A STUDY ON BIOCHEMICAL ANALYSIS OF SOME TRADITIONALLY USED HEPATOPROTECTIVE MEDICINAL PLANTS & *IN-VIVO* ASSAY IN RODENT MODEL

A THESIS SUBMITTED TO BODOLAND UNIVERSITY, KOKRAJHAR, ASSAM FOR THE AWARD OF THE DOCTOR OF PHILOSOPHY IN THE DEPARTMENT OF BIOTECHNOLOGY, UNDER THE FACULTY OF SCIENCE AND TECHNOLOGY.

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Certificate

This is to certify that the Ph.D thesis entitled **"A study on biochemical analysis of some traditionally used hepatoprotective medicinal plants & *in-vivo* assay in rodent model"** is a bonafide record of Ph.D work done by Mr. Hankhray Boro under my supervision during the year 2013 to 2019. He has been registered under Bodoland University vide registration no: 001915 of 2013-14 and the Ph.D enrollment no: PHDBT13003 along with the final registration no: FINAL/03BIO0003 of 2013-2014. The thesis has not been previously submitted for the award of any degree, diploma or other research title.

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ABSTRACT

Indigenous community/ healers have their own conventional medicine system which may use single or combination of different plant parts. The plant *Morus indica* L. used for hepatoprotective purposes by local tribes of Bodoland Territorial Area District (BTAD), Assam was well supported by the *in-vivo* experimental model conducted on Carbon Tetrachloride (CCl_4) induced acute liver damage in wistar albino rats. Treatment with different doses of root of *Morus indica*-ethanolic extracts (RoMi-EE) (100 mg and 200 mg), the elevated levels of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) were markedly decreased and increased concentration of total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), total bilirubin, gamma-glutamyltransferase (GGT), creatinine were notably checked and depleted levels of high-density lipoprotein (HDL), albumin and total protein were recovered considerably. Meanwhile, significant increase in the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) activity and reduction of malondialdehyde (MDA) content upto 48% was noted in the RoMi-EE and silymarin treated groups. The histopathological study after treatment with different concentrations of RoMi-EE, the severity of CCl_4 induced liver and kidney intoxication was reduced in a dose-dependent manner, although the treatment with silymarin showed much better result.

In-silico molecular docking with 1NFK & 3LN1 proteins revealed that the ligand 2, 6, 10-Dodecatrien-1-ol, 3, 7, 11- trimethyl-9-(phenyl sulfonyl)–(E,E)- showed best docking score (ΔG binding affinity) of -4.95 (-45.35 kcal/mol) which is also comparable with that of silymarin and -9.78 (-27.8172 kcal/mol) respectively.

The *in-vivo* test so performed showed good results which are attributed to higher contents of phenolics, flavonoids, total antioxidant capacity and higher activity in total reducing power assay were obtained in the plant extract. Increase in radical scavenging activity was observed with increase in concentration in the *in-vitro* antioxidant tests of DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and H_2O_2 (hydrogen peroxide) having lowest IC_{50} values observed in the RoMi-EE (130.57 ± 12.46), RoAc-EE (7.94 ± 1.33) and RoAc-EE (12.67 ± 1.58) µg/ml respectively. For ICC (iron chelating capacity) and FRAP (ferric reducing antioxidant power) assay, the lowest EC_{50} value and highest $FeSO_4.7H_2O$ concentration was observed in RoPt-EE (535.16 ± 121.56 µg/ml) and RoAc-AE (2512.7 ± 157.37 µM/mg) respectively. Presence of various phytochemicals *viz*: phenols, flavonoids, tannins, resins, terpenoids, glycosides and steroids have been detected from the selected plants through qualitative test.

The GC-MS analysis have revealed the presence of various bioactive compounds that are reported for the first time from the ethanolic extract of RoMi and reported to have various biological activities such as antibacterial, anti-inflammatory, anti-diabetic, anticancer, anti-arthritic, hepatoprotective, effective against asthma and HIV-1 entry.

From the study, it is evident that RoMi-EE, showed high contents of antioxidant and *in-vivo* activity, making it highly potential as an alternative for treating liver disorders.

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CHAPTER-I

1. INTRODUCTION

The herbal medicines are known to mankind as an oldest form of healthcare system considered to be of great importance among different rural and indigenous communities in many developing countries (Gosh 2003).

People prefer herbal medicines all over the world more than conventional medicines. Plants are always been a major source of drugs and most of the existing drugs which are available at present have been derived from plants directly or indirectly (Dasgupta *et al.* 2013). According to the WHO 2002, in the developing countries about 80% of the population relies solely on traditional medicine for their daily healthcare and in India about 60% of the population living in rural areas use herbal medicines. According to Stickel & Schuppan 2007, the utilization of herbal supplements has increased from 2.5% to 12% during the recent years. The Indian system of traditional medicine is based on Ayurveda, Unani, Siddha, etc., which still provide primary healthcare at large, especially to rural folk (Sharma *et al.* 2012). Around the world, revival of traditional knowledge has gained major importance from conservation perspective as well as sustainable development and search for new formulation or utilization patterns of the plant resources is still on (Pan *et al*. 2013). Traditional medicine system also includes the traditional knowledge, skill and practices truly based on theories as well as experiences of traditional healers/folk communities to sustainably manage their health problems. Many indigenous communities/ traditional healers have their own conventional medicine system with diverse medicinal plants or plant parts and many conventional therapies for untreatable diseases (Sharma *et al.* 2012). A huge number of both wild and cultivated medicinal plants are being used for the cure of various ailments, thus a significant amount of medicinal plants information are available with the traditional healers.

Currently the scenario in America about the improvisation and research on herbal medicine is improving. As reported by Pelkonen *et al.* 2014, in USA, National Center for Complementary and Alternative Medicine (NCCAM) has been funded a sum of US$ 50-128.8 million per annum to complementary and alternative medicines including the herbal medicine. After Asia, Europe is the second largest import/export market of the herbal products (De Vos 2010) and in China alone approximately 100,000 herbal formulae and over 11,000 individual medicinal plants have been documented, which are generally regarded as

rich natural resources for developing new drugs including new type of multi-component drugs (Wang *et al.* 2008; Kuhn & Wang 2008).

Indian forests are rich in medicinal plant species with two mega reserves (Hot spots) of biodiversity (Eastern Himalayas and Western Ghats) having wide spectrum of potential resources of healing properties. The rich diversity of Eastern Himalayan region of India is witnessed by herbs showing potential virtue with regards to hepatoprotective disorders. Traditional Indian system of medicine is therefore well developed due to this richness of bio-resources. More than 7500 plant species are referred in Indian folklore and only about 1700 plant species are mentioned in the documented form of ancient or past literature, out of which only 700 species (approx) have been investigated pharmacologically and chemically (Maiti 2004; Patil & Patil 2010). The turnover of Indian herbal industry is estimated to be around 4000 crore per annum (Nirmal *et al.* 2013; Patil & Patil 2010). The current use of medicinal plant resources on the basis of market signals is however inefficient and inadequate. According to Patil & Patil 2010, nearly 500 plant species are being used by the pharmaceutical industries in India and most of the medicinal plants, especially the medicinal tree species are presently collected from the wild. Recently, utilization of medicinal plants has increased multifold due to ever increasing market of herbal industry.

Liver diseases cause some of the major mortality and morbidity across worldwide. According to estimates made by WHO 2008, prevalence of hepatitis A, B and C virus infection cases is reported to be about 1.4 million, 2 billion and 130-170 million respectively. About 350 million people live with chronic liver infection and almost 6 lakhs and 3.5 lakhs people die every year due to the consequences of hepatitis B (acute or chronic) & hepatitis C respectively (Sharma *et al.* 2012). Thus, impact of the liver disorders on overall population of the world is significant and remains to be one of the severe health problems. Jaundice is not a disease but a symptom which indicate the liver malfunctioning in which yellowing of the skin as well as mucous membranes occurs due to an increased 'bilirubin' a bile pigment in the blood, which is a global concern & most frequent (Fatma & Uphadhyay 2015; Sharma *et al.* 2012). There are various different causes of jaundice *viz*., hepatitis (A, B, C, D, and E), inflammation of the liver, liver cirrhosis, alcoholic liver disease, obstruction of bile ducts, yellow fever, gallstones, typhoid, malaria, haemolytic anaemia, pancreatic cancer, tuberculosis, (Riyaz 1997; Fatma & Uphadhyay 2015; Sharma *et al.* 2012).

Despite of remarkable advancement, the conventional or synthetic drugs used to treat liver diseases are unsatisfactory because they can cause serious long-term side effects

(Velioglu *et al*. 1998; Giacometti *et al*. 2016). It is sometime very difficult to find a protective drug/agents at present for common ailment of liver. Due to lack of reliable hepatoprotective drugs in allopathic system of medical practices, herbs play a significant role in the management of various liver disorders (Sharma *et al.* 2012). A large number of plants and its formulations is known to posses hepatoprotective activity (160 phyto-constituents from 101 different plants) as reported by Handa *et al.* 1986.

1.1 Need for herbal medicine based research

A. Most chronic diseases are not single entities. Instead, mostly there are several etiological factors and multiple mechanisms within numerous molecular pathways (Pelkonen *et al.* 2014). Preventing and treating these major chronic diseases have led to the use of multiple drugs to tackle different targets and various symptoms. It seems that one of the reasons for the less satisfactory success of drug development during the recent decades has been the single-target-single-compound or one-disease-one drug paradigm (Hopkin 2008). Herbal plants have very diverse group of compounds that can solve the problem of single-target-single-compound or one-disease-one drug paradigm (Hopkin 2008).

B. Traditional system of medicine continues to be widely practised on many accounts. Population rise, inadequate supply of drug, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of traditional herbal medicine/plant materials claim by the traditional healers as a source of medicines for a wide variety of human ailments which otherwise lack scientific validation (Pathare & Wagh 2012).

C. North-East India is the richest reservoir of plant diversity in India with almost 50% of India's total plant diversity (Mao & Hynniewta 2000) and fall under Eastern Himalayan biodiversity hotspots region. Kokrajhar, Assam is one of the gateway to the seven sisters of North-East India (NER). Ethnic communities of Kokrajhar and the other parts of North-East Region of India have always generated, refined and passed on traditional knowledge from generation to generation and still plays a vital role in the daily lives of these people.

D. Due to rapid depletion of bio-resources because of anthropogenic activities and urbanization coupled with improper scientific documentation on herbal medicinal

plants, urgent systematic investigation is required by using biotechnological tools to authenticate and develop new novel drugs from the NE region.

It is reflected that most of the degenerative human diseases have their origin from the deleterious free radical reactions (Florence 1995; Liu *et al*. 2018). Medicinal plants have high contents of natural phyto-constituents viz., polyphenols, flavonoids, terpenoides, tannins, beta-carotene, vitamin C and E etc., which are excellent antioxidants that act as radical scavenger and can prevent incidence of several human disorders (Anderson *et al.* 2001; Alam *et al*. 2013; Ksouri *et al*. 2015). Plant phyto-constituents are chemical compounds that are produced through primary or secondary metabolism by the plants. They play a big role in plant biological system like growth or defence against pathogens (Molyneux *et al.* 2007). Therefore, it is crucial to understand the role of herbal medicines as an alternative to cure liver disorder.

1.2 Research problem and gap

The preliminary work including ground survey & review of essential literature reflected the lack of proper documentary evidence regarding the availability of sufficient scientific work to evaluate the efficacy of candidate medicinal plants used by traditional healer of Kokrajhar to correlate with the efficiency of the same.

1.3 Objectives of the study

The research gap indicated the necessity of such work which can minimise the knowledge gap and boost the utility of candidate plants. Hence the present study is anticipated with the following objectives:

1. To collect and identify traditionally used hepatoprotective medicinal plants by Bodo tribe of Kokrajhar, Assam.
2. To study the *In-vitro* antioxidants property of some hepatoprotective medicinal plants.
3. To study the phytochemical characterization of the candidate medicinal plant by GC-MS analysis.
4. To study *in-vivo* efficacy of the extract on CCl_4 induced hepatotoxicity in rodent model.
5. To study the activity of some specific liver enzymes and histopathological evaluation of the liver & kidney.
6. To understand the *in-vivo* activity of plant bioactive compounds by *in-silico* molecular docking model if any.

CHAPTER-II

2. LITERATURE REVIEW

In recent years, medicinal plants & plant extracts have gained immense importance, since they are the major source of herbal drugs. Medicinal plants like *Moringa oliefera* (Ghasi *et al.* 2000; Arabshahi-Delouee *et al.* 2007; Urooj & Reddy 2010), *Allium sativum* (Yeh & Liu 2001), *Solanum melongena* (Sudheesh *et al.* 1997), *Apium graviolans, Achyranthes aspera, Terminalia arjuna, Zinziber officinalis, Phylanthus niruri, Momordica charantia, Ocimum sanctum* (Phadke 2007), *Trigonella foenum graecum* (Shashikumar *et al.* 2018), *Azardiracta indica* (Igwenyi *et al.* 2017), *Amaranthus spinosus* (Zeashan *et al.* 2009), *Launaea procumbens* (Khan *et al.* 2012) and numerous other plants are scientifically proven during the last few decades as natural antioxidants, nutraceuticals, lipid lowering as well as hepatoprotective agents.

2.1 Survey review

In a survey reported by Kpodar *et al.* 2016, traditional healers of the Togo utilize a total of 99 plant species belonging to 88 genera and 49 families that are used for hepatic diseases. Among the reported families *Caesalpiniaceae* was the highest with 8 species, followed by *Euphorbiaceae* with 7 species, *Apocynaceae* and *Asteraceae* with 6 species each. The plant parts used mainly were the leaves, followed by roots, whole plant, rhizome and the bark accounting for more than 10% each. The herbal medicines for the treatment of liver disorder were prepared mostly by decoction and were administered through orally.

A total of 40 medicinal plants were reported by Sharma *et al.* 2012, which belongs to 31 families and 38 genera were recorded to be used by the Bhoxa, nomadic Gujjars and Tharu communities in 45 formulations (single plant or in combination of plants) that uses 15, 23 and 9 plants, respectively as a remedy of jaundice. Survey shows that: *Amaranthus spinosus* L., *Cissampelos pareira* L., *Ehretia laevis* Roxb., *Holarrhena pubescens* Wall., *Ocimum americanum* L., *Physalis divaricata* D. Don, *Solanum incanum* L. and *Trichosanthes cucumerina* L. were reported for the first time in India. The literature survey by Sharma *et al.* 2012, revealed that total of 214 plants (under 181 genus & 78 families) are used as internal, 19 plants (under 18 genus & 12 families) are used as external and 14 plants (under 14 genus & 11 families) are used as magico-religious remedy for jaundice by various communities in different parts of India.

Hepatoprotective medicinal plants survey by Thockchom *et al.* 2018, in 13 different villages (under Bishnupur, Kakching and Thoubal districts) of Manipur, India, have reported a total of 34 different families and 52 plant species. They have also calculated Disease Consensus Index (DCI) on the basis of information collected to determine most significant plants. DCI value was found to be highest in *Engelhardtia spicata*, which is followed by *Saccharum officinarum, Averrhoa carambola, Andrographis paniculata, Justicia adhatoda* and *Cuscuta reflexa*.

2.2 Plant phytochemicals and antioxidant review

Medicinal plants have high contents of natural phyto-constituents viz., polyphenols, flavonoids, terpenoids, tannins, beta-carotene, vitamin C and E, etc., which are excellent antioxidants that can prevent incidence of several free radical induced human disorders viz; cancer, atheroschlerosis, retinopathy, liver disorder, ulcerative colitis, diabetes, hypertension, chirrosis, allergy arthritis, inflammation, etc. (Anderson *et al.* 2001; Alam *et al.* 2013; Ksouri *et al.* 2015). Several studies investigated that polyphenols are directly attached with biological activity such as hepatoprotective activity (Santillan *et al*. 2014; Wu *et al*. 2017).

Presence of phenolic, flavonoids, terpenoides, saponins, tannins and reducing sugar from the ethanolic extracts of different medicinal plants viz; leaves of *Psidium guajava, Carica papaya, Vernonia amygdalina*, stem bark of *Magnifera indica* (Ayoola *et al*. 2008), *Enicostemma littorale* (Selvaraj *et al.* 2014) and leaves, stem, bark of (Ganesan *et al*. 2016). In addition to that, *Enicostemma littorale* & *Datura stramonium* were also found to have steroids, alkaloids and glycosides. Further the DPPH radical scavenging activity showed IC_{50} values of 0.04, 0.313, 0.58, 2.3 and 0.054 mg/mL from *P. guajava, M. indica, C. papaya, V. amygdalina* and vitamin C respectively.

In a different research, various *in-vitro* antioxidant activities were studied taking experimental models like iron (III) reducing capacity, total antioxidant capacity, DPPH, FRAP & H_2O_2 radical scavenging activity, β-carotene bleaching assay, total phenolic, flavonoids, ascorbic acid content, nitric oxide and inhibition of ferrous sulphate-induced oxidation of lipid system by various researchers with different extracts on the plants *viz*; *Morus indica* (leaves), *Averrhoa carambola* (green & ripe fruit peel, stem, tart & honey type fruits), *Averrhoa bilimbi* (different types), *Parkia javanica* (seed) and leaves of *Phlogacanthus thyrsiflorus* (Delouee & Urooj 2007; Lim & Lee 2013; Sindhu *et al.* 2013; Asna & Noriham 2014; Chanu *et al*. 2012). Based on the outcome of above research, it was

found that the antioxidant activity of *M. indica* leaves remained unchanged at 50°C and was maximum at pH-7. *A. carambola* fruit antioxidant capacities were increased significantly with ripening, except for the total ascorbic acid content. The ripe star fruit peel contained higher total polyphenol, flavanol and ascorbic acid contents than green star fruit peel. Ripe star fruit peel also demonstrated stronger FRAP and DPPH (75% inhibition) values than the green star fruit peel. *A. carambola* tart type possessed highest total phenolic, flavonoids and strong DPPH radical scavenging activity and the *A. carambola* honey type showed higher value in FRAP and β-carotene bleaching assay. *A. carambola* stem showed strong DPPH, Nitric oxide, H_2O_2 radical scavenging activity in ethanolic extract which showed IC_{50} values of 878.06 µg/mL, 391.69 µg/mL, 83.29 µg/mL respectively. Methanolic leave extract of *P. thyrsiflorus* showed highest value of 61.07 ± 0.61 and 77.29 ± 0.51 mg Trolox equivalent/g in DPPH and FRAP assay respectively whereas ethanolic extract caused maximum inhibition of lipid peroxidation at 200 µg/mL concentration (81.28 ± 0.1%) and *P. javanica* water extract showed highest phenolic content with 51.09 ± 0.78 GAE mg/g of extract.

Study by Chatterjee *et al.* 1983 and Balasubramanian *et al.* 2005, revealed that *Morus indica* (root and leaves) extracts significantly inhibited carrageenan-induced oedema in wistar rat. Root extract inhibited histamine, serotonin, bradykinin induced oedema, hyaluronidase-induced oedema and also reduces the intensity of peritoneal inflammation by decreasing exudation of plasma protein. The average mean ±SE value of total protein exudate in artificial peritoneal inflammation induced by acetic acid was 39.33 ± 3.5 mg, but with prior administration of 400 mg/kg & 800 mg/kg of *M. indica* root extract, the average mean (± SE) amounts of protein exudation were 32.75 ±1.8 mg and 20.9 ± 1.9 mg, respectively. The leave extract also reduced carrageenan induced edema by 56.76% on oral administration of 100 mg/kg, whereas indomethacin 10 mg/kg inhibited edema by 64.86% as compared to untreated control group. The extract also inhibited leukocyte migration, pleural exudates reduction and decreased granuloma weight in the cotton pellet granuloma method. The inhibition of *M. indica* extract and indomethacin drug was found to be 53.1 and 64.4%, respectively.

