# Comparative Insights into the Pharmacological Significance of Selected Pakistani *Datura* Species




**PhD Thesis**

**By**

**BAKHT NASIR**

**Department of Pharmacy**
**Faculty of Biological Sciences**
**Quaid-i-Azam University**
**Islamabad, Pakistan**

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# Comparative Insights into the Pharmacological Significance of Selected Pakistani *Datura* Species

Thesis Submitted by

**BAKHT NASIR**

**Registration No. 03331513001**

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**Department of Pharmacy**
**Faculty of Biological Sciences**
**Quaid-i-Azam University**
**Islamabad, Pakistan**
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**Supervisor:** _____

**Dr. Ihsan-ul-Haq**
Professor Dr,
Department of Pharmacy,
Quaid-i-Azam University,
Islamabad

**External Examiner I:** _____

**External Examiner II:** _____

**Chairperson:** _____

**Dated:** _____

*This thesis is dedicated to;*

*My Father*

*Mr. Bakht Biland Khan*

*And my mother*

*Who never once, to my knowledge, stopped believing in my abilities*

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# Acknowledgements

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**Bakht Nasir**

# List of Tables

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# List of Abbreviation

| Abbreviation | Description |
|---|---|
| $^{13}$C NMR | $^{13}$Carbon nuclear magnetic resonance |
| 1D-NMR | One dimensional nuclear magnetic resonance |
| $^{1}$H NMR | Proton nuclear magnetic resonance |
| 2D-NMR | Two-dimensional nuclear magnetic resonance |
| ADHP | 10-acetyl-3,7-dihydroxyphenoxazine |
| ALP | Alkaline phosphatase |
| ALT | Alanine transferase |
| ALL | Acute lymphocytic leukemia |
| AML | Acute myelogenous leukemia |
| AP-1 | Activator protein-1 |
| Api | Apigenin |
| ArC | Aromatic carbon |
| ArH | Aromatic hydrogen |
| AST | Aspartate transferase |
| BAX | Bcl-2 associated X protein |
| BCD | Bowman's capsule's disruption |
| Br | Broad |
| BUN | Blood urea nitrogen |
| C1 | Compound 1 |
| C2 | Compound 2 |
| c-NOS | Constitutive nitric oxide synthase |
| $CD_3OD$ | Deuterated methanol |
| CLL | Chronic lymphocytic leukemia |
| CMC | Carboxy methyl cellulose |
| COSY | Correlation spectroscopy |
| D | Doublet |
| Da | Dalton |
| DAB | 3,3' Diaminobenzidine |
| Dd | Double doublet |
| DI | *Datura innoxia* |
| DIF-Dw | *Datura innoxia* fruit distlled water extract |
| DIF-EA | *Datura innoxia* fruit ethyl acetate extract |
| DIF-M | *Datura innoxia* fruit methanol extract |
| DIF-NH | *Datura innoxia* fruit n-hexane extract |
| DIL-Dw | *Datura innoxia* leaf distlled water extract |
| DIL-EA | *Datura innoxia* leaf ethyl acetate extract |
| DIL-M | *Datura innoxia* leaf methanol extract |
| DIL-NH | *Datura innoxia* leaf n-hexane extract |
| DIR-Dw | *Datura innoxia* root distlled water extract |
| DIR-EA | *Datura innoxia* root ethyl acetate extract |
| DIR-M | *Datura innoxia* root methanol extract |

| Abbreviation | Description |
|---|---|
| DIR-NH | *Datura innoxia* root n-hexane extract |
| DIS-Dw | *Datura innoxia* stem distlled water extract |
| DIS-EA | *Datura innoxia* stem ethyl acetate extract |
| DIS-M | *Datura innoxia* stem methanol extract |
| DIS-NH | *Datura innoxia* stem n-hexane extract |
| DMEM | Dulbecco's modified eagle medium |
| DMSO-*d6* | Deuterated dimethyl sulfoxide |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| DRT | Degenerated renal tubules |
| DS | *Datura stramonium* |
| DSF-Dw | *Datura stramonium* fruit distilled water extract |
| DSF-EA | *Datura stramonium* fruit ethyl acetate exract |
| DSF-M | *Datura stramonium* fruit methanol extract |
| DSF-NH | *Datura stramonium* fruit n-hexane extract |
| DSL-Dw | *Datura stramonium* leaf distilled water extract |
| DSL-EA | *Datura stramonium* leaf ethyl acetate exract |
| DSL-M | *Datura stramonium* leaf methanol extract |
| DSL-NH | *Datura stramonium* leaf n-hexane extract |
| DSR-Dw | *Datura stramonium* root distilled water extract |
| DSR-EA | *Datura stramonium* root ethyl acetate exract |
| DSR-M | *Datura stramonium* root methanol extract |
| DSR-NH | *Datura stramonium* root n-hexane extract |
| DSS-Dw | *Datura stramonium* stem distilled water extract |
| DSS-EA | *Datura stramonium* stem ethyl acetate exract |
| DSS-M | *Datura stramonium* stem methanol extract |
| DSS-NH | *Datura stramonium* stem n-hexane extract |
| DTNB | 1,2-dithio-bis nitrobenzoic acid |
| FC | Folin ciocalteu |
| FRSA | Free radical scavenging activity |
| GAE | Gallic acid equivalent |
| GST | Glutathione S transferase |
| HCT-15 | Human colon adenocarcinoma cancer cell line |
| HMQC | Heteronuclear multiple quantum coherence |
| HSQC | Heteronuclear single quantum coherence |
| HTAB | Hexadecyltrimethylammonium bromide |
| HYL | Hyalinization |
| IL-1β | Pro-interleukin-1 beta |
| iNOS | Inducible nitric oxide synthase |
| IUPAC | International union of pure and applied chemistry |
| *J* | Coupling constant |
| MCF-7 | Human breast adenocarcinoma |
| MDA | Malondialdehyde |

| Abbreviation | Description |
|---|---|
| MMP | Matrix metalloproteinases |
| MTT | Dimethylthiazol-diphenyltetrazolium bromide |
| Myr | Myricetin |
| NLRP3 | NLR family pyrin domain containing 3 |
| Nrf2 | nuclear factor erythroid 2 (NFE2)-related factor 2 |
| p-JNK | Phosphorylated-Jun-N-terminal kinase |
| PARP-1 | Poly adenosine diphosphate ribose polymerase-1 |
| PC-3 | Human prostate cancer cell line |
| PHA | Phytohaemagglutinin |
| PPAR-γ | Peroxisome proliferator-activated receptor gamma |
| QE | Quercetin equivalent |
| Quer | Quercetin |
| RPMI | Roswell Park memorial institute medium |
| SAR | Structure activity relationship |
| SIRT1 | Sirtuin 1 |
| SRB | Sulforhodamine B |
| STAT3 | Signal transducer and activator of transcription 3 |
| TBA | Thiobarbituric acid |
| TBARs | Thiobarbituric acid reactive substances |
| TBST | Tris buffered saline with tween 20 |
| Ti | Initial reaction time |
| TMS | Tetramethyl silane |
| TST | Tail suspension test |
| VLDL | Very low-density lipoprotein |
| ZOI | Zone of inhibition |

# Abstract

Medicine derived from plant sources have been used by human populations for their healthcare needs since antiquity. Phytopharmaceuticals developed from traditional medicine can provide effective therapeutic alternatives to conventional medications. The current study was designed to provide comparative insights into the pharmacological significance of two *Datura* species i.e., DS and DI (*Datura stramonium* Linn. and *Datura innoxia* Mill.). Initially, 32 extracts of root (R), stem (S), fruit (F) and leaf part (L) of both species were procured by successive extraction in solvents of increasing polarity i.e., n-hexane (NH), ethyl acetate (EA), methanol (M) and distilled water (Dw) using ultra-sonication assisted maceration process. The extracts were then examined through phytochemical and *in vitro* biological assays to determine the bioactive plant parts and most effective solvents for preparative scale extraction in latter phase of the study. Phenolic and flavonoid contents were estimated through colorimetric assays. The EA extracts of both species were the most proficient in terms of their phenolic and flavonoid contents, followed by NH and M extracts. The same trend was observed in antioxidant assays including total antioxidant capacity (TAC) and total reducing power (TRP) of the crude extracts. RP-HPLC analysis was done to detect and quantify selected polyphenols. Presence of pharmacologically significant polyphenolic compounds i.e., gallic acid, rutin, apigenin, myricetin, kaempferol and catechin was confirmed in EA and M extracts of DS and DI plants. EA leaf and fruit extracts of DS (DSL-EA and DSF-EA) while EA leaf and stem extracts of DI (DIL-EA and DIS-EA) were found to be most abundant in selected polyphenols. The preliminary *in vitro* cytotoxicity and anticancer potential was investigated using brine shrimp lethality and protein kinase inhibition assay respectively. Significant cytotoxicity was observed against brine shrimp larvae, 75% of DS extracts resulted in $LC_{50}$ values of < 25 µg/ml while $LC_{50}$ values < 23 µg/ml were recorded for 75% of DI extracts. In protein kinase inhibition assay, the EA leaf extract of both species showed significant results with DIL-EA and DSL-EA resulting in highest bald phenotype zones i.e., 19 and 12.50 mm respectively. Preliminary investigations revealed the noteworthy bioactivity of medium to low polarity extracts of both species, leaf part showed comparatively stronger bioactivity in these experiments and Dw extracts were excluded from further screening. The anticancer properties of remaining extracts (NH, EA and M) of both species were investigated by determining their cytotoxicity against PC-3,

MDA-MB 231 and MCF-7 cancer cell lines. DIL-EA was the most potent extract with $IC_{50}$ < 3 µg/ml against each cancer cell line while among DS extracts, DSR-EA and DSL-EA displayed slight cytotoxicity. The extracts were then further shortlisted, and NH and EA leaf extracts of both species (DSL-NH, DSL-EA, DIL-NH and DIL-EA) were selected for the next phase of the study. The safety profile of these extracts was established *via* MTT assay against isolated human lymphocytes and rat macrophages and none of the extracts revealed any significant cytotoxicity against normal cells. DSL-EA at 20 µg/ml showed significant (72.65±1.98%) nitric oxide (NO) scavenging potential followed by DIL-EA (41.72±1.33%). The *in vivo* anti-inflammatory (acute carrageenan induced paw edema and croton oil induced anal edema), anti-depressant (tail suspension test) and analgesic potential (hot plate method) of these extracts was also investigated in Balb/c mice. DSL-EA and DIL-EA exhibited significant anti-inflammatory activity and mild antidepressant and analgesic action. These results helped in selection of two most potent extracts for chronic *in vivo* studies, i.e., EA extracts of the leaf part of DS and DI (DSL-EA and DIL-EA). These two extracts were extensively examined in benzene induced leukemia and carbon tetrachloride ($CCL_4$) induced liver damage in Sprague Dawley rats through a series of hematological, biochemical, histological and immunohistochemistry studies. Significant alleviative effects of DSL-EA led to the selection of this extract for preparative scale extraction and isolation of bioactive compounds. Three compounds, stigmasterol (compound a), ferulic acid (compound b) stigmasterol-D-glucoside (compound c) were isolated from DSL-EA using normal phase column chromatography. The structure of these compounds was confirmed using 1D and 2D nuclear magnetic resonance (NMR) techniques. The compounds selected for *in vivo* evaluation were stigmasterol-D-glucoside (C1) and ferulic acid (C2). The antileukemic and anti-inflammatory potential of C1 and C2 was extensively investigated in Balb/c mice. Marked improvement in hematological, biochemical, histological and regulation of molecular markers of cancer and inflammation was observed in C1 and C2 treated groups. The extensive evaluation of the pharmacological potential of DS and DI in our study has helped in the scientific validation of their ethnomedicinal claims. Both species, particularly DS, proved to be a very prominent source of anticancer and anti-inflammatory agents.

# CHAPTER 1

# INTRODUCTION

# 1. INTRODUCTION

Ethnomedicine has been in practice since ancient times. Ethnomedicine utilizes medicinal plants as the source of chemicals to treat various diseases. Such chemicals are the secondary metabolites that have diversity in chemical and biological attributes. The variety of genera and plant species produce multitude of secondary metabolites that may not be necessary for plant's growth but are essential source of bioactive molecules capable of producing different pharmacological actions. These bioactive molecules are termed as "natural products" obtained either as single phytoconstituent or a combination of multiple constituents. Research on natural products is ongoing keeping in view the complex nature of molecules as well as multiple druggable targets to fulfill the current and future medical need (Gray *et al.,* 2012). Various drugs isolated from plants have been successful in combating different ailments. For example, *Catharanthus roseus, Cinchona spp*., *Taxus brevifolia* and *Artemisia annua* were the source of vincristine & vinblastine, quinine, taxol and artemisinin (Pal *et al.,* 2014). Out of all drugs approved by the European Medical Agency (EMA) and Food and Drug Administration (FDA), 25% drugs are obtained from plants. This accounts for the limited fraction of plant species that have been studied for pharmacological (6%) properties (Gray *et al.,* 2012). On the contrary, huge numbers of plants and natural products have not been investigated either in part or whole for potential pharmacological activities that can provide effective drug candidates for different diseases.

High throughput screening (HTS) is the mainstay of current research practices that provides vigorous screening method for a particular druggable target using specific drug or natural product. Arrays of assays are conducted on both plant extracts and purified isolated compounds for evidence of therapeutic profile. This is successfully steered with access to the libraries and databases and a sustainable source of natural products (Gray *et al.,* 2012). HTS fast-track drug discovery process and aids in characterizing natural products libraries both qualitatively and quantitatively (Fox *et al.,* 2006). For HTS, it is imperative to select a particular disease and molecular targets to evaluate effectiveness of natural products. Although, there are multiple diseases that are affecting people worldwide; however, some pathological features are consistent among disease that majorly contribute to their pathogenesis. Two of these features are inflammation and oxidative stress.

Oxidative stress and inflammation intertwine in various chronic diseases such as cardiovascular disease, renal diseases, diabetes, neurodegenerative diseases, depression and cancer (Biswas *et al.,* 2007; Ambade *et al.,* 2012; Biswas, 2016). Experimental and epidemiological studies have provided evidence of chronic low-grade inflammatory process and oxidative stress in the pathogenesis of many diseases (Biswas, 2016). These two processes instigate the basic symptoms of fever and pain as the first indication of a disease. Among all diseases, cancer is the enigma that has no cure and impacts human lives both physically and psychologically. Cancer grows inside the body like a parasite, eating away nutrients and damaging neighboring cells. Once metastatic, it spreads to various places inside the body, and activates inflammatory cells and reactive oxygen species (ROS). Inflammatory cells and ROS in turn provide tumor microenvironment sustainable for cancer cells and the cycle goes on. Researchers have considered targeting inflammation and ROS in cancer to impede its progression (Reuter *et al.,* 2010).

Medicinal plants have shown promising results in combating oxidative stress and inflammation. Considering the importance of medicinal plants in general and *Datura* in particular, two species *Datura stramonium* and *Datura innoxia* were selected in the current work for evaluation of biological activites and isolation of purified bioactive compounds. It was aimed to investigate and screen different extracts of both plants for antioxidant, anti-inflammatory and anticancer activities. Our work demonstrated good biological profile of both plants with superior results of *D. stramonium* leaf extracts in *in vitro* and *in vivo* inflammatory, cancer and behavioral models.



## 1.1. Cancer

Cancer is described by aberrant growth and proliferation of cells leading to the formation of a tumor. It can be carcinoma, lymphoma, leukemia or sarcoma depending on the cell characteristics (Hanahan and Weinberg, 2011). Carcinogenesis is defined by the stages of initiation, promotion, progression, and metastasis (Figure 1.1). Cancer is initiated by carcinogens that cause permanent DNA mutations and breaks. Such damage is transmitted to the progeny during cell division and is accompanied by activation of oncogenes like *Ras, c-myc, MAPK, Akt* etc., and silencing of tumor suppressor genes including *p53, pRb7, Pten APC, BRCA1-2, INK4* etc. (Cooper *et al.,* 2007).

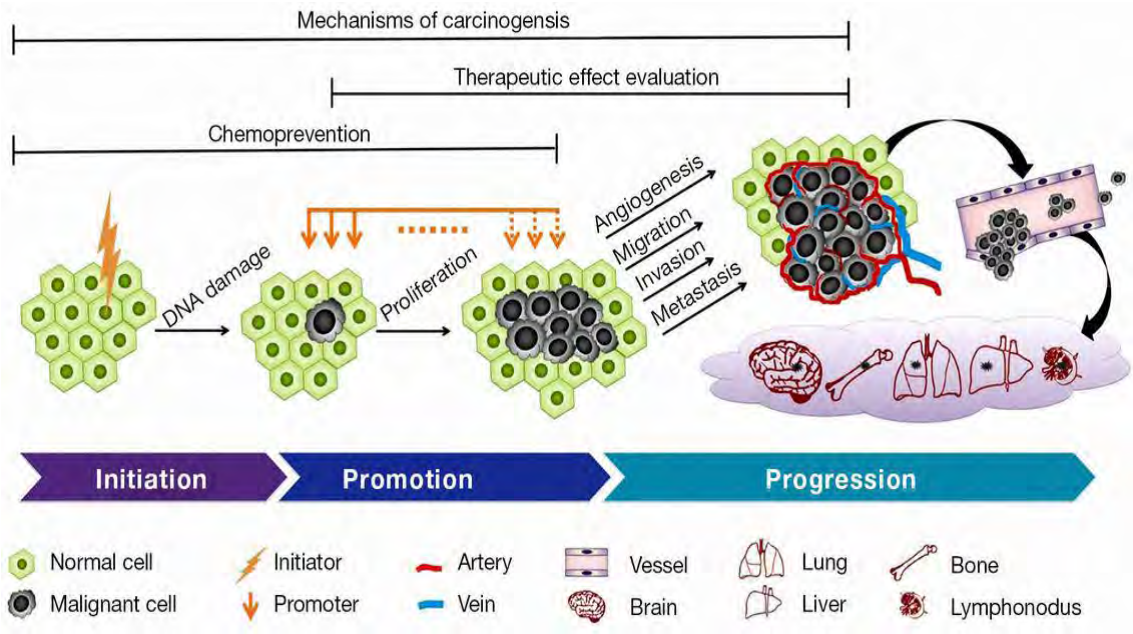


**Figure 1.1** Phases of carcinogenesis.

***Note:*** *Carcinogenesis comprises of three phases; initiation, represented by DNA mutations; promotion, exhibiting boosted cell proliferation varied expression of tumor suppressor genes and proto-oncogenes, and progression, encompassing angiogenesis, migration, invasion and metastasis of cancer cells. The figure is adapted from Quantitative imaging in medicine and surgery with permission* (Liu Yewei *et al.,* 2015).



Cancer is promoted to the second stage of unchecked proliferation due to aberrant cell cycle function, constant replication and translation of proliferation proteins. These mitogenic transmitters include growth factors; upregulated receptor mediated signaling, activated cell cycle cyclins, polymerases, mitogen activated protein kinases (MAPKs) and cytoskeleton proteins. Evidence shows increased expression of telomerase in immortalized cancer cells, disruption of negative feedback mechanism such as mutations in Ras GTPase activity, and formation of proinflammatory signals instigating sustained proliferation signals. Generation of inflammatory molecules in tumor microenvironment causes a paradoxical effect supplementing tumorigenesis (Hanahan and Weinberg, 2011). This aids in progressing tumor from benign to malignant forming blood vessels for continuous supply of nutrients and initiate metastasis. Here again, inflammatory and immune cells trigger angiogenesis and facilitates invasion of surrounding cells through activation of integrins and epithelial-mesenchymal transition (Liu Yewei *et al.,* 2015).

### 1.1.1. Role of inflammation and oxidative stress

Inflammation and oxidative stress are interlinked in adding fuel to the progression of cancer (Vaidya *et al.,* 2020). Literature evidenced that oxidative stress and

inflammation supports various oncogenic events such as cell proliferation, metabolic reprogramming, angiogenesis, migration, and avoidance of programmed cell death in cancer cells. These add to the advancement of cancer in a unanimous arrangement with noteworthy cellular signaling response and consequences. Oxidative stress is the imbalance between generation and damage by ROS and protection by endogenous antioxidants. It can distort macromolecules and dysregulate complex cell signaling. Sustained oxidative stress triggers DNA mutation, genome instability and neoplastic transformation (Liou *et al.,* 2010; Trachootham *et al.,* 2008). It stimulates chronic inflammation by actuating multiple transcription factors such as nuclear factor kappa-B (NF-κB), activator protein-1 (AP-1), Wnt/β-Catenin, p53, phosphorylated Jun-N-terminal kinase (p-JNK), peroxisome proliferator-activated receptor gamma (PPAR-γ), hypoxia inducible factor 1-alpha (HIF-1α), and nuclear factor erythroid-2 related factor 2 (Nrf2). These transcription factors induce reformed expression of growth factors, cell cycle regulatory proteins, oncogenes, tumor suppressor genes, pro-inflammatory cytokines, and chemokines (Vaidya *et al.,* 2020).

Once inflammation accelerates, it can recruit mast cells and leukocytes to the site of tumor. This causes a burst of metabolites of arachidonic acid, nitric oxide synthase cytokines i.e., [tumor necrosis factor (TNF), interleukin-1 and 6 (IL-1, IL-6)], and chemokines [IL-8 and C-X-C chemokine receptor type 4 (CXCR4)] leading to accumulation of more ROS. Furthermore, ROS and inflammation affect expression of matrix metalloproteinases (MMP). For example, sustained hydrogen peroxide exposure activates MMP2 and promotes cell invasion. Reports have shown the role of Ras, ERK1/2 activation, and inactivation of phosphatases by inflammatory cells to modulate MMP expression (Reuter *et al.,*2010). Thus, persistent inflammatory vs oxidative environment primes a vicious cycle that can harm normal surrounding epithelial and stromal cells and lays foundation of carcinogenesis (Nagarsheth *et al.,* 2017).

### 1.1.2. Pain: an indicator of cancer

Cancer pain is a debilitating condition that adds to the overall suffering of the patient. Pain can be a presenting symptom and lead to the diagnosis of cancer. Cancer pain is the most invalidating symptoms in approximately 66% of cancer patients, where 19% patients suffer from neuropathic pain and 39% have both neuropathic and nociceptive components (Svendsen *et al.,* 2005). With the growth of tumor, cytokines and inflammatory mediators form a necrotic center that can damage the nerve ending in the

tumor microenvironment. The tumor cells can also directly harm the sensory nerves by compression or by prompting hyperinnervation or denervation in the local microenvironment. It has been observed that tumor cells can induce reorganization of sensory and sympathetic nerve fibers forming neuroma like structures. These can generate ectopic pain episodes associated with augmented release of nerve growth factor. Furthermore, variation in expression of substance P and calcitonin gene-related peptide (CGRP), P2X purinoceptor 3 (P2X3), transient receptor potential cation channel subfamily V member 1 (TRPV1), acid sensing ion channel 3 (ASIC3), activating transcription factor 3 (ATF3) and sodium-channel-anchoring molecule p11 have been found at the site of primary tumor and metastatic lesions. Such changes cause hyperexcitable pain responses to mechanical, thermal, and electrical stimuli (Falk *et al.,* 2014).

### 1.1.3. Behavioral and psychological changes in cancer patients

Cancer diagnosis is distressful for patients that can affect their emotional and mental health. Cancer is considered a threat to the general sense of security and has a negative stigma that can affect a patient's response to diagnosis and treatment. Psycho-oncology addresses the impact of cancer on behavioral and psychological aspects of a patient. It is common for cancer patients to suffer from psychological problems such as anxiety, distress, adjustment disorder, depression, posttraumatic stress disorder, delirium, sexuality dysfunctions and phobias (Dankert *et al.,* 2003). This is accompanied by suicidal thoughts, indecisiveness, fear of pain, abandonment etc., as well as loss of hope, body image, social interactions, self-identity, and self-control. It is estimated that 70% of cancer patients suffer from depression in one or another form. Such clinical depression is associated with anhedonia, dysphoric mood, anorexia, weight loss, fatigue, and other vegetative symptoms. Moreover, anxiety augments pain sensitivity and negatively affects the overall quality of life (Gregurek *et al.,* 2010).

Studies suggest that psychological implications are associated with aberrant response of immune and endocrine systems in cancer patients. Cytokines have been considered the basic modulators of behavioral changes since DNA damage and malignant transformation incite a load of cytokines that can ultimately weaken the body defense mechanisms (Cleeland *et al.,* 2003). Preliminary clinical and psychophysical studies revealed the correlation between increased levels of cytokines (INF, TNF-α, IL-6, IL-8) and behavioral disturbances such as fatigue, cognitive impairment, and reduced

quality of life. This necessitates psychotherapy along with pharmacotherapy for effective management of cancer patients.

## 1.2. Medicinal Plants or Natural Products as Anti-Inflammatory, Antioxidant and Anticancer Agents

Plants are the unlimited source of various bioactive molecules. Almost every medicinal plant has the benefit of providing defense against oxidative stress, dominantly due to the presence of polyphenols. Phytochemicals including carotenoids, terpenoids, flavonoids, polyphenols, tannins and saponins have been observed to prompt antioxidant activities (Shaikh *et al.,* 2014). In a study, water extracts of *Alpinia officinarum, Spatholobus suberectus, Salvia officinalis, Tussilago farfara, Salvia miltiorrhiza* and *Uncaria rhyncophylla* with significant levels of phenolics and flavonoids content showed high antioxidant activities. The *S. officinalis*, *Drosera indica*, *Paeonia suffuticosa*, *U. rhyncophylla* and *Rhodamnia rubescens* exhibited inhibition of nitric oxide and TNF-α mediated inflammation (Ravipati *et al.,* 2012). Moreover, *Curcuma longa* that has abundant curcuminoids, has been commonly used as an anti-inflammatory agent (Joshi *et al.,* 2020). In addition, medicinal plants are valuable in the management of cancer. According to an estimate, around 50% of anticancer agents either approved or in clinical trials are plant derived compounds (Shaikh *et al.,* 2014). Commonly used anticancer drugs such as vinblastine, vincristine, etoposide, teniposide, paclitaxel, camptotecin and irinotecan have been obtained from plants. In short, medicinal plants and natural products are crucial in the prevention and management of cancer. There is still a large reservoir of medicinal plants that need extensive research for potential anticancer drug candidates.

### 1.2.1. Genus *Datura*

*Datura* (family: Solanaceae), a genus of wild weeds, is known for its medicinal and hallucinogenic properties. In Sanskrit, the term “Dhutra” means divine and genus *Datura* is named after it based on its healing properties. This genus comprises of various medicinal and toxic plants including *D. stramonium, D. innoxia*, *D. wrightii*, and *D. metel.* More than 30 alkaloids have been identified from *Datura* pertaining to its medicinal value (Vermillion *et al.,* 2011a) with reports of antiviral, antifungal, antibacterial, anticancer, anxiolytic, antiulcer, antiperspirant, immunomodulatory, hypoglycaemic, antiseptic, and wound healing properties (Maheshwari *et al.,* 2012). Considering the wide distribution and folklore medicinal properties of *Datura*, two

plants *D. stramonium* and *D. innoxia* were investigated in the present work for therapeutic potential particularly in cancer and cancer associated maladies.

#### *1.2.1.1. Datura stramonium*

*Datura stramonium* L. (Family *Solanaceae*), commonly recognized as Devils Trumpet, Locoweed and Jimson weed is widely used in Ayurvedic medicine. It is a wild-growing shrub (3–4 ft high) that has large whitish roots; green or purple, cylindrical smooth stem that branches in a forked manner; and green to brown fruits (Figure 1.2). Single flowers and leaves grow through the forked branches. Seeds are irregular and dark-colored with their surface either pitted or slightly reticulated (Noé, 2002; Kuete, 2014).

*D. stramonium* contains alkaloids hyoscyamine and scopolamine, acetyl derivatives of caffeic, p-coumaric and ferulic acid, steroidal glycosides, daturataturins A and B, flavonoids and withanolides. Traditionally, *D. stramonium* is used for the treatment of inflammatory disorders such as wounds, ulcers, rheumatism and gout; sciatica pain and tooth ache; fever and respiratory disorder like asthma and bronchitis (Noé, 2002; Kuete, 2014). They are also used to treat insomnia, induce sedation in psychotic patients and manage Parkinsonism and hemorrhoids (Guarrera, 1999). Literature shows that ethanolic extract of *D. stramonium* leaves exhibited antibacterial, anti-inflammatory and anticancer activities. The extracts reduced growth of Gram positive and Gram-negative bacteria (Reddy 2010), inhibited carrageenan induced inflammation in rats (Sonika *et al.,* 2010) and blocked proliferation of breast (MDA- MB231), head and neck (FaDu), and lung (A549) cancer cells (Ahmad *et al.,* 2009).

**Figure 1.2** *Datura stramonium.*
***Note:*** *Kingdom; Plantae, Division; Magnoliophyta, Class; Magnoliopsida, Order; Solanales, Family; Solanaceae, Genus; Datura, Species; Datura Stromonium, Common name; Devil Trumpet, and Binomial name; Datura stramonium L.*



### *1.2.1.2. Datura innoxia*

*Datura innoxia* Mill. from family Solanaceae, is also known as Angel's-trumpet, Thorn Apple, Downy Thorn Apple and Indian Apple. This shrub has been traditionally used across the world and widely distributed in Mexico, United States, China, Asia and Caribbean Islands (Maheshwari *et al.,* 2013). It is called Dhatura in Pakistan and can be found at roadsides and weedy places. It is a greyish plant owing to soft/short grayish hairs over leaves and stem that attains a height of 0.6-1.5 meters. It has trumpet-shaped, white, 12–19 cm long flowers, spiny egg-shaped, 5 cm in diameter fruit whose capsule splits open when it is fully ripped (Figure 1.2). *D. innoxia* has both medicinal and toxic properties based on the dose of the plant extract. In Ayurveda, all parts of the plant are used for the management of behavioral disturbances, skin diseases like leprosy, rabies etc. Furthermore, acute poisoning due to higher dose may result in delirium and death. Biologically active phytoconstituents of *D. innoxia* are hyoscyamine, withanolides, tropanes, atropine and scopolamine (Vermillion *et al.,* 2011a).

**Figure 1.3** *Datura innoxia*.
***Note:*** *Kingdom; Plantae, Unranked; Angiosperm, Unranked; Eudicotes, Unranked; Asterids, Order; Solanales, Family; Solanaceae, Genus; Datura, Species; Innoxia, Common name; Dhatura, and Binomial name; Datura innoxia Mill.*



Beside traditional uses, many studies reported antimicrobial, antioxidant and anticancer activities of *D. innoxia*. Aqueous and organic extracts of *D. innoxia* have been shown to be effective in reducing growth of Gram positive and Gram-negative bacteria, where maximum antibacterial activity was shown by methanol extract of leaves (Kaushik *et al.,* 2008). Another study endorsed the antibacterial activity of aerial parts of the plant. Moreover, *D. innoxia* seed extracts were superior in scavenging free radicals as compared to other *Datura* species (Ramadan *et al.,* 2007). In the same lines, dinoxin B, a withanolide, isolated from methanol extract of leaves of *D. innoxia* effectively inhibited the growth of human cancer cell lines making it a potential anticancer drug candidate (Vermillion K. *et al.,* 2011a). Methanol extract of *D. innoxia* leaves also induced apoptosis in human larynx (Hep-2) and human colon adenocarcinoma (HCT 15) cancer cell lines (Arulvasu *et al.,* 2010).

## 1.3. Toxicity Analysis: An Important Component of Natural Products

Preclinical studies on natural products involve efficacy, pharmacokinetic and toxicological studies using *in vitro* and *in vivo* models. Estimates of 200,000 natural products from plants are known and continue to be discovered over time (Ifeoma *et al.,* 2013). Nonetheless, their origin does not promise their safety, as chemical constituents

found in plants may exhibit toxic effects, since every biochemical is safe up to a particular level of dose (Sharwan *et al.,* 2015). Lack of adequate research about the safety profile of natural products could be hazardous if administered in very high doses or repeatedly over a period of time. Additionally, literature showed that the common toxic effects produced by natural products include nephrotoxicity (Asif 2012), hepatotoxicity, allergic reactions, neurotoxicity and cardiotoxicity toxicity (Tariq *et al.,* 2018). This makes toxicity testing imperative for the development of new drugs and to extend the efficacy of existing natural products (Parasuraman 2011). The standard for toxicity testing is to check the effect of the extract or compound under study in *in vitro* models and *in vivo* laboratory animals. In general, acute, sub-chronic and chronic toxicity studies employ wide range of tests in different animals subjected to long term administration of the test compound, monitoring of physiological and biochemical parameters and detailed gross or histological examination (Arome *et al.,* 2013). In the current study, toxicity testing was conducted initially on crude extracts using *in vitro* models at cellular level such as cytotoxicity assay on macrophages and isolated lymphocytes. Moreover, it was analyzed as part of the efficacy study in animal models.

### 1.4. Extraction, Isolation and Characterization Methods

Production of biologically active natural products from plants is a multistep process that comprises of plant validation, preparation of crude extracts with solvents of wide polarity range and isolation and characterization of purified compounds. Extraction of phytoconstituents from dried plant material depends on various factors such as the nature of extraction solvents and extractives as well as extraction methods. Knowledge about the specific features of extractives like stability and polarity and extraction solvents (volatility, toxicity, viscosity, and purity) is necessary to obtain maximum yield of the desired extract (Bucar *et al.,* 2013). Furthermore, the final natural product is selected owning to the biological activity, purity and quantity of the purified compound. This makes purification process as a rate-limiting step in the detection of biologically active natural product (Chami *et al.,* 2018; Wang Zheng *et al.,* 2019).

Generally, two approaches can be followed for isolation process (Figure 1.4). First is the bioassay guided isolation where isolation of purified compound is directed by bioactivity data at each step. Only fractions with significant biological profile are processed for isolation of respective compounds. Second is structure-based isolation process where compounds are isolated from all fractions of plant extract that may or

may not have any bioactivity. This strategy is time consuming and results in excessive utilization of chemicals. Thus, bioassay guided isolation is preferable in current research practice (Bucar *et al.,* 2013). Moreover, in present study bioassay guided isolation was used along with different chromatography and compound characterization techniques as discussed below.

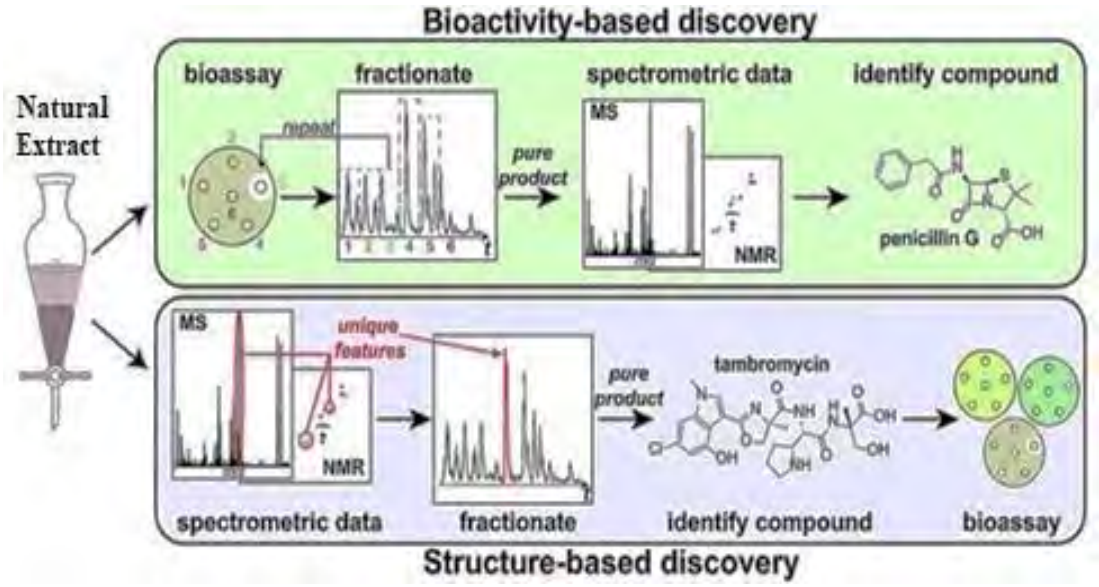


**Figure 1.4** Scheme for bioactivity based and structure-based isolation of compounds from natural source (Henke *et al.,* 2016).

### 1.4.1. Classical solvent extraction procedure

The classical solvent extraction method, also called ultra-sonication assisted maceration method, works on the principle of soaking dried powder of plant material in solvent with sonication cycles. Sonicating the soaked material at regular intervals allows cellular breakdown making it easier for the solvent to get absorbed in material and dissolve the phytoconstituents. It provides higher extraction yields within limited extraction time (Bucar *et al.,* 2013).

### 1.4.2. Solvent-solvent extraction

The principle of solvent-solvent extraction is to distribute a solute between two layers of immiscible solvents based on the polarity and partition coefficient of solute. Generally, two solutes with significant difference in partition coefficients are easily separated between solvents of distinct polarities. Hence, polarity of solvent is also a critical factor since non-specific solvent tends to extract various solutes (Rydberg 2004) and multiple steps are necessary to isolate solutes of close polarities. Despite being easy and inexpensive, it is difficult to validate and is time consuming (Berk 2018).

### 1.4.3. Fractionation and isolation

Successive extraction by maceration using different solvents can generate multiple fractions of extracts with variable extractives. These fractions are processed through liquid chromatography (LC) techniques to isolate and purify the natural product. Various chromatography techniques from simple column chromatography to high performance liquid chromatography (HPLC), medium pressure liquid chromatography (MPLC) and vacuum liquid chromatography (VLC) can be used. Furthermore, combination of chromatography with mass spectrometry (MS) like LC-MS, GC-MS, tandem mass spectrometry (MS/MS) techniques facilitate the parallel isolation and characterization of natural products. This has been beneficial in generating a wide library of natural products from plants with known medicinal value (Gray *et al.,* 2012).

### 1.4.4. Column chromatography and its types

Column chromatography is the most widely used chromatography technique where a mobile phase runs over a stationary phase filled in glass column under gravity. The sample is separated based on the partition coefficient and adsorption affinities between stationary and mobile phases. The particle size and porosity of stationary phase are 10-200 μm and 50 nm, respectively. Stationary phases used can be silica (cyano, amino, hydroxyl and nitro), polyacrylamide beads, crossed linked dextrans (Sephadex), alumina and styrene divinylbenzene (Coskun, 2016). There are different types of column chromatography including flash chromatography (FC), VLC and pressure liquid chromatography (PLC). These techniques differ based on the method by which solvent is forced through the column. FC, VLC and PLC utilize compressed air or nitrogen (ca. 1 bar above atmospheric pressure), vacuum or low to high (≤5 to ≥20 bar) pressure pumps to run the mobile phase through the column (Ghisalberti, 2008). Columns can be manually prepared, or prepacked cartridges or columns can be used. Sample is loaded on the normal or reverse phase, stationary phase- and run-in step gradient elution. Sample size in VLC and PLC ranges from few milligrams to 50 g and 200 mg to 300 g, respectively. The particle size of stationary phase in low and medium PLC can be in the range of 40-60 μm and 25-40 μm, respectively (Ghisalberti, 2008).

#### *1.4.4.1. Thin layer chromatography*

In thin layer chromatography (TLC), a film of silica as stationary phase is coated on glass or alumina based planar support. Multiple spots of samples applied on silica layer are eluted using a mobile phase where solvent runs based on capillary action. TLC

elution can be one dimensional, two dimensional, horizontal or vertical (Coskun, 2016). Components in a sample travel and are separated due to different binding affinities with the stationary phase. The distance travelled by each component with respect to solvent defines its relative mobility (R*f)* that serves to indicate whether the sample has single or multiple compounds (Sherma *et al.,* 2003). R*f* values are not reproducible due to multiple factors such as type and size of TLC chamber, characteristics of mobile phase, chemical nature and thickness of stationary phase, elution dimension, environmental conditions like humidity, chamber saturation and sample preparation technique (Sherma *et al.,* 2003). Nevertheless, it is a convenient and effective method to determine the purity of fractions and samples.

#### *1.4.4.2. High performance liquid chromatography*

HPLC is a type of pressure liquid chromatography that uses pressures of >20 bar to pump the mobile phase through column. It is the widely used commercial chromatography technique for isolation, purification, qualitative/quantitative measurements, high resolving power and analysis of natural compounds (Gupta and Shankar, 2011). HPLC can be categorized as normal or reverse phase based on polarities of stationary or mobile phases and isocratic or gradient HLPC based on composition of mobile phase. In isocratic HPLC, mobile phase with a constant composition is used throughout the elution process. On the other hand, the composition of mobile phase varies gradually or continuously during the elution process. Column diameter, sample load and flow rates are important determinants of effective separation (Gupta and Shankar, 2011).

A typical unit of HPLC has components including pumps, reservoir, column, degasser, injector/autosampler, guard column, fraction collectors, detector and recorder. Different types of detectors linked to HPLC are electrochemical detector (non-specific; high detection level), fluorescence detector (specific; low detection level), infrared detector, mass spectrometer, photodiode array detector (λ 190-800 nm), scattering detector and UV-visible detectors (Gupta and Shankar, 2011). HPLC has made structural/functional analysis and purification of several biologically active molecules convenient and time saving (Coskun, 2016).

### 1.4.5. Nuclear magnetic resonance

NMR techniques have advanced the characterization and structure elucidation of chemical compounds. It has high detection efficiency in determining the structure of individual compounds from a solution (Bucar *et al.,* 2013). NMR works on the principle that all nuclei are electrically charged and this charge in the form of energy is transferable from base energy to higher energy level on the application of external magnetic field. NMR can be one dimensional (1D) or two-dimensional (2D) NMR based on number of frequency axes in the spectra. The 1D NMR spectra are presented on x and y axes corresponding to frequency and intensity of measurements. On the other hand, 2D NMR spectra have frequency on vertical and horizontal axes and intensities are plotted topographically as contour plots thus providing detailed information on a compound as compared to 1D NMR spectra (Friebolin *et al.,* 2005).

#### *1.4.5.1. One dimensional NMR (1D-NMR)*

1.4.5.1.1. Proton NMR ($^{1}$H-NMR)

$^{1}$H-NMR measures the number of hydrogen atoms with respect to the signal generated by the chemical shifts in hydrogen nuclei. Each signal is measured in parts per million (ppm) and represented as δ (delta) scale. $^{1}$H-NMR spectrum gives detail of the structure of compound based on number, position, intensity and spin-spin splitting of signals. These represent type of protons, specific location of proton such as located on C=C double bond or methylene group, number of same type protons and total protons on carbon atom close to absorbing proton (Friebolin *et al.,* 2005). NMR signal for sample is determined relative to a reference compound tetramethyl silane (TMS) using the following equation;

$$\delta = \frac{V\mathrm{o} - V\mathrm{o}^{TMS}}{V\mathrm{o}^{TMS}}$$

Where $V\mathrm{o}$ is frequency of peak of interest and $V\mathrm{o}^{TMS}$ is frequency of TMS

1.4.5.1.2. Carbon NMR ($^{13}$C-NMR)

$^{13}$C-NMR estimates the nature of carbon atoms in a compound. $^{13}$C-NMR provides signal over a broader range of 0-220 ppm (Friebolin *et al.,* 2005).

#### *1.4.5.2. Two-dimensional NMR (2D-NMR)*

1.4.5.2.1. Heteronuclear single- or multiple-quantum coherence spectroscopy (HSQC or HSMQ)

It identifies in real time the hydrogen atoms correlated to nuclei of heteroatom (C-atoms). It permits relocation of hydrogen atom to C-skeleton allowing detection of better signal dispersion in $^{13}C$ dimension. HSQC technique aids in measuring $^{13}C$-NMR chemical shifts of all protonated carbon atoms with greater sensitivity than direct measurement. $^{13}C$-NMR frequencies can be run through databases having information on similar structures to identify the compound of interest (Bross-Walch *et al.,* 2005).

1.4.5.2.2. Heteronuclear multiple bond correlation spectroscopy (HMBC)

HMBC technique determines associations between H and C atoms based on two or three bond scalar coupling. As occurrence of such couplings is usually less (<10 Hz); therefore, HMBC only detect correlations of equitably sharp lines. HMBC helps in obtaining chemical shifts of C-atoms without H-atoms (e.g., carbonyl groups) as well as to associate different fragments. Combining correlated spectroscopy **(**COSY) with HSQC/HMBC makes it easier to characterize highly complex molecules having few H-atoms (Bross-Walch *et al.,* 2005).

1.4.5.2.3. Correlated spectroscopy

It is the prototype 2D-NMR technique that identifies associations of J-coupled H-atoms. COSY provides data about the geminal and vicinal coupling partners in the sample. Couplings are then resolved to obtain the cross peaks. This helps in establishing the structures of fragments in the sample (Bross-Walch *et al.,* 2005).



## 1.5. *In Vitro* Assays

### 1.5.1. Phytochemical screening

A basic step in research on plants extracts is to determine the phytoconstituents present in it. Bioassay guided extraction and isolation of natural products require information on the nature of the constituent to annotate the bioactivity to that particular chemical. Such chemicals can be polyphenols, carotenoids, flavonoids, phytosterols, terpenoids, lignans, alkaloids and organosulphur compounds (Upadhyay *et al.,* 2015). These have been proved beneficial in preventing various diseases such as heart diseases, hypercholesterolemia, neoplasia and metabolic disorders (Dharajiya *et al.,* 2017). Therefore, phytochemical analysis of *D. stramonium* and *D. innoxia* extracts was conducted.

#### *1.5.1.1. Total phenolics content (TPC)*

Polyphenols are the major components of plants that provide defense to the plant itself from oxidative damage. These can be simple phenol moieties to complex polymerized molecules (mol wt > 30,000 Da) (Cheynier *et al.,* 2015). Polyphenols can scavenge free radicals and singlet oxygen thereby reducing the oxidative stress. (Stevanato *et al.,* 2014). Analysis of polyphenols in plant extract can be done using Folin ciocalteu (FC) reagent. Phenolic compounds can inhibit the oxidation of the reagent. These are oxidized in the basic medium resulting in the formation of superoxide ions. Which then react with phosphomolybdate to form molybdenum oxide that has intense absorbance. Ketonic, hydroxyl and methoxy groups may be responsible for inhibiting the oxidation of FC reagent. (Andrew *et al.,* 2017).

#### *1.5.1.2. Total flavonoids content (TFC)*

Flavonoids are abundant (more than 4000 identified) in plants and can exist as anthocyanins, catechins, flavanones, flavones, flavonones and isoflavonoids. These exhibit various pharmacological activities such as antioxidant, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic (Gomes *et al.,* 2016). Reaction of flavonoids with aluminum chloride helps in qualitative and quantitative estimation of total flavonoid content in plant extract. Aluminum chloride forms acid stable complexes with C-4 keto group and C-3 or C-5 hydroxyl group of flavonoids. In addition, aluminum chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A or B ring of flavonoids. The more the functional groups in sample, the stronger the absorption at 415nm after reaction with aluminum chloride (Kumar *et al.,* 2015).

### 1.5.2. Antioxidant assays

Oxidative stress is responsible for damaging the body at cellular and molecular levels. It can attack cells 10,000 times per day where lack of protective antioxidant mechanism can induce many pathologies (Charles 2012). Oxidative stress is induced by ROS and reactive nitrogen species (RNS) ($RO^{\bullet}$, $O_2^{\bullet -}$, $^{\bullet}OH$, $ROO^{\bullet}$, $ONOO^{-}$, $^{\bullet}NO_2$, $^{\bullet}NO$) that are produced in the body by normal biological process or induced by external factors (Kowaltowski *et al.,* 2009). These free radicals can lead to oxidative stress induced DNA mutation, protein damage, inactivation of enzymes etc. This becomes a triggering force for many diseases like cardiovascular, endocrine, cancer, renal and hepatic disorders (Apak *et al.,* 2016). Medicinal plants are storehouses of antioxidant

constituents that can relieve oxidative stress in the human body. Use of herbal teas is a common practice to benefit from their antioxidant effects due to the presence of phenols and flavonoids (Phaniendra *et al.,* 2015). Quantification of antioxidant potential can give an idea about the strength of extract or natural product. Multiple assays can be used for such analysis as 2,2-diphenyl-1-picrylhydrazyl (DPPH; %FRSA) assay, total reducing power (TRP) and total antioxidant capacity (TAC).

DPPH free radical is stable and cell permeable that is quenched by antioxidant constituents in sample to generate hydrazine. The extent of discoloration of purple color depicts the antioxidant capacity in reaction mixture (Adjimani *et al.,* 2015). Another test uses formation of green phosphomolybdate complex in acidic medium to determine the total antioxidant capacity of the sample. The greater the TAC value, higher the ability to reduce Mo (VI) to Mo (V) and intensity of green color. Furthermore, measurement of redox potential is another way to test antioxidant capacity in terms of total reducing power of the sample. In this method, the sample reduces potassium ferricyanide to potassium ferrocyanide by donating hydrogen atom (Mir *et al.,* 2018a). Beside these assays, determining the effect of natural products on endogenous antioxidant system of the body can provide useful information about the activity of natural products. Relief of stress over the endogenous antioxidant system by plant extracts or natural products proves their beneficial effect in reducing the overall oxidative stress in the body. Catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) and glutathione S-transferase (GST) are key relievers of endogenous oxidative stress. CAT converts hydrogen peroxide into water and oxygen, SOD uses redox mechanism to dismutate superoxide anion radical and POD induces the catalytic reduction of lipid peroxides and hydrogen peroxide (Batool *et al.,* 2017a). Likewise, GST enzymes are responsible for detoxification of xenobiotics and other toxic compounds (Veal *et al.,* 2002). The activity of natural products on myeloperoxidase and lipid peroxidation is another way to measure their antioxidant potential. Myeloperoxidase assay uses the reaction between hydrogen peroxide and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) to produce detectable fluorescent compound resorufin (Pulli *et al.,* 2013). Moreover, thiobarbituric acid (TBA) assay works to measure the red adduct produced by interaction of malondialdehyde (MDA) with TBA (Garcia *et al.,* 2005). All these methods were used in present work to annotate the antioxidant potential of *D. stramonium* and *D. innoxia* extracts.

### 1.5.3. Brine shrimp lethality assay

Cytotoxicity of a natural product can be measured as an indicator of its toxicity in normal cells or potential use against cancer cells (Olowa *et al.,* 2013). Generally, a simple way is to measure lethal effects of test samples in brine shrimps to assess the bioactivity. It is a rapid, reliable and convenient method for preliminary assessment of toxicity of the plant extracts by using a small amount of sample (Krishnaraju *et al.,* 2005). Literature shows the successful use of this assay as an *in vitro* test for bioassay-guided fractionation of cytotoxic and anticancer agents (Hamidi *et al.,* 2014).

### 1.5.4. Dimethylthiazol-diphenyltetrazolium bromide (MTT) assay

Another way to measure cytotoxicity of natural products or plant extracts is to use MTT assay. This test can be performed on both normal and cancer cells. Viable cells can reduce yellow tetrazolium salt to insoluble blue/purple formazan crystals in the mitochondria by mitochondrial succinate dehydrogenase enzyme. The greater the intensity of blue color, higher the number of viable cells (Ahmed Madiha *et al.,* 2017a). In the present study, this test was used to assess the cytotoxicity of extracts on isolated lymphocytes, macrophages and cancer cells.

### 1.5.5. Protein kinase inhibition assay

Protein kinases are the driving forces in cells for cell division, proliferation, migration and apoptosis. These tyrosine and serine-threonine protein kinases transfer phosphoryl groups onto respective proteins to activate or deactivate downstream processes. Dysfunctional activity of protein kinases can induce oncogenesis leading to various tumors such as gastrointestinal stromal tumors, chronic myelogenous leukemia (Gschwind *et al.,* 2004), ovarian cancer (Cheng *et al.,* 1992), prostate cancer and some non-malignant disorders. Over the past decades, research has been focused on protein kinase inhibitors as anticancer drug candidates. Till now FDA has approved 37 kinase inhibitors for treatment of cancers such as breast and lung cancer. Additionally, around 150 kinase inhibitors are in clinical trials and various are being studied in the preclinical phase of drug development (Bhullar *et al.,* 2018). Following these lines, protein kinase inhibition activity of *D. stramonium* and *D. innoxia* extracts was conducted to determine their potential as kinase inhibitor agents. For that purpose, *Streptomyces* 85E strain was used whose protein kinases closely resemble their eukaryotic counterparts. Inhibition of hyphae formation of *Streptomyces* 85E after treatment with the extracts

served as an indicator of kinase inhibition since that is essential for hyphae development in the selected strain.

## 1.6. Acute *In Vivo* Assays

### 1.6.1. *In vivo* models of acute inflammation

As discussed earlier, inflammation plays important role in the pathogenesis of many diseases including cancer. Tumor microenvironment is filled with inflammatory cells that damage the surrounding cells and matrix to allow the spread of tumor cells. Generation of oxygen derived free radicals and activation of various transcription factors attract chemokines and other inflammatory cells at the site of injury, cancer cells or any other cellular damage (Reuter *et al.,* 2010). Transcription factors such as AP-1, HIF-1α, Wnt/β-Catenin, PPAR-γ, p53, NF-κB and Nrf2 play pivotal role in controlling over 500 genes associated with inflammation (Reuter *et al.,* 2010; Simmonds *et al.,* 2008). Thus, targeting inflammation can mitigate cancer mediated cellular damage. Various plants have shown potential in reducing inflammation. Plants, including *Zizyphus jujube, Ruta graveolens, Acacia modesta* and *Caralluma attenuate* have shown anti-inflammatory properties in animal models (Al-Reza *et al.,* 2010; Bukhari *et al.,* 2010; Ramesh *et al.,* 1998; Ratheesh *et al.,* 2010). The most commonly used animal model is carrageenan induced paw edema in rodents. Carrageenan induces acute inflammation in a biphasic manner. The products of arachidonic acid metabolism; cyclooxygenase-2 and reactive oxygen species, mediate its localized inflammatory response in rodents' paws (Mulla *et al.,* 2010). Histamine, serotonin and kinins are involved in the early inflammatory phase that initiates immediately and persists until one hour of the application of carrageenan. The second phase is initiated after one to two hours of administration and is mediated by the release of prostaglandins, oxygen-derived free radicals and production of inducible COX-2 (Panthong *et al.,* 2004; Mulla *et al.,* 2010). Inflammation either localized or systemic is also associated with increased levels of pro-inflammatory cytokines i.e., TNF-α, IL-1, and IL-6 (Sethi *et al.,* 2008). Two of the most widely recognized mice models to evaluate the anti-inflammatory potential of medicinal agents are carrageenan induced paw edema and croton oil induced anal edema inhibition tests (Yim *et al.,* 2009). These models were used to measure the anti-inflammatory activity of extracts in current work since these are simple and reliable assays to measure the biological response.

### 1.6.2. Heat mediated inflammatory pain

Pain is a marker of inflammatory process that is accompanied by redness, heat and swelling. The peripheral nociceptors are sensitized during inflammation and develop ectopic discharges upon nerve injury or disease (Schaible, 2007). Some of the chemicals that excite pain are serotonin, acetylcholine, histamine, bradykinin, potassium ions, acids and proteolytic enzymes. Cancer patients suffer from chronic pain associated with injury and inflammation. Plants and natural products that reduce pain are a blessing for cancer patients (Maroon *et al.,* 2010). A simple and acute method to assess pain relieving, called analgesic, activity of extracts is heat mediated inflammatory pain method. It uses a hot-plate to incite pain in rodents' paws and their response to painful stimulus is measured. Rodents exposed to thermal pain stimuli, show avoidance, and retain pain-related information in their memory that is related to higher brain functions. Rodents show reaction by reflex withdrawal and licking of paws. Exposure to heat on hot-plate is limited for a period of 30-60 seconds to avoid nerve injury (Schaible, 2007). It was the method of choice in present work to measure the analgesic activity of *Datura* extracts.

### 1.6.3. Tail suspension test

The potential antidepressant action of drugs can be effectively tested with the help of tail suspension test (TST). This simple test can also give information about certain manipulations that are known to play a role in depression like behavioral response (Can *et al.,* 2012b). TST was introduced some 20 years ago and since then it has become widely accepted as an efficient tool to determine the antidepressant activity of drug samples in mice. The test relies on the fact that when animals are exposed to a short term, unavoidable state of being suspended head down by their tail, will go into an immobile posture. Medicinal agents with antidepressant potential will shorten the period of immobility and in the contrary, motivate the animal to adopt an escape related behavior. In TST, the stress is imparted by the hemodynamic discomfort resulting from the manner in which the animals are hung upside down from their tail. The stronger the antidepressant properties of a drug the longer the duration of the escape directed behavior of the test animals pretreated with that drug (Cryan *et al.,* 2005). This simple test was included in the current study to determine the effect of chosen plant extracts on the behavioral aptitude of study animals.

## 1.7. Chronic *In Vivo* Assays

### 1.7.1. Benzene induced leukemia

Leukemia is a cancer with an excess number of white blood cells associated with dysfunctional bone marrow and lymphatic system. Leukemia can be acute lymphocytic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML) (Zhao *et al.,* 2021). Animal models of leukemia can be used to assess the bioactivity of plant extracts. An easy method is to use benzene as leukemogenic agent. Benzene is a toxic chemical that has been reported to cause AML and other hematological malignancies. It can be used to develop rodent model of secondary leukemia. Benzene has been reported to induce oxidative stress, dysregulation of aryl hydrocarbon receptor and cell cycle and reduced immunosurveillance over the generation and clonal evolution of leukemic stem cells to leukemia. It also has been found to promote leukemia development in transgenic mice having mutation in Trp53 or Ras. Literature shows that exposure to approximate 300 ppm of benzene is required to induce AML in mice (Kawasaki *et al.,* 2009; Zhao *et al.,* 2021). In the present study, benzene induced leukemia model was used to assess the anticancer activity of *Datura* extracts and isolated compounds.

### 1.7.2. Carbon tetrachloride induced inflammation

A model of chronic inflammation is useful in assessing the role of plant extracts for the treatment of long-term inflammation. This further strengthens possible use of extracts in managing cancer associated inflammation. One such model is carbon tetrachloride ($CCL_4$) induced renal and hepatic inflammation in rodents. $CCL_4$ causes hepatic and renal damage by generating reactive metabolites and free radicals. Hepatic injury is associated with fat accumulation and centrilobular necrosis. It is assumed that $CCL_4$ mediated free radicals cause lipid peroxidation by binding to the macromolecules (Raucy *et al.,* 1993). Briefly, the P450-enzyme system breaks C-Cl bond of $CCL_4$ by single electron transfer forming chloride ($Cl^{\bullet}$) and trichloromethyl ($CCl_3^{\bullet}$) radicals. The $CCL_3^{\bullet}$ radical rearranges into chloroform that covalently binds to macromolecules or enoic fatty acids of endoplasmic reticulum membranes. In turn, trichloromethyl peroxyl radical is formed that accelerates cellular damage. Alternatively, reductive biotransformation of $CCL_4$ breeds carbene-type radicals under anaerobic conditions that can damage nucleic acids and proteins (Raucy *et al.,*1993). Free radicals and lipid peroxidation causes hepatotoxicity (Al-Assaf 2013), loss of cytochrome P450

monooxygenase system and glucose-6-phosphatase (Boll *et al.,* 2001) that leads to the lack of transport of triglyceride-rich low-density lipoprotein into the plasma ensuing in lipid accumulation. Additionally, $CCL_4$ declines NADPH cytochrome P450 and glutathione in renal microsomes, and glutathione alone in renal cortex and mitochondria. This is accompanied by ROS and lipid peroxidation leading to glomerular diseases, acute renal failure, pyelonephritis, obstructive nephropathy and progressive renal failure. These are indicated by the increased concentrations of BUN and creatinine in serum (Moneim *et al.,* 2013).

## 1.8. Other Methods

### 1.8.1. Immunohistochemistry

Immunohistochemistry method utilizes the principles of antibody protein interaction and detection either by chromogens or fluorescent system. A set of protein specific primary antibody and conjugated secondary antibodies are used to detect the presence of specific protein in the sample. Immunohistochemistry is performed on the tissue section obtained from biopsies either from patients or animal models. Tissue sections are dissevered, fixed, implanted in paraffin and layered on microscopic slides. These sections are hydrated and probed with the protein specific primary antibodies. Later, it is incubated with secondary antibody conjugated with horseradish peroxidase (HRP) or alkaline phosphatase (AP). A collection of chromogenic, fluorogenic and chemiluminescent substrates can be used with either enzyme. Antibody–HRP conjugates are better than antibody–AP conjugates with respect to high turnover rate, good stability and low cost. A chromogen system called 3,3'-diaminobenzidine (DAB) is commonly used to intensify the reaction of protein and antibody. DAB deposits brown residues on tissue sections after reaction with protein of interest. Intensity of the brown color corresponds to the extent of expression of the protein (De Matos *et al.,* 2010).

### 1.8.2. Western blotting

Western blotting (WB) is used to detect the expression of a specific protein in the mixture of extracted protein sample from the cells or tissues. It is useful to evaluate expression of proteins from various clinical models after the treatment with natural products or plant extracts. It also aids in validating the signaling pathways associated with a disease and treatment (Kurien *et al.,* 2006). Western blotting has many benefits. First, gel and membranes are flexible and easy to handle. Second, different ligands can

be used to access the proteins restricted on the membrane. Third, many replicas of gel are feasible. Fourth, it is possible to store membranes for longer durations. Fifth, multiple analyses can be done on one membrane (Kurien *et al.,* 2006).

WB process can be divided into four major steps including separation of different sized proteins by gel electrophoresis, relocation of proteins from gel to a nitrocellulose membrane by electroblotting, binding with protein specific monoclonal antibodies and imagining the bands of protein by an imaging system. Briefly, proteins are segregated based on molecular weight on sodium dodecyl sulfate (SDS) polyacrylamide gels (4-20%). Blocking-buffer is used to block the non-specific binding sites after transferring to nitrocellulose membrane and probed with protein specific primary antibody. Primary antibody is then bound with a horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugated secondary antibody obtained from goat, rabbit, mouse etc. Development of the enzyme-conjugated secondary antibodies with appropriate substrates will produce color or fluorescence for detection of the target protein (Kurien *et al.,* 2006). Knowledge of molecular weights of proteins and thickness and intensity of the band is necessary to correctly interpret the expression patterns.

## 1.9. Hypothesis and Problem Statement

Historically, medicinal plants have been a reliable and an effective source of drugs that are used in treatment of inflammation and cancer. However, medicinal plants are a large reservoir of bioactive constituents, which require thorough investigations either for new bioactive molecules or drug repurposing. On this basis, *D. stramonium* and *D. innoxia* that have numerous ethnomedicinal uses particularly in inflammatory conditions as well as in cancer, were studied in the current project. It is hypothesized to find drug candidates, other than the extensively investigated tropane alkaloids, from either of these plants that can be effective for the treatment of inflammation, cancer and cancer associated psychological changes.

