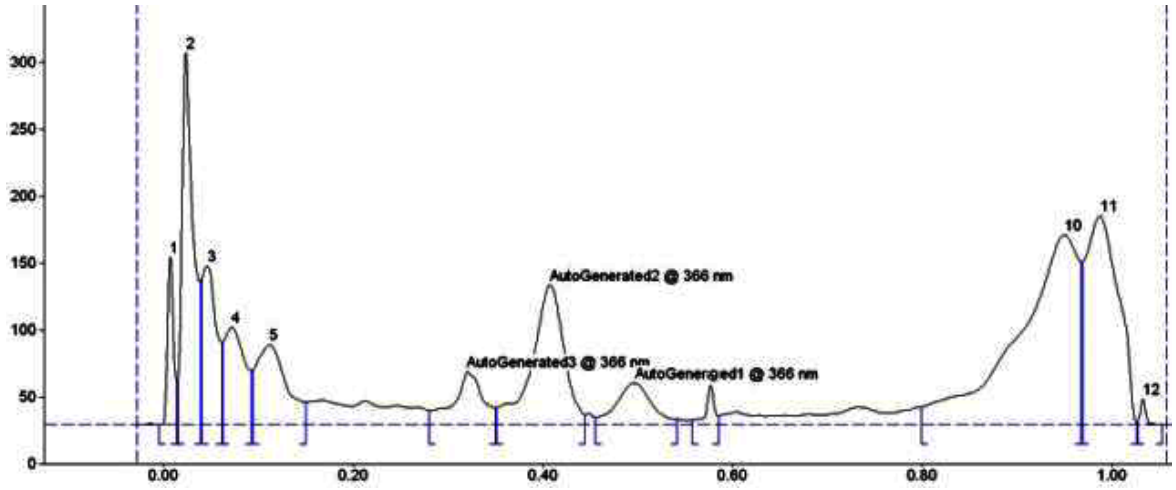


Figure Chromatogram of mixture of EAA, ESX and EHI

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.00	0.4	0.01	124.7	10.63	0.01	31.4	753.0	2.69
2	0.02	32.1	0.03	277.2	23.63	0.04	107.3	3119.8	11.15
3	0.04	108.9	0.05	118.4	10.09	0.06	61.8	1696.6	6.06
4	0.06	62.1	0.07	72.7	6.20	0.09	40.3	1478.7	5.28
5	0.09	40.7	0.11	59.7	5.09	0.15	16.8	1774.4	6.34
6	0.28	10.7	0.32	39.6	3.38	0.35	12.6	1133.3	4.05
7	0.35	12.7	0.41	104.1	8.87	0.44	7.2	3252.7	11.62
8	0.46	5.7	0.50	31.1	2.65	0.54	3.8	1072.9	3.83
9	0.56	3.7	0.58	29.0	2.48	0.59	6.6	238.5	0.85
10	0.80	13.3	0.95	141.6	12.07	0.97	121.4	8394.3	29.99
11	0.97	121.5	0.99	155.8	13.28	1.03	1.5	4978.3	17.79
12	1.03	2.2	1.03	19.1	1.63	1.05	0.3	97.9	0.35

Figure 5.54 Chromatogram details of mixture

The Developed and scanned band of mixture showed good resolution of 12 bands when scanned ay UV_{366}. As showed in figure 5.54, the chromatogram of mixture fingerprinting for all three herbs. Figure 5.50 (A) and (B) produced well resolved peak in developed HPTLC plate.

Figure 5.55 showed Overlain Chromatogram of EAA, ESX, EHI and Mixture while figure 5.56 explain 3D image for Chromatogram of EAA, ESX, EHI and Mixture