2.3 Hepatoprotective activity

Hepatic disease is a term that indicates damage to the cells, tissues, structure, or liver function and this damage can be induced by biological factors (bacteria, virus, and parasite infection), autoimmune diseases (immune hepatitis, primary biliary cirrhosis), as well as by the action of different chemicals/ drugs (high doses of paracetamol and antitubercular drugs),

toxic compounds (carbon tetrachloride, thioacetamide, dimethylnitrosamine, *D*-galactosamine/lipopolysaccharide, and unquestionably, excessive consumption of alcohol (Upur *et al*. 2009; Kumar *et al.* 2013; Santillan *et al.* 2014). Unfortunately, conventional or synthetic drugs used to treat liver diseases are unsatisfactory because they can exert serious long-term side effects (Velioglu *et al*. 1998; Giacometti *et al*. 2016).

Oh *et al.* 2010, studied immunomodulatory and hepatoprotection effect induced by CCl_4 from *Morus indica* Linn. (MIL) glycoproteins and found that reduced activities of ALT, LDH and TBARS in serum and reduced activity of Cyclooxigenase-2 (COX-2) and expression of TNF-α and IL-1β in liver from CCl_4-treated mice were reported. Moreover MIL glycoprotein also suppressed the stress-activated protein kinase/c-jun N-terminal kinase phosphorylation and activator protein-1 transcriptional activation in liver from CCl_4-treated mice. The result indicated that MIL glycoprotein protects against liver injury effectively by down-regulation of oxidative stress and also by the inflammatory response.

In several studies, significant elevation of ALP, AST, ALT, GGT, LDH, bilirubin, glucose, urea, cholesterol level and subsequent liver injury with decreased levels of liver antioxidant enzymes (SOD, CAT, GSH-Px, GSH) content are observed in liver toxicity induced by CCl_4 (Dutta *et al.* 2018; Bahashwan *et al.* 2015; Rahmat *et al.* 2014; Ahsan *et al.* 2009). Various studies hepatoprotective activities have shown that medicinal plants/ plant extracts which are traditionally being used in many liver disorders were reported to be very much useful in maintaining the various liver enzymes activities and also in restoring the liver histopathological structure (Maheshwari *et al.* 2011; Rahmat *et al.* 2014; Dutta *et al.* 2018; Reddy & Urooj 2017; Ahsan *et al.* 2009).

Amaranthus spinosus whole plant ethanolic extract was found to contain 336 ±14.3 mg/g gallic acid equivalent total polyphenolics, while the reducing capacity was 2.26 times of BHA, whereas significant antioxidant activity in DPPH assay (IC_{50} of 29 µg/mL), superoxide scavenging assay (IC_{50} ~66–70 µg/mL), hydrogen peroxide assay (IC_{50} ~120–125 µg/mL), hydroxyl radicals assay (IC_{50} ~140–145 µg/mL) and nitric oxide assay (IC_{50} ~135–140 µg/mL) were observed in ASE. ASE (6-10 µg/mL) was able to normalise the levels of biochemical parameters in isolated rat hepatocytes intoxicated with CCl_4. The effect of the ASE at 10 µg/mL was found to be comparable to control group. The investigation carried out in human liver derived HepG2 cells against CCl_4 induced damage also confirmed the hepatoprotective nature of the ASE in a dose dependent manner (Zeashan *et al.* 2009).

Hepatoprotective activity of *Averrhoa carambola* stem ethanolic extract (ACSEE) was evaluated by CCl_4 induced hepatotoxicity in rats and estimated the serum hepatic enzyme levels (Eswaraiha *et al.* 2013). Treated animals with ACSEE (250 mg/kg) and ACSEE (500 mg/kg) showed reduction in serum enzyme levels and were comparable with standard silymarin. Histopathological studies of normal rat liver showed normal hepatocytes, sinusoids. Liver section of rat treated with CCl_4 exhibited severe necrosis, disappearance of hepatocytes and area of inflammation with increased sinusoidal spaces. Liver section of rat treated with ACSEE (250 mg) and CCl_4 exhibited mild degree of necrosis, reduced sinusoidal dilation and less inflammation. Liver section of rat treated with ACSEE (500 mg) and silymarin showed normal hepatocyte, sinusoids with no inflammation.

2.4 GC-MS review of study conducted by using GC-MS

The combination of an ideal separation technique (GC) with the best identification technique (MS) made GC–MS an ideal technique for qualitative and quantitative for volatile and semi-volatile compounds (Lordache *et al*. 2009). Because of which, in recent years, gas chromatography and mass spectrography (GC–MS) has been applied unambiguously to identify the structures of different phyto-constituents from plant extracts and biological samples with great success. GC-MS is a reliable technique to identify the phytoconstituents of volatile matter, long-chain branched hydrocarbons, alcohols, acids, esters, etc. Peak area, retention time and molecular formula were used for the confirmation of phytochemical compounds (Venkatesh *et al*. 2014; Rukmini & Devi 2014).

The *Morus alba* leave extract components were identified using gas chromatography and decomposition products were characterized by mass analyser detector GC-MS which shows three components viz; 9,12,15-octadecatrienoic acid ethyl ester, linolenic acid ethyl ester and gibberallic acid respectively (Emniyet *et al*. 2014). Saravanan *et al.* 2014, reported 13 different compounds from GC-MS analysis of *Ficus religiosa,* that were found to have antioxidant and antimicrobial activity: phenol, 4-methoxy phenol, ethyl isoallocholate and octadecanoic acid. In another study, GC-MS analysis showed the presence of 9,12,15-octadecatrienoic acid in *Vitis sitosa,* that possesses many biological activities which includes anti-inflammatory, cancer preventive, hepatoprotective, etc., (Gobalakrishnan *et al*. 2014).

2.5 Docking review

Molecular docking is a tool of molecular modelling by which we can understand the mechanism of action/interaction of medicinal plant's bioactive compounds that allows to

present antagonist/agonist the best suitable orientation for most stable complex formation and the best position for molecular binding (ligand) with target proteins (Sousa *et al*. 2013; Kahraman *et al*. 2007; Lu *et al.* 2010; Ganguly & Panigrahi 2009). Docking reduce expenses and time due to carrying out the procedure that is similar to high-performance biological screening and is also possible to calculate the strength of binding (affinity) between them (Kitchen *et al.* 2004). Affinity is equal to the concentration of the ligand, in which half of the targets binds with the ligand (Meng *et al*. 2004; Morris *et al*. 2005; Zsoldos 2007). The measure of biological activity depends on ligand concentration at which the cell response is equal to half the maximum. Therefore, ligands with the highest affinity provided will block or activate the molecular target in biological experiments best of all (Cerqueira *et al*. 2009; Scigress explorer ultra, 2012; Huang *et al*. 2010). Glushchenko *et al*. 2015, stated that ligand affinity in relation to the receptor is assessed both by geometric criteria of surface complementarity of the ligand with that of receptor cavity and by physico-chemical criteria (like electrostatic interaction, van der Waals repulsion, hydrogen bonds formations etc.).

Molecular docking analysis with PDB- 3i7h complexed with H-Box motif of HBX, it was observed that the compound α-amyrin which was identified by the GC-MS analysis in the leaf extract of *Croton bonplandianus* Baill. have shown better potentiality to protect hepatocellular damages than the standard drug Silymarin (Dutta *et al*. 2018).

Gunalan *et al*. 2014, have studied docking analysis by the GC-MS compounds identified from *Bauhinia variegate* ethanolic leaf extract to inhibit COX-2 and iNOS. Among the 33 ligands of the active fractions, four ligands were found to have best glide score. The ligand: Benzofuranone was found to have better binding affinity with glide score of -3.147224 (-24.739633 glide energy Kcal/mol) in COX-2 biotarget and in case of iNOS the ligand Dioctylphthalate have shown better binding affinity with glide score of -9.359683 (-48.046527 glide energy Kcal/mol).

Glushchenko *et al.* 2015, conducted the docking studies by the method of a flexible molecular docking using the SCIGRESS software in order to determine the possible mechanisms of the hepatoprotective effect of *Bupleurum aureum* plant components for the inhibition of protein NFκB (nuclear factor kappa B) (code 2I9T). The best docking score of -35.050, -37.870 and -28.004 was obtained from the flavonoids (quercetin), alcohols (xylitol) and monoterpenoids (lolioside) respectively from *B. aureum* plant.

CHAPTER-III

3. DISCRIPTION OF PLANTS

3.1 Sample selection

The roots of *Morus indica* L., *Averrhoa carambola* L. and *Phlogacanthus thyrsiflorus* Nees. were selected on the basis of availability of the plant on the region and literature review of the plants/plant parts. The root of *M. indica*, *A. carambola* and *P. thyrsiflorus* (***Appendix-G***) were collected from the Bashbari (Bismuri) having Lat: 26˚33'42.6"N / Lon: 90˚16'17.2"E, Purana Titaguri having Lat: 26˚26'43.44"N / Lon: 90˚17'16.07"E and Sukhanjhara having Lat: 26˚23'26.18"N / Lon: 90˚12'18. 61"E respectively.

3.2 Scientific classification of *Morus indica* L.

Kingdom: Plantae

Clade: Angiosperms

Clade: Eudicots

Clade: Rosids

Order: Rosales

Family: Moraceae

Tribe: Moreae

Genus: *Morus*

Species: *indica*

Discription: The *Morus indica* is a fast growing deciduous, woody, small perennial tree with small fruits and grows upto 5-12 metres in height and are extensively cultivated as the leaves are food for silkworms. The plant is well branched and small. The plants are generally dioecious with catkin inflorescence bearing unisexual flowers in scaly clusters. Barks are rough and grey brown. The leaves are simple, alternate, usually smaller, ovate, stipulate, petiolate, entire or lobed and the number of lobes varies from one to five, have long tip and thin, uneven deeply cut edge, rough above and are about 5-10 cm long. The flowers are separate, slender hanging spikes. Fruit are small (upto 2 cm), cylindrical and dark red colour.

Distribution: Commonly found in South-East Asia.

3.3 Scientific classification of *Averrhoa carambola* L.

Kingdom: Plantae

Clade: Angiosperms

Clade: Eudicots

Clade: Rosids

Order: Oxalidales

Family: Oxalidaceae

Genus: *Averrhoa*

Species: *carambola*

Discription: It is a small tree which grows about 5-12 meters in height. The branches are flabby and the woods are white that turn reddish and are generally bushy shaped due to lots of branches producing rounded crown like appearance. Leaves are soft, medium-green, 4-10 cm in length & 2-5 cm wide, spirally arranged around the branches in an alternate fashion. The pinnate leaves have a single terminal leaflet and 5 to 11 nearly opposite leaflet. The leaflets are ovate or ovate-oblong in shape. Flowers are rose to red-purple, small, bell-shaped and have five petals with whitish edges. Under tropical condition, the flowers are produced throughout the year. Fruits are oblong shape: they are longitudinally 4 to 5 angled in general and 6–12 cm long and up to 9 cm wide, have a thin waxy skin and is orange-yellow colored.

Distribution: This evergreen tree is native to South-East Asia and the Indian subcontinent.

3.4 Scientific classification of *Phlogacanthus thyrsiflorus* Nees:

Kingdom: Plantae

Clade: Angiosperms

Clade: Eudicots

Clade: Rosids

Order: Lamiales

Family: Acanthaceae

Genus: *Phlogacanthus*

Species: *thyrsiflorus*

Discription: It is a shrub that grows upto 3-7 ft in height. The leaves are lance shaped, hairless, large with 2-4 cm wide and 8-16 cm long having tapering at both ends. Flowers are borne in thyrses at branch end or on short lateral branches with 10-25 cm long. Sepals are 6-8 mm long, densely velvet-hairy, linear. Flowers are 2 lipped, orange, wide tube shaped and closely hairy. Tube is broad from the base, curve; upper lip nearly erect and the lower lip spreading. Stamens are hairless or slightly hairy near the base of filament. Capsules are generally hairless, 3 cm long and 4 mm wide, somewhat quadrangular, 12-14 seeded. Seeds are much compressed, circular in outline and densely hairy.

Distribution: Native to South-East Asia.

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Study area

Kokrajhar (89.46' E to 90.38' E longitudes and 26.19" N to 26.54" N latitudes) is one of the twenty-seven districts of Assam and head quarter of Bodoland Territorial Council, described as the gateway to the north-eastern region of India located on the north bank of the river Brahmaputra dominated by the Boro tribe. It is surrounded by Bhutan on the north, the Sonkosh river and Jalpaiguri district of West Bengal on the west and tracts of Brahmaputra valley (Dhubri and Goalpara district) on the south and Chirang & Bongaigaon district on the east. It covers a total area of 3,169.22 sq. km; 2001 census of India, put population of Boro as 9,30,404, whereas Brahma *et al.* 2001, stated that Boro population is as high as 52 lakhs.

4.2 Topography

The Kokrajhar district land surface is generally slope from north to the south. Phukan 1990, has divided the area into two distinct physiological zones- (a) North zone are in foothills of the Himalaya and (b) the plain area towards the south. Kokrajhar has alluvial soil type and may vary from sandy loam to silty clay loam, silty clay and clay. In northern part, it is mostly covered with light gravel that originates from alluvial soil of old mountain valley and towards the southern part, varying proportion of sand and clay were found ranging from poor sand stiff clay. The soil is silt in organic matter and acidic in nature (Bhowmick *et al*. 2015). The district is situated in sub-tropical climate with moderately hot summer and cold winter with high humidity. Generally, the months of December – February is the coldest months of the year with less than 11 ºC and temperature goes up to 32 ºC during summer season with heavy rainfall during the month of June- September ranging up to 747 mm (Kour & Sharma 2016).

4.3 Collection and identification:

An ethno-botanical survey was carried out between March 2014 and May 2017 to obtain relevant information about medicinal plants used in the treatment of liver disorder in Kokrajhar, Lower Assam, India, through a series of oral interviews with traditional healers as well as elderly people of the villages who still practice/have knowledge of the indigenous system of medicine. The oral interview was accomplished in their local dialect, with the help of questionnaires attached in '***Appendix A***' (Willcox *et al.* 2011). The traditional healers were requested to accompany to collect the plant specimens from adjoining forest areas. In some

cases when the experienced and familiar persons were unable to accompany in the exploration visit to the forest area, the fresh specimens were collected from the target location with details of their vernacular name, habit, habitat, height of the plant, fragrance, taste, flower, fruit nature, etc., and consequently shown to them to obtain accurate information (Sharma *et al.* 2012).

The voucher specimens were prepared for all collected plants and deposited to get identified at B.S.I. Shillong, Meghalaya, India prior to initiation of the said work.

4.4 Extract preparation of Sample:

The collected samples were washed properly with water and were oven dried at 40-45 ºC for 7-14 days. After that, they were crushed up and ground to get homogeneous fine powder by a grinder and stored at air tight container for further use (Oyedemi & Afolayan 2011). 50 g of dried root powder is extracted twice in 200 mL of ethanol (70%) and acetone for 48 hrs and filtered with whatmann's filter paper number 42. Filtrate was concentrated by evaporating in rota-evaporator until fully dried (extract) and store at 4 °C for further studies.

4.5 Phytochemical screening

The qualitative phytochemical screening of roots of *Morus indica*, *Averrhoa carambola* and *Phlogacanthus thyrsiflorus* by 70% ethanolic and acetonic solvents were accomplished. The following methods were adopted for the phytochemical screening:

A. Detection of phenol (Hussein *et al.* 2012):

In a test tube, 5 mL of each previous filtered extracts were taken and 1 mL of $FeCl_3$ (1%) and 1 mL $K_3[Fe(CN)_6]$ (1%) were added. The appearance of fresh radish blue color indicated the presence of polyphenols.

B. Detection of flavonoids (Hussein *et al.* 2012):

Two solutions A and B from both parts of the plant extracts were prepared. The solution A contains 5 mL of ethanolic extract previously prepared. The solution B consists of 5 mL of ethanolic solvent added to 5 mL of KOH (50%). Then two solutions A and B were mixed together. The presence of flavonoids is indicated by the appearance of yellow color.

C. Detection of tannins (Ganesan *et al.* 2006):

About 2 mL of the extract and a few drops of 1% lead acetate were added. A yellow precipitate formation indicates the presence of tannins.

D. Detection of resins (Hussein *et al.* 2012):

10 mL of each previous filtered extracts were taken and 20 mL of HCl 4% were added. The appearance of turbidity indicates the presence of resins in the extracts.

E. Detection of terpenoids (Ayoola *et al.* 2008):

To 0.5 g each of the extract was added 2 mL of Chloroform. And again 3 mL of concentrated H_2SO_4 was carefully added to form a layer. The presence of reddish brown on the interface indicated the presence of terpenoids.

F. Detection of alkaloids (Hussein *et al.* 2012):

0.2 g of the powder extract of root of the plant was dissolved in 10 mL of 1% HCl. Then, they were transferred to a water bath for few minutes. After, 1 mL of the filtrated extract was treated with 2-4 drops of Dragendorff's reagent. The presence of alkaloids is indicated by the appearance of an orange reddish precipitation.

G. Detection of glycosides (Ganesan *et al.* 2006):

The extract was hydrolysed with HCl for few hours on a water bath. To the hydrolysate, 1 mL of pyridine was added and a few drops of Na-Nitroprusside solution was added and then it was made alkaline with NaOH solution. Appearance of pink to red colour shows the presence of glycosides.

H. Detection of cardiac glycosides (Ganesan *et al.* 2006):

To 0.5 g of extract diluted to 5 mL in water was added 2 mL of Glacial acetic acid containing one drop of ferric chloride solution. This was under layered with 1 mL of conc. H_2SO_4. A brown ring at the interface indicated the presence of a deoxy-sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring.

I. Detection of reducing sugar (Kabesh *et al.* 2015):

To 1 mL of extract, added 1 mL of Fehling's – A solution and 1 mL of Fehling's-B solution. Formation of red colour indicates the presence of sugar.

J. Detection of steroids (Kabesh *et al.* 2015):

To 1 mL of extract, mix with 1 mL of chloroform and concentrated H_2SO_4 sidewise. A red color presence at the lower chloroform layer indicates presence of steroids.

K. Detection of anthraquinones (Ganesan *et al.* 2006):

About 5 mL of extract solution was hydrolysed with dilute H_2SO_4 extracted with benzene. 1 mL of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.

L. Detection of saponins (Sathi & Sengottuvel 2016):

About 0.5 mg of the extract was shaken with 5 mL of distilled water. Formation of frothing (appearance of creamy miss of small bubbles) shows the presence of saponins.

4.6 *In-vitro* antioxidant tests

4.6.1 Total phenolic content

The folin–ciocalteau method (Maheswari *et al.* 2011) was used for the determination of total phenolic content. Briefly, in a test tube 100 µL of the extract was taken, to which 1600 µL of doubled distilled water was added and then 100 µL of folin-ciocalteau reagent (0.25 N) were added and mixed properly. The above sample mixture was allowed to react for 3 minutes. After that, 150 µL of Na_2CO_3 (1 N) solution was added. The mixture was incubated at room temperature in the dark for 2 hrs. The absorbance was taken at 725 nm using a PC based double beam spectrophotometer (Systronics) by taking Gallic acid as standard and were expressed in milligram of gallic acid equivalent (GAE)/g of dried extract.

4.6.2 Total flavonoid content

Total flavonoid content was determined by aluminum chloride method (Hsish *et al.* 2016). From all the extracts, 0.1 mg/mL of extracts were prepared in double distilled water and were reacted with 1.5 mL ethanol (95%), 0.1 mL aluminum chloride hexahydrate (10%), 0.1 mL potassium acetate (1 M) and 2.8 mL of doubled distilled water for 40 minutes at room temperature. Finally, the absorbance of the above mixture was measured at 415 nm taking quercetin as standard.

4.6.3 Total reducing power assay

The reducing power assay was determined by the method adopted by Hsieh *et al.* 2016. An aliquot of 1 mL of sample was reacted with 0.5 mL of phosphate buffer (0.2 M) and 0.5 mL of potassium ferricyanide (1%). The above reaction mixture was then incubated at 50 ºC for 20 minutes. After the cooling, 0.5 mL of trichloroacetic acid (10%) was added. Now, 2 mL of distilled water was mixed with 2 mL of the above reaction liquid and 0.2 mL of iron (III) chloride (0.1%). Finally, the absorbance was measured at 700 nm. BHA was used as a positive control.

4.6.4 Total antioxidant capacity

Phosphomolybdate method was adopted for the determination of total antioxidant capacity (Shah *et al.* 2013). In brief, 0.3 mL of each extracts was added to 3 mL of phosphomolybdate reagent (0.6 M H_2SO_4, 0.028 M sodium phosphate, 0.004 M ammonium molybdate). This reaction mixture was incubated at 95 °C in water bath for 90 minutes. After cooling to room temperature, the absorbance was taken at 765 nm. Ascorbic acid served as standard. Results were expressed in mg of ascorbic acid equivalent (AAE)/g of dried extract.