## 1.10. Aim and Objectives

This study was aimed to perform a comparative analysis of the pharmacological benefits of *D. stramonium* and *D. innoxia* in cancer and inflammation. Our specific objectives were as follows;

- To perform polarity based successive extraction from different parts of *D. stramonium* and *D. innoxia* plants
- To evaluate and compare the *in vitro* antioxidant, anti-inflammatory and anticancer activities of *D. stramonium* and *D. innoxia* extracts
- To scrutinize, shortlist and select the potent crude extracts of both species for acute and chronic *in vivo* assays
- To isolate, characterize and elucidate the structure of bioactive compounds from the most potent crude extract
- To investigate the effect of isolated compounds in cancer and inflammation using chronic *in vivo* models

# CHAPTER 2

# MATERIAL AND METHODS

# 2. MATERIALS AND METHODS

## 2.1. Material

### 2.1.1. Chemicals and reagents

All chemicals and reagents used in current study were of analytical grade. Solvents i.e., n-hexane, ethyl acetate, methanol, chloroform and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Folin Ciocalteu reagent and 2,2-diphenyl-1-picrylhydrazyl (DPPH), formaldehyde, Triz ma base, acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), ammonium per sulfate (APS), glycine, 2X laemmli buffer, histopaque-1077, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, surfactin, ferrous chloride, sea, salt, sodium hydroxide, doxorubicin sodium hydroxide, aluminum chloride, ascorbic acid, quercetin, gallic acid, rutin, caffeic acid, kaempferol, myricetin and (+)-catechin, cyclophosphamide, silymarin, canola oil, ethyl methane sulfonate, were acquired from Sigma–Aldrich (Steinheim, Germany). Griess reagent, tween 80, thiobarbituric acid, tricholoroacetic acid, zinc sulphate, sodium nitrite, ammonium molybdate, ferric chloride and phenazine methosulphate were acquired from Sigma (Chemicals Co. St. Louis, USA). Sodium carbonate, sulphuric acid, hydrogen peroxide, potassium ferricyanide, sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Merck KGaA (Darmstadt, Germany). Phosphate buffer saline, RNA weight solution, tissue protein extraction reagent, protein assay dye reagent, 2x laemmli sample buffer, trizma base, acrylamide, bisacrylamide, sodium dodecyl sulfate, ammonium persulphate, glycine, skimmed milk, sodium chloride, potassium chloride, Low melting agarose, normal melting agarose, ethidium bromide, ibuprofen, tramadol and fluoxetine were purchased from Sigma-Aldrich (USA).

Heat inactivated fetal bovine serum (HIFBS: Biowest, South America), RPMI-1640 (Gibco BRL, Life Technologies, Inc), and dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Merck Millipore) were purchased from respective sources. Pre-coated silica gel 60 $F_{254}$ TLC plates, normal phase silica gel 60 (63-200 µm particle size), silica gel 60 (40-60 µm particle size) and chromatography columns were purchased from Merck (Germany). Medium ISP4 was constituted in the laboratory.

Stacking buffer, tetramethylethylenediamine (TEMED), monomer solution, running solution, Tris buffered saline with tween 20 (TBST), total protein extraction reagent

(TPER) solution, RNA wait solution, poly-vinylidene fluoride (PVDF) membranes, enhance chemiluminescence (ECL) solution, laemmli buffer, glycine and protein assay dye reagent were purchased from Sigma Aldrich (USA) and DaeJung Chemicals & Metals Co. Ltd (South Korea).

### 2.1.2. Primary and secondary antibodies for western blot analysis

Primary antibodies i.e., Nrf-2 (sc-365949), p-JNK (sc-6254), NF-ƙB (sc-8008), PARP-1 (sc-8007), TNF-α (sc-52746), SIRT1 (sc-74504), NLRP3 (sc-134306), Caspase-1 (sc-56036), IL-1β (sc-32294), β-Actin (sc-47778) were purchased from Santa Cruz Biotechnology, Paso Robles, CA USA, while the anti-mouse HRP conjugated secondary antibody (0000375517) was purchased from PROMEGA, USA. All chemicals were of pharmaceutical grade.

### 2.1.3. Apparatus and equipment

Erlenmeyer flask, muslin cloth, whatman filter paper, beaker, funnel, tripod stand, petri plates, micropipette (Sartorius, France), Vernier calliper (Tailin, Japan), pasteur pipette, bi-compartment perforated tray, sterile transparent 96 well-plate (SPL life science, Korea), sonicator (Sweepzone technology, USA), incubator IC83 (Memmert, Germany), microplate reader (Elx 800, Biotek, USA), HPLC system (Shimadzu LC-20AT) equipped with auto-sampler (SIL-20A), column oven (CTO-20A) and diode array detector (SPD-M20A), analytical column (Nucleosil C18, 5 µm 100 A°, 250x4.60 mm, Phenomenex), guard column (KJO-4282, Phenomenex), HPLC system (Agilent Chem station Rev. B.02-01-SR1) equipped with Agilent 1200 series binary gradient pump, analytical column (Zorbax-C8, 4.6 × 250 mm, 5 µm particle size, Agilent, USA), rotary evaporator (RE-200 Bibby Sterlin, UK), centrifuge (B. Bran, Germany), compound light microscope (Irmeco, Germany), neubaeur chamber (Marien, Germany) and 5% $CO_2$ incubator (Sanyo MCO-17AIC, Japan), freezer -80°C (So-low, Cincinnati, Ohio, USA), Eddy's hot plate, UGO Basile Plethysmometer (7140), glass tank (46 cm height, 21 cm diameter and 30 cm depth), and fluorescent microscope. Nuclear Magnetic Resonance (NMR) spectrophotometer; Bruker Avance II 600 and Bruker Avance III 400 NMR spectrophotometer. Gel electrophoresis assembly and trans-Blot (Bio-Rad Germany).

### 2.1.4. Cultures, cell lines and animals

Brine shrimp (*Artemia salina*) eggs were acquired from Oceans Star International USA (O.S.I®). *Streptomyces 85E* strain maintained in the laboratory was used for protein kinase assay. Sprague Dawley rats (150-250g; 6-8 weeks), Balb/c mice (25-30 g; 6-8 weeks. Males) were purchased from National Institute of Health (NIH), Islamabad, Pakistan.

Prostate cancer PC-3 (ATCC® CRL-1435) and breast cancer cell lines MDA-MB 231 (ATCC# HTB-26™) and MCF-7 (ATCC#HTB-22™) were purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in RPMI-1640 supplemented with 2.2 g/l $NaHCO_3$ and 10% v/v HIFBS (pH 7.4) in a humidified $CO_2$ (5%) incubator at 37 °C.

## 2.2. Animal Ethical Statement

Animal studies were performed according to the guidelines of ethical committee of Quaid-i-Azam University and NIH, Islamabad, Pakistan. Prior approval from the ethical committee was obtained for experiments (letter no. QAU-PHM-023/2016) and animal care (letter no. QAU-PHM-017/2016). Blood samples for isolation of lymphocytes were obtained from healthy volunteers after approval from the review board of Quaid-i-Azam University (letter no. IRB-QAU-116; Dated 4/11/2016) and informed consent from the volunteers. Animals were kept in aluminum cages at 25±1 °C and air humidity of 45±5% with a 12 h light/dark cycle. All animals were provided standard laboratory feed and water ad libitum.



## 2.3. Methods

32 extracts for each *D. stramonium* and *D. innoxia* were prepared using four plant parts and four extraction solvents. These 32 extracts were subjected to preliminary phytochemical and *in vitro* biological evaluation. Based on the results, 12 extracts from each plant (total 24) were selected and further screened for *in vitro* anticancer activity using prostate and breast cancer cell lines. Next, 4 selected extracts (2 from each plant) were evaluated for *in vitro* toxicity and *in vivo* acute biological activities. From the results of these experiments, one extract from each of *D. stramonium* and *D. innoxia* was subjected to chronic *in vivo* studies. Lastly, the most effective extract of *D. stramonium* (ethyl acetate leaf extract) was employed for the isolation of compounds

that were again tested for their activities in pathological and behavioral models of cancer and inflammation.

### 2.3.1. Collection and successive solvent extraction

#### *2.3.1.1. Collection and identification*

*D. stramonium* and *D. innoxia* were collected from Quaid-i-Azam University, Islamabad and Oghi Town, Dictrict Mansehra, Khyber Pakhtunkhwa, Pakistan, respectively in August 2016. Since none of the plants is an endangered species; therefore, could easily be collected from their wild habitat. Both plants were verified by Prof. Dr. Rizwana Aleem Qureshi, Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University. Later, dried sample of the plants were deposited at the Herbarium of Quaid-i-Azam University under voucher numbers PHM-504 and PHM-487 for *D. stramonium* and *D. innoxia*, respectively.

#### *2.3.1.2. Preparation of crude extract*

The plants were thoroughly washed with water to remove debris and contaminants. Their leaves, fruits, stems and roots were carefully separated, and shade dried at with adequate ventilation. Each plant part was then separately crushed into fine powder. Next, both plants were subjected to successive extraction by ultra-sonication assisted maceration at room temperature using four solvents including n-hexane, ethyl acetate, methanol and distilled water. After extracting each part twice using the same solvent, the extracts were filtered to separate the marc using Whatmann No.1 filter paper, concentrated in a rotary evaporator and dried in a vacuum oven at 40 °C. Percent extract recovery (%w/w) was calculated based on the weight of dry extract (A) and powdered plant material (B) using the following formula;

$$\% \text{ Extract recovery (\% w/w)} = (A / B) \times 100$$

### 2.3.2. Preliminary screening of extracts of *D. stramonium* and *D. innoxia*

Preliminary screening was performed on the total 32 extracts of *D. stramonium* and *D. innoxia* plants (Table 2.1).

#### *2.3.2.1. Phytochemical analysis*

2.3.2.1.1. Qualitative estimation of total phenolics content (TPC)

TPC was quantitated using Folin-Ciocalteu reagent following standard protocol (Nasir *et al.,* 2020) and is given in annexure 1. Estimated TPC was expressed as µg gallic acid equivalent per mg extract (µg GAE/mg extract)

2.3.2.1.2. Qualitative estimation of total flavonoids content (TFC)

Aluminum chloride colorimetric assay was used for the quantification of TFC using previously reported procedure (Nasir *et al.,* 2020) as described in annexure 1. TFC were expressed as µg ascorbic acid equivalent (AAE) per mg extract.

**Table 2.1** Extracts of *D. stramonium and D. innoxia.*

| Sr. No. | Solvent | Plant part | Code |
|---|---|---|---|
| ***D. stramonium*** | | | |
| **1** | n-hexane | Root | DSR-NH |
| **2** | Ethyl acetate | Root | DSR-EA |
| **3** | Methanol | Root | DSR-M |
| **4** | Distilled water | Root | DSR-Dw |
| **5** | n-hexane | Fruit | DSF-NH |
| **6** | Ethyl acetate | Fruit | DSF-EA |
| **7** | Methanol | Fruit | DSF-M |
| **8** | Distilled water | Fruit | DSF-Dw |
| **9** | n-hexane | Stem | DSS-NH |
| **10** | Ethyl acetate | Stem | DSS-EA |
| **11** | Methanol | Stem | DSS-M |
| **12** | Distilled water | Stem | DSS-Dw |
| **13** | n-hexane | Leaf | DSL-NH |
| **14** | Ethyl acetate | Leaf | DSL-EA |
| **15** | Methanol | Leaf | DSL-M |
| **16** | Distilled water | Leaf | DSL-Dw |
| ***D. innoxia*** | | | |
| **17** | n-hexane | Root | DIR-NH |
| **18** | Ethyl acetate | Root | DIR-EA |
| **19** | Methanol | Root | DIR-M |
| **20** | Distilled water | Root | DIR-Dw |
| **21** | n-hexane | Fruit | DIF-NH |
| **22** | Ethyl acetate | Fruit | DIF-EA |
| **23** | Methanol | Fruit | DIF-M |
| **24** | Distilled water | Fruit | DIF-Dw |
| **25** | n-hexane | Stem | DIS- NH |
| **26** | Ethyl acetate | Stem | DIS-EA |
| **27** | Methanol | Stem | DIS-M |
| **28** | Distilled water | Stem | DIS-Dw |
| **29** | n-hexane | Leaf | DIL-NH |
| **30** | Ethyl acetate | Leaf | DIL-EA |
| **31** | Methanol | Leaf | DIL-M |
| **32** | Distilled water | Leaf | DIL-Dw |



2.3.2.1.3. Quantitative estimation through RP-HPLC analysis

The detection and quantification of polyphenols in selected *Datura* species was conducted by RP-HPLC analysis. Procedure reported by (Nasir *et al.,* 2020) was used with slight modifications as given in annexure 1.

### *2.3.2.2. In vitro biological evaluation*

A stock solution of 20 mg was prepared for each extract. It was further diluted into different concentrations and used in following biological assays.

#### 2.3.2.2.1. Antioxidant potential assessment

a. Total antioxidant capacity (TAC)

TAC of the *Datura* extracts was estimated by phosphomolybdenum based method as described (Nasir *et al.,* 2020) in annexure 1.

b. Total reducing power (TRP)

TRP determines the reducing capacity of samples, and it was performed by a previously reported method (Nasir *et al.,* 2020) using potassium ferricyanide. It is given in annexure 1.

c. Percent free radical scavenging assay (% FRSA)

#### 2.3.2.2.2. Free radical scavenging activity

It was determined using DPPH radical according to the standard protocol (Nasir *et al.,* 2020) given in annexure 1.

#### 2.3.2.2.3. Brine shrimp cytotoxicity assay

The effect of extracts on viability of *Artemia salina* larvae is a good way to determine their cytotoxicity. It was performed according to the procedure detailed in annexure 1. (Nasir *et al.,* 2020)

#### 2.3.2.2.4. Protein kinase inhibition assay

Inhibition of hyphae formation of *Streptomyces 85E* strain after exposure to *Datura* extracts was considered as the protein kinase inhibitory activity of the test extracts. It was estimated by standard disc diffusion method (Nasir *et al.,* 2020) as described in annexure 1. The preliminary *in vitro* screening of 32 extracts discussed in the preceding sections enabled us to select the potentially potent ones and exclude the less active ones from further evaluation.

#### *2.3.2.3. Cancer cell lines mediated screening of extracts of D. stramonium and D. innoxia*

Based on the results of above assays, 24 extracts were selected for further evaluations. These were tested in prostate and breast cancer cell lines to determine their potential cytotoxic activity.

##### 2.3.2.3.1. MTT assay

PC-3, MDA-MB 231 and MCF-7 cells were treated with different concentrations (20, 10, 5, 2.5 and 1.25 µg/ml) of the extracts and subjected to MTT assay as previously described (Nasir *et al.,* 2020). It is provided in detail in annexure 1.

#### *2.3.2.4. In vitro toxicity and acute in vivo studies of selected extracts*

Toxicity and acute *in vivo* studies were performed on 4 extracts including DSL-NH, DSL-EA, DIL-NH and DIL-EA, selected based on the results obtained from cancer cells-based assay.

##### 2.3.2.4.1. *In vitro* toxicity assays

a. Toxicity against isolated lymphocytes

Lymphocytes were freshly isolated from the blood of human volunteers and treated with 20 µg/ml concentration of the extracts in the presence of phytohaemagglutinin (PHA). MTT was performed as previously described (Nasir *et al.,* 2020) and given in annexure 1.

b. Toxicity against isolated macrophages

Macrophages were isolated from the peritoneal fluid of albino rats and plated in a 96-well plate at a density of $1x10^6$ cells per well (Farooq *et al.,* 2020). The plate was incubated in a 5% $CO_2$ incubator at 37ºC for 24 h. Later, the cells were treated with 20 µg/ml concentration of the extracts or DMSO as vehicle control and allowed to grow for a period of 24 h. Cytotoxicity was estimated by MTT assay.

##### 2.3.2.4.2. Acute *in vivo* toxicity study

Sprague Dawley rats were used for acute toxicity analysis of NH and EA extracts of leaves of *D. stramonium and D. innoxia.* Rats (n= 6) were randomly divided into test and control groups. The test groups orally received single boaster dose of extracts (150, 300, 500, 1000 and 2000 mg/kg) while the rats in the control group were given normal saline (10 ml/kg of animals). Animals were observed daily for a period of two weeks

for physical signs of toxicity and mortality. Toxicity signs include changes in skin color or texture, body secretions, i.e., lacrimation, salivation, nasal discharge, urination and defecation, any injury to organ sites and behavioral aberrations like imbalance, aggression and sleep disturbances. Guidelines provided by Organization for Economic Cooperation and Development (OECD) were followed during acute toxicity study.

2.3.2.4.3. Acute *in vivo* assays

Acute efficacy studies were performed on DSL-NH, DSL-EA, DIL-NH and DIL-EA extracts. Balb/c mice (25-30 g; male) were housed in standard metallic cages and provided with standard diet and water *ad libitum.* Animals were acclimatized to the environment before the start of the experiment. Mice were randomly divided into 11 groups (n = 6) as given in table 2.2.

**Table 2.2** Animal groups for acute *in vivo* assays.

| Group | Title | Description |
|---|---|---|
| **I** | Normal control | Untreated, standard food only |
| **II** | Vehicle control | 10% DMSO in olive oil or CMC |
| **III** | Positive control | Standard drug specific for each test |
| **IV** | DSL-NH-LD | Low dose 150 mg/kg |
| **V** | DSL-NH-HD | High dose 300 mg/kg |
| **VI** | DSL-EA-LD | Low dose 150 mg/kg |
| **VII** | DSL-EA-HD | High dose 300 mg/kg |
| **VIII** | DIL-NH-LD | Low dose 150 mg/kg |
| **IX** | DIL-NH-HD | High dose 300 mg/kg |
| **X** | DIL-EA-LD | Low dose 150 mg/kg |
| **XI** | DIL-EA-HD | High dose 300 mg/kg |

a. Carrageenan induced inflammation

Carrageenan induced paw inflammation model was used to determine the anti-inflammatory activity of extracts according to previously reported protocols with slight modifications (Saha *et al.,* 2007). Balb/c mice were orally administered extracts and controls one hour prior the administration carrageenan. Ibuprofen (10 mg/kg) was used as positive control. Later, 0.05 ml of 1% carrageenan solution in sterile water for

injection was injected into the sub-planter region of right hind paw of mice. Paw volume was measured immediately after the injection and then at regular intervals for up to 4 hours. Change in paw volume represented the inflammatory edema induced by carrageenan. Change in paw volume was measure by using formula $EV = PVa - PVi$, where *EV, PVi and PVa* represents edema volume, initial paw volume before injection and paw volume after carrageenan injection, respectively. Results are expressed as percent edema inhibition and calculated as follows;

$$\text{Percent edema inhibition} = [\frac{\text{EVc} - \text{EVt}}{\text{EVc}}] \text{ x } 100$$

where, *EVc* = Edema volume of normal control mice, *EVt* = Edema volume of sample mice.

b. Croton oil induced anal edema

Another method to assess the anti-inflammatory activity of selected n-hexane and ethyl acetate extracts was croton oil induced anal edema in mice (Majid *et al.,* 2018). Balb/c mice were orally administered the vehicle, ibuprofen (10 mg/kg) and low and high doses of selected extracts one hour before the induction of anal inflammation. Later, a croton swab soaked in 200 µl of 6% croton oil in diethyl ether was softly inserted into the anus of the mice for 10 seconds. Afterwards, anal edema was measured every hour for 4 hours using a vernier caliper. The extent of prevention of edema formation in treatment groups was considered as the anti-inflammatory activity of test sample when compared with the control. Percent inhibition of anal inflammation was calculated using the following equation.

$$\% \text{ inhibition of edema} = \left(\frac{\text{VEc} - \text{VEt}}{\text{VEc}}\right) \times 100$$

Where, VEc and VEt represent edema volume in normal control and treatment groups, respectively.

c. Motivational tail suspension test

The motivational effect of the selected extracts of *Datura* species on behavior of animals in response to depressive stimulus was assessed by tail suspension test. Mice were orally administered extracts 10% DMSO in CMC (vehicle control) and saline (negative control). Moreover, fluoxetine (positive control) was administered via i.p route (20 mg/kg). One hour later, each mouse was suspended by tail 7.5 cm above the surface of table that was 70 cm above the ground. Mouse tail was suspended on a solid support using an adhesive tape placed 1 cm away from the tip of the tail. Mice were

observed for a period of 6 minutes and the time these were immobile during this phase was recorded. Immobility recorded in terms of passive hanging without any motion was considered a sign of decreased motivation or depression in response to inability to escape the suspension. Results were presented as mean±SD of immobility time in seconds (Can *et al.,* 2012a).

d. Heat mediated inflammatory pain

Inflammation at the site of nerve endings can enhance pain sensitivity to thermal stimulus. This phenomenon was used to measure the analgesic activity of extracts on heat mediated inflammatory pain using hot plate method (Dzoyem *et al.,* 2017). In this method (Ismail *et al.,* 2015), mice are placed on hot plate set at 55±2°C and their response to thermal pain was noted in terms of paw liking or paw withdrawal or jumping. After recording the initial reaction time (Ti), mice were orally administered with extracts and DMSO (vehicle) while tramadol was used as a positive control (12.5 mg/kg) given intraperitoneally (i.p). One hour later, animal reaction time (Tf) to thermal pain on hot plate was recorded every hour for a period of 4 hours. A cutoff time of 30 seconds was set to avoid damage to paw nerve endings. Percent analgesic activity in response to inflammatory pain was calculated by the following formula:

$$\text{Percent analgesic activity} = \left(\frac{Tf - Ti}{Ti}\right) \times 100$$

Ethyl acetate extracts of leaves of *D. stramonium* (DSL-EA) *and D. innoxia* (DIL-EA) were found effective among all others. These two extracts were selected for subsequent acute toxicity and chronic *in vivo* efficacy studies.

### 2.3.3. Chronic *in vivo* models

#### *2.3.3.1. Benzene induced leukemia*

Benzene induced leukemia model was used to test the anticancer activity of ethyl acetate extracts of *D. stramonium and D. innoxia* leaves. Leukemic rats were exposed to the two doses of extracts (low dose, LD; high dose, HD), observed and euthanized at the end of study. Blood and tissue samples were collected, and tests were performed according to the protocols described by (Nasir *et al.,* 2020). It is described in detail in annexure 1. Animals' groups are given in table 2.3.

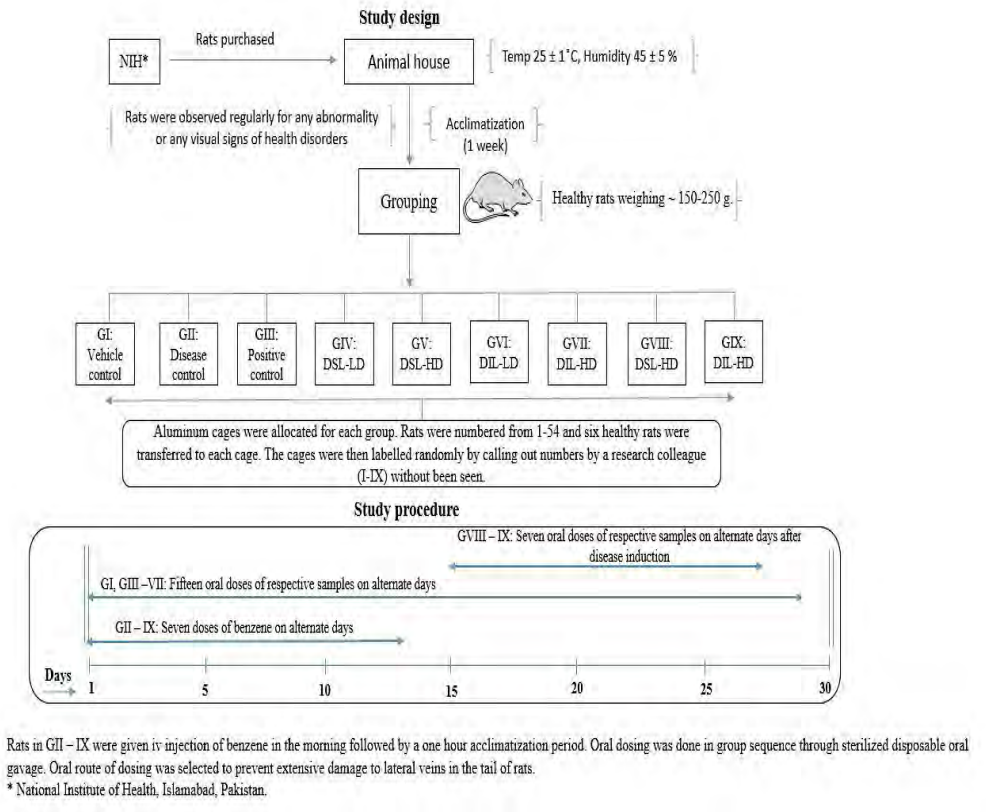


**Figure 2.1** A schematic diagram of study design and experimental timeline of *in vivo* antileukemic activity performed using male Sprague Dawley rats.
**Note:** Clip art images used in the figure were made using ChemDraw Professional v19.0.

**Table 2.3** Animal groups for benzene induced leukemia.

| Group | Title | Description |
|---|---|---|
| **I** | Vehicle control | 10% DMSO in water |
| **II** | Disease control | 0.2 ml Benzene, 1:10 in water for injection |
| **III** | Positive control | Cyclophosphamide 10 mg/kg, 0.2 ml Benzene |
| **IV** | DSL-EA-LD | Low dose 100 mg/kg, 0.2 ml Benzene, preventive |
| **V** | DSL-EA-HD | High dose 200 mg/kg, 0.2 ml Benzene. Preventive |
| **VI** | DIL-EA-LD | Low dose 100 mg/kg, 0.2 ml Benzene, preventive |
| **VII** | DIL-EA-HD | High dose 200 mg/kg, 0.2 ml Benzene, preventive |
| **VIII** | DSL-EA-HD | High dose 200 mg/kg, 0.2 ml Benzene, treatment |
| **IX** | DIL-EA-HD | High dose 200 mg/kg, 0.2 ml Benzene, treatment |

2.3.3.1.1. Serum hematological analysis

Serum analysis for red blood cells, white blood cells, platelets, erythrocyte sedimentation rate and hemoglobin were done as given in annexure 1. (Nasir *et al.,* 2020)

2.3.3.1.2. Biochemical analysis

Alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine phosphokinase (CPK), total serum protein, urea and creatinine were estimated as described by (Nasir *et al.,* 2020). Annexure 1.

2.3.3.1.3. Estimation of endogenous antioxidant enzymes

Catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and glutathione S-transferase (GST) were estimated as described by (Nasir *et al.,* 2020) Annexure 1.

2.3.3.1.4. Estimation of lipid peroxidation and nitric oxide

Thiobarbituric acid reactive substances (TBARs) and nitric oxide levels were calculated as described by (Nasir *et al.,* 2020) Annexure 1.

2.3.3.1.5. Histopathology

Hematoxylin and eosin stain slides of organs were prepared and evaluated according to procedure given in annexure 1. (Nasir *et al.,* 2020)

### *2.3.3.2. $CCL_4$ induced inflammation*

Inflammation is highly active in cancer state. Thus, anti-inflammatory activity of DIL-EA and DSL-EA extracts was estimated in $CCL_4$ induced inflammation model (Batool *et al.,* 2017a; Moneim *et al.,* 2013). Sprague Dawley rats (male, age: 6-8 weeks, ~150-250 g) were housed in aluminum cages with wood shavings as bedding under standard 12 h light/dark cycle. The temperature and humidity were maintained at 25±1°C and 45±5%, respectively. Rats were provided with standard feed and water ad libitum.

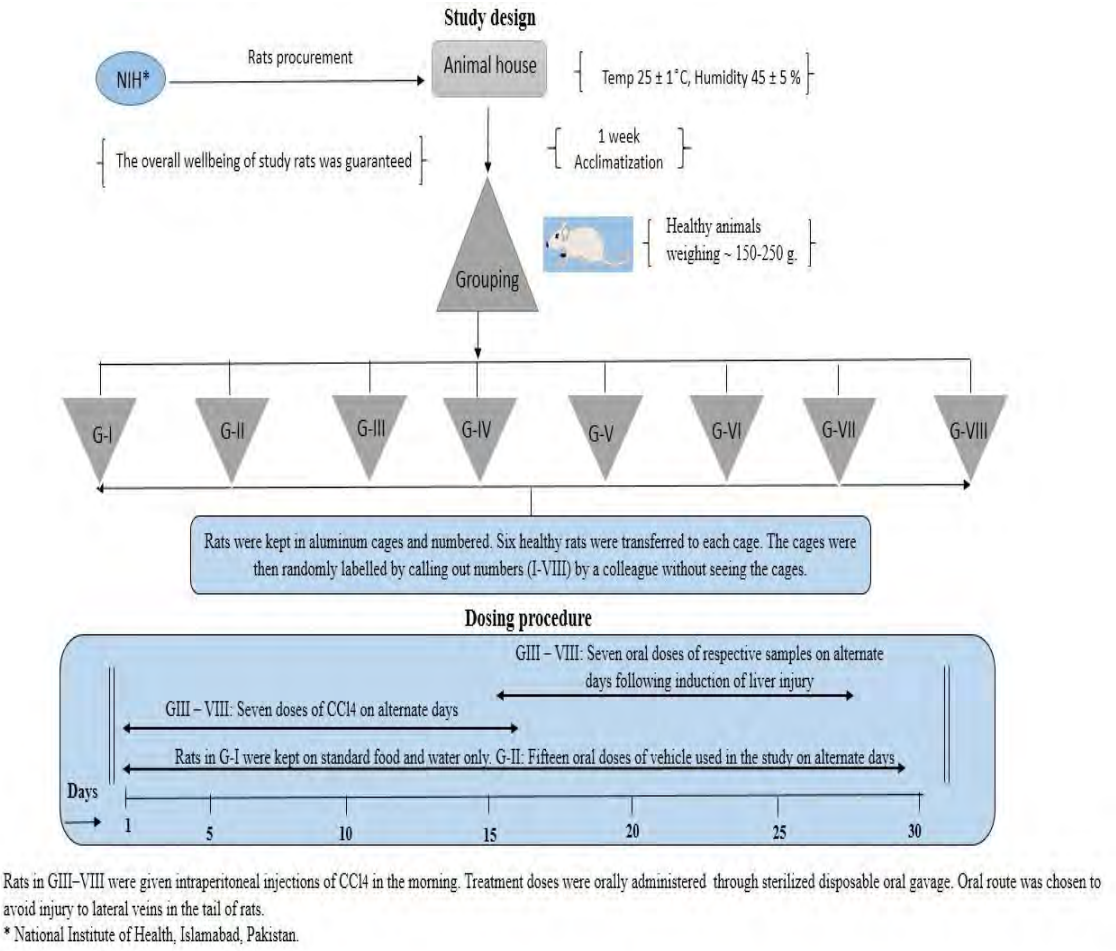


**Figure 2.2** A schematic diagram of study design and experimental timeline of *in vivo* anti-inflammatory activity performed using male Sprague Dawley rats.
**Note:** Clip art images used in the figure were made using ChemDraw Professional v19.0.

Rats were divided into 8 groups with each having 6 rats. Groups I and II received no treatment (normal control) and 10% DMSO in olive oil (vehicle control), respectively. These were not subjected to any inflammation inducer. Hepatotoxicity in rats among groups III-VIII was induced by intra-peritoneal injection of 0.2 ml 30% $CCL_4$ in olive oil. A total of 7 doses were administered on alternate days over a period of 14 days. Animals were observed for clinical and toxicological symptoms including abdominal swelling, dark urine color, pale stool color, swelling in paws, loss of appetite and abnormal bruising. After disease induction, from day 15, rats in groups IV-VIII were either treated with extracts or standard drug Silymarin (50 mg/kg). Two dosing schedules, i.e., low dose and high dose were used for both DIL-EA and DSL-EA as given in table 2.4. All samples were administered in the maximum volume of 1 ml via sterile oral gavage. The vehicle 10% DMSO in olive oil was used to constitute

suspensions of the extracts. All doses (7 in total) to the respective groups were given in the morning on alternate days.

**Table 2.4** Animal groups for $CCL_4$ induced inflammation.

| Group | Title | Description |
|---|---|---|
| **I** | Normal control | Untreated, standard food only |
| **II** | Vehicle control | 10% DMSO in olive oil |
| **III** | Disease control | 1 ml/kg of 30% $CCL_4$ in olive oil |
| **IV** | Positive control | 1 ml/kg of 30% $CCL_4$ + Silymarin 50 mg/kg |
| **V** | DSL-EA-LD | Low dose 150 mg/kg, 1 ml/kg of 30% $CCL_4$ |
| **VI** | DSL-EA-HD | High dose 300 mg/kg, 1 ml/kg of 30% $CCL_4$ |
| **VII** | DIL-EA-LD | Low dose same as DSL-EA-LD |
| **VIII** | DIL-EA-HD | High dose same as DSL-EA-HD |

After 7 doses of each sample, rats were fasted for 24 h and blood sample was drawn under chloroform anesthesia. Later, all animals were euthanized by cervical dislocation and liver was harvested. Different *in vitro* assays and histological evaluations were performed on animal samples to determine the anti-inflammatory activity of *Datura* extracts.

2.3.3.2.1. Collection of blood and liver samples

Sufficient amount of blood was collected from abdominal aorta in sampling tubes free from any anticoagulant. Blood samples were centrifuged at 6000 rpm for 15 minutes at 4°C to separate the serum and stored at −20°C until used for further tests. Next, liver tissues were harvested from each rat and a portion was immediately flashed frozen, while the other portion was fixed in 10% formaldehyde solution to maintain liver histology. These flash frozen liver samples were homogenized in 10X buffer containing 100 mM potassium phosphate buffer and 1 mM ethylenediaminetetraacetic acid at pH 7.4. Liver homogenates were centrifuged at 12,000×g for 30 minutes at 4°C. The supernatant was carefully removed in clean tubes and stored at −20°C until further evaluations. Various hematological, biochemical and enzymatic analyses were performed on serum and liver homogenates.

2.3.3.2.2. Serum/hematological analysis

Serum analysis for red blood cells, white blood cells, platelets, erythrocyte sedimentation rate and hemoglobin were done as given in annexure 1 (Nasir *et al.,* 2020).

2.3.3.2.3. Biochemical analysis

Alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, albumin and creatinine were estimated as described by (Nasir *et al.,* 2020). Annexure 1.

2.3.3.2.4. Estimation of endogenous antioxidant enzymes

Catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and glutathione S-transferase (GST) were estimated as described by (Nasir *et al.,* 2 020). Annexure 1.

2.3.3.2.5. Estimation of lipid peroxidation and nitric oxide

Thiobarbituric acid reactive substances and nitric oxide levels were calculated as described by (Nasir *et al.,* 2020). Annexure 1. TBARs were estimated in liver tissue. Concisely, liver tissue homogenate (250 µl) in PBS was incubated for 1 h at 37°C in a water bath followed by adding 250 µl of 5% trichloroacetic acid and 500 µl of 0.67% thiobarbituric acid. Absorbance was recorded at 535 nm and results among treatment and control groups were compared.

Nitric oxide levels were measured in serum and liver tissue homogenates using Griess reagent as previously described (Nasir *et al.,* 2020).

2.3.3.2.6. Estimation of myeloperoxidase activity

The myeloperoxidase (MPO) activity was determined in liver tissue samples following hexadecyltrimethylammonium bromide (HTAB) buffer and o-dianisidine method as previously reported (Palić *et al.,* 2007). Briefly, MPO from the cells was released by treatment of tissue sample with HTAB in 50 mM PBS (pH 6). It was followed by subjecting the tissue to freeze thaw cycles thrice and centrifuging to settle the debris. The supernatant containing MPO was mixed with hydrogen peroxide and o-dianisidine in 96 well plates. Later, it was scanned at 540 nm using a micro plate reader. Results between control and treatment groups were compared.

2.3.3.2.7. Histopathology

The liver tissue was fixed in buffered 10% formaldehyde (pH 7.4) for 12 hours. Later, it was embedded in paraffin and small pieces (3-5 µm thickness) of the embedded tissue were sectioned to prepare slides. For staining, slides were washed with different ethanol concentrations (50, 70, 90 and 100%) to remove the traces of infiltrated wax. Slides were stained with Eosin and Hematoxylin (Nasir *et al.,* 2020) for evaluation of liver tissue histology and inflammation status. Moreover, Masson's trichome staining was performed to assess fibrosis in liver tissue (Khan *et al.,* 2020). All slides were examined under Nikon Eclipse 80i microscope (Japan).

2.3.3.2.8. Immunohistochemistry

The effect of DSL-EA and DIL-EA extracts on expression of inflammatory mediators Nrf2 and iNOS was assessed in liver tissue by immunohistochemistry staining as previously described (Ruiz *et al.,* 2013). Briefly, tissue slides were washed with xylene, hydrated with decreasing concentrations of ethanol (100, 90, 70, 50%) and treated with proteinase-k. It was treated with normal goat serum and primary antibodies for Nrf2 and iNOS and left overnight to allow the binding of antibodies to their respective sites. Next, days, slides were washed in wash buffer and treated with secondary antibody for 2 hours. The result was detected using 3,3'-diaminobenzidine (DAB) staining.

### 2.3.4. Preparative scale extraction, fractionation and isolation of compounds

Ethyl acetate was selected as the solvent to be used for the preparative scale extraction from leaf portion of *D. stramonium* based on the results from the preliminary screening of extracts.

#### *2.3.4.1. Preparative scale extraction*

The selected plant part was collected, sorted to remove wilted leaves and any adulterations and finally shade dried in a well-ventilated area. Fully dried leaves were pulverized to fine powder with the help of a commercial mill. Total 4.4 kg dried powdered leaf portion of *D. stramonium* was used for bulk extraction. Ultrasonication assisted maceration was initiated with NH to follow an identical extraction pattern to the one used initially as stated in section 2.3.1.2. The solvent used in the second phase was EA and the extraction yields were recorded. The extracts were stored at -20°C till further use.

### *2.3.4.2. Fractionation of DSL-EA using solid phase extraction*

Crude extracts of plants can be effectively fractionated using solid phase extraction (SPE) technique. DSL-EA was fractionated by dissolving 100 g extract in ethyl acetate and latter adsorbed on 200 g silica gel 60 (70-230 µm). The sample to silica ratio was kept at 1:2. The sample, once fully adsorbed on silica was dried in vacuum oven at 45°C. Dried silica having the sample adsorbed was then loaded into a glass column packed with silica. A 2 cm protective layer of fine white sand was used over the top of the sample loaded silica. The column was eluted with various solvents including NH, EA and M used alone or in combination in order of increasing polarity as depicted in figure 2.3. A total of 33 fractions of 350 ml each (DSL-EA 1-33) were collected and dried at 35° in a rotary evaporator.

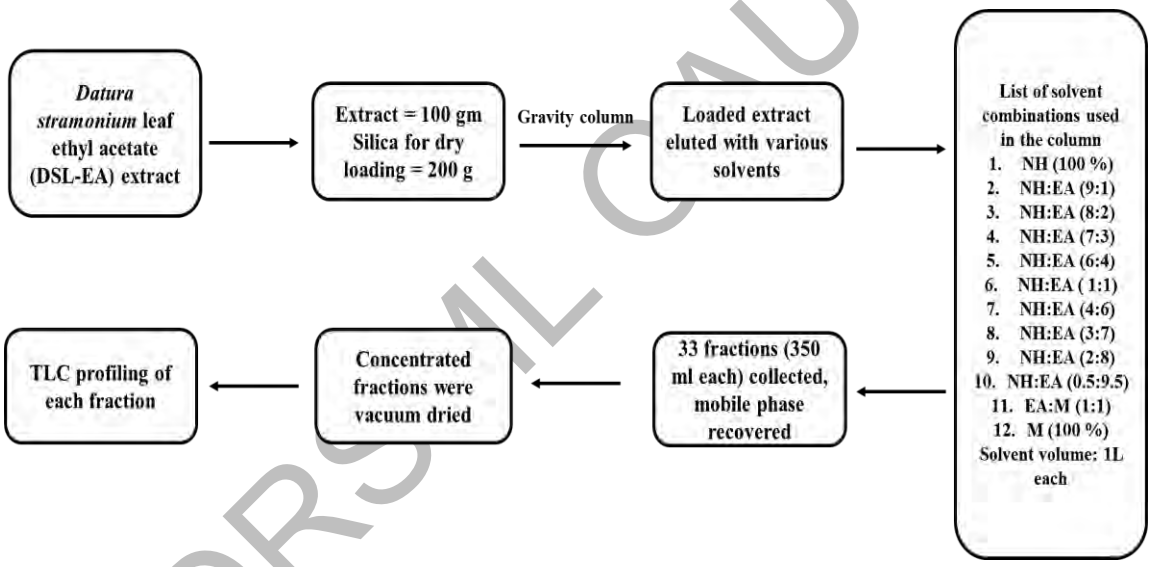


**Figure 2.3** Extraction scheme for the fractionation of DSL-EA.

### *2.3.4.3. Isolation and characterization of compounds from DSL-EA*

Isolation and purification of compounds from the fractions of DSL-EA was guided by TLC profiling of the fractions prior to initiation of purification columns. Normal phase liquid chromatography was majorly used to isolate individual pure compounds. The appropriate mobile phase was chosen for column chromatography by extensive fingerprinting using TLC analysis of DSL-EA fractions using multiple combinations of NH, EA and M. TLC was done using Merck silica gel F254 pre-coated aluminum sheet. Ultraviolet light at 254 and 365 nm wavelength was used along with a variety of spray reagents including phosphomolybdenum and Dragendorrf's reagent for the visualization of compounds.

The isolation scheme of pure compounds fulfilling the following criteria will be described in this section,

- The quantity of isolated moieties was sufficient for structural characterization and biological evaluation
- The compounds were pure enough to be considered for further biological screening.

2.3.4.3.1. Isolation of compound a

Briefly, DSL-EA-10 (2.6 g) was selected following initial fingerprinting using TLC and the solvents resulting in most efficient separation was employed in the purification column. The elution of singular spots was detected on the TLC plate using NH and EA as solvents in the presence of 0.2% formic acid in the solvent combination. Initially, 200 mg from DSL-EA-10 was dissolved in EA and adsorbed on silica gel 60 (70-230 µm; 400 mg). The sample, once fully loaded on the silica, was dried at 45°C in a vacuum oven. A column packed with normal phase silica gel 60 (230-400 µm) was then dry packed with the sample and a 2 cm protective layer of sand was also added above the sample in order to avoid any irregularities in the elution process. The column was eluted using medium pressure with solvents i.e., NH (100%) + 0.2% formic acid to NH:EA (2:8) + 0.2% formic acid as shown in figure 2.4. A total of 45 sub fractions were procured each having an approximate volume of 30 ml. The sub fractions were then monitored using TLC technique and based on similarity and singularity of detected spots on the TLC plates, sub fraction 14-17 were combined. The combined weight of these sub fractions was 64 mg, and a second purification column was planned. The sample was now dissolved in EA and adsorbed on to silica gel (70-230 µm; 128 mg) and after drying the adsorbed sample as mentioned previously, it was dry packed in a glass column containing normal phase silica gel 60 (230-400 µm). After addition of a 2 cm protective layer of sand, the column was eluted with NH (100%) + 0.2% formic acid to NH:EA (1:9) + 0.2% formic acid. This resulted in 38 further sub fractions, each approximately of 10 ml volume. TLC profiling of each resultant fraction showed the presence of a pure compound in sub fraction 14-19, the contents were mixed and dried which resulted in appearance of 21 mg white amorphous powder material. The isolation sketch is presented in 2.4.

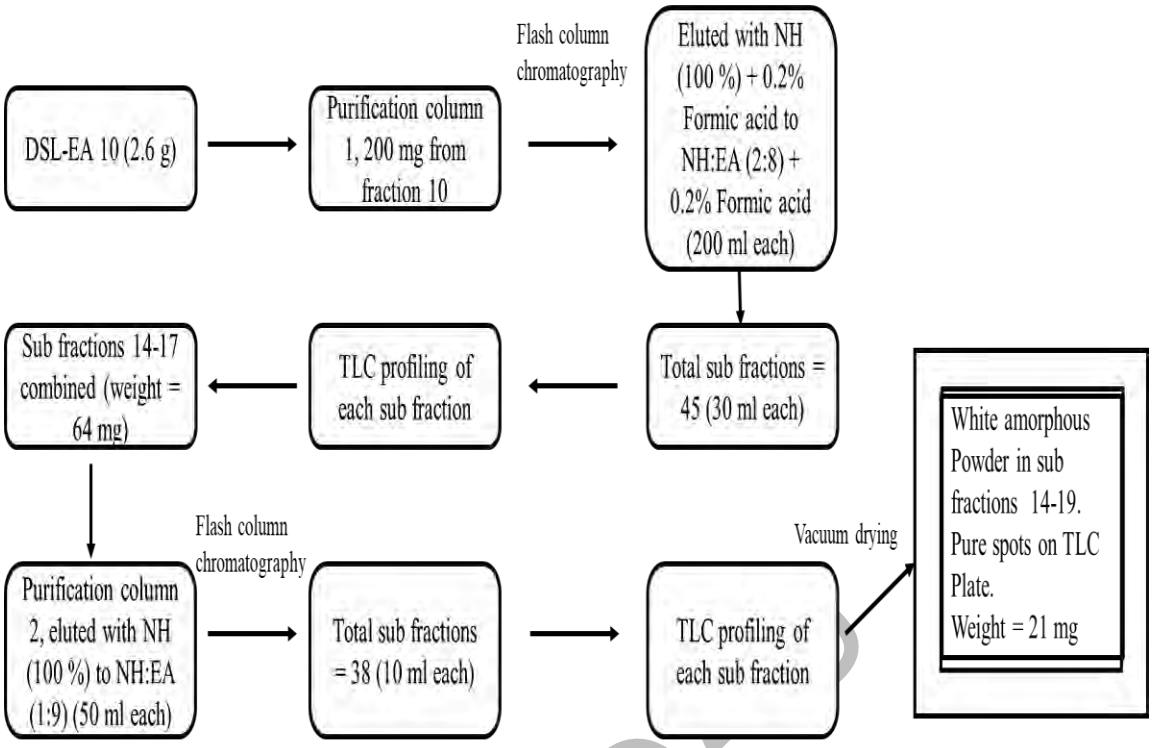


**Figure 2.4** Isolation scheme of compound a

***Note:*** *TLC; Thin layer chromatography.*

2.3.4.3.2. Isolation of Compound b

DSL-EA 25-26 from the fractionation step as described in section 2.3.4.2 were mixed based on their identical TLC profiles. The combined weight was 5.8 g. At first, 50 mg was taken from the combined fraction, and it was dissolved in EA and latter adsorbed on silica gel 60 (70-230 µm; 300 mg). The adsorbed sample was then dried at 45°C in a vacuum oven. A glass column packed with normal phase silica gel 60 (230-400 µm) was used for the isolation process. The sample loaded silica was dry packed into the column and a 2 cm protective sand layer was added to the top. The column was gradually eluted with solvents of varying polarity ranging from NH (100%) + 0.2% formic acid to NH:EA (2:8) + 0.2% formic acid. The first purification column yielded 46 sub fractions of 30 ml each. Following TLC profiling of all the sub fractions, contents of sub fractions 15-18 were mixed and dried which resulted in a dry weight of 123 mg. A second purification column was performed using normal phase silica of same specifications for sample adsorption and packing of the glass column. The dried sample (123 mg) was dissolved in EA and loaded on silica (250 mg). After drying the sample loaded silica, the column was eluted with NH (100%) + 0.2% formic acid to NH:EA (1:9) + 0.2% formic acid. The second purification column resulted in 84 sub

fractions (10 ml each). The presence of pure compound was detected in sub fractions 67-73 using TLC profiling of all the sub fractions. The contents were mixed and dried, a single defined spot at the TLC plate further confirmed the purity. The dried weight of compound was 24 mg. The details of the isolation sketch are given in figure 2.5.

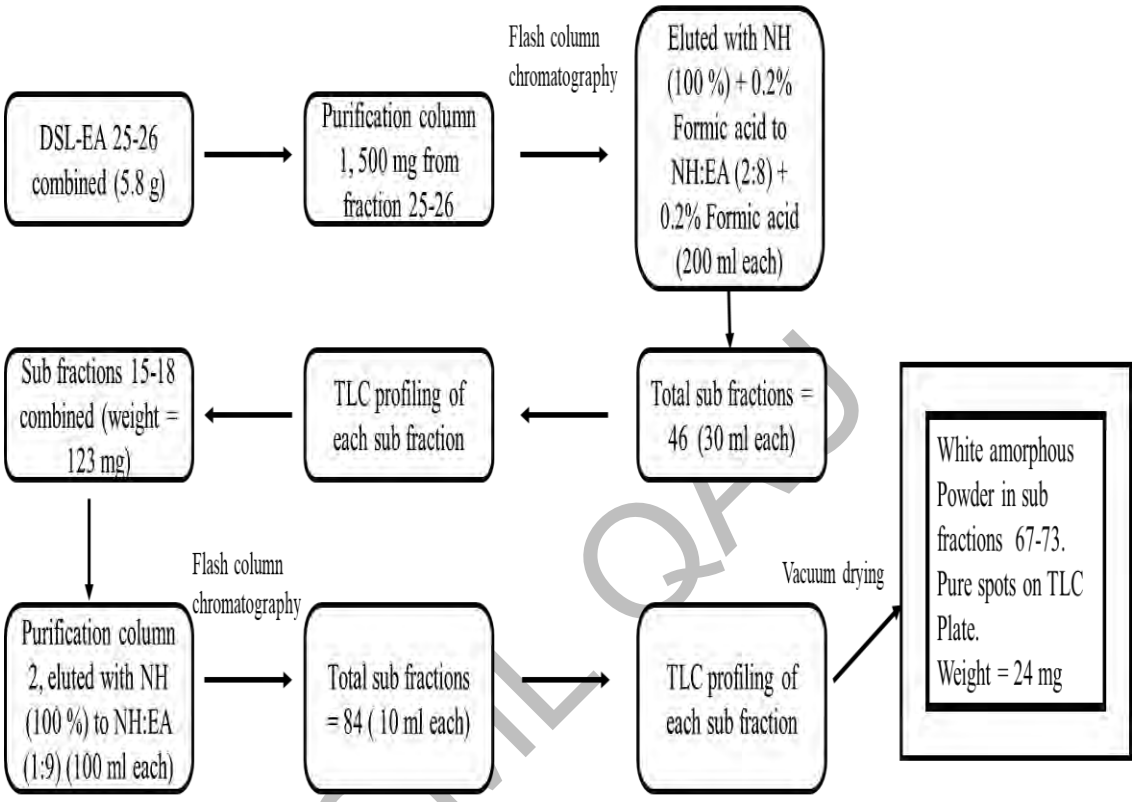


**Figure 2.5** Isolation scheme of compound b

2.3.4.3.3. Isolation of Compound c

Fraction DSL-EA-31 from the fractionation step (2.3.4.2) was then selected from the list of fractions with potentially pure compounds based on TLC profiling. The weight of DSL-EA-31 was 7.1 g. For isolation of compounds from this fraction, 600 mg was weighed, dissolved in EA and adsorbed on silica gel (1.2 g). The specifications of silica gel for sample adsorption and column packing were the same as discussed in previous sections. The dried sample loaded silica was then added to the pre-packed glass column and a 2 cm protective layer of sand was introduced at the top. The column was eluted with a series of solvent combinations of increasing polarity. The TLC profile of DSL-EA-31 showed that the compounds are of polar nature so the elution was initiated with NH:EA (1:1) + 0.2% formic acid and the highest polarity solvent combination used in this purification column was EA:M (1:1) + 0.2% formic acid. The details are given in figure 2.6. The purification column yielded 30 sub fractions of 50 ml each. The

appearance of white precipitates was observed in sub fractions 7-10. The precipitates were allowed to settle, and the supernatants were collected in a separate flask. The white amorphous powder from sub fractions 7-10 were combined and washed multiple times with NH:EA(7:3). TLC was performed after drying the powder and the purity level was determined. The total weight calculated was 27 mg.

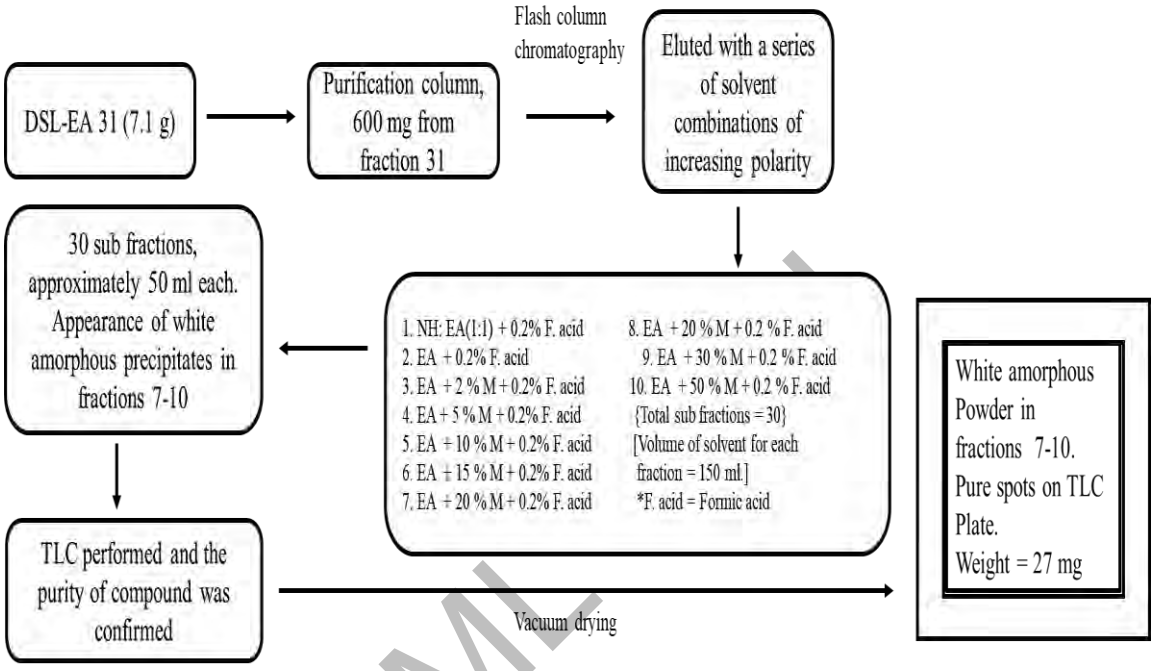


**Figure 2.6** Isolation scheme of compound c. F.acid; Formic acid.

### 2.3.5. Characterization of isolated compounds via NMR

#### *2.3.5.1. Nuclear magnetic resonance (NMR) spectroscopy*

Nuclear Magnetic Resonance (NMR) spectra were obtained mainly with the help of a Bruker Avance II 600 and Bruker Avance III 400 NMR spectrophotometer. The analysis was performed at 25 °C and the spectra were analysed using ACD/NMR Processor Academic Edition software version12.01. The abbreviations used during NMR assignments are given in table 2.5.

**Table 2.5** Abbreviations used in assignment of NMR peaks.

| Abbreviation | Description |
|---|---|
| S | Singlet |
| D | Doublet |
| T | Triplet |
| Q | Quartet |
| M | Multiplet |
| Br | Broad |
| Dd | Double doublet |
| ArH | Aromatic hydrogen |
| ArC | Aromatic quaternary carbon |
| ArCH | Aromatic carbon having hydrogen attached |

Selection of compounds for further biological studies was determined by certain factors i.e., chemical class of isolated compounds, their quantity and purity as well as lack of sufficient scientific data in the literature related to their pharmacological evaluation. Keeping in view the above-mentioned selection parameters, two compounds were taken forward in the current study and were referred to as C1 and C2 in the following sections.

### 2.3.6. Biological evaluation of isolated compounds

#### *2.3.6.1. Animals and treatment groups*

The isolated compounds C1 and C2 were tested in chronic models of leukemia and inflammation to assess their anticancer and anti-inflammatory activities. Male Balb/c mice (25-30 g) were housed in the animal house at Department of Pharmacy, Quaid-i-Azam University as per the guidelines of the ethical committee of Quaid-i-Azam University. All animals were provided food and water ad libitum and were acclimatized to the laboratory environment before the start of experiments. Mice were divided into seven groups with 6 mice per group for both benzenes induced leukemia and $CCL_4$ induced inflammation models. Moreover, two doses of the compounds labelled as low dose and high dose were tested in the subject animals. Animal groups for chronic studies are given in table 2.6.

**Table 2.6** Animal groups for chronic diseases models.

| Groups/Model | $CCL_4$ induced inflammation | Benzene induced leukemia |
|---|---|---|
| **Group I** | Vehicle control | Vehicle control |
| **Group II** | Disease control | Disease control |
| **Group III** | Positive control | Positive control |
| **Group IV** | C1 LD 30 mg/kg | C1 LD 30 mg/kg |
| **Group V** | C1 HD 60 mg/kg | C1 HD 60 mg/kg |
| **Group VI** | C2 LD 10 mg/kg | C2 LD 10 mg/kg |
| **Group VII** | C2 HD 20 mg/kg | C2 HD 20 mg/kg |

#### *2.3.6.2. Benzene induced leukemia*

C1 and C2 were tested in benzene induced leukemia model as per previously described protocol (Nasir *et al.,* 2020). Mice were divided into 7 groups as given in table 2.6. Leukemia was induced by intra-peritoneal injection of 20 µl of benzene (2:10 in water for injection). A total of 7 doses were administered on alternative days from Day 1 to Day 14. From Days 15-28 of the study duration, total 7 doses of 100 µl of vehicle, compounds or positive control were orally administered on alternate days. Cyclophosphamide 10 mg/kg and distilled water with 0.05% tween 20 were used as positive and vehicle controls, respectively. The disease control group did not receive any treatment. Animals were periodically weighed and observed for any signs of disease induction and toxicity during the study. At the end of the study, mice were euthanized using cervical dislocation and blood was drawn from abdominal aorta for analysis of serum, biochemical and other parameters depicting leukemia and inflammation in mice as given in section 2.2.8. Furthermore, liver samples were collected and processed for western blot analysis of proteins. The antileukemic activity was calculated from the difference in values of parameters between sample and disease control groups (Nasir *et al.,* 2020).

#### *2.3.6.3. $CCL_4$ induced inflammation*

The anti-inflammatory effect of C1 and C2 compounds was determined by $CCL_4$ induced inflammatory model in mice. Male Balb/c mice (25-30 g) were randomly divided into 7 groups with 6 animals per group as given in table 2.5. Two doses, low and high, of both compounds were tested. Liver inflammation in groups II-VIII was induced by intraperitoneal administration of 90 µl of $CCL_4$ (in olive oil). Silymarin (positive control; 100 mg/kg), vehicle (distilled water with 0.05% tween 20) and extracts were orally administered on alternate days for 14 days. Similar procedure was followed as given in section 2.3.3(b).

### *2.3.6.4. Evaluation of animal samples from chronic disease models*

Blood samples from mice of both chronic disease models were collected from abdominal aorta of mice in anticoagulant free tubes and centrifuged at 6000 rpm for 15 minutes at 4ºC to separate the serum. The serum was stored at -20ºC and later analyzed for the following parameters. Liver and kidney tissues were harvested from mice of benzene induced leukemia and $CCL_4$ induced inflammation model. A portion of the harvested organs was immediately flushed frozen while the other portion was fixed in 10% formaldehyde solution for histological evaluation.

#### 2.3.6.4.1. Serum/hematological analysis

Serum analysis for red blood cells, white blood cells, platelets, erythrocyte sedimentation rate and hemoglobin were done as given in annexure 1 (Nasir *et al.,* 2020).

#### 2.3.6.4.2. Biochemical analysis

ALT, AST, ALP, total serum protein, urea and creatinine were estimated as described by (Nasir *et al.,* 2020) Annexure 1.

#### 2.3.6.4.3. Estimation of endogenous antioxidant enzymes

GSH, GST, SOD, CAT and POD as described by (Nasir *et al.,* 2020). Annexure 1.

#### 2.3.6.4.4. Expression of biomarkers of oxidative stress (TBARs, MPO and NO)

TBARs and NO levels were calculated as described by (Nasir *et al.,* 2020). Annexure 1. TBARs were estimated in liver homogenates samples of mice from benzene induced leukemia and $CCL_4$ induced inflammation models. The myeloperoxidase (MPO) activity was estimated using hexadecyltrimethylammonium bromide (HTAB) method (Palić *et al.,* 2007).

#### 2.3.6.4.5. Histopathology

Hematoxylin and eosin stain slides of organs from animals of benzene induced leukemia and $CCL_4$ induced inflammation models were prepared and evaluated according to procedure given in annexure 1 (Nasir *et al.,* 2020). Masson's trichome staining was also performed to assess fibrosis in liver tissue obtained from animals of $CCL_4$ induced inflammation model (Khan *et al.,* 2020). All slides were examined under Nikon microscope (Eclipse 80i Japan).

2.3.6.4.6. Immunohistochemistry

The immunohistochemistry staining was performed to examine the effect of the extracts on nuclear factor erythroid 2 (Nrf2) and inducible nitric oxide synthase (iNOS) following liver injury caused by $CCL_4$ toxicity. Paraffin embedded staining protocol was followed. The tissue was washed with xylene and ethanol followed by treatment with proteinase-k, normal goat serum (NGS), primary and secondary antibodies (Nrf2 and iNOS) (Ruiz *et al.,* 2013).

2.3.6.4.7. Western blotting on samples from chronic disease models

Western blotting was performed to assess the expression of different proteins in tissue (liver) homogenates from animals of benzene induced leukemia and $CCL_4$ induced inflammation models. Flashed freeze organ samples were processed for separation and quantification of proteins (Shah *et al.,* 2015; Shah *et al.,* 2017).

a. Preparation of solutions

Running buffer was prepared by dissolving 36.3 g of Trizma base in 200 ml of distilled water at pH 8.8. The monomer solution was prepared by dissolving 60 g acrylamide and 1.6 g bis-acrylamide in 200 ml of distilled water. SDS solution was constituted by mixing 10 g of SDS in 100 ml of distilled water. For stacking buffer, 3 g Trizma base was dissolved in 50 ml distilled water (pH 6.8). Ammonium per sulfate 10% solution was freshly prepared in water. Glycine 144 g, SDS 10 g and Trizma base 30 g were dissolved in 1000 ml of distilled water to make 10X tank buffer (pH 8.3). It was then diluted to 1X tank buffer by adding 900 ml distilled water in 100 ml 10X tank buffer. For 5X transfer buffer, 29 g trizma base, 14.4 g glycine and 1.85 g SDS were dissolved in 1000 ml distilled water. Later, 200 ml of 5X transfer buffer was mixed with 150 ml methanol and 650 ml distilled water to make 1X transfer buffer. Sodium chloride 320 g, potassium chloride 8 g and Trizma base 120 g were mixed in 2000 ml of distilled water (pH 7.4) to make 20X Tri buffer saline tween (TBST). 1X TBST was prepared by adding 96 ml 20X TBST, 4 ml tween and 1900 ml distilled water. Distilled water 3.6 ml, running buffer 3.75 ml, monomer solution 7.5 ml, SDS 150 µl, APS 150 µl and Temed 9.9 µl were mixed to make 15% running gel. Distilled water 4 ml, stacking buffer 1.6 ml, monomer solution 880 µl, APS 60 µl, SDS 133.6 µl and Temed 13.6 µl were mixed to prepare stacking gel.

b. Preparation of protein lysates and quantification

The organs including liver and kidney from $CCL_4$ model and liver and spleen from leukemia model were mixed with 400 µl of tissue protein extraction reagent and homogenized using Omni tissue homogenizer at 4ºC to avoid protein denaturation. Rod of the homogenizer was thoroughly rinsed with phosphate buffer saline and distilled water before and after each sample. Afterwards, samples were centrifuged at 14000 rpm for 25 minutes at 4ºC. The supernatant was collected in clean eppendorf tubes placed on ice and the resultant protein was quantified using protein assay kit. Briefly, a solution of protein assay dye reagent in distilled water (1:4) was prepared. One ml of this solution was mixed with 1 µl of protein lysate sample in cuvettes. Blank contained 1 µl of water instead of protein lysate. Subsequently, optical density was measured by UV spectrophotometer at a wavelength of 595.8 nm. The result was recorded as µg/ml of protein in each sample.

c. Gel electrophoresis and blot detection

For gel electrophoresis, 30 µg of protein pipetted based on the ratio of its respective optical density was vortexed with equal volume of 2X laemmli buffer, briefly centrifuged and incubated at 96°C for 10 minutes. Samples were then placed on ice and briefly vortexed and centrifuged before use. Denatured samples were loaded on gel fixed in BioRad electrophoresis tank that was filled with running buffer. The apparatus was fixed, and protein were initially run down the stacking gel at 50 mA for 30 minutes and then separated on 12-15% running gel at 120 volts for 90-100 minutes. Afterwards, polyvinylidene fluoride (PVDF) membrane was activated in methanol and proteins on the gel were transferred to using transblot apparatus set at 1.6 A, 10 Volts and 5 Watt for 45 minutes. Next, non-specific protein sites on blots were blocked by 5% skimmed milk solution prepared in TBST buffer, washed, and probed with appropriate primary monoclonal antibody overnight at 4ºC on orbital shaker. Later, blots were washed for 5 min, probed with specific anti-HRP secondary antibody (mouse IgG) for 3-4 hours at room temperature. Blots were again washed with 1X TBST buffer and transferred on X-ray film and were developed using a chemiluminescence solution in the dark room. Protein bands were detected by chemiluminescence autoradiography using ChemDoc™ MP imaging system (Shah *et al.,* 2017; Shah *et al.,* 2015).