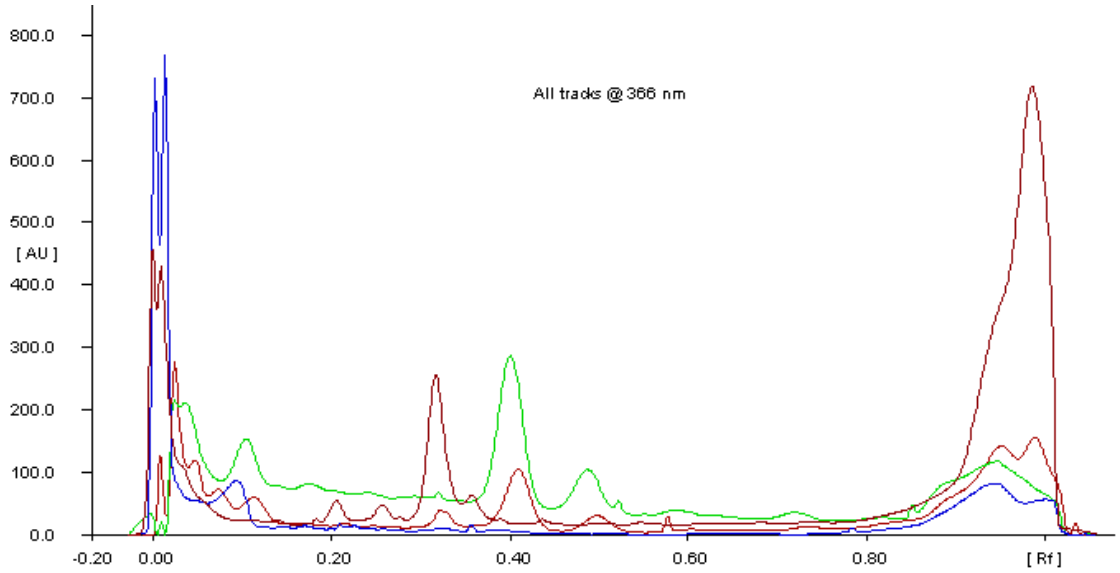


Figure 5.55 Overlain Chromatogram of EAA, ESX, EHI and Mixture

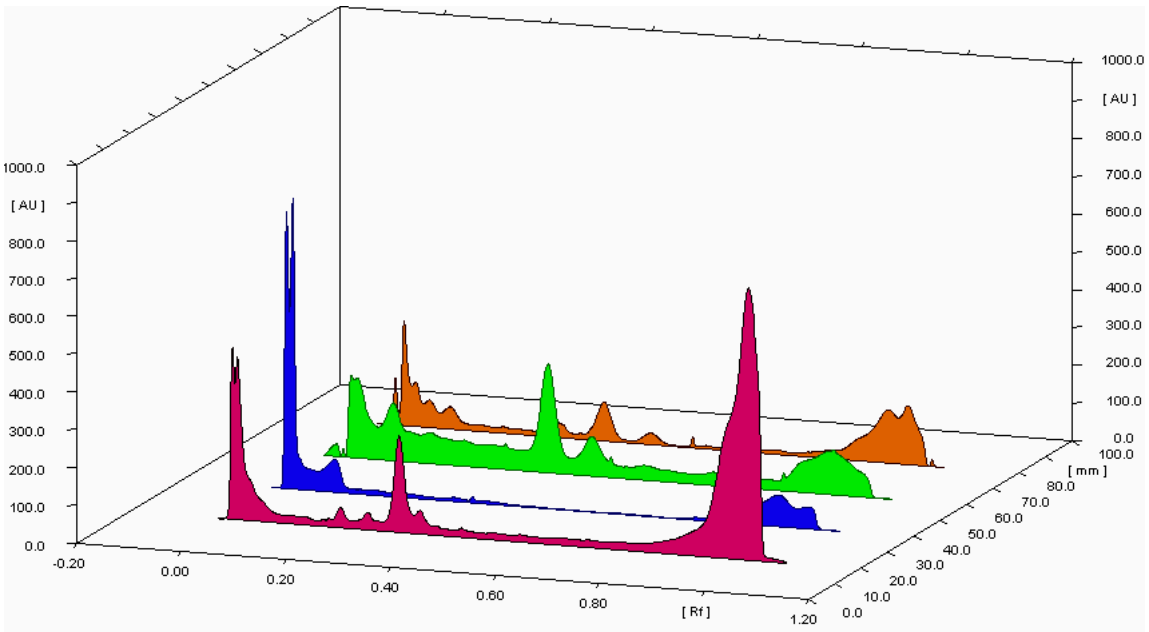


Figure 5.56 3DChromatogram of EAA, ESX, EHI and Mixture

Discussion…

6. Discussion

Steroids are most helpful drug candidates in treatment of anti-inflammatory diseases. In present study, selection of plants containing steroidal moiety done based on availability of plant material and its reported uses. Many herbal plant steroids are investigated for their benefits but no systematic study was reported for evaluation of steroidal contents as corticoids.

Plant and its part selected as major source of herbal steroid were Fenugreek (*Trigonella foenum graecum*) seed, Wild Yam (*Dioscorea alata*) underground tuber, Indian Sarsaparilla (*Hemidesmus indicus*) root, *Agave americana* leaf and Kantakari (*Solanum xanthocarpum*) fruits. The plant materials herbariums (No. SU/DPS/407-411, dated 14/10/2011) were identified and authentifed by centre for advanced studies in plant biotechnology and genetic engineering, Saurashtra University, Rajkot. The report for the identification and authentification attached in Annexure.

Fenugreek (*Trigonella foenum graecum*) seed, Wild Yam (*Dioscorea alata*) underground tuber, Indian Sarsaparilla (*Hemidesmus indicus*) root, *Agave americana* leaf and Kantakari (*Solanum xanthocarpum*) fruits were procured from authentic source and evaluated for macroscopic characters as per references given in previous section. All selected plant material were packed and stored properly.

The steroid hormone such as the adrenocorticoids is present in the body only in very low concentrations (e.g., 0.1-1.0 nM). Yet, these hormones exert potent physiologic effects on sensitive tissues at those low concentrations. Over the past several decades, the general mechanism of steroid hormone action has been extensively studied (David *et al.*, 2002).

The corticoid steroids may be classified as those having important effects on intermediary metabolism (glucocorticoids) and those having principally salt-retaining activity (mineralocorticoids) (Katzung *et al.*, 2007).

Glucocorticoids dramatically reduce the manifestations of inflammation. This is due to their profound effects on the concentration, distribution, and function of

peripheral leukocytes and to their suppressive effects on the inflammatory cytokines and chemokines and on other lipid and glucolipid mediators of inflammation. The benefits obtained from use of the glucocorticoids vary considerably. Use of these drugs must be carefully weighed in each patient against their widespread effects on every part of the organism (Mehdi *et al.*, 2007).

The major undesirable effects of the glucocorticoids are the result of their hormonal actions, which lead to the clinical picture of iatrogenic Cushing's syndrome and many other side effects. The study protocol was approved by Institutional animal ethics committee and the protocol no. is SU/DPS/IAEC/1001 date 11/02/2010 as attached in annexure.