4.6.5 DPPH radical scavenging assay

The antioxidant ability of root extracts were determined by the method of Shukla *et al*. 2016, by their capacities to neutralize radicals of DPPH (di (phenyl)- (2,4,6-trinitrophenyl) iminoazanium). The antioxidants in the sample scavenge the free radical and turn it into yellow in colour. A working solution of DPPH (0.004%) was prepared freshly in methanol. 1 mL of sample and standard dilution of various concentrations (10, 20, 40, 80 and 160 µg/mL) was added to 3 mL of DPPH working solution. After 30 minutes of incubation in dark at room temperature 25 °C ±2, change in colour from violet to yellow was recorded at 517 nm with UV-VIS Spectrophotometer (Systronic). Ascorbic acid was used as a positive control. 1 mL of methanol with 3 mL of working DPPH solution serves as control. The ability to scavenge DPPH radical activity was calculated by-

$$\% \text{ inhibition} = Ac - As / Ac \times 100$$

Where, Ac is the absorbance of the control and As is the absorbance of samples or standard. Lower values represent higher antioxidant ability.

4.6.6 ABTS radical cation scavenging activity

ABTS (2, 2 azobis, 3-ethyl benzothiozoline-6- sulphonic acid) radical cation scavenging activity was determined by Shah *et al*. 2013 methodology. Briefly, ABTS (7 mM) solution was allowed to react with potassium persulfate (2.45 mM) overnight in dark for generation of dark colored ABTS radicals. For the analysis, the ABTS solution was diluted with 50% ethanol to obtain initial absorbance of 0.7 ± 0.05 at 745 nm. For the determination 100 µL sample of different dilution was added to 1 mL of ABTS solution. The decrease in absorbance was measured at 745 nm after 1 minute and 6 minutes of mixing. The difference was calculated and compared with control. The BHT was taken as positive control. ABTS radical % inhibition was calculated by the formula:

$$\% \text{ inhibition} = Ac - As / Ac \times 100$$

4.6.7 Iron chelating capacity

For the evaluation of ferrous ion chelating potential of the extracts were done by the method adopted by Sasikumar and Kalaisezhiyen 2014. In a reaction mixture, 1 mL of various concentration of extracts (200-1000 µg/mL) and 2 mM $FeCl_2$ (0.05 mL) was taken. Control contains all the reagents except for the sample. The reaction was initiated after the addition of 5 mM Ferrozine (0.2 mL). Shaken vigorously and left in the room temperature for

10 minutes. The absorbance of both reaction mixture and control was taken at 562 nm. Lower the absorbance higher will be ferrous ion chelating potential. EDTA was taken as standard.

% inhibition = Ac – As/ Ac × 100

4.6.8 H_2O_2 radical scavenging assay

H_2O_2 radical scavenging activity of the extracts was measured by the method adopted by Narzary *et al.* 2016. 20 mM H_2O_2 solution was prepared by mixing 226 µL from 30% H_2O_2 in 99.8 mL of 0.1 mM phosphate buffer saline having pH 7.4. In a different sample/standard concentration (2, 4, 6, 8 and 10 µL/mL), 2 mL of 20 mM H_2O_2 solution was added and incubated in dark for 10 minutes. The absorbance of the scavenging activity was taken at 230 nm using UV-VIS double beam spectrophotometer (Systronics). Phosphate buffer saline was used as blank and BHA as positive control. The amount of H_2O_2 inhibited by the extract was calculated from the equation:

% inhibition = Ac – As/ Ac × 100

4.6.9 Ferric reducing antioxidant assay

Ferric reducing antioxidant power of was determination by the method of Song *et al.* 2010. The FRAP reagent is freshly prepared by mixing 300 mmol/L sodium acetate buffer (pH 3.6), 10 mmol/L TPTZ solution in 40 mmol/L HCl and 20 mmol/L iron (III) chloride solution in a ratio of 10:1:1 to generate FRAP reaction solution, which should be warmed to 37 °C in a water bath before use. After that, 100 µL of the diluted sample was mixed with 3 mL of the FRAP reaction solution. It was then incubated in dark for 4 minutes at room temperature and the absorbance of the reaction mixture and standard was recorded at 593 nm. The standard curve was constructed using $FeSO_4.7H_2O$ solution, and the results were expressed as µmol Fe (II)/g dry extract. All experiments were performed in triplicate.

4.7 Gas Chromatography coupled with Mass Spectrometry analysis

The analysis of hydro-alcoholic and acetonic extracts RoMi were performed at Sophisticated Instrumentation Facility, VIT, Vellore, using Perkin Elmer gas chromatography (Clarus 680) coupled with mass spectrometry (Clarus 600 EI) employed with fused silica column and packed with capillary column Elite- 5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df). The components were separated using Helium as carrier gas at a constant flow of 1 mL/minute. The injector temperature was set at 260 °C during the chromatographic run. 1 µL of extract sample was injected into the instrument. The oven temperature was programmed at: 60 °C (2 minutes); then increased to

300 °C for 6 minutes (at the rate of 10 °C per minute). The mass detector conditions were: transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. 40 to 600 Da fragment size were scanned. The spectrums of the components were compared with database of spectrum of known components stored in GC-MS NIST (2008) library.

4.8 *In-vivo* animal model experiment

4.8.1 Experimental animals

Thirty male wistar albino rats weighing 210-250 g were used for the experiment. The animals were housed in standard cages (46 × 24 × 20 cm) (Tarson, India), with six animals per cage in a standard environmental condition (25 ± 3 ºC, 60 ± 1% relative humidity, and light control room with a 12-h dark-light cycle). They were allowed to acclimatize for a period of 1-2 weeks and were fed with standard pellet feed (Lipton India Ltd., Bangalore) and water *ad libitum* (Vuda *et al.* 2012; Maheshwari *et al.* 2011). All the experiments were performed in strict accordance with standard guidelines accepted internationally, and the ethical approval was obtained from Maharani Lakshmi Ammanni College Ethical Committee (1368/ac/10/CPCSEA), Bangalore, for the care and use of laboratory animals.

4.8.2 Acute toxicity study

OECD guidelines (test 423: Acute oral toxicity-Acute toxic class method; 2002) were followed to study the acute toxicity of the RoMi ethanolic extract (OECD Library, 2002). All the animals (12 male Wistar albino rats) were kept on fasting overnight prior to the experiment. The RoMi-EE was administered orally in an increasing dose of 250, 500, 1000 and 2000 mg/kg body weight (BW). The followed concentrations are determined as per OECD guideline. After feeding of various doses of formulation, all the rats were carefully observed for the development of clinical or toxicological symptoms at 30 minutes and then at 2, 4, 8, 24 and 48 hours. Finally, the rats were observed for the development of clinical or toxicological symptoms till 14 days.

4.8.3 Experimental design

To evaluate the hepatoprotective effect of the RoMi-EE, the rats were divided into five groups containing six rats each. 3 mL/kg of CCl_4 was administered orally once on the first day. RoMi-EE was dissolved in milipore water and two different concentrations 100 mg/kg & 200 mg/kg BW (Khan *et al.* 2012) of RoMi-EE were administered orally everyday

in morning with the help of 16g gavage needle while the control group was maintained on distilled water. Body weights were monitored throughout the experiment.

Group 1 was administered distilled water + dietary supplement served as positive control.

Group 2 was administered CCl_4 (on the first day) + dietary supplement served as negative control.

Group 3 was administered CCl_4 (on the first day) + 100 mg/kg (BW) of RoMi-EE orally + dietary supplement for 7 days.

Group 4 was administered CCl_4 (on the first day) + 200 mg/kg (BW) of RoMi-EE orally + dietary supplement for 7 days.

Group 5 was administered CCl_4 (on the first day) + 25 mg/kg (BW) of silymarin orally + dietary supplement for 7 days.

Twenty-four hours after the last treatment, all rats were weighed and then euthanized through intraperitoneal injection using Xylazine (30 mg/kg BW) and Ketamine (300 mg/kg BW) ratio (1:10) as proposed by Committee for the Purpose of Control and Supervision of Experiments in Animal (CPCSEA). The blood was collected by retro-orbital puncture in an EDTA-containing tube from all the experimental rats. The liver and kidneys of each rat were removed, weighed and perfused in the ice-cold phosphate buffer of pH 7.0. A portion of liver was preserved in 10% formaldehyde solution for hisptopathological evaluation and from the remaining portion, the homogenate was prepared by centrifuging at 1000 × g for 10 minutes at 4 ºC. The supernatant was collected after centrifugation and was used for *in-vivo* enzymatic assays (Khan *et al.* 2012).

4.8.4 Assessment of serum biochemical assays

The activities of serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase, total bilirubin, tri-glycerides, HDL-cholesterol, LDL-cholesterol and total cholesterol were estimated using standard AMP Diagnostic Kits (Graz, Austria). Liver damage was assessed by estimating the enzymatic activities of serum AST, ALT, and ALP, as well as serum TC, TG, and albumin level, using the corresponding commercial kits, respectively. Silymarin was procured from indigenous pharmacy store, Bangalore, India. The kidney damage was assessed by creatinine level. The results were expressed according to the manufacturer recommendation.

4.8.5 Assessment of levels of antioxidant enzymes

The liver tissues were homogenized (1%) in 10 mL volumes of 100 mM KH_2PO_4 buffer containing 1 mM ethylenediamine tetra-acetic acid (EDTA; pH 7.4) and centrifuged at

12,000 × g for 30 minutes at 4 ºC (Khan *et al*. 2012). The supernatant was collected and used for the assessment of antioxidant enzymes. Protein concentrations in the supernatants of liver tissue homogenates were determined using crystalline bovine serum albumin (BSA) as standard. All chemicals used in enzymatic analysis were purchased from Sigma-Aldrich.

4.8.5.1 Superoxide dismutase (SOD) assay

SOD assay was measured by the method of Misra and Fredovich 1972. A volume of 880 µL of 0.05 M carbonate buffer (pH 10.2) containing 0.1 mmol EDTA and 20 µL of 30 mmol epinephrine in 0.05% acetic acid were added to the tissue extract of 100 µL and changes in activity were measured at 480 nm for 4 minutes. Activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equivalent to one unit and is expressed in terms of units/mg protein.

4.8.5.2 Catalase assay

Catalase activity was determined using the method of Bears and Sizer 1952. The reaction solution of catalase activity contained 1 mL of 59 mmol/L H_2O_2 (dissolved in 50 mmol phosphate buffer, pH 7.0) and 0.1 mL of hepatic supernatant were added to 1.9 mL deionized water. Changes in the absorbance of the reaction solution at 240 nm were determined every 1 minute up to 3 minutes (using Kinetics spectrometer). One unit of catalase activity was defined as an absorbance change of 0.01 as units/minute.

4.8.5.3 Gluthathione peroxidase

GPx activity was measured at 37 °C by the method of Middha *et al.* 2011. The reaction mixture consisted of 500 µL of phosphate buffer, 100 µL of 0.01 M reduced glutathione (GSH), 100 µL of 1.5 mM NADPH and 100 µL of GR (0.24 U). 100 µL of tissue extract was added to the reaction mixture and incubated 37 ºC for 10 minutes. 50 µL of 12 mM t-butyl hydro-peroxide was added to 450 µL of tissue reaction mixture and measured at 340 nm for 180 seconds in a biospectrometer (Eppendorf, Model BL 192). A molar absorptivity of 6.22 × 103 M/cm was used to determine enzyme activity. One unit of activity is equal to µM NADPH oxidized/minute/mg protein.

4.8.5.4 Gluthathione assay

GSH was estimated using the method of Jollow *et al.* 1974. A total of 1.0 mL of homogenate was precipitated with 1.0 mL of 4% sulfosalicylic acid. Samples were kept at 4 °C for 1 hour and then centrifuged at 1200 × g for 20 minutes at 4° C. The total volume of 3.0 mL assay mixture contained 0.1 mL of a filtered aliquot, 2.7 mL of phosphate buffer (0.1

mol; pH 7.4) and 0.2 mL of DTNB (100 mmol). The yellow color that developed was read immediately at 412 nm on a SmartSpec™ Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA). It was expressed as µmol GSH/g tissue.

4.8.5.5 Lipid-peroxidation

Malondialdehyde (MDA), a marker of LPx was assessed by the method of Okhawa *et al.* 1979, using 1, 1, 3, 3- tetramethoxy propane as standard. Briefly, 8.1% SDS was added to the tissue homogenate and incubated for 10 minutes at room temperature, followed by boiling with 20% acetic acid and 0.6% thiobarbituric acid (TBA) for 1 hour in a water bath. After cooling, butanol: pyridine solution (15: 1 v/v) was added and the mixture was centrifuged at 600 × g for 5 minutes. Absorbance of the upper colored layer was measured at 532 nm and the concentration of MDA was expressed in terms of nM /mg protein.

4.8.5.6 Total protein assay

The total protein assay was done by the method of Lowry *et al.* 1975, taking BSA as standard. In brief, to the supernatant of 100 µL, 500 µL of alkaline copper sulphate was added and allowed to incubate for 10 minutes at room temperature. After incubation, 60 µL of diluted Folin Catecholamine reagent (1:1) was added and incubated for another 30 minutes at room temperature. The absorbance was measured at 660 nm against the reagent blank.

4.9 Histopathological study

A small portion of the liver and kidney was excised and washed with normal saline and processed separately for histopathological observation. Initially, the liver tissues were fixed in 10% buffered neutral formalin for at least 48 h, dehydrated in gradual ethanol (50-100%), cleared in xylene, and embedded in paraffin. The 4 µm sections were prepared using microtome. Then liver sections were dewaxed in xylene, rehydrated in a series of different grades of alcohol and then washed with distilled water for 5 minutes. Sections were prepared and stained with hematoxylin (40 s) & eosin (20 s) dye (Maheshwari *et al.* 2011). The sections were examined in Olympus microscope at 40× magnification (Khan *et al.* 2012) for any histopathological changes, including cell necrosis, fatty changes and vacuolation.

4.10 Molecular docking with hepatoprotective biotarget.

4.10.1 Ligand selection

The molecules that were detected in GC-MS analysis from the root of *Morus indica* are selected for the docking study with the NFκB (Nuclear factor kappa B) and COX-2

(Cyclooxyginase-2) proteins which are responsible for the hepatotoxicity and inflammation of the liver. For the docking analysis, molecules having 160-500 Da were selected for the ligand docking.

4.10.2 Selection of target proteins

The proteins were selected from the literature data available on the mechanism of hepatoprotective and anti-inflammatory effect (Glushenko *et al.* 2015; Lamie *et al.* 2015). The proteins (1NFK & 3LN1) having resolution of 2.3 & 2.4 Å was downloaded from https://www.rcsb.org. Both the proteins 3LN1 and 1NFK are from the organism *Mus musculus*. In the 3LN1 protein, Celecoxib is bound to the COX-2 active site and 1NFK is the nuclear factor kappa-B P50 homodimer. The X-ray crystal structure of protein 1NFK is complexes with DNA molecule.

4.10.3 Ligand molecules

The compounds that were identified by the GC-MS analysis of ethanolic root extracts of *Morus indica* were used as ligand molecules for the docking analysis and were downloaded from https://pubchem.ncbi.nlm.nih.gov in SDF format.

4.10.4 Ligand preparation

The ligands were prepared using "LigPrep" software for ligand preparation from Maestro Schrödinger Suite (Maestro, 2015). 3D conformers were generated using tautomers and ionising state at pH 7.0 followed by the optimization process using the force field OPLS2003e. The Relative Mean Square Deviation 0.30 Å (RMSD) was used to select the best conformation. The charges of the molecule were obtained using the Macro Model software module default setting from Schrödinger package. The stereochemistry was performed with maximum of 32 stereoisomers (default) per ligand.

4.10.5 Preparation of receptor

The crystallized proteins (1NFK & 3LN1) were processed in the Maestro (Schrödinger) using the protein preparation wizard facility. The following preparations were completed stepwise (Maestro, 2015):

1. Protein structures were preprocessed using the OPLS3e force field.
2. The protein missing side chains were added using prime.
3. Geometry refinement and optimization of the protein were completed. In the process the hydrogen atoms were added to heavy atoms of the protein.

4. The minimization of the protein were carried out using optimized potentials for liquid simulations (OPLS) 2005 imposing an RMDS (root mean square deviation) of 0.3 Å for the heavy atom coverage.
5. If the standard ligand in the active site is not present, then active binding site of the protein were generated using Site Map module with reports up to 5 different binding site based on their site score. The best site score were selected for the study.
6. Finally the protein molecules of best site score were grid generated (with grid box of 40 × 40 × 40) with a space of 0.375 Å and grid centre of x, y, z coordinates 9.740, 64. 640 and 15.986 respectively using receptor grid generation from Glide, Maestro.

4.11 Molecular docking

The docking study was carried out using Glide, Ligand Docking of Maestro Schrödinger software suite 2018. The prepared LigPrep molecules and the grid generated proteins were incorporated into the workspace. The 3D images of the ligands (pre-processed), proteins (minimized) and all the ligand-protein binding interactions were saved in JPEG format. The docking study was conducted at Intel (R) Core (TM) i5, CPU (3.2 GHz) having Windows 10 operating system.

4.12 ΔG Binding affinity

Binding affinity of molecules with that of proteins was performed using Molecular Mechanics/Generalized Born Surface Area (MM-GBSA) module of Maestro Schrödinger.

4.13 Adsorption distribution metabolism & excretion (ADME) property of ligands

Physically significant descriptors and pharmaceutically relevant properties of all the lead compounds of both the fractions were analysed using 'Molsoft' prediction tool (Gunalan *et al.* 2014; Tamilvanan & Hopper 2013). Molecular weight, log P Octanol/water partition coefficient, H-bond donors, H-bond acceptors, Mol Log S and their positions according to Lipinski's rule of five (Lipinski *et al.* 1997; Lipinski 2000) were obtained using the tool. It also evaluates acceptability of analogues according to Lipinski's rule of five (Lipinski *et al.* 1997), which are essential for rational drug design (Tamilvanan & Hopper 2013).

4.14 Statistical analysis

Results of all the experiments were presented as mean ± SD of triplicate experiment (n= 3) and ± SD (n= 6) for animal model experiments. Relative significant differences among the means were determined by one-way ANOVA test ($p \leq 0.05$) and Tukey's multiple comparison tests using Origin pro 8.5 software.

CHAPTER- V

5. RESULTS

5.1 Hepatoprotective medicinal plants

In the survey of hepatoprotective medicinal plants utilized traditionally by local tribe, a total of 40 plants (**Table 1** & ***Appendix-G***) from 26 families and 40 different genus have been identified (having authentication number- BSI/ERC/2014/Plant authentication/538; BSI/ERC/ Tech./ Plant Iden./ 2015/68; BSI/ERC/ Tech./ Plant Iden./ 2015/513; BSI/ERC/ Tech./ Plant Iden./ 2017/309) and documented. From the survey (**Figure 1**) it was revealed that the tribe uses 29 types of leaves, 10 types of roots, 3 types of whole shoot, 2 types of flowers and fruit, one type of bark and seed respectively. For the process of making herbal medicine, formulations may depend on traditional healers (Ojha), who may use single or combination of different plants or plant parts for various liver disorders. From the **Table 1,** it was evident that the traditional healers mostly prefer single plant formulation, whereas only 3 different formulations of plant combinations were recorded.