## 2.4. Statistical Analysis

Statistical analysis of results from all assays was carried out using (SPPS version 10.0, Chicago, IL) and GraphPad Prism 5. The data is presented as means of respective values with SDs of individual replicates. Regression analysis was done to calculate $IC_{50}$ values. T-test and ANOVA with suitable post hoc test were used to calculate *p* values where required.

# CHAPTER 3

# RESULTS

# 3. RESULTS

The comparative pharmacological assessment of *D. stramonium* and *D. innoxia* was carried out initially by selecting the root, fruit, stem and leaf parts of both plants. Phytoconstituents were extracted successively with solvents of increasing polarity. Different polarity indices of each solvent led to extraction of a distinct set of phytoconstituents. Therefore, when these extracts were subsequently assessed *via* a diverse set of phytochemical and *in vitro* biological assays, it helped in selection of most potent extracts for further biological evaluation using *in vivo* models as well as isolation of potentially active moieties responsible for the observed effects. The results of the aforementioned study scheme are stated in this chapter.

## 3.1. Extract Recovery for Extraction Optimization

A unique trend in the percent extract yield was observed in case of selected plant parts of *D. stramonium* and *D. innoxia.* It was due to the diverse set of phytoconstituents procured by using solvents of different polarities. The strategy of successive extraction with solvents of increasing polarity has several advantages including partitioning of the constituents based on their chemical nature and affinity for the solvents as well as exhaustive extraction of maximum moieties from the powdered plant material. Highest extraction recovery in *D. stramonium* extracts was observed when Dw was used as the solvent (DSL-Dw: 12.10%, DSF-Dw: 11.51%), followed by M (DSL-M: 6.39%), EA (DSL-EA: 2.34%) while NH resulted in minimum extraction yield (DSS-NH: 0.34%). *D. innoxia* followed the same trend with maximum extract recovery observed in Dw extracts (DIF-Dw: 11.50%, DIR-Dw: 9.75%), next in line in terms of most effective solvents were MeOH (DIL-M: 8.85%) and EA (DIL-EA: 2.59%) while NH proved to be the least effective (DIS-NH: 0.31%) The results are further elaborated in figure 1. Results in this section as well as preliminary screening of extracts will be presented in a comparative manner in order of increasingly polar extracts of *D. stramonium* root, fruit, stem and leaf parts followed by *D. innoxia* extracts.

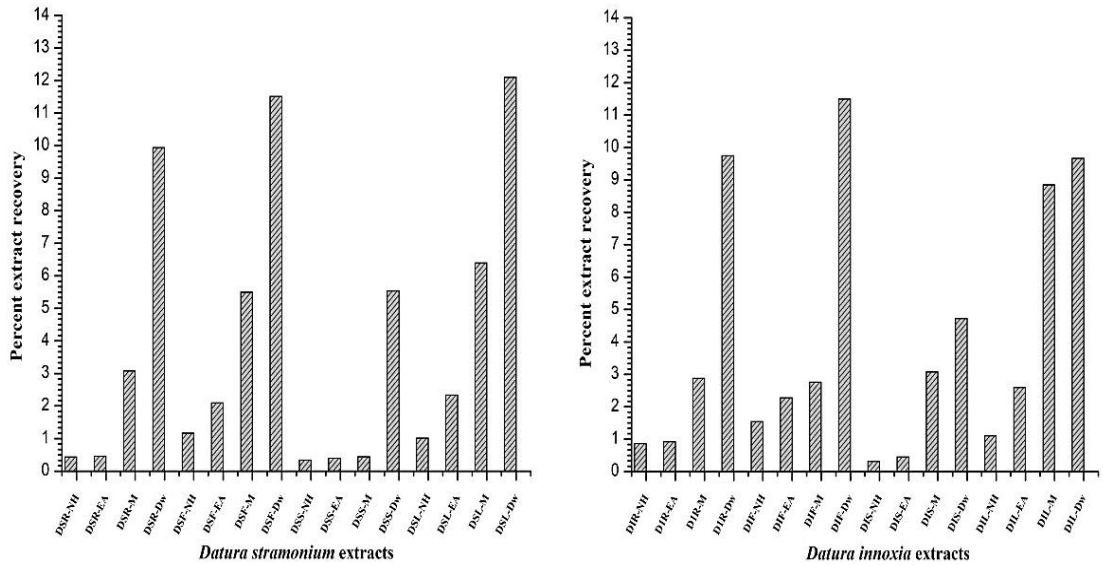


**Figure 3.1** Extract recovery of *D. stramonium* (DS) and *D. innoxia* (DI) extracts.
***Note:*** *Solvents are used in increasing order of polarity i.e., NH: n-hexane, EA: Ethyl acetate, M: Methanol and Dw: Distilled water. D. stramonium extracts: DSR: D. stramonium root, DSF: D. stramonium fruit, DSS: D. stramonium stem and DSL: D. stramonium leaf. D. innoxia extracts: DIR: D. innoxia root, DIF: D. innoxia fruit, DIS: D. innoxia stem and DIL: D. innoxia leaf.*

## 3.2. Preliminary Screening of Extracts of *D. stramonium* and *D. innoxia*

### 3.2.1. Phytochemical analysis

#### *3.2.1.1. Total phenolic content*

The total phenolic content (TPC) of *D. stramonium* and *D. innoxia* crude extracts expressed as µg gallic acid equivalent (GAE) per mg extract are presented in figure 3.2. Highest TPC among *D. stramonium* extracts in terms of µg GAE/mg extract was recorded in DSF-EA (41.78±2.13) while lowest content was estimated in DSS-Dw (7.49±0.47). In case of *D. innoxia* extracts, maximum TPC was recorded in DIL-EA (27.69±1.12) while DIS-Dw showed lowest TPC content i.e., 7.39±0.24 µg GAE/mg extract. Overall, the EA extracts of both plant species showed better results while Dw extracts showed minimum TPC. The results are further elaborated in figure 3.2.

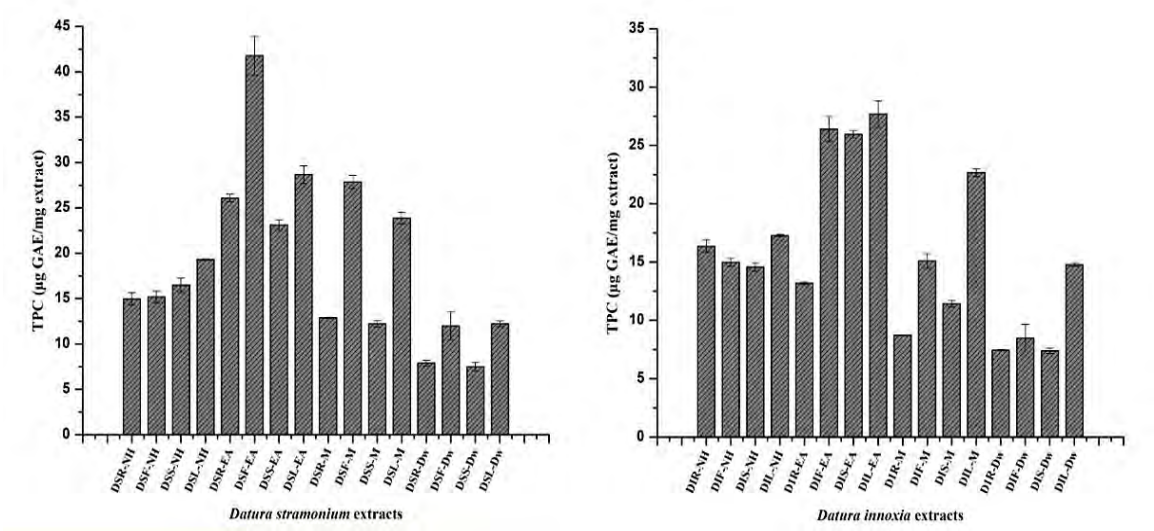


**Figure 3.2** Total phenolic content of *D. stramonium* and *D. innoxia* extracts.

### *3.2.1.2. Total flavonoid content*

The total flavonoid content (TFC) of extracts expressed as µg quercetin equivalent per mg extract (µg QE/mg extract) are presented in figure 3.3. Maximum TFC (µg QE/mg extract) in *D. stramonium* extracts was estimated in DSL-EA (16.16±0.06) while DSR-NH showed minimum content (1.09±0.06). *D. innoxia* extracts also showed somewhat identical trends and highest and lowest values were observed in DIL-EA (20.17±0.17) and DIS-NH extracts (1.31±0.3) µg QE/mg extract respectively. Significant TFC were recorded in EA extracts of both plant species, while NH and Dw extracts showed minimum flavonoid content. The details are listed in figure 3.3.

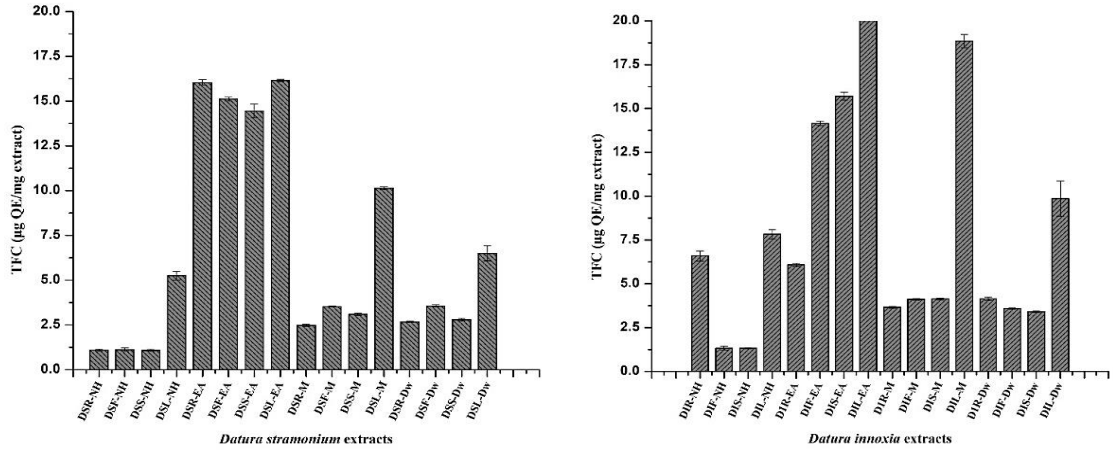


**Figure 3.3** Total flavonoid content (TFC) of *D. stramonium* and *D. innoxia* extracts.

### *3.2.1.3. RP-HPLC analysis*

The quantitative analysis of selected plant phenolics was done using reverse phase HPLC-DAD based profiling of EA and M extracts of both Datura species. The chromatographic fingerprinting of detected polyphenols was performed by comparing the UV spectra and retention time of the test samples with reference compounds used in the HPLC analysis. A significant amount of gallic acid, rutin, catechin, apigenin, myricetin and kaempferol were detected and quantified in some of the extracts used in the study. Amongst *D. stramonium* extracts, maximum number of polyphenols were quantified in DSF-EA (gallic acid, rutin, apigenin, myricetin and kaempferol), followed by DSL-EA and DSR-EA (gallic acid, rutin, catechin and apigenin). *D. innoxia* extracts also showed presence of numerous polyphenols i.e., rutin, catechin and kaempferol. The chromatograms of standards as well as polyphenols detected in EA and M extracts of *D. stramonium* and *D. innoxia* are presented in figure 3.4. The calibration equation parameters of standards are presented in table 3.1(a) while the quantified values are presented in table 3.1(b).

**Table 3.1(a)** Retention time and calibration curve equations of standard polyphenols.

| **Standard** | **Retention time** (min) | **Calibration curve equation** | **Correlation coefficient ($r^2$)** |
|---|---|---|---|
| Rutin | 12.6 | Y = 8.3367x + 22.217 | 0.9966 |
| Gallic Acid | 3.6 | Y = 24.857x - 45.174 | 0.9979 |
| Catechin | 6.8 | Y = 7.9854x - 17.565 | 0.9995 |
| Caffeic Acid | 9.0 | Y = 26.097x + 95.435 | 0.9924 |
| Apigenin | 21.3 | Y = 18.111x + 25.565 | 0.997 |
| Kaempferol | 20.4 | Y = 9.9944x + 15.261 | 0.9998 |
| Myricetin | 14.9 | Y = 5.2278x - 6.3043 | 0.9988 |
| Quercetin | 17.7 | Y = 12.21x - 20.348 | 0.9978 |

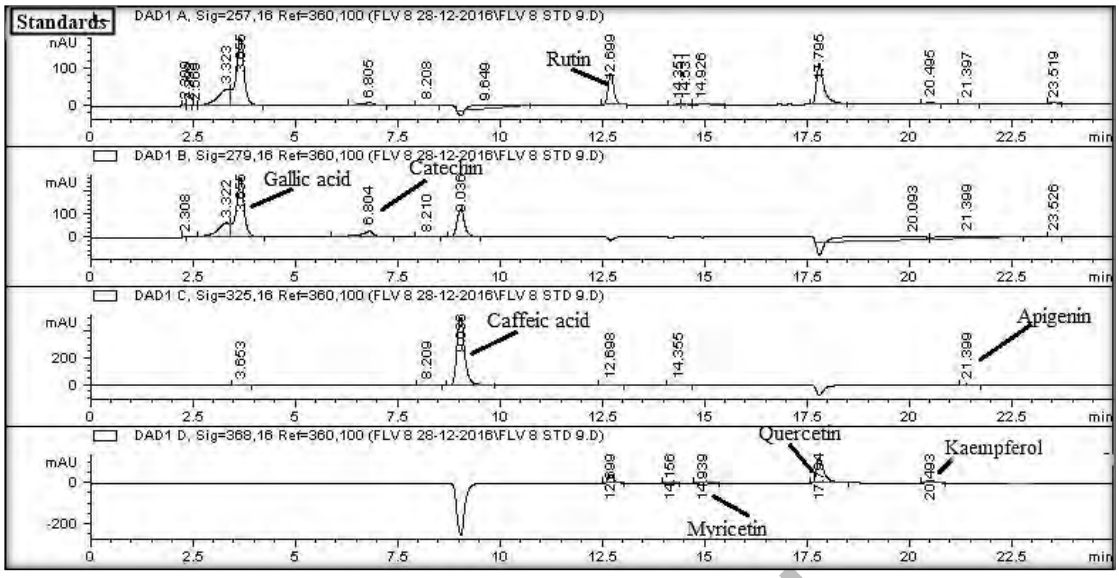


**Figure 3.4(a)** Chromatograms of standard polyphenols.


**Figure 3.4(b)** Chromatograms of polyphenols detected in ethyl acetate extracts of root (DSR-EA), fruit (DSF-EA), stem (DSS-EA) and leaf part (DSL-EA) of *D. stramonium*.

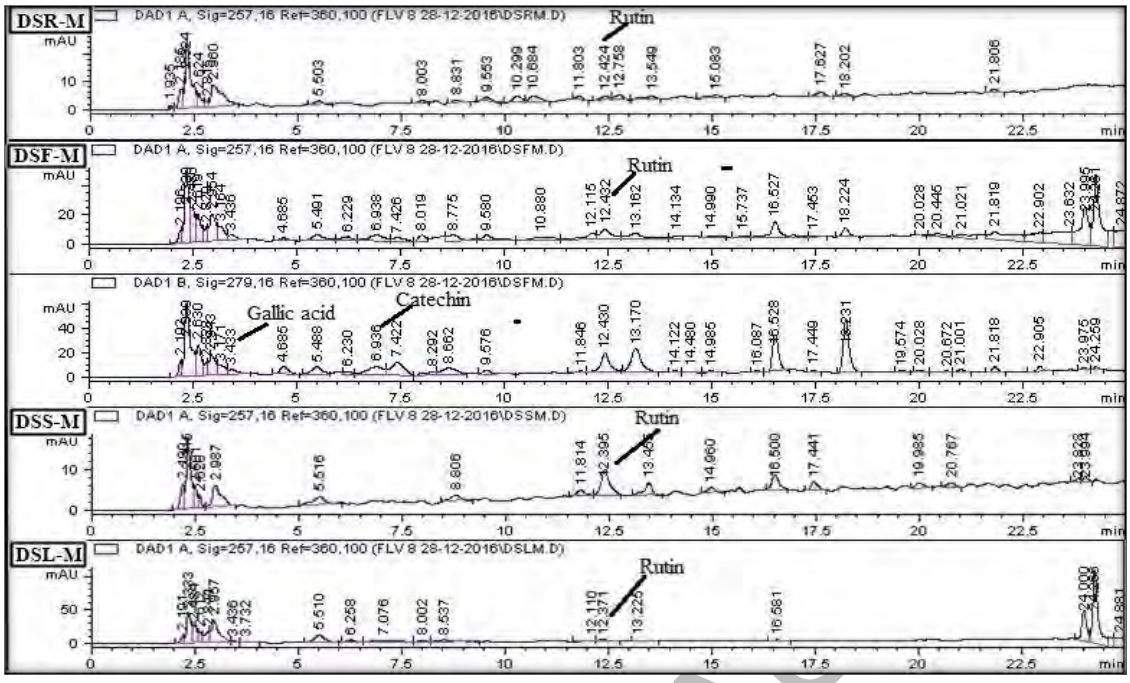


**Figure 3.4(c)** Chromatograms of polyphenols detected in methanol extracts of root (DSR-M), fruit (DSF-M), stem (DSS-M) and leaf part (DSL-M) of *D. stramonium*.

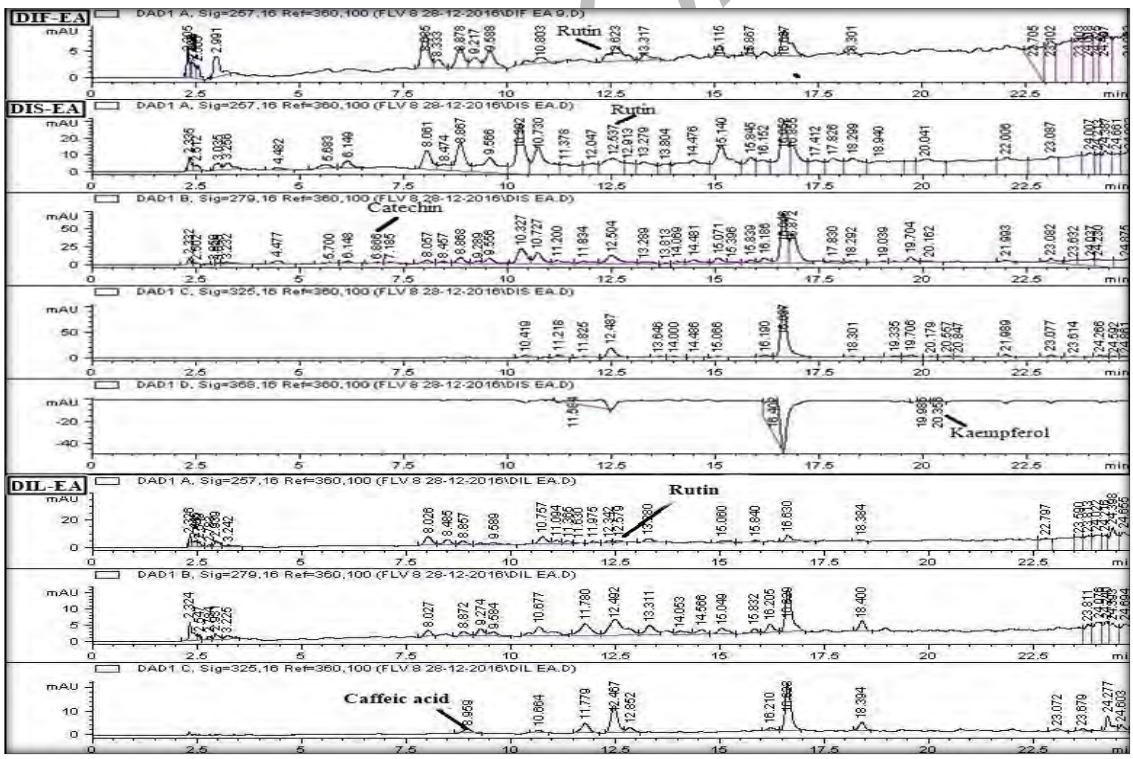


**Figure 3.4(d)** Chromatograms of polyphenols detected in ethyl acetate extracts of fruit (DIF-EA), stem (DIS-EA) and leaf part (DIL-EA) of *D. innoxia*.

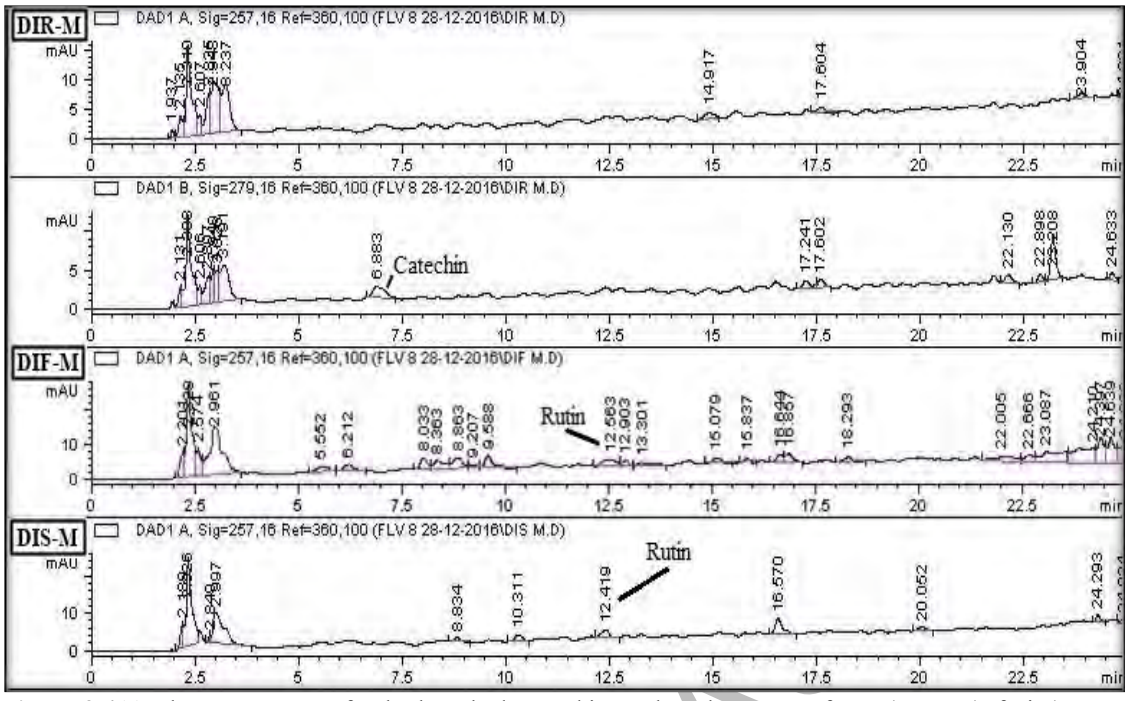


**Figure 3.4(e)** Chromatograms of polyphenols detected in methanol extracts of root (DIR-M), fruit (DIF-M) and stem part (DIS-M) of *D. innoxia.*

**Table 3.1(b)** Chemical profiling of EA and M extracts of *D. stramonium* and *D. innoxia* using HPLC-DAD.

| Extracts | Polyphenols (µg/mg extract) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Phenolic acid | Flavonol glycoside | Hydroxy cinnamate | Flavan-3-ol | Flavone aglycone | Flavonol flavonoid | | |
| | GA | Rutin | CA | Catec | Api | Myr | Quer | Kaemp |
| | ***D. stramonium* extracts** | | | | | | | |
| **DSR-EA** | 0.29±0.02** | 1.73±0.11*** | -- | 1.50±0.97*** | 0.23±0.05** | -- | -- | -- |
| **DSF-EA** | 0.29±0.03** | 3.16±0.21*** | -- | -- | 2.01±0.18*** | 0.96±0.08** | -- | 1.26±0.15** |
| **DSS-EA** | 0.530±0.04** | 2.56±0.21*** | -- | 1.19±0.12** | -- | -- | -- | -- |
| **DSL-EA** | 0.35±0.07** | 0.89±0.03*** | -- | 0.24±0.02** | 0.29±0.09** | -- | -- | -- |
| **DSR-M** | -- | 0.059±0.01* | -- | -- | -- | -- | -- | |
| **DSF-M** | 0.44±0.03** | 1.51±0.13*** | -- | 3.10±0.23*** | -- | -- | -- | -- |
| **DSS-M** | -- | 0.87±0.08** | -- | -- | -- | -- | -- | -- |
| **DSL-M** | -- | 0.407±0.07** | -- | -- | -- | -- | -- | -- |
| | ***D. innoxia* extracts** | | | | | | | |
| **DIR-EA** | -- | -- | -- | -- | -- | -- | -- | -- |
| **DIF-EA** | -- | 0.64±0.07** | -- | -- | -- | -- | -- | -- |
| **DIS-EA** | -- | 4.27±0.32*** | -- | 0.53±0.09** | -- | -- | -- | 0.06±0.009* |
| **DIL-EA** | -- | 0.036±0.004* | -- | 0.27±0.03** | -- | -- | -- | -- |
| **DIR-M** | -- | -- | -- | 0.65±0.04** | -- | -- | -- | -- |
| **DIF-M** | -- | 0.25±0.04** | -- | -- | -- | -- | -- | -- |
| **DIS-M** | -- | 0.12±0.02* | -- | -- | -- | -- | -- | -- |
| **DIL-M** | -- | -- | -- | -- | -- | -- | -- | -- |

***Note:*** *GA: Gallic acid, CA: Caffeic acid, Catec: Catechin, Api: Apigenin, Myr: Myricetin, Quer: Quercetin, Kaemp: Kaempferol.*

**** Significant, ** Fair, * Slight concentration, -- Not detected.*

### 3.2.2. *In vitro* biological evaluation

#### *3.2.2.1. Antioxidant potential*

3.2.2.1.1. Total antioxidant capacity

The total antioxidant capacity (TAC) of extracts was appraised using phosphomolybdenum based method and results were expressed as µg ascorbic acid equivalent (AAE) per mg extract. Maximum TAC (AAE/mg extract) amongst *D. stramonium* extracts was exhibited by DSL-EA (160.92±3.00 while the lowest was recorded in DSR-Dw 8.34±0.95). *D. innoxia* extracts also showed significant TAC. DIL-EA exhibited highest TAC i.e., 140.44±5.6 while DIS-Dw showed the lowest activity i.e., 15.60±1.98, both expressed in terms of µg AAE/mg extract. Overall, NH, EA and M extracts of both plant species showed appreciable TAC as compared to Dw extracts. The results are represented in figure 3.5.

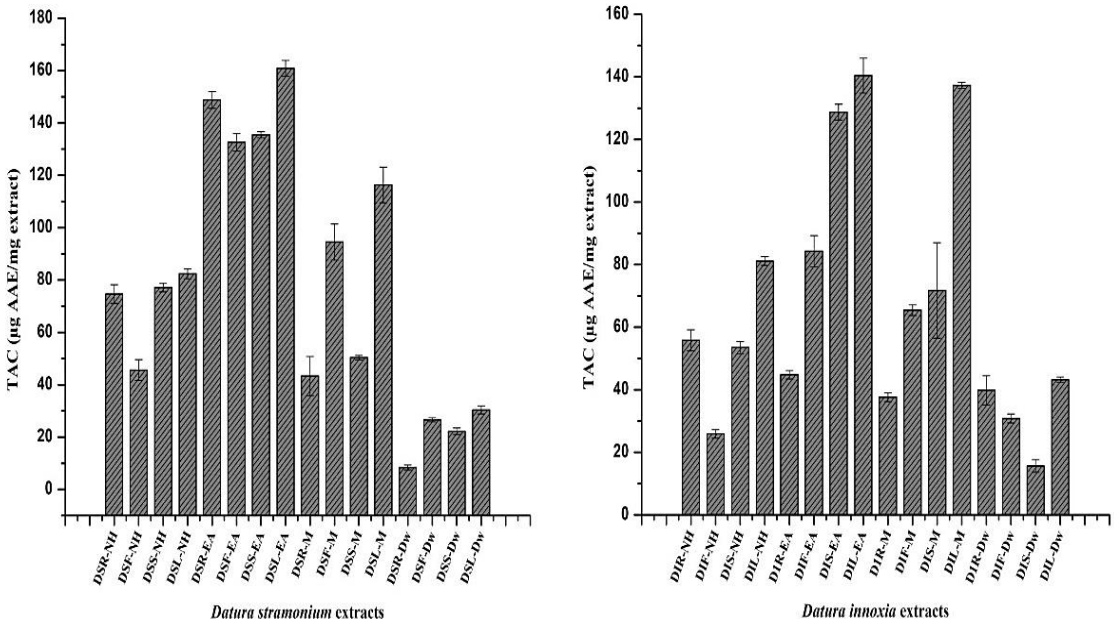


**Figure 3.5.** Total antioxidant capacity of *D. stramonium* and *D. innoxia* extracts.

3.2.2.1.2. Total reducing power

Total reducing power (TRP) was manifested by evaluating the ferric ion (Fe3+) conversion to ferrous ion (Fe2+) in the presence of known concentration of extracts. DSL-EA exhibited slightly better antioxidant assertiveness in terms of reducing power with highest and lowest values estimated in DSR-EA and DSR-Dw (i.e., 64.70±0.81 and 16.87±0.87 µg AAE/mg extract respectively). Maximum reducing power amongst

*D. innoxia* extracts was shown by DIR-EA i.e., 49.86±4.22 and lowest was recorded in DIF-Dw i.e., 17.25±0.30µg AAE/mg extract. The results of this experiment also affirmed that Dw extracts were devoid of significant antioxidant moieties and this is in accordance with the findings of phytochemical assays. The details are presented in figure 3.6

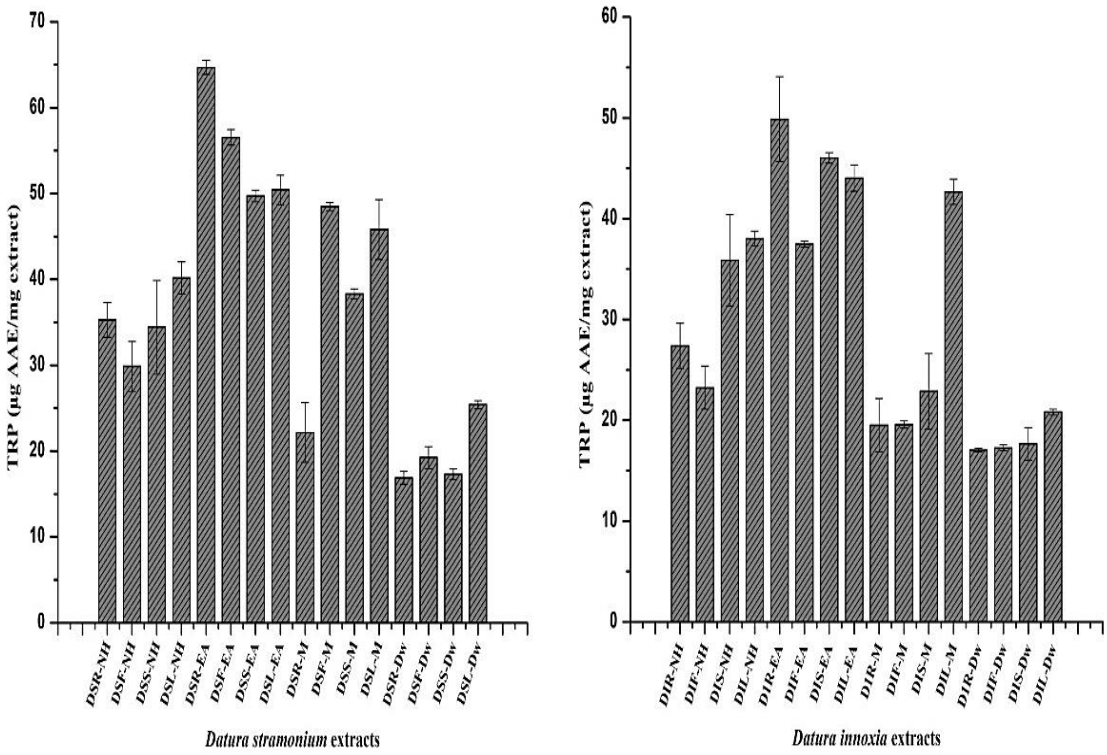


**Figure 3.6** Total reducing power of *D. stramonium* and *D. innoxia* extracts.

3.2.2.1.3. Free radical scavenging assay

The percent free radical scavenging activity (% FRSA) was measured by discoloration of DPPH solution. Extracts of both species did not show any significant action. DSL-EA showed maximum scavenging activity (43.72±3.37%) while in *D. innoxia* extracts, DIL-M exhibited highest FRSA (36.67±0.86%). Results are elaborated in figure 3.7.

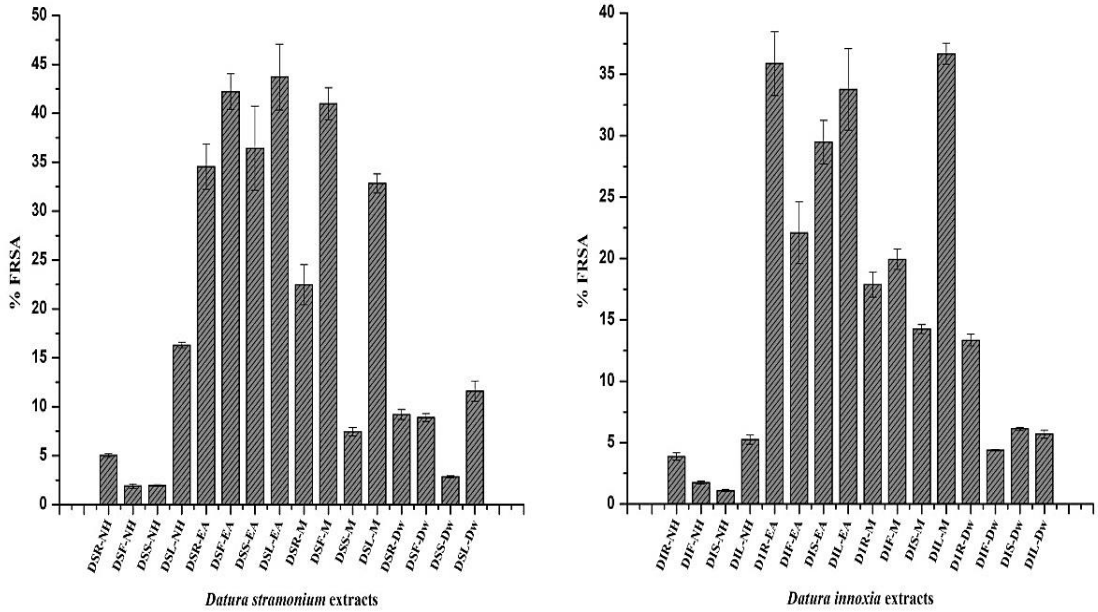


**Figure 3.7** Percent free radical scavenging potential of *D. stramonium* and *D. innoxia* extracts.

### *3.2.2.2. Brine shrimps cytotoxicity assay*

Cytotoxicity profile was evaluated with the aid of brine shrimp lethality assay. Numerous extracts of both *Datura* species showed remarkable activity. The extracts have shown concentration dependent cytotoxic action with the activity tapering down at lower concentrations. It was observed that non-polar and moderately polar extracts have shown greater cytotoxic potential while Dw extracts did not possess any notable activity against brine shrimps. In case of *D. stramonium* extracts, DSL-M exhibited the strongest activity at 200 µg/ml concentration (100±5.57% mortality) with an $LC_{50}$ value of 7.86±0.13 µg/ml. *D. innoxia* extracts revealed an even stronger action with DIR-EA extract resulting in 100±0.0% lethality at 200 µg/ml and an $LC_{50}$ value of 4.70±0.12 µg/ml. Doxorubicin was used as a standard and its $LC_{50}$ value was recorded to be 5.43 µg/ml. Results are shown in Table 3.2.

### *3.2.2.3. Protein kinase inhibition assay*

The capability of extracts to inhibit protein kinase and subsequently the aerial hyphae formation by extracts was gauged based on appearance of bald zones on cultured plates. The trend in the results somewhat continued from the previous assays with nonpolar to moderately polar extracts exhibiting better results and Dw extracts showing little to no activity. Amongst all extracts EA leaf extracts of both species showed significant protein kinase inhibitory potential, DSL-EA exhibited a bald phenotype zone of 12.50 mm at a concentration of 100 µg/disc (MIC: 100 µg/disc) while DIL-EA exhibited a

bald zone of 19 mm at the same concentration (MIC: 25 µg/disc). Surfactin being used as a positive control exhibited a 26.5 mm zone while DMSO (negative control) did not show any inhibitory potential. The results are further demonstrated in table 3.3.

### 3.3. Inclusion/Exclusion of Extracts Base on their Bioactivities

Preliminary screening of 32 extracts of *D. stramonium* and *D. innoxia* in four different solvents have assisted in selection of only those samples which have shown considerable number of polyphenols, significant antioxidant potential as well as brine shrimps cytotoxicity and protein kinase inhibitory potential. It was found that Dw extracts failed to exhibit any noteworthy pharmacological potential so extracts of both species derived using Dw solvent were excluded from further studies. The remaining extracts (24) were further screened for their cytotoxic action against selected breast and prostate cancer cell lines.

#### 3.3.1. Cytotoxicity against cancer cell lines

Cytotoxicity against PC-3, MDA-MB 231 and MCF-7 was determined initially as percent inhibition at 20 µg/ml final concentration followed by determination of IC50 values of potent extracts. *D. stramonium* extracts showed mediocre cytotoxic potential, the most potent was DSR-EA with cytotoxicity against PC-3, MDA-MB 231 and MCF-7 cell lines estimated to be 47.21±2.43, 51.58±3.39 and 49.98±1.98% respectively. DSL-EA also showed mild cytotoxic potential with 14.34±0.18% inhibition against PC-3, 37.67±2.10% in case of MDA-MB 231 and 40.56±1.89% against MCF-7 cell line. The most promising activity was shown by DIL-EA against each cancer cell line with $IC_{50}$ values of 2.86±0.1 µg/ml against PC-3, 1.56±0.16 µg/ml against MDA-MB 231 and 2.45±0.04 µg/ml in case of MCF-7 cell line. It was followed by DIL-M with $IC_{50}$ values of 14.30±1.87 (PC-3), 18.56±0.98 (MDA-MB 231) and 17.89±1.35 (MCF-7) µg/ml. Doxorubicin was the positive control used and it exhibited $IC_{50}$ values of 2.95±0.1, 3.2±0.11 and 2.45±0.24 µg/ml against PC-3, MDA-MB 231 and MCF-7 cell lines respectively. Details are given in table 3.4

**Table 3.2** Brine shrimps cytotoxicity assay of *D. stramonium and D. innoxia* extracts.

| Brine shrimps cytotoxicity assay | | | | | |
|---|---|---|---|---|---|
| **Samples** | ***D. stramonium* extracts** | | Samples | ***D. innoxia* extracts** | |
| | % Mortality 200 **µ**g/ml | $LC_{50}$ µg/ml | | % Mortality 200 **µ**g/ml | $LC_{50}$ µg/ml |
| DSR-NH | 100±10 | 15.02±1.21 | DIR-NH | 100±5.77 | 6.89±0.39 |
| DSF-NH | 100±5.77 | 13.69±0.87 | DIF-NH | 100±0.0 | 8.76±0.62 |
| DSS-NH | 100±5.77 | 10.27±0.76 | DIS-NH | 100±10 | 7.69±0.41 |
| DSL-NH | 100±10 | 8.644±1.11 | DIL-NH | 100±10 | 11.54±0.63 |
| DSR-EA | 100±0.0 | 10.27±0.13 | DIR-EA | 100±0.0 | 4.707±0.12 |
| DSF-EA | 100±5.77 | 15.02±0.41 | DIF-EA | 100±5.77 | 6.56±0.21 |
| DSS-EA | 100±5.77 | 11.38±0.72 | DIS-EA | 100±0.0 | 11.61±0.27 |
| DSL-EA | 96.96±5.25 | 12.04±0.21 | DIL-EA | 100±0.0 | 10.37±0.56 |
| DSR-M | 100±5.77 | 15.93±0.71 | DIR-M | 100±10 | 7.82±0.24 |
| DSF-M | 100±5.77 | 24.62±0.82 | DIF-M | 100±5.77 | 21.25±0.65 |
| DSS-M | 90±10 | 19.75±0.19 | DIS-M | 100±0.0 | 22.26±0.73 |
| DSL-M | 100±5.77 | 7.86±0.13 | DIL-M | 100±10 | 11.66±0.32 |
| DSR-Dw | 40±5.77 | > 200 | DIR-Dw | 40±10 | >200 |
| DSF-Dw | 70±10 | 140.01±3.87 | DIF-Dw | 60±10 | 100±4.23 |
| DSS-Dw | 40±5.77 | >200 | DIS-Dw | 30±5.77 | >200 |
| DSL-Dw | 40±5.77 | >200 | DIL-Dw | 30±5.77 | >200 |
| **Controls** | | | | | |
| Doxorubicin (4 µg/ml) | 100±0.00[a] | 5.74±0.18 | | | |
| DMSO (1%) | --- | --- | | | |

*Values are presented as mean±SD of triplicate analysis. Results of standards and/or controls are given in respective rows.*

**Table 3.3** Protein kinase inhibitory potential of *D. stramonium and D. innoxia* extracts.

| Protein kinase inhibition assay | | | | | | | |
|---|---|---|---|---|---|---|---|
| Samples | ***D. stramonium* extracts** | | | Samples | ***D. innoxia* extracts** | | |
| | Diameter at 100 µg/disc (mm) | | MIC **µ**g/disc | | Diameter at 100 µg/disc (mm) | | MIC **µ**g/disc |
| | Clear zone | Bald zone | | | Clear zone | Bald zone | |
| DSR-NH | 7±0.58 | …. | | DIR-NH | 8±0.58 | 10±0.58 | |
| DSF-NH | 7±1 | …. | | DIF-NH | 8±0.58 | 9±1 | |
| DSS-NH | 8±0.58 | …. | | DIS-NH | 8±0.58 | 9±1 | |
| DSL-NH | 8±1 | 11±1 | 100 | DIL-NH | 9±1 | 10±0.58 | |
| DSR-EA | 8±1 | …. | | DIR-EA | 11±1 | …. | |
| DSF-EA | 8±0.58 | …. | | DIF-EA | 10±0.58 | …. | |
| DSS-EA | 8±0.58 | …. | | DIS-EA | 9±1 | …. | |
| DSL-EA | 7.5±0.58 | 12.5±0.58 | 100 | DIL-EA | 9±1 | 19±0.58 | 25 |
| DSR-M | 7±0.58 | …. | | DIR-M | 7±0.58 | 12±1 | 100 |
| DSF-M | 7±0.58 | 10±1 | | DIF-M | 7±1 | 10±0.58 | |
| DSS-M | 7±1 | 11±1 | 100 | DIS-M | 7±1 | …. | |
| DSL-M | 7±0.58 | …. | | DIL-M | 7±0.58 | …. | |
| DSR-Dw | …. | …. | | DIR-Dw | …. | …. | |
| DSF-Dw | …. | …. | | DIF-Dw | …. | …. | |
| DSS-Dw | 7±0.58 | …. | | DIS-Dw | …. | …. | |
| DSL-Dw | …. | …. | | DIL-Dw | …. | …. | |
| **Controls** | | | | | | | |
| Surfactin (20 µg/disc) | ……. | 26.50±1 | | | | | |
| DMSO | …. | …. | …. | | | | |

*Values are presented as mean±SD of triplicate analysis. Results of standards and/or controls are given in respective columns.*

**Table 3.4.** Cytotoxicity of *Datura* extracts against cancer cell lines.

| Cytotoxicity against cancer cell lines | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | ***D. stramonium* extracts** | | | | | | **Samples** | ***D. innoxia* extracts** | | | | | |
| | **PC-3** | | **MDA-MB 231** | | **MCF-7** | | | **PC-3** | | **MDA-MB 231** | | **MCF-7** | |
| | % inhibition 20 µg/ml | $IC_{50}$ µg/ml | % inhibition 20 µg/ml | $IC_{50}$ µg/ml | % inhibition 20 µg/ml | $IC_{50}$ µg/ml | | % inhibition 20 µg/ml | $IC_{50}$ µg/ml | % inhibition 20 µg/ml | $IC_{50}$ µg/ml | % inhibition 20 µg/ml | $IC_{50}$ µg/ml |
| DSR-NH | 9.63±0.16 | …. | 3.45±0.24 | …. | 4.56±0.18 | …. | DIR-NH | …. | …. | 10.31±0.09 | …. | 12.45±0.44 | …. |
| DSF-NH | 16.75±1.21 | …. | 20.48±0.39 | …. | 25.56±0.96 | …. | DIF-NH | 8.53±0.34 | …. | 17.87±1.01 | …. | 4.56±0.19 | …. |
| DSS-NH | 4.70±0.14 | …. | …. | …. | …. | …. | DIS-NH | …. | …. | 8.95±0.28 | …. | 2.89±0.41 | …. |
| DSL-NH | 19.16±0.21 | …. | 27.94±1.24 | …. | 31.89±0.37 | …. | DIL-NH | 27.75±1.18 | …. | 39.45±1.32 | …. | 25.65±1.43 | …. |
| DSR-EA | 47.21±2.43 | …. | 51.58±3.39 | …. | 49.98±1.98 | …. | DIR-EA | …. | …. | …. | …. | …. | …. |
| DSF-EA | 21.60±0.89 | …. | 36.84±2.10 | …. | 30.05±3.32 | …. | DIF-EA | …. | …. | 50.56±2.84 | …. | 42.15±2.74 | …. |
| DSS-EA | 22.53±0.76 | …. | 11.24±0.62 | …. | 15.89±1.06 | …. | DIS-EA | 21.08±1.65 | …. | 33.16±0.89 | …. | 23.56±1.72 | …. |
| DSL-EA | 14.34±0.18 | …. | 38.03±1.38 | …. | 40.02±1.89 | …. | DIL-EA | 100.11±4.54 | 2.86±0.14 | 98.56±3.78 | 1.54±0.08 | 99.32±4.85 | 2.45±0.21 |
| DSR-M | …. | …. | …. | …. | …. | …. | DIR-M | 8.88±0.14 | …. | 1.54±0.32 | …. | …. | …. |
| DSF-M | …. | …. | …. | …. | …. | …. | DIF-M | …. | …. | 11.54±0.52 | …. | 2.35±0.11 | …. |
| DSS-M | 8.71±0.54 | …. | 5.65±0.22 | …. | …. | …. | DIS-M | …. | …. | 2.53±0.08 | …. | 4.56±0.67 | …. |
| DSL-M | 18.76±0.28 | …. | 3.15±0.08 | …. | …. | …. | DIL-M | 99.24±3.06 | 14.30±1.87 | 76.54±2.75 | 18.56±0.98 | 80.25±2.71 | 17.89±1.35 |
| **Controls** | | | | | | | | | | | | | |
| **Doxorubicin** | 99.43±0.15 | 2.95±0.11 | 98.11±0.48 | 3.2±0.11 | 98.62±0.42 | 2.45±0.24 | | | | | | | |
| **DMSO** | …. | …. | …. | …. | …. | …. | | | | | | | |

*Values are presented as mean±SD of triplicate analysis. Results of standards and/or controls are given in respective columns.*

## 3.4. *In Vitro* Toxicity and Acute *In Vivo* Assays

The samples shortlisted for toxicity studies and acute and chronic *in vivo* assays were NH and EA extracts of *D. stramonium* and *D. innoxia* leaf portion. The decision was made by keeping numerous factors in mind i.e., significant extraction yield from the leaf portion, overall promising results in majority, if not all the assays performed in the preceding section and the predetermined scheme of progressing our comparative study in a symmetric manner, thus two solvents and one identical plant part is selected from each specie. It left us with the four most promising crude extracts i.e., DSL-NH, DSL-EA, DIL-NH and DIL-EA to carry out our next group of assays.

### 3.4.1. *In vitro* toxicity and nitric oxide scavenging assays

#### *3.4.1.1. Toxicity against isolated lymphocytes*

The cytotoxic nature of selected extracts was also evaluated against normal lymphocytes isolated from human blood. Percent inhibition was calculated at 20 µg/ml final concentration (Table 3.5). No significant activity was depicted by either of the four extracts against normal lymphocytes. None of the extracts exhibited more than 30% cytotoxicity at the tested concentration, DSL-EA extract proved to be the least toxic with merely 4±0.35% toxicity against isolated lymphocytes. DIL-NH showed the greatest percent inhibition (29.72±1.43%). Observed cytotoxicity was significantly lower in comparison to that observed against cancer cells. This selective cytotoxic action of used extracts is extremely beneficial in targeting cancerous cells while sparing the normal ones. Vincristine was used as a positive control, and it exhibited an $IC_{50}$ value of 6.98±0.19 µg/ml.

#### *3.4.1.2. Toxicity against isolated macrophages*

The extracts were also investigated for percent cell viability by performing methyl thiazole tetrazolium (MTT) assay. Cytotoxicity against isolated macrophages was determined and the results were expressed in terms of % viable cells in the test wells having predetermined concentration of the extracts and designated controls. Results have shown that the tested extracts, even at a maximum concentration of 20 µg/ml yielded cell viability greater than 85%. DSL-EA again proved to be the safest in terms of % inhibitory action against isolated macrophages with only 1.07±0.08% inhibition estimated at 20 µg/ml. Doxorubicin was the positive control used in the assay and it exhibited an $IC_{50}$ value of 17.08±0.37 µg/ml. Results are listed in table 3.5.

**Table 3.5** Toxicity of *Datura* extracts against isolated lymphocytes and macrophages.

| | ***In vitro* toxicity assays** | | | |
|---|---|---|---|---|
| **Samples** | **Lymphocyte toxicity20 µg/ml** | **$IC_{50}$ µg/ml** | **Macrophage toxicity20 µg/ml** | **$IC_{50}$ µg/ml** |
| DSL-NH | 18.32±0.34 | …. | 9.46±0.11 | …. |
| DSL-EA | 4±0.35 | …. | 1.07±0.08 | …. |
| DIL-NH | 29.72±1.43 | …. | 10.14±0.20 | …. |
| DIL-EA | 23±1.45 | …. | 14.59±0.45 | …. |
| Vincristine | 79±2.55 | 6.89±0.19 | …. | …. |
| Doxorubicin | …. | …. | 82.21±0.27 | 17.08±0.37 |

*Values are presented as mean±SD of triplicate analysis.*

#### *3.4.1.3. In vitro NO scavenging potential*

The *in vitro* NO scavenging action was determined to further establish the efficacy of selected extracts before initiating the acute and sub chronic in vivo studies. The extracts were tested for their NO scavenging potential at a maximum concentration of 20 µg/ml. DSL-EA curbed LPS induced NO generation by more than 70% at a concentration of 20 µg/ml and the response tapered down at lower concentrations proving a concentration dependent scavenging response. The NO scavenging action of tested extracts followed the following trend; DSL-EA>DIL-EA>DSL-NH>DIL-NH. The positive control used in this assay was piroxicam and it inhibited NO production by 82.68±2.12% at 100 µM concentration. The details are presented in table 3.6.

**Table 3.6** NO scavenging potential of *Datura* extracts.

| **Nitric oxide scavenging assay** | | | | | |
|---|---|---|---|---|---|
| **Extracts** | **% Scavenging** | | | | **$IC_{50}$ µg/ml** |
| | **20 µg/ml** | **10 µg/ml** | **5 µg/ml** | **2.5 µg/ml** | |
| DSL-NH | 35.55±0.82 | 23.10±0.42 | 11.32±0.21 | 9.75±0.44 | > 20 |
| DSL-EA | 72.65±1.98 | 16.61±0.23 | 9.92±0.30 | 6.02±0.36 | 7.625±0.51 |
| DIL-NH | 31.95±1.10 | 56.45±0.61 | 41.73±0.52 | 32.38±0.63 | > 20 |
| DIL-EA | 41.72±1.33 | 32.88±0.62 | 19.18±0.22 | 12.03±0.38 | > 20 |

*Values are presented as mean±SD of triplicate analysis.*

### 3.4.2. Acute *in vivo* studies

#### *3.4.2.1. Acute toxicity study*

Acute toxicity was checked after a single booster dose (150-2000 mg/kg) to rats divided into different groups. The rats were kept under surveillance for a period of two weeks. No deaths were recorded during the said time period and the rats did not experience any abnormal changes in the behavior. Normal physiology and all the senses were intact

which showed that DSL-NH, DSL-EA, DIL-NH AND DIL-EA were safe to be administered up to the highest dose of 2000 mg/kg with no risk of any kind of toxicity.

#### *3.4.2.2. Non-invasive in vivo assays*

##### 3.4.2.2.1. Carrageenan induced paw edema

The anti-inflammatory potential of selected extracts was evaluated by calculating its edema inhibitory effect in carrageenan induced hind paw edema test. DSL-EA was most effective in alleviating edema induced by carrageenan. The extract acted in a dose and time dependent manner high dose of DSL-EA showing more than 70% inhibitory effect while low dose exhibited moderate activity with the effect peaking at 4th hr. i.e., 51.63±5.49% reduction noted in hind paw edema. DIL-EA also showed moderate anti-inflammatory action with high and low doses resulting in 47.03±6.09 and 56.18±7.09% reduction in edema respectively as per readings collected at 4th hr. The NH leaf extracts of both plant species were not able to produce pharmacologically significant results. Ibuprofen, the standard drug used in the study reduced paw edema by 82.6±9.4% as evident from the results elaborated in figure 3.8.

##### 3.4.2.2.2. Croton oil induced anal edema

A Croton oil induced anal edema test was also performed for further validation of the anti-inflammatory potential of selected extracts. A substantial steady decline in percent edema volume was observed over the course of the study duration and maximum activity in terms of edema inhibition was observed at 4th hr. in case of DSL-EA-HD i.e., 67.62±7.56%. Low dose of the extract curbed the edema by slightly less than 43% as of 4th hr. reading. As observed in paw edema test, DIL-EA proved to be the second in line in terms of % edema reduction with low and high dose extract resulting in 38.22±4.86 and 44.77±6.03% edema reduction respectively. Ibuprofen was the standard drug used in the study and it showed 80.1±5.1% edema inhibition at 3rd hr. The details can be seen in figure 3.9.

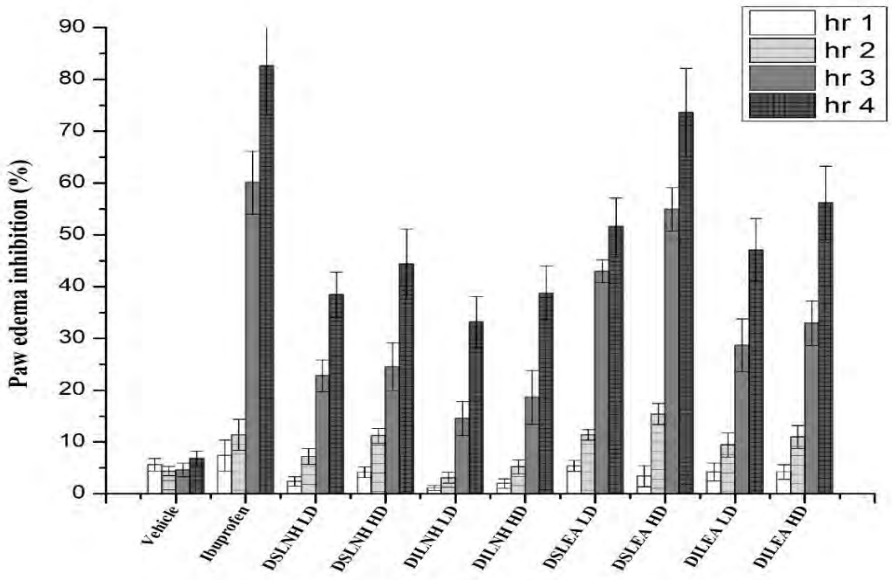


**Figure 3.8** Effect of *Datura* extracts on carrageenan induced paw edema
***Note:*** *In Balb/c. Data values shown represent mean±SD (n=6).*

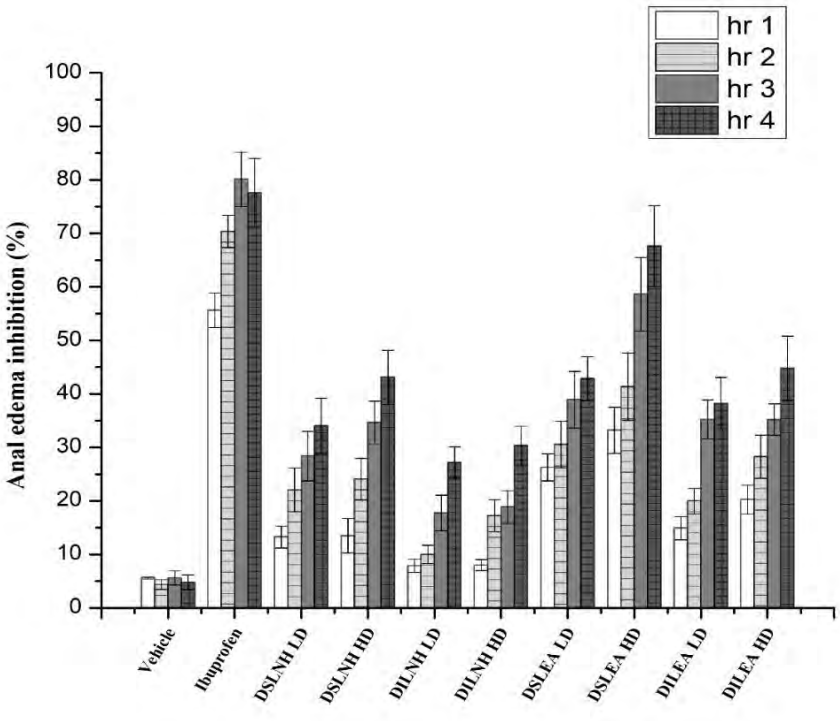


**Figure 3.9** Effect of *Datura* extracts on croton oil induced anal edema in Balb/c mice.
***Note:*** *Data values shown represent mean±SD (n=6).*

a. Tail suspension test

The potential antidepressant action of extract was estimated using tail suspension test. Fluoxetine was the standard drug used in this assay and it significantly reduced the immobility time in mice (66.66±11.37 sec) in comparison to the vehicle control group (168.33±14.04 sec). NH leaf extracts of both plant species failed to reduce the

immobility time in BALB/c mice in a significant manner. EA extracts on the other hand showed slight improvement and the most effective extracts were DSL-EA-HD (117±6.93 sec) and DIL-EA-HD (128.89±10.72 sec). The results are stacked in figure 3.10.

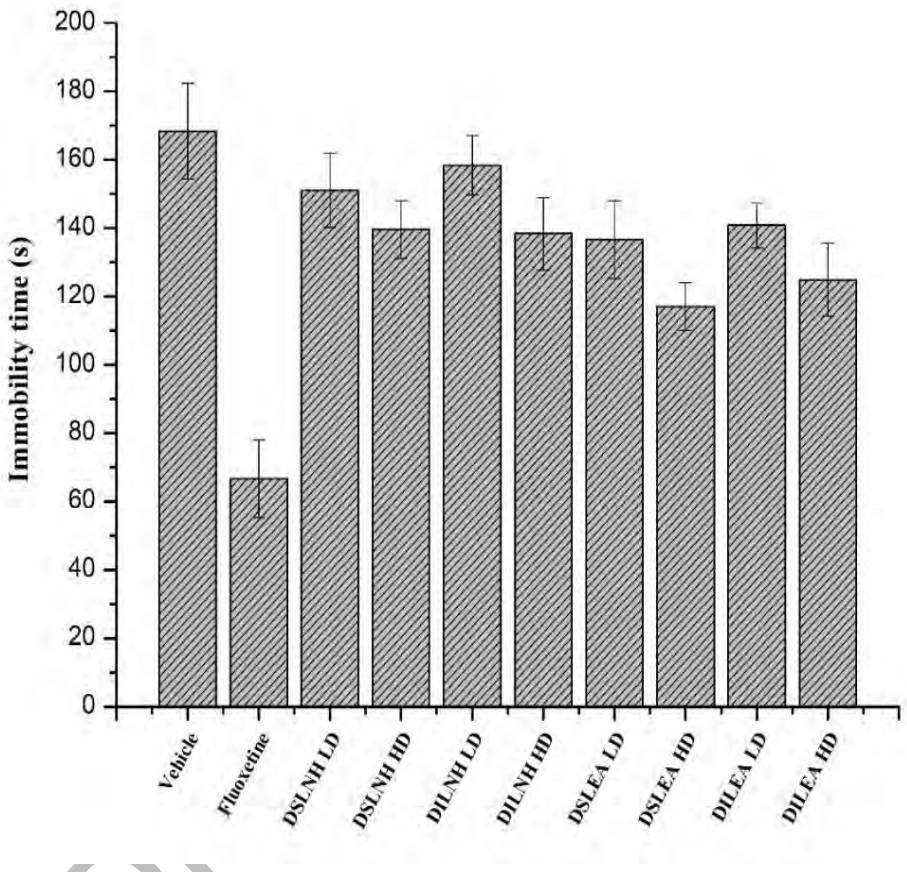


**Figure 3.10**. Tail suspension test and effect of *Datura* extracts on the immobility time (seconds) in Balb/c mice.
***Note:*** *Data values shown represent mean±SD (n=6).*

b. Hotplate test

The analgesic property of DSL-EA was also determined against thermally induced pain using hotplate method. The analgesic effect was evaluated by observing the % increase in latency period and the results were compared with standard drug i.e., tramadol. The maximum analgesic action of tramadol was observed at 3rd hr. i.e., 91.66±9.74% increase in latency period. Moderate elevation in latency period was observed in EA extracts with DSL-EA-HD resulting in maximum increase of 59.4±7.6% observed at 4th hr. DIL-EA-HD's analgesic action was slightly weaker i.e., 51.23±6.50% increase in latency period at 4th hr. The results are further elaborated in figure 3.11.