The aim of present study was to investigate effect of steroidal saponins and indexed them as glucocorticoid and mineralocorticoid. The selection of solvent performed based on solubility of steroidal content of plant. The plant materials were pre-socked for 12 hrs with water to enhance its extraction through soxhlet hot extraction. The common solvent and common procedure for all extract applied to performed comparative evaluation of all plant materials. Literature survey reveals that the steroidal saponins are best extracted with ethanol.

Evaluation of steroidal content of selected plant material is important and it was important task to check presence of steroidal content in present ethanolic extracts. The plant extract were evaluated for qualitative estimation of steroidal by chemical test and thin layer chromatography.

Many chemical tests in literature reported for saponins and plant steroids (Hostettamann *et al.,* 1995). In present study four representative chemical tests were selected and performed to check presence of steroidal content. Saponin containing plants produce foam when they are shaken in water solutions. saponin like compound produces heamolysis in RBC showed clear RBC rupture under microscope.

Libermann Bruchard test commonly used to check presence of steroidal moiety in plant material. Unsaturated and hydroxylated triterpenes and steroids give a red, blue or green coloration with acetic anhydride and sulphuric acid (Abisch *et al.*

1960). Since terpenoid saponins tend to produce a pink or purple shade and steroidal saponins a blue-green coloration. In another chemical test with antimony trichloride, all plant material extracts produced formation of pink which indicate presence of steroids (Hostettamann *et al.*, 1995).

The qualitative analysis of saponins by TLC is of great importance for all aspects of saponin investigations. TLC plates (usually silica gel) can handle both pure saponins and crude extracts, are inexpensive, rapid to use and require no specialized equipment. A number of visulization reagents are available for spraying onto the plates. Here in present study two TLC visualization reagents, Lieberman Burchard reagent and 20% H_2SO_4 solution were used (Hostettamann *et al.*, 1995).

The Libermann Bruchard reagent showed clear indication for presence of steroidal molecule in all plant extracts when pre coated TLC plates observed under white light. In ETF spot, major steroidal spot were visualized with dark purple and pink color. In EDA spot, major steroidal spot were found light purple and dark purple coloration. EHI spot gave presence of steroidal spot with dark purple and dark pink tint. In EAA spot, major steroidal spot showed dark purple, green and dark green coloration. ESX spot gave major steroidal band of dark purple and light purple tint. Another TLC plate was developed in 20% H_2SO_4 solution which gives red to pink color for triterpenoids and saponins (Abisch *et al.*, 1960).

TLC observed in UV_{366} UV_{254} serve additional basis for qualitative evaluation by TLC method. The TLC developed with spraying reagent 20% H_2SO_4 solution and observed in UV_{254} shown blue appearance because of destruction of illuminating substance of HPTLC plate by H_2SO_4.

The ANOVA tests the null hypothesis that samples in two or more groups are drawn from the same population. When ANOVA shows a significant difference between groups, if one of the groups is a control (reference) group, Dunnett's Test is used as a *post hoc* test. This multiple comparison test can be used to determine the significant differences between a single control group mean and the remaining treatment group means in an analysis of variance setting. It is one of the least conservative *post hoc* tests (Anscombe *et al.*, 1948).

Adjuvant arthritis in rats

Utilizing the footpad for immunization of rat may be necessary in particular studies where the isolation of a draining lymph node, as a primary action site, is required. The well being of subject animals should be addressed such as limiting the quantity of adjuvant-antigen solution injected into the footpad, the use of only one foot per experimental animal, and housing on soft bedding rather than screens. In instances where there is no evidence indicating a specific requirement for footpad inoculation, this technique should not be used for routine immunization of rat. If scientific justification is provided, the recommended maximum footpad injection volumes are 0.01-0.05 in mice and 0.10 ml for rats (Jackson et. al. 1995).

Adjuvant arthritis (AA) is an induced form of (sub) chronic arthritis. Strains of rats have a varying genetic susceptibility to AA, whereas mice generally are not susceptible. Adjuvant arthritis (AA) was initially observed by accident when complete Freund's adjuvant (CFA) was used for immunization (Pearson *et al.*, 1956). An injection of complete Freund's adjuvant into the rat paw induces inflammation as primary lesion with a maximum after 3 to 5 days. In this protocol, adjuvant arthritis (AA) is induced in albino Wistar rats in the classical manner using ground, heat-killed Mycobacterium tuberculosis H37Ra suspended in incomplete freund's adjuvant in left paw of rat.

The choice of the animal strain has been found to be very important for the performance of this test. Wistar rats have been proven to be very suitable in contrast to other sub strains. In present study albino Wistar rat were used as test animal and reference (Rekha *et. al.* 2010, Mingxing *et. al.* 2005, and David *et. al.* 1980) available for adjuvant arthritis animal model.

Secondary lesions occur after a delay of approximately 12 days which are characterized by inflammation of non-injected sites (hind leg, forepaws, ears, nose and tail), a decrease of weight and immune responses. The procedure has been modified by several authors in order to differentiate between anti-inflammatory and immunosuppressive activity (Perper *et al.* 1971). Anti-inflammatory compounds do not inhibit secondary lesions, which are prevented or diminished by

immunosuppressive agents. The result of this study gives important direction for preparation of glucocorticoid and mineralocorticoid comparative index of selected herbs.

All animal were grouped (n=6) as control, HC, ETF, EDA, EHI, EAA and ESX. Dose 300 mg/kg per rat kept common for all other treatment group for comparative evaluation of relative potencies. Paw volumes of both sides and body weight were recorded on the day of injection, day 5, day 12, day 17 and day 21 whereby paw volume is measured plethysmographically.