Name of places under Kokrajhar district area with GPS location where the plant samples have been collected:

1. Bashbari:	Lat: 26˚33’42.6”N,	Lon: 90˚16’17.2”E
2. Karigaon:	Lat: 26˚33’7.71”N,	Lon: 90˚20’34.36”E
3. Tinali:	Lat: 26˚29’11.92”N,	Lon: 90˚18’32.28”E
4. Khagrabari:	Lat: 26˚28’3.66”N,	Lon: 90˚13’27.13”E
5. Khagrabari(Part1):	Lat: 26˚28’3.65”N,	Lon: 90˚13’27.14”E
6. Salbari:	Lat: 26˚23’22.07”N,	Lon: 90˚12’46.54”E
7. Sukhanjhara:	Lat: 26˚23’26.18”N,	Lon: 90˚12’18.61”E
8. Kokrajhar Main Bazar:	Lat: 26˚23’55.46”N,	Lon: 90˚16’3.06”E
9. Naigaon:	Lat: 26˚21’57.80”N,	Lon: 90˚22’28.32”E
10. Khargaon:	Lat: 26˚24’46.66”N,	Lon: 90˚17’8.70”E
11. Bowbazar:	Lat: 26˚24’50.00”N,	Lon: 90˚16’34.12”E
12. Purana Titaguri:	Lat: 26˚26’43.44”N,	Lon: 90˚17’16.07”E
13. Choto gendrabil:	Lat: 26˚23’0.58”N,	Lon: 90˚17’5.33”E
14. Debargaon:	Lat: 26˚28’11.62”N,	Lon: 90˚17’42.92”E
15. Tipkai Doloagaon:	Lat: 26˚17’12.59”N,	Lon: 90˚1’56.07”E
16. Sundaari:	Lat: 26˚31’26.79”N,	Lon: 90˚27’51.80”E

Table 1: The medicinal plants with vernacular name, family name, scientific name, parts used and formulation for medicines are described below:

Family Name	Scientific name	Vernacular name	Parts Used	Mode of Use
1.Moraceae	*Morus indica* L.	Gonger thaisib	Leaves & Roots	Small pieces of roots are made hollow cylinder by removing the mid portion and are put around the neck with white/red thread. Leaves are used in bathing with mixture of other medicinal plant leaves (Herbal formulation 3) after boiling. Roots mixture with other plant roots are also used (Herbal formulation 2).
2.Moraceae	*Ficus religiosa* L.	Fakhri fifang	Bark	Bark is cut 1 inch deep in V shape & dipped in water along with three tulsi leaves, $2^{1/2}$ black pepper & kept overnight. Extract along with raw milk (kept overnight) are mixed 1:1 ratio & drink in the morning.
3.Moraceae	*Artocarpus heterophyllus* Lam.	Khanthal fifang	Leaves	Leaves are boiled in water with mixture of other medicinal plant leaves (Herbal formulation 3) and used for bathing.
4.Rutaceae	*Glycosmis pentaphyla* (Retz.)DC	Amai fifang	Leaves	1 cup water extract is mixed with rock sugar & allowed to drink 2-3 times a day for 3 days.
5.Rutaceae	*Murraya koenigii* (L.) Spreng.	Nwrshing	Leaves	Leaves are boiled in combination with other plant leaves and are used in bath.
6.Rutaceae	*Citrus medica* L.	Nareng lebu	Leaves	Leaves are used in jaundice. Mixed with leaves of other plants & boiled. Boiled water is used for bathing.
7. Rubiaceae	*Paederia foetida* L.	Khifi bendwng	Leaves	Leaves are boiled in water with mixture of other medicinal plant leaves (Herbal formulation 3) and are used in bathing. It is also eaten as vegetable.
8. Rubiaceae	*Oldenlandia diffusa* Willd. (Roxb)	Dausri athing	Leaves	Leaves are boiled in water with mixture of other medicinal plant leaves (Herbal formulation 3) and the patient is advised to take bath.
9. Apiaceae	*Hydrocotyle sibthorpioides* Lam.	Mani-muni fisa	Leaves	Boiled in water with mixture of other medicinal plant leaves (Herbal formulation 3) and used in bathing. Also eaten fresh in early morning.
10. Apiaceae	*Centella asiatica* L.	Mani-muni gidir	Leaves	Leaves are boiled with mixture of other medicinal plant leaves (Herbal formulation 3) and are used in bathing. It can be also eaten fresh early morning.
11. Acanthaceae	*Justicia*	Barsikha gufur	Leaves, roots	Root powders are mixed with other plants root (Herbal formulation 1) &

	adhatoda L.		and flowers	rice grain powder and were advised to eat 2-3 times a day. Leaves are boiled with mixture of other medicinal plant leaves (Herbal formulation 3) and are used in bathing. Flowers are edible and are good for jaundice.
12. Acanthaceae	*Phlogacanthus thyrsiflorus* Nees.	Barsikha gwja	Leaves, roots and flowers	Leaves are used for bathing after boiling with mixture of other medicinal plant leaves (Herbal formulation 3). Root powders are mixed with rice grain & other plant roots (Herbal formulation 1) and are eaten 2-3 times/day. Flowers are edible as vegetable.
13. Solanaceae	*Solanum indicum* L.	Khunthai nara	Roots.	Root powder along with other plant roots (Herbal formulation 1) & rice grain powder is mixed and advised to take 2-3 times per day.
14. Solanaceae	*Physalis minima* L.	Ganga thofa	Roots.	Root powder along with other plant roots (Herbal formulation 2) are grounded together, placed inside the cocoon of Eri worm and tied in red thread and are put in neck for one complete week.
15. Menispermaceae	*Stephania japonica* (Thunb.) Miers	Phanel khuga	Whole shoot	Leaves are separated and only shoots are allowed to put around neck before sleep at night and must be thrown in next morning. It can be also placed bellow the pillow at night instead of wearing in neck. The leaves can be boiled with mixture of other medicinal plant leaves (Herbal formulation 3) and also be used in bathing.
16. Cuscutaceae	*Cuscuta reflexa* Roxb.	Gwmw bendwng	Whole plant	Whole plant is boiled with mixture of other medicinal plant leaves (Herbal formulation 3) & used for bathing in specific day like Tuesday and Saturday only.
17. Dilleniaceae	*Dillenia indica* L.	Thaigir fifang	Leaves & Fruits	Leaves are boiled in combination with the leaves of other plants (Herbal formulation 3) and are advised to take bath before sunrise and after sunset. Fruits are boiled & filtered fruit water is taken fresh.
18. Verbenaceae	*Clerodendrum cordatum* (D.Don)	Lwkhwna	Leaves & roots	The leaves are boiled with mixture of other medicinal plant leaves (Herbal formulation 3) & used in bathing. In other formulation, dried roots are used along with other plant roots (Herbal formulation 1 & 2).
19. Costaceae	*Costus*	Buri thokon	Roots	Rhizome juice is used as medicine for jaundice. The juice is allowed to

	speciosus Koen ex. Retz			drink in empty stomach early in the morning. Its young shoots are also eaten as vegetable by local tribe.
20. Plumbaginaceae	*Plumbago zeylanica* L.	Agar sitha	Roots	Roots are grounded in water and a white thread is mixed with the powder and advised to put in right hand. The remaining grounded powder is applied in forehead for 3 hours. Its roots are also used with other plants root (Herbal formulation 2).
21.Hypericaceae	*Hypericum japonicum* Thunb.	Sona puli	Leaves	Boiled leaves along with other plant leaves (Herbal formulation 3) are used for bathing.
22. Carryophyllaceae	*Stellaria media* L.	Na bikhi	Leaves	Leaves are used in taking bath after boiling with leaves of other plants (Herbal formulation 3).
23. Lamiaceae	*Leucas indica* (L.) R.Br ex. Vatke	Kangsinsha	Leaves	Leaves are boiled in water with mixture of other medicinal plant leaves (Herbal formulation 3) & are used for bathing. It is also used as vegetable.
24. Myrtaceae	*Psidium guajava* L.	Sumfram	Leaves	Leaves are boiled in combination with other plant leaves (Herbal formulation 3) and are allowed to take bath. Fresh young leaves are also eaten to cure stomach ailment.
25. Asteraceae	*Artemisia vulgaris* L.	Na deona	Leaves	Leaves are boiled in water in combination with leaves of other medicinal plants (Herbal formulation 3) & used for bathing.
26. Lamiaceae	*Pogostemon plectranoides* Desf.	Swimakhitangth-ari	Leaves	Leaves are boiled in water with the mixture of other plant leaves (Herbal formulation 3) and are used for taking bath.
27. Scrophulariaceae	*Scoparia dulcis* L.	Sini fifang	Leaves	Leaves of the plant along with other plant leaves (Herbal formulation 3) are boiled together and the patient is advised to take bath.
28. Saururaceae	*Houttuynia cordata* Thunb.	Maisundari	Leaves	Leaves are boiled in water with the mixture of other plant leaves (Herbal formulation 3). The boiled water is used for bathing.
29. Meliaceae	*Azadirachta indica* A. Juss.	Neem fifang	Leaves	Leaves are boiled in water along with the leaves of other plants (Herbal formulation 3) & are used for bathing.
30. Oxalidaceae	*Averrhoa carambola* L.	Khwrdwi / Khambrenga	Fruits, leaves & roots	Ripe fruits are eaten fresh & also taken as curry. Leaves are boiled with other plants leave (Herbal formulation 3) & used in bathing. Root water extract is taken twice after meal.

31. Anacardiaceae	*Mangifera indica* L.	Thaijwo fifang	Leaves & seeds	Leaves are mixed with other medicinal plant leaves (Herbal formulation 3) & boiled in water and advised to take bath.
32. Thelypteridaceae	*Amphineuron opulentum* (Kaulf.)	Bis-dingkia	Roots.	Dried root powder along with roots of other plants (herbal formulation 1) are mixed with rice grain powder and are advised to eat 2-3 times/day.
33. Clusiaceae	*Garcinia cowa* Roxb.	Thaikha	Leaves	Leaves are boiled in combination with other plant leaves (Herbal formulation 3) and are advised to take bath with it.
34. Verbenaceae	*Premna herbacea* Roxb.	Kheradapkhini	Leaves	Leaves are boiled in water along with mixtures of other plant leaves (Herbal formulation 3) and are allowed to take bath. The leaves are also taken as vegetable.
35. Fabaceae	*Cajanus cajan* L. Millsp.	Khokling	Leaves	Fresh leaves are ground and allowed to drink.
36. Asteraceae	*Spilanthes paniculata* Wall. ex DC.	Usumwi	Leaves	Leaves are boiled in water along with mixtures of other plant leaves (Herbal formulation 3) and are allowed to take bath. The leaves are also taken as vegetable.
37. Acanthaceae	*Hygrophila phlomoides* Nees.	Rwda gangra	Root	Fresh roots are put on the hand.
38. Rubiaceae	*Morinda angustifolia* Roxb.	Asho	Leaves	Leaves are used but no proper formulation was obtained.
39. Molluginaceae	*Mollugo pentaphylla* L.	Rupa fuli	Whole shoot	Leaves are boiled in water along with mixtures of other plant leaves (Herbal formulation 3) and are allowed to take bath.
40. Acanthaceae	*Andrographis paniculata* (Burm.f.) Nees	Khalmegh	Whole shoot	Whole shoots are eaten fresh.

5.2 Some herbal formulation

1. Roots (dried powder) of *Phlogacanthus thyrsiflorus*, *Amphineuron opulentum*, *Justicia adhatoda*, *Clerodendrum cordatum* and *Solanum indicum* were mixed along with rice grain powder and were advised to take 2-3 times a day.
2. In some other formulation, the roots of *Morus indica, Plumbago zeylanica, Clerodendrum cordatum, Stephania japonica* and *Physalis minima,* are ground together. The ground powder are placed inside the cocoon of Eri worm and tied around the neck with the help of red thread for one week.
3. In another formulation the leaves of *Garcinia cowa*, *Justicia adhatoda*, *Hydrocotyle sibthorpioides, Centella asiatica, Averrhoa carambola, Morus indica, Artocarpus*

heterophyllus, Mangifera indica, Azadirachta indica, Stephania japonica, Scoparia dulcis, Psidium guajava, Murraya koenigii, Leucas indica, Stellaria media, Citrus medica, Houttuynia cordata, Oldenlandia diffusa, Hypericum japonicum, Pogostemon plectranoides, Artemisia vulgaris, Dillenia indica Clerodendrum cordatum, Cuscuta reflexa, Mollugo pentaphylla, Paederia foetida, Permna herbacea, Spilanthes paniculata and Phlogacanthus thyrsiflorus were washed and boiled together. After that, the patient is advised to take bath before sunrise or after sunset.

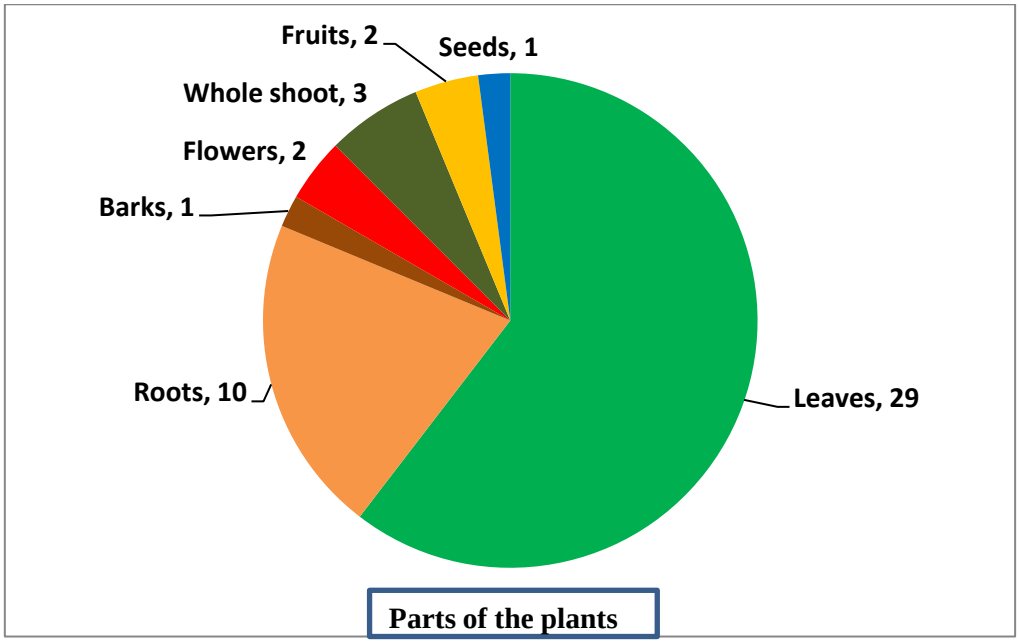


Fig 1: Hepatoprotective medicinal plant parts used.

5.3 Extract yield

The roots of *Morus indica* L., *Phlogacanthus thyrsiflorus* Nees. and *Averrhoa carambola* L., produced a total of 16.5%, 12% and 15.76% (w/w) yield from the ethanolic extracts and 11.78%, 9% and 10.2% (w/w) yield from that of acetone extract respectively.

5.4 Phytochemical screening

Qualitative screening showed positive for phenols, flavonoids, tannins, resins, terpenoids, glycosides and steroids in all the root extracts of *P. thyrsiflorus* (RoPt), *M. indica* (RoMi) and *A. carambola* (RoAc). Presence of alkaloids was seen in ethanolic extracts of RoMi, ethanolic and acetone extracts of RoPt. Cardiac glycosides was absent in both extracts of RoMi. Reducing sugar was detected only in acetone extract of RoMi. Except in the extracts of RoPt, presence of anthraquinone were seen in all RoMi & RoAc extracts. All the extracts showed positive for saponin except acetone extract of RoPt, **Table 2**.

Table 2: Qualitative tests

Phytochemical Tests	**RoPt**		**RoMi**		**RoAc**	
	70% EE	AE	70% EE	AE	70% EE	AE
Phenols	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Resins	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+
Alkaloids	+	+	+	—	—	—
Glycosides	+	+	+	+	+	+
Cardicac Glycosides	+	+	—	—	+	+
Reducing sugar	—	—	—	+	—	—
Anthraquinone	—	—	+	+	+	+
Saponins	+	—	+	+	+	+
Steroids	+	+	+	+	+	+

NB: (+) represents presence and (⁻) indicates absence of phytochemical compound.

5.5 Total phenolic and flavonoid contents

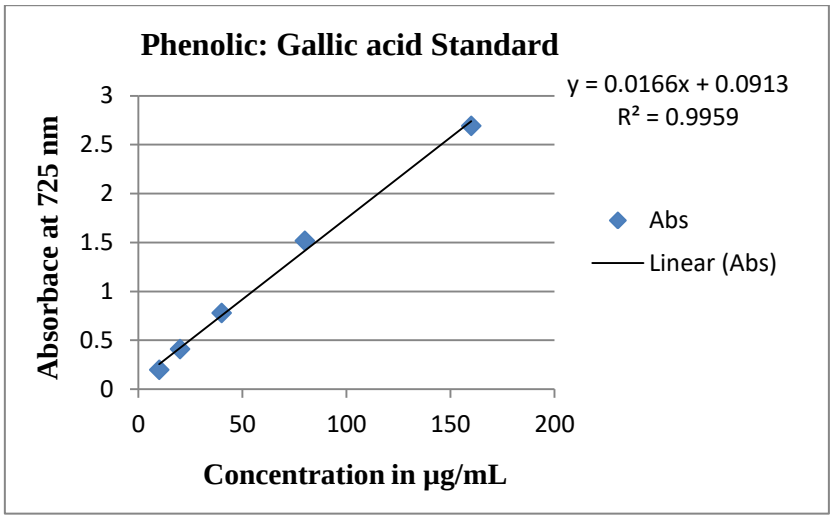


Fig 2: Total phenolic content: standard linear graph of gallic acid.

Result for the total phenolic content (extracts of RoMi, RoAc and RoPt) are presented in **Figure 3.** Phenolic content in the ethanolic and acetone extracts were determined from linear curve of standard gallic acid (y = 0.0166x + 0.0913; R^2 = 0.9959), **Figure 2**. Highest content of TPC were found in ethanolic extract of RoAc with 235.26 ± 11.91 mg GAE/g, followed by ethanolic extracts of RoMi with 214.71 ± 2.21 mg GAE/g of dried extract. The lowest content was found in acetone extract of RoPt with 84.21 ± 4.82 mg GAE/g of dried extract. Other results are RoAc-AE: 213.91 ± 11.18; RoMi-AE: 190.61 ± 2.88 and RoPt-EE: 101.26 ± 2.52 mg GAE/g of dried extract.

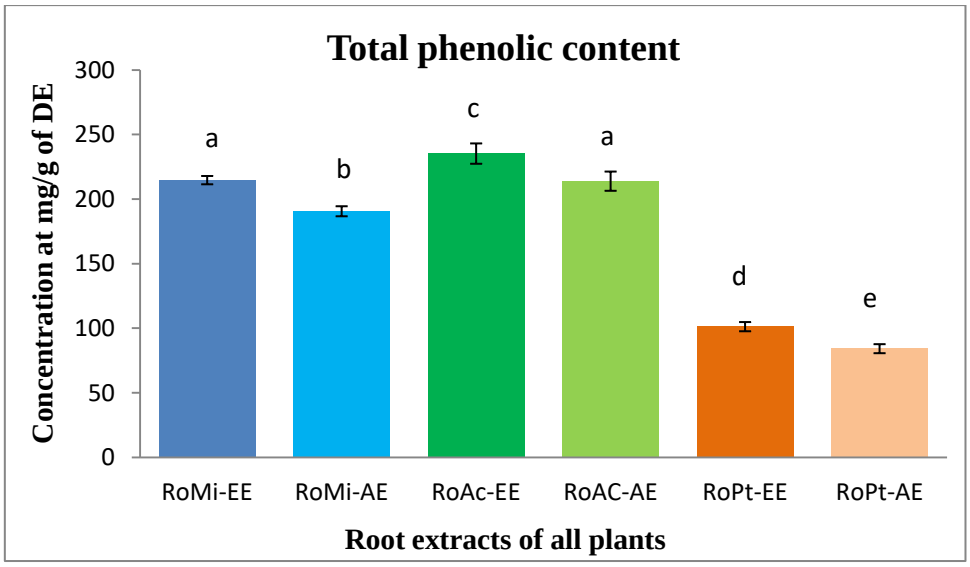


Fig 3: Showing the TPC in all extracts of RoMi, RoAc & RoPt. Results are mean value of ±SD (3n). Different letters indicate statistically significant (ANOVA-Tukey, $p<0.05$).