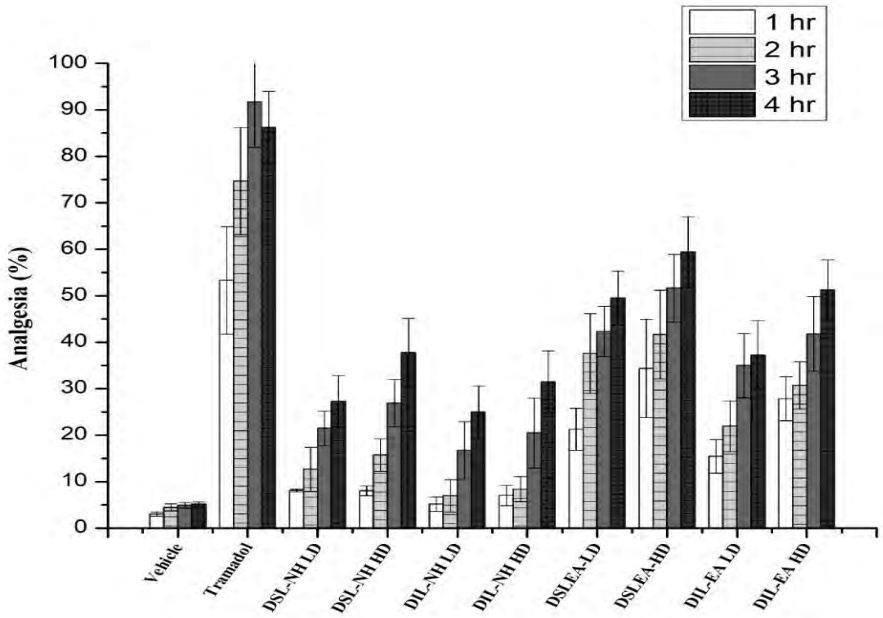


**Figure 3.11** Percent analgesic effects of *Datura* extracts in Balb/c mice using hot plate method.
***Note:****. Data values shown represent mean±SD (n=6).*

Toxicity studies and acute *in vivo* assays mentioned in preceding section revealed the safety as well as efficacy profile of tested extracts. It was concluded that EA extract of *D. stramonium* was the safest and most effective of all the samples followed by DIL-EA. Furthermore, NH extracts of both species were omitted from further involvement in chronic *in vivo* studies. It left us with the two most effective crude extracts i.e., DSL-EA DIL-EA to carry out next group of *in vivo* assays.

### 3.4.3. Chronic *in vivo* models

#### *3.4.3.1. Benzene induced leukemia*

DSL-EA and DIL-EA were selected for estimation of *in vivo* antileukemic activity. Extracts were standardized based on their significant phenolics and flavonoids content, noteworthy antioxidant, protein kinase inhibitory and cytotoxic potential as well assessment of their safety profile and also based on the findings of acute *in vivo* studies.

Following intravenous administration of predetermined doses of benzene to preventive, treatment and disease control groups, obvious signs of leukemia induction were observed including, unusual bleeding, excessive bruising, skin rashes, weakness and weight loss. Efficacy of extracts in treatment or prevention of disease progression was measured through a series of hematological, biochemical and histological studies.

3.4.3.1.1. Hematological studies

Results of benzene induced hematological variations are displayed in Table 3.7. As evident from the data, the carcinogen used has caused a whole lot of hematological aberrations, most eminent of which are decline in RBCs (4.33±0.065 × 106/µl), platelets (344±3.19 × 103/µl) and hemoglobin levels (5.9±0.26 g/dl) while increase in ESR (9.6±0.12 mm/h) and WBCs count (7.78±0.012 × 103/µl) was also observed in disease control rats. Findings of hematological investigation revealed that the positive control, vehicle control, preventive and treatment groups are significantly different ($p < 0.05$) from the disease control group. The average RBC and platelets count in preventive and treatment groups of both plant species were raised up to 5.78±0.23 × 106/µl and 529±20.17 × 103/µl respectively, while average hemoglobin levels were elevated to 7.43±0.25 g/dl. High and low doses of both species of *Datura* normalized the WBCs count and ESR with average values of 3.95±0.26 × 103/µl and 6.08±0.24 mm/h respectively.

**Table 3.7** Hematological investigations of experimental rats of all study groups.

| Groups | RBCs ($\times10^6$)/µl | WBCs ($\times10^3$) /µl | Platelets ($\times10^3$) /µl | Hb (g/dl) | ESR (mm/h) |
|---|---|---|---|---|---|
| **Vehicle control** | 6.23±0.035$^{a}$ | 3.55±0.018$^{f}$ | 581±2.72$^{a}$ | 9.78±0.62$^{a}$ | 4.4±0.067$^{g}$ |
| **Positive control** | 6.03±0.095$^{a}$ | 3.85±0.067$^{d}$ | 495±2.72$^{e}$ | 8.38±0.62$^{b}$ | 5.4±0.067$^{f}$ |
| **Disease control** | 4.33±0.065$^{e}$ | 7.78±0.012$^{a}$ | 344±3.19$^{f}$ | 5.9±0.26$^{e}$ | 9.6±0.12$^{a}$ |
| **DSL LD (P)** | 5.73±0.071$^{ab}$ | 3.61±0.025$^{de}$ | 538±1.98$^{c}$ | 7.7±0.35$^{cd}$ | 5.7±0.071$^{e}$ |
| **DSL HD (P)** | 5.95±0.083$^{ab}$ | 3.72±0.038$^{de}$ | 556±2.59$^{b}$ | 7.7±0.47$^{bc}$ | 5.9±0.066$^{d}$ |
| **DIL LD (P)** | 5.86±0.037$^{ab}$ | 3.86±0.027$^{d}$ | 523±3.07$^{d}$ | 7.34±0.72$^{cd}$ | 6.2±0.034$^{c}$ |
| **DIL HD (P)** | 6.02±0.065$^{a}$ | 4.01±0.011$^{c}$ | 541±2.63$^{c}$ | 7.12±0.58$^{cd}$ | 6.12±0.081$^{c}$ |
| **DSL HD (T)** | 5.36±0.069$^{b}$ | 4.23±0.044$^{b}$ | 517±1.98$^{d}$ | 7.2±0.28$^{d}$ | 6.21±0.036$^{c}$ |
| **DIL HD (T)** | 5.73±0.081$^{b}$ | 4.25±0.035$^{b}$ | 499±2.94$^{e}$ | 7.52±0.79$^{cd}$ | 6.34±0.097$^{b}$ |

*Values are presented as mean±SD (n = 6, where n = number of rats analysed in each group). Means with different superscripts (a-g) in the columns are significantly ($p < 0.05$) different from one another.*

3.4.3.1.2. Biochemical analysis

Various enzymatic and biochemical tests performed on serum acquired from rats of study groups have clearly shown the deleterious effects of benzene on vital organs including liver and kidney in the disease control group. Administration of low and high doses of DSL-EA and DIL-EA have curbed the harm instigated by benzene to a great extent as evident from the enzymatic and biochemical findings of test groups. Liver enzymes and CPK levels of disease control group were significantly higher ($p < 0.05$) than all other groups. ALT, AST, ALP and CPK levels estimated in disease control rats

were 93.02±2.64, 67.80±2.05, 356±5.69 and 234±4.97 U/L respectively. Moreover, total proteins estimated in the serum were expressively lower in the case of disease control rats (Albumin 1.20±0.07, globulin 0.94±0.07 and total protein 2.14±0.11 g/dl) confirming the harm caused to liver by benzene. Low and high doses of tested extracts have reverted the liver damage in identical manner, average values of ALT, AST, ALP, CPK and total proteins of 6 groups (both preventive and treatment) are; 41.56±2.59, 27.99±1.98. 141±8.46, 148.1±7.98 U/L and 6.54±0.38 g/dl respectively. The details are given in Table 3.8(a) and (b).

**Table 3.8(a)** Enzymatic investigation of control and leukemic rats.

| Groups | ALT (U/L) | AST (U/L) | ALP (U/L) | CPK (U/L) |
|---|---|---|---|---|
| **Vehicle control** | 41.3±1.65$^{e}$ | 23±0.26$^{g}$ | 127±3.28$^{b}$ | 133±4.04$^{g}$ |
| **Positive control** | 47.3±1.87$^{b}$ | 28±0.63$^{d}$ | 143±3.57$^{b}$ | 139±3.28$^{ef}$ |
| **Disease control** | 93.20±2.64$^{a}$ | 67.80±2.05$^{a}$ | 356±5.69$^{a}$ | 234±4.97$^{a}$ |
| **DSL LD (P)** | 45.31±2.28$^{c}$ | 24.65±1.01$^{f}$ | 148±3.26$^{b}$ | 154±2.32$^{c}$ |
| **DSL HD (P)** | 42.54±1.79$^{d}$ | 26.81±0.86$^{e}$ | 139±4.02$^{b}$ | 146±2.86$^{d}$ |
| **DIL LD (P)** | 42.81±1.66$^{d}$ | 29.34±1.28$^{c}$ | 152±3.89$^{b}$ | 159±3.47$^{b}$ |
| **DIL HD (P)** | 40.76±2.26$^{e}$ | 28.11±0.94$^{d}$ | 143±2.93$^{b}$ | 151±3.63$^{c}$ |
| **DSL HD (T)** | 39.64±1.95$^{f}$ | 29.06±0.68$^{c}$ | 135±3.61$^{b}$ | 141±2.79$^{e}$ |
| **DIL HD (T)** | 38.32±1.58$^{g}$ | 30.01±1.14$^{b}$ | 129±4.01$^{b}$ | 138±3.34$^{f}$ |

*Results are represented as mean±SD (n = 6, where n = number of rats analysed in each group). Mean values with different superscripts (a-g) in the columns are significantly ($p < 0.05$) different from one another.*

Creatinine, urea and bilirubin levels were markedly high in disease control rats confirming acute leukemic condition and kidney damage. High and low doses of selected plants have notably reversed the abuse inflicted by benzene as evident in Table 3.8(b). Statistically significant difference ($p < 0.05$) was observed when positive control and other groups were compared with disease control group.

**Table 3.8(b)** Biochemical investigation of control and leukemic rats.

| Groups | Urea (mg/dl) | Creatinine (mg/dl) | Bilirubin (mg/ml) | Albumin (g/dl) | Globulin (g/dl) | Total protein (g/dl) |
|---|---|---|---|---|---|---|
| Vehicle control | 29±1.76$^{g}$ | 1.01±0.11$^{b}$ | 2.7±0.17$^{e}$ | 3.9±0.18$^{c}$ | 2.8±0.13$^{b}$ | 6.70±0.32$^{c}$ |
| Positive control | 26±1.01$^{h}$ | 1.08±0.14$^{b}$ | 2.67±0.21$^{e}$ | 4.07±0.24$^{c}$ | 2.79±0.16$^{b}$ | 6.86±0.38$^{b}$ |
| Disease control | 72.2±2.76$^{a}$ | 2.89±0.54$^{a}$ | 7.56±0.97$^{a}$ | 1.20±0.07$^{e}$ | 0.94±0.07$^{g}$ | 2.14±0.11$^{f}$ |
| DSL LD (P) | 36.21±0.9$^{b}$ | 1.19±0.09$^{b}$ | 3.21±0.09$^{b}$ | 4.56±0.15$^{a}$ | 2.05±0.09$^{f}$ | 6.61±0.53$^{c}$ |
| DSL HD (P) | 32.34±1.7$^{e}$ | 1.06±0.12$^{b}$ | 2.89±0.64$^{d}$ | 4.01±0.21$^{c}$ | 2.98±0.18$^{a}$ | 6.99±0.59$^{a}$ |
| DIL LD (P) | 35.14±0.8$^{c}$ | 1.12±0.11$^{b}$ | 3.04±0.33$^{c}$ | 4.32±0.19$^{b}$ | 2.56±0.15$^{c}$ | 6.88±0.63$^{ab}$ |
| DIL HD (P) | 34.12±1.2$^{d}$ | 1.08±0.08$^{b}$ | 2.98±0.26$^{c}$ | 4.27±0.26$^{b}$ | 2.32±0.13$^{d}$ | 6.59±0.49$^{c}$ |
| DSL HD (T) | 30.09±2.05$^{f}$ | 1.01±0.10$^{b}$ | 2.48±0.08$^{f}$ | 3.64±0.17$^{d}$ | 2.39±0.08$^{d}$ | 6.03±0.37$^{e}$ |
| DIL HD (T) | 28.20±1.8$^{g}$ | 1.02±0.07$^{b}$ | 2.42±0.39$^{f}$ | 3.96±0.23$^{c}$ | 2.19±0.11$^{e}$ | 6.15±0.42$^{d}$ |

*Values are represented as mean±SD (n = 6, where n = number of rats analysed in each group). Means with different superscripts (a-h) in the columns are significantly ($p < 0.05$) different from one another.*

3.4.3.1.3. Effect on Endogenous antioxidant enzymes

The activity level of endogenous antioxidant enzymes in serum is presented in table 3.9. A significant ($p < 0.05$) decline in CAT, POD, SOD and GST was estimated in disease control group when compared with vehicle and positive control groups. Activity levels of CAT, POD, SOD and GST in serum drawn from disease control rats were 0.5±0.04, 1.00±0.04 U/min, 0.96±0.05 U/mg protein and 81.01±0.44 nM/min/ml respectively. A dose dependent increase in CAT, SOD, POD and GST level was observed in test groups. Significant ($p < 0.05$) increase was observed in case of high doses of selected plants in preventive and treatment groups. High doses of DIL raised the activity levels of CAT, POD, SOD and GST to a reasonably greater extent as compared to similar doses of DSL in treatment mode i.e., 2.5±0.15, 3.9±0.10 U/min, 2.27±0.09 U/mg protein and 162.03±1.54 nM/min/ml respectively. Low doses given to preventive groups showed a slight increase in level of antioxidant enzymes.

**Table 3.9** Effect of *Datura* extracts on activity levels of endogenous antioxidant enzymes.

| Groups | CAT (U/min) | POD (U/min) | SOD (U/mg protein) | GST (nM/min/ml) |
|---|---|---|---|---|
| **Vehicle control** | 1.7±0.02$^{c}$ | 2.1±0.03$^{e}$ | 4.00±0.05$^{a}$ | 216.04±1.46$^{c}$ |
| **Positive control** | 2.2±0.04$^{b}$ | 3.1±0.06$^{c}$ | 3.51±0.03$^{b}$ | 236.04±1.46$^{b}$ |
| **Disease control** | 0.5±0.04$^{e}$ | 1±0.04$^{g}$ | 0.96±0.05$^{g}$ | 81.01±0.44$^{g}$ |
| **DSL LD (P)** | 0.9±0.07$^{d}$ | 1.7±0.02$^{f}$ | 2.42±0.02$^{f}$ | 108.03±0.63$^{f}$ |
| **DSL HD (P)** | 1±0.006$^{d}$ | 2.2±0.01$^{e}$ | 2.83±0.04$^{d}$ | 162.02±0.70$^{d}$ |
| **DIL LD (P)** | 1.8±0.06$^{c}$ | 1.6±0.04$^{f}$ | 2.79±0.02$^{d}$ | 108.02±1.03$^{f}$ |
| **DIL HD (P)** | 2.0±0.13$^{b}$ | 4.2±0.03$^{a}$ | 3.18±0.03$^{c}$ | 297.05±1.60$^{a}$ |
| **DSL HD (T)** | 2.1±0.08$^{b}$ | 2.8±0.09$^{d}$ | 2.65±0.10$^{e}$ | 135.02±0.63$^{e}$ |
| **DIL HD (T)** | 2.5±0.15$^{a}$ | 3.9±0.10$^{b}$ | 2.72±0.09$^{e}$ | 162.03±1.54$^{d}$ |

*Results are expressed as mean±SD (n = 6, where n = number of rats analysed in each group). Values with different superscripts (a-g) in the columns indicate they are significantly (p < 0.05) different form each other.*

3.4.3.1.4. Oxidative stress markers (TBARs and NO estimation)

After completion of the assay, TBARs and NO levels were recorded in serum obtained from test rats of each group. Benzene treated disease control group showed escalated level of TBARs i.e., 133.75±2.61 nM/min/mg protein. Treatment groups of DSL-EA and DIL-EA have significantly ($p < 0.05$) lowered the level of TBARs i.e., 83.49±and 92.25±1.17 nM/min/mg protein respectively. Estimated NO level was markedly high in disease control group with value 93.81±2.88 µM/ml while positive control group showed significant decrease in NO level i.e., 61.74±1.98 µM/ml. Low and high doses of DSL-EA and DIL-EA exhibited expressively reduced NO levels compared to disease control group with values not higher than 65 µM/ml in any of the study groups. Results are given in Table 3.10.



**Table 3.10** TBARs and NO estimation of controls and leukemic rats.

| Groups | TBARs (nM/min/mg protein) | NO (µM/ml) |
|---|---|---|
| **Vehicle control** | 88.90±1.87$^{f}$ | 60.74±2.23$^{e}$ |
| **Positive control** | 85.80±1.09$^{g}$ | 61.74±1.98$^{d}$ |
| **Disease control** | 133.75±2.61$^{a}$ | 93.81±2.88$^{a}$ |
| **DSL LD (P)** | 113.77±2.21$^{b}$ | 60.56±1.62$^{e}$ |
| **DSL HD (P)** | 100.82±1.48$^{d}$ | 62.52±2.25$^{c}$ |
| **DIL LD (P)** | 113.12±2.32$^{b}$ | 62.81±2.73$^{c}$ |
| **DIL HD (P)** | 105.82±1.62$^{c}$ | 64.89±2.64$^{b}$ |
| **DSL HD (T)** | 83.49±1.19$^{h}$ | 62.22±1.76$^{c}$ |
| **DIL HD (T)** | 92.25±1.77$^{e}$ | 63.41±1.59$^{b}$ |

*Results are expressed as mean±SD (n = 6, where n = number of rats analysed in each group). Mean values with different superscripts (a-h) in individual columns specify significance at p <0.05.*

3.4.3.1.5. Histology

Histological investigations have verified and endorsed the effects of EA extracts of selected *Datura* species on the studied biochemical parameters (particularly LFTs). The liver and kidney of the vehicle and positive control groups (group I and III) exhibited normal morphological features i.e., intact hepatocytes, sinusoids, typical central veins, Bowman's capsule and glomerular tuft. Benzene treated disease control group caused significant damage to liver and kidney tissues i.e., necrosed hepatocytes, cellular hypertrophy, hyalinization of glomerular tuft and marked degeneration of renal tubules as evident in Figure 3.12 and 3.13. Low and high doses of administered extracts alleviated benzene induced damage to a greater extent as evident in the figure (Group IV-IX). These images further reinforce the findings of biochemical and enzymatic parameters.

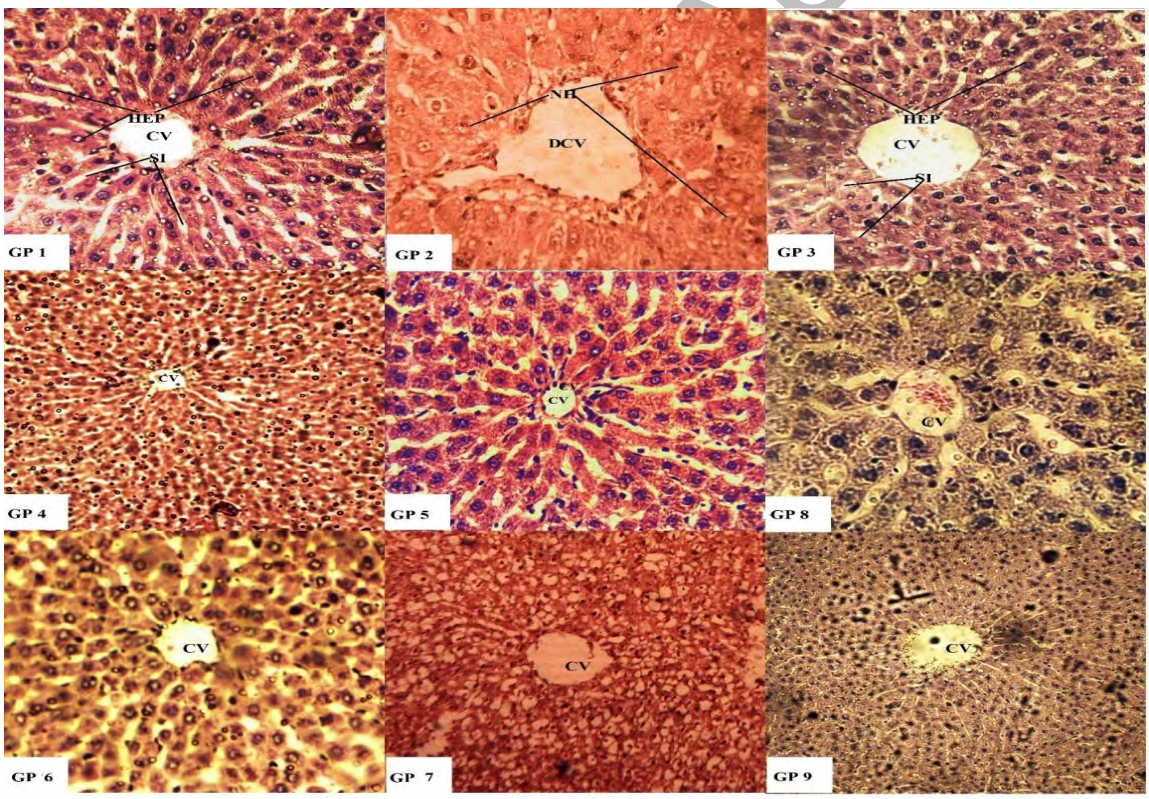


**Figure 3.12** Hematoxylin-eosin stain.

***Note:*** *Histopathological observations (40X) for the protective potential of ethyl acetate extracts of D. stramonium and D. innoxia on liver in rat. Gp I: Vehicle control (10% DMSO in water), Gp II: Disease control (0.2 ml Benzene), Gp III: Positive control (Cyclophosphamide 10 mg/kg), Gp IV: DSL-LD (100 mg/kg, Preventive), Gp V: DSL-HD (200 mg/kg, Preventive), Gp VI: DIL-LD (100 mg/kg, Preventive), Gp VII: DIL-HD (200 mg/kg, Preventive), Gp VIII: DSL-HD (200 mg/kg, Treatment) and Gp IX: DIL-HD (200 mg/kg, Treatment). BC- Bowman's capsule, CV- Central vein, DCV- Damaged central vein, HEP-Hepatocytes, NH- Necrosed hepatocytes.*

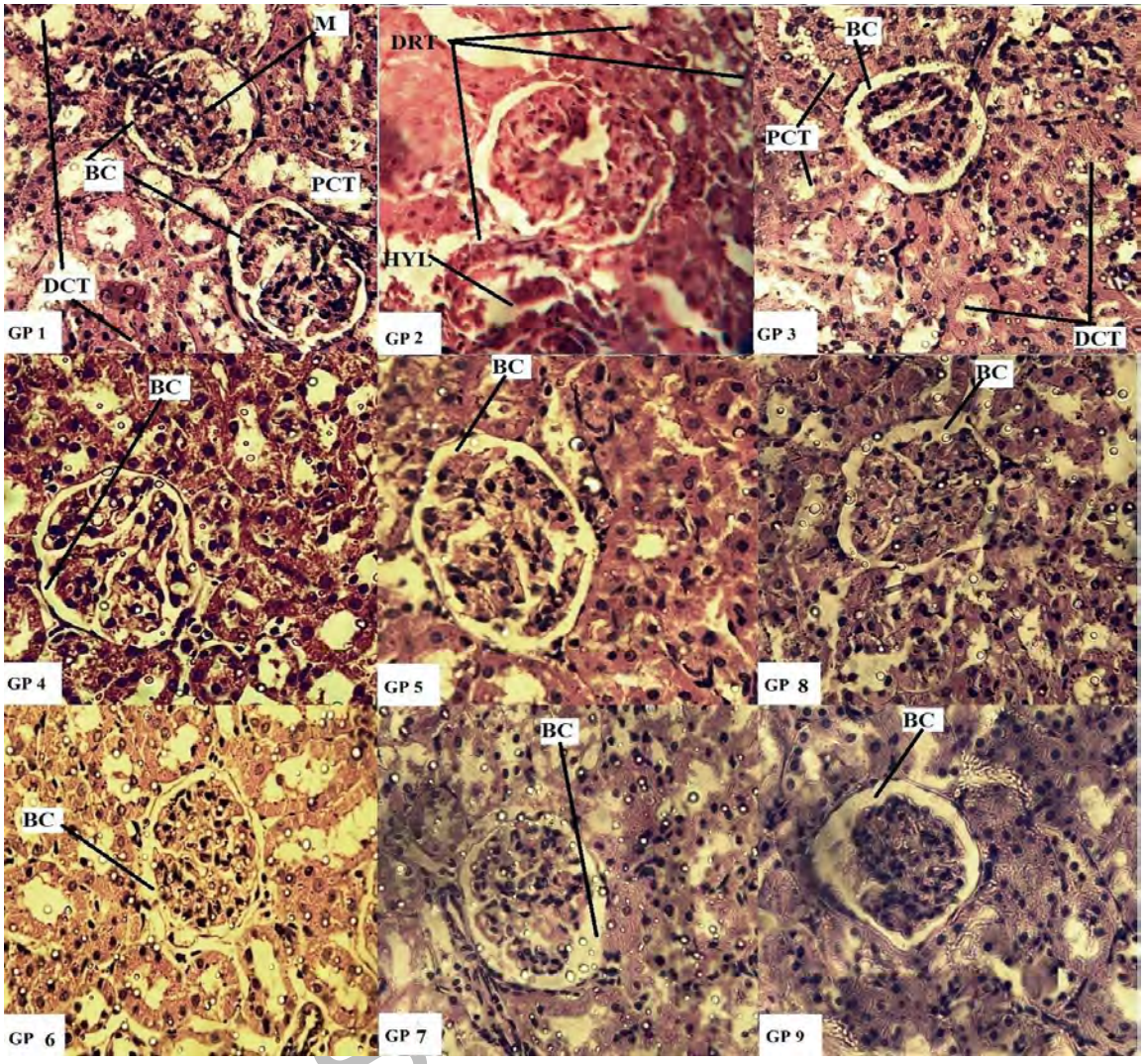


**Figure 3.13** Hematoxylin-eosin stain.

***Note:*** *Histopathological observations (40X) for the protective potential of ethyl acetate extracts of D. stramonium and D. innoxia on kidney in rat. Gp I: Vehicle control (10% DMSO in water), Gp II: Disease control (0.2 ml Benzene), Gp III: Positive control (Cyclophosphamide 10 mg/kg), Gp IV: DSL-LD (100 mg/kg, Preventive), Gp V: DSL-HD (200 mg/kg, Preventive), Gp VI: DIL-LD (100 mg/kg, Preventive), Gp VII: DIL-HD (200 mg/kg, Preventive), Gp VIII: DSL-HD (200 mg/kg, Treatment) and Gp IX: DIL-HD (200 mg/kg, Treatment). BC- Bowman's capsule, DCT: Distal convoluted tubules, DRT- Degenerated renal tubules, HYL- Hyalinization (of glomerular tuft), M- Mesangium, PCT- Proximal convoluted tubules.*

3.4.3.1.6. Alleviative effects of Datura extracts in benzene induced leukemia

The antileukemic effects of DSL-EA and DIL-EA are summarized in Table 3.11. The percentage alleviating effects of both extracts against benzene induced leukemia are collectively illustrated in the table. Apart from the activity levels of antioxidant enzymes i.e., CAT, POD, SOD and GST, where DIL-EA exhibited an expressively greater curative effect, all other parameters were stabilized in a comparative and significant manner by both extracts.

**Table 3.11** Alleviative effects of DSL-EA and DIL-EA extracts in benzene induced leukemia.

| Evaluation parameters | Study Groups | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Disease control | Positive control | DSL-LD (P) | DSL-HD (P) | DIL LD (P) | DIL HD (P) | DSL HD (T) | DIL HD (T) |
| | | Percent alleviative effect | | | | | | |
| **Hematological parameters** | | | | | | | | |
| **RBCs ($\times10^6$)/µl** | 4.33±0.065 | 41.80±2.20$^{a}$ | 32.90±1.65$^{c}$ | 38.71±1.92$^{ab}$ | 35.64±0.85$^{bc}$ | 39.66±1.51$^{ab}$ | 23.79±1.62$^{d}$ | 32.74±1.88$^{c}$ |
| **WBCs ($\times10^3$) /µl** | 7.78±0.012 | 50.53±0.87$^{b}$ | 53.54±0.33$^{a}$ | 52.14±0.49$^{a}$ | 50.39±0.35$^{b}$ | 48.46±0.15$^{c}$ | 45.67±0.57$^{d}$ | 45.28±0.46$^{d}$ |
| **Platelets ($\times10^3$)/µl** | 344±3.19 | 44.08±0.79$^{e}$ | 56.42±0.58$^{b}$ | 61.81±0.75$^{a}$ | 52.37±0.89$^{c}$ | 58.15±0.76$^{b}$ | 49.76±0.58$^{d}$ | 45.25±0.86$^{e}$ |
| **Endogenous antioxidant enzymes** | | | | | | | | |
| **CAT (U/min)** | 0.5±0.04 | 342.67±8.33$^{b}$ | 84.67±15.53$^{d}$ | 100±1.20$^{d}$ | 274±12.49$^{c}$ | 285.3±27.15$^{c}$ | 315.3±17.47$^{bc}$ | 410±30.27$^{a}$ |
| **POD (U/min)** | 1±0.04 | 209±6.56$^{b}$ | 70.33±2.52$^{e}$ | 122±1.73$^{d}$ | 63.33±4.16$^{e}$ | 317.67±3.21$^{a}$ | 183.67±9.07$^{c}$ | 301±10.15$^{a}$ |
| **SOD (U/mg protein)** | 0.96±0.05 | 261.81±3.66$^{a}$ | 154.17±2.08$^{e}$ | 198.96±4.17$^{c}$ | 190.63±2.08$^{cd}$ | 232.29±3.76$^{b}$ | 176.7±10.43$^{d}$ | 191.6±10.26$^{cd}$ |
| **GST (nM/min/ml)** | 81.01±0.44 | 191±1.81$^{b}$ | 33.72±0.78$^{e}$ | 99.22±0.87$^{c}$ | 34.43±1.28$^{e}$ | 266.29±1.98$^{a}$ | 67.07±0.78$^{d}$ | 101.08±1.91$^{c}$ |
| **Biochemical parameters** | | | | | | | | |
| **ALT (U/L)** | 93.20±2.64 | 50.67±2.01$^{b}$ | 50.83±2.45$^{b}$ | 54.36±1.92$^{ab}$ | 52.47±1.78$^{ab}$ | 56.96±2.43$^{a}$ | 58.13±2.10$^{a}$ | 57.46±1.70$^{a}$ |
| **AST (U/L)** | 67.80±2.05 | 59.73±0.94$^{bc}$ | 65.23±1.50$^{a}$ | 61.91±1.27$^{ab}$ | 54.72±1.89$^{de}$ | 58.58±1.39b$^{cd}$ | 56.96±1.01$^{cde}$ | 53.79±1.69$^{e}$ |
| **ALP (U/L)** | 356±5.69 | 60.69±1.01$^{bcd}$ | 58.38±0.92$^{de}$ | 61.14±1.14$^{abc}$ | 57.28±0.81$^{e}$ | 59.43±0.35$^{cde}$ | 62.15±1.01$^{ab}$ | 63.38±1.13$^{a}$ |
| **Urea (mg/dl)** | 72.2±2.76 | 62.59±1.41$^{a}$ | 51.01±1.37$^{c}$ | 57.02±2.44$^{ab}$ | 50.15±1.18$^{c}$ | 52.29±1.71$^{bc}$ | 61.52±2.84$^{a}$ | 59.65±2.60$^{a}$ |
| **Creatinine (mg/dl)** | 2.89±0.45 | 61.01±5.04$^{a}$ | 60.21±3.34$^{a}$ | 61.82±4.19$^{a}$ | 60.32±3.89$^{a}$ | 62.40±2.78$^{a}$ | 61.25±3.51$^{a}$ | 61.94±2.50$^{a}$ |
| **Bilirubin (mg/ml)** | 7.56±0.97 | 65.21±2.82$^{a}$ | 58.38±1.23$^{a}$ | 63.10±8.54$^{a}$ | 59.79±4.37$^{a}$ | 60.76±3.44$^{a}$ | 68.17±1.07$^{a}$ | 68.08±5.16$^{a}$ |
| **Total proteins (g/dl)** | 2.14±0.11 | 214.02±18.19$^{a}$ | 185.51±23.36$^{a}$ | 224.45±27.63$^{a}$ | 213.55±29.55$^{a}$ | 210.28±23.22$^{a}$ | 186.29±17.50$^{a}$ | 195.02±19.61$^{a}$ |
| **Lipid peroxidation assay** | | | | | | | | |
| **TBARs (nM/min/mg protein)** | 133.7±2.61 | 35.74±0.82$^{a}$ | 15.08±1.65$^{d}$ | 24.27±1.11$^{c}$ | 15.12±1.74$^{d}$ | 21.39±1.22$^{c}$ | 37.41±0.89$^{a}$ | 31.01±1.32$^{b}$ |

*Results of disease control are expressed as mean±SD (n = 6, where n = number of rats analysed in each group). Alleviative effects of remaining groups are expressed as percent increase (black) or decrease (red) of tested parameters in comparison to the disease control group. Groups are compared statistically to the positive control group and superscripts (a-e) in individual rows specifying significance at $p < 0.05$.*

### *3.4.3.2. $CCL_4$ induced liver inflammation*

The second major *in vivo* model targeted in our project was $CCL_4$ induced liver injury model in male Sprague Dawley rats. Following intra-peritoneal administration of predetermined doses of $CCL_4$ to rats in group III-VIII [disease control, silymarin treated, DSL-EA-LD, DSL-EA-HD, DIL-EA-LD and DIL-EA-HD] and induction of hepatic toxicity, groups were treated with respective drugs and samples. The efficacy of low and high doses of DSL-EA and DIL-EA in alleviation of the hepatic toxicity was measured through extensive hematological, biochemical and histological examinations. The effects produced by crude extracts were compared with the controls used in the study.

#### 3.4.3.2.1. Hematological studies

Results of $CCL_4$ induced hematological variations are shown in table 3.12. As obvious from the data, $CCL_4$ has caused significant aberrations i.e., decline in RBC (6.07±0.32 × 106/$mm^3$) and HGB (8.02±1.02 g/dL) while significant hike in WBC (13.12±0.03 × 103/$mm^3$), neutrophils (62.04±1.31%), monocytes (12.12±1.03%), eosinophils (0.90±0.22%) and basophils (0.81±0.05%) in disease control rats. Hematological parameters of DSL-EA treated groups were significantly different from the disease control group ($p < 0.05$, 0.01 and 0.001). A dose dependent restorative effect was observed in case of DSL-EA extract and HD group showed the best results i.e., RBC (10.06±0.23× 106/$mm^3$), HGB (12.10±1.03 g/dL), WBC (8.34±0.32 × 103/$mm^3$), neutrophils (36.14±0.32%), monocytes (7.64±1.09%), eosinophils (0.761±0.05%) and basophils (0.521±0.12%). The results were almost comparable to the standard control 'silymarin' used in the study. DSL-EA-LD also showed good results, while DIL-EA extracts affected the hematological parameters in a dose dependent manner, though less significantly than DSL-EA.

**Table 3.12** Effect of the *Datura* extracts on the complete blood profile.

| Hematological parameters | Normal control (mean±SEM) | Vehicle control (mean±SEM) | Disease control (mean±SEM) | Silymarin treated (mean±SEM) | DSL-EA-LD (mean±SEM) | DSL-EA-HD (mean±SEM) | DIL-EA-LD (mean±SEM) | DIL-EA-HD (mean±SEM) |
|---|---|---|---|---|---|---|---|---|
| **HGB (g/dL)** | 12.86±0.45 | 12.67±0.92 | 8.02±1.02### | 11.98±0.087* | 10.34±0.23* | 12.10±1.03* | 9.97±0.54* | 11.12±0.98* |
| **RBC (x10$^6$/mm$^3$)** | 10.01±0.34 | 10.2±0.04 | 6.07±0.32### | 9.98±0.22* | 8.78±0.43* | 10.06±0.23* | 7.78±0.64* | 7.91±0.48* |
| **WBC (x10$^3$/mm$^3$)** | 8.078±1.21 | 8.11±0.04 | 13.12±0.03### | 8.98±0.03* | 9.43±0.23* | 8.34±0.32* | 11.2±0.42* | 9.44±0.36* |
| **Neutrophils (%)** | 33.7±4.06 | 31.3±1.07 | 62.04±1.31### | 45.8±0.72** | 59.87±1.23* | 36.14±0.32** | 55±1.42** | 38±2.31** |
| **Monocytes (%)** | 5.83±0.70 | 5.43±0.70 | 12.12±1.03### | 7.09±0.35* | 8.92±1.12* | 7.64±1.09* | 9.12±0.92* | 6.92±1.11* |
| **Eosinophils (%)** | 0.78±0.10 | 0.73±0.05 | 0.90±0.22### | 0.78±0.12** | 0.85±0.01** | 0.761±0.05** | 0.88±0.03** | 0.79±0.09** |
| **Basophils (%)** | 0.45±0.02 | 0.461±0.12 | 0.81±0.05### | 0.57±0.14* | 0.598±0.051* | 0.521±0.12* | 0.63±0.08* | 0.58±0.12* |

*All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group. Results were analyzed by Two-way ANOVA followed by Dunnett's test.*

**Table 3.13** Effect of *Datura* extracts on the biochemical parameters.

| Biochemical parameters | Normal control (mean±SEM) | Vehicle control (mean±SEM) | Disease control (mean±SEM) | Silymarin treated (mean±SEM) | DSL-EA-LD (mean±SEM) | DSL-EA-HD (mean±SEM) | DIL-EA-LD (mean±SEM) | DIL-EA-HD (mean±SEM) |
|---|---|---|---|---|---|---|---|---|
| **ALT (U/L)** | 25.32±1.1 | 25.9±1.50 | 188.73±3.24### | 56.45±1.24** | 78.62±2.81** | 51.32±2.18** | 61.08±3.26** | 54.19±4.03** |
| **AST (U/L)** | 28.9±1.53 | 30.1±2.40 | 231.98±2.32### | 76.2±4.12*** | 96.71±3.12** | 80.35±4.00*** | 111.52±4.68*** | 93.78±5.32** |
| **ALP (U/L)** | 45.12±2.10 | 47.56±1.90 | 172.74±4.41### | 67.54±1.21*** | 92.42±2.32** | 62.81±2.90*** | 122.45±5.97** | 98.32±4.88** |
| **Bilirubin (mg/dL)** | 1.023±0.02 | 1.1±0.011 | 2.99±0.04### | 1.870±0.09*** | 2.09±0.087** | 1.54±0.71*** | 2.59±0.13** | 2.33±0.24*** |
| **Albumin (g/L)** | 16.34±1.32 | 15.32±0.87 | 4.28±0.09### | 12.45±0.10*** | 8.12±0.84** | 13.5±0.09*** | 6.32±0.41* | 11.56±1.18** |
| **Creatinine (mg/dL)** | 0.276±0.03 | 0.284±0.10 | 1.08±0.04### | 0.41±0.089** | 0.73±0.13* | 0.378±0.10** | 0.89±0.09* | 0.53±0.05** |

*Values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group. Results were analyzed by Two-way ANOVA followed by Dunnett's test.*

3.4.3.2.2. Biochemical analysis

The deleterious effects of CCL4 on liver and kidneys were further confirmed by numerous biochemical tests performed using serum acquired from rats of each study group. Liver function tests showed significantly higher level of liver enzymes ($p < 0.05$, 0.01 and 0.001) in comparison to other study groups. ALT, AST, ALP and bilirubin levels recorded in serum of disease control rats were 188.73±3.24, 231.98±2.32, 172.74±4.41 (U/L), and 2.99±0.04 mg/dL respectively. Furthermore, albumin level was markedly lower i.e., 4.28±0.09 g/L while creatinine level was elevated in disease control group (1.08±0.04 mg/dL). There was no statistically significant difference ($p <$ 0.05, 0.01 and 0.001) in the estimated biochemical parameters of positive control, DSL-EA-LD and DSL-EA-HD groups. CCL4 induced damage to liver functionality was reverted by the extracts in a dose dependent manner. The restorative effect of tested extracts can be graded as follows; DSL-EA-HD > DIL-EA-HD > DSL-EA-LD > DIL-EA-LD. The results are further elaborated in table 3.13

3.4.3.2.3. Endogenous antioxidant enzymes and GSH levels

The effect of DSL-EA and DIL-EA treatment on endogenous antioxidant enzymes and GSH levels of the study groups is presented in Figure 13. A significant ($p < 0.05$, 0.01 and 0.001) reduction in activity level of GST, GSH, SOD, CAT and POD was observed in disease control group further confirming the damage inflicted by CCL4 dosing. High dose treatments with DSL-EA curbed the damage and elevated the level of aforementioned antioxidant enzymes and GSH to a similar extent as observed in silymarin treated group. The% activity levels of tested antioxidant defense system in liver tissues was restored by the extracts in a dose dependent manner in the following order; DSL-EA-HD > DIL-EA-HD > DSL-EA-LD > DIL-EA-LD as presented in figure 3.14. As observed in the previous investigations, DSL-EA was slightly dominant in terms of the restorative effects following $CCL_4$ induced liver injury.

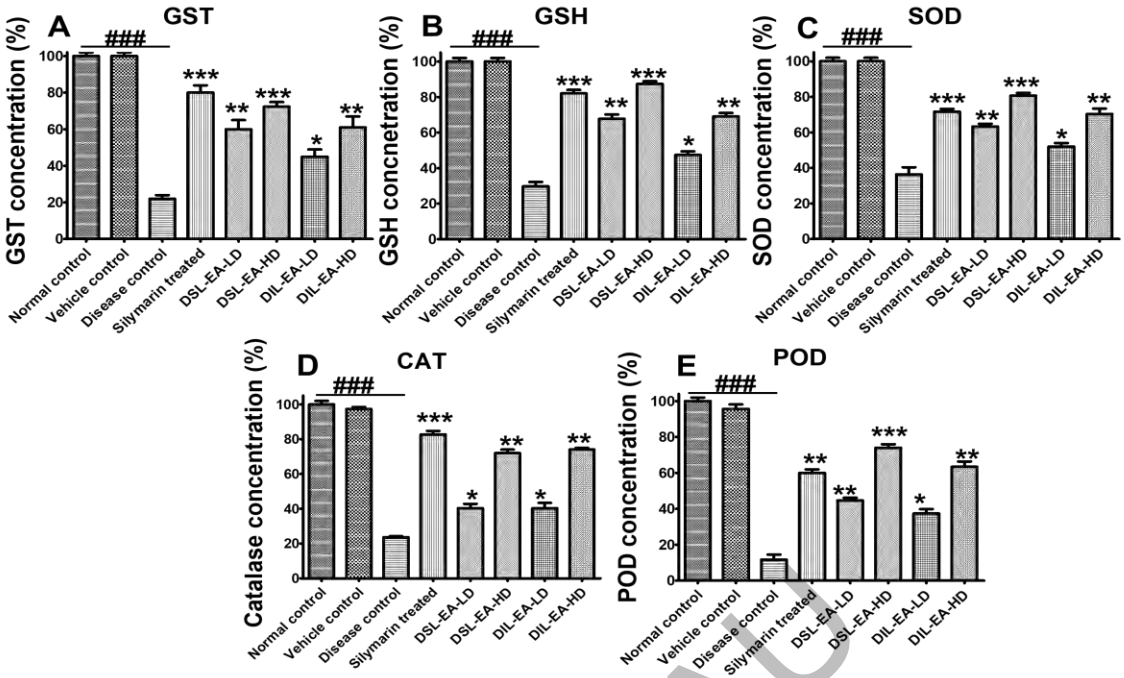


**Figure 3.14** Effect of DSL-EA and DIL-EA on glutathione S-transferase (GST) [A], glutathione (GSH) [B], superoxide dismutase (SOD) [C], Catalase [D] and peroxidase (POD) [E] levels in liver tissue compared to the disease control group.
***Note:*** *All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

3.4.3.2.4. Oxidative stress markers (TBARs, MPO and NO estimation)

The oxidative stress markers investigated in current study were NO, TBARs and MPO and their levels were greatly elevated in liver tissues of disease control group. NO levels were assessed both in plasma and liver tissue since it is an important mediator of inflammation influenced by iNOS gene. As presented in figure 3.15, % NO levels both in plasma and tissue homogenates were excessively elevated in the disease control group. DSL-EA-HD and silymarin decreased the NO levels in a significant ($p < 0.05$, 0.01 and 0.001) and comparable manner while DSL-EA-LD and DIL-EA-HD treatments also yielded very good results.

There was statistically significant ($p < 0.05$, 0.01 and 0.001) difference between the % TBARs level of disease control rats and groups treated with extracts. Dose dependent decline in the oxidative stress was observed in treatment groups which further support the results of % enzyme activity and antioxidant status as discussed earlier. High dose treatments of both extracts reduced the TBARs levels in liver tissues by more than 50% while low dose treatment also resulted in moderate improvement of the overall oxidative status of test rats. The details are presented in figure 3.15.

The MPO expression level in liver tissue acts as marker of neutrophilic infiltration during inflammation. As expected in $CCL_4$ induced liver injury, MPO expression was markedly increased in the liver tissues of disease control rats. Silymarin, DSL-EA-LD and DSLA-EA-HD and DIL-EA-HD decreased the % MPO activity in a statistically significant and comparable manner ($p < 0.05$, 0.01 and 0.001) as shown in figure 3.15. The reduced MPO activity greatly helps in curbing neutrophilic infiltration and inflammation in liver tissues.

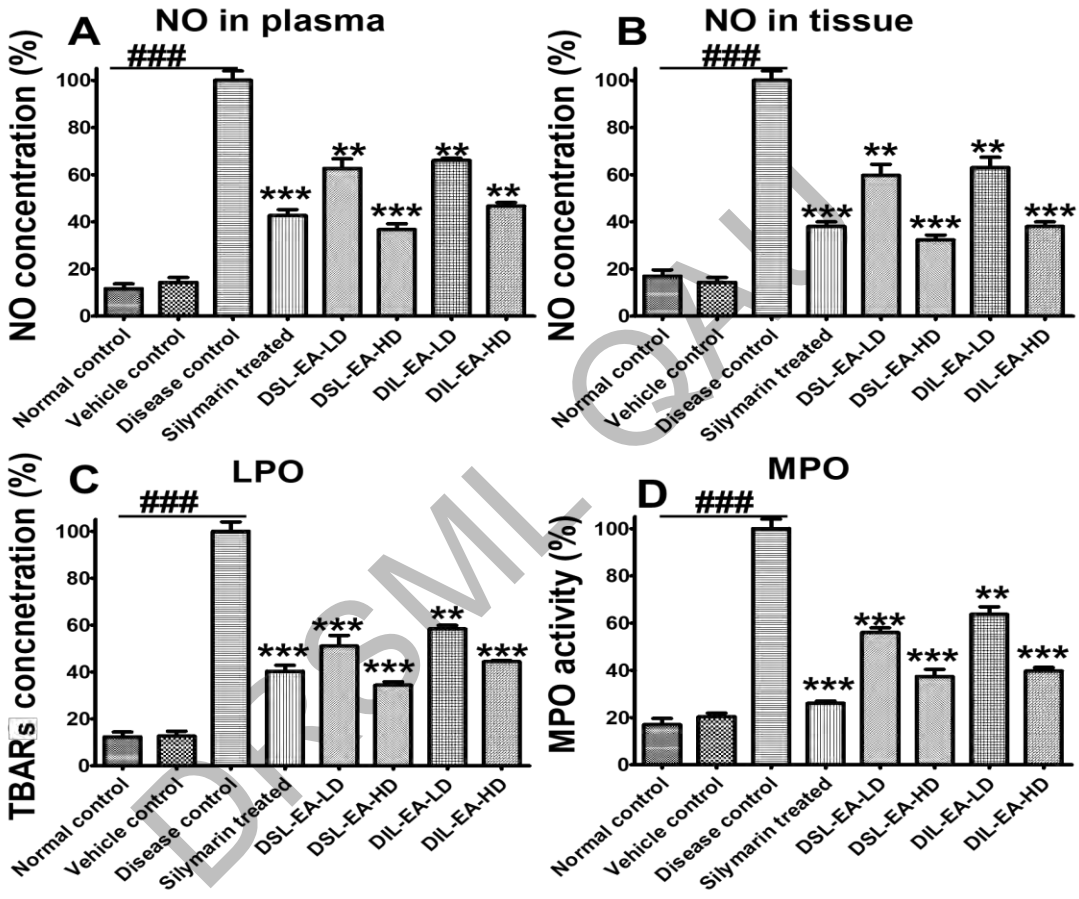




**Figure 3.15** Effect of the *Datura* extracts on the oxidative stress markers.
***Note:*** *Nitric oxide (NO) in plasma [A], NO in liver tissue [B], malondialdehyde (MDA) [C] and myeloperoxidase (MPO) [D] concentration in liver tissues compared to disease control group. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001. LPO; lipid peroxidation assay.*

3.4.3.2.5. Histology (H & E and Masson's trichrome staining)

Histological examination has further ratified the findings of biochemical tests performed previously. Liver tissues of normal and vehicle control groups had normal morphological features i.e., unharmed hepatocytes, central veins and sinusoids. The disease control group incurred severe liver injury due to $CCL_4$ injections. The most obvious signs of liver damage were immune cell infiltration, fibrosis, necrosed

hepatocytes and edema as evident in figure 3.16 [(H&E) staining]. In an identical manner to effects of DSL-EA and DIL-EA observed in biochemical parameters, there was a significant ($p < 0.05$, 0.01 and 0.001) dose dependent restorative effect on liver tissues. Low dose of administered extracts tapered the $CCL_4$ induced liver injury which is clearly visible from the reduced number of necrotic hepatic cells in liver histology images. Aberrations in liver's histoarchitecture were significantly curtailed by high doses of DSL-EA and DIL-EA and there was no significant difference between the liver histology score of silymarin and groups that received high dose treatments of both extracts. The details can be seen in figure 3.16.

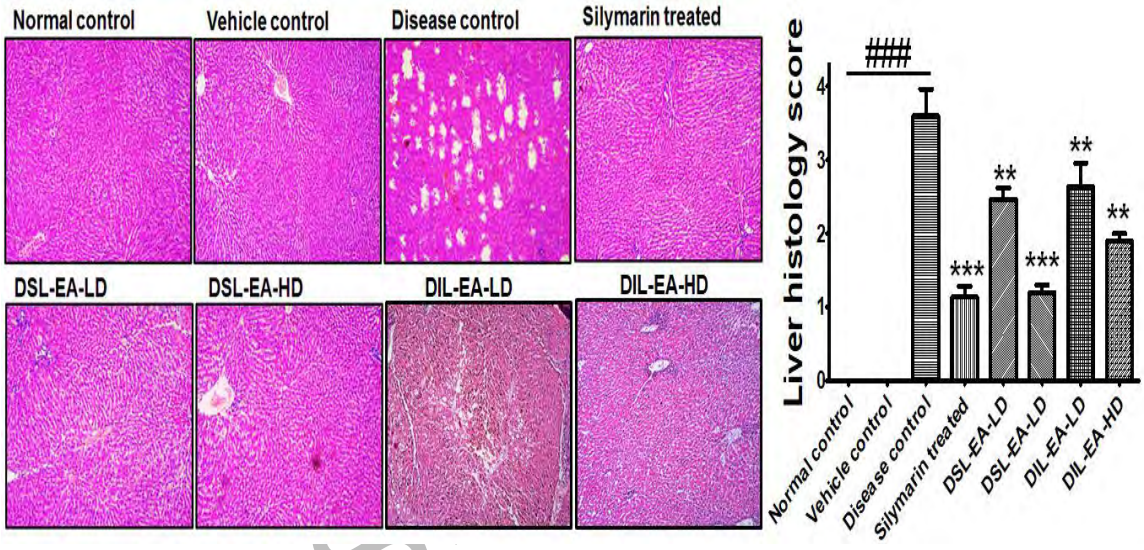


**Figure 3.16** Hematoxylin-eosin staining of the liver tissue.
***Note:*** *The restorative effect of Datura extracts on liver tissues following $CCL_4$ induced liver injury. The extracts markedly improved the histological parameters such as immune cell infiltration, fibrosis and edema compared to the disease control. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

The excessive accumulation of collagen and subsequent liver damage i.e., fibrosis was confirmed by Masson's trichrome staining. The results were in complete accordance with H & E-stained slides of liver tissue. Marked reduction in accumulation of extracellular matrix proteins (collagen) was observed in DSL-EA-HD, DIL-EA-HD treated rats and likewise in positive control group. Low dose of the extracts also resulted in alleviating the injury caused by $CCL_4$ compared to the disease control group as shown in figure 3.17.

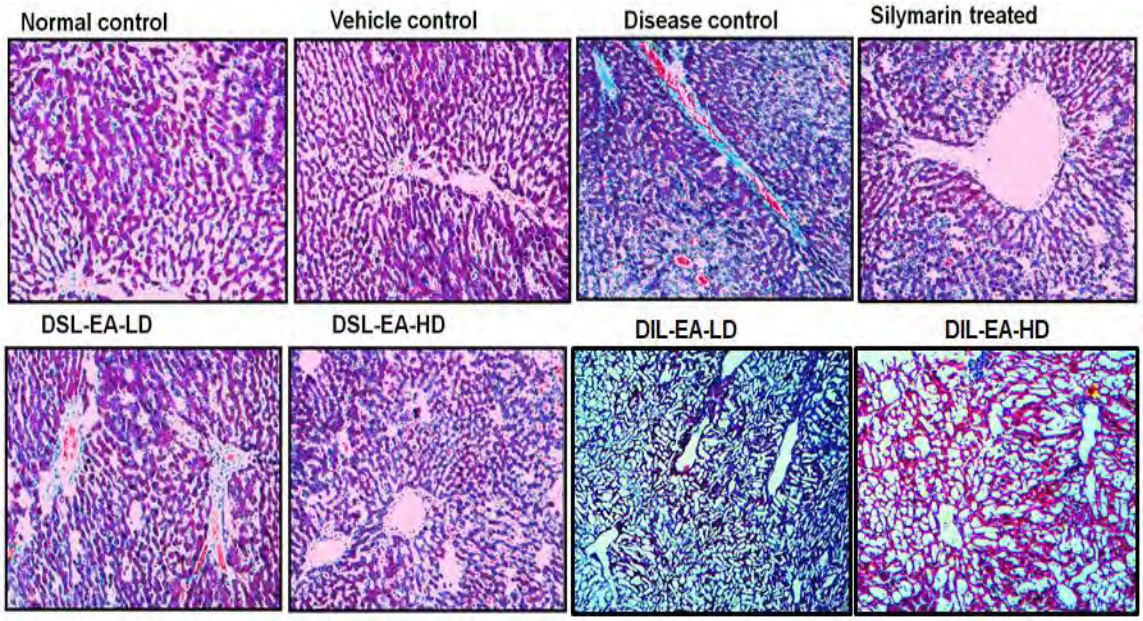


**Figure 3.17** Masson's trichome staining of the liver tissues.
***Note:*** *The extracts markedly improved the histological parameters and inhibited the liver fibrosis compared to the disease control.*

3.4.3.2.6. Effect on Nrf2 and iNOS expression using immunohistochemistry

The expression of nuclear factor erythroid 2 (Nrf2) and inducible nitric oxide synthase (iNOS) was investigated using immunostaining. There was marked reduction in Nrf2 expression in CCL4 treated disease control group confirming the diminished resistance to oxidative stress. The expression level of iNOS on the other hand was increased due to obvious signs of tissue damage in disease control rats. As shown in figure 3.18, the extracts have restored the normal Nrf2 and iNOS expression levels in a significant manner ($p < 0.05$, 0.01 and 0.001), though DSL-EA proved to be the dominant of the two selected extracts, DIL-EA given in high dose also displayed good restorative effects. As observed in hematological and biochemical investigations, the effect was dose dependent, with DSL-EA-HD and silymarin treated groups revealing identical immunoreactivity scores (figure 3.18).

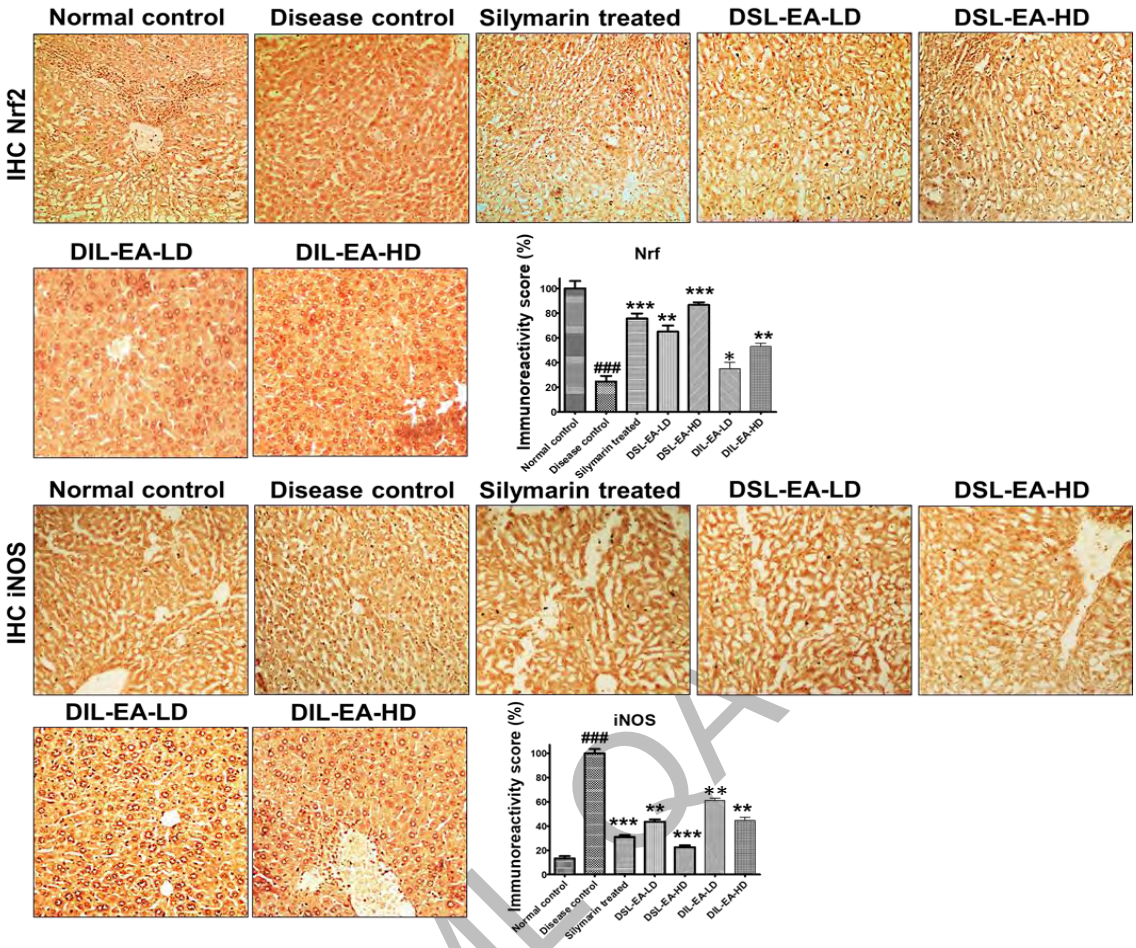


**Figure 3.18** Effect of high and low dose treatment of DSL-EA and DIL-EA on expression level of nuclear factor erythroid 2 and inducible nitric oxide synthase in the liver tissue.
***Note:*** *Using immunohistochemistry analysis. The extracts induced the expression of Nrf2, while inhibited the iNOS expression in a dose dependent manner compared to the disease control. IHC: Immunohistochemistry. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

Chronic *in vivo* studies stated in preceding section presented a comprehensive comparative analysis of the selected extracts. The preclinical anticancer and anti-inflammatory potential of EA leaf extracts of both species was extensively evaluated. It was concluded that EA extract of *D.stramonium* was comparatively dominant as far as its *in vivo* response against benzene induced leukemia and $CCL_4$ induced liver inflammation is concerned. It was therefore decided to carry DSL-EA forward to the next stage of our study i.e., preparative scale extraction and isolation of potentially active moieties therefrom.

## 3.5. Preparative Scale Extraction, Isolation and Characterization of Compounds

### 3.5.1. Bulk extraction and isolation of compounds

DSL-EA, owing to its significant activity in *in vitro* studies, safety profile and *in vivo* anticancer as well as ant-inflammatory studies, was selected from the pool of tested crude extracts. Therefore, DSL-EA was the extract used for preparative scale extraction and isolation of compounds. The % extraction recovery recorded with NH solvent was 0.71% and in case of EA it was 2.61%. Preparative scale extraction yielded 31.3 gm DSL-NH and 115 gm DSL-EA. Isolation of compounds from DSL-EA extract was carried out *via* column chromatography involving a series of both gravity and flash chromatography columns. Three compounds were isolated from DSL-EA in our study and characterized using selected characterization tools. Based on the quantity and chemical nature of isolated compounds they were subjected to comprehensive preclinical pharmacological assessment using the chronic in vivo models employed in the earlier section.

### 3.5.2. Characterization of isolated compounds

The isolated compounds were characterized using 1D and 2D NMR techniques and the details are given as under.

#### *3.5.2.1. Structure elucidation of "compound a"*

(3S,8S,9S,10R,13R,14S,17R)-17-[(E,2R,5S)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H cyclopenta[a]phenanthren-3-ol) or Stigmasterol.

The "compound a" isolated from DSL-EA was analysed by 1D and 2D NMR techniques and the molecular formula $C_{29}H_{48}O$ was determined. The structure (figure 3.27) was assigned by comparison of NMR data with already published reports in the literature.

The $^{1}$H-NMR spectrum (figure 3.19) of "compound a" present a total proton count of forty-eight and revealed the presence of three olefinic methine protons resonating at 5.04 ppm (dd, J= 15.2, 8.8 Hz, 1 H, H-21), 5.17 ppm (dd, J= 15.2, 8.8 Hz, 1 H, H-20) and 5.37 ppm (br.d, J= 5.6 Hz, 1 H, H-6). The signal at 3.55 ppm (m) corresponds to CH proton directly attached to the OH group. Furthermore, the $^{1}$H-NMR spectrum also confirmed the presence of six methyl groups resonating at 0.72 ppm (s, 3 H, Me-29), 0.81 ppm (d, J= 7.6 Hz, 3 H, Me-26), 0.83 ppm (t, J= 2 × 6.8 Hz, 3 H, Me-24), 0.87

ppm (d, J= 6.4 Hz, 3 H, Me-27), 1.03 ppm (s, 3 H, Me-28) and 1.05 ppm (overlapping-d, J= 7.6 Hz, 3 H, Me-19). All the corresponding carbons were identified using the heteronuclear single quantum coherence (HSQC) spectrum.

The $^{13}C$ NMR spectrum (figure 3.20) of "compound a" also indicated the resonances for twenty-nine carbons while distortionless enhancement by polarization transfer (DEPT-135) spectrum (figure 3.21) showed nine $CH_2$ groups pointing downwards with resonating frequencies of 21.1 ppm (C11), 24.4 ppm (C15), 25.4 ppm (C23), 28.9 ppm (C16), 31.7 ppm (C2, C7), 37.3 ppm (C1), 39.7 ppm (C12) and 42.3 ppm (C4). Three quaternary carbons were identified by comparing DEPT-135 with $^{13}C$ NMR spectrum resonating at 36.5 ppm (C10), 42.23 ppm (C13) and 140.8 ppm (C5). All the remaining protons and carbons were identified using HSQC (figure 3.22, 3.23 and 3.24) and heteronuclear multiple bond correlation (HMBC) spectra (figure 3.25 and 3.26) of the compound.