Decrease of weight of normally observed with catabolic steroids in rat. After the completion of study, on day 21 the weight were measure and change in weight compare with initial weight (day 1, before injection of CFA).

The injection of catabolic steroid, hydrocortisone provoked steroid like action marked by decrease in body weight. The decrease in body weight by hydrocortisone on day 5 and day 12 found significant ($p<0.05$) when compared to control. The significant weight loss of any plant extracts observed after day 17. The weight reduction by hydrocortisone and ESX on day 17 found extremely significant ($p<0.001$) and significant ($p<0.05$) when compared to control.

On day 21 all treatment groups except ETF and EDA showed significant weight loss as compare to control group. The reduction in weight loss by HC and ESX were extremely significant ($p<0.001$) and highly significant ($p<0.01$) while EHI and EAA showed significant ($p<0.05$) weight loss when compared to control.

Catabolic steroids have some noted side effects including weight gain. The hydrocortisone treated group showed weight loss which is because of more calcium per unit length of bone and decrease in the skeletal structure (Yasumura et. al. 1976).

Corticoids produce anti-inflammatory and Immunosuppressant activity and both activity can be differentiate in adjuvant arthritis in rat (Vogel *et al.*, 2002). The inhibition of paw volume at injected site after 5 days is because of its anti-inflammatory potential while inhibition of paw volume at non-injected site after 12

days and inhibition of arthritic lesion are because of its potential to modulate immune response.

Paw volume measured on day 5 for left (injected) and right (non injected) paw in ml for control, HC, ETF, EDA, EHI, EAA and ESX. No group showed significant change in paw volume as compare to control ($P<0.05$). The result after day 5 suggests mild anti inflammatory activity by all treatment groups.

On day 12, the change in left (injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were calculated. The reduction in edema for HC found highly significant and EAA significant ($p<0.05$). The change in right (non-injected) paw volume (ml) on day 12 for control, HC, ETF, EDA, EHI, EAA and ESX were calculated and The reduction in edema for HC found highly significant while EDA, EHI, EAA and ESX significantly reduced paw edema ($p<0.05$).

Edema (Paw volume) measured on day 17 for left and right paw in ml. On day 17, the change in left (injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were calculated and The reduction of edema for HC and EAA were highly significant while ETF, EDA, EHI and ESX showed significant decrease in paw volume ($p<0.05$). On day 17, the change in right (non-injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were calculated. The reduction of edema for only HC found significant ($p<0.05$).

Paw volume measured on day 21 for left and right paw in ml. On day 21, the change in left (injected) paw volume (ml) for control, HC, ETF, EDA, EHI, EAA and ESX were expressed. The reduction of edema for HC EAA, EHI and ESX were highly significant and the reduction of edema for EDA was significant ($p<0.05$). The change in right (non-injected) paw volume (ml) on day 21 for control, HC, ETF, EDA, EHI, EAA and ESX were calculated and The reduction in paw volume for HC, ETF, EDA, EHI and ESX found highly significant while EAA significantly reduced paw edema ($p<0.05$).

The immunosuppressive activity can also express by inhibition of lesion compare to control on day 21. The arthritic lesion on day 21 for control, HC, ETF,

EDA, EHI, EAA and ESX were calculated as per the scheme given in materials and method. The suppression of arthritic lesion on day 21 for HC, EHI, EAA and ESX found highly significant while ETF and EDA showed significantly inhibition of arthritic lesion ($p<0.05$).

Primary lesions expressed by change in paw volume by injected left paw on day 5. All treatment groups showed mild anti inflammatory action on day 5 when we recorded change in left (injected) paw volume as compare to day 1.

Secondary lesions (immune response) expressed by change in paw volume by non injected right paw on day 12. All treatment groups except ETF showed significant immunosuppressive action on day 12 when we recorded change in right (non-injected) paw volume as compare to day 1.

The immunosuppressive action also expressed by inhibition of arthritic lesion on day 21 and all treatment group showed significant reduction in lesion ($p<0.05$).

The total percentage change calculated by addition of percentage inhibition of left paw on day 5, percentage inhibition of right paw on day 12 and percentage inhibition of arthritic score on day 21. The total percentage change for HC, ETF, EDA, EHI, EAA and ESX were calculated which shows overall potential of plant extract as herbal steroids.

Liver glycogen deposition

The liver glycogen deposition test, as described by Stafford *et al.* (1955) is a simple and specific test for Glucocorticoid activity.

A well-established action of adrenal-cortical steroids in the intact animal is to stimulate hepatic glycogen deposition, as may be inferred from the decline in liver glycogen after Adrenalectomy (Vogel *et al.* 2002). This effect of glucocorticoid is due to activation of glycogen synthetase to produce the form, as shown by measurements of pathway intermediates and of enzyme activities. Conversely, in starved adrenalectomized animals, activation of synthetase to the form is impaired, and the capacity for glycogen deposition is diminished. This lesion in the synthetase system

may be corrected by glucocorticoid and also by administration of glucose or insulin to intact animals, and is impaired in insulin deficient animals, suggesting that the impairment in glycogen synthesis may reflect insulin insufficiency (Patricia et. al. 1976).

The classical evaluation of hormone functions is the surgical ablation of the hormone producing endocrine gland and the substitution with exogenously administered substances. Most studies on the physiological role of adrenocortical hormones and the pharmacological effects of corticosteroids were performed in adrenalectomized rats. This procedure involved complete removal of both, adrenal gland cortex and medulla. As a results the glucose, glycogen and mineral metabolism of rat altered and their ability to store minerals, glucose, glycogen etc. impaired. Therefore this animals model is useful for the wide range of metabolic studies including hormone metabolism, obesity, drug metabolism etc. This model frequently used in studies related to endocrinology and neurology (Stafford *et al.*, 1955).