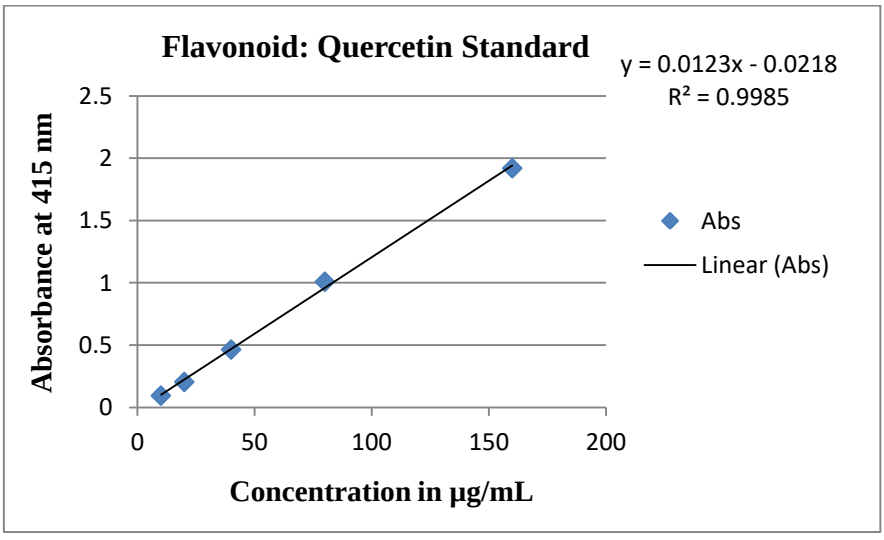


Fig 4: Total flavonoid content showing linear curve of Quercetin standard.

Flavonoid content in the ethanolic and acetone extracts were determined from linear curve of standard quercetin (y = 0.0123x - 0.0218; R^2 = 0.9985), **Figure 4**. Result for the total flavonoid contents are presented in **Figure 5**. Highest TFC was found in RoMi-EE with 123.39 ± 2.04 followed by RoMi-AE with 113.09 ± 7.25 and the least amount of flavonoid was observed in RoPt-AE with 68.22 ± 4.82 mg QE/g of dried extract respectively. Flavonoid contents of other extracts showed RoAc-EE: 101.96 ± 6.87; RoPt-EE: 99.92 ± 5.49 and RoAc-AE: 82.81 ± 5.94 mg QE/g of dried extract.

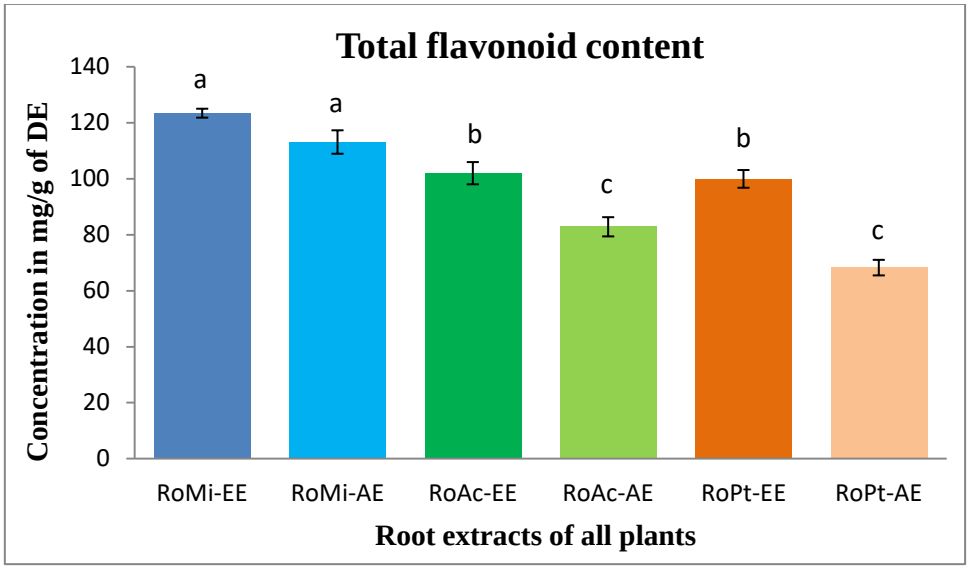


Fig 5: Showing TFC in all extracts of RoMi, RoAc & RoPt. Results are mean value of ±SD (3n). Different letters indicate statistically significant (ANOVA-Tukey, $p<0.05$).

5.6 Total reducing power assay

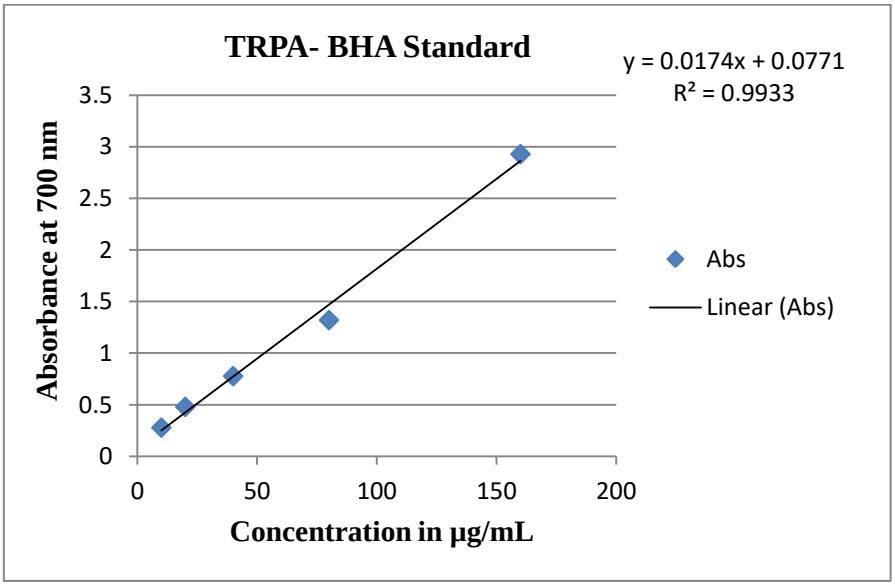


Fig 6: Total reducing power assay showing linear curve of BHA standard.

The reducing power activity of the extracts were determined from the standard linear curve of butylated hydroxyanisole (y= 0.0174x + 0.0771; R^2 = 0.9933), **Figure 6**. Reducing power assay of the extracts was carried out by taking three different concentrations (50, 100 and 200 µg/mL). RoAc-EE showed highest reducing power activity with the absorbance 0.907 ± 0.015 followed by RoMi-EE with 0.878 ± 0.035 and the lowest reducing power assay was found in RoPt-AE which showed 0.395 ± 0.015 absorbance value at the concentration of 200 µg/mL of dried extract. The standard BHA was found to be better reducing power activity than the extracts which showed absorbance of 2.928 at only 160 µg/mL of concentration. The results of reducing power assay were presented in the **Table 3**.

Table 3: Showing reducing power activity of all extracts of RoMi, RoAc and RoPt.

Concentration	RoMi-EE	RoMi-AE	RoAc-EE	RoAc-AE	RoPt-EE	RoPt-AE
50 µg/mL	0.324 ± 0.021 a	0.162 ± 0.016 b	0.355 ± 0.006 a	0.265 ± 0.008 c	0.175 ± 0.011 b	0.149 ± 0.009 b
100 µg/mL	0.541 ± 0.016 a	0.293 ± 0.023 b	0.605 ± 0.007 c	0.472 ± 0.014 d	0.268 ± 0.013 b	0.246 ± 0.012 b
200 µg/mL	0.878 ± 0.035 a	0.498 ± 0.03 b	0.907 ± 0.015 a	0.732 ± 0.014 c	0.421 ± 0.013 d	0.395 ± 0.015 d

Result represents mean ± SD value of triplicate experiment. Different letters indicate statistically significant in the same row (ANOVA-Tukey, $p<0.05$).

5.7 Total antioxidant property as per phosphomolybdate assay.

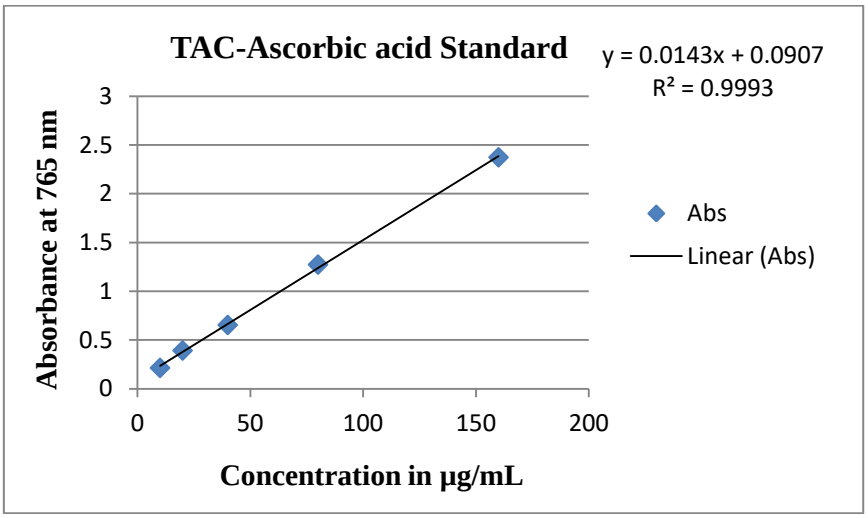


Fig 7: Total antioxidant property showing linear curve of ascorbic acid standard.

The total antioxidant property of root extracts were determined from the standard linear curve of ascorbic acid (y= 0.0143x + 0.0907; R^2 = 0.9993), **Figure 7**. In the present study, the highest antioxidant property was found in RoMi-EE having 584.98 ± 22.28 and is

followed by RoAc-EE with 512.87 ± 29.72 in respect to RoPt-AE which showed lowest value of only 189.94 ± 16.72 mg Ascorbic acid equivalent (AAE)/g of the dried extracts. The data obtained are presented in the **Figure 8**. The other plant extracts showed RoAc-AE: 478.57 ± 24.99; RoMi-AE: 287.3 ± 17.3 and RoPt-EE: 198.35 ± 18.25 mg AAE/g of dried extracts.

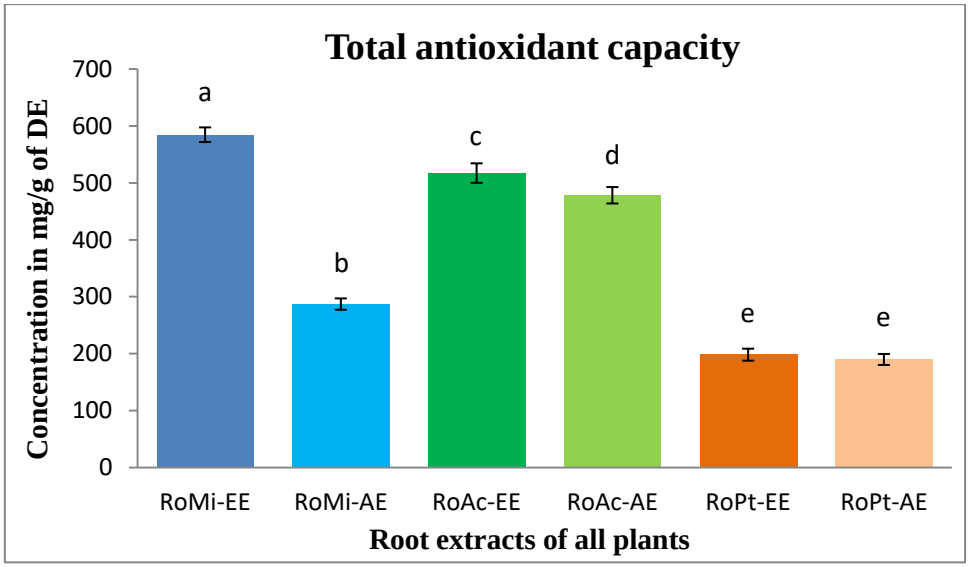


Fig 8: Showing TAC in all extracts of RoMi, RoAc & RoPt. Results are mean value of ±SD (3n). Different letters indicate statistically significant (ANOVA-Tukey, $p<0.05$).

5.8 DPPH radical scavenging activity

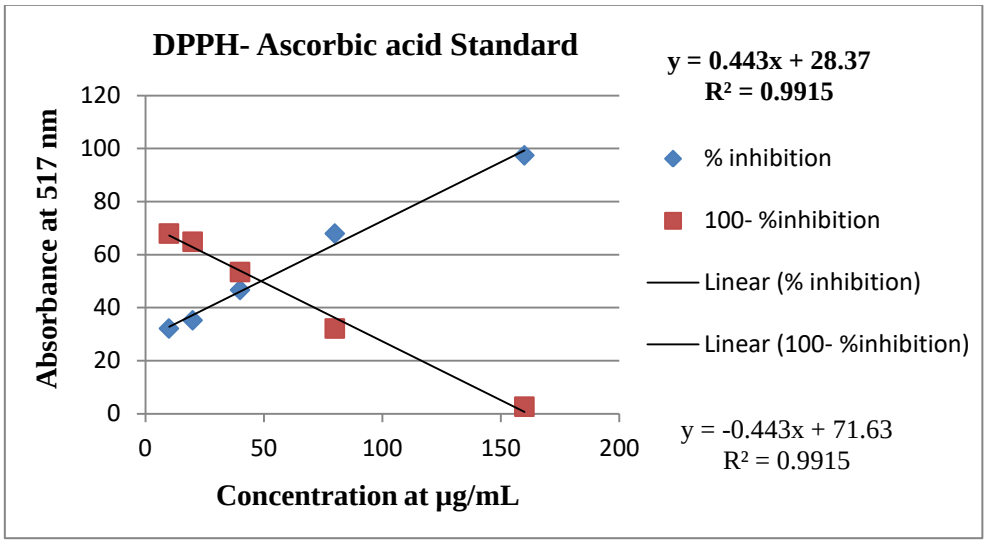


Fig 9: Showing DPPH free radical scavenging activity of ascorbic acid.

Increase in DPPH radical scavenging activity was observed notably with increased concentration of the extracts/standard. Among the extracts highest percent inhibition of 54.36 ± 2.15% was seen in the 160 µg/mL concentration of RoMi-EE (with IC_{50} value of 130.57 ±

12.46 µg/mL) and the RoPt-AE showed lowest inhibition of 30.05 ± 3.56% with IC_{50} value 302.55 ± 35.68 µg/mL. The IC_{50} values & increased in percent inhibition with increase in concentration of the extracts and ascorbic acid are furnished in the **Table 4** & **Figure 10**. Results obtained in this study suggest that DPPH scavenging activity of ascorbic acid is quite better than all extracts which showed 97.62% inhibition at 160 µg/mL concentration with 48.93 µg/mL IC_{50} value. Result of IC_{50} values of other plant extracts are RoAc-EE (138.66 ± 11.41), RoAc-AE (174.1 ± 21.18), RoMi-AE (233.92 ± 14.46) & RoPt-EE (265.87 ± 17.58).

Table 4: Presenting the DPPH radical scavenging activity by extracts/standard.

Samples	% inhibition at different concentration of sample/standard at µg/mL					
	10 µL	20 µL	40 µL	80 µL	160 µL	IC_{50}
AA	31.87	34.84	46.73	68.13	97.62	48.93
RoMi-EE	25.76 ±1.91	28.22 ±2.13	33.81 ± 2.1	42.77 ±2.86	54.36 ±2.15	130.57 ±12.46
RoMi-AE	16.09 ±0.92	17.95 ±1.13	22 ± 0.97	28.7 ± 1.1	38.41 ±1.25	233.92 ±14.46
RoAc-EE	25.01 ± 3.53	27.35 ±3.94	31.94 ±3.23	40.98 ±3.34	52.91 ±1.63	138.66 ±11.41
RoAc-AE	29.69 ± 3.22	31.03 ±3.05	34.36 ±2.32	39.52 ±2.79	47.8 ± 2.68	174.1 ± 21.18
RoPt-EE	14.35 ± 2.11	15.5 ± 1.98	17.68 ±1.69	25.13 ±1.91	34.88 ±1.89	265.87 ±17.58
RoPt-AE	9.35 ± 2.39	10.9 ± 2.58	15.42 ±2.18	20.89 ±3.15	30.05 ±3.56	302.55 ±35.68

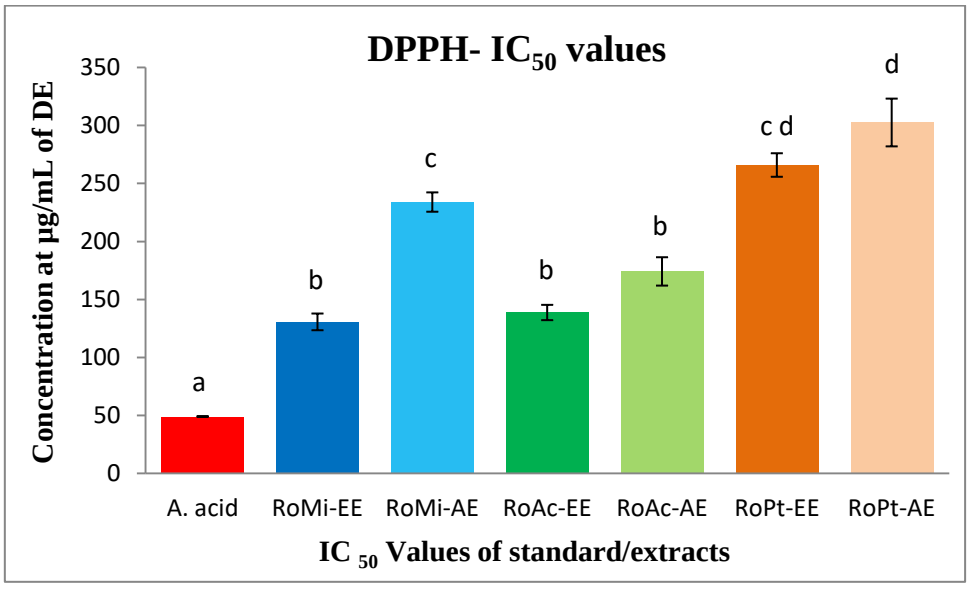


Fig 10: Showing DPPH IC_{50} values of all extracts of RoMi, RoAc, RoPt & Ascorbic acid. Results are mean value of ±SD (3n). Different letters indicate statistically significant (ANOVA-Tukey, $p<0.05$).

5.9 ABTS radical scavenging activity

The percent inhibition and IC_{50} values of ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] free radical scavenging activity of both the extracts and standard butylated hydroxytoluene (BHT) are plotted in **Table 5** and **Figure 12**.

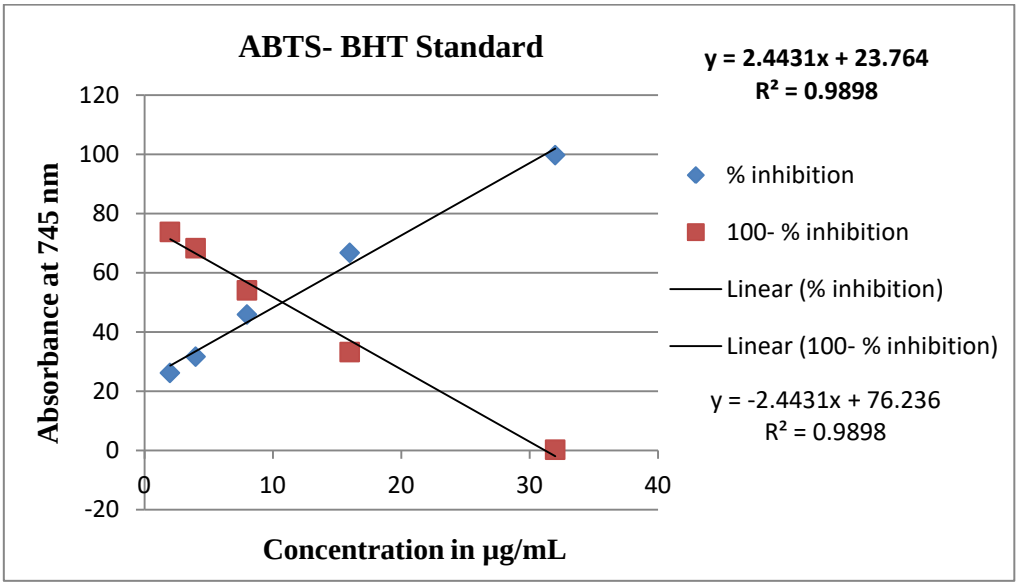


Fig 11: ABTS radical scavenging activity of standard BHT.