**$^{1}H$ NMR (400 MHz, CHLOROFORM-d) □ ppm:** 0.72 (s, 3 H, Me-29), 0.81 (d, J= 7.6 Hz, 3 H, Me-26), 0.83 (t, J= 2 × 6.8 Hz, 3 H, Me-24), 0.87 (d, J= 6.4 Hz, 3 H, Me-27), 0.95 (m, 1 H, H-9), 1.02 (m, 1 H, H-14), 1.03 (s, 3 H, Me-28), 1.05 (overlapping-d, J= 7.6 Hz, 3 H, Me-19), 1.08 (m, 1 H, H-15a), 1.10 (m, 1 H, H-1a), 1.13 (m, 1 H, H-17), 1.18 (m, 2 H, H-23a,b), 1.24 (m, 1 H, H-12a), 1.29 (m, 1 H, H-16a), 1.46 (m, 1 H, H-2a), 1.47 (m, 1 H, H-25), 1.48 (m, 1 H, H-11a), 1.49 (m, 1 H, H-8 ), 1.51 (m, 1 H, H-11b), 1.55 (m, 1 H, H-22 ), 1.57 (m, 1 H, H-7a), 1.58 (m, 1 H, H-15b), 1.72 (m, 1 H, H-16b), 1.86 (m, 1 H, H-2b), 1.88 (m, 1 H, H-1b), 1.98 (m, 1 H, H-12b), 2.03 (m, 1 H, H-7b), 2.08 (m, 1 H, H-18), 2.29 (m, 2 H, H-4a,b), 3.55 (m, 1 H, H-3), 5.04 (dd, J= 15.2, 8.8 Hz, 1 H, H-21), 5.17 (dd, J= 15.2, 8.8 Hz, 1 H, H-20), 5.37 (br.d, J= 5.6 Hz, 1 H, H-6).

**$^{13}C$ NMR (101 MHz, CHLOROFORM-d) □ ppm:** 12.1 (C29), 12.3 (C24), 18.9 (C26), 19.4 (C28), 21.1 (C11), 21.1 (C27), 21.2 (C19), 24.4 (C15), 25.4 (C23), 28.9 (C16), 31.7 (C2, C7), 31.9 (C8, C25), 36.5 (C10), 37.3 (C1), 39.7 (C12), 40.5 (C18), 42.2 (C13), 42.3 (C4), 50.2 (C9), 51.3 (C22), 55.9 (C17), 56.9 (C14), 71.8 (C3), 121.7 (C6), 129.3 (C21), 138.3 (C20), 140.8 (C5).

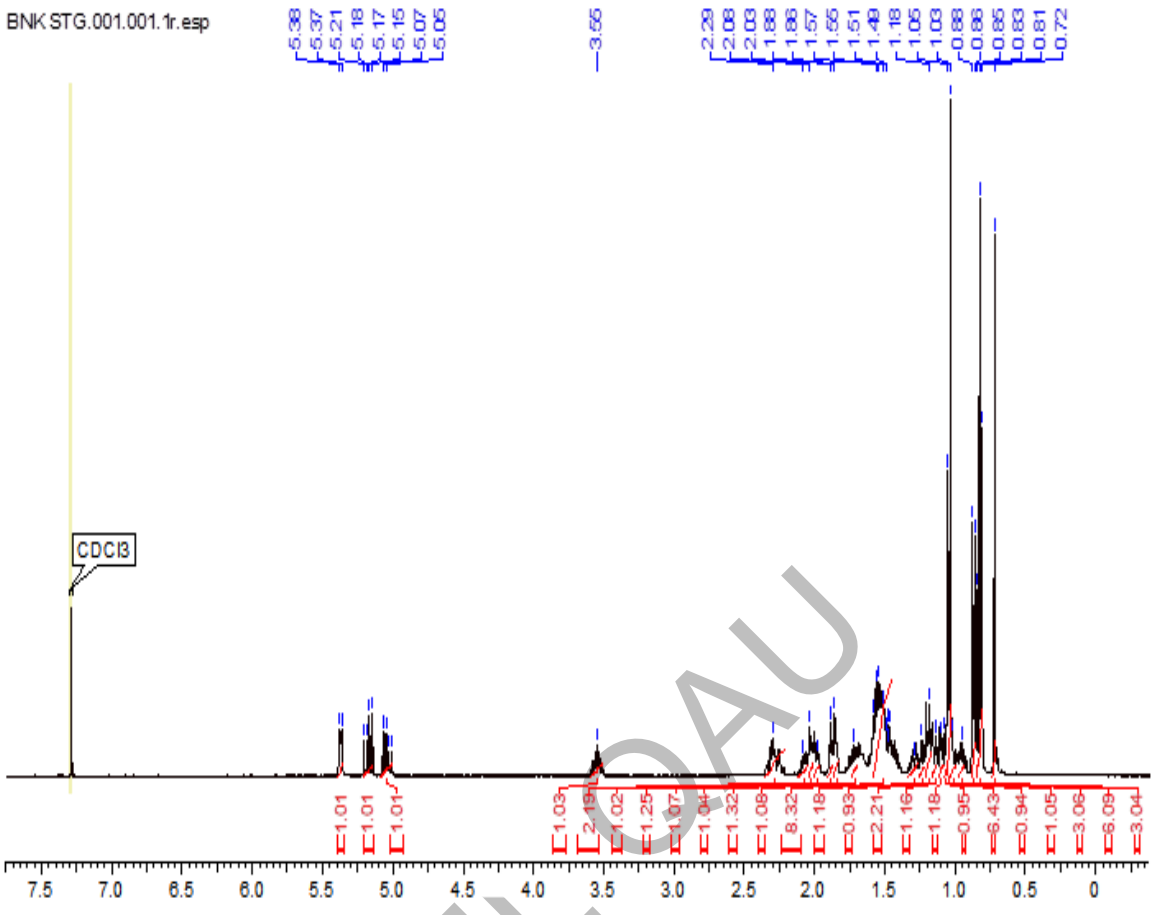


**Figure 3.19** $^{1}$H NMR (400 MHz, chloroform-d) spectrum of "compound a".

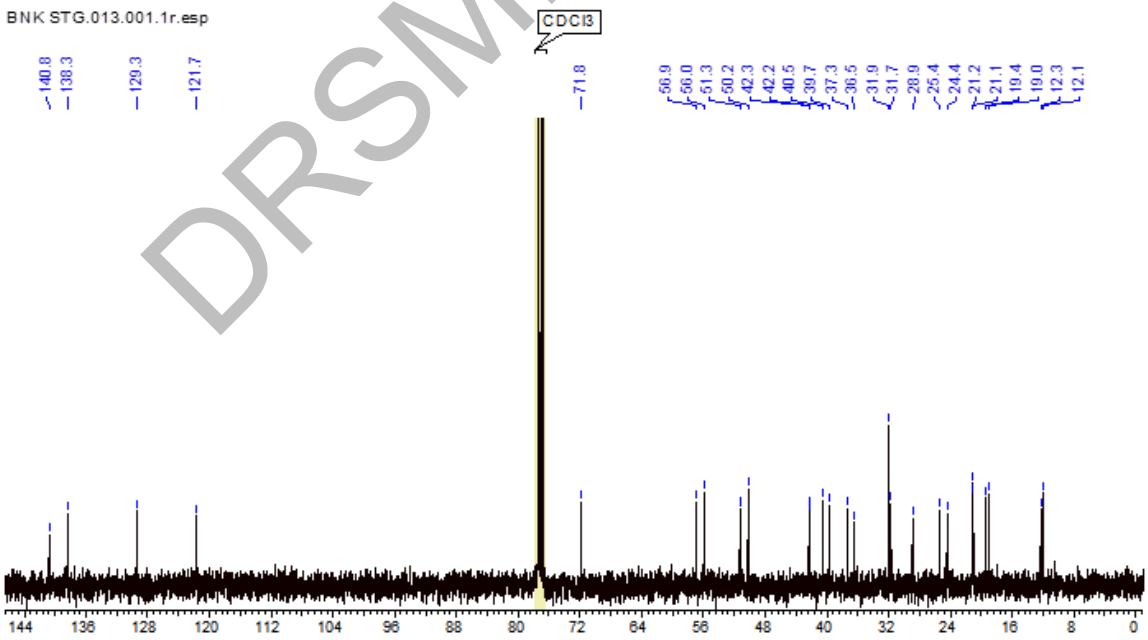


**Figure 3.20** $^{13}$C NMR (101 MHz, chloroform-d) spectrum of "compound a".

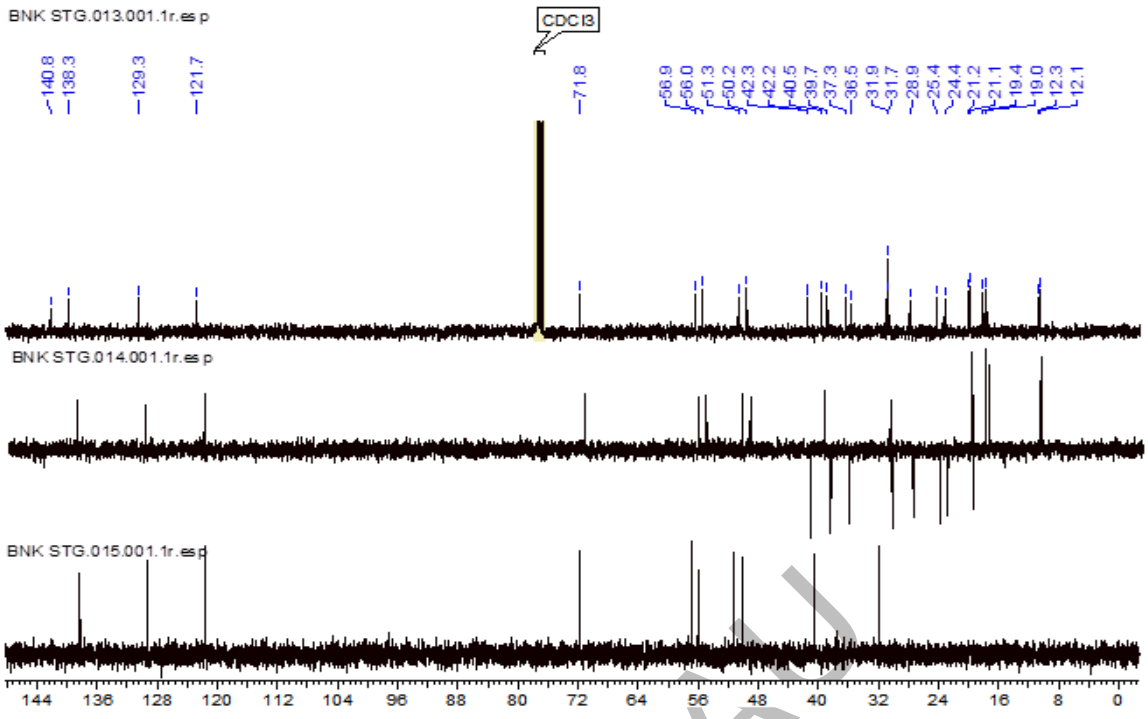


**Figure 3.21** $^{13}$C NMR, DEPT-90 and DEPT-135 spectra of "compound a".

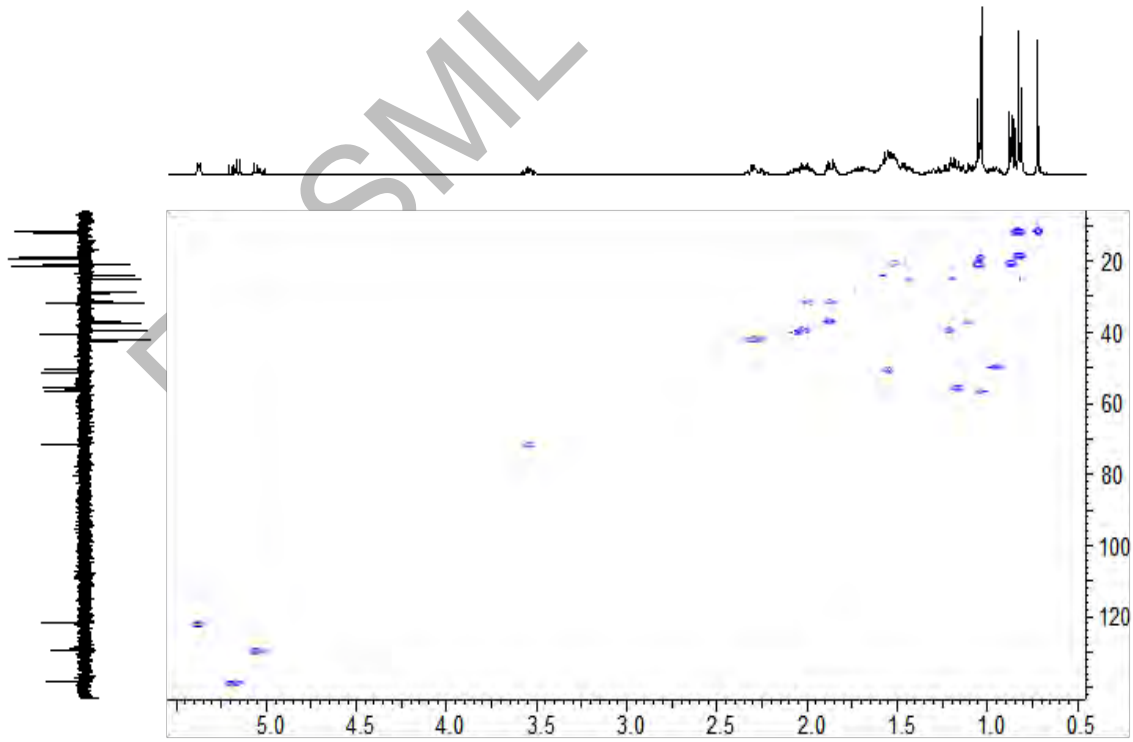


**Figure 3.22** HSQC spectrum of "compound a".

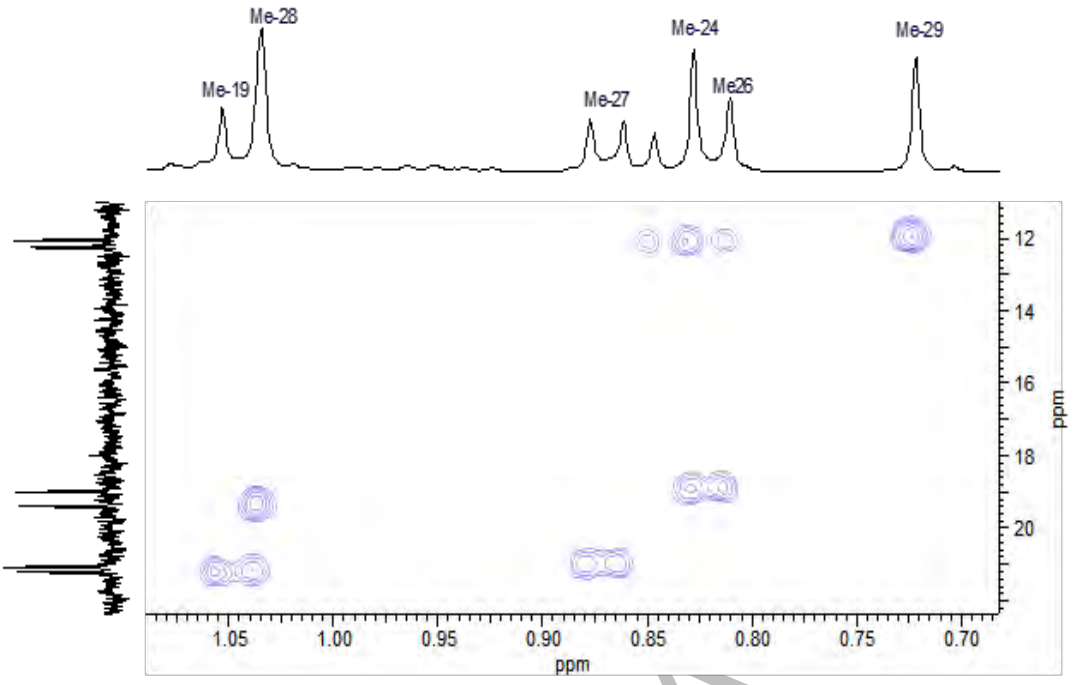


**Figure 3.23** Expansion of selected region of HSQC spectrum of "compound a", showing six methyl groups.

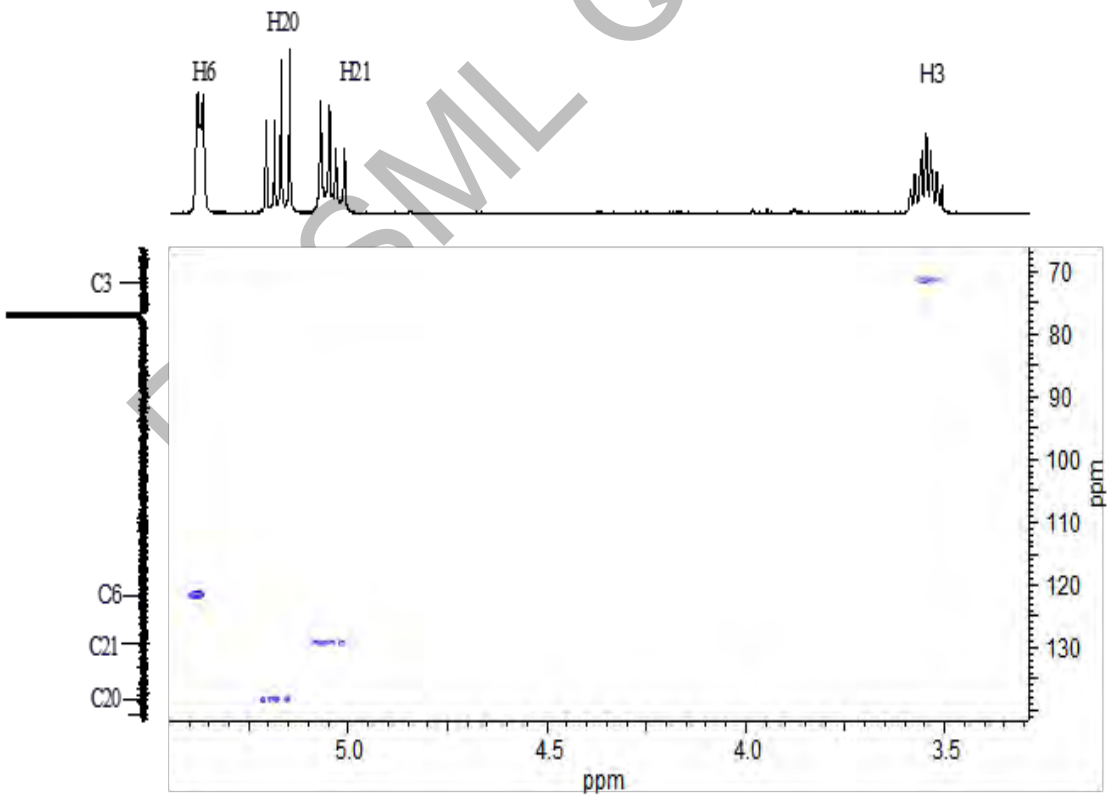


**Figure 3.24** Expansion of selected region of HSQC spectrum of "compound a".

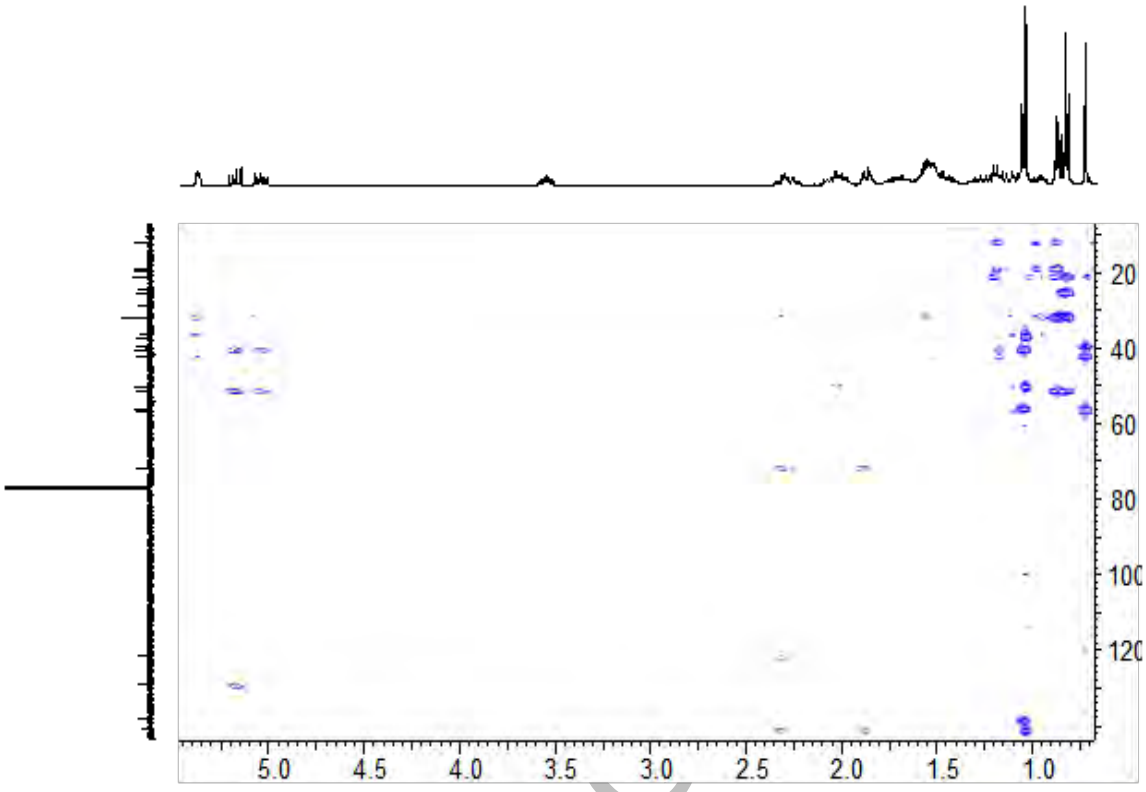


**Figure 3.25** HMBC spectrum of "compound a".

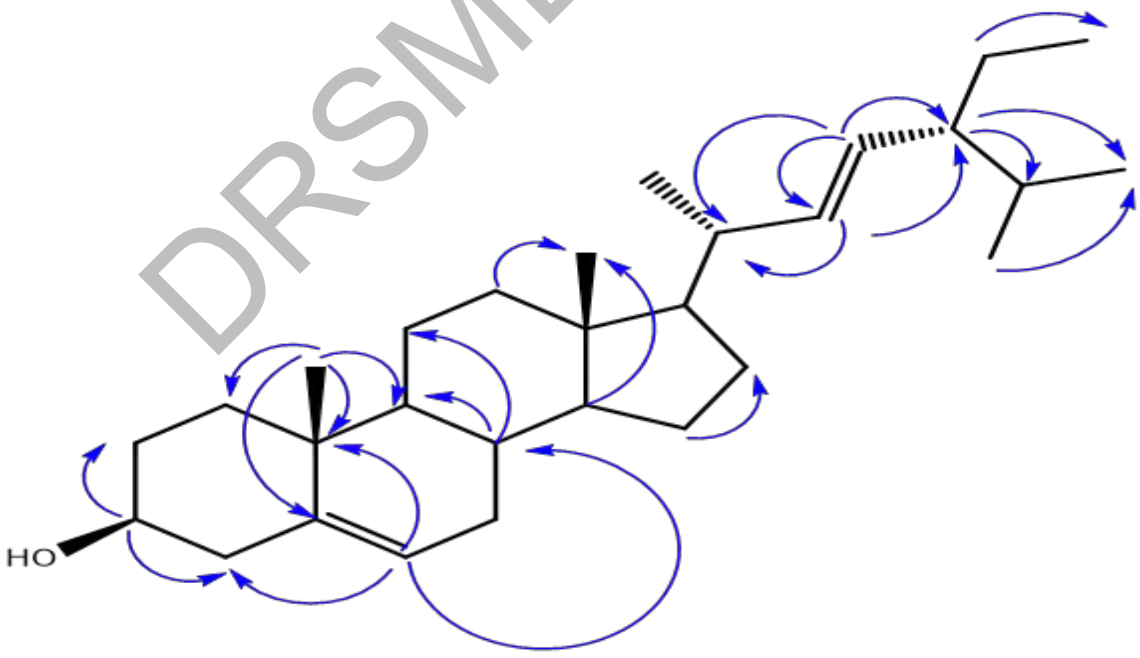


**Figure 3.26** HMBC correlations of compound a.

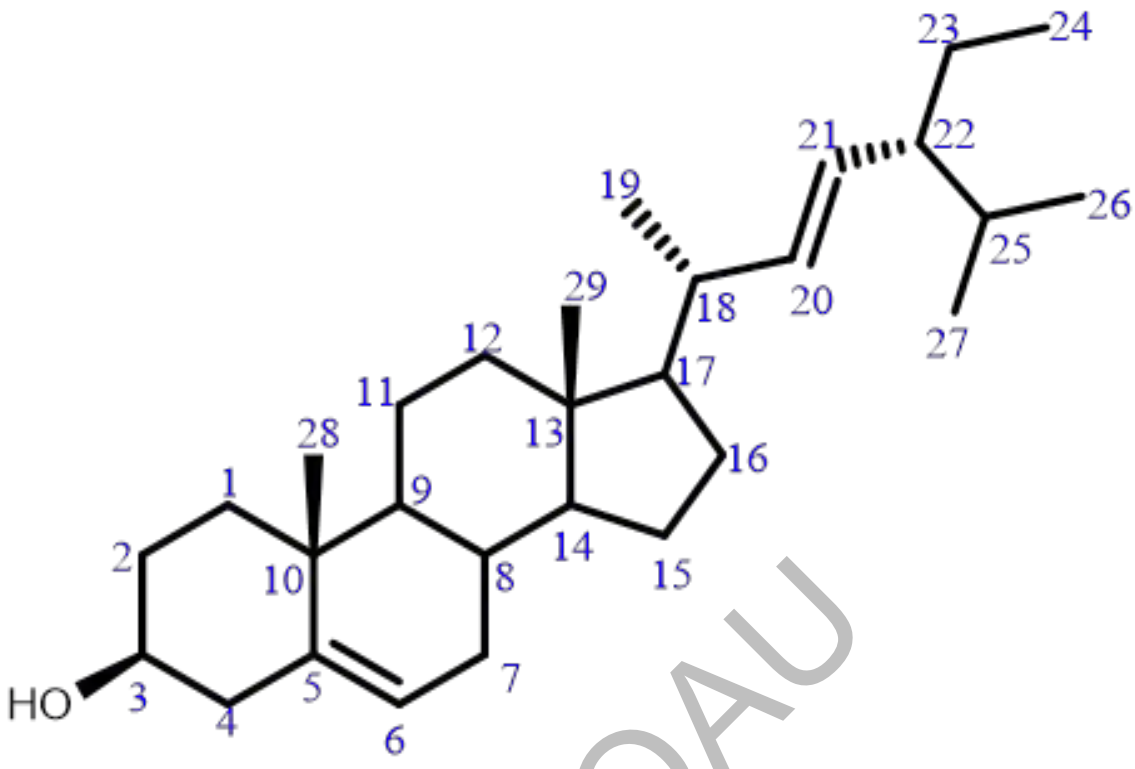


**Figure 3.27** Elucidated structure of "compound a".

### *3.5.2.2. Structure elucidation of "compound b"*

[3-(4-hydroxy-3-methoxyphenyl) prop-2-enoic acid] or Ferulic acid

For the structural confirmation of "compound b", a combination of 1D and 2D NMR techniques were used. The $^{1}$H NMR spectrum (figure 3.28) represents a count of 8 protons. Starting with the $^{1}$H NMR spectrum, the most up field signal appearing as singlet at 3.82 ppm represents the methoxy protons. The next signal appearing as doublet at 6.20 ppm with an integral value of 1 corresponds to the acrylic acid side chain proton H8. Moving further downfield towards the aromatic region, the next two doublets at 6.78 and 6.94 ppm each with an integral value of 1 represent the two aromatic protons H5 and H6 respectively. Both H5 and H6 are distinguished by the fact that in HMBC spectrum (figure 3.31 and 3.32) the H6 proton shows coupling to C2 carbon while H5 shows coupling to C3 carbon. The remaining aryl proton H2 was found to be resonating as a singlet at 7.03 ppm while the last and most downfield signal appearing as doublet at 7.46 ppm represents the acrylic acid side chain proton H7. All the corresponding carbons were identified using HSQC spectrum (figure 3.30). The elucidate structure of "compound b" is given in figure 3.33.

The $^{13}C$ spectrum (figure 3.29) confirmed the presence of all expected 10 carbons. The carbonyl carbon C9 was found resonating at 168.6 ppm while the quaternary carbons C1, C3 and C4 were found resonating at 126.2 ppm (C1), 148.1 ppm (C3) and 149.3 ppm (C4).

**$^{1}H$ NMR (600 MHz, DMSO-d6) δ ppm:** 3.82 (3 H, s, OCH3), 6.20 (1 H, d, CH=CH, H-8, J = 15.8 Hz), 6.78 (1 H, d, ArH, H-5, J = 8.4 Hz), 6.94 (1 H, d, ArH, H-6, J = 8.4 Hz), 7.03 (1 H, s, ArH, H-2), 7.46 (1 H, d, CH=CH, H-7, J = 15.8 Hz).

**$^{13}C$ NMR (151 MHz, DMSO-d6) δ ppm:** 55.9 (OCH3), 110.7 (ArCH, C2), 115.7 (CH=CH, C8), 115.8 (ArCH, C5), 122.8 (ArCH, C6), 126.2 (ArC, C1), 144.7 (CH=CH, C7), 148.1 (ArC, C3), 149.3 (ArC-OH, C4), 168.6 (COOH, C9).

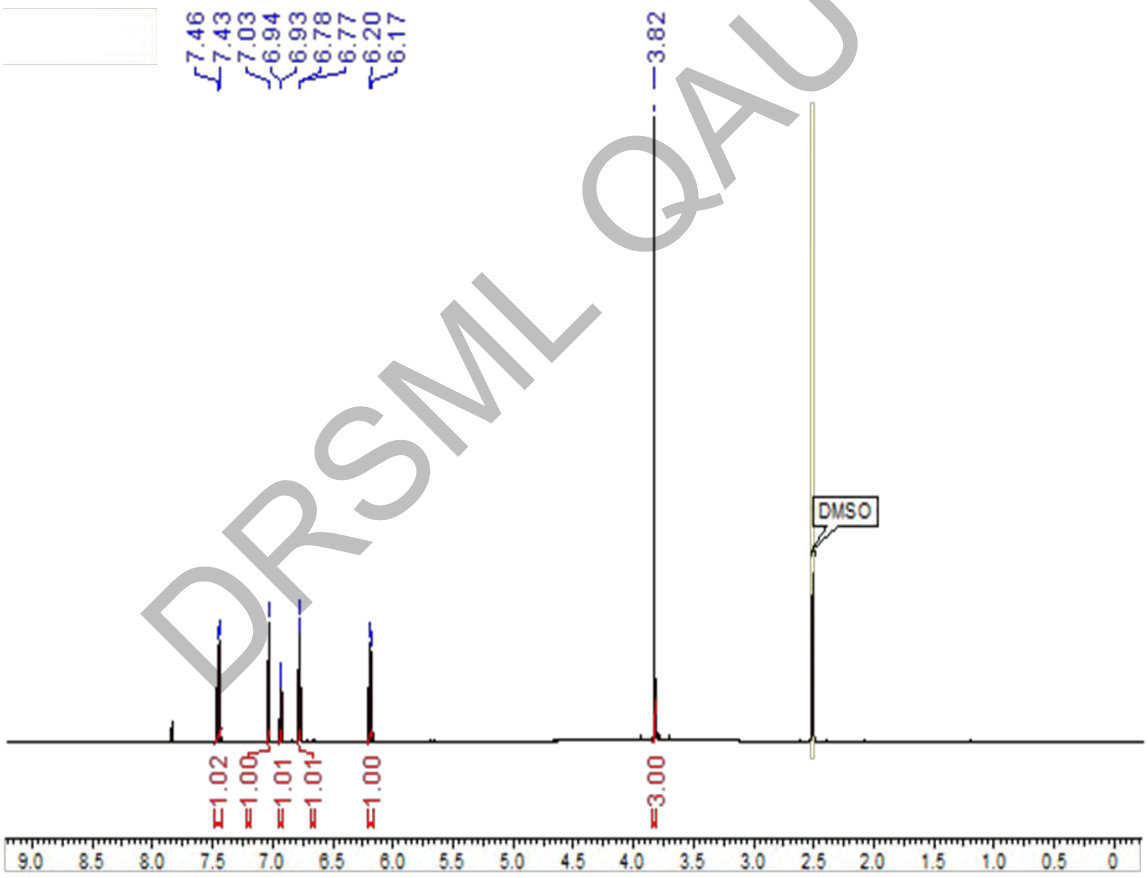




**Figure 3.28** $^{1}H$ NMR (400 MHz, DMSO) spectrum of "compound b".

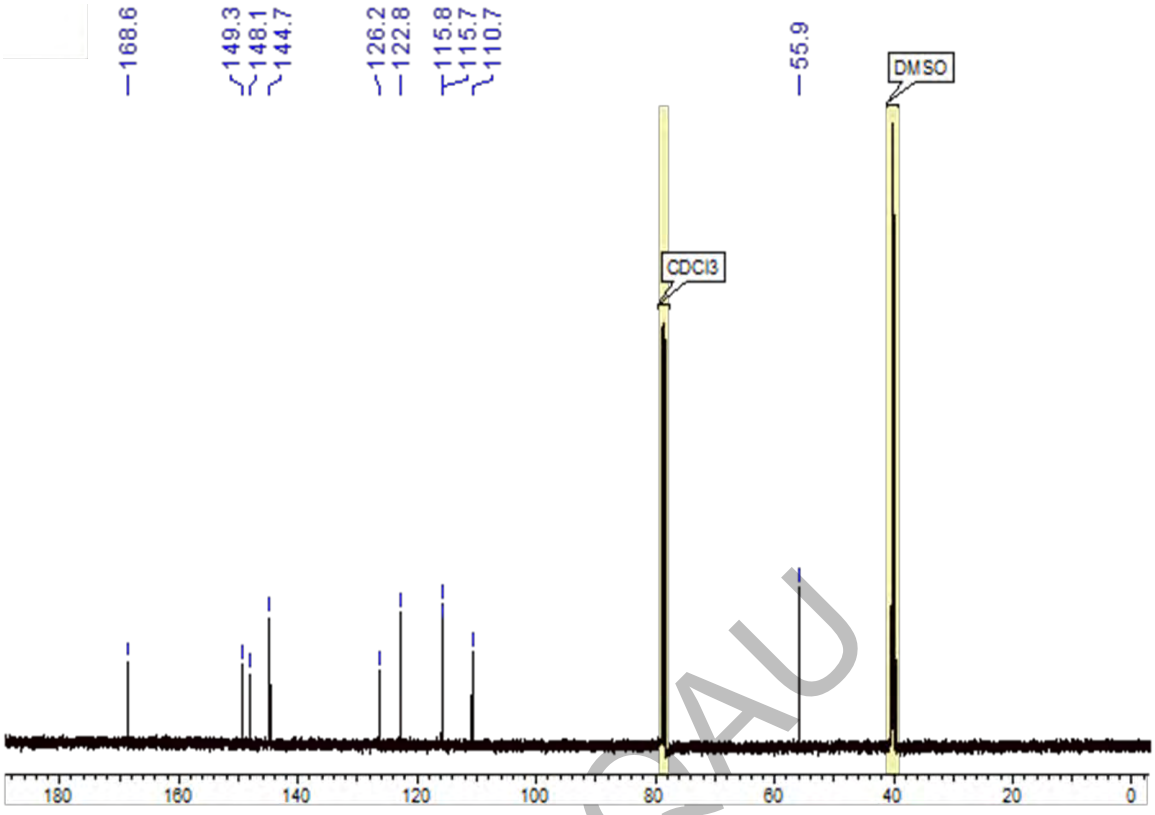


**Figure 3.29** $^{13}$C NMR (DMSO, chloroform-*d*) spectrum of "compound b". DSMO; Dimethyl sulfoxide.

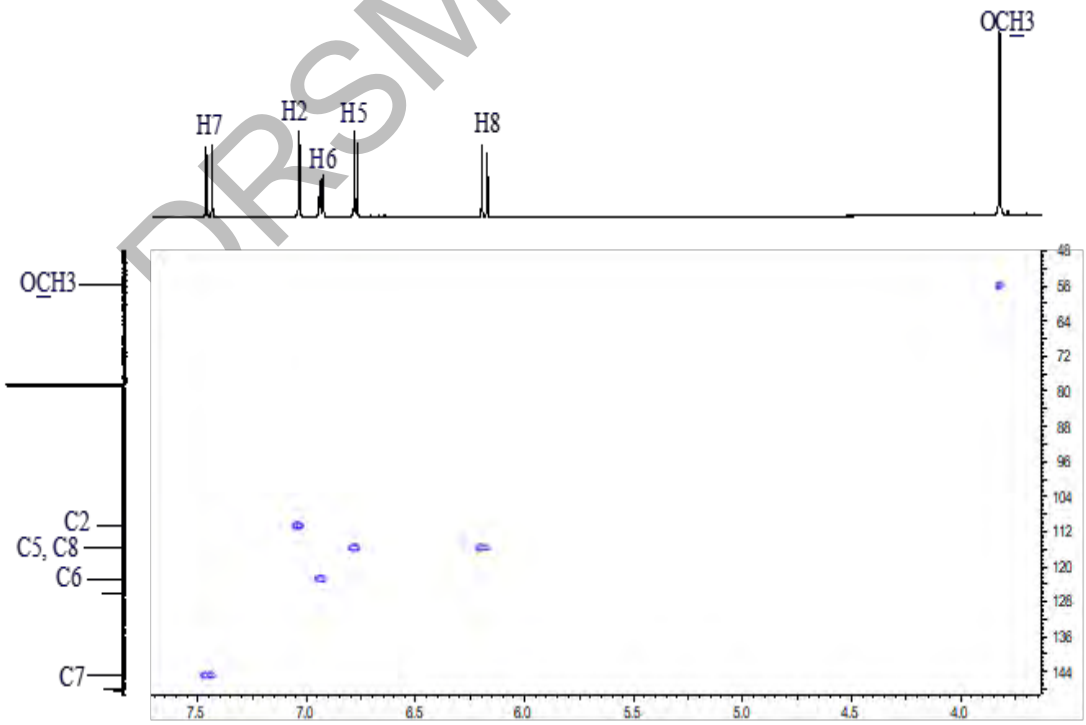


**Figure 3.30** HSQC spectrum of "compound b".

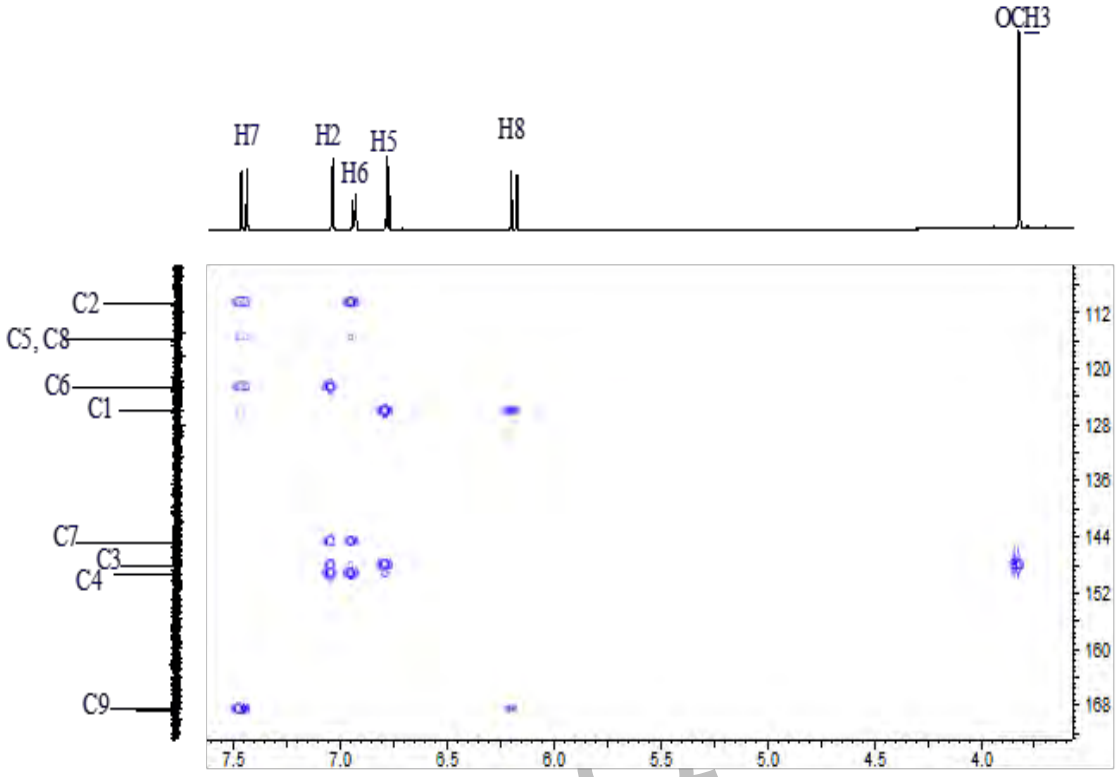


**Figure 3.31** HMBC spectrum of "compound b".

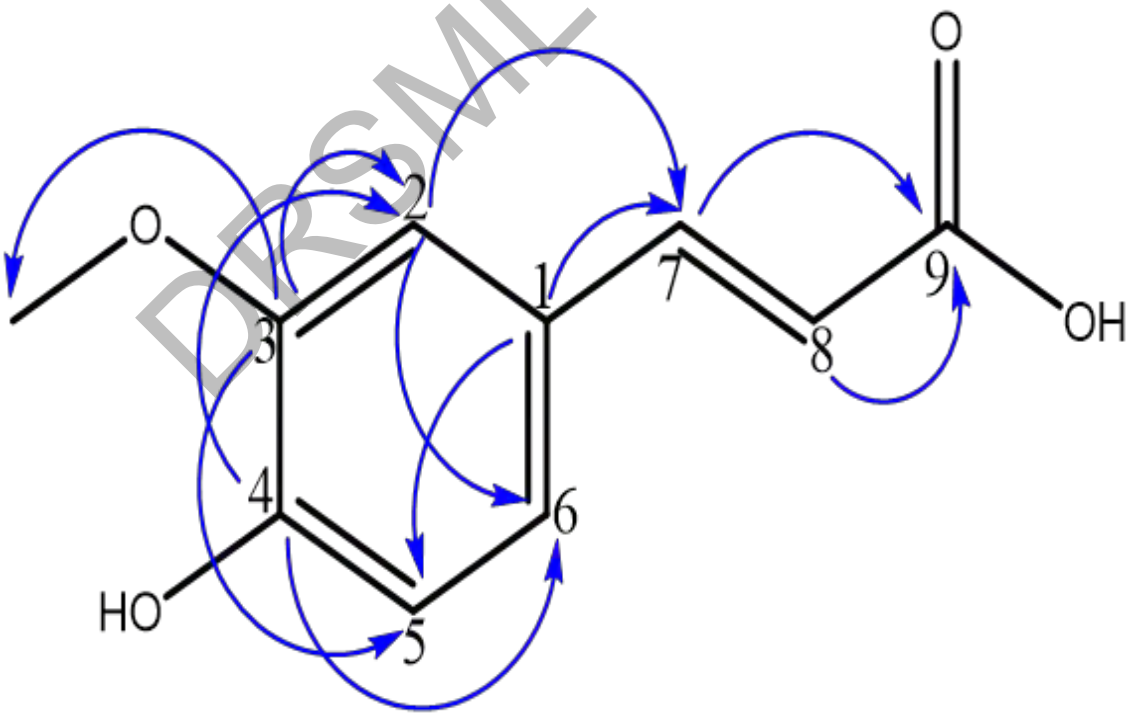


**Figure 3.32** HMBC correlations of "compound b".

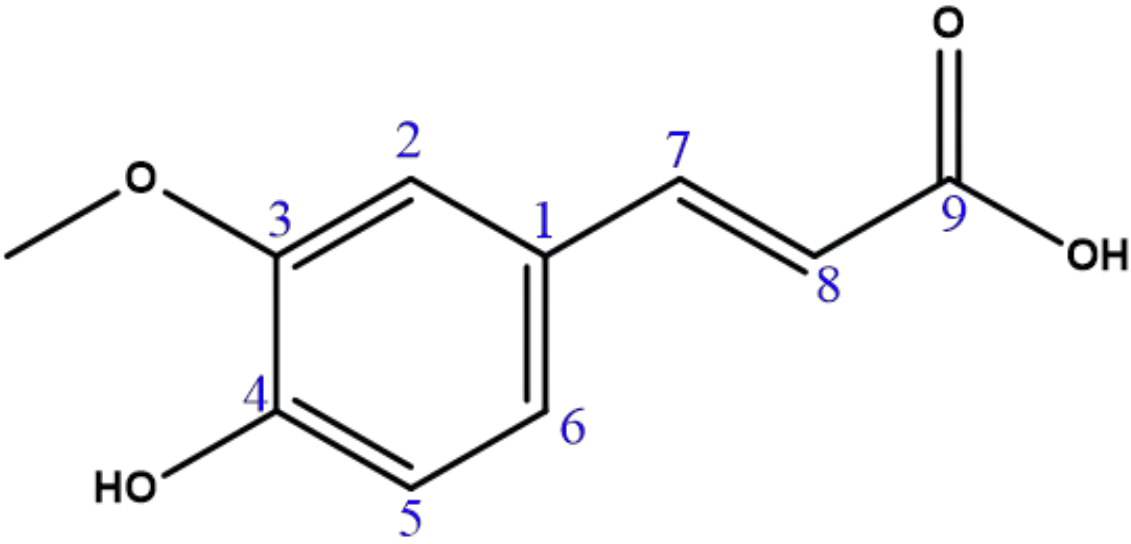


**Figure 3.33** Elucidated structure of "compound b".

### *3.5.2.3. Structure elucidation of compound c*

(3β,22E)-*Stigmasta*-5,20-dien-3-yl β-*D-glucopyranoside*) or Stigmasterol-D-glucoside The structural elucidation of "compound c" isolated from DSL-EA was done with the help of 1D and 2D NMR techniques. The $^{1}$H-NMR spectrum (figure 3.34) of compound C showed a total proton count of fifty-eight and revealed the presence of three olefinic methine protons resonating at 5.03 (m, 1 H, H-21), 5.16 (m, 1 H, H-20) and 5.33 (br.d, *J*= 5.6 Hz, 1 H, H-6). The $^{1}$H-NMR spectrum (figure) also indicated the presence of four OH group resonating at 4.43 ppm (t, *J*= 2 × 6 Hz, 1 H, OH-6′), 4.86 ppm (overlapping-d, *J*= 4.8 Hz, 1 H, OH-4′), 4.87 ppm (overlapping-d, *J*= 4.8 Hz, 1 H, OH-2′) and 4.89 ppm (d, *J*= 4.8 Hz, 1 H, OH-3′). Furthermore, the $^{1}$H-NMR spectrum also confirmed the presence of six methyl groups resonating at 0.66 (Me-29), 0.79 (Me-26), 0.81 (Me-24), 0.83 (Me-19), 0.90 (Me-27) and 0.96 ppm (Me-28). All the corresponding carbons were identified using the HSQC (figure 3.36 and 3.37) spectrum.

The $^{13}$C NMR spectrum (figure 3.35) suggested a sterol-based skeleton. The $^{13}$C NMR spectrum, showed the presence of thirty-five carbons, being three quaternary carbons at 36.7, 42.3 and 140.9 ppm, six methyl carbons at 12.2, 12.3, 18.6, 19.0, 19.6 and 20.5 ppm, ten methylene carbons at 21.1, 23.1, 24.3, 28.3, 29.7, 31.9, 37.3 38.8, 39.6 and 61.6 ppm and three olefinic methine carbons at 121.7, 129.3 and 138.5 ppm. The$^{13}$C NMR spectrum (figure) also showed the presence of six signals between 70 to 102 ppm correspond to five methine carbons of glucopyranoside moiety (C1', C2', C3', C4', C5')

and a C3 of stigmasterol skeleton and all signals are shifted downfield due to the influence of the oxygen atoms. All other remaining carbons and protons were identified using H-H COSY, HSQC and HMBC spectra. The elucidated structure of "compound c" is given in figure 3.38.

**$^{1}$H NMR (600 MHz, DMSO-*d*$_6$) □ ppm:** 0.66 (s, 3 H, Me-29), 0.79 (d, *J*= 6.6 Hz, 3 H, Me-26), 0.81 (overlapping-t, *J*= 6.2 Hz, 3 H, Me-24), 0.83 (overlapping-d, *J*= 7.2 Hz, 3 H, Me-19), 0.89 (m, 1 H, H-9), 0.90 (overlapping-d , *J*= 6.4 Hz, 3 H, Me-27), 0.96 (s, 3 H, Me-28), 0.98 (m, 1 H, H-14), 0.99 (m, 1 H, H-1a), 1.01 (m, 1 H, H-17), 1.05 (m, 1 H, H-15a), 1.14 (m, 1 H, H-12a), 1.23 (m, 2 H, H-23a,b), 1.26 (m, 1 H, H-16a), 1.39 (m, 1 H, H-25), 1.40 (m, 1 H, H-8), 1.41 (m, 1 H, H-11a), 1.48 (m, 1 H, H-2a), 1.49 (m, 1 H, H-11b), 1.53 (m, 1 H, H-15b), 1.55 (m, 1 H, H-22), 1.79 (m, 1 H, H-16b),1.81 (m, 1 H, H-1b), 1.82 (m, 1 H, H-2b), 1.91 (m, 1 H, H-7a), 1.94 (m, 1 H, H-7b), 1.95 (m, 1 H, H-12b), 2.02 (m, 1 H, H-18), 2.13 (m, 1 H, H-4a), 2.36 (m, 1 H, H-4b), 2.89 (td, *J*= 2 × 8.4, 4.8 Hz, 1 H, H-2'), 3.02 (td, *J*= 2 × 8.4, 4.8 Hz, 1 H, H-4'), 3.07 (m, 1 H, H5'), 3.13 (td, *J*= 2 × 8.2, 4.6 Hz, 1 H, H-3'), 3.41 (dt, *J*= 11.7, 2 × 5.9 Hz, 1 H, H-6'a), 3.47 (m, 1 H, H-3), 3.65 (ddd, *J*= 11.6, 5.6, 1.8 Hz, 1 H, H-6'b), 4.22 (d, *J*= 7.8 Hz, 1 H, H-1'), 4.43 (t, *J*= 2 × 6 Hz, 1 H, OH-6′), 4.86 (overlapping-d, *J*= 4.8 Hz, 1 H, OH-4′), 4.87 (overlapping-d, *J*= 4.8 Hz, 1 H, OH-2′), 4.89 (d, *J*= 4.8 Hz, 1 H, OH-3′), 5.03 (m, 1 H, H-21), 5.16 (m, 1 H, H-20), 5.33 (br.d, *J*= 4.8 Hz, 1 H, H-6).

**$^{13}$C NMR (151 MHz, DMSO-*d*$_6$) □ ppm:** 12.2 (C29), 12.3 (C24), 18.6 (C26), 19.0 (C27), 19.6 (C28), 20.5 (C19), 21.1 (C11), 23.1 (C23), 24.3 (C15), 28.3 (C16), 29.7 (C2), 31.8 (C25), 31.8 (C7), 31.9 (C8), 36.7 (C10), 37.3 (C1), 38.8 (C4), 39.6 (C12), 40.4 (C18), 42.3 (C13), 50.1 (C9), 51.1 (C22), 56.7 (C14), 56.7 (C17), 62.6 (C6'), 70.6 (C4'), 73.9 (C2'), 77.2 (C5'), 77.2 (C3'), 77.4 (C3), 101.3 (C1'), 121.7 (C6), 129.3 (C21), 138.5 (C20), 140.9 (C5).

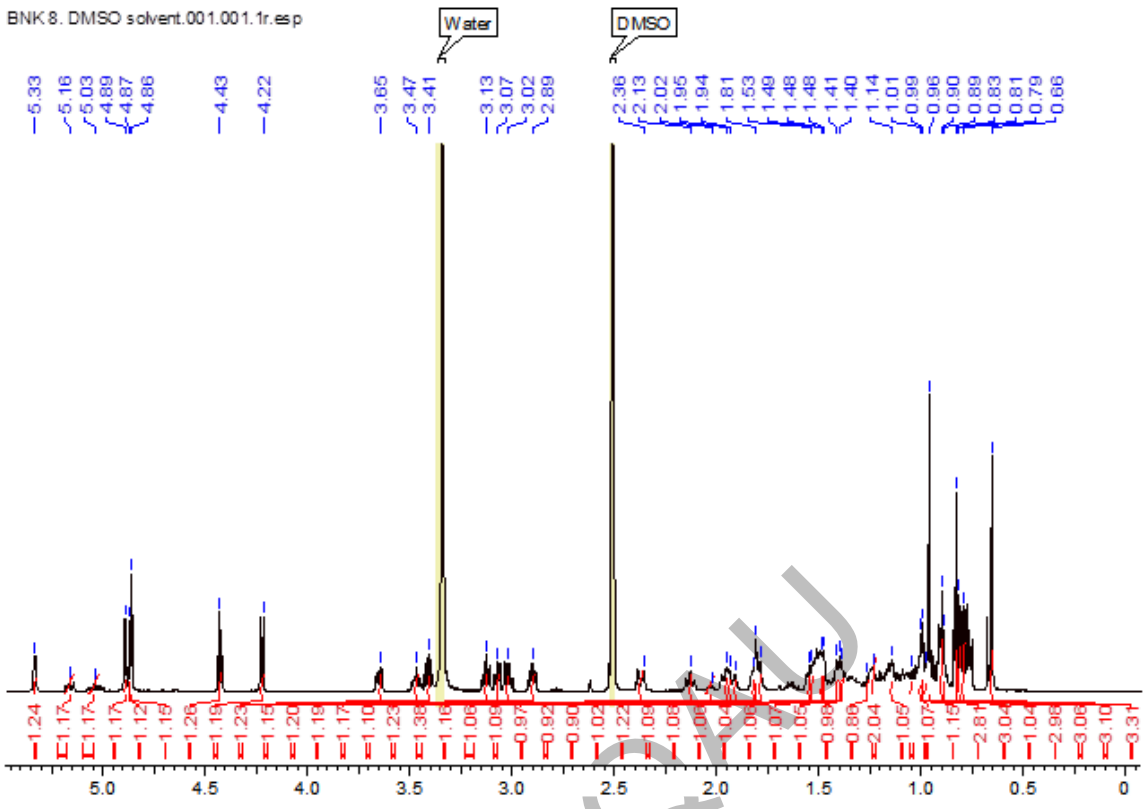


**Figure 3.34** $^{1}$H NMR spectrum of "compound c"

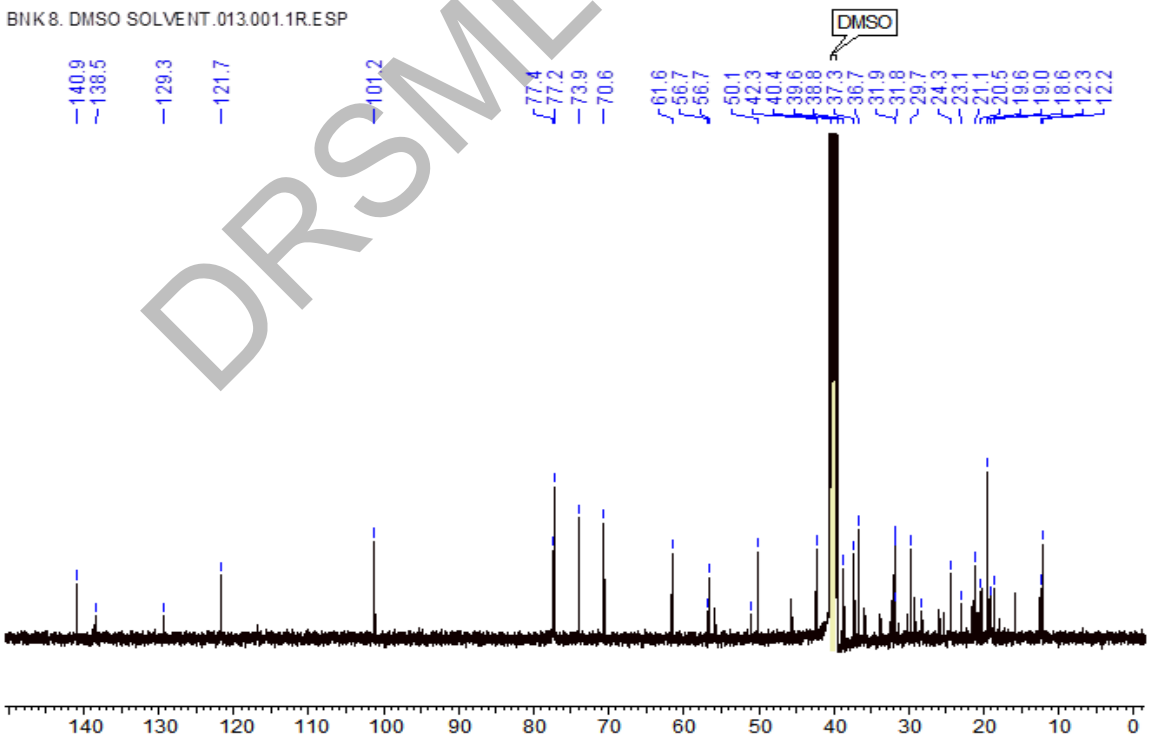


**Figure 3.35** $^{13}$C NMR spectrum of "compound c"

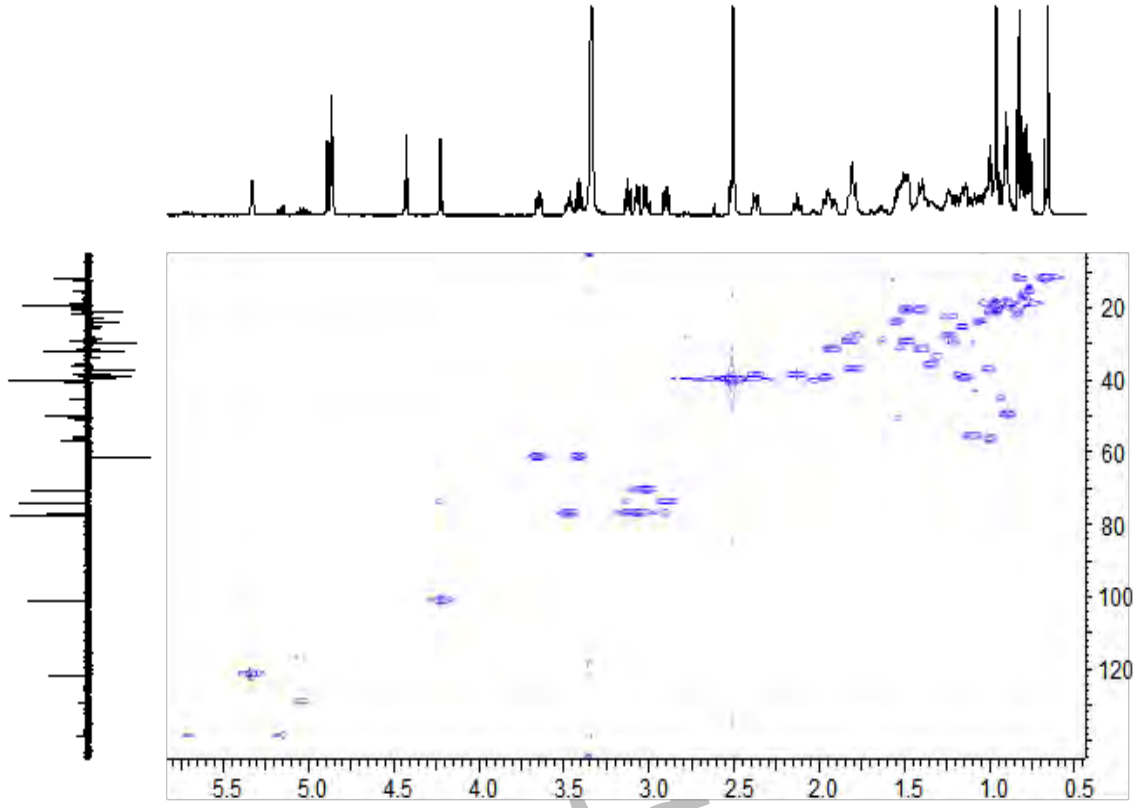


**Figure 3.36** HSQC spectrum of "compound c"

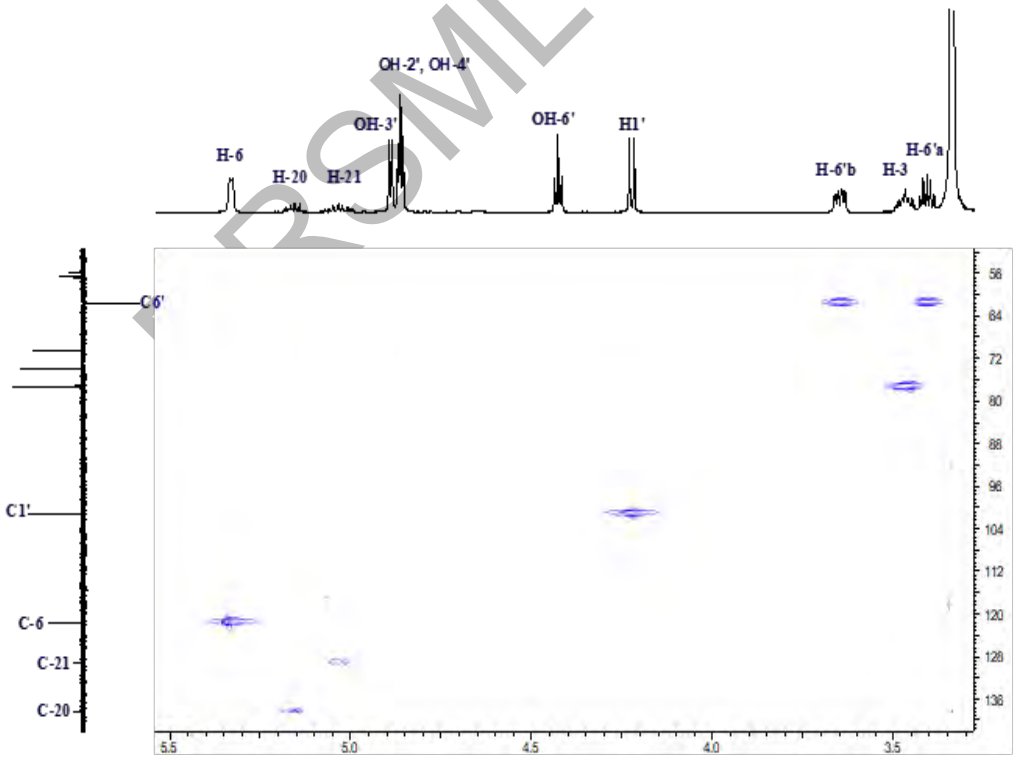


**Figure 3.37** Expansion of selected region of HSQC spectrum of "compound c".

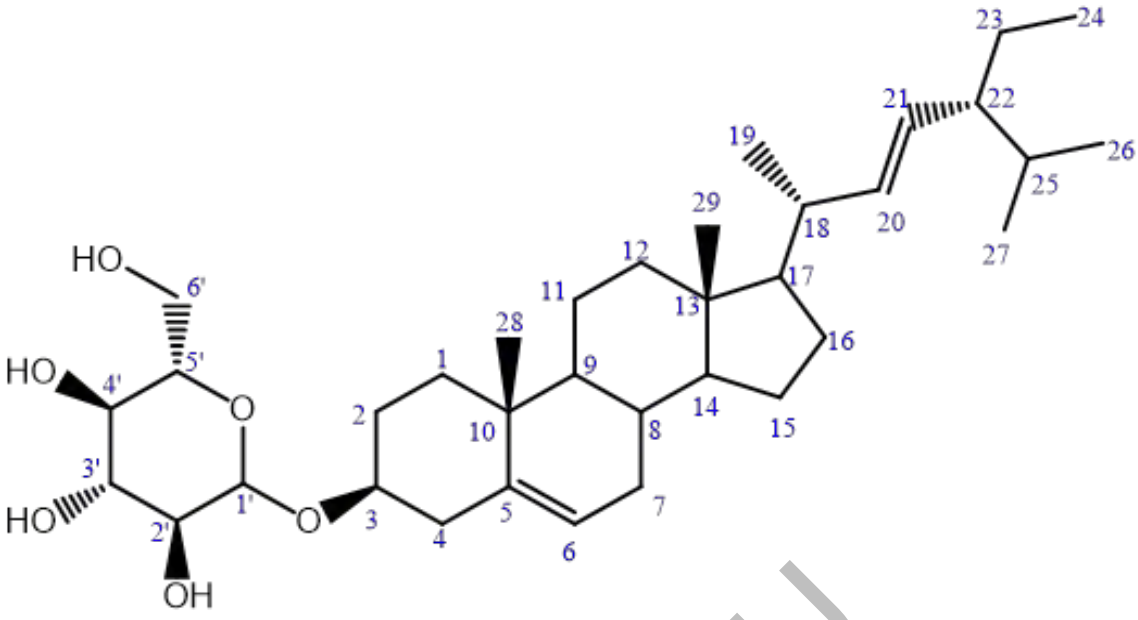


**Figure 3.38** Elucidated structure of "compound c".

The selection of compounds from the available pool was established by certain factors i.e., quantity, purity, retrospective safety data and pharmacological significance as determined by their chemical class and literature review. This screening led us to select two compounds from the available options as presented in the previous section i.e., Stigmasterol-d-glucoside, the steroidal glucoside (referred to as C1 hereafter) and ferulic acid, the polyphenol (referred to as C2 hereafter). These compounds were tested for their anticancer and anti-inflammatory potential using Balb/c mice as the test animal.