Carbohydrate exists as free sugars and polysaccharides. The basic units of carbohydrates are the monosaccharide which cannot be split by hydrolysis into simpler sugars. The carbohydrate content can be measured by hydrolysing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharide. Carbohydrates are first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green colored product with an absorption maximum at 620 nm (Vogel *et al.* 2002).

The Calibration curve of glucose for estimation of liver glycogen was prepared and linearity range, co-relation coefficient (r^2) and linearity equation were 20-100 µg/ml, 0.986 and Y = 0.006X + 0.103.

The care should be taken while mixing 30% KOH solution with anthrone reagent in sulphuric acid (95%) which laid to effervescence of fumes. The liver glycogen deposition was measure on day 5 after seven hrs post treatment from calibration curve of glucose. The glycogen deposition in liver was expressed as liver glycogen in mg per gm of liver. The increase in liver glycogen for HC was found

highly significant. The gain in liver glycogen for EHI and EAA was found moderately significant while ESX was found significant when compared with control ($P<0.05$).

Glucocorticoids exert minor effects on hemoglobin and erythrocyte content of blood, as evidenced by the frequent occurrence of polycythemia in Cushing's syndrome and of normochromic, normocytic anemia in adrenal insufficiency (Wardlaw *et al.,* 2001). More profound effects are seen in the setting of autoimmune hemolytic anemia, in which the immunosuppressive effects of glucocorticoids can diminish the self-destruction of erythrocytes. The administration of glucocorticoids leads to a decreased number of circulating lymphocytes, eosinophils, monocytes, and basophils. The hematological evaluation for blood cells, SGPT, SGOT, serum calcium and serum potassium was performed for control group and treatment groups.

The red blood corpuscle (RBC) count (10^6/cumm) for control, HC, EHI, EAA and ESX were calculated and The increase in RBC for HC was found to be significant as compare to control ($P<0.05$). The white blood cells (WBC) count per cu mm for control, HC, EHI, EAA and ESX were measured and the gain in WBC for ESX was found to be moderately significant as compare to control which support its immunomodulatory activity. The increase in WBC for HC was found to be significant as compare to control ($P<0.05$). The platelets count per (10^3/cumm) for control, HC, EHI, EAA and ESX were calculated and only EAA showed significant decrease in platelet counts as compare to control ($P<0.05$).

Aldosterone is by far the most potent endogenous corticosteroid with respect to fluid and electrolyte balance. Mineralocorticoid act on the distal tubules and collecting ducts of the kidney to enhance reabsorption of Na^+ from the tubular fluid, they also increase the urinary excretion of K^+ and H^+. Conceptually, it is useful to think of aldosterone as stimulating a renal exchange between Na+ and K^+ or H^+ (Bernard *et al.*, 2008).

In addition to their effects on monovalent cations and water, glucocorticoids also exert multiple effects on Ca^{2+} metabolism. Steroids interfere with Ca^{2+} uptake in the gut and increase Ca^{2+} excretion by the kidney. These effects collectively lead to decreased total body Ca^{2+} stores (Bernard *et al.*, 2008).

The serum calcium level (mg/dl) for control, HC, EHI, EAA and ESX were calculated. The HC shown moderately significant increase in serum calcium level as compare to control while EAA shown significant gain in serum calcium level as compare ($P<0.05$). The serum potassium level (mMol/liter) for control, HC, EHI, EAA and ESX were calculated. The HC shown highly significant increase in serum potassium level as compare to control ($P<0.05$).

Liver has to perform different kinds of biochemical, synthetic and excretory functions, so no single biochemical test can detect the global functions of liver. Enzymes that are often measured in LFTs include gamma-glutamyl transferase (GGT); alanine aminotransferase (ALT or SGPT); aspartate aminotransferase (AST or SGOT); and alkaline phosphatase (ALP). LFTs also may include prothrombin time (PT), a measure of how long it takes for the blood to clot (Henderson *et al.* 2001).

Alanine aminotransferase (ALT), formerly called serum glutamate pyruvate transaminase, or SGPT, is an enzyme necessary for energy production. It is present in a number of tissues, including the liver, heart, and skeletal muscles, but is found in the highest concentration in the liver. Aspartate aminotransferase (AST), formerly called serum glutamic-oxaloacetic transaminase, or SGOT, is another enzyme necessary for energy production. It, too, may be elevated in liver and heart disease. In liver disease, the AST increase is usually less than the ALT increase. However, in liver disease caused by alcohol use, the AST increase may be two or three times greater than the ALT increase (Henderson *et al.* 2001).

Serum glutamate pyruvate transaminase, or SGPT (ALT) level (IU/L) for control, HC, EHI, EAA and ESX were calculated and The HC shown moderately significant increase in SGPT as compare to control ($P<0.05$). SGOT or Serum glutamic-oxaloacetic transaminase (AST) level (IU/L) for control, HC, EHI, EAA and ESX were calculated the HC shown significant increase in SGOT level as compare to control ($P<0.05$).

Liver glycogen was determined under standard conditions on intact and adrenalectomized rats. The liver uses glucose as a fuel and also has the ability to store it as glycogen and synthesize it from noncarbohydrate precursors (gluconeogenesis).