Table 5: Presenting the ABTS radical scavenging activity by extracts/standard.

Samples	% inhibition at different concentration of sample/standard at µg/mL					
	2 µL	4 µL	8 µL	16 µL	32 µL	IC_{50}
BHT	33.8	42.11	57.18	74.1	99.72	7.04
RoMi-EE	28.85 ±3	37.59 ±3.08	53.3 ±3.39	72.53 ±3.49	97.29 ±2.79	8.82 ± 1.42
RoMi-AE	15.43 ±4.61	25.6 ±4.18	42.08 ±2.65	66.68 ±5.88	95.65 ±3.81	12.75 ±1.61
RoAc-EE	30.87 ±3.08	40.41 ±2.35	54.96 ±2.91	74.03 ±3.45	97.2 ± 2.28	7.94 ± 1.33
RoAc-AE	29.77 ±3.49	38.1 ±2.88	52.7 ±3.28	71.99 ±5.04	96.34 ± 3.6	8.81 ± 1.66
RoPt-EE	18.78 ±1.14	26.81 ±1.28	35.35 ±1.39	49.06 ±1.36	72.82 ±3.39	17.89 ±1.18
RoPt-AE	19.15 ±1.62	26.34 ±1.69	31.74 ±1.55	41.46 ±2.53	58.97 ±2.19	24 ±1.61

The BHT showed highest scavenging activity at 32 µg/mL concentration, which inhibited 99.72% of ABTS free radicals and having the IC_{50} value of only 7.04 µg/mL. While in the same concentration the RoAc-EE showed high percent inhibition of 97.2 ± 2.28 showing best IC_{50} value of 7.94 ± 1.33 µg/mL and is followed by RoMi-EE with percent

inhibition of 97.29 ± 2.79 having IC_{50} value of 8.82 ± 1.42 µg/mL. The acetone extract of RoPt showed lowest percent inhibition of 58.97 ± 2.19 with IC_{50} value of 24 ± 1.61 µg/mL. Concentration depended inhibition were observed in all the extract as well as standard.

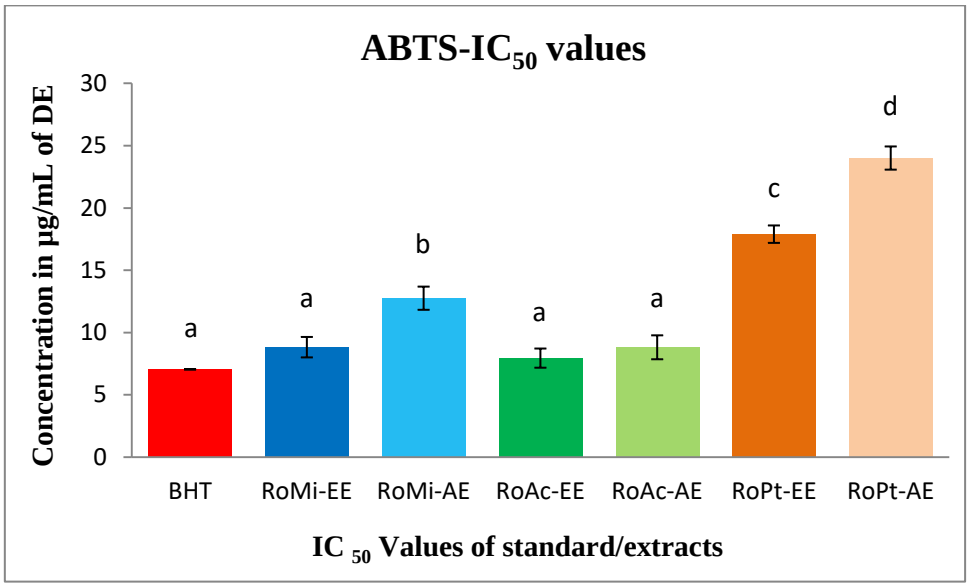


Fig 12: Showing ABTS IC_{50} value of all extracts of RoMi, RoAc, RoPt & BHT. Results are mean value of ±SD (3n). Different letters indicate statistically significant (ANOVA-Tukey, $p<0.05$).

5.10 Iron chelating capacity

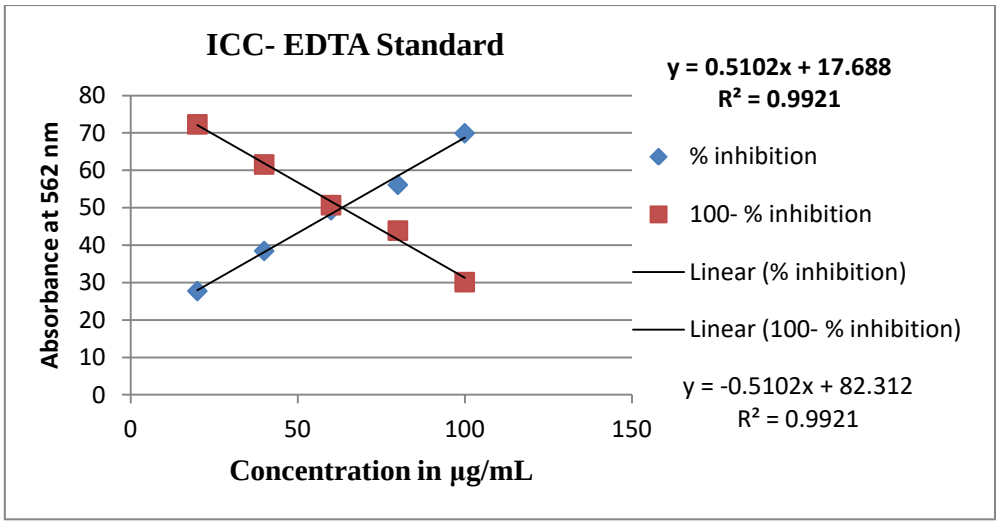


Fig 13: Showing iron chelating capacity of EDTA standard.

The result obtained in the study was presented in **Table 6** & **Figure 14**. The result indicates that highest chelating activity was observed in RoPt-EE at 1000 µg/mL having 72.06 ± 6.69% chelating capacity which also showed very good EC_{50} value with 535.16 ±

121.56 µg/mL followed by RoMi-EE with 50.06 ± 6.08 percent inhibition & having 1038.6 ± 143.97 µg/mL EC_{50} value, whereas lowest chelating activity was observed in RoMi-AE with 24.65 ± 1.91 percent inhibition and having EC_{50} value of 2006.9 ± 170.4 µg/mL of dried extract respectively. The chelating ability of extracts increases with increased concentration of extracts/EDTA. The EC_{50} value of EDTA was found to be lowest with 63.33 µg/mL, showing 69.9% inhibition at only 100 µg/mL concentration.

Table 6: Presenting the ICC radical scavenging activity by extracts/standard.

Samples/ Standard	% inhibition at different concentration of sample/standard at µg/mL					
	200 /20 µL	400 /40 µL	600 /60 µL	800 /80 µL	1000/100µL	EC_{50}
EDTA	27.72	38.44	49.32	56.12	69.9	63.33
RoMi-EE	21.31 ±4.51	27.02 ±4.29	31.27 ±3.91	41.77 ± 4.1	50.06 ± 6.08	1038.6 ±143.97
RoMi-AE	3.72 ±0.49	7.1 ±0.35	11.64 ±1.51	18.16 ±0.99	24.65 ± 1.91	2006.9 ± 170.4
RoAc-EE	3.68 ±0.97	8.8 ±1.07	16.84 ±1.39	24.02 ±2.76	32.78 ± 2.83	1500.43 ±130.1
RoAc-AE	2.85 ±1.04	7.2 ±0.97	13.39 ±1.93	21.18 ±2.93	25.72 ± 2.83	1817.3 ±183.26
RoPt-EE	34.58 ±3.14	44.56 ±5.66	54.11 ±6.97	61.65 ±8.32	72.06 ± 6.69	535.16 ±121.56
RoPt-AE	3.06 ±0.61	10.33 ±2.12	19.1 ± 2.25	25.83 ± 1.9	32.12 ± 2.15	1471.32 ±91.7

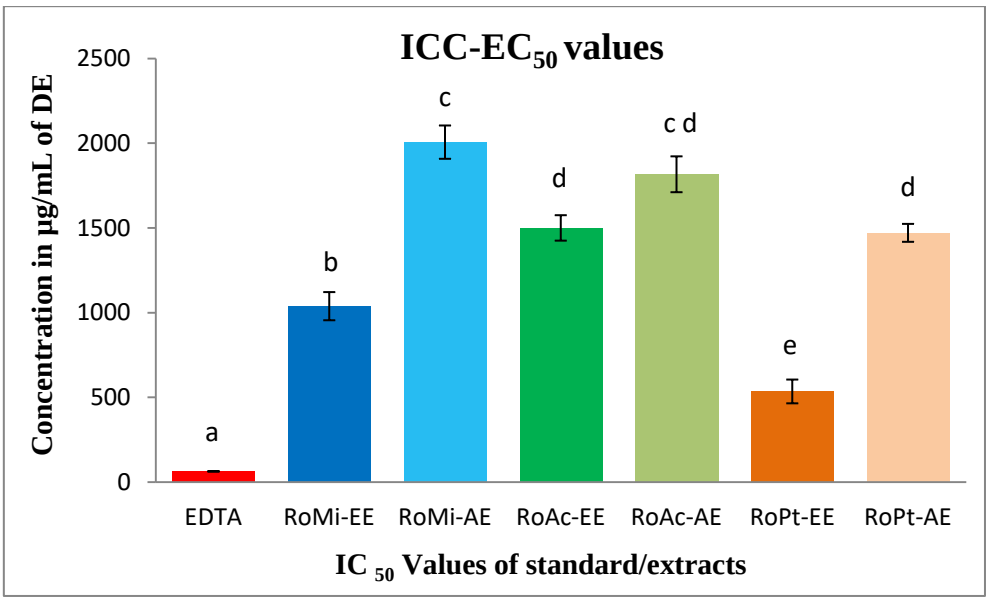


Fig 14: Showing ICC EC_{50} value of all extracts of RoMi, RoAc, RoPt & EDTA. Results are mean value of ±SD (3n). Different letters indicate statistically significant (ANOVA-Tukey, $p<0.05$).

5.11 H_2O_2 radical scavenging activity

The ability of ethanolic & acetone extracts of RoMi, RoAc and RoPt to scavenge hydrogen peroxide radicals is presented in **Table 7** & **Figure 16**, using BHA as standard. The **Figure 15** shows the ability of Butylated hydroxyanisole to scavenge the free radicals of hydrogen peroxide.

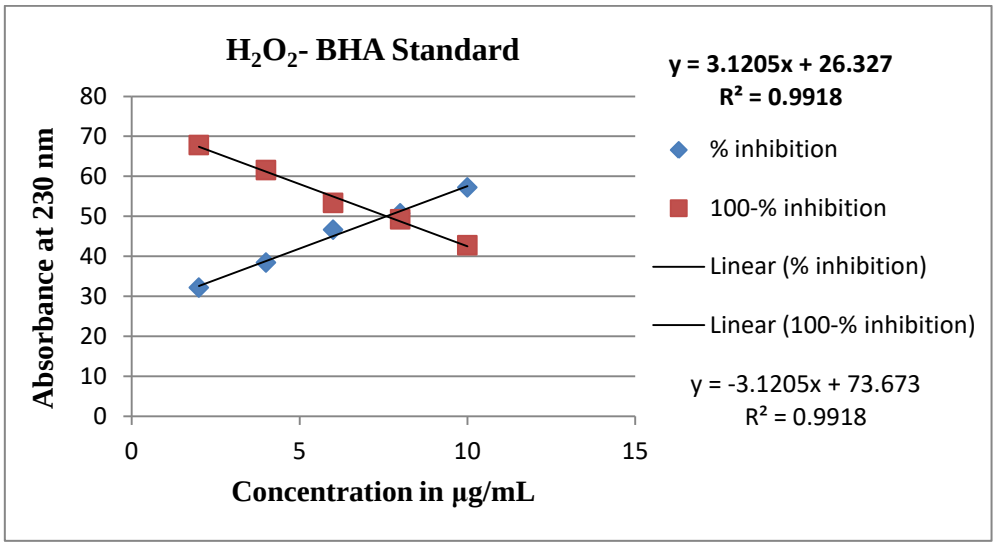


Fig 15: Showing hydrogen peroxide radical scavenging activity of Butylated hydroxyanisole.

Table 7: Presenting the H_2O_2 radical scavenging activity by extracts/standard.

Samples	% inhibition at different concentration of sample/standard at µg/mL					
	2 µL	4 µL	6 µL	8 µL	10 µL	IC_{50}
BHA	32.18	38.44	46.65	50.75	57.23	7.59
RoMi-EE	4.89 ±2.38	13.75 ±4.11	22.87 ±2.35	29.89 ±2.81	37.85 ±6.23	12.88 ±1.54
RoMi-AE	3.67±2.4	10.56 ±3.52	16.2 ±3.9	22.62 ±4.29	28.75 ±4.01	16.9 ±1.8
RoAc-EE	30.6 ±2.29	32.52 ±1.61	38.12 ±2.26	41.75 ±2.04	44.8 ±2.93	12.67 ±1.58
RoAc-AE	20.74 ±1.42	27.09 ±0.79	29.51 ±1.12	34.01 ±1.71	38.2 ±3.3	15.82 ±2.13
RoPt-EE	16.56 ±1.65	21.38 ±3	23.97 ±2.86	28.51 ±2.19	34.13 ±1.89	17.89 ±1.05
RoPt-AE	12.82 ±1.83	18.43 ±1.87	22.75 ±0.66	27.14 ±1.39	31.68 ±0.54	18 ±1.87

The result indicated a concentration dependent activity in BHA and all the root extracts with highest inhibition of 57.23% (at only 10 µg/mL concentration) was observed in BHA having IC_{50} value of only 7.59 µg/mL. Among the plant extracts at same concentration, the highest inhibition of 44.8 ± 2.93% was found in RoAc-EE which showed the IC_{50} value

of 12.67 ± 1.58, followed by RoMi-EE which showed 37.85 ± 6.23 percent inhibition (at 10 µg/mL concentration) with IC_{50} value of 12.88 ± 1.54 µg/mL of dried extract. The lowest IC_{50} value of 18 ± 1.87 µg/mL was obtained in RoPt-AE. The IC_{50} values of hydrogen peroxide radical scavenging activity of other extracts are RoAc-AE (15.82 ± 2.13), RoMi-AE (16.9 ± 1.8) and RoPt-EE (17.89 ± 1.05).

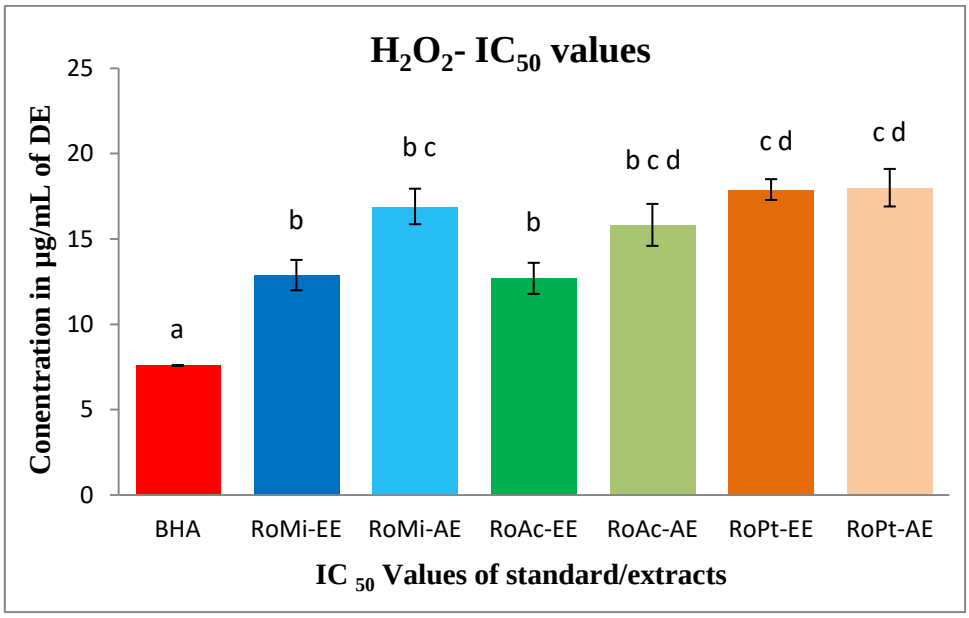


Fig 16: Showing H_2O_2 IC_{50} values of all extracts of RoMi, RoAc, RoPt & BHA. Results are mean value of ±SD (3n). Different letters indicate statistically significant (ANOVA-Tukey, $p<0.05$).

5.12 Ferric reducing antioxidant property

In FRAP assay, the antioxidant capacity was evaluated on the basis of the extract's ability to reduce ferric (III) ion to ferrous (II) ion. The result of ferric reducing antioxidant properties were evaluated from the standard linear curve of $FeSO_4.7H_2O$ ($0.0051x - 0.0408$; $R^2 = 0.9965$) **Figure 17**.

The results of FRAP antioxidant capacity is presented in **Figure 18**. Present study have revealed that acetone extract of RoAc showed better antioxidant capacity with 2512.7 ± 157.37 µmol Fe^{2+}/g followed by RoAc-EE having 2484.27 ± 135.3 µmol Fe^{2+}/g of dried extract. The lowest ferrous ion concentration was observed in RoPt-EE with 751.67 ± 85.48 µmol Fe^{2+}/g of dried extract. Result for other root extracts showed ferrous ion concentration of 1116.4 ± 98.56 in RoMi-EE, 1027.9 ± 115.03 in RoMi-AE and RoPt-AE with 820 ± 110.63 µmol Fe^{2+}/g of dried extract.

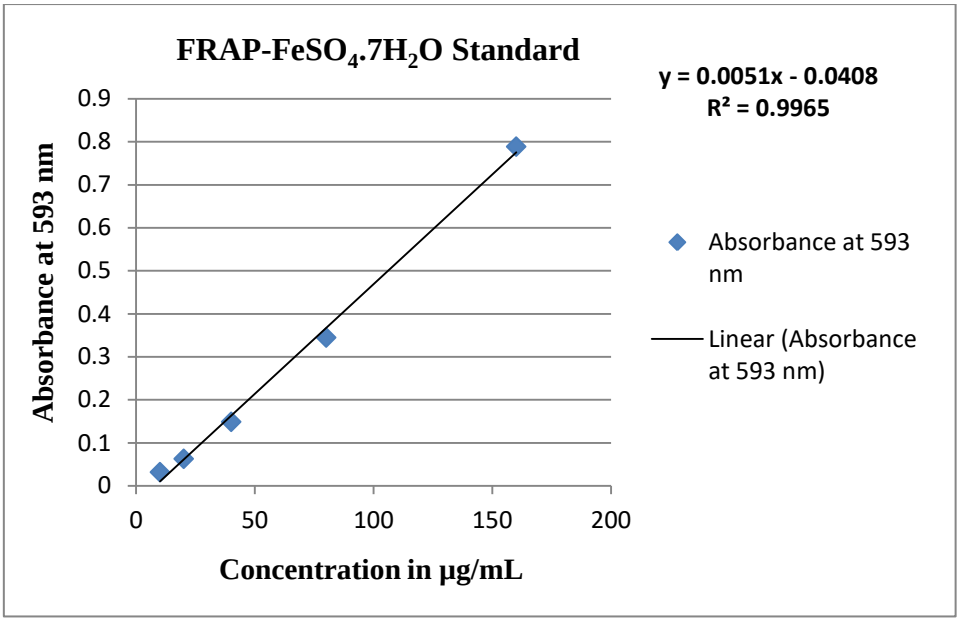


Fig 17: FRAP radical scavenging activity showing linear curve of $FeSO_4.7H_2O$ standard.