### 3.6. Evaluation of Anticancer and Anti-inflammatory Potential of C1 and C2

The selection of isolated compounds for chronic *in vivo* screening was based on their chemical class, reported pharmacological significance and established safety profile as well as the quantity procured in the isolation process. Keeping the mentioned parameters in mind, we selected compound 1 and 2 for the following *in vivo* assays.

#### 3.6.1. Benzene induced leukemia

Following intra-peritoneal administration of predetermined doses of benzene to study groups elaborated in chapter 2, obvious signs of leukemia induction were observed including, unusual bleeding, excessive bruising, skin rashes, weakness and weight loss. Efficacy of compounds in mitigating the disease was measured through a series of

hematological, biochemical, oxidative stress markers and histological investigations. Moreover, the expression level of selected proteins was also confirmed via western blotting analysis.

#### *3.6.1.1. Hematological studies*

Results of benzene induced hematological variations are displayed in Table 3.14. Benzene's devastative effects were evident from hematological investigations of the disease control group. A significant decline in RBCs (3.6±0.24 x106/µl), platelets (234±4.25 x103/µl) and hemoglobin (8.1±0.53 g/dL) levels while marked increase in WBCs count (15.3±0.06 x103/µl) was observed in disease control mice. Findings of hematological investigation revealed that the normal control, positive control, C1 and C2 treated groups are significantly different ($p < 0.05$) from the disease control group. Hemoglobin levels (g/dL) were significantly raised by high doses of C1 (15.3±0.87) and C2 (14.2±0.29), the levels matched the findings of normal (14.8±1.12) and positive control (14.5±0.26) groups. High dose treatments of both compounds resulted in a noteworthy increase in RBCs and platelets count as well. The expressively raised WBCs level was lowered by high and low doses of C1 and C2 with C1-HD showing maximum effect (7.7±0.08 x 103/µl) followed by C2-HD (8.3±0.07 x 103/µl). The results are further elaborated in table 3.14.

#### *3.6.1.2. Effect on biochemical parameters*

Serum acquired from mice of study groups was used for investigation of biochemical parameters. The results provide clear evidence of damaging effects of benzene on liver and kidney in the disease control group. Liver and kidney functionality as well as and lipid profiles of C1 and C2 treated groups were compared with the controls used in the study. Low and high doses of C1 and C2 have restricted the harm initiated by benzene to a great extent as evident from the enzymatic and biochemical findings. ALT, AST and ALP levels estimated in disease control mice were 61±3.30, 55±5.01, 283±58.71 and 234±4.97 U/L respectively. C1 and C2 given in high doses resulted in statistically identical ($p < 0.05$) restoration of liver enzymes. Total protein levels dropped significantly (2.5±0.25 g/L), while triglycerides (296±44.96 mg/dL) and cholesterol levels (211±33.06 mg/dL) were markedly higher in disease control mice further confirming benzene induced liver damage. Results observed in C1 and C2 treated groups proved the pharmacological significance of these compounds by virtue of the

fact that the tested parameters in these groups were closely identical ($p < 0.05$) to normal and positive control groups.

Bilirubin **(**1.4±0.01 mg/dL**)**, urea (63±6.37 mg/dL) and creatinine (0.98±0.24 mg/dL) levels were distinctly elevated in disease control mice confirming acute leukemic condition and kidney damage. The compounds managed to restore serum bilirubin levels in a mediocre way. Urea levels were significantly ($p < 0.05$) improved while creatinine levels were prominently improved by C2-LD (0.52±0.08), C2-HD (0.74±0.24) and C1-HD (0.76±0.17 mg/dL). Statistically significant difference ($p < 0.05$) was observed when positive control and other groups were compared with disease control group. The results are presented in table 3.15.

#### *3.6.1.3. Effect on endogenous antioxidant enzymes and GSH levels*

The activity level of endogenous antioxidant enzymes and GSH expressed in terms of % concentration in liver tissues is presented in figure 3.39. A significant ($p < 0.05$) decline in GST, GSH, SOD, CAT and POD concentration was estimated in disease control group when compared with normal and positive control groups. C1 and C2 restored the levels of aforementioned parameters in a dose dependent manner. Both compounds showed almost identical restorative action and high doses significantly ($p < 0.05$) improved the % concentration of endogenous antioxidant enzymes and GSH in liver tissues of mice in respective groups. Low doses of C1 and C2 also resulted in a slight increase. The results are further elaborated in figure 3.39.

**Table 3.14** Effect of C1 and C2 on the complete blood profile.

| Hematological parameters | Normal control (mean±SEM) | Disease control (mean±SEM) | Cyclophosphamide treated (mean±SEM) | C1-LD (mean±SEM) | C1-HD (mean±SEM) | C2-LD (mean±SEM) | C2-HD (mean±SEM) |
|---|---|---|---|---|---|---|---|
| **HGB (g/dL)** | 14.8±1.12$^{ab}$ | 8.1±0.53$^{d}$ | 14.5±0.26$^{ab}$ | 11.6±1.04$^{c}$ | 15.3±0.87$^{a}$ | 11±0.65$^{c}$ | 14.2±0.29$^{b}$ |
| **RBC (x10$^{6}$/µl)** | 6.21±0.32$^{a}$ | 3.6±0.24$^{c}$ | 5.11±0.42$^{ab}$ | 5.1±0.28$^{b}$ | 5.4±0.53$^{ab}$ | 4.7±0.35$^{b}$ | 4.9±0.36$^{b}$ |
| **WBC (x10$^{3}$/µl)** | 5.12±0.21$^{i}$ | 15.3±0.06$^{a}$ | 7.4±0.09$^{f}$ | 9.2±0.06$^{c}$ | 7.7±0.08$^{e}$ | 12.2±0.11$^{b}$ | 8.3±0.07$^{d}$ |
| **Platelets (x10$^{3}$/µl)** | 632±8.32$^{b}$ | 234±4.25$^{g}$ | 454±5.10$^{e}$ | 421±6.56$^{f}$ | 442±3.38$^{e}$ | 679±7.29$^{a}$ | 605±6.65$^{c}$ |

All values are expressed as mean±SEM (n=6), (n = 6, where n = number of rats analysed in each group). Means with different superscripts (a-i) in the columns are significantly ($p < 0.05$) different from one another

**Table 3.15** Effect of C1 and C2 on the biochemical parameters.

| Biochemical parameters | Normal control (mean±SEM) | Disease control (mean±SEM) | Cyclophosphamide treated (mean±SEM) | C1-LD (mean±SEM) | C1-HD (mean±SEM) | C2-LD (mean±SEM) | C2-HD (mean±SEM) |
|---|---|---|---|---|---|---|---|
| **ALT (U/L)** | 34±4.93$^{d}$ | 61±3.30$^{a}$ | 44±2.26$^{bc}$ | 48±3.03$^{bc}$ | 41±2.01$^{cd}$ | 53±4.43$^{ab}$ | 42±1.92$^{cd}$ |
| **AST (U/L)** | 36±4.35$^{c}$ | 55±5.01$^{a}$ | 39±3.26$^{bc}$ | 47±4.06$^{ab}$ | 34±3.26$^{c}$ | 51±4.54$^{a}$ | 33±2.94$^{c}$ |
| **ALP (U/L)** | 189±28.16$^{bc}$ | 283±58.71$^{a}$ | 154±33.21$^{c}$ | 174±19.87$^{bc}$ | 187±24.90$^{bc}$ | 251±33.45$^{ab}$ | 196±29.32$^{bc}$ |
| **Bilirubin (mg/dL)** | 0.4±0.02$^{d}$ | 1.4±0.01$^{a}$ | 0.5±0.04$^{d}$ | 1.3±0.03$^{ab}$ | 1.0±0.06$^{c}$ | 1.1±0.13$^{bc}$ | 1.2±0.21$^{abc}$ |
| **Total protein (g/L)** | 6.3±0.43$^{a}$ | 2.5±0.25$^{c}$ | 5.6±0.38$^{ab}$ | 4.4±0.49$^{b}$ | 5.3±0.42$^{ab}$ | 5.3±0.61$^{ab}$ | 5.8±0.32$^{a}$ |
| **Urea (mg/dL)** | 33±3.88$^{b}$ | 63±6.37$^{a}$ | 24±3.02$^{b}$ | 26±2.65$^{b}$ | 23±4.41$^{b}$ | 29±3.39$^{b}$ | 26±3.22$^{b}$ |
| **Creatinine (mg/dL)** | 0.63±0.15$^{ab}$ | 0.98±0.24$^{a}$ | 0.44±0.12$^{b}$ | 0.93±0.22$^{ab}$ | 0.76±0.17$^{ab}$ | 0.52±0.08$^{b}$ | 0.74±0.24$^{ab}$ |
| **Triglycerides (mg/dL)** | 121±23.87$^{c}$ | 296±44.96$^{a}$ | 100±17.76$^{c}$ | 149±14.93$^{bc}$ | 148±22.31$^{bc}$ | 191±19.58$^{b}$ | 149±20.38$^{bc}$ |
| **Cholesterol (mg/dL)** | 143±41.50$^{b}$ | 211±33.06$^{a}$ | 137±17.87$^{b}$ | 125±10.28$^{b}$ | 146±24.37$^{b}$ | 111±9.31$^{b}$ | 95±8.96$^{b}$ |

*Values are expressed as mean±SEM (n = 6, where n = number of rats analysed in each group). Means with different superscripts (a-d) in the columns are significantly ($p < 0.05$) different from one another.*

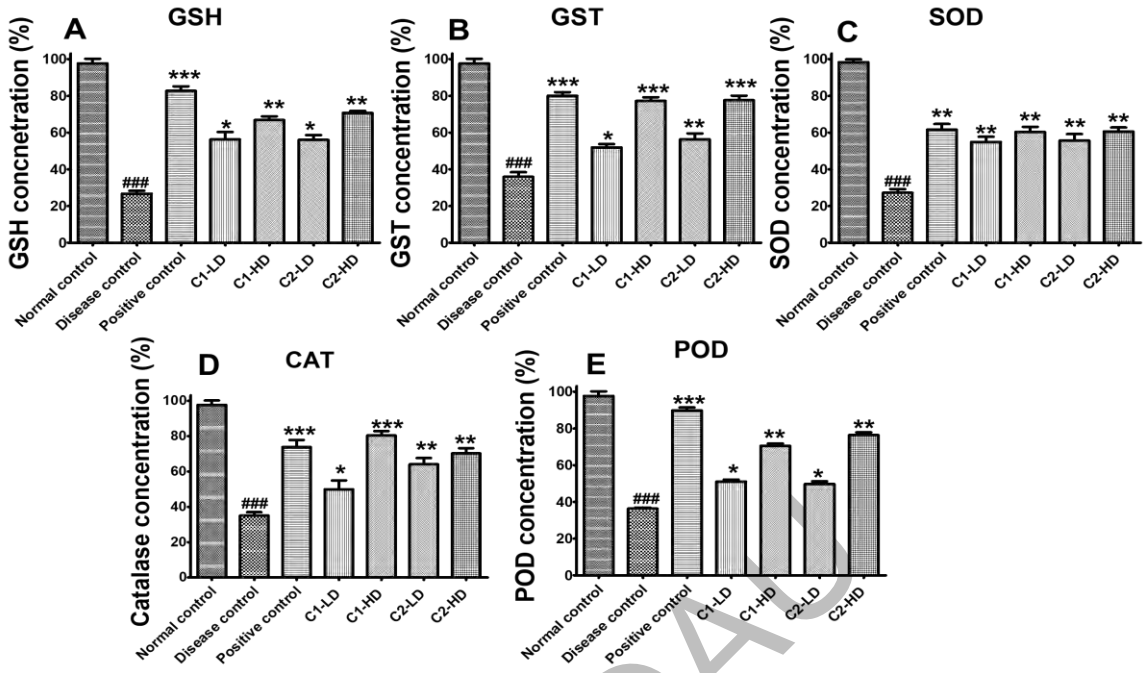


**Figure 3.39** Effect of C1 and C2 on glutathione (GSH) [A], glutathione S-transferase (GST) [B], superoxide dismutase (SOD) [C], Catalase [D] and peroxidase (POD) [E] levels in liver tissue compared to the disease control group.
***Note:*** *All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

### *3.6.1.4. Effect on oxidative stress markers*

NO and TBARs levels of each group were estimated to get an overall view of the oxidative stress status in the study mice. The results have shown a great increase in the levels of these stress markers in disease control mice. NO, an important mediator of inflammation influenced by iNOS gene, was greatly elevated (almost two folds as compared to normal control) in liver tissues of disease control group. Dose dependent response of C1 and C2 was observed in the results with HD groups showing statistically identical ($p < 0.05$) results to the positive control group. The results are presented in figure 3.40.

The TBARs level in liver tissues of benzene treated disease control group was almost four folds higher than that of normal control group. This excessively higher TBARs level further confirmed the damage caused by benzene used as an inducer since TBARs are the end products of lipid peroxidation reactions. Both compounds have shown excellent results and up to 70% reduction in TBARs level was observed in high dose groups of the compounds as shown in figure 3.40.

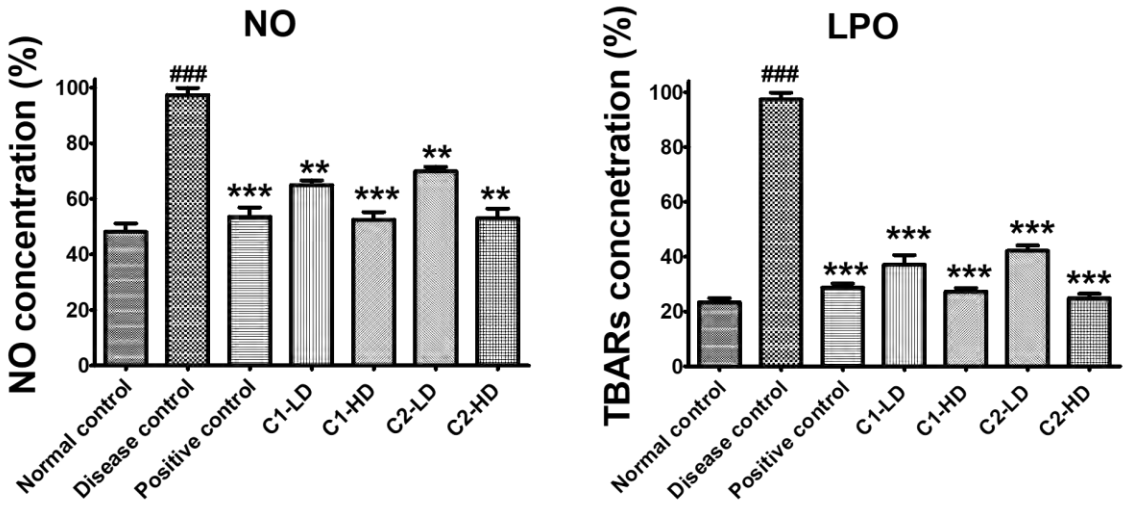


**Figure 3.40** Effect of the C1 and C2 on the oxidative stress markers.
***Note:*** *Nitric oxide (NO) and thiobarbituric acid reactive substances (TBARs) in liver tissues compared to disease control group. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001. LPO; lipid peroxidation assay.*

### *3.6.1.5. Histopathology (H & E and Masson's trichrome staining)*

The effects of C1 and C2 on liver and kidney functionality as revealed in the biochemical screenings are further supported by the histological investigations of liver and kidney of mice in each study group. Liver tissues isolated from normal control had normal morphological features (H & E staining) i.e., intact hepatocytes, sinusoids, and normal central veins. Similarly, kidney tissues revealed normal histoarchitecture and displayed normal Bowman's capsule and glomerular tuft. Benzene has been known to cause serious liver and kidney damage and the histology of disease control mice revealed significant tissue damage including but not limited to cellular hypertrophy, necrosed hepatocytes, degenerated renal tubules and hyalinized glomeruli. The toxicity caused by benzene to liver and kidney tissues was alleviated to a great extent by C1 and C2 as evident in the figure 3.41 and 3.42. Findings from the histological studies are in accordance with the results of biochemical and enzymatic investigations presented in the preceding sections.

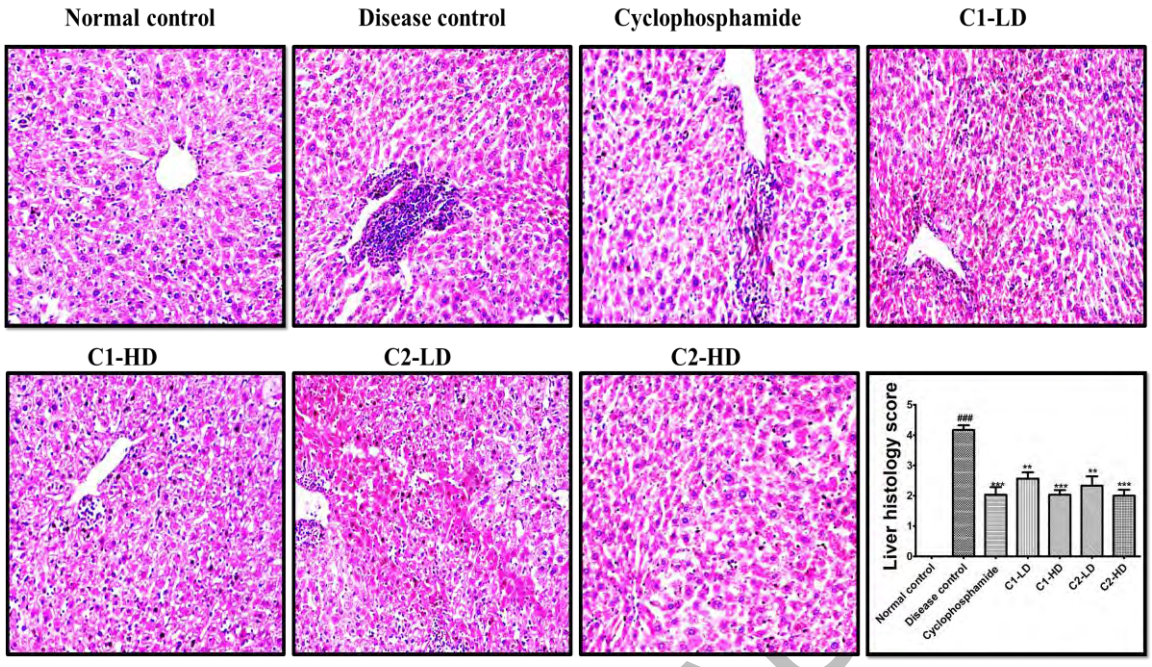


**Figure 3.41** Hematoxylin-eosin staining of the liver tissue.
***Note:*** *The restorative effect of C1 and C2 on liver tissues following benzene induced toxicity. The extracts markedly improved the histological parameters such as immune cell infiltration, fibrosis and edema compared to the disease control. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

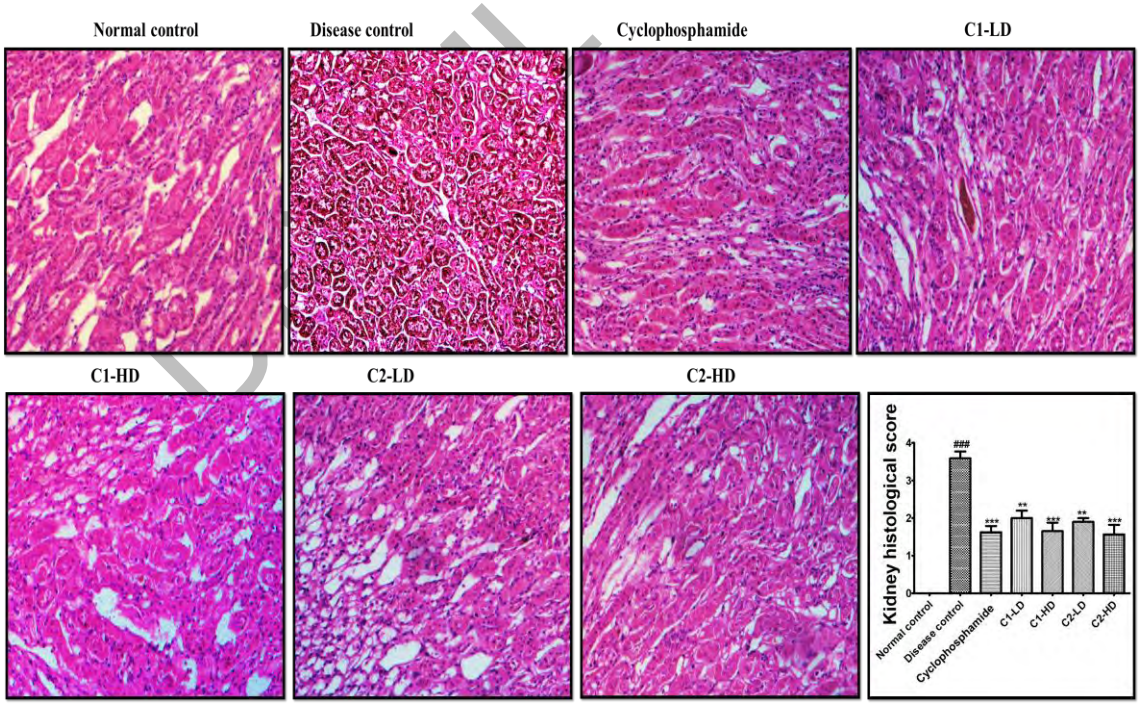


**Figure 3.42** Histopathological observations for the protective potential of C1 and C2 isolated from *D. stramonium* on kidney tissues.
***Note:*** *All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

Masson's trichrome staining showed an identical pattern of results as observed in H & E staining. Collagen was stained blue while the hepatocyte nuclei appeared as dark

purple or red spots within the cells. Liver tissues of disease control mice revealed greater collagen deposition around the portal triads and close to hepatocytes and sinusoidal boundaries. The damage was curbed to a great extent by high doses of C1 and C2 as evident in figure 3.43.

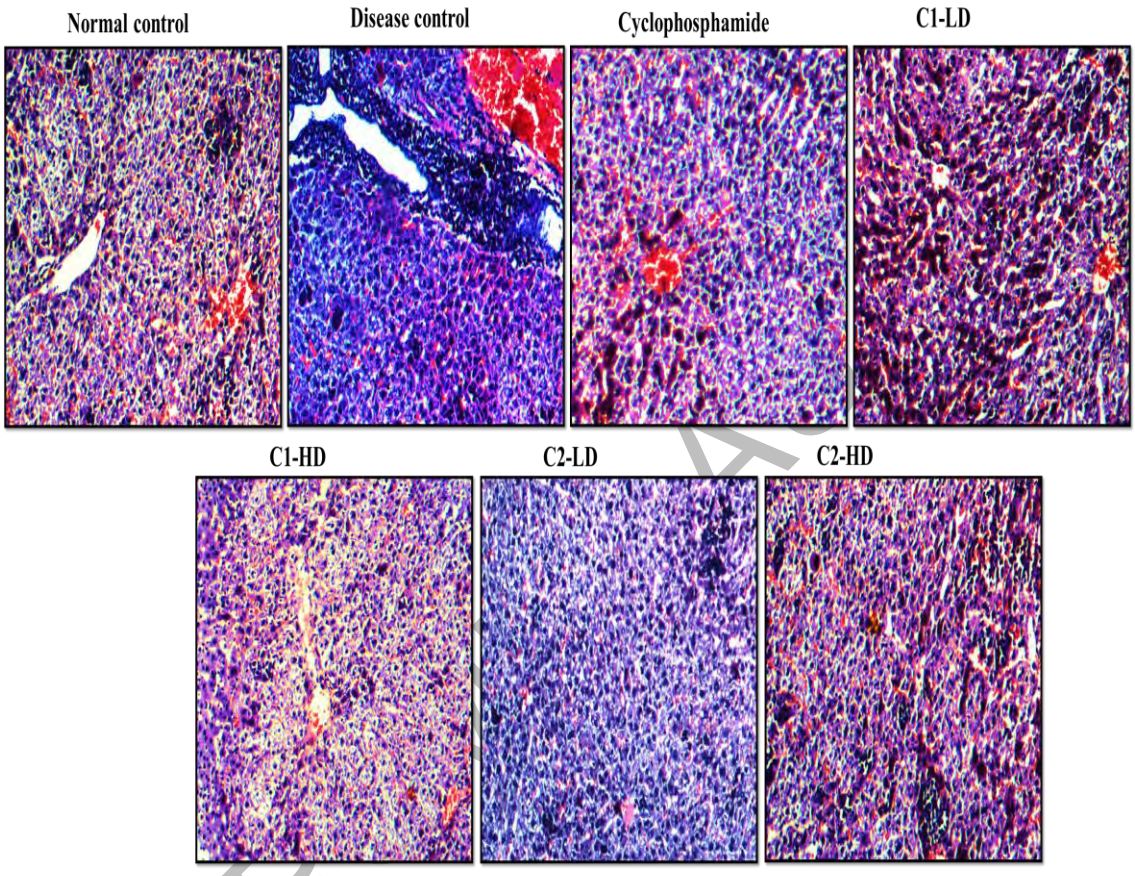


**Figure 3.43** Masson's trichome staining of the liver tissues.
***Note:*** *C1 and C2 markedly improved the histological parameters and inhibited liver fibrosis compared to the disease control.*

The trichrome staining of the kidney tissues of study mice also showed ominous signs of benzene induced toxicity in the disease control group including glomerular fibrosis. Excessive collagen deposition is known to be the hallmark of fibrosis and Masson's trichrome staining was used to assess the collagen in kidney tissues stained as blue. The disease control mice showed exaggerated deposition while C1 and C2 led to marked improvement much in alliance with the results of H & E staining as well as the biochemical findings presented in preceding sections. The details are given in figure 3.44.

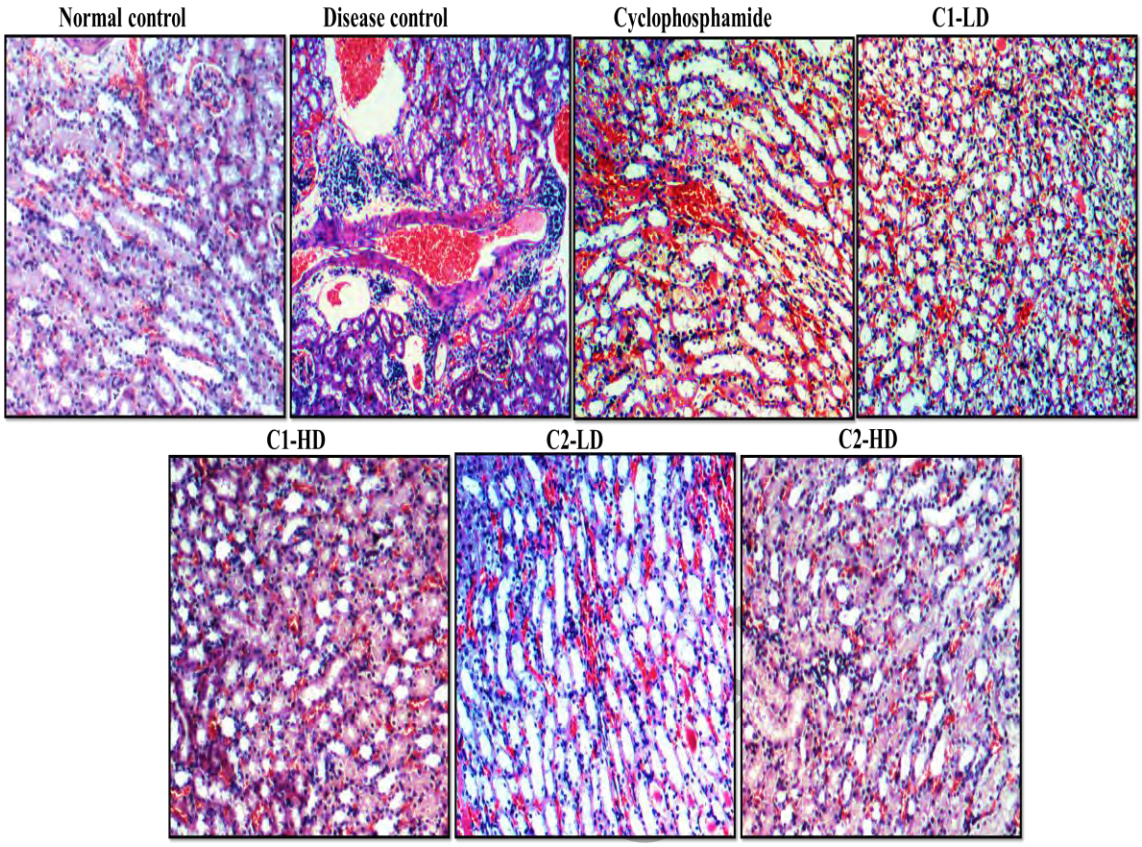


**Figure 3.44** Masson's trichome staining of the kidney tissues.
***Note:*** *C1 and C2 markedly improved the histological parameters and significantly inhibited fibrosis compared to the disease control.*



### *3.6.1.6. Immunohistochemistry*

The expression of NF-ƙB and COX-2 was investigated using immunostaining. The expression level of NF-ƙB and COX-2 was markedly higher in liver and kidney of disease control mice as evident from the increased antibody binding in the immunohistochemistry images. This gave an indication of the hematological malignancy initiated by benzene administration since both NF-ƙB and COX-2 are reported to be overexpressed in such conditions. C1 and C2 have restored the normal expression levels in a dose dependent manner and the high dose groups showed a statistically ($p < 0.05$) identical immunoreactivity score to the positive control group as shown in figure 3.45 and 3.46.

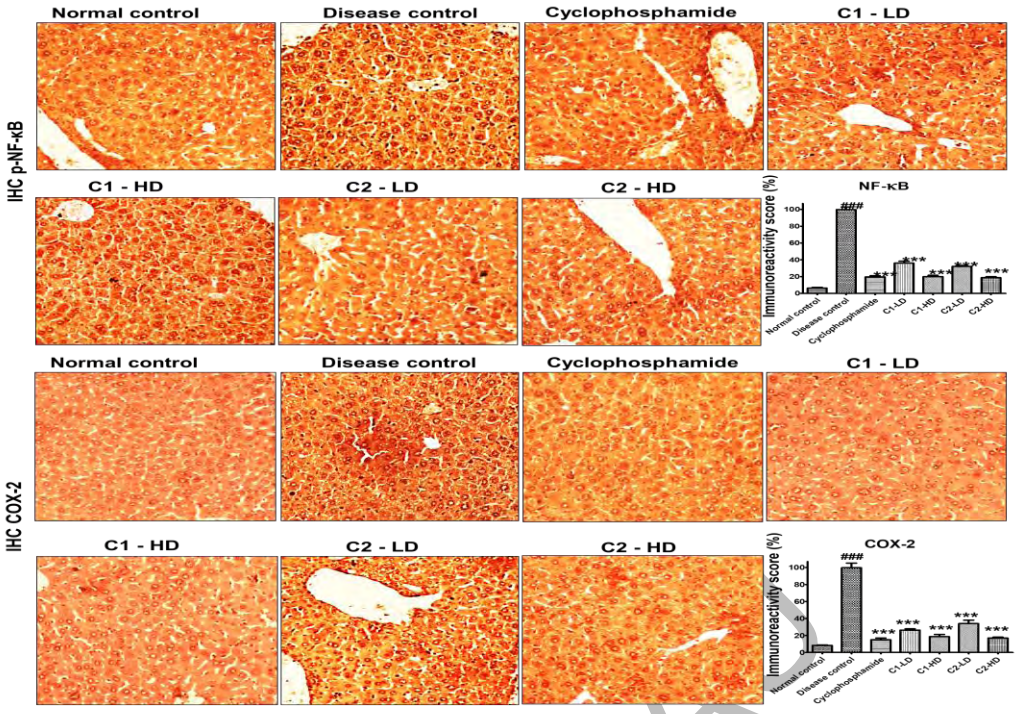


**Figure 3.45** Effect of high and low dose treatment of C1 and C2 on expression level of nuclear factor kappa-B and cyclooxygenase 2 in the liver tissue.

***Note:*** *Using immunohistochemistry analysis. The extracts inhibited the expression of NF-κB and COX-2 in a dose dependent manner compared to the disease control. IHC: Immunohistochemistry. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

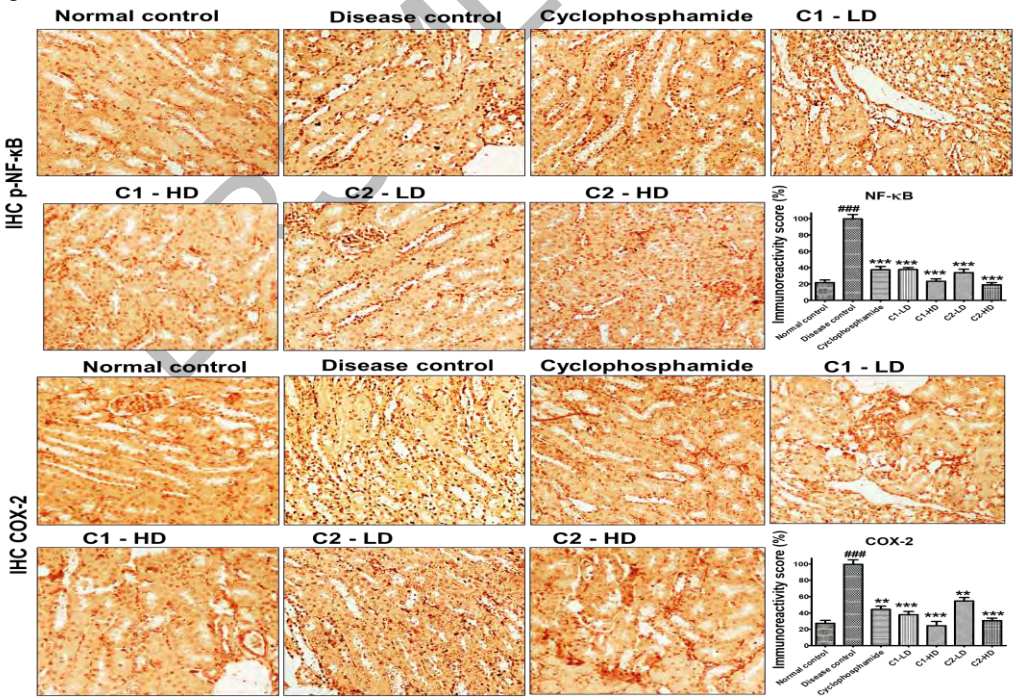


**Figure 3.46** Effect of high and low dose treatment of C1 and C2 on expression level of nuclear factor kappa-B and cyclooxygenase 2 in the kidney tissues.

***Note:*** *Using immunohistochemistry analysis. The extracts inhibited the expression of NF-κB and COX-2 in a dose dependent manner compared to the disease control. IHC: Immunohistochemistry. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

### *3.6.1.7. Western blotting studies*

The underlying molecular mechanism of the significant alleviative effects observed with C1 and C2 in benzene induced leukemia was investigated by estimating the expression of numerous proteins i.e., NRF-2, p-JNK, NF-κB, PARP-1 and IL-1β in liver homogenates of the mice *via* western blot technique (Figure 3.47 and 3.49). Western blot analysis provided clear evidence that treatment with C1 and C2 resulted in a significant overexpression of NRF-2. The expression of p-JNK was also significantly down-regulated by both the compounds. The compounds also resulted in a substantial downregulation of NF-κB, PARP-1 and TNF-α. This further validated the anticancer and anti-inflammatory potential of C1 and C2. Fold change in protein expression has been plotted using Image J and given in Figure 3.48 and 3.50.

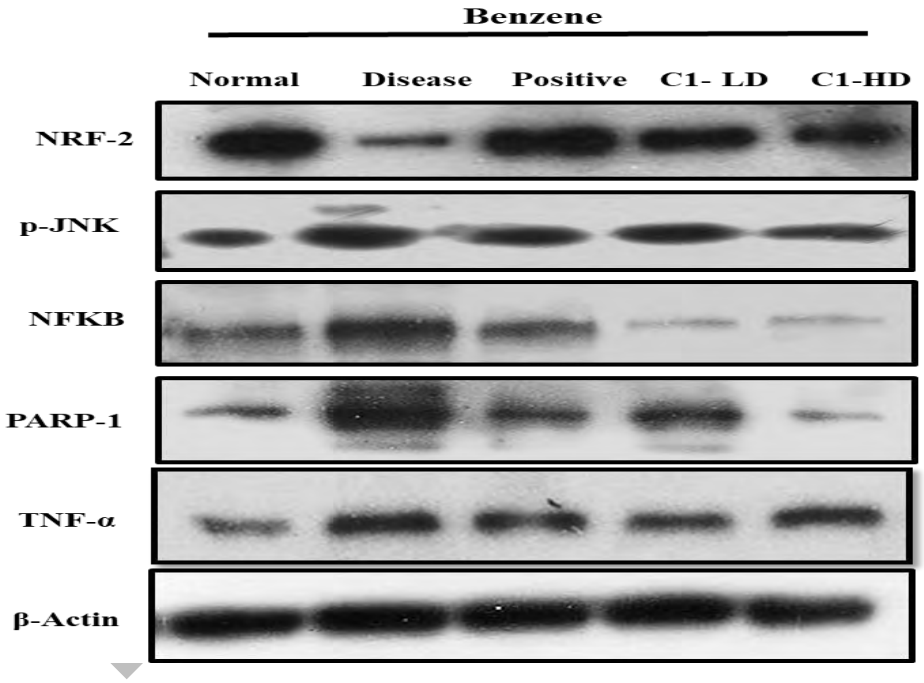


**Figure 3.47** Western blot analysis of proteins targeted by C1 in benzene induced leukemia.
***Note:*** *Low and high doses of C1 (30 and 60 mg/kg) have resulted in upregulation of NRF-2 while p-JNK, NF-κB, PARP-1 and TNF-α were downregulated in a dose dependent manner. ß-Actin was used a loading control. NRF2- Nuclear factor-erythroid factor 2, p-JNK; Phosphorylated- Jun-N-terminal kinase, NF-κB; Nuclear factor kappa B, PARP-1; Poly adenosine diphosphate ribose polymerase-1, TNF- α; Tumor necrosis factor-alpha.*

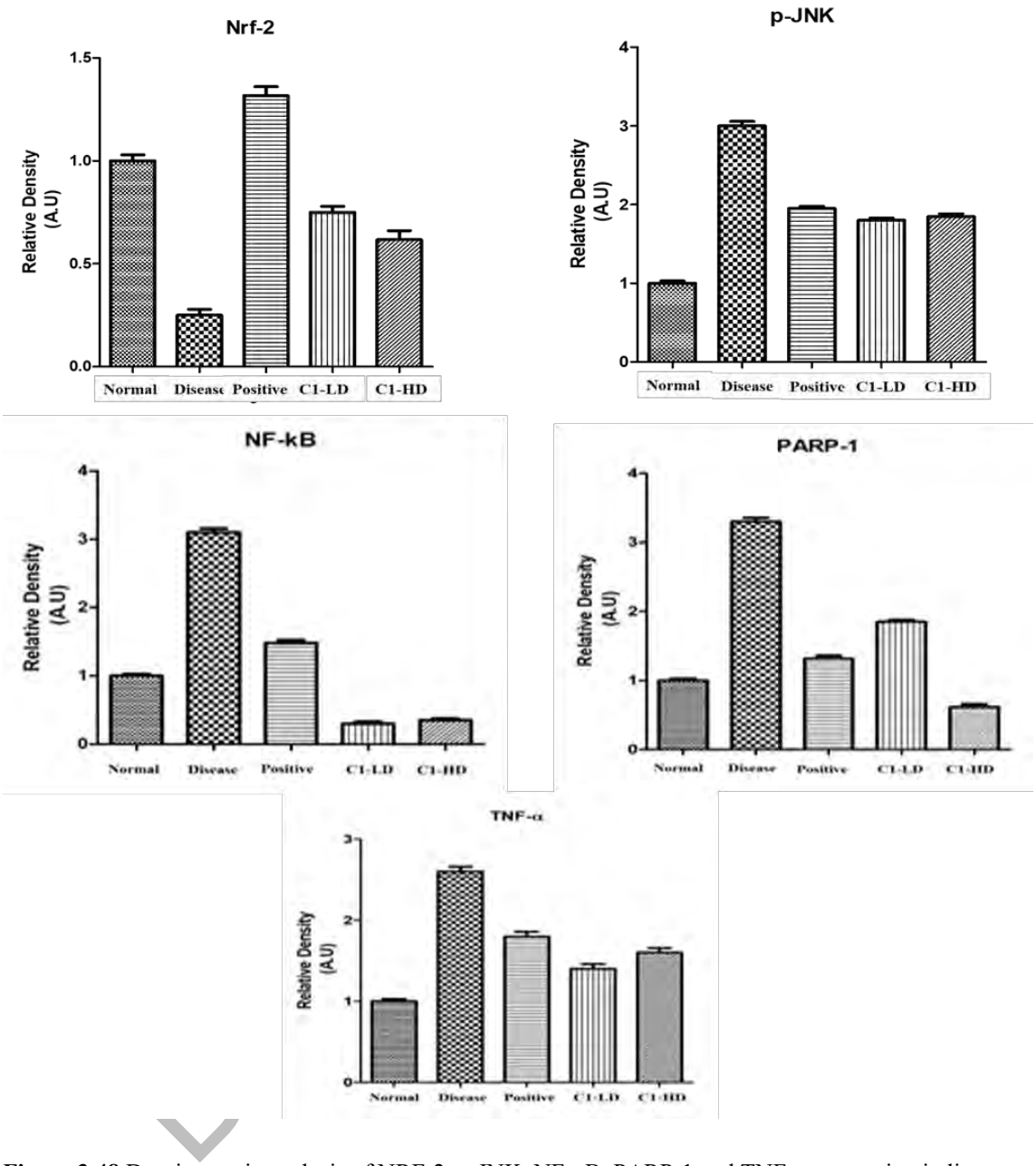


**Figure 3.48** Densitometric analysis of NRF-2, p-JNK, NF-κB, PARP-1 and TNF-α expression in liver tissue.
***Note:*** *Role of C1 in proteins expression is plotted. Data is presented as mean±SD (n=6).*

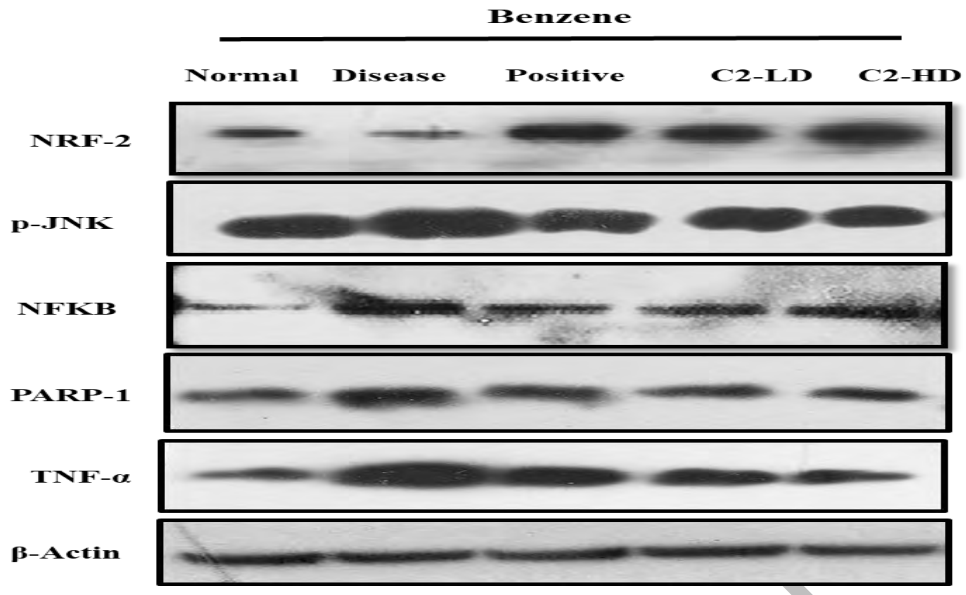


**Figure 3.49** Western blot analysis of proteins targeted by C2 in benzene induced leukemia.
***Note:*** *Low and high doses of C2 (10 and 20 mg/kg) have resulted in upregulation of NRF-2 while p-JNK, NF-κB, PARP-1 and TNF-α were downregulated in a dose dependent manner. ß-Actin was used a loading control.*

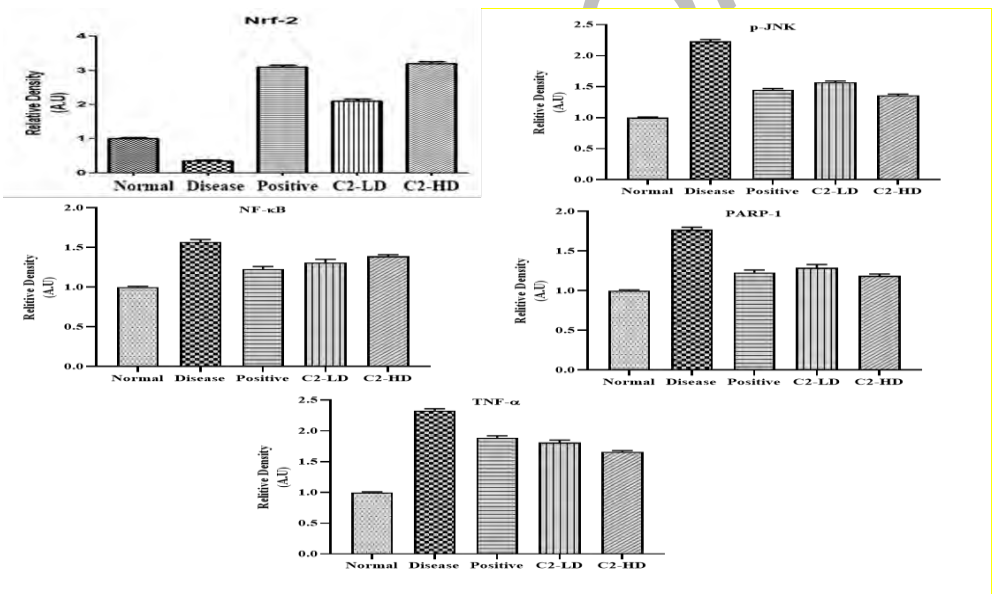


**Figure 3.50** Densitometric analysis of NRF-2, p-JNK, NF-κB, PARP-1 and TNF-α expression in liver tissue.
***Note:*** *Role of C2 in proteins expression is plotted. Data is presented as mean±SD (n=6).*

### 3.6.2. $CCL_4$ induced liver inflammation

Following intra-peritoneal administration of predetermined doses of $CCL_4$ to study groups elaborated in chapter 2, obvious signs of liver injury and inflammation were observed including abdominal swelling, dark urine color, pale stool color, swelling in paws, loss of appetite and abnormal bruising. Efficacy of compounds in mitigating the disease was measured through a series of hematological, biochemical, oxidative stress

markers and histological investigations. Moreover, the involvement of certain proteins was also confirmed *via* western blotting analysis.

#### *3.6.2.1. Hematological studies*

The chemical inducer of liver inflammation has adversely affected the blood parameters as evident from the results presented in table 3.16. RBCs (4.1±0.27 x106/µl), platelets (284±3.90 x103/µl) and hemoglobin levels (11.1±0.66 g/dL) in disease control mice were greatly reduced. WBCs level (12.2±0.31 x103/µl) of disease control mice was recorded to be the highest in comparison to other groups. Hematological parameters of C1-HD and C2-HD treated groups are significantly different from the disease control group ($p < 0.05$). Compounds isolated from *D. stramonium* regulated the hematological irregularities in a dose dependent manner identical to silymarin given at 50 mg/kg dose. The details can be seen in table 3.16.

#### *3.6.2.2. Effect on biochemical parameters*

Results of numerous biochemical tests performed using serum acquired from mice involved in the study revealed the damaging effects of $CCL_4$ on liver and kidneys. Disease control mice showed maximum toxicity and their liver function tests showed markedly elevated level ($p < 0.05$) of liver enzymes when compared to other study groups. The ALT, AST and ALP levels recorded in serum of disease control mice were 112±11.06, 96±9.88 and 344±49.98 U/L respectively. C1 and C2 given in high doses resulted in statistically significant ($p < 0.05$) restoration of liver enzymes almost identical to silymarin treated group. A pronounced decline in total protein level (3.1±0.63g/L) while elevated triglycerides (253±32.45 mg/dL) and cholesterol (196±36.85 mg/dL) levels observed in disease control mice further confirmed the liver damage initiated by $CCL_4$ administration. As observed in results of benzene induced leukemia, C1 and C2 administration, particularly high doses, reverted the aberrations and the said parameters were restored in a statistically identical manner ($p < 0.05$) to the standard drug used in the study.

The excessively higher bilirubin (1.6±0.13 mg/dL), urea (56±7.32 mg/dL) and creatinine (0.98±0.18 mg/dL) levels in disease control mice confirmed $CCL_4$ induced kidney damage. C2-HD restored these parameters even more effectively than silymarin, while C1-HD also managed to restore kidney functionality in a significant manner ($p < 0.05$). The results are compiled in table 3.17.

### *3.6.2.3. Effect on endogenous antioxidant enzymes and GSH levels*

The effect of C1 and C2 treatment on endogenous antioxidant enzymes and GSH levels of the study groups is presented in Figure 3.51. A significant ($p < 0.05$) reduction in activity level of GST, GSH, SOD, CAT and POD was observed in disease control group further confirming the $CCL_4$ perpetrated damage to liver tissues. There was statistically significant ($p < 0.05$) difference between the results of disease control group when compared to the other groups included in the study which further confirm the restorative effects of C1 and C2 in the event of $CCL_4$ induced liver inflammation. The % activity levels of tested antioxidant defense system in liver tissues was restored by the compounds in a dose dependent manner and high doses of C1 and C2 generated almost identical results to silymarin treated group. The results are shown in figure 3.51.

### *3.6.2.4. Effect on oxidative stress markers*

The levels of NO and TBARs in liver tissues of mice included in the study were estimated since they provide a useful indicator of the oxidative stress within the physiological system. The disease control mice revealed significantly elevated levels of these markers. The compounds have clearly helped in restoring the normal NO levels indicating a strong anti-inflammatory action of C1 and C2. Tissue NO levels of high dose groups were identical to silymarin treated group while low dose groups also showed good results as illustrated in figure 2.52. $CCL_4$ being a prototypical lipid peroxidative agent did induce lipid peroxidation in liver as confirmed by excessively raised TBARs level in disease control mice. An approximate threefold reduction in TBARs level in liver tissues of C1 and C2 treated mice was recorded and surprisingly, the results were not dose dependent, both low and high dose groups revealed a significant ($p < 0.05$) decline in lipid peroxidation end product i.e., TBARs. The details are presented in figure 2.52.

**Table 3.16** Effect of C1 and C2 on the complete blood profile.

| Hematological parameters | Normal control (mean±SEM) | Disease control (mean±SEM) | Silymarin treated (mean±SEM) | C1-LD (mean±SEM) | C1-HD (mean±SEM) | C2-LD (mean±SEM) | C2-HD (mean±SEM) |
|---|---|---|---|---|---|---|---|
| **HGB (g/dL)** | 14.8±1.12$^{ab}$ | 11.1±0.66$^{cd}$ | 13.5±0.73$^{abc}$ | 12.3±1.31$^{bcd}$ | 13.4±1.04$^{abc}$ | 10.4±0.38$^{d}$ | 15.2±1.43$^{a}$ |
| **RBC (x10$^6$/µl)** | 6.21±0.32$^{a}$ | 4.1±0.27$^{c}$ | 5.2±0.19$^{b}$ | 3.8±0.36$^{c}$ | 5.6±0.54$^{ab}$ | 3.9±0.33$^{c}$ | 4.6±0.49$^{bc}$ |
| **WBC (x10$^3$/µl)** | 5.12±0.21$^{f}$ | 12.2±0.31$^{a}$ | 5.8±0.07$^{e}$ | 7.1±0.09$^{c}$ | 6.9±0.11$^{cd}$ | 9.6±0.08$^{b}$ | 6.5±0.20$^{d}$ |
| **Platelets (x10$^3$/µl)** | 632±8.32$^{c}$ | 284±3.90$^{f}$ | 701±5.56$^{b}$ | 444±6.38$^{e}$ | 712±4.22$^{b}$ | 504±4.27$^{d}$ | 805±7.25$^{a}$ |

All values are expressed as mean±SEM (n = 6, where n = number of rats analysed in each group). Means with different superscripts (a-f) in the columns are significantly ($p < 0.05$) different from one another.

**Table 3.17** Effect of C1 and C2 on the biochemical parameters.

| Biochemical parameters | Normal control (mean±SEM) | Disease control (mean±SEM) | Silymarin treated (mean±SEM) | C1-LD (mean±SEM) | C1-HD (mean±SEM) | C2-LD (mean±SEM) | C2-HD (mean±SEM) |
|---|---|---|---|---|---|---|---|
| **ALT (U/L)** | 34±4.93$^{d}$ | 112±11.06$^{a}$ | 58±4.87$^{d}$ | 80±6.43$^{bc}$ | 67±5.54$^{cd}$ | 93±7.06$^{ab}$ | 76±6.68$^{bcd}$ |
| **AST (U/L)** | 36±4.35$^{d}$ | 96±9.88$^{a}$ | 52±5.80$^{cd}$ | 88±8.01$^{ab}$ | 61±5.69$^{c}$ | 82±8.59$^{ab}$ | 68±7.76$^{bc}$ |
| **ALP (U/L)** | 189±38.16$^{b}$ | 344±49.98$^{a}$ | 183±28.76$^{b}$ | 236±35.54$^{b}$ | 219±29.86$^{b}$ | 226±22.47$^{b}$ | 203±19.97$^{b}$ |
| **Bilirubin (mg/dL)** | 0.4±0.02$^{e}$ | 1.6±0.13$^{a}$ | 0.7±0.04$^{d}$ | 1.2±0.17$^{b}$ | 0.8±0.07$^{cd}$ | 0.9±0.09$^{c}$ | 0.7±0.05$^{cd}$ |
| **Total protein (g/L)** | 6.3±0.43$^{a}$ | 3.1±0.63$^{c}$ | 6.7±0.48$^{a}$ | 6.1±0.90$^{ab}$ | 7.2±0.66$^{a}$ | 4.8±0.43$^{b}$ | 6.4±0.50$^{a}$ |
| **Urea (mg/dL)** | 33±3.88$^{a}$ | 56±7.32$^{c}$ | 37±3.21$^{a}$ | 38±4.03$^{ab}$ | 35±3.90$^{a}$ | 25±4.33$^{b}$ | 19.6±3.84$^{a}$ |
| **Creatinine (mg/dL)** | 0.63±0.15$^{bc}$ | 0.98±0.18$^{a}$ | 0.61±0.09$^{bc}$ | 0.6±0.23$^{bc}$ | 0.5±0.08$^{bc}$ | 0.7±0.21$^{bc}$ | 0.3±0.06$^{c}$ |
| **Triglycerides (mg/dL)** | 121±23.87$^{b}$ | 253±32.45$^{a}$ | 108±14.89$^{b}$ | 155±19.84$^{b}$ | 123±12.22$^{b}$ | 149±20.09$^{b}$ | 125±18.68$^{b}$ |
| **Cholesterol (mg/dL)** | 143±41.50$^{ab}$ | 196±36.85$^{a}$ | 142±21.35$^{ab}$ | 138±19.76$^{ab}$ | 128±15.90$^{ab}$ | 148±22.69$^{ab}$ | 105±18.95$^{b}$ |

*Values are expressed as mean±SEM (n = 6, where n = number of rats analysed in each group). Means with different superscripts (a-e) in the columns are significantly ($p < 0.05$) different from one another.*

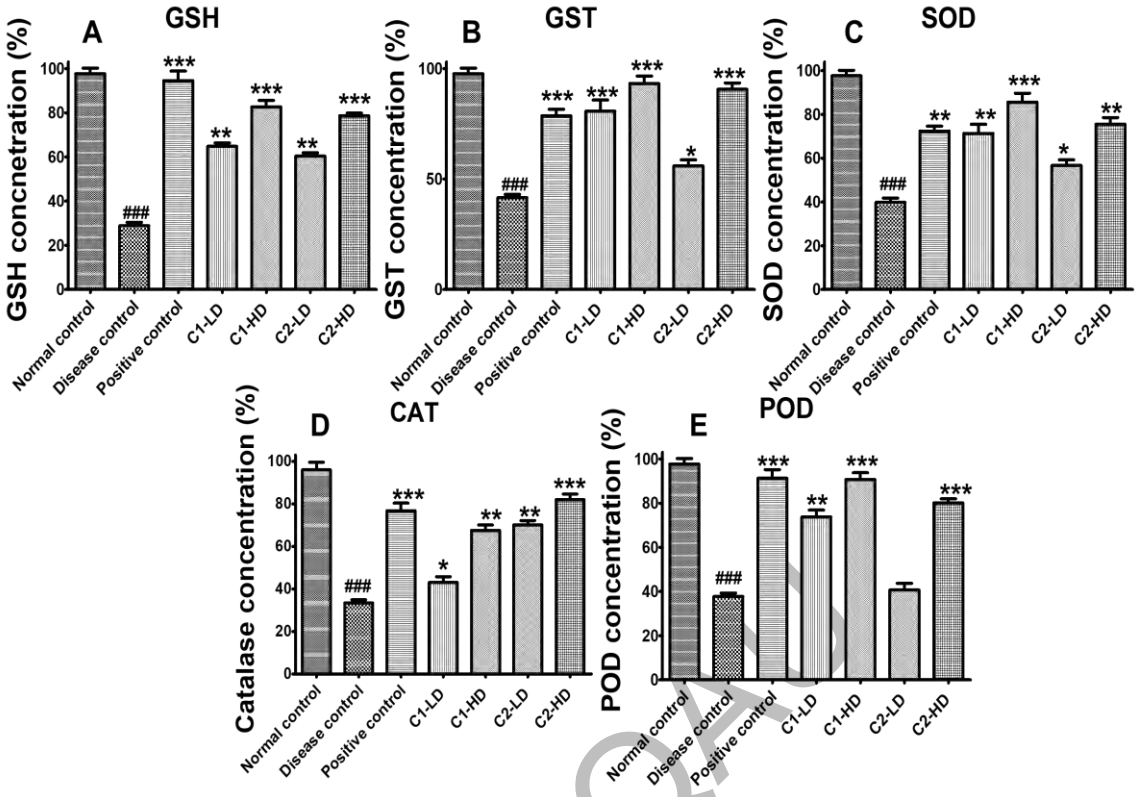


**Figure 3.51** $CCL_4$ induced liver injury. Effect of C1 and C2 on GSH (A), GST (B), SOD (C), CAT (D) and POD (E) levels in liver tissue compared to the disease control group.
***Note:*** *All values are expressed as mean±SEM (n=6), *P < 0.05, ** P < 0.01, *** P < 0.001.*

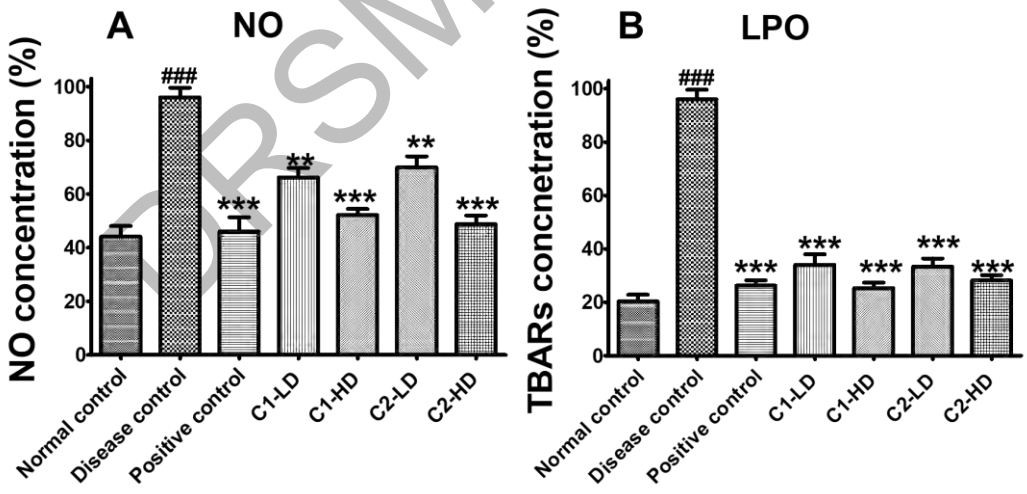


**Figure 3.52** $CCL_4$ induced liver injury.
***Note:*** *Effect of C1 and C2 on oxidative stress markers i.e., NO (A) and TBARs (B) levels in liver tissues compared to the disease control group. All values are expressed as mean±SEM (n=6), *P < 0.05, ** P < 0.01, *** P < 0.001. LPO; lipid peroxidation assay.*

### *3.6.2.5. Histopathology (H & E and Masson's trichrome staining)*

Histological examination has indorsed the findings of biochemical tests performed previously. Liver and kidney tissues of mice in normal control group showed normal

morphological features under the microscope. $CCL_4$ injections caused severe injury to liver and kidneys of disease control mice. Fibrosis, necrosed hepatocytes, edema and immune cell infiltration was noticed in liver histology of disease control mice while severely damaged glomeruli and Bowman's capsule were the most obvious signs of kidney damage in the same study group (H & E staining, figure 3.53 and 3.54). As observed in biochemical and enzymatic studies, a dose dependent alleviative effect was observed and the restoration of liver and kidney histoarchitecture was more significant in case of high dose groups of C1 and C2. As evident in figures 3.53 and 3.54, the histology score of high dose groups of both C1 and C2 was statistically similar to the positive control used in the study.