Periodic acid-Schiff (PAS) is a staining method used to detect glycogen and other polysaccharides in tissues. The reaction of periodic acid oxidizes the diol functional groups in glucose and other sugars, creating aldehydes that react with the Schiff reagent to give a purple-magenta color. A suitable basic stain is often used as a counterstain. Wax blocks were made and section was made by microtome, stained by Periodic acid-Schiff (PAS) and photographed (Ulusoy *et al.,* 2006).

The effect of HC, EHI, EAA and ESX on histopathology of liver during liver glycogen deposition study carried out and the Histopathology of liver treated with HC clearly showed more deposition of glycogen in hepatocytes. The Glycogen deposition was comparatively observed more in HC, EHI and EAA while ESX showed least signs for glycogen deposition.

Mineralocorticoid activity in rat

Corticoid hormones exert their physiological action by binding to receptors that belong to a transcription factor superfamily, which also includes some of the proteins regulating steroid synthesis. Steroids stimulate sodium absorption by the activation and/or de novo synthesis of the ion-gated, amiloride-sensitive sodium channel in the apical membrane and that of the Na/K -ATPase in the basolateral membrane. Receptors, channels, and pumps apparently are linked to the cytoskeleton and are further regulated variously by methylation, phosphorylation, ubiquination, and glycosylation, suggesting a complex system of control at multiple checkpoints. Mutations in genes for many of these different proteins have been described and are known to cause clinical disease. Mineralocorticosteroids enhance sodium retention and potassium excretion. The sodium excretion in adrenalectomized rats is dose-dependently decreased. This parameter can be used for mineralocorticoid activity of test compounds (Chrousos *et al.,* 1995).

Wistar rats have been proven to be very suitable in contrast to other sub strains (Vogel et al., 2002). In present study albino Wistar rat were used as test animal and many references available for determination of electrolyte excretion in mineralocorticoid activity model. The mineralocorticoid activity by measurement of sodium excretion carried out only for all herb extracts and standard drug. Along with

sodium excretion, urine analysis also performed for urine chlorides, urine potassium and urine Creatinine (Vogel *et al.* 2002).

The urine chloride (Cl^-) level (mMol/L) for control, HC, EHI, EAA and ESX were calculated and HC shown moderately significant decrease in urine chloride level as compare to control ($P<0.05$). The urine Creatinine level (mg/dl) for control, HC, EHI, EAA and ESX were calculated and The HC showed moderately significant gain in urine creatinine level as compare to control ($P<0.05$).

The urine sodium level (mMol/L) for control, HC, EHI, EAA and ESX were calculated and The HC showed highly significant fall in urine sodium level as compare to control while EAA showed significant reduction in urine sodium level as compare to control ($P<0.05$). The urine potassium level (mMol/L) for control, HC, EHI, EAA and ESX were calculated and HC showed moderately significant increase in potassium excretion level as compare to control ($P<0.05$).

The Na^+/K^+ ratio for control, HC, EHI, EAA and ESX were expressed and The HC showed moderately significant fall in Na^+/K^+ ratio as compare to control ($P<0.05$) which clearly suggest reduction in sodium excretion and increase in potassium excretion.

Relative Potencies of Hydrocortisone, EHI, EAA and ESX were calculated and Glucocorticoid potential of (Liver glycogen deposition) HC, EHI, EAA and ESX were found to be 1.0, 0.61, 0.58 and 0.55 while Mineralocorticoid potential (Na+ excretion) of HC, EHI, EAA and ESX were found to be 1.0, 0.57, 0.73 and 0.36

All plant extracts exert insignificant action on vital hematological parameter except reduction in platelets count and increase serum calcium by EAA. Except EAA, both EHI and ESX have not shown any significant sigh on urine electrolyte in evaluation as mineralocorticoid. Final conclusion drawn from study is that combined extract of EHI and ESX can be used as alternative for synthetic steroids in steroid therapy while EAA can be used in condition like Addison's disease.

Quality control

In general, the methods for quality control of herbal medicines involve sensory inspection (macroscopic and microscopic examinations) and analytical inspection using instrumental techniques such as thin layer chromatography and High performance thin layer chromatography. TLC is still frequently used for the analysis of herbal medicines since various pharmacopoeias such as American Herbal Pharmacopoeia, Chinese Drug Monographs and Analysis; Pharmacopoeia of the People's Republic of China etc. still use TLC to provide first characteristic fingerprints of herbs. Rather, TLC is used as an easier method of initial screening with a semi quantitative evaluation together with other chromatographic techniques (Kotadia *et al.,* 2005).

High performance thin layer chromatography (HPTLC) is an enhanced form of thin layer chromatography (TLC). A number of enhancements can be made to the basic method of thin layer chromatography to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements. Automation is useful to overcome the uncertainty in droplet size and position when the sample is applied to the TLC plate by hand. One recent approach to automation has been the use of piezoelectric devices and inkjet printers for applying the sample (Hostettamann *et al.,* 1995).