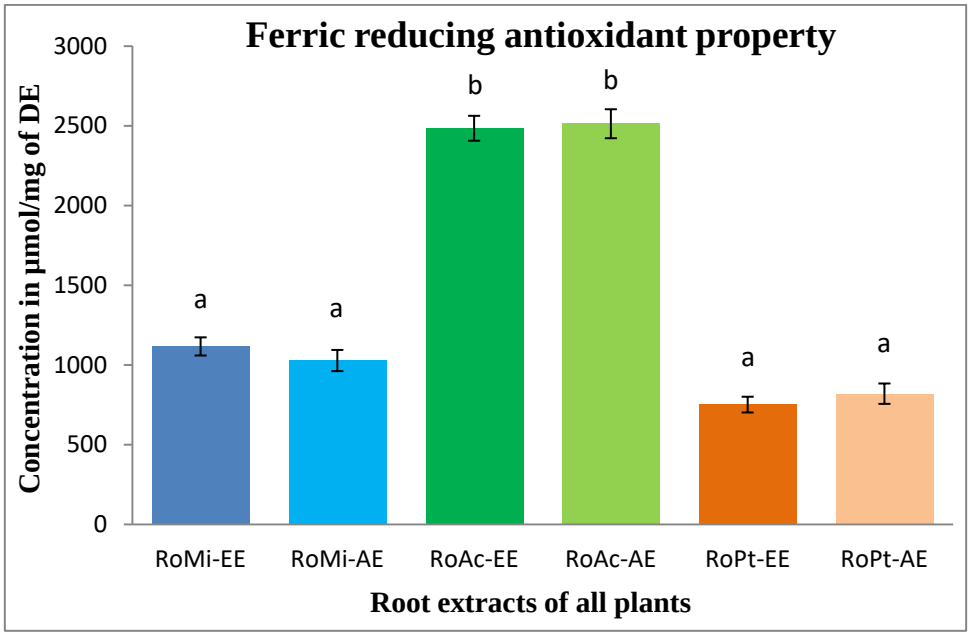
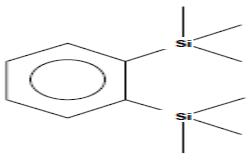
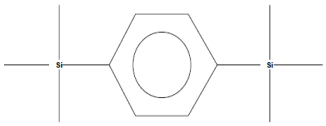
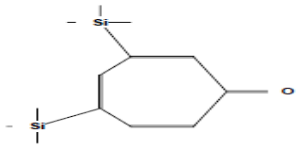
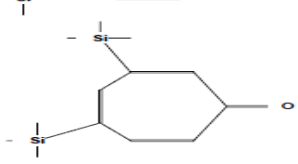
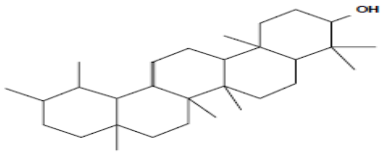


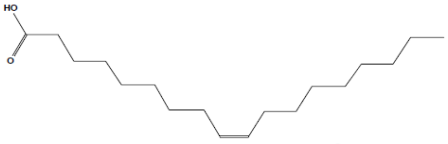
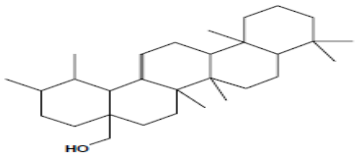
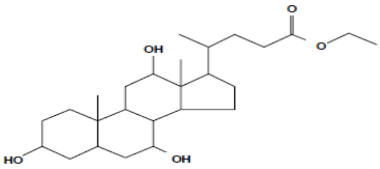
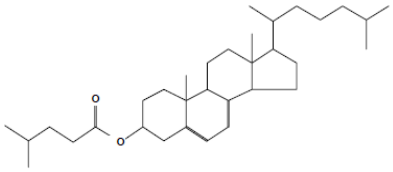
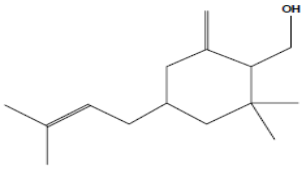
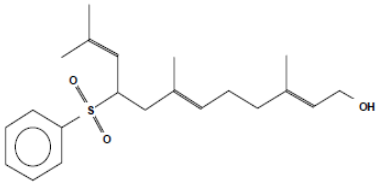
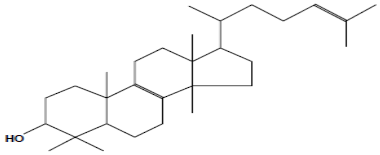
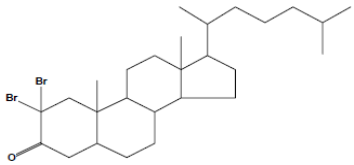
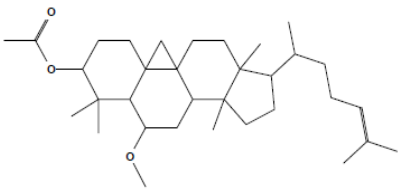
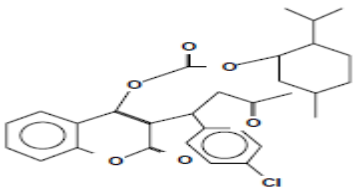
Fig 18: Extracts showing $FeSO_4.7H_2O$ concentration in µM/mg of DE. Results are mean value of ±SD (3n). Different letters indicate statistically significant (ANOVA-Tukey, $p<0.05$).

5.13 GC-MS analysis

The GC-MS chromatograms of hydro-alcoholic extract of *Morus indica* root are presented in ***Appendix-B***. A set of peaks were observed that indicates the existence of the diverse phytochemical components. The active components, molecular weight, retention time, peak area in percentage and pubchem CID are presented in **Table 8**.

Table 8: Bioactive compounds identified by GC/MS analysis of RoMi-EE.

Compound names (RoMi-EE)- 1st Chromatogram.	2D Structure	PubChem ID	MW	RT	Area (%)
1,2-Bis(Trimethylsilyl) Benzene (CAS: 17151-09-6)		519794	222	30.609	6.953
Silane, 1,4-Phenylenebis [Trimethyl- (CAS: 13183-70-5)		25771	222	30.634	5.191
2,4,6-Cycloheptatrien-1-One, 3,5-Bis-Trimethylsilyl- (CAS: 900161-21-8)		610038	250	30.674	5.699
2,4,6-Cycloheptatrien-1-One, 3,5-Bis-Trimethylsilyl- (CAS: 900161-21-8)		610038	250	31.035	27.563
Alpha.-Amyrin (CAS: 638-95-9)		73170	426	31.755	54.594
Results from the 2nd chromatogram					
Octadecanoic acid, ethyl ester (111-61-5)		8122	312	18.175	2.276
N-Hexadecanoic Acid (CAS: 57-10-3)		985	256	18.815	14.195
Eicosanoic Acid- (CAS: 506-30-9)		10467	312	19.040	1.993
9,12-Octadecadienoic Acid (Z,Z)- (CAS: 60-33-3)		3931	280	19.455	4.831
1-Octadecyne (CAS: 629-89-0)		69425	250	20.300	14.539
Pentadecanoic Acid- (CAS: 1002-84-2)		13849	242	20.465	4.612

Oleic Acid- (CAS: 112-80-1)		445639	282	20.585	2.097
Urs-12-En-28-Ol- (CAS: 10153-88-5)		22213452	426	25.918	6.621
Ethyl Iso-Allocholate- (CAS: 900043-05-3)		6452096	436	26.318	1.878
7-Dehydrocholesteryl Isocaproate- (CAS: 900251-07-6)		312789	484	28.408	3.384
1-Methylene-2b-Hydroxy methyl-3,3-Dimethyl-4b-(3-Methylbut-2-Enyl)-C (CAS: 900144-10-6)		550196	222	28.889	3.849
2,6,10-Dodecatrien-1-Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E) (CAS: 57683-67-7)		5368759	362	29.034	6.163
Lanosterol- (CAS: 79-63-0)		246983	426	29.339	5.678
2,2-Dibromo- Cholestanone (CAS: 97370-79-1)		22212696	542	29.884	2.343
3-O-Acetyl-6- Methoxy -Cycloartenol- (CAS: 900286-40-9)		537607	498	30.114	7.021
2-Isopropyl-5- Methylcyclo hexyl -3-(1-(4-Chlorophenyl)-3-Oxobutyl)-C- (CAS: 900143-59-5)		537118	524	30.409	1.976

5.14 *In-vivo* hepatoprotective activity

5.14.1 Acute toxicity study

The RoMi ethanolic extract was administered orally and no fatalities were examined in the investigational animals at 2000 mg/kg dose. Therefore, $1/10^{th}$ (200 mg/kg) of the maximum dose and $1/20^{th}$ (100 mg/kg) were considered safe for the *in-vivo* studies. There were no sign of clinical or toxicological symptoms or delay death in experimental animals observed after 14 days.

5.14.2 Effect of interventions on Biochemical parameters

5.14.2.1 Effect of RoMi on the indices of hepatotoxicity by liver marker enzymes

Administration of CCl_4 markedly increased the levels of liver serum enzymes such as ALT, AST, and ALP in the control group as compared with the normal group (**Table 9**). Elevation in the secretion of these enzymes was significantly decreased with the treatment of RoMi-EE; however, the higher concentration (200 mg/kg) showed better results.

5.14.2.2 Effect of RoMi on TC, TG, HDL, LDL, and VLDL level

The administration of CCl_4 has increased TC (6.40 ± 0.02 mmol/L), TG (1.78 ± 0.01 mmol/L), LDL (162.00 ± 10.81 mg/dL), and VLDL (29.01 ± 0.87 mg/dL), and decreased HDL (27.11 ± 1.42 mg/dL); whereas in the groups that received silymarin and test drug RoMi-EE, the levels of TC, TG and LDL have significantly decreased and that of HDL was increased slightly (**Table 9**). Higher concentration of RoMi-EE shows better results when compared with the low concentration group. The major fold changes were seen in LDL (1.2 folds) followed by TC (1.1 folds), VLDL (0.99 folds) and TG (0.73 folds) after the treatment with a higher dose of RoMi-EE as compared to the CCl_4 treated animals. The level of HDL increased with the administration of RoMi-EE and showed better result at higher concentration (34.20 ± 2.83 mg/dL). These results of high dose treatment are also comparable with the standard drug silymarin which was effective in reverting the biochemical parameters in diseased animals.

5.14.2.3 Effect of interventions on Creatinine

The serum biochemical assay of current investigation has been tabulated in **Table 9**. In normal group, creatinine level was noted to be 0.68 ± 0.01 mg/dL. CCl_4 administration significantly increased the plasma creatinine level (0.79 ± 0.01) as compared to the normal group. Treatment with CCl_4 shoot up the creatinine level by almost 1.2 folds. When the

experimental rats were treated with standard drug silymarin and different dosage of RoMi-EE, it was noticed that in the case of silymarin the creatinine level almost clocked back to the normal (0.7 ± 0.01 mg/dL). Upon treatment with RoMi-EE, the high dose was found more effective (0.71 ± 0.04 mg/dL) than the lower dose (0.77 ± 0.02 mg/dL) and was found statically significant in both of the cases.

Table 9: Biochemical parameters of Wistar rat blood from control and treatments

Tests	Normal	Negative Control	Silymarin (25 mg)	RoMi-EE (100 mg)	RoMi-EE (200 mg)
Albumin (g/dL)	39.14 ±1.21	25.7 ±1.76a	29.6 ±1.98b	26.02 ±1.90a	30.25 ±0.25b
ALT (U/L)	26.5 ±0.98	118 ±9.43a	88.1 ±9.31b	108.3 ±11.42a	66.33 ±4.73b
AST (U/L)	27.5 ±1.31	208 ±24.94a	98.2 ±10.21b	149.67 ±11.93c	101.33 ±11.2c
ALP (U/L)	14 ±0.45	27 ±1.20a	15.5 ±0.8b	22.33 ±2.52a	21 ±1.31b
TC (mmol/L)	4.1 ±0.03	6.4 ±0.02a	4.3 ±0.2b	5.6 ±0.01c	4.9 ±0.01b
TG (mmol/L)	1.09 ±0.01	1.78 ±0.01a	1.31 ±0.01b	1.5 ±0.01a	1.38 ±0.01b
HDL (mg/dL)	51.19 ±0.06	27.11 ±1.42a	37.92 ±0.3b	29.12 ±0.39a	34.2 ±2.83b
LDL (mg/dL)	44.98 ±0.21	162 ±10.81a	91.00 ±11.91b	131.00 ±11.3c	109.0 ±11.4b
VLDL (mg/dL)	10.90 ±0.09	29.01 ±0.87a	21.91 ±1.31b	28.90 ±0.02a	23.41 ±1.92b
Bilirubin (mg/dL)	0.6 ±0.01	1.88 ±0.02a	1.09 ±0.01b	1.51 ±0.01a	1.29 ±0.01b
GGT (U/L)	4.53 ±0.12	11.87 ±1.10a	6.81 ±0.31b	9.01 ±0.43c	8.93 ±0.8b
Creatinine (mg/dL)	0.68 ±0.01	0.79 ±0.01a	0.7 ±0.01b	0.77 ±0.02a	0.71 ±0.04b
Total Protein (g/dL)	6.16 ±0.23	5.98 ±0.11a	6.11 ±0.34b	6.09 ±0.05b	6.13 ± 0.17b

Values are mean ± S.E. (n= 6 animals /group). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p<0.05$. Those which are not sharing the same letters in a row are significantly different. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alanine phosphatase; TC: Total Cholestrol; TG: Triglyceride; HDL: High-density lipoproteins; LDL: Low-density lipoproteins; VLDL: Very-low-density lipoproteins; GGT: γ-glutamyl transferase

5.14.2.4 Effect of interventions on Total Protein and Albumin

As shown in **Table 9**, CCl_4 administration decreased the albumin and total protein levels, although the decrease in total protein was not significant. The albumin was decreased to 65.6% (as compared to the normal group). Treatment with RoMi-EE at a higher dose (200 mg/kg) and silymarin significantly increased the albumin to 77.3% and 75.62% respectively. There were no significant changes (p<0.05) was observed in the levels of total protein in the treatment groups as compared to the normal group.

5.14.2.5 Effect of interventions on Bilirubin and GGT

The control group that received CCl_4 showed an elevation in the levels of serum bilirubin (313.3%) and GGT (262.03%) as compared to the normal group (**Table 9**). After the treatment, the bilirubin was lessened to 251.6% (100 mg of RoMi-EE), 215% (200 mg of RoMi-EE) and 181.6% (25 mg silymarin); whereas, GGT was reduced to 198.9% (100 mg of RoMi-EE), 197.1% (200 mg of RoMi-EE), and 150.3% (25 mg silymarin).

5.14.3 Effect of RoMi on the parameters of oxidative stress in liver

5.14.3.1 Effect of interventions on antioxidant enzymes SOD, CAT and GPx

The administration of CCl_4 resulted in a decrease of hepatic antioxidant enzymes such as SOD, CAT, and GPx in the liver (**Figure 19-21**).

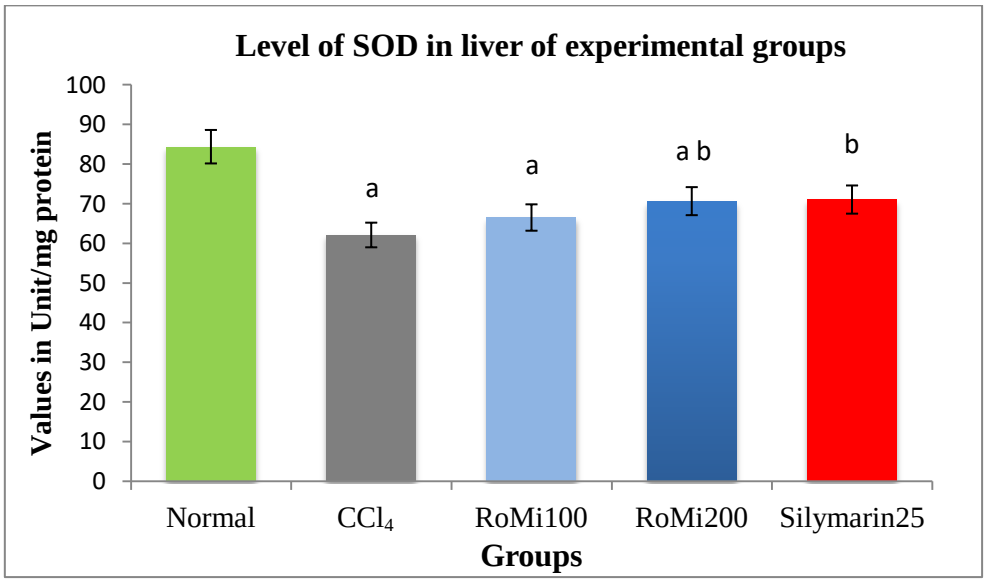


Fig 19: Effect of RoMi-EE on the levels of Superoxide dismutase. Values are mean ±SE (n= 6 animals /group). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at p<0.05. Those which are not sharing the same letters are significantly different.

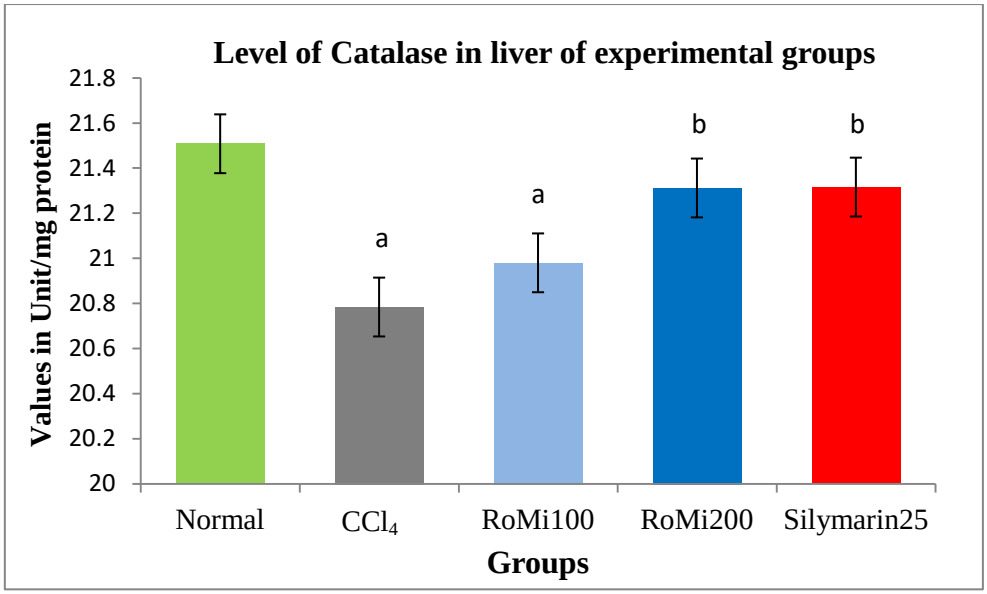


Fig 20: Effect of RoMi-EE on the levels of Catalase. Values are mean ±SE. (n= 6 animals /group). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p<0.05$. Those which are not sharing the same letters are significantly different.

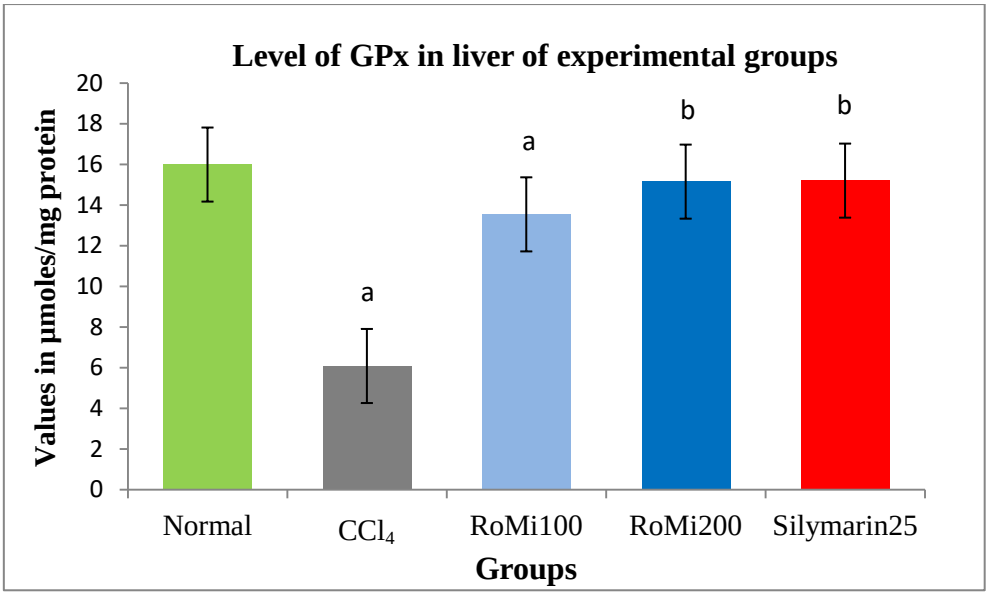


Fig 21: Effect of RoMi-EE on the levels of Glutathione peroxidase. Values are mean ±SE. (n= 6 animals /group). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p<0.05$. Those which are not sharing the same letters are significantly different.