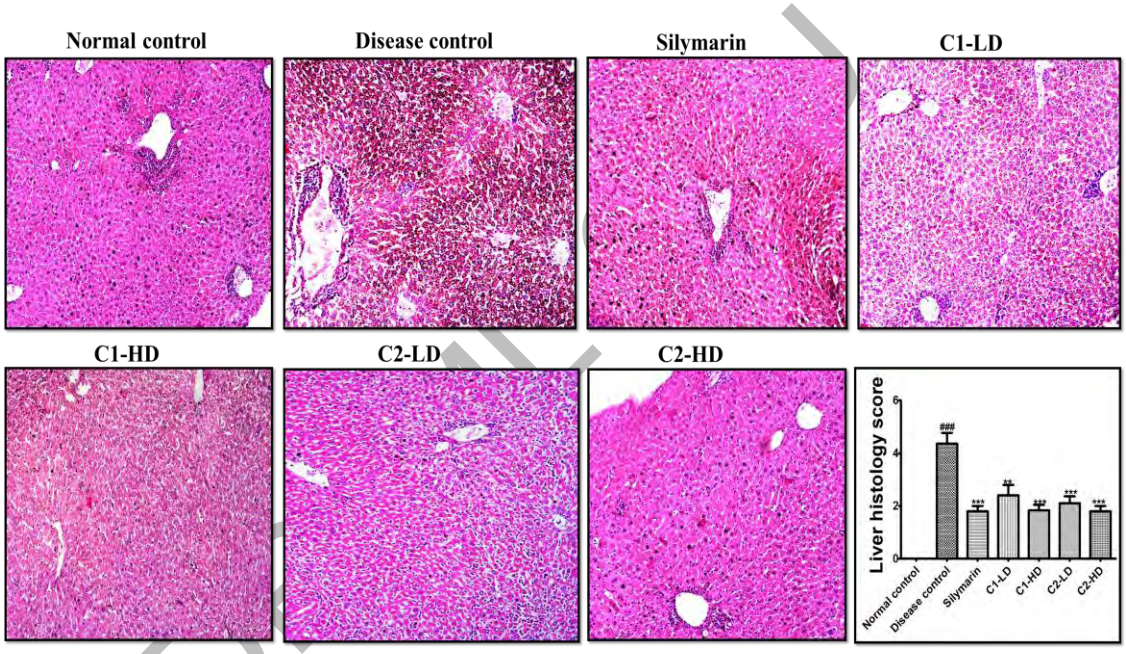


**Figure 3.53** $CCL_4$ induced liver injury. Hematoxylin-eosin **(**H and E) staining of the liver tissue.
***Note:*** *The restorative effect of C1 and C2 on liver tissues following benzene induced toxicity. The extracts markedly improved the histological parameters such as immune cell infiltration, fibrosis and edema compared to the disease control. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

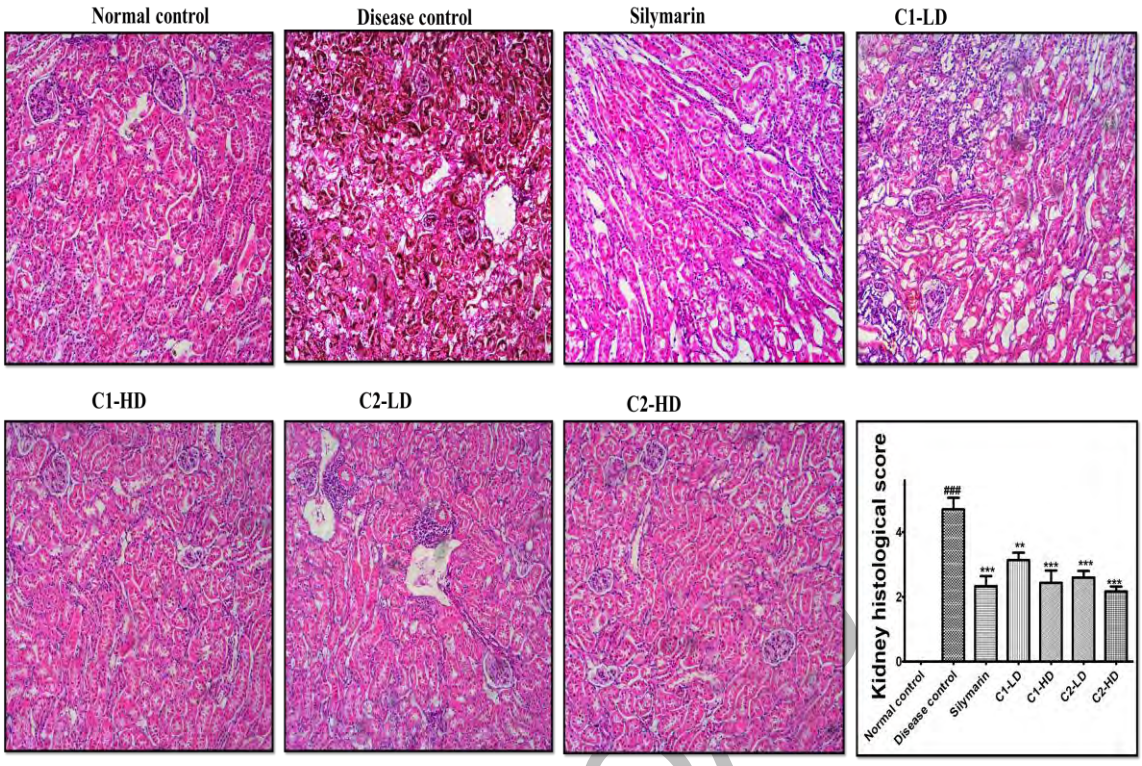


**Figure 3.54** $CCL_4$ induced liver injury. Histopathological observations for the protective potential of C1 and C2 isolated from *D. stramonium* on kidney tissues.
***Note:*** *All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

Masson's trichrome stain under light microscope was used to evaluate the accumulation of collagen and subsequent liver damage. Moreover, mesangial cell abnormalities and deposition of proteinaceous matter in the kidney can also be evaluated using this technique. $CCL_4$ is a noxious chemical that has widely documented reports of liver and kidney damage. The damage was revealed in the histology slides of disease control mice. The results were thoroughly in line with the observations made from H & E stained slides of liver and kidney tissues. A significant drop in accumulation of collagen was observed in C1-HD, C2-HD treated mice and equally so in silymarin treated group. The results are further elaborated in figure 3.55 and 3.56.

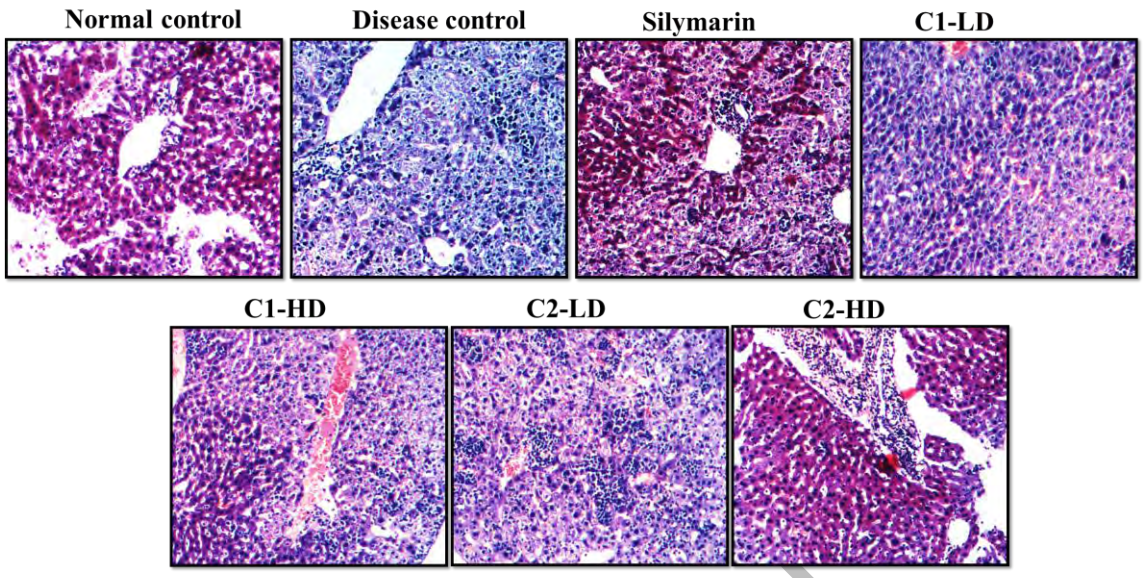


**Figure 3.55** $CCL_4$ induced liver injury. Masson's trichome staining of the liver tissues.
***Note:*** *C1 and C2 markedly improved the histological parameters and inhibited liver fibrosis compared to the disease control*

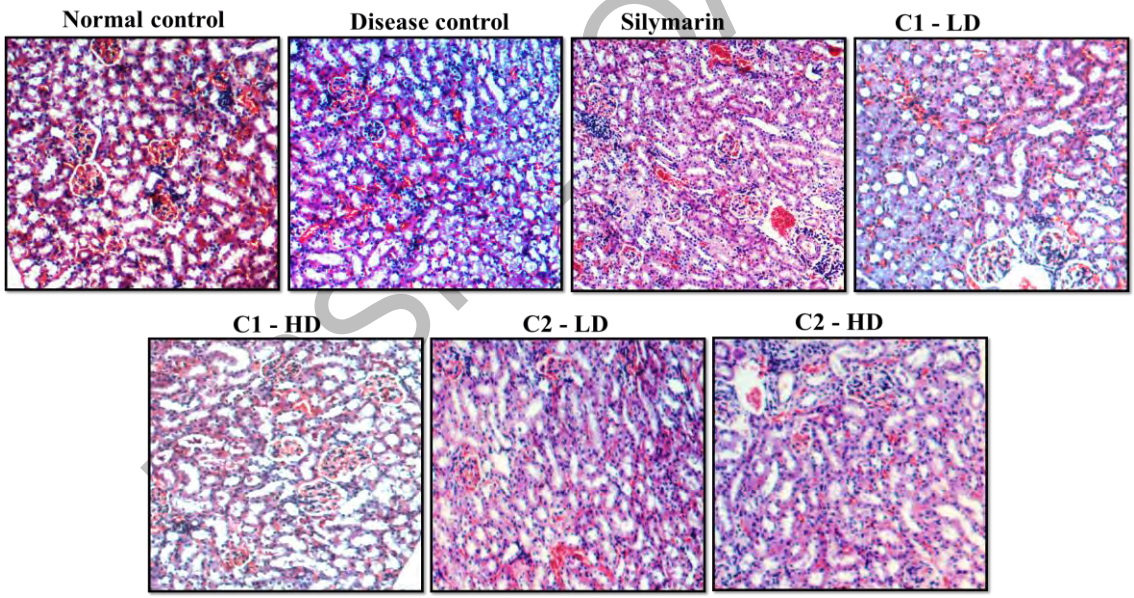


**Figure 3.56** $CCL_4$ induced toxicity. Masson's trichome staining of the kidney tissues.
***Note:*** *C1 and C2 markedly improved the histological parameters and significantly inhibited fibrosis compared to the disease control.*

### *3.6.2.6. Immunohistochemistry*

Immunostaining was used to determine the expression of NF-ƙB and COX-2 in liver and kidney tissues. The excessive ROS generation due to $CCL_4$ intoxication and subsequent inflammatory conditions resulted in up regulation of NF-ƙB and COX-2 signaling. It was confirmed by the excessively higher antibodies binding in the immunostained slides of liver and kidney tissues of disease control mice. As evident in

figure 3.57 and 3.58, the immunoreactivity score of silymarin was matched by high doses of C1 and C2. The results are consistent with the investigations done in preceding sections suggesting significant anti-inflammatory action of C1 and C2 in $CCL_4$ induced hepatic injury model.

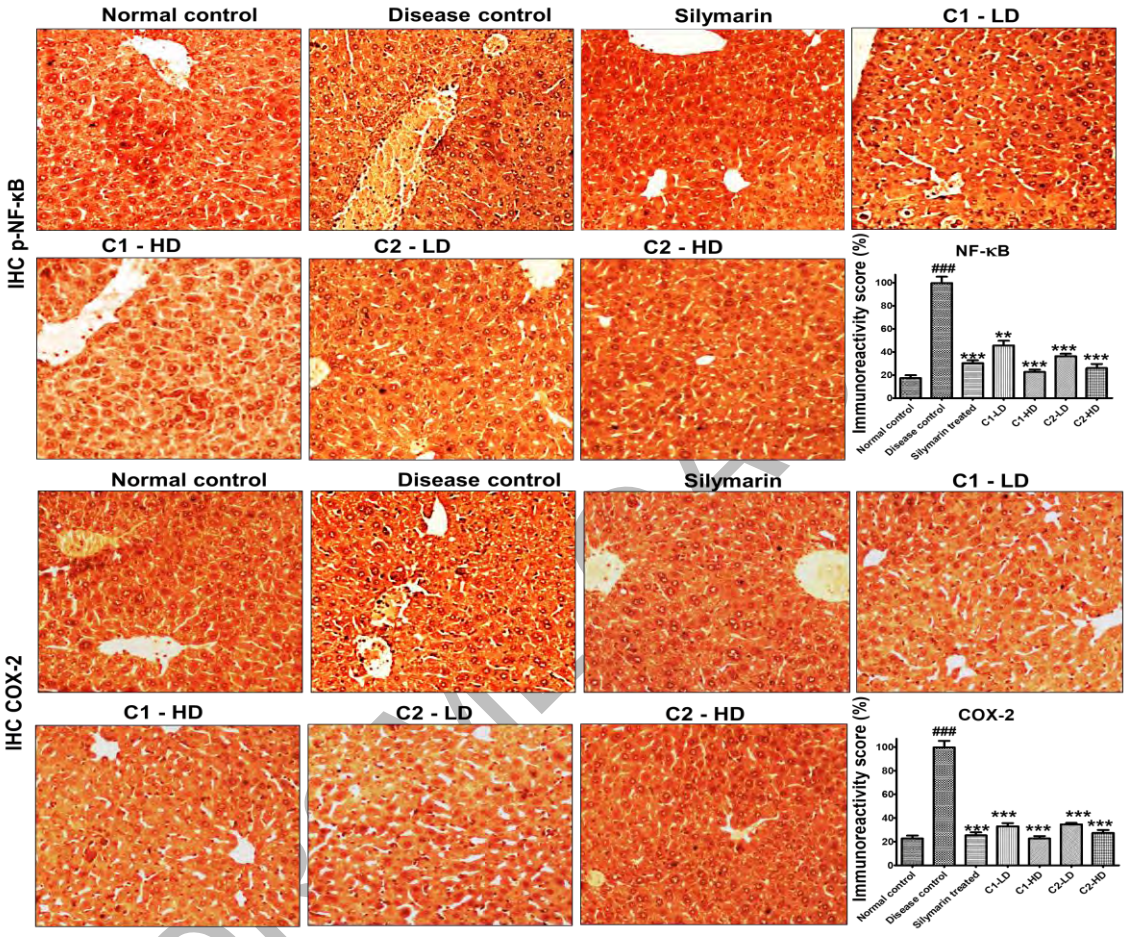


**Figure 3.57** $CCL_4$ induced liver injury. Effect of high and low dose treatment of C1 and C2 on expression level of nuclear factor kappa-B (NF-κB) and cyclooxygenase 2 (COX-2) in the liver tissue.
***Note:*** *using immunohistochemistry analysis. The extracts inhibited the expression of NF-κB and COX-2 in a dose dependent manner compared to the disease control. IHC: Immunohistochemistry. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

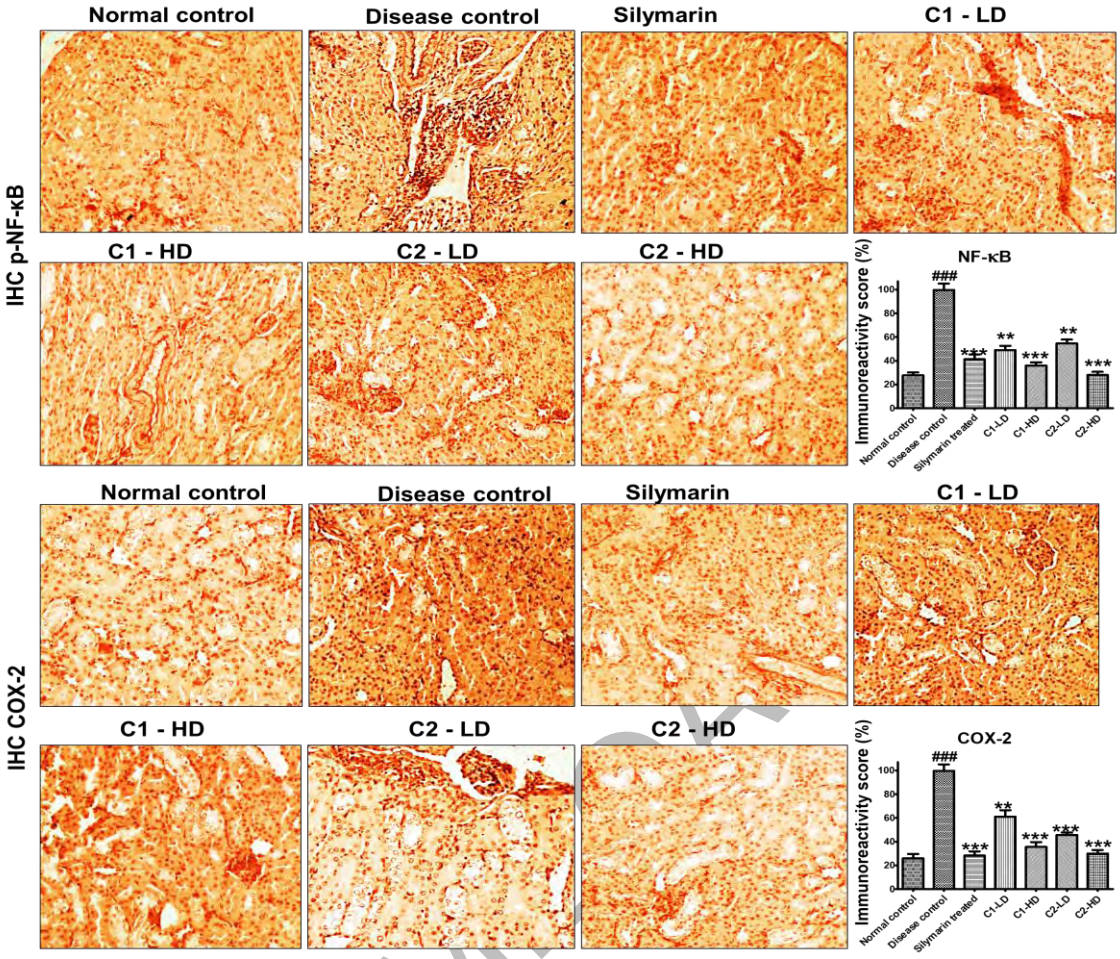


**Figure 3.58** $CCL_4$ induced toxicity. Effect of high and low dose treatment of C1 and C2 on expression level of nuclear factor kappa-B (NF-κB) and cyclooxygenase 2 (COX-2) in the kidney tissues.
***Note:*** *Using immunohistochemistry analysis. The extracts inhibited the expression of NF-κB and COX-2 in a dose dependent manner compared to the disease control. IHC: Immunohistochemistry. All values are expressed as mean±SEM (n=6), *P < 0.05, **P < 0.01, ***P < 0.001 as compared to disease control group.*

### *3.6.2.7. Western blotting studies*

To explore the molecular mechanism of significant hepatoprotective potential of C1 and C2 in $CCL_4$ induced hepatic toxicity was investigated by estimating the expression of numerous proteins i.e., SIRT1, p-AKT, NLRP3, ACS, Caspase-1 and TNF-α in liver homogenates of the mice *via* western blot technique (Figure 3.59 and 3.61). β-actin served as loading control. Western blot analysis provided clear evidence that treatment with C1 and C2 resulted in a significant dose dependent up-regulation of SIRT1 expression. AKT/PI3K pathway was regulated by both compounds *via* suppression of NF-kB mediated transcription of inflammatory cytokines to minimize $CCL_4$ induced cellular intoxication. Significant down-regulation of proteins associated with assembly and activation of inflammasomes including NLRP3, Caspase-1, IL-1β and ASC have been observed in western blot analysis. Western blot analysis confirmed the finding of

previous assays done in this study and further proved that that C1 and C2 possess significant potential to regulate the inflammatory pathways involved in the initiation and aggravation of cellular damage caused by toxins like $CCL_4$. Fold change in protein expression has been plotted using Image J and given in Figure 3.60 and 3.62.

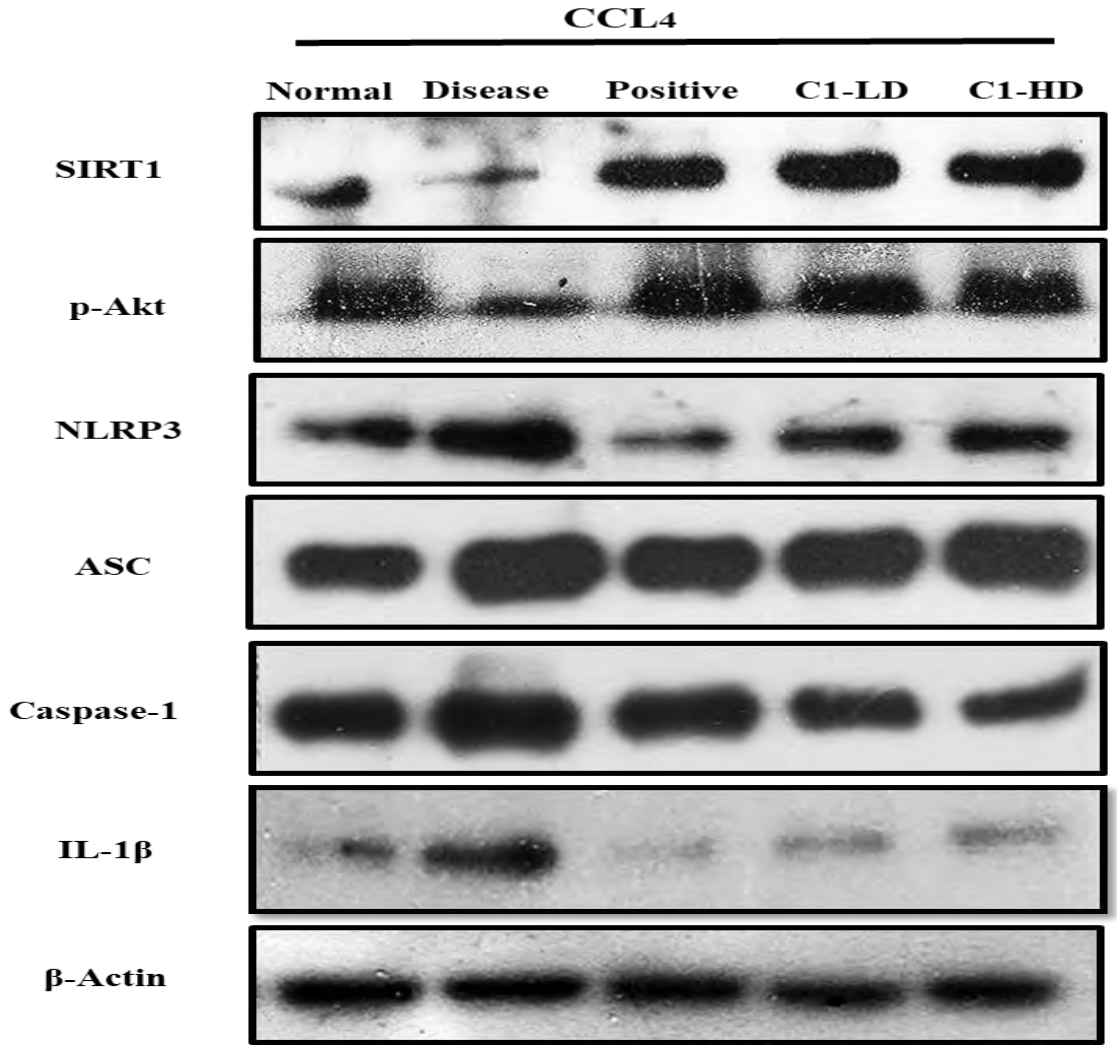


**Figure 3.59** Western blot analysis of proteins targeted by C1 in $CCL_4$ induced hepatic inflammation.
***Note:*** *Low and high doses of C1 (30 and 60 mg/kg) have resulted in upregulation of SIRT1 and p-Akt while NLRP3, ASC, Caspase-1 and IL-1ß were downregulated in a dose dependent manner. ß-Actin was used a loading control. SIRT1; Sirtuin 1, p-Akt; Protein kinase B, NLRP3; NLR family pyrin domain containing 3, ASC; Apoptosis associated speck like protein containing a C-terminal caspase recruitment domain, IL-1β pro-interleukin-1 beta.*

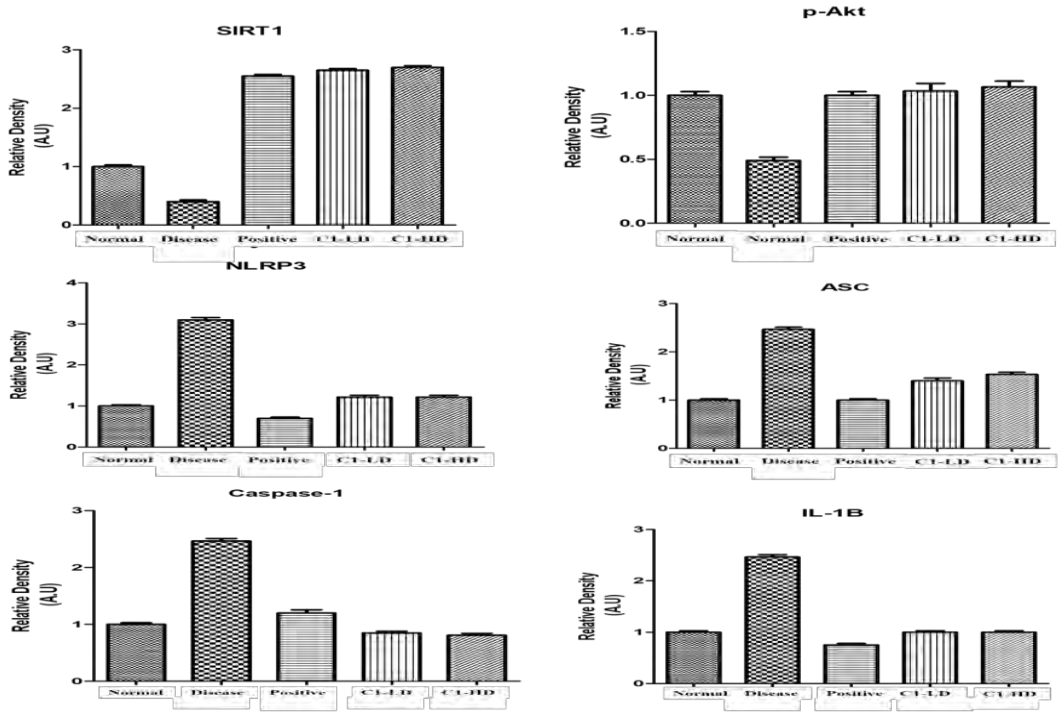


**Figure 3.60** Densitometric analysis SIRT1, p-Akt, NLRP3, ASC, Caspase-1 and IL-1ß expression in liver tissue.
***Note:*** *Role of C1 in proteins expression is plotted. Data is presented as mean±SD (n=6).*

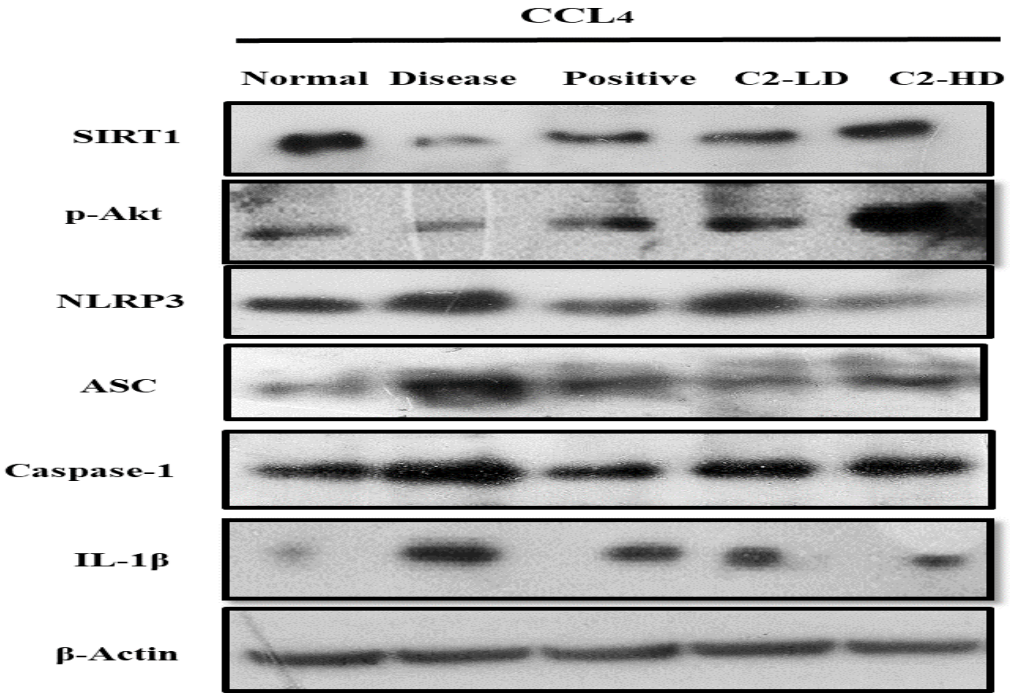


**Figure 3.61** Western blot analysis of proteins targeted by C2 in $CCL_4$ induced hepatic inflammation.
***Note:*** *Low and high doses of C2 (10 and 20 mg/kg) have resulted in upregulation of SIRT1 and p-Akt while NLRP3, ASC, Caspase-1 and IL-1ß were downregulated in a dose dependent manner. ß-Actin was used a loading control.*

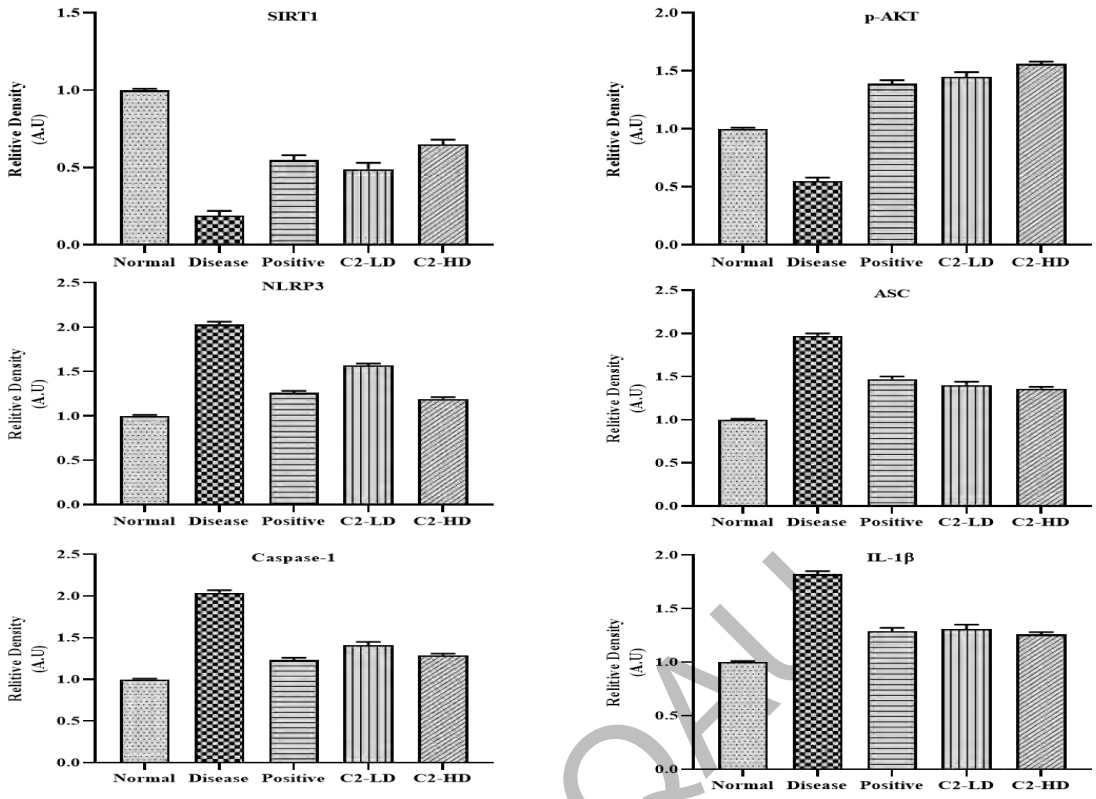


**Figure 3.62** Densitometric analysis SIRT1, p-Akt, NLRP3, ASC, Caspase-1 and IL-1ß expression in liver tissue samples of study mice.
***Note:*** *Role of C2 in proteins expression is plotted. Data is presented as mean±SD (n=6).*

# CHAPTER 4

# DISCUSSION

# 4. DISCUSSION

Natural products have been used by humans for the prevention, inhibition and treatment of numerous diseases. Due to the ever-growing demand of newer and safer drugs as well as problems faced due to incidents of resistance against marketed drugs, the development of innovative drug discovery approaches is a highly important research area in the field of medical sciences (Patil, 2011). Several moieties derived from plant sources have shown efficacy against numerous infectious and other chronic ailments (Duraipandiyan *et al.,* 2006). The ease of availability and cost effectiveness of ethnomedicine is acknowledged by the World Health Organization (WHO) and it is claimed that up to 60-80% population of developing countries rely on plant-based remedies to resolve their primary health issues. Pharmaceutical industry round the globe has shown great interest in natural product drug discovery from plant sources and researchers have dedicated their efforts in the development of plant-based remedies with improved efficacy and safety profile (Calixto, 2000).

Medicine derived from natural origin has gained immense interest and popularity in recent years owing to their great potential to prevent and treat several health issues linked to oxidative stress in a much safer and effective way (Aruoma, 2003). Plants are considered the earliest source of drug discovery and plant-based drugs have played a vital role in the healthcare system around the world (McRae *et al.,* 2007). Most medicinal plants have been known to be useful in mitigating more than one disease condition. This is because of the fact that plants do possess a cocktail of constituents each possessing their own pharmacological effects. These constituents act *via* diverse mechanisms and some of these have synergistic effects while others have distinct therapeutic effects elicited through numerous receptors.

The presence of structurally diverse secondary metabolites with numerous medicinal benefits imparts great significance to plants in the area of novel drug discovery, these natural sources can be used either as medicinally beneficial extracts or as potential sources of pure drug molecules. The compounds derived from plants have complex chemical makeup comprised of distinct functional groups and this complexity is the result of evolutionary processes spanning over many millennia. This makes them far superior candidates to interact with molecular targets within the body (McChesney *et al.,* 2007). The selection and identification of plants for extensive pharmacological

screening is based on several parameters including is ethnopharmacological utility, chemotaxonomic data and ecological observations (Fabricant *et al.,* 2001). This approach has led to the discovery of numerous drug compounds of unparalleled medicinal benefits including vincristine, vinblastine, morphine, taxol and ephedrine etc. It is safe to say that the drug discovery approach based on ethnopharmacological data is one of the most effective strategies in identification of potentially active drug molecules.

In the current study, we have chosen two *Datura* species (*Datura stramonium* and *Datura innoxia*) having known traditional uses and designed our study to evaluate their pharmacological potential by employing a wide spectrum of *in vitro* and *in vivo* assays. Preliminary phytochemical screening and *in vitro* assessment of the antioxidant potential of 32 (16 from each species) extracts of root, stem, leaf and fruit parts of selected *Datura* species was the starting point of this study. It was followed by selection of the most active extracts based on observed results. After toxicity studies of chosen extracts, they were utilized in predetermined doses for the estimation of *in vivo* antileukemic and anti-inflammatory activity. Subsequently, preparative scale extraction was employed using the most bioactive crude extract followed by isolation of pure compounds. The purified compounds were characterized by different analytical techniques. These pure compounds were then finally selected for *in vivo* screening based on their safety profile, chemical class, quantity and purity and their pharmacological potential was determined.



### 4.1. Effect of Extraction Solvent on Extraction Yield

A total of 4 solvents of escalating polarity were utilized to prepare 32 extracts of root, stem, leaf and fruit parts of selected species. The extraction method adopted in the study was “ultrasonication assisted maceration”. The choice of extraction method having high efficiency in terms of time/extraction yield ratio is very crucial to procure and quantify the phytoconstituents in a reproducible manner. An ideal extraction method should have the capability to achieve optimal tissue disruption, enhanced intra-particle diffusion and surface mass transfer as well as attaining sufficient yield and limiting the extraction time as much as practically possible (Vilkhu *et al.,* 2008). The ultrasonication based extraction method makes use of ultrasonic pressure waves to generate greater collisions within the particles. This causes a reduction of the particle size of the material; greater surface area as well enhances the exposure of extraction solvent to higher number of

particles. Ultrasonication also enables swelling of the cells resulting in hydration, enlargement and widening of the pores in the cell wall (Vinatoru 2001). This technique has wide implications in enhancing the extraction efficiency of numerous phytochemicals including anthocyanins, polyphenols, aromatic compounds and polysaccharides etc. (Zhang *et al.,* 2012; Ji *et al.,* 2006; Hromadkova *et al.,* 2003; Vila *et al.,* 1999). Another factor that plays a key role in optimizing the extraction yield is the solvent used in the extraction process. The separation and segregation of compounds of interest also depends on the solvent properties and its interaction with the solute particles. There are studies to confirm that polar solvents i.e., water, methanol and ethanol tend to give greater extraction yield as compared to lesser polar or non-polar solvents i.e., n-hexane. Successive extraction technique using solvents with increasing order of polarity was used for exhaustive extraction of phytoconstituents. The same decreasing trend in extract yield along with decreasing polarity of extraction solvent has been observed in the current study. Highest extraction efficiency was observed when Dw was used as the solvent, i.e., DSL-Dw: 12.10% and DIF-Dw: 11.50% while the least polar solvent used was n-hexane and it resulted in lowest extraction yield i.e., DSS-NH: 0.34% and DIS-NH: 0.31%. Better extraction yield does not always mean procurement of biologically significant constituents. The chemical nature of the extracted constituents is exclusively responsible for their potential therapeutic effects. It is thus widely possible that a lesser polar solvent may possess the capacity to extract a pharmacologically significant compound. So, the crude extracts acquired were screened using a range of phytochemical and biological assays.

## 4.2. Preliminary Screening

### 4.2.1. Phytochemical analysis

Phytochemical analysis of medicinal plants plays a vital role in establishing the chemotaxonomic data of these plant species. Phytochemicals are basically non-nutritive components of plants, they play a vital role in combating diseases and are proven beneficial both for plants and animals (Fatima *et al.,* 2015).

The phytochemicals assessment of selected parts of *D. stramonium and D. innoxia* was done using colorimetric assays for the estimation of phenols and flavonoids. The total gallic acid equivalent phenols in *D. stramonium* extracts ranged from 41.78±2.13 to 7.49±0.47 µg GAE/mg extract with the highest level quantified in EA extract of fruit part while lowest level estimated in Dw extract of stem. In case of *D. innoxia* extracts,

DIL-EA showed the highest content and the lowest was recorded again in DIS-Dw. Largely, the total phenolic content is reported to be higher in extracts prepared using moderately polar to polar solvents. Though, in our study successive extraction method with Dw used as the final extraction solvent has rendered the Dw extracts slightly less abundant in phenolics. Phenolics are ubiquitous molecules and are known to be present in almost all plant parts. These represent a structurally diverse group of compounds and have a variety of medicinal benefits. The antioxidant properties of phenolics are widely reported and they act *via* a diverse set of mechanisms including free radical scavenging, $H^+$ donation, quenching of singlet oxygen, chelation of metal ions as well acting as a substrate for superoxide. They can also inhibit lipid peroxidation by the termination of free radical reactions within the body (Robards *et al.,* 1999). Numerous studies have reported the presence of medicinally significant phenolic compounds in different species of *Datura* (Al-Snafi, 2017). These compounds are known to exhibit useful pharmacological actions including but not limited to their anticancer, anti-inflammatory, hepatoprotective, neuroprotective, anti-antherogenic and antimicrobial actions (Trivellini *et al.,* 2016).

Flavonoids are hydroxylated phenolic compounds and they comprise one of the most abundant sub-groups of polyphenols. The degree of reactivity and chemical nature of flavonoids is dependent on several factors including their chemical structure as well as extent of polymerization and hydroxylation. The $OH^-$ groups in the flavonoid skeleton are majorly responsible for their antioxidant activity (Kumar *et al.,* 2013). In our study, the maximum flavonoid content was estimated in EA extracts of the leaf part of both species, giving an indication about the potential medicinal worth of these extracts.

Furthermore, the quantification of several phenolics was done using RP-HPLC based analysis. The presence of specific polyphenolic compounds was confirmed by comparison of UV spectra and retention time of the standard compounds used in the analysis with EA and M extracts of *D. stramonium* and *D. innoxia*. A significant amount of gallic acid, rutin, catechin, apigenin, myricetin and kaempferol was quantified in some of the analyzed extracts. Amongst *D. stramonium* extracts, DSF-EA, DSL-EA and DSR-EA revealed the presence of numerous polyphenols including gallic acid, rutin, apigenin, myricetin, kaempferol and catechin while *D. innoxia* extracts also contained several pharmacologically significant molecules i.e., rutin, catechin and kaempferol. Detection of all these polyphenols further potentiates the

medicinal value of selected *Datura* species. The polyphenols detected and quantified in selected extracts have established pharmacological uses i.e., rutin is known for its anticancer, antidiabetic and antioxidant properties (Chua, 2013), apigenin has known anticancer and anti-inflammatory potential (Singh *et al.,* 2004) and it is also known to induce autophagy in leukemia cells (Fatima *et al.,* 2015). Gallic acid has numerous medicinal uses particularly its application as antiangiogenic, anticancer, anti-inflammatory and as an antimicrobial agent (Choubey *et al.,* 2015). Antioxidant properties of caffeic acid are extensively studied, in one such study it surpassed numerous other antioxidants in curbing aflatoxin generation by more than 95% (Dai *et al.,* 2010). Apart from antioxidant properties, these polyphenols have documented likelihood to impede cancer cell proliferation, activation of pro-carcinogens and inhibit growth signaling pathways and thus prevent cancer cell proliferation (Hussain *et al.,* 2016; Amin *et al.,* 2015). A study has reported substantial correlation between the occurrence of polyphenols and *in vivo* tumor growth inhibition via regulating MAPK/ERK, PI3K/AKT and STAT3 pathways in lung metastasis and breast cancer stem cells (Vuong *et al.,* 2016).

### 4.2.2. *In vitro* biological evaluation

#### *4.2.2.1. Antioxidant assays*

The total antioxidant capacity (TAC) of *D. stramonium* and *D. innoxia* extracts was evaluated by phosphomolybdenum based colorimetric assay. The method is based on the reduction of Molybdenum (VI) to Molybdenum (V) by the antioxidant moieties that results in the formation of a phosphate/Mo (V) complex. This complex is green colored and gives maximal absorption at 695 nm during UV analysis (Jafri *et al.,* 2017). Of all the *D. stramonium* extracts, maximum TAC expressed as µg AAE/mg extract) was exhibited by DSL-EA (160.92±3.00 µg AAE/mg extract. While in case of *D. innoxia* extracts DIL-EA showed maximum TAC i.e., 140.44±5.6 µg AAE/mg extract. Natural antioxidants of plant origin are of paramount importance in avoiding cellular injuries caused by oxidative stress and thus have a positive impact on overall human wellbeing and health. Some of the most effective antioxidants of plant origin include phenolic acids (caffeic, protocatechuic, rosmadial, gallic and rosmarinic acids), flavonoids (catechin, quercetin, kaempferol and naringenin) and phenolic diterpenes (rosmanol, carnosol and carnosic acid etc.) (Brewer, 2011).

The antioxidant potential of *Datura* extracts was also evaluated by using ferric ion reducing power assay. This assay relies on the transformation of $Fe^{3+}$ to $Fe^{2+}$ (ferric ion in potassium ferricyanide) in the presence of antioxidant molecules within the test samples. The antioxidant moieties form a complex with potassium ferricyanide in acidic conditions which results in appearance of greenish color in the test solution. This change in color is analyzed through spectrophotometric method to quantify the antioxidants presence within the test samples (Majid *et al.,* 2015). As described in chapter 3, DSL-EA displayed stronger reducing potential (64.70±0.81 µg AAE/mg extract) as compared to other extracts of *D. stramonium.* Moreover, significant antioxidant action in terms of reducing power was revealed by medium polar extracts of *D. innoxia.* Initial phytochemical screening using HPLC analysis quantified considerable amounts of polyphenols i.e., rutin, gallic acid, catechin, apigenin and caffeic acid in numerous extracts of our study plants. Presence of these pharmacologically significant polyphenols with widely reported antioxidant activities might be responsible for the strong antioxidant capacity and reducing potential of the moderately polar extracts of *D. stramonium* and *D. innoxia.*

The percent free radical scavenging activity (% FRSA) was estimated by discoloration of DPPH solution. Slightly less significant radical scavenging action was observed in this assay. The maximum scavenging action was shown by DSL-EA (43.72±3.37%) while in *D. innoxia* extracts, the highest FRSA scavenging action was observed with DIL-M (36.67±0.86%). The results of preliminary *in vitro* biological evaluation affirmed that Dw extracts were devoid of significant antioxidant moieties, and this is in accordance with the findings of the phytochemical assays as discussed earlier.

#### *4.2.2.2. Brine shrimps cytotoxicity assay*

The evaluation of cytotoxic potential of extracts tend to help in initial screening of their potential anticancer, antitumor and antimicrobial activities. Brine shrimps (*Artemia salina*) larvae are a well-recognised model for the preliminary investigation of the cytotoxic, anticancer and antitumor properties of samples (Nguta *et al.,* 2012). The cytotoxicity of *D. stramonium* and *D. innoxia* extracts was tested against brine shrimp larvae to determine their lethality profile. Out of a total of 32 extracts of both *Datura* species screened for cytotoxic activity against brine shrimp larvae, several extracts showed significant activity. The extracts have shown a concentration dependent lethality with higher concentrations showing greater cytotoxicity as compared to lower

ones. It was evident from the results that extracts procured using low to medium polarity range solvents showed greater cytotoxicity as compared to Dw extracts which resulted in negligible cytotoxic effect. The $LC_{50}$ values of *D. stramonium* and *D. innoxia* NH, EA and M extracts of each plant part were less than 25 µg/ml. On the contrary, 75% of Dw extracts of both species had an $LC_{50}$ value greater than 200 µg/ml. The $LC_{50}$ values of the test samples can give a clear indication about their potency, and it is reported that a plant extract with $LC_{50}$ value lower than 1000 µg/ml can be considered as cytotoxic in nature (Nguta *et al.,* 2012). In our study, 75% of *D. stramonium* extracts resulted in $LC_{50}$ values of less than 25 µg/ml while 75% of *D. innoxia* extracts had $LC_{50}$ values < 23 µg/ml. These results strongly suggested the occurrence of compounds with cytotoxic properties in the tested extracts. The findings of this experiment paved the way for further exploration of the cytotoxic potential of selected *Datura* species using other *in vitro* anticancer studies including against cancer cell lines.

#### *4.2.2.3. Protein kinase inhibition assay*

Extracts were also screened for their protein kinase inhibitory potential. *Streptomyces* 85E strain used in the assay requires protein kinases for their aerial hyphae formation which is the basic principle behind the performance of this assay. Kinases play a key role in cell proliferation, division and are considered as critical factors in carcinogenesis (Kandel *et al.,* 1999; Gschwind *et al.,* 2004). This assay was thus performed for the determination of kinase inhibitory and ultimately anticancer action of the extracts. The results of this experiment followed an identical trend as observed in brine shrimp cytotoxicity assay in terms of the potency of extracts. Extracts derived using nonpolar and medium polar solvents showed better activity as far as appearance of bald zones on cultured plates is concerned while Dw extracts of both species did not reveal any significant activity. The EA leaf extract of both species was particularly phenomenal with DIL-EA at 100 µg/disc resulting in highest bald phenotype zone i.e., 19 mm and DSL-EA followed with an inhibitory zone of 12.50 mm. The development of protein kinase inhibitors from natural sources particularly from plants has generated renewed interest in targeting kinases. It is because of the fact that protein kinases have a major role to play in cell growth and development, progression of cell cycle and signals transduction across the nuclear membrane (Nasir *et al.,* 2017). Mutations in genes specifically important for cell growth, differentiation and death result in elevated kinase

activity at serine/threonine residues usually found in human cancers (Nasir *et al.,* 2017). Serine/threonine kinases play a critical role in carcinogenesis (Kandel *et al.,* 1999). Likewise, a study has shown alteration in a subfamily of serine/threonine kinases in ovarian carcinomas (Cheng *et al.,* 1992). Tyrosin kinases are involved in regulation of cellular functions of normal cells, additionally they play a key role in oncogenesis (Gschwind *et al.,* 2004). It is for this reason that kinase inhibitors have emerged as auspicious targets for cancer treatment (Yao *et al.,* 2011). A definite advantage of using whole cell *Streptomyces* is that the cytotoxic activity of samples can be readily identified using this assay. Furthermore, inhibitors of signal transduction for numerous applications i.e., antitumor, anti-infective and anti-mycobacteria can also be identified with the aid of this simple assay (Waters *et al.,* 2002).

**Summary of preliminary screening and exclusion of DW extracts**

The preliminary *in vitro* screening of 32 crude extracts of both *Datura* species enabled us to exclude the less potent extracts from further analysis. The extracts were evaluated based on the findings of a series of phytochemical and biological assays and NH, EA and M extracts of both species were further screened against certain breast and prostate cancer cell lines.

## 4.3. Cytotoxicty Against Cancer Cell Lines and Selection of Most Potent Extracts for Further Studies

Anticancer potential of *Datura* extracts was further assessed against selected cancer cell lines. PC-3 is a human prostate cancer cell line and is used in exploration of numerous biochemical variations in prostate cancer cells and assessment of their response to different anticancer agents. Prostate cancer is one of the most widespread cancers diagnosed in men and developing new chemotherapeutic agents for its treatment has been quite a challenge (Nasir *et al.,* 2020). Human breast cancer cell lines MDA-MB 231 and MCF-7 were also used in the current study. Breast cancer is a major health problem in women, one in ten of all reported cancers annually is a cancer of female breast (Ferlay *et al.,* 2010).

Amongst all the extracts included in the experiment, EA leaf extracts of both species showed good results. DIL-EA exhibited remarkable activity against each of the three tested cancer cell lines with $IC_{50}$ values lower than 3.00 µg/ml in each case. DSL-EA was the second most effective in terms of the cytotoxicity against selected cancer cell

lines, although it displayed comparatively lesser activity. The anticancer potential of aerial parts and seeds of *Datura* species against cancer cell lines has been explored previously by numerous researchers using different solvent extracts. DS methanol extract of seeds when tested against MCF-7 cell line displayed an $IC_{50}$ value of 113.05 µg/ml and the observed activity was postulated to be due to the detected antioxidant potential of the same extract (Iqbal *et al.,* 2017). In another study DS water extract of leaf part was tested at a concentration of 1 mg/ml against numerous cell lines including MDA-MB 231, and 24-hours exposure led to a significant decline in cell survival rate (Ahmad *et al.,* 2009). *D. innoxia* methanolic leaf extract and one of its constituents from the extract named 'Dinoxin B' were tested for anti-proliferative action against a range of normal and human cancer cell lines. Sub-micromolar $IC_{50}$ values were observed against different cancer cells and the most sensitive ones were various breast cancer cell lines. The activity was accredited to the presence of Dinoxin B found specifically in leaf portion of *D. innoxia* (Vermillion Karl *et al.,* 2011b). Dinoxin B is from the withanolide class of natural products. Keeping many of the previously reported studies in sight, we can infer that antioxidant moieties as well as different withanolides reported in *Datura* species might well be responsible for the remarkable anticancer activity of DIL-EA as well as the modest activity profile of DSL-EA.

Keeping in view certain factors i.e., extraction yield and promising results in preliminary *in vitro* assays, NH and EA extracts of *D. stramonium* and *D. innoxia* leaf portion (DSL-NH, DSL-EA, DIL-NH and DIL-EA) were shortlisted for further assessments including *in vitro* toxicity studies as well as acute and chronic *in vivo* assays.



## 4.4. *In Vitro* Toxicity, NO Scavenging and Acute *In Vivo* Assays

### 4.4.1. *In vitro* toxicity assessment

The toxicity profile of the extracts was evaluated, and no significant cytotoxic action was observed against isolated normal human lymphocytes. None of the extracts revealed greater than 30% inhibition at 20 µg/ml final concentration. Toxicity was further gauged by determining their action against macrophages isolated from rat peritoneum. As discussed in the results section, even at the highest used concentration of 20 µg/ml, none of the extracts showed any cytotoxic action against the isolated macrophages which further proved the selective nature of their cytotoxic action. Keeping in view the escalating demand to discover new anticancer and anti-

inflammatory drug moieties, it is quite imperative to identify potent molecules with clinically proven safety profile.

### 4.4.2. NO scavenging potential

The nitric oxide scavenging potential of DSL-NH, DSL-EA, DIL-NH and DIL-EA extracts in LPS challenged murine macrophages was estimated and at concentration of 2.5-20 µg/ml, significant inhibition of NO production was observed. DSL-EA proved to be the most efficient with an $IC_{50}$ value of 7.625±0.51 µg/ml. Macrophages activated by immune response generate NO at a higher rate at inflammatory sites which then play a major role as immune regulators and neurotransmitters in various tissues (Bogdan *et al.,* 2000; Jin *et al.,* 2007). Scavenging the excessive NO radicals thus constitutes a prominent therapeutic approach for curbing inflammatory disorders.

### 4.4.3. Acute *in vivo* toxicity

Following *in vitro* screening of the extracts, acute toxicity was assessed in rats at doses ranging from 150-2000 mg/kg. Observation of no harmful and damaging effects on any of the groups over a period of two weeks further confirmed the safety of the four shortlisted crude extracts within the specified dose range and it led to the designing of acute non-invasive and sub chronic anti-cancer and anti-inflammatory *in vivo* assays.

### 4.4.4. Acute *in vivo* studies – non invasive

In the initial phase of *in vivo* studies, the extracts were screened for their anti-inflammatory, antidepressant and analgesic potential using Balb/c mice. Two of the most widely recognized mice models to evaluate the anti-inflammatory potential of potential medicinal agents are carrageenan induced paw edema and croton oil induced anal edema inhibition tests (Yim *et al.,* 2009). Mediators effecting acute inflammatory responses generally work in three different phases with histamine and serotonin been released in first phase (first 1.5 hr), second phase involves bradykinin release (1.5-2 hr) while prostaglandins are involved in the third and last phase (2.5-6 hr) (Aboluwodi *et al.,* 2017). Carrageenan induced edema is a biphasic model and edema induction in the first two hours is due to bradykinin, serotonin and histamine release while in the latter stages (3-5 hr), prostaglandins are primarily responsible for edema (Sakat *et al.,* 2014). Moreover, croton oil induced inflammatory responses are primarily characterized by edema, greater vascular permeability, neutrophil infiltration and prostaglandins production (Rao *et al.,* 1993). Considerable reduction in edema volume was observed

at 4th hr following administration of predetermined doses of DSL-EA in both models of inflammation used in current study. The anti-inflammatory response was in a dose dependent manner and previously published data support our findings (Aboluwodi *et al.,* 2017; Sonika *et al.,* 2010). The second in line in terms of efficacy was DIL-EA extract which also appeared to reduce the edema to a notable extent in both models of inflammation. It can be inferred from the pattern of observed edema inhibitory action that the test samples, particularly ethyl acetate leaf extracts have a tendency to limit the production of certain inflammatory mediators and pro-inflammatory cytokines. The prevalence of depression is very common in cancer patients. The possibility of co-occurrence of these commodities increases with increase in the severity of disease symptoms including pain, aches and fatigue. The provision of timely psychological support is known to improve the survival time, quality of life and severity of symptoms in cancer patients (Spiegel *et al.,* 2003).

Inflammation is reckoned to play a critical role in promoting susceptibility to depression, so treating inflammation, regardless of its type and cause, can be of great therapeutic benefit in improving the overall health status (Beurel *et al.,* 2020). There is ample data to support the notion that depression is accompanied by elevated oxidative and nitrosative stress, it also has a strong association with chronic inflammatory response (Moylan *et al.,* 2013; Maes *et al.,* 2012). Several pathways are involved in carrying signals to the brain in the event of peripheral inflammation i.e., cytokine transport system, vagus nerve and leaky regions in blood brain barrier (Quan *et al.,* 2007) and peripheral cytokines then disturb the synthesis and reuptake of neurotransmitters including, serotonin, dopamine and norepinephrine (Miller *et al.,* 2013).

In current study, tail suspension test was used to predict the effect of *Datura* extracts on the behavior of test animals when they were exposed to testing conditions. The tail suspension test is a commonly used behavioral test in rodents and is used to assess the clinical effectiveness of antidepressant agents (Cryan *et al.,* 2005). The efficacy of test samples is usually scaled by observing the reduction in immobility time i.e., the state of helplessness displayed by test animals. Although the NH leaf extracts did not show any significant effect, the EA extracts, particularly high dose (HD) of DSL-EA resulted in some degree of improvement. DSL-EA HD, when compared with the positive control (fluoxetine), displayed mild effect while slight reduction in the immobility time was

observed in the low dose group animals. *D. stramonium* has reported ethno-medicinal uses in epilepsy and depression when used internally while in form of ointments it was utilized by ancient communities in rheumatism and burns (Guarrera, 1999) but there is lack of latest comprehensive data about its antidepressant potential.

Hot plate test is a prominent nociceptive test using thermal stimuli to assess the centrally mediated analgesic action of test samples (Barrot 2012). Scientific evidence supports the notion that drug molecules causing increase latency period possibly possess central analgesic activity (Ibironke *et al.,* 2007). Ethnopharmacological use of leaves of selected *Datura* species as an analgesic was further validated by conducting the hot plate assay using mice model. Moderate elevation in latency period was observed in case of DSL-EA HD, while low dose revealed slight analgesic response as per readings taken at $4^{th}$ h of the experiment. EA leaf extract of *D. innoxia* resulted in a weaker analgesic action at high doses while NH extract of both species did not show any prominent activity. *D. stramonium* leaves have been used traditionally for the management of pain externally as topical preparations (Njoroge 2012), the plant also has reported pharmacological use as an analgesic in variety of inflammatory disorders and pain (Soni *et al.,* 2012; Gaire *et al.,* 2013). Our observations have further reinforced this ethnomedicinal and folklore use of *D. stramonium.*

Based on the findings of non invasive *in vivo* studies, chronic *in vivo* studies were undertaken using the extracts with promising pharmacological benefits. The NH extracts of both species were thus excluded and the anticancer and anti-inflammatroy potential of DSL-EA and DIL-EA extracts was assessed wth two distinct animal models as discussed in the following sections.



## 4.5. Chronic *In Vivo* Models Using DSL-EA and DIL-EA Extracts

### 4.5.1. Benzene induced leukemia

Leukemia is amongst the most prominent cancer forms both in infants and adults and is characterized by unrestrained formation, multiplication and accumulation of malignant white blood cells in bone marrow and peripheral blood (Gopinath *et al.,* 2017). The exact cause of leukemia is fundamentally unknown but occupational and environmental factors are considered as significant contributors to the onset and progression of the disease. Mutations in DNA because of oncogenes activation or deactivation of tumor suppression genes is considered the main causative factor of

leukemia. Apart from sporadic mutations, few occur as a result of certain predisposing factors like exposure to different carcinogens (Ross *et al.,* 2002). One such carcinogen is benzene which is associated with many health hazards. The induction of hematological toxicity by benzene has been reported over a century ago and fresh studies have also linked benzene to hematotoxicity even at concentrations less than 1 ppm in air (Lan *et al.,* 2004; Qu *et al.,* 2002). Benzene causes acute myeloid leukemia (AML) and myelodysplastic syndrome and is also a plausible cause of other hematological disorders including non-Hodgkin lymphoma (Khalade *et al.,* 2010; Steinmaus *et al.,* 2008). The postulated mode of action of benzene briefly consists of five major steps: (a) benzene metabolism to its metabolite, benzene oxide; (b) its interaction with target cells in the bone marrow; (c) initiation of mutations in bone marrow cells; (d) designated clonal proliferation of these mutated cells and (e) abnormal growth and multiplication of target cells in bone marrow resulting in neoplasia (leukemia) (Meek *et al.,* 2010).

The antileukemic potential of DSL-EA and DIL-EA on male Sprague Dawley rats bearing benzene induced leukemia was assessed on the basis of results acquired from a series of hematological, biochemical, enzymatic and histological studies. Significantly decreased RBCs, platelets and hemoglobin levels while elevated WBCs and ESR in disease control rats established the fact that leukemia was induced in this group. Marked elevation in WBCs count is one of the prominent signs of acute leukemic condition. Results revealed a 40-50% decrease in WBCs count in study groups when compared to negative control. Leukemia induction in rats by benzene administration has been previously reported in numerous studies (Olufemi *et al.,* 2012; Akanni *et al.,* 2017; Akanni *et al.,* 2014; Cronkite *et al.,* 1984). Comparison of study groups clearly revealed the protective effect of low and high doses of *Datura* extracts. There was no statistically significant difference ($p < 0.05$) between the calculated hematological parameters of treatment and preventive groups when compared with the positive control group. It showed that administered doses of extracts have shown comparable antileukemic activity to the standard drug used in current study. Polyphenols detected in phytochemical investigation might be responsible for potentiating the vasoprotective and vasotonic effects of DSL-EA and DIL-EA.

Exposure to benzene has been reported to cause liver damage since it is the major organ responsible for compound metabolism into inert and potentially toxic metabolites.

Enzymatic conversion of benzene to reactive species can lead to oxidative stress. These species can interact with tissues and cell membranes causing lipid peroxidation which can further aggravate liver injury (Al-Olayan *et al.,* 2014; Winn 2003; Abd El-Shakour *et al.,* 2015). Abnormalities in level of serum enzymes and macromolecules signifying liver functionality, including ALT, AST and ALP as well as bilirubin and total proteins clearly indicated liver injury in disease control rats. The incidence of liver injury is very common in hematological diseases, particularly leukemia (Walz-Mattmüller *et al.,* 1998). Preventive and treatment groups of both extracts reverted liver damage to a great extent as evident by their statistically significant ($p < 0.05$) difference from disease control group. Although the biochemical mechanism for regeneration of liver enzymes and functionality is not clear, nevertheless inhibition of lipid peroxidation and free radical scavenging properties of polyphenolic compounds might be responsible for curbing the damage caused to liver by benzene (Fatima *et al.,* 2015; Nasir *et al. ,*2017).