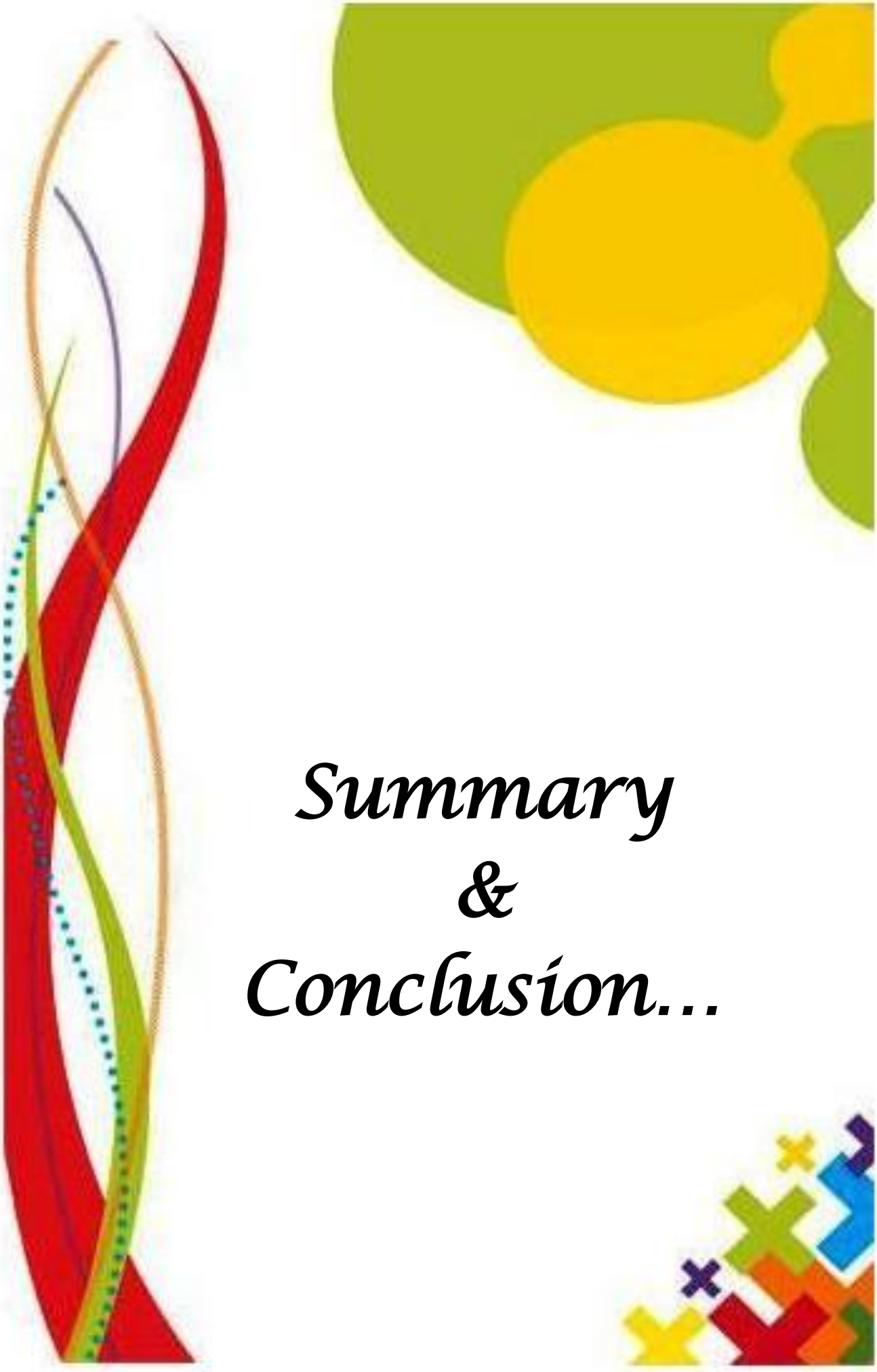
High performance thin layer chromatography (HPTLC) is an invaluable quality assessment tool for the evaluation of botanical materials. It allows for the analysis of a broad number of compounds both efficiently and cost effectively. Additionally, numerous samples can be run in a single analysis thereby dramatically reducing analytical time. With HPTLC, the same analysis can be viewed using different wavelengths of light thereby providing a more complete profile of the plant than is typically observed with more specific types of analyses. HPTLC finger printing provides important information to evaluate composition of any herbal extract as single or in mixture (Kotadia *et al.,* 2005).

In present study we developed a HPTLC method for fingerprinting of EHI, EAA and ESX. The mixture also prepared in same proportion for EHI, EAA and ESX

and the HPTLC fingerprinting developed for mixture. Based on result obtained after mobile phase optimization, it clearly showed that mobile phase Toluene/Ethyl acetate/Methanol, 6:1:0.8 v/v/v resolved more number of peaks for ETF, EDA, EHI, EAA and ESX.

HPTLC performed only for EHI, EAA and ESX, those herbs which showed significant activity as steroids in adjuvant arthritis activity. Selected mobile phase, Toluene/Ethyl acetate/Methanol, 6:1:0.8 v/v/v used for further investigation of plant extracts. In instrumental component winCATS planner chromatography system manager used along with Camag linomat 5 semi automatic applicator and Camag scanner 3 with quantitative evaluation. TLC Aluminum sheets of silica gel 60 F_{254} pre washed with methanol before HPTLC analysis and the saturation time optimized to 45 min.

The Developed and scanned band of EAA showed good resolution of 7 bands, ESX showed good resolution of 6 bands and EHI showed good resolution of 13 bands when scanned ay UV_{366}. Overlain Chromatogram of EAA, ESX, EHI and Mixture while figure 5.56 explain 3D image for Chromatogram of EAA, ESX, EHI and Mixture for HPTLC fingerprinting.



Summary & Conclusion…

7. Summary and Conclusion

The adrenocorticoids are mainly used because of its glucocorticoid and mineralocorticoid action. The use of sterids is almost unavoidable in many clinical situations like chronic inflammation, arthritis, Addison's disease etc. which lead to toxic manifestation. Selection of corticoids depends on its potential as glucocorticoid and mineralocorticoid. The use of adrenocorticoids can be replaced with herbal steroids. To generate clinical evidence for herbal steroids we need to established herbal steroids as glucocorticoid and mineralocorticoid.

Pharmacologic activities of steroid containing plants like Wildyam, Kantakari, agave spices, Indian Sarsaparilla and fenugreek have been reported and this may be a hint for investigating use of herbal steroids as therapeutic agents. Hence, in order to find a safer alternate steroid management for various ailments.

Literature survey revealed that no systematic study has been reported to classify herbal steroids as glucocorticoid and mineralocorticoid. The indexing of herbal steroids based on its corticoid potential, it can be used to replace allopathic steroids and adverse manifestation can be overcome.

Identified and authentified plant herbal steroid were *Trigonella foenum graecum* seed, *Dioscorea alata* underground tuber, *Hemidesmus indicus* root, *Agave americana* leaf and *Solanum xanthocarpum* fruits. The common solvent and common procedure for all extract applied to performed comparative evaluation of all plant materials. Literature survey reveals that the steroidal saponins are best extracted with ethanol. The presence of steroids in plant extract were ensure by test like foam test, haemolysis test, libermann bruchard test and antimony trichloride test. The all plant extracts were also by checked for its steroidal content by TLC and visualized in Libermann Bruchard reagent and 20% H_2SO_4 solution.

Adjuvent arthritis in rat (n=6) used as animal model to evaluate steroidal potential of selected herbs. The total percentage change was calculated for all extracts and top

three results against control group were 96.8, 90.33 and 86.88 for ESX, EAA and EHI respectively.

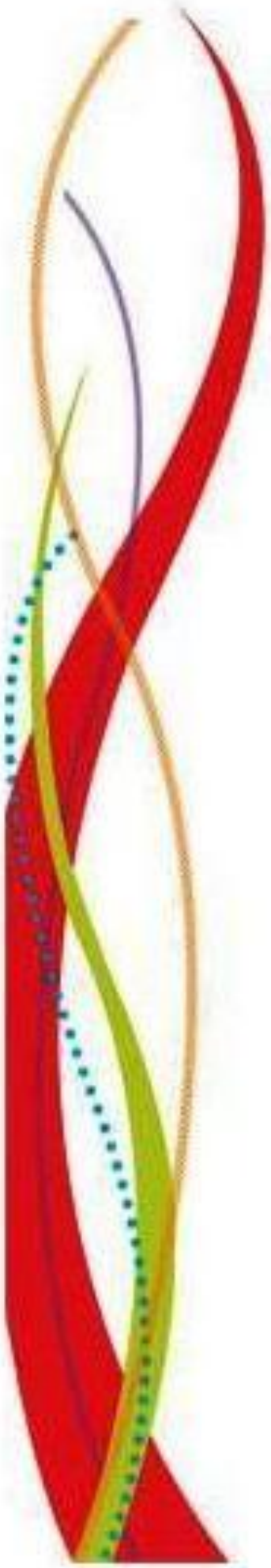
The proved extracts of EHI, EAA and ESX further investigated for glucocorticoid potential by liver glycogen deposition in rat (n=6) and mineralocorticoid potential by measuring sodium excretion in urine.

All herbal extracts showed significant glycogen deposition in rat with clear indication in histopathology. All plant extracts exert insignificant action on vital hematological parameter except reduction in platelets count and increase serum calcium by EAA.

Except EAA, both EHI and ESX have not shown any significant sigh on urine electrolyte in evaluation as mineralocorticoid. Final conclusion drawn from study is that combined extract of EHI and ESX can be used as alternative for synthetic steroids in steroid therapy while EAA can be used in condition like Addison's disease.

The said work can be extend in following direction

- More herbal extracts should be screened for its glucocorticoid and mineralocorticoid potential.
- Polyherbal formulation of glucocorticoid herbal steroids should be evaluated for synergistic action and clinical trials in chronic inflammation like rheumatoid arthritis.
- Polyherbal formulation of mineralocorticoid herbal steroids should be evaluated for synergistic action and clinical trials in condition like Addison's disease.



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Annexure…

CPBGE
Centre For Advanced Studies In Plant Biotechnology & Genetic Engineering

SU|Bio|FAP|37
14|10|11.

Ref. No. SU/DPS/422/2011

Prof. & Head
Department of
Pharmaceutical Sciences
Saurashtra University
Rajkot – 360 005.
Gujarat.

Kindly refer to your letter dated 14/10/2011 for identification of given five Herbarium Specimens (No. SU/DPS/407-411) have been identified as given below.

Sl No	Sample received as	Part	Sample identified as	Remarks
SU/DPS/407	*Trigonella foenum graecum*	Whole plant	*Trigonella foenum graecum* L.	O.K.
SU/DPS/408	*Dioscorea alata*	Whole plant	*Dioscorea alata* L. Syn. *Dioscorea rubella* Roxb.	O.K.
SU/DPS/409	*Hemidesmus indicus*	Roots with photograph	*Hemidesmus indicus* L.	O.K.
SU/DPS/410	*Agave americana*	Leaves	*Agave americana* L. with yellow margins on the leaves.	O.K.
SU/DPS/411	*Solanum xanthocarpum*	Whole plant	*Solanum xanthocarpum* Schrad. & Wendl.	O.K.

With regards

Dr. V. S. Thaker
Programme Co-ordinator
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Saurashtra University
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