The kidney acts as one of the major organs responsible for detoxification and waste elimination rendering it highly susceptible to toxins induced injury. Benzene induced nephrotoxicity has been documented previously (Abd El-Shakour *et al.,* 2015). Glomerulonephritis and nephrotic syndrome without evident kidney failure have been reported in leukemia and lymphoma. Tumor lysis and cell bursting in acute leukemic conditions result in elevated potassium, phosphate and decreased calcium level along with acid-base imbalance. All these anomalies result in kidney failure characterized initially by significantly elevated serum urea and creatinine level (Luciano *et al.,* 2014). Benzene administration posed significant nephrotoxic effects. Disease control rats were the most affected ones based on highest estimated values of selected nephrotoxicity markers. *Datura* species at low and high doses lowered urea and creatinine levels in a manner comparable to the positive control. The nephroprotective effect observed in the current study further strengthened the potential antileukemic action of used extracts.

The role played by oxidants in different stages of carcinogenesis is well documented (Cerutti 1994). Cells using aerobic metabolism for their energy needs inevitably generate ROS (Goto *et al.,* 2007). Extensive accumulation of ROS mediates harm to biomolecules and causes deleterious effects resulting in numerous diseases including hematopoietic malignancies (Valko *et al.,* 2006). There is a well-documented association between oxidative stress and leukemia, higher amounts of ROS are generated by leukemic cells as compared to normal cells because they are under a

recurrent state of oxidative blockade (Al-Gayyar *et al.,* 2007). The damaging effects mediated by ROS can be nullified by the action of enzymatic and non-enzymatic antioxidants. Endogenous antioxidant enzymes including CAT, POD, SOD and GST play a significant role in combating oxidative stress (Valko *et al.,*2006). Conversion of $H_2O_2$ to $H_2O$ and $O_2$ via catalytic conversion is triggered by CAT, which is a major antioxidant enzyme present in aerobic cells. SOD, on the other hand dismutates superoxide anion radical ($2O_2$) to less toxic $H_2O_2$ and molecular oxygen/$O_2$ by redox mechanism. A selenoprotein prevalent in cytosol and mitochondrial matrix, POD, is involved in the catalytic reduction of lipid peroxides and $H_2O_2$ (Batool *et al.,*2017a). GSTs are evolutionarily conserved enzymes that play a key role in detoxification of numerous xenobiotics. The conjugation of reduced glutathione (GSH) to electrophilic substrates is catalyzed by GSTs resulting in production of easily soluble and less toxic compounds (Veal *et al.,* 2002). The level of these endogenous enzymes is disturbed due to oxidative overload. There are contradictory reports in the literature regarding the level of endogenous antioxidant enzymes in various types of cancers (Battisti *et al.,* 2008). In our study, benzene treated disease control group showed severely reduced activity levels of CAT, POD, SOD and GST. These findings reinforce the observed aberrations perpetrated by the leukemogenic chemical used in current study. Excessive ROS generation and extended oxidative stress following benzene doses and leukemia induction might have led to exhaustion of endogenous antioxidant defense system particularly the antioxidant enzymes. Furthermore, the expression of endogenous antioxidant enzymes might also be adversely affected. Administration of EA leaf extracts of both plants ameliorated the oxidative stress in a dose dependent manner. Low doses yielded mild restorative effects while greater results were observed in case of high doses of both preventive and treatment modes. The restorative effect might well be accredited to the presence of useful polyphenols and other antioxidant moieties within *Datura* species.

Weakened antioxidant defense system results in increased lipid peroxidation with consequent deterioration in cellular membrane integrity and cellular functions. There are numerous end products of lipid peroxidation including TBARs. These are considered as effective markers of oxidative disorders and numerous cancers including leukemia (Akanni *et al.,* 2014; Mansour *et al.,* 2006). As revealed by the estimated biochemical parameters, the antioxidant defense system of disease control rats was

highly affected. Inflated oxidative degradation of lipids was thus an anticipated outcome. Both extracts expressively lowered TBARs level in treatment mode, while high doses in preventive mode also resulted in mild recovery. Escalated NO level in disease control rats further press the possibility of an irreversible damage to lipoproteins and cellular membrane owing to the tendency of NO to react with $\cdot O_2$ and generation of highly volatile peroxynitrite. These aberrations were also curbed by high and low doses of extracts in identical manner.

Detection of medicinally important phytochemicals, endowed with antioxidant activity in current study and the documented evidence of occurrence of compounds i.e., withanolides (Zhang *et al.,* 2014) may be responsible for the observed anticancer action of *Datura* extracts. Withanolides have proven cytotoxic action in numerous cancer cell types including leukemia. These compounds act through diverse molecular mechanisms i.e., induction of apoptosis via down regulation of akt phosphorylation (Oh *et al.,* 2008), or through activation of p38 mitogen activated protein kinase (MAPK) signaling cascade resulting in elevated levels of BAX (Bcl-2- associated X protein) and ultimately, initiation of mitochondrial cell death (Mandal *et al.,* 2008). Findings of our study further deduce the alleviating effects of selected species of *Datura* in benzene induced leukemia. Even though animal models have numerous common properties with human physiology, due diligence must be performed when trying to extrapolate findings from an animal model of a disease to a clinical trial setting.

### 4.5.2. $CCL_4$ induced liver inflammation

In the next phase of our study, the anti-inflammatory potential of DSL-EA and DIL-EA was evaluated using $CCL_4$ induced liver injury model in male Sprague Dawley rats. Liver is continuously exposed to exogenous moieties derived from food, drugs, chemicals and microbiota in the gut even under normal physiological conditions. Liver parenchymal and non-parenchymal cells are susceptible to harm instigated by oxidative stress and prolonged stress can lead to changes in the composition of parenchymal cells as well as hepatic extra cellular matrix. The cascade of events then causes recruitment of inflammatory and immune cells at the site of injury which further result in activation of non-parenchymal cells i.e. stellate and hepatic kupffer cells (Cederbaum *et al.,* 2009). This is followed by a significant elevation in the levels of cytokines, chemokine and growth hormones leading to liver fibrosis, the gateway to numerous hepatic abnormalities including HCC (Dey *et al.,* 2015). Regardless of the intrinsic

dissimilarities between numerous etiological factors responsible for fibrosis, cirrhosis and HCC, the preservation of wound healing response triggered by parenchymal cell death and the subsequent inflammatory cascade is a common denominator (Bishayee 2014).

$CCL_4$ is a toxicant linked to liver damage via generation of oxidative stress and injury of cellular components (Ullah *et al.,* 2020). In the current study, the effect of low and high doses of DSL-EA and DIL-EA extracts in $CCL_4$ induced liver inflammation was assessed through a series of hematological, biochemical, enzymatic and histological studies. Moreover, immunohistochemistry studies were also incorporated to further scrutinize the anti-inflammatory potential of tested samples. Hematological investigations are a useful prognostic tool for underlying inflammatory conditions and consequent oxidative stress in vital organs including liver. $CCL_4$ is known to cause hematological aberrations including lysis of RBCs and anemia following its metabolism and ROS production (Abuelgasim *et al.,* 2008a). Distinctly decreased RBCs and hemoglobin levels and higher WBCs count in the disease control rats indicated the toxic effects of chemical toxin used in the study. DSL-EA treated groups revealed significant alleviative effects, the results were statistically identical ($p <$ 0.05, 0.01 and 0.001) to the positive control group (silymarin) especially in case of DSLA-EA HD treated rats. DIL-EA extracts also showed a dose dependent effect on the hematological parameters, though less significant than DSL-EA. The presence of pharmacologically significant secondary metabolites including polyphenols, terpenoids, witahnolides, steroidal glycosides and alkaloids might be responsible for the vasotonic effects of tested extracts.

Estimation of serum levels of enzymes i.e., ALT, AST and ALP are considered as important indicators of the functional integrity of hepatocellular membranes. These are cytosolic enzymes and seep out into the plasma in the event of hepatic injury accounting for their raised levels in the serum (Batool *et al.,* 2017b; Pari *et al.,* 2005) as observed in the disease control rats in our study due to $CCL_4$ intoxication. Elevated bilirubin level in serum is also an indication of underlying liver damage and subsequent obstruction in bile excretion thus serving as a useful confirmative test (Hassan *et al.,* 2012). Similarly, reduced level of serum albumin is also an indication of ROS mediated inflammatory condition within the body due to protein oxidation and lipid peroxidation type reactions (Rashid *et al.,* 2016). The extracts showed

remarkable restorative effects on the tested biochemical parameters and there was significant difference between the findings of DSL-EA-LD, DSL-EA-HD and DIL-EA-HD treated groups and the disease control rats. While the mechanism of restorative effect on liver functionality is not clear, this might be accredited to free radical scavenging and lipid peroxidation inhibitory potential of polyphenolic compounds present in the selected plants (Fatima *et al. ,*2015).

Oxidative stress due to $CCL_4$ intoxication damages the antioxidant defense mechanism by deactivating the cellular antioxidant enzymes. Trichloromethyl peroxy radicals ($CCl_3OO^-$) derived from $CCL_4$ cause lipid peroxidation and inhibition of oxidative enzymes thus leading to over accumulation of $O_2^-$ and $H_2O_2$ resulting in massive outpouring of free radicals causing hepatic injury (Tsai *et al.,* 2009). The major antioxidant enzymes responsible for neutralization of free radicals are GST, SOD, CAT and POD. Moreover, a non-enzymatic antioxidant, GSH also play a major role in shielding hepatocytes by scavenging hydrogen peroxides and lipid peroxides as well as through its role as a substrate in catalytic action of glutathione peroxide (Martensson *et al.,* 1991). As observed in the previous investigations, DSL-EA was slightly superior to DIL-EA extract in terms of the restorative effects following $CCL_4$ induced liver injury.

The restoration of mentioned antioxidant defense system by *Datura* extracts, specifically in high dose groups of both species was remarkable and the results were almost comparable to positive control used in the study. The findings of *in vitro* antioxidant and anti-inflammatory assays in our study are further validated with these results. *D. stramonium* extracts yet again revealed slightly dominant effects as far as the restoration of antioxidant defense system was concerned. Medicinally important phytoconstituents of *D. stramonium* leaf i.e., terpenes (Silva *et al.,* 2014), essential oils (Aboluwodi *et al.,*2017), polyphenols, steroids, tannins (Sreenivasa *et al.,* 2012) and steroidal glycosides (Shobha *et al.,* 2014) might be responsible for the effects of DSL-EA observed in our study.

Oxidative stress markers addressed in the current study are TBARs, MPO and NO levels. In inflammatory conditions, NO is synthesized in excessive amounts, surpassing the normal physiological NO level by almost 1000 folds and this exceedingly higher NO production can result in ROS mediated tissue damage (Sharma *et al.,* 2007). A debilitated antioxidant defense setup results in dire

consequences including greatly increased lipid peroxidation and loss of cellular membrane integrity. There are several end products of lipid peroxidation including TBARs. Furthermore, there exists a close association between MPO enzyme level and oxidative stress (Schindhelm *et al.,* 2009). Increase in MPO level serves as an indirect indicator of neutrophilic infiltration and inflammation (Kothari *et al.,* 2011). Estimating the effect of DSL-EA and DIL-EA on the levels of the abovementioned markers thus provided a stout indication of its overall anti-inflammatory potential. The results observed were in accordance with the findings of the biochemical and antioxidant enzymes level. NO production was observed to be higher both in plasma and liver tissue of disease control rats. Moreover, TBARs and MPO levels in liver tissue were greatly elevated, indicating the antioxidant defense system to be in dire straits due to $CCL_4$ intoxication. Considerable reduction in % concentration of NO and TBARs was observed in extracts treated groups (more effective reduction in high dose groups of both *D. stramonium* and *D. innoxia*). Our results further support the findings of previously reported study on *D. stramonium* leaf extract in curbing oxidative stress (Nasir *et al.,* 2020). MPO level was also reduced in treatment groups as compared to significantly elevated level in disease control rats.

Biotransformation of $CCL_4$ is carried out by endoplasmic reticulum (ER) which is one of the chief cellular organelles responsible for normal cellular functions (Lee *et al.,* 2011; Slater *et al.,* 1985). $CCL_4$ is known to disrupt the normal function of ER within the hepatocytes leading to centrilobular necrosis and fatty degeneration of liver (Ritesh *et al.,* 2015). $CCL_4$ intoxication is normally associated with damaged ER and cellular membrane, immune cell infiltration and necrosed hepatocytes and all these collectively result in severely disfigured hepatocyte ultrastructure (Altinoz *et al.,* 2018). In the present study, H and E staining of the liver tissue of disease control group revealed immune cell infiltration, fibrosis, necrosed hepatocytes and edema. High dose treatments of both extracts, in a manner identical to silymarin, restored the normal histoarchitecture of liver tissues. A marked improvement in the histological indices was also evident with the low dose extracts. The findings are in true agreement with a range of investigations performed in current study proving significant anti-inflammatory action of the plant extract. Masson's trichrome staining is used effectively to measure the extent of liver fibrosis and necrosis by detecting collagen in liver tissues (Wang *et al.,* 2016). The presence of hyperplastic fibrous tissue due to $CCL_4$ induced liver

damage was confirmed by Masson's staining. Clear improvement was observed in DSL-EA-HD and DIL-EA-HD treated groups in terms of detected collagen content and fibrosis, further confirming the results of preceding investigations done in current study.

Numerous *in vivo* studies have proved the pivotal role played by Nrf2 in inflammatory diseases including liver damage. The $Nrf2^{-/-}$ animals used in these studies have shown aggravated tissue damage and symptoms of inflammation. It is thus postulated that Nrf2 signaling pathway has a definite protective role in inflammatory disorders (Mo *et al.,* 2014; Ahmed *et al.,* 2017b). Nrf2 signaling helps in curbing inflammatory insults by regulation of endogenous antioxidant enzymes and pro-inflammatory cytokines (Ahmed *et al.,*2017b). The progression of an early phase liver injury to fibrosis usually is preceded by an inflammatory phase with building up of continuous oxidative stress and under these circumstances there is up regulation of iNOS and consequential generation of greater amounts of NO (Diesen *et al.,* 2011). In our study, reduced Nrf2 and elevated iNOS expression was observed in liver tissue of $CCL_4$ treated disease control rats using immunohistochemistry analysis. Nrf2 expression was elevated by DSL-EA in a dose independent manner and both low and high dose groups exhibited notable improvement ($p < 0.05$, 0.01 and 0.001). The expression level of iNOS on the other hand was distinctly reduced by high dose of the DSL-EA extract and the positive control used in the study. DIL-EA showed a comparatively weaker response than DSL-EA.

Outcomes of our study have scientifically validated the folkloric usage of *D. stramonium* in inflammatory diseases.

Out of the two species included in the chronic in vivo assays, *D. stramonium* has outperformed *D. innoxia* at many fronts. The preparative scale extraction, isolation and characterization of pure chemical compounds was thus initiated from *D. stramonium* keeping in view the significant potential this specie in the field of natural product drug discovery.

### 4.6. Preparative Scale Extraction, Fractionation, Isolation and Characterization of Compounds from DSL-EA

The extraction optimization done in the initial phase of our study led us to the conclusion that out of the four selected plant parts of *D. stramonium*, leaf part was the

most efficient in terms of its bioactivity profile as well as the extraction yield. Therefore, to stay in line with the highly prudent approach of "bioactivity guided isolation of active compounds", preparative scale extraction and isolation of compounds was planned using leaf part of *D. stramonium.* The solvent selected for the preparative scale extraction was "ethyl acetate" keeping n view the tremendous potential shown by DSL-EA in numerous preliminary phytochemical, antioxidant, anticancer and anti-inflammatory assay done in earlier segments of our study. Furthermore, extraction was performed using sonication aided maceration technique.

### 4.6.1. Fractionation

Solid phase extraction (SPE) was employed as the separation method for the fractionation of DSL-EA crude extract. A silica packed glass column was used to elute the compounds from extract loaded over silica with numerous organic solvents in increasing order of polarity. Several fractions obtained thus contained mixture of compounds separated based on the difference in their polarity. A total of 33 fractions i.e., DSL-EA 1-33 were procured from DSL-EA, each eluted with a solvent system of increasing order of polarity.

### 4.6.2. Isolation of compounds

Liquid column chromatography was used to isolate and purify compounds from the fractions of DSL-EA. It is one of the most efficient and cost-effective isolation techniques used in natural product drug discovery labs. TLC profiling of procured fractions facilitated the planning of all the purification columns. Pure compounds were isolated by using a combination of various normal phase liquid chromatography techniques i.e., gravity column, medium pressure and vacuum column chromatography.

**Summary of entire fractionation and isolation process**

As mentioned in earlier sections, DSL-EA was the crude extract shortlisted for the isolation and purification of potentially bioactive components based on its prolific activity in the preliminary *in vitro* and *in vivo* experiments. DSL-EA extract was thus procured in bulk and partitioned into phytochemically distinct fractions using gravity and flash column chromatography. The fingerprinting of each fraction was done using TLC and the ones with identical chemical makeup were combined. Furthermore, small scale purification columns were run using the gradient elution strategy with solvents of increasing polarity. The resultant sub fractions were again profiled using normal phase

TLC plates. The presence of pure compounds was confirmed using several spray reagents and observation under short and long UV light. TLC profiling assisted in identification of pure compounds based on their distinct R*f* values and it also helped in method development for large scale isolation of the compounds to be used in selected *in vivo* experiments later in the study. The pure compounds obtained at the end of extensive isolation and purification columns were then characterized using 1D and 2D NMR techniques.

### 4.6.3. Characterization of isolated compounds

A total of three compounds (Compounds a, b and c) were isolated using normal phase gravity, vacuum and medium pressure column chromatography as the isolation techniques. The compound-a was isolated from fraction 10 of DSL-EA preparative crude extract, compound-b from DSL-EA- 25-26 and compound-c from DSL-EA-31. NMR (1D and 2D) spectroscopy was used for the characterization of the isolated compounds and their structure elucidation. Isolated compounds were identified as an unsaturated phytosterol i.e., stigmatsterol (compound-a), a phenolic phytochemical i.e., ferulic acid (compound-b) and a steroidal saponin /sterol glucoside i.e. (3β,22E)-*Stigmasta*-5,20-dien-3-yl β-*D-glucopyranoside (compound-c)*

Phytosterols represent a group of lipophilic compounds and more than 40 phytosterols are known so far. The majorly reported ones are; β-sitosterol, stigmasterol, campesterol and avenasterol. A bioactive fraction of *D. stramonium* was reported to induce human immune cells mediated cytotoxicity against certain cancer cell lines including MCF-7 breast cancer and A549 lung carcinoma. The presence of sitosterol, stigmasterol, daturaolone and daturadiol was confirmed by LC-MS analysis of this fraction of *D. stramonium* (Gupta and Shankar, 2011). The presence of different phytosterols including cholesterol like compounds i.e., stigmasterol, 5-ergostenol, cholestane and campsterol was previously confirmed using GC-MS analysis of leaves and callus of *D. stramonium* (Bhardwaj *et al.,* 2014). Stigmasterol has been detected in other species of *Datura* as well. HPLC analysis of crude n-hexane extract of *D. metel* revealed the presence of numerous phytosterols, the sterol indicators used in this study were β-sitosterol, stigmasterol, lanosterol, sitostanol and D5-avenasterol (Ramadan *et al.,*2007). The structure of stigmasterol isolated from the leaf of *D. stramonium* in current study was identified by comparing the NMR spectra with already reported data (Cordeiro *et al.,* 2013).

The second compound isolated from *D. stramonium* leaf was ferulic acid. It is rated as one of the most highly cited hydroxycinnamic acids in the plant kingdom (Mathew *et al.,* 2004). Ferulic acid is known for is tremendous medicinal benefits including its antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial, antiallergic, antiviral, antithrombotic and hepatoprotective properties The isolated molecule was fairly pure, and its structure was confirmed by comparing the NMR spectral details with previously published data (Kumar *et al.,* 2014).

The steroidal glycoside/saponin, (3β,22E)-*Stigmasta*-5,20-dien-3-yl *β*-D-glucopyranoside was isolated from the most polar sub fraction of DSL-EA owing to the presence of a sugar moiety in its structure. The structure was identified by comparison of the NMR spectra of compound-c with previously published data (KF *et al.,* 2014). Stigmasterol glucoside has been previously isolated from different plant species including, *Atriplex nummularia,* 10), *Cissus javana12*), *Ambroma augusta* 13), *Cassia petersiana,* 9) and *Thalassodendron ciliatum* 11). It is also known as stigmasterol-3-o-β-d-glucopyranoside and is reported to have anti-bacterial, cytotoxic, anti-inflammatory activities (Kamei *et al.,* 2018). We have reported the isolation of this steroidal/saponin glucoside for the first time from *D. stramonium.*

The selection and shortlisting of compounds isolated in our study for further pharmacological evaluation and screening of their medicinal uses was done keeping in view certain factors i.e., their purity, quantity and chemical class. The two compounds thus selected were stigmasterol glucoside and ferulic acid and referred to as C1 and C2 respectively in the following sections.

### 4.7. Evaluation of Anticancer and Anti-Inflammatory Potential of C1 and C2 (Benzene Induced Leukemia and $CCL_4$ Induced Inflammation)

In order to determine the role that C1 and C2 have played in the significant pharmacological potential of DSL-EA extract in leukemia and liver inflammation models as discussed in the previous sections, these chronic *in vivo* assays were performed again on Balb/c mice. The selected compounds were tested at predetermined concentrations keeping the study design as identical as possible to the extract based *in vivo* studies. The compounds selected for the assessment of *in vivo* anticancer and anti-inflammatory potential and their structurally related meloecules have been previously used in different *in vivo* experimenst using mice and rats as lab animals. The safety profile of these moities is therefore established. The concentrations to be tested in the

mentioned experiments were also decided based on previously published data (Xu *et al.,* 2018; Kumar *et al.,* 2014).

### 4.7.1. Benzene induced leukemia

As discussed in earlier sections, DSL-EA extract was selected out of all the tested samples based on prolific results in numerous *in vitro* and *in vivo* assays that were designed to determine its anticancer potential. C1 and C2 isolated from DSL-EA are also tested in benzene induced leukemia in Balb/c mice in order to find out their possible role in the alleviative effects of the extract tested earlier in our study. The anticancer potential of *D. stramonium* plant is evaluated to fill in the void that is still there in scientific literature related to anticancer potential of this plant and its compounds. Although there is data available on the anti-inflammatory and antimicrobial effects of stigmasterol glucoside (Kamei *et al.,*2018), a comprehensive study on the anticancer benefits or more specifically, the antileukemic potential of this compound has not been performed yet to the best of our knowledge. These factors make our current study more prudent and at the same time, exclusive.

Leukemia is a cancer type that involves mainly the WBCs. In leukemia, the body generates undeveloped WBCs which are unable to perform normal function. These leukemic cells get replaced with normal cells in the bone marrow of the effected person. This uncontrolled increase of leukemic cells causes them to enter the blood stream and ultimately penetrate important organs of the body. This shrinks the room for mature and functional RBCs, WBCs and platelets (Lackritz 2001). The effect of benzene administration was clearly evident from the results of hematological parameters of the disease control mice used in the study. The compounds have shown vasotonic and vasoprotective effects and both C1 and C2 were effective in alleviating the damage inflicted by benzene to a great extent. While data on C1 is relatively scarce, ferulic acid (C2) has been widely reported to have protective effects on the hematopoietic system. It has been previously shown to promote the recovery of the bone marrow cells and help in the mitigation of hematopoietic injuries (Ma *et al.,* 2011).

Liver and kidneys, being the major organs responsible for metabolism of numerous chemicals and subsequently, the detoxification of the entire body, are more exposed to aggressive insults from different endogenous and exogenous threats. Exposure to benzene is associated with severe deleterious effects including impairment of liver and renal functions. This ultimately aggravates the risk of carcinogenesis (Abd El-Shakour

*et al.,*2015; D'Andrea *et al.,* 2014). Irregularities in the levels of ALT, AST and ALP, as well as other macromolecules signifying liver and kidney functionality i.e., bilirubin, total proteins, urea, creatinine, triglycerides and cholesterol clearly indicated liver injury in disease control mice. High dose groups of both C1 and C2 were found to be phenomenal in restoring the tested parameters in a statistically identical manner ($p < 0.05$) to the standard drug used in the experiment (cyclophosphamide). Not much has been reported about C1 and its role in mitigating liver injury due to underlying diseases i.e., cancer. Nonetheless, studies have shown the benefits of other compounds from the class of steroidal saponins to have alleviative effects in liver damage (Zhao *et al.,* 2012). Moreover, C2, being a polyphenolic compound has tremendous antioxidant potential and it helps in decreasing the damage caused by oxidative stress (Aruoma 2003; McRae *et al.,*2007). There is data to support the positive effects of C1 and C2 on the lipid profile of the study groups. C1 has a more hydrophilic moiety in its structure (the sugar part), which is thought to impede the esterification of cholesterol and consequent inhibition of cholesterol entry into the blood vessels (Khatun *et al.,* 2012). C2 is also known to act as a competitive inhibitor of hydroxymethylglutaryl coenzyme A (HMG-COA) reductase in the liver and inhibit the synthesis of cholesterol and promote the excretion of acidic sterol (Kim *et al.,* 2003; Ou *et al.,* 2001).

The role played by antioxidant enzymes and GSH in combating oxidative stress and the fluctuations in their activity levels in disease conditions i.e., leukemia is well explained in earlier sections. The activity levels of GSH, GST, SOD, CAT and POD in disease control mice expressed in terms of % concentrations in liver tissue experienced a significant decline as compared to the mice in the normal control group. The compounds managed to restore the normal concentration levels of these antioxidant defense molecules in a dose dependent manner. C1 and C2 resulted in almost identical restoration, and this can be one of the reasons of their pharmacological response in the event of benzene induced leukemic condition. Steroidal saponins (and/or sterol glucosides) are known to modulate oxidative stress and compounds belonging to this class have been known to up regulate the expression of antioxidant enzymes including CAT, SOD, GST as well as GSH (Son *et al.,* 2007). Likewise, C2 owing to its strong antioxidant properties has also played a role in avoiding the exhaustion of natural antioxidant system within the mice and our results are in accordance with previously

published reports confirming the role of ferulic acid in replenishment of endogenous antioxidant defense system (Baskaran *et al.,* 2010).

Studies have shown the role of oxidative stress in various ailments such as rheumatoid arthritis, ischemic heart disease, several autoimmune disorders and cancer. NO• performs in multiple dimensions i.e., it acts as an intracellular messenger, also it is a lethal agent in many pathophysiological, inflammatory and autoimmune diseases including cancer. Chronic inflammation can trigger the formation of NO•, which can cause DNA damage directly, or indirectly by generating more importunate RNS (Liu *et al.,* 1995). The presence of cNOS and iNOS in numerous human cancers provides proof for the involvement of NO• in carcinogenesis (Thomsen *et al.,* 1995; Rosbe *et al.,* 1995). ROS/RNS have a role in initiation and promotion of multistep carcinogenesis. They may damage DNA, trigger pro-carcinogens, instigate lipid peroxidation, disable enzyme systems and change the cellular antioxidant defense system (Sun 1990). Elevation of oxidative stress can cause peroxidation of membrane lipids with the production of peroxides that can be degraded to numerous mutagenic carbonyl products. Lipid peroxides (LPA) and TBARs are well-defined lipid peroxidation end products. They both are believed to be involved in mutagenesis and carcinogenesis (Zhang *et al.,* 2002). They can also alter the expression of genes associated with tumor development (Cerutti, 1985). Higher levels of lipid peroxidation products suggests that cancer cells generate free radicals in huge amount (Cerutti, 1994) and that there is a link between malignancies and radical activity (Dormandy, 1983). Very much in alliance with previously published reports, the estimated % concentrations of NO and TBARs in the liver tissues of disease control mice were significantly ($p< 0.05$) greater than the mice in normal control group. There are numerous studies done in a variety of human cancers where identical results have been observed as far as the level of these oxidative stress markers is concerned (Ray *et al.,* 2000; Manju *et al.,* 2002; Geetha *et al.,* 2001). Naturally derived steroid saponins are known to decrease lipid peroxidation by downregulating the peroxidation reaction as well as upregulating the endogenous antioxidant defense system (Jagadeesan *et al.,* 2012). C1 belongs to the same class of natural compounds and although there are not enough studies supporting its mode of action, it is most likely that C1 will act through one of the mechanisms shared by its structurally related cousin molecules. C2, the polyphenol has strong NO scavenging activity (Ogiwara *et al.,* 2002). The possible mechanism of reaction of ferulic acid with

NO is the radical adduct formation at the C=C double bond (Lu *et al.,* 2022). The findings of our study are also in accordance with a previously published report stating the inhibitory effect of ferulic acid on lipid peroxidation and consequent decrease in the levels of TBARs (Balasubashini *et al.,* 2004).

The pathological changes caused by benzene were verified from the hisopathology test results of the liver and kidney samples of the test groups. Benzene is known to cause damage to the liver and kidney tissues (Golabi-Habashi *et al.,* 2021). It is transported in blood and absorbed to different extents by various tissues. It is estimated that about half of the absorbed dose may get eliminated unchanged while the remaining half is metabolized in the liver (Raj *et al.,* 2001). As observed in the results of biochemical investigations, urea and creatinine were greatly increased in the disease control mice, excessive accumulation of these metabolic wastes indicated significant kidney damage along with inevitable damage to the tubular epithelium. In the disease control group, many aberrations were observed in liver and kidney tissues including necrosed hepatocytes, cellular hypertrophy, infiltration of inflammatory cells, degenerated renal tubules and hyalinized glomeruli etc. Co treatment with C1 and C2 has managed to alleviate the hepatocellular damage and restored the normal kidney histoarchitecture. The physiological integrity of liver and kidney tissues was well conserved by both the compounds as observed in the finding of other biochemical tests discussed in previous sections. The effects of C1 and C2 on liver and kidney tissues are in agreement with previous studies undertaken to determine the restorative effect of plant sterols/steroid terpenoids, sterol glucosides and ferulic acid in the event of toxicity caused by various toxins (Sparg *et al.,* 2004). Additionally, to detect the deposition of collagen in tissues, Masson's trichrome staining was used. This technique has been used for many decades and it is very effective in staining the collagen in various tissues (Chen *et al.,* 2017). The presence of hyperplastic fibrous tissues were confirmed by Masson's staining, the damage was more evident in the disease control mice and equally alleviated by higher doses of C1 and C2. The results were in alliance with the earlier findings of our study discussed in previous sections i.e., biochemical and H & E staining.

Nuclear factor κB (NF-κB) signaling has a pivotal role to play in regulation of numerous cellular functions associated with cancer progression i.e., inflammation, cell survival as well as proliferation (Mantovani *et al.,* 2008). Abnormal NF-κB signaling is therefore a key factor in development and progression of numerous cancers (Hanahan

and Weinberg, 2011). In chronic lymphocytic leukemia (CLL) patients, NF-κB pathway is expressively activated and is known to play a significant role in the progression of the disease condition. The molecular mechanism of its up regulation still not fully clear, but there still is evidence drawn from certain epigenomic and transcriptomic data which suggests that the abnormal activation of NF-κB pathway is triggered by external stimuli that ultimately leads to the disease progression (Cahill *et al.,* 2013; Niemann *et al.,* 2013; Tuveson *et al.,* 2015). During disease condition, the NF-κB becomes free from the inhibitory influence of the IκB and translocate to the nucleus. Following nuclear translocation, the NF-kB translocate to the nucleus to induce the expression of the concerned genes such as inflammatory cytokines (Dotan *et al.,* 2011; Newcomb *et al.,* 2007). Various pro-inflammatory proteins are induced, COX-2 is one of them. The increased COX-2 not only aggravates the underlying condition but also positively regulate the activation of other signaling proteins including but not limited to MAPKs (Schett *et al.,* 2008; Kazmi *et al.,* 2020). In the present study, the expression levels of NF-κB and COX-2 were monitored in the liver and kidney tissues by performing immunohistochemistry. A marked increase in the expression of these two markers was noticed in the liver and kidney tissues of the disease control mice further confirming the induction of benzene induced malignancy. High doses of C1 and C2 managed to restore the activity levels/ expression of NF-κB and COX-2 and the immunoreactivity score noticed in both tissues was almost identical to the positive control mice. These results are consistent with numerous hematological, biochemical, histological studies done prior to the immunohistochemistry analysis in the current project. There is not sufficient data in the scientific literature about the effect of stigmasterol glucoside (C1) on the expression of NF-κB and COX-2 in the event of diseases i.e., cancer. Ferulic acid (C2), on the other hand, is known to have a modulative role on the expression of these markers in toxins induced cancers in lab animals. C2 is thus reported to down regulate the expression of NF-κB and COX-2 (Manoharan *et al.,* 2014). The findings of our study are in agreement with these studies.

#### *4.7.1.1. Molecular expression analysis*

Leukemia may result after prolonged exposure to the carcinogen benzene (Snyder 2002). The primary site exposed to benzene is the bone marrow. Benzene and its metabolites are toxic to hematopoietic stem cells (HSCs) at various phases of development in the bone marrow, altering gene expression, apoptosis, oxidative stress,

DNA repair, and epigenetic control of HSCs (Snyder 2002; Wang *et al.,* 2012; Murugesan *et al.,* 2013; Billet *et al.,* 2010). Particularly, oxidative stress has been identified as a critical event in the development of benzene-induced toxicity (McHale *et al.,* 2012). The benzene metabolites phenol, benzoquinone, and hydroquinone were discovered to stimulate ROS formation in HSCs cells, which resulted in DNA and mitochondrial damage (Wang *et al.,* 2012).

NRF2 is a transcription factor that controls the expression of antioxidants and phase II detoxification enzyme genes. Oxidative stress induces modifications in the cysteine residues of KEAP1 which triggers a decline in proteasomal degradation of NRF2, enabling direct translocation of NRF2 into the nucleus. More than 200 downstream antioxidant enzymes including GSTs and quinone oxidoreductase (NQOs) are activated by NRF2 coupled with SMAF proteins, following their binding to antioxidant response elements (AREs), this results in a wider control of oxidative stress (Zenkov *et al.,* 2017; Chiu *et al.,* 2017; DeNicola *et al.,* 2011). Several studies have shown that NRF2-ARE pathway is essential for protection against hydroquinone and benzoquinone induced toxicity (Rubio *et al.,* 2011). C-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinase (MAPK) family of enzymes that plays a role in cancer cell proliferation, growth, and progression. Certain cues inhibit JNK-mediated cell death and activate the cell survival effect of JNK. The role of JNK in cancer cell survival is mediated by autophagy, since JNK may activate autophagy to prevent apoptosis. Numerous studies have shown that JNK-mediated pro-survival autophagy increases cancer cell resistance to chemotherapy. Thus, JNK is a promising target for chemotherapeutic agents with kinase inhibitor properties.

NF-kB is a family of proteins that regulate gene transcription, expression of certain cytokines and cell survival. To prevent apoptosis, NF-kB controls anti-apoptotic genes such as tumor necrosis factor associated factors (TRAF1/TRAF2) and inhibits the caspase enzyme family. Cell proliferation and tumor growth are linked to overexpression of NF-kB. Consequently, medicinal agents that may inhibit NF-kB, by activating caspases and promoting apoptosis can be useful therapeutic options (Chau Shuk-Ling *et al.,* 2020). Apoptosis is mainly governed by intrinsic (mitochondrial) and extrinsic (Fas and Fas ligand) pathways. Extrinsic or intrinsic pathways cause caspase activation by the activation of caspase-9 and caspase-8 respectively. These can then lead to activation or cleavage of caspase-3 which then provoke apoptosis. Likewise, the

ADP-ribolsyting enzyme, poly ADP-ribose polymerase (PARP) is critical for the initiation of various DNA repair mechanisms, and it has been shown to be overexpressed in a variety of malignancies, including neuroblastoma, testicular tumors, malignant lymphoma, leukemia, breast, and colon cancers (Li Xia *et al.,* 2018).

Tumor necrosis factor-alpha (TNF-α) is a multifunctional cytokine that is involved in apoptosis, cell survival and inflammation. Several studies have reported that TNF-α promotes tumor initiation and progression in various blood cancers including B-cell lymphoma, cutaneous T-cell lymphoma, megakaryoblastic leukemia and adult T-cell leukemia as well as in breast, and colon cancers (Sethi *et al.,*2008).

In the present research, protein expression analysis through western blot indicates that the studied compounds (C1 and C2) have anticancer potential. Considerable damage was recorded in the disease control group but the antioxidant and anti-inflammatory capabilities of C1 and C2 greatly suppressed cell proliferation and cancer development. C1 and C2 work by activating the KEAP1-NRF-2 induced defensive response and preventing the toxicity triggered by benzene metabolites. Thus, the increase of NRF-2 enzymes indicates that the chemicals have a protective impact against ROS caused by benzene metabolites. Similarly, p-JNK expression was substantially raised in disease control group, suggesting its role in WBCs proliferation and progression towards leukemic condition. C1 and C2 on the other hand, at high and low doses, significantly suppressed p-JNK, suggesting its strong kinase inhibitory potential. The anticancer properties of C1 and C2 have been further validated by significant downregulation of NF-κB, PARP and TNF-α when compared to the disease control group.



### 4.7.2. $CCL_4$ induced hepatic inflammation

The hematological study offers an extrapolative measure of numerous inflammatory conditions such as oxidative stress in liver, kidney and other important body organs. $CCL_4$ when metabolized, generates intensively reactive species, and initiates the breakdown of RBCs which results in causing anaemia (Abuelgasim *et al.,* 2008b). High number of WBCs may cause a defensive reaction against toxicity, systemic inflammation and renal dysfunction. Another key parameter is insufficient or low platelet level which is responsible for causing anaemia. Decreased number of platelets might also responsible for the action of uremic toxins and increased NO production (van Bladel *et al.,* 2012). The aberrations in hematological indices observed in $CCL_4$ treated group, is the indication of toxin induced oxidative stress. In the current study,

C1 and C2 notably recovered the disturbed hematological parameters and the observations made were similar to those of the standard (silymarin) treated and control groups. This states the biological significance of C1 compound in reinstating oxidative stress relating hematological parameters. On the other hand, as mentioned previously, ferulic acid has known benefits in restoring the normal hematopoietic aptitude within the body. It has proven bone marrow cells recovery properties (Ma *et al.,* 2011) and our results are in alignment with these reports.

Reducing the harm done by any toxins to the hepatic cells and preservation of standard hepatic environment are the two major factors upon which the effectiveness any hepatoprotective drug is based. $CCL_4$ stimulated a rise in the hepatic stress markers i.e., ALT, ALP, AST and bilirubin while decline in total proteins level further supported the already published reports about the toxicity of $CCL_4$. The increased level of enzymatic stress markers indicates the derangement of hepatocyte's cellular membrane. Mainly, these are the cytosol residing enzymes and when cellular membrane is disrupted, these enzymes get emptied into the blood circulation (Kazmi *et al.,* 2018). Considerably reduced level of these enzymes in groups treated with C1 and C2 supports the notion that serological levels of these transaminases stabilize with the revival of hepatic cells and parenchyma. The compounds used in this experiment at high doses have shown tremendous restorative effects as far as the activity levels of mentioned cytosolic enzymes are concerned. There is a void in scientific literature regarding studies on measuring the extensive pharmacological evaluation of C1 in liver diseases. Nonetheless, structurally related steroidal saponins have been studied and their alleviative effects are well reported (Zhao *et al.,*2012). C2 has known antioxidant potential as it belongs to the class of polyphenolic compounds and these molecules are known to have curative effects in oxidative stress induced liver injuries (Aruoma 2003; McRae *et al.,*2007).

In chronic kidney and liver ailments, there is reasonably higher content of triglyceride in serum whereas in end stage renal disorder (ESRD), comparatively higher level of cholesterol content is observed. In addition to this increase, an increase in serum concentration and impaired clearance of very low-density lipids (VLDL) is also observed. Also, there is reduction in serum high density lipid (HDL) content. Liver is the main metabolic organ for lipid metabolism. It is responsible for the uptake, development, and distribution of lipoprotein to the circulation. Numbers of chronic

hepatic disorders are progressed due to alteration in lipid metabolism. In contrast, liver diseases affect lipid metabolism followed by altered serum lipid levels hence causing dyslipidemia, a condition in which there is lower HDL and higher LDL levels (hypercholesteremia) (Longo *et al.,* 2001).

The expression level of hepatic cholesterol 7a-hydroxylase (CYP7A1) can be reduced by $CCL_4$ intoxication causing hypercholesterolaemia. The link between the higher serum lipid level and $CCL_4$ exposure is many-dimensional which can either be because of increased lipoprotein production or their impaired clearance. Moreover, $CCL_4$ can disturb the normal functionality of enzymes involved in lipid metabolism in the liver which reduce the formation of bile acids. Bile acids are responsible for eradicating cholesterol from the biological system (Li *et al.,* 2015).

In this study, increased values of serum triglycerides and cholesterol in the $CCL_4$ intoxicated group were viewed. When treated with C1 and C2, they were reinstated to normal levels. Owing to the presence of a sugar moiety in the structure of C1, it is known to hinder cholesterol esterification and its entry into blood vessels (Khatun *et al.,* 2012). Moreover, C2 has been reported to inhibit cholesterol synthesis as well as to promote excretion of acidic sterol (Kim *et al.,* 2003; Ou *et al.,* 2001). The significant improvement in the lipid profile of mice treated with high doses of C1 and C2 might be due to the underlying mechanisms mentioned above.

A variety of enzymatic and non-enzymatic antioxidants are contained in a biological antioxidant defense system. SOD isoforms are the primary intrinsic enzymes responsible for fighting against oxidative stress. They are accountable for the dismutation of superoxide into $H_2O_2$ and oxygen (Ratliff *et al.,* 2016). $H_2O_2$ is neutralized by CAT, which is a hem containing enzyme distributed all over the cytosol and peroxisomes. For the process of neutralization of CAT, presence of any cofactor is not required which makes it the main consumer of $H_2O_2$. CAT performs its catalytic activity together with another selenoprotein, glutathione peroxidase (POD). This enzyme is profusely spread in the nucleus, mitochondria and cytoplasm and is involved in the regulation of cellular redox status. POD, at the expenditure of its substrate GSH, consumes even very negligible number of peroxides (Hassan *et al.,* 2012). GST is a family of enzymes principally responsible for the detoxification of reactive species by instigating the conjugation of a huge variety of substrates to reduced glutathione (Strange *et al.,* 2001). GSH, an endogenous antioxidant, is a non-enzymatic tripeptide

which guards the cells by wiping out the peroxides. All these antioxidants participate in restraining the ROS induced macromolecular abnormalities.

In this study, the reduced functionality of enzymatic antioxidants in mice intoxicated with $CCl_4$, signals that they are excessively consumed in combating the high free radical generation. These results are in alignment with the results of a formerly published study (Dutta *et al.,* 2018). There was a dose dependent restorative effect of C1 and C2 on the activity levels of these antioxidant defense molecules. High doses of C2 revealed slightly stronger restorative effect as compared to C1. Compounds from steroidal saponin class have previously been reported to up regulate the expression of CAT, SOD, POD, GST as well as GSH (Son *et al.,* 2007). Our results are also in compliance with previous studies on the role of ferulic acid (C2) in combating oxidative stress and restoring the activity levels of endogenous antioxidant defense molecules (Baskaran *et al.,* 2010).

$CCL_4$ gets metabolized into highly active $CCl_3^{\bullet}$ *via* CYP450 enzymes system in the liver. These extremely active free radicals having a short life span instantly start peroxidation in hepatocyte's cell membrane, elevating the circulatory levels of TBARS and $H_2O_2$ (Elgawish *et al.,* 2015). Moreover, $CCl_3^{\bullet}$ boosts the nitric oxide ($NO^{\bullet}$) and dioxide ($1^{-}$) which have the ability to augment peroxynitrate anions ($ONOO^{-}$) induced cellular destruction (Dong *et al.,* 2015). The LPO, TBARS, $H_2O_2$ and $NO^{\bullet}$ levels in blood are thus increased by the long term $CCL_4$ intoxication.

Steroidal saponins derived from natural sources have the potential to cause down regulation of lipid peroxidation and enhance the performance of endogenous antioxidant defense system (Jagadeesan *et al.,* 2012). It is very likely that the significant activity shown by C1 will be through the stated mechanism shared by structurally identical steroidal saponins. Additionally, the effect of C2 on the oxidative stress markers in the current study further support the findings of studies done previously illustrating the role of ferulic acid in alleviating oxidative stress induced lipid peroxidation (Balasubashini *et al.,* 2004).

$CCL_4$ is lipophilic in nature due to which enables its interaction with cell membrane and generation of $OH^{\bullet}$ which results in oxidation of lipid bilayer and eventually rupture the hepatocellular membrane and alter its ultrastructure (Go *et al.,* 2016). The accretion of fats within the liver cells is another main aspect of hepatotoxicity. Accumulation of

triglycerides within the liver cells result in severe inflammation, vacuolization, steatosis, hepatic tissue fibrosis, and necrosis (Zaulet *et al.,* 2017). Histological values revealed that in contrast to $CCL_4$ intoxicated group, no visible inflammation, fibrosis or steatosis was observed in liver tissue of C1 and C2 treated mice. The varying degree of hepatoprotective nature of C1 and C2 at their high and low doses has been approved by the histoarchitectural inspection.

The histological examination of $CCL_4$ treated mice kidneys showed adverse consequences such as glomerular congestion, vacuolization, tubular degeneration, Bowman's capsule disruption and brush borders erosion. These effects are the result of free radical generation following $CCL_4$ metabolism. Evidence of renal damage in $CCL_4$ treated mice were also reported by (Moneim *et al.,* 2012) as glomerular inflammation, congestion and vacuolization of convoluted tubules, epithelial cells atrophy, pyknotic nuclei, disruption of a typical cytoplasm and depletion of brush border.

In our study, improvement of the above stated adverse effects can be examined in a dose dependent manner in groups treated with C1 and C2. These outcomes were similar to those of the standard (silymarin) and the normal control groups. Treatments of mice with low and high doses of C1 and C2 have alleviated the hepatocellular and kidney damage and restored the normal histoarchitecture. These effects are in accordance with the results of biochemical tests performed in these experiments. Steroidal saponins and polyphenolic compounds are reported to restore the normal liver and kidney tissue functionality in case of toxicity caused by numerous toxins and our results further support the findings of these studies (Sparg *et al.,*2004; Sanjeev *et al.,* 2019; Mir *et al.,* 2018b). The chronic *in vivo* experiments have also managed to fill the void in literature as far as the extensive pharmacological evaluation of stigmasterol glucoside is concerned.

Moreover, Masson'strichome staining confirmed the extensive fibrosis in liver and kidney tissues which was significantly alleviated by C1 and C2 in higher doses.

In the event of oxidative stress led inflammatory disorders, immune cells are recruited to the site of inflammation and if the oxidative damage is prolonged it leads to chronic inflammation and that can pave way for cancer (Oving *et al.,* 2002; Chau *et al.,* 2006; Dotan *et al.,* 2011). NF-κB signaling is activated in inflammatory conditions and this can further exacerbate the underlying condition and plays a role in disease progression

and evolution (Rustgi, 2007). Increased expression of NF-κB result in generation of numerous pro-inflammatory cytokines including COX-2 and TNF-α to name a few (Charalambous *et al.,* 2009). COX-2 is one of the key pro-inflammatory mediators and it further aggravates the inflammatory condition by prompting the synthesis of prostaglandins (Sawhney *et al.,* 2007). In the current study, immunostaining of liver and kidney tissues the mice revealed a marked increase in the expression of both NF-κB and COX-2 in the disease control group confirming the inflammatory condition caused by $CCL_4$ intoxication. As discussed in the previous sections, C1 and C2 at high doses resulted in significant restoration of the physiological indices in a statistically identical manner to the positive control used in the study. Our study has aided in the scientific validation of the role of C1 in inflammation. As discussed in the earlier sections, there is data to support the role of C2 in regulating the expression of NF-κB and COX-2 in inflammation and cancer. Moreover, there are numerous studies emphasizing the role of COX-2 and NF-Kb regulating agents in improving the overall quality of life of patients suffering from inflammatory disorders (Zwerina *et al.,* 2005).

#### *4.7.2.1. Molecular expression analysis western blotting*

Sirtuin 1 (SIRT1), a mammalian Silent Information Regulator 2 (Sir2) protein family homologue, is a class III histone deacetylase that has been found to function on a variety of histone and non-histone substrates. Several studies have shown that SIRT1 affects essential metabolic and physiological processes i.e., senescence, stress tolerance, metabolism, and apoptosis. The SIRT1 protein is found in almost every body organ, including heart, liver, kidney, brain, pancreas, spleen, skeletal muscle, endothelium tissue, and white adipose tissue (Wang *et al.,* 2006). SIRT1 expression levels have been shown to be altered in a variety of diseases, including metabolic disorders, neurological diseases, cancer, and ageing. Current findings have shown a link between SIRT1 downregulation and disease progression with increase in oxidative stress and inflammation (Elibol *et al.,* 2018). Therefore, pharmacologic stimulation of SIRT1 could be a potential treatment for inflammatory disorders.

The NLRP3 inflammasome is an important part of the innate immune system. It promotes caspase-1 activation and the release of pro-inflammatory cytokines i.e., pro-interleukin-1 (IL-1) and pro-interleukin-1 beta (IL-1β) in the event of infection or cell injury. The NLRP3 inflammasome is activated by a diverse set of stimuli and molecular events including mitochondrial distortion, ionic flux ROS generation and lysosomal

damage. This results in the upregulation of NLRP3, Caspace-1, and IL-1β *via* the activation of transcription factor NF-κB (Kelley *et al.,* 2019). Apoptosis associated speck like protein containing a C-terminal caspase recruitment domain (ASC or Pycard) is another key protein involved in the assembly and activation of inflammasomes. It is required by Caspase-1 for its activation in response to signals initiated during disease conditions. ASC, along with the inflammasome sensor NLRP3 and caspase-1, is expressed in immature immune cells (CD4+ T lymphocytes) (Javanmard *et al.,* 2019). Thus, useful insights into disease pathogenesis and identification of therapeutic interventions can be obtained from the study of inflammasome pathways.

In the present work, C1 and C2, which have considerable antioxidant and anti-inflammatory properties, substantially activated SIRT1 in a dose-dependent way when compared to silymarin, a typical naturally derived hepatoprotective agent. Western blot examination of SIRT1 expression validates the *in vivo* hepatoprotective potential of C1 and C2. Western blot analysis revealed downregulation of proteins involved in inflammasome assembly and activation, such as NLRP3, Caspase-1, IL-1β and ASC. These results support the antioxidant and anti-inflammatory properties of C1 and C2 as discussed in earlier sections of current study. Western blot research clearly revealed that C1 and C2 have the capacity to shut off the inflammatory pathways involved in the initiation and exacerbation of cellular damage induced by xenobiotic i.e., $CCL_4$.

## 4.8. Research Outcomes

- Although *Datura* species have well documented therapeutic benefits, our project on the analysis of *D. stramonium* and *D. innoxia* is the first extended, systematic and in-depth comparative study of the chosen species.
- Despite several ethnomedicinal claims, the current study is one the first attempts to scientifically validate the anecdotal traditional uses of selected *Datura* species, particularly in cancer and inflammation.
- Extraction optimization and preliminary *in vitro* phytochemical and biological screening using different solvents and four plant parts of each specie.
- Quantification of medicinally useful polyphenolic compounds i.e., gallic acid, rutin, catechin, apigenin, myricetin and kaempferol in root, fruit, stem and leaf parts of both species using RP-HPLC basis analysis.
- Two chronic *in vivo* models of cancer and inflammation are executed to assess the activity of potent crude extracts shortlisted in the preliminary screening stage.
- The current study is the first to report the comparative antileukemic potential of EA leaf extracts of *D. stramonium* and *D. innoxia*.
- Additionally, the comparative anti-inflammatory potential of EA leaf extracts in $CCL_4$ induced hepatic injury model is also exclusively recorded in the present study.
- Isolation of bioactive components from the most potent extract i.e., DSL-EA. Although stigmasterol and ferulic acid have been isolated previously, our study has provided a new and simpler isolation scheme of these two compounds from the leaf portion of *D. stramonium*.
- The isolation of steroidal glucoside (stigmasterol glucoside) is reported for the first time from *D. stramonium.*
- The *in vivo* antileukemic potential of stigmasterol glucoside and ferulic acid in male Balb/c mice is reported for the first time in the current study.
- The *in vivo* anti-inflammatory action of stigmasterol glucoside in $CCL_4$ induced hepatic injury is also reported for the first time.
- This the first report on the extensive molecular mechanism of pharmacological effect of stigmasterol glucoside and ferulic acid in the event of benzene induced leukemia and $CCL_4$ induced hepatic inflammation.

## 4.9. Study Limitations

Some of the factors that affected the current study are listed as under;

- Some of the compounds eluted with very close Rf values in the isolation process could not be fully purified and thus excluded from the study.
- Biological evaluation of all compounds isolated in current study was not possible due to certain restraints i.e., purity and quantity.
- Since both compounds selected for chronic *in vivo* experiments have an established safety profile, due to the limitation of quantity of the purified compounds, multi dose toxicity assessment was not included in the current study.

# CONCLUSIONS

- The most proficient solvent for the extraction of phytoconstituents from different parts of *D. stramonium* and *D. innoxia* was distilled water (Dw), followed by methanol (M), ethyl acetate (EA) and n-hexane (NH).
- The quantitative phytochemical analysis and preliminary *in vitro* antioxidant assays of the crude extracts revealed that the extracts procured using NH and EA solvents were significantly active while Dw extracts of both species were devoid of any significant bioactive principles.
- HPLC profiling of EA and M crude extracts of both species confirmed the presence of pharmacologically significant polyphenolic compounds i.e., gallic acid, rutin, apigenin, myricetin, kaempferol and catechin. The crude extracts most abundant in the selected polyphenols were the EA extracts of *D. stramonium* fruit and leaf (DSF-EA and DSL-EA) and EA extracts of *D. innoxia* leaf and stem (DIL-EA and DIS-EA).
- The *in vitro* cytotoxicity potential was tested *via* brine shrimp lethality assay, while the preliminary anticancer activity was assessed with protein kinase inhibition assay. Medium to low polarity extracts of both plant species showed significant results. The leaf part of both species showed comparatively stronger bioactivity in these experiments.

Based on the findings of preliminary screenings, Dw extracts were omitted from any further experiments.

- The cytotoxicity of NH, EA and M extracts of the two *Datura* species was assessed against PC-3, MDA-MB 231 and MCF-7 cancer cell lines. DIL-EA was the most active crude extract with $IC_{50}$ values recorded to be less than 3 µg/ml against each cancer cell line. Amongst *D. stramonium* extracts, the EA root and leaf extracts (DSR-EA and DSL-EA) were the most active in terms of cytotoxicity against the stated cancer cell lines.

Following the cytotoxicity assay, the extracts were further shortlisted based on certain factors i.e., signficant extraction yield and overall promising results in majority of the preliminary assays. So, NH and EA leaf extracts of both species were selected for the next phase of the study.

- The safety profile of shortlisted crude extracts (DSL-NH, DSL-EA, DIL-NH and DIL-EA) was established by testing their cytotoxicity using MTT assay

against isolated human lymphocytes and rat macrophages. DSL-EA proved to be the least toxic extract in terms of % cytotoxicity.

- DSL-EA extract also revealed the strongest nitric oxide scavenging potential followed by DIL-EA.
- Among the NH and EA leaf extracts of *D. stramonium* and *D. innoxia,* DSL-EA and DIL-EA exhibited significant anti-inflammatory activity in acute carrageenan induced paw edema and croton oil induced anal edema models in Balb/c mice. These extracts also showed good results in acute *in vivo* models of depression (tail suspicion test) and analgesia (hot plate method).

Results of *in vitro* toxicity and acute *in vivo* studies showed that DSL-EA was the safest and most effective of all the samples followed by DIL-EA. These two crude extracts were selected to be screened *via* chronic *in vivo* models of cancer and inflammation.

- Antileukemic action as appraised by hematological, biochemical and histological evaluation as well as endogenous antioxidant enzymes levels clearly indicated the significant ameliorating effects of DSL-EA and DIL-EA against benzene induced leukemia in male Sprague Dawley rats.
- The anti-inflammatory potential of DSL-EA and DIL-EA in $CCl_4$ induced liver damage in male Sprague Dawley rats was also estimated. DSL-EA in a dose dependent manner showed significant alleviative effects. This claim is supported by results procured from a series of investigations including hematological, biochemical, oxidative stress markers, histological and immunohistochemistry studies.

It was concluded that DSL-EA was comparatively dominant as far as its *in vivo* response in benzene induced leukemia and $CCL_4$ induced liver inflammation is concerned. So, this extract was finalized for the next phase of this study, i.e., isolation of bioactive compounds.

- Three compounds were isolated from DSL-EA using normal phase column chromatography.
- Structure elucidation of the compounds was done using NMR (1D and 2D) technique and they were characterized as stigmasterol (compound a), ferulic acid (compound b) and stigmasterol glucoside (compound c).

- Two compounds, stigmasterol glucoside (C1) and ferulic acid (C2) were selected for further pharmacological evaluation using *in vivo* models of cancer (benzene induced leukemia) and inflammation ($CCL_4$ induced liver inflammation) in Balb/c mice.
- The compounds showed significant alleviative effects in both disease models. The activity of compounds was gauged through numerous hematological, biochemical, histological, immunohistochemical and western blot analysis.

# FUTURE PROSPECTIVES

- The compounds isolated in the current study could provide effective therapeutic options in cancer and inflammatory disorders.
- In depth toxicity studies could be carried out using both *in vitro* and *in vivo* models to establish the safety profile of isolated compounds.
- The influence of climate, collection time and plant part on the concentration of isolated compounds within the plant could provide useful insights in optimization of the isolation process.
- Plant parts other than the leaf that have shown promising results in preliminary screening (i.e., root, stem and fruit of *D. stramonium* and *D. innoxia*) could be utilized for the isolation of bioactive compounds.
- Formulation studies of the isolated compounds by experts of pharmaceutical dosage form designing could provide significant assistance in establishing the pharmacokinetic profile of these compounds.
- Advanced isolation techniques could be used to separate and purify compounds having close *Rf* value from DSL-EA extract.
- The leaf portion of *D. stramonium* and *D. innoxia,* owing to the tremendous pharmacological potential witnessed in the current study, could be further screened for presence of bioactive compounds of different chemical classes.
- Mechanistic studies targeting the molecular mechanism of stigmasterol glucoside in disease conditions i.e., leukemia and hepatic inflammation could provide further information about the possible mode of action of this compound.
- *Datura* species possess immense potential in the field of plant-based drug discovery and further research in exploring the pharmacological relevance of leaf, root and stem parts of these plants could lead to the discovery of several medicinally important compounds.

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# ANNEXURE I

# Annexure I

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RESEARCH ARTICLE Open Access

# Preclinical anticancer studies on the ethyl acetate leaf extracts of *Datura stramonium* and *Datura inoxia*

Bakht Nasir[1], Muhammad Waleed Baig[1], Muhammad Majid[1,2], Syeda Masooma Ali[1], Muhammad Zafar Irshad Khan[1], Syeda Tayyaba Batool Kazmi[1] and Ihsan-ul Haq[1*]

**Abstract**

**Background:** Cancer is a horrific disease relentlessly affecting human population round the globe. Genus *Datura* encompasses numerous species with reported medicinal uses. However, its potential as a source of natural anticancer agents is yet to be determined. *Datura stramonium* (DS) and *Datura inoxia* (DI) are the two species chosen for this study.

**Methods:** Total phenolic and flavonoid content (TPC and TFC) as well as antioxidant activity were assessed through colorimetric method. Polyphenolic quantification was done by RP-HPLC. Following extract standardization ethyl acetate leaf extracts of both species (DSL-EA and DIL-EA) were chosen for anticancer studies. In vitro cytotoxicity using various models including cancer cell lines was monitored. Following toxicity studies, benzene (0.2 ml) was used to induce leukemia in Sprague-Dawley rats. Extracts were orally administered to preventive (100 and 200 mg/kg) and treatment (200 mg/kg only) groups. The antileukemic potential of extracts was assessed through haematological, biochemical, endogenous antioxidants and histological parameters.

**Results:** Significant TPC and TFC were estimated in DSL-EA and DIL-EA. RP-HPLC quantified (μg/mg extract) rutin (0.89 ± 0.03), gallic acid (0.35 ± 0.07), catechin (0.24 ± 0.02) and apigenin (0.29 ± 0.09) in DSL-EA while rutin (0.036 ± 0.004) and caffeic acid (0.27 ± 0.03) in DIL-EA. Both extracts exhibited significant brine shrimp cytotoxicity ($LC_{50}$ < 12.5 μg/ml). DIL-EA exhibited greater cytotoxicity against PC-3, MDA-MB 231 and MCF-7 cell lines ($IC_{50}$ < 3 μg/ml in each case) as well as higher protein kinase inhibitory action (MIC: 25 μg/disc) compared to DSL-EA. Leukemia induced in rats was affirmed by elevated serum levels of WBCs (7.78 ± 0.012 (× $10^3$) /μl), bilirubin (7.56 ± 0.97 mg/dl), Thiobarbituric acid reactive substances (TBARs) (133.75 ± 2.61 nM/min/mg protein), decreased RBCs (4.33 ± 0.065 (× $10^6$)/μl), platelets (344 ± 3.19 (× $10^3$)/μl), total proteins (2.14 ± 0.11 g/dl), Glutathione S-transferases (GST) (81.01 ± 0.44 nM/min/ml), endogenous antioxidant enzymes levels and abnormal liver and kidney functionality in disease control rats. Both species revealed almost identical and significant ($p < 0.05$) alleviative effects in benzene induced leukemia.

(Continued on next page)

* Correspondence: ihsan99@yahoo.com; ihaq@qau.edu.pk
[1]Department of Pharmacy, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad 45320, Pakistan
Full list of author information is available at the end of the article

## List of Publications

### From this project

- **Bakht Nasir**, Muhammad Waleed Baig, Muhammad Majid, Syeda Masooma Ali, Muhammad Zafar Irshad Khan, Syeda Tayaba Batool Kazmi and Ihsan-ul-Haq. *Preclinical Anticancer Studies on the Ethyl acetate Leaf Extracts of Datura stramonium and Datura inoxia.* BMC Complement. Med. Therap.. 2020. 20.188. (https://doi.org/10.1186/s12906-020-02975-8).

- **Bakht Nasir,** Ashraf Ullah Khan, Muhammad Waleed Baig, Yusuf S. Althobaiti, Muhammad Faheem, and Ihsan-Ul Haq. *Datura stramonum leaf extract exhbits anti-inflammatory activity in CCL4 induced hepatic injury model by modulating oxidative stress markers and iNOS/Nrf2 expression.* BioMed Research International. Volume 2022. (https://doi.org/10.1155/2022/1382878).

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- Muhammad Waleed Baig, Madiha Ahmed, Nosheen Akhtar, Muhammad K. Okla, **Bakht Nasir**, Ihsan-Ul Haq, Jihan Al-Ghamdi, Whaida H Al-Qahtani and Hamada Abdelgawad. *Caralluma tuberculata N.E.Br Manifests Extraction Medium Reliant Disparity in Phytochemical and Pharmacological Analysis.* Molecules. 26 (24) 2021. (DOI: 10.3390/molecules26247530).
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