The activities of SOD, CAT, and GPx were decreased respectively by 27.3%, 3.53%, and 63.1%. The groups treated with RoMi-EE at two different doses and silymarin

significantly increased ($p<0.05$) in the activities of the enzymes, and the protective effect of RoMi-EE treatment at 200 mg/kg was similar to that of silymarin treatment.

5.14.3.2 Effect of interventions on the activity of GSH

From **Figure 22**, it is evident that CCl_4 reduced the activity of GSH by 25% as compared to the normal group. The activity was ameliorated upon treatment with RoMi-EE at both the concentrations as well as silymarin group. The 100 mg RoMi-EE showed 9.11% increase in GSH concentration. However, 200 mg RoMi-EE have better GSH concentration that showed 12.42% increase and was comparable to that of silymarin group with 12.83% increase when compared to the CCl_4 group. The higher concentration of RoMi-EE and silymarin showed significant increase ($p<0.05$).

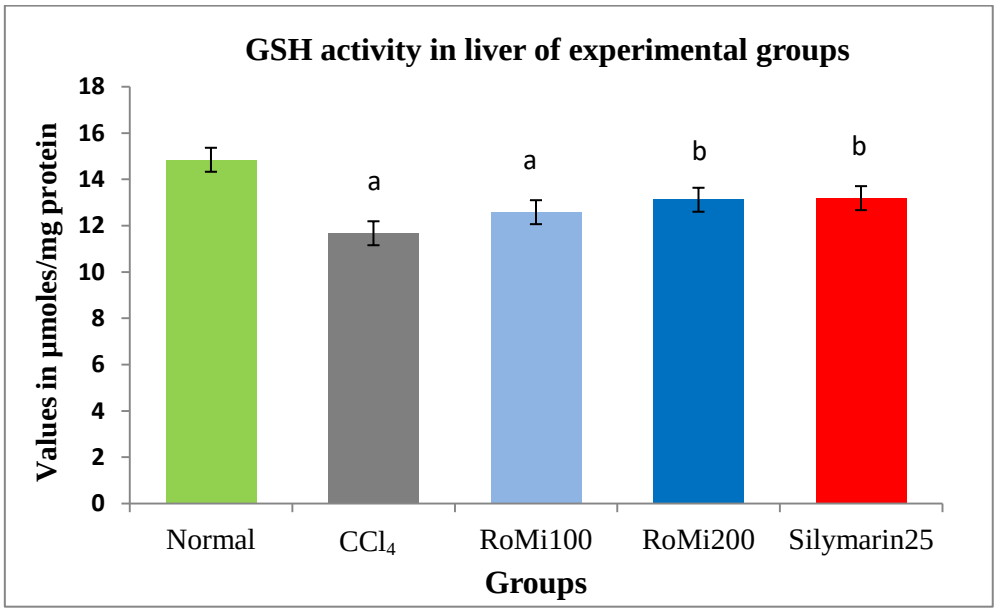


Fig 22: Effect of RoMi-EE on the levels of GSH. Values are mean ±SE. (n= 6 animals /group). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p<0.05$. Those which are not sharing the same letters are significantly different.

5.14.3.3 Effect of interventions on Lipid Peroxidation

Lipid peroxidation was accessed by evaluating the level of Malondialdehyde (MDA) and was expressed in nmol/mg of protein. **Figure 23**, shows the effects RoMi-EE treatment on the CCl_4-induced alteration of MDA level. The MDA content was significantly elevated by 48.53% in the CCl_4-treated group when compared with that of the normal group ($p<0.05$). However, the treatment with RoMi-EE and silymarin significantly reduced MDA level.

Lower concentration of RoMi-EE have reduced the elevated level of MDA by 19.12%. However, RoMi ethanolic extract at higher concentration markedly decreased the CCl_4-induced elevation of lipid peroxidation by 33.82% showing notably better result than the silymarin group which reduced the same by 22.08% ($p<0.05$).

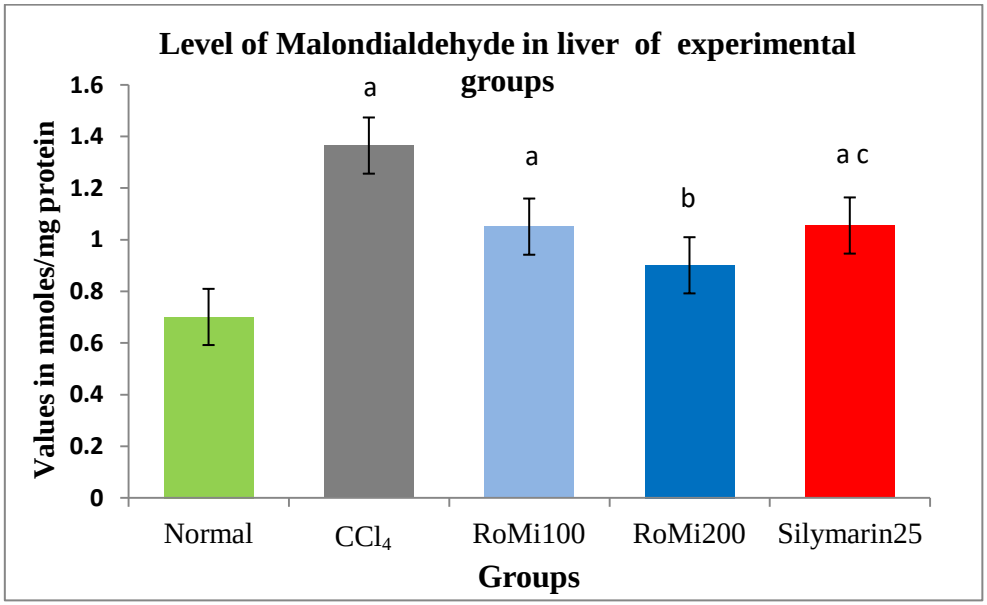


Fig 23: Effect of RoMi-EE on lipid peroxidation (the level of MDA). Values are mean ±SE. (n= 6 animals /group). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p<0.05$. Those which are not sharing the same letters are significantly different.

5.15 Effect of interventions on Histopathological changes in Liver

Histopathological evaluation of the rat livers (**Figure 24**) showed that the hepatocytes of a healthy rat had a normal architecture (**Figure 24-A**), whereas, in contrast, the CCl_4 induced severe hepatocyte necrosis, inflammation, hemorrhage, biliary cirrhosis, vacuolation, microvesicular steaosis and broad infiltration of kupffer cells around the central vein (**Figure 24-B**). After treatment with RoMi ethanolic extract, the severity of CCl_4-induced liver intoxication was reduced in a dose-dependent manner. Animal treated with CCl_4 and lower concentration of RoMi-EE showed mild sinusoidal dialatation in centrizonal area, necrosis recovery around the central vein and the regenerating hepatocytes are observed (**Figure 24-C**). Animal treated with CCl_4 and higher concentration (200 mg) of RoMi-EE showed no centrilobular necrosis, higher recovery of hepatocytes around the central vein and proper sinusoid texture (**Figure 24-D**), although the treatment with silymarin showed much better result showing normal hepatocytes and proper central vein. (**Figure 24-E**).

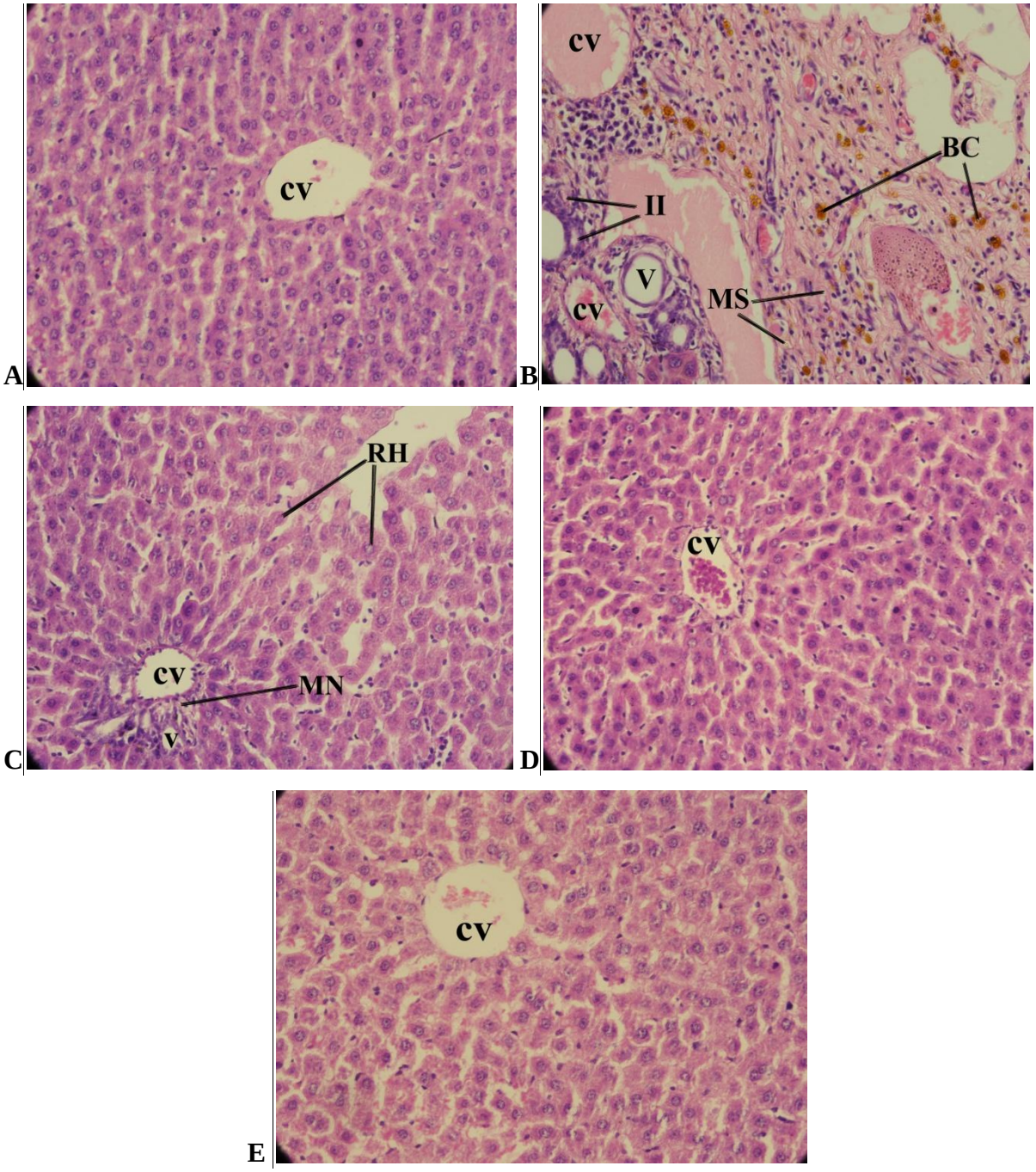


Fig 24: Histopathologic section of liver (40× magnification): A. Normal group: showing arrangement of hepatocytes in the liver lobule. B. CCl_4 group: showing hepatocyte necrosis, inflammatory infiltration (II), biliary cirrhosis (BC), microvesicular steatosis (MS), broad infiltration of kupffer cells and vacuolation (V). C. CCl_4 & 100 mg treated group: showing mild sinusoidal dialatation in centrizonal area, necrosis recovery/ mild necrosis (MN) around the central vein (CV) and regenerative hepatocytes (RH). D. CCl_4 & 200 mg treated gFroup: showing no centrilobular necrosis, hepatocytes with normal texture around the central vein and proper sinusoid texture. E. CCl_4 & silymarin treated group: showing normal hepatocytes and proper central vein.

5.16 Effect of interventions on Histopathological changes in kidney

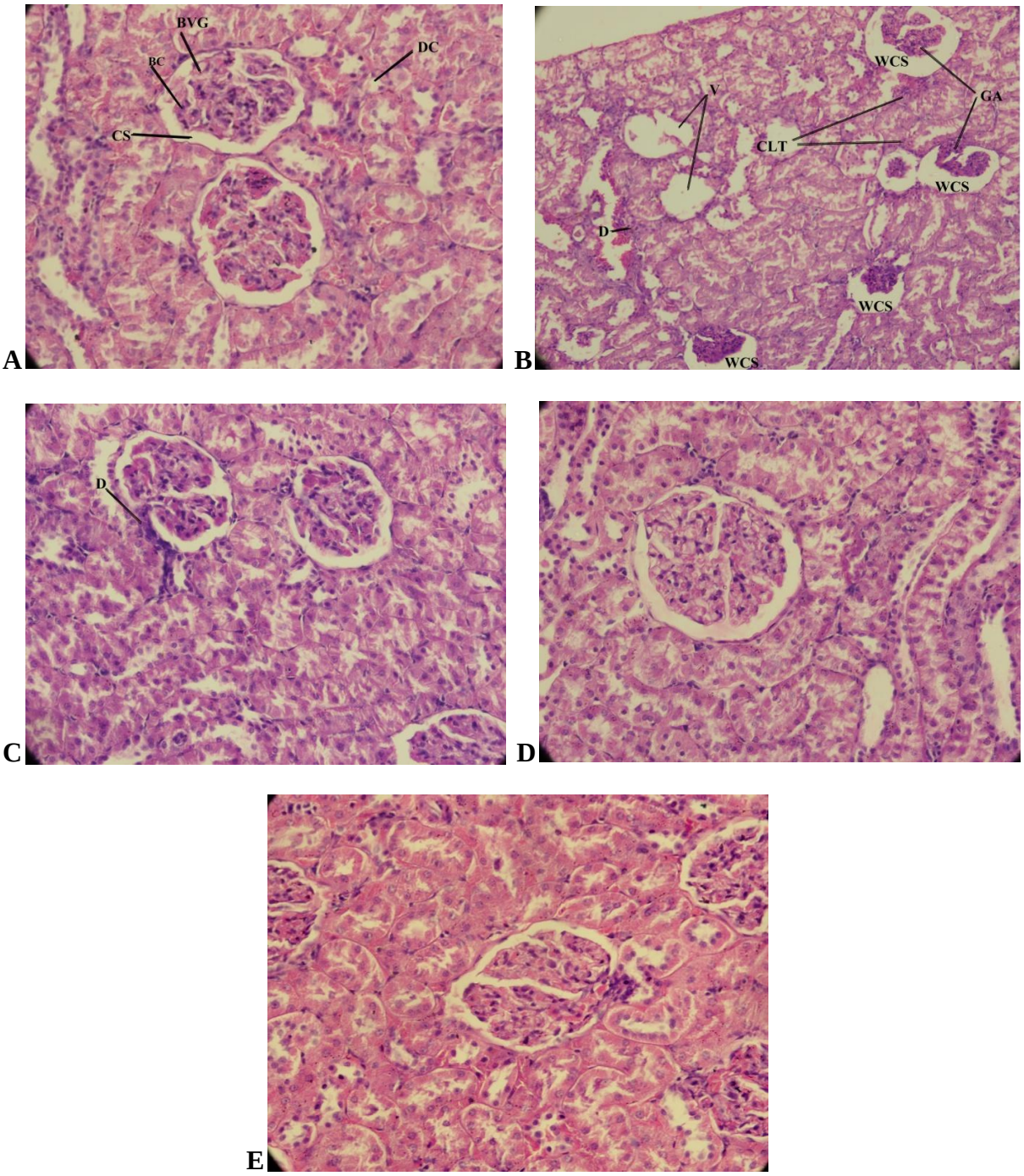


Fig 25: Cross section through kidney cortex (40× magnification): A. Positive control B. Negative control (CCl_4 induced), C. CCl_4 & 100 mg, D. CCl_4 & 200 mg and E. CCl_4 & 25 mg Silymarin. Normal cell structure with blood vessel glomeruli (BVG), distal convoluted tubule (DC), capsule space (CS) and bowman's capsule was observed in A, D & E. Disrupted cells with vacuolation (V), glomerular atrophy (GA), widening of capsule space (WCS), Cell layer thickening and degeneration of cells was seen in B. Recovery of glomerular atrophy, capsule space decrease and degeneration (D) of cell was observed in C.

The histopathology of cross section through wistar rat cortex kidney in CCl_4 group showed vacuolation, glomerular atrophy, widening of capsule space, cell layer thickening and degeneration of cells. The recovery of glomerular atrophy, decrease in capsule space and less degeneration of cell was observed in 100 mg RoMi-EE experimental drug and the 200 mg RoMi-EE experimental drug. Whereas silymarin showed identical structure and was similar to that of normal group.

5.17 Docking analysis of NFκB (1NFK) protein

5.17.1 Receptor Sitemap and site score of 1NFK

Since there is no information about the binding site or standard ligand in a target of interest (reference protein), a putative binding site has been identified by computational means and the druggability of the target was also identified by druggability score [**Table 10**].

A.

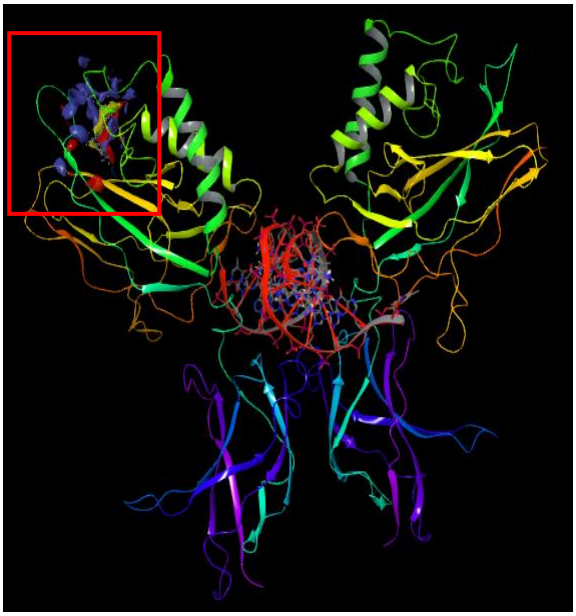


Fig 26: 1NFK protein, A. Showing Sitemap (Site 4) in the protein NFκB.

Table 10: Site score of the 1NFK protein

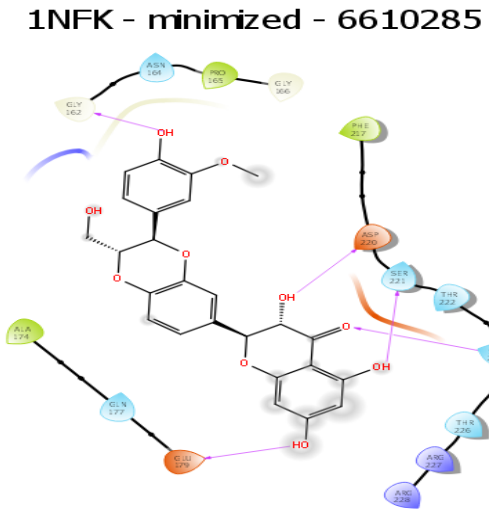
Sites of 1NFK	Site Score	Drug ability Score
Site 1	1.043	0.986
Site 2	0.981	1.015
Site 3	0.993	1.04
Site 4	1	1.04
Site 5	0.868	0.674

The results of site score (**Table 10**) shows that Site 4 as best since the site is showing site score and drug ability score of ≥ 1. Hence the Site 4 was docked with selected ligands.

5.17.2 Ligand docking with 1NFK

As a result of docking a number of values of consensus scoring functions has been obtained. These values assess the quality and energy of binding of molecules studied with the 1NFK-having residues of Site-4 (Chain B): 92, 161, 162, 163, 164, 165, 166, 167, 174, 176, 177, 178, 179, 180, 181, 182, 183, 217, 223, 224, 225, 226, 227, 228. The values of docking score of all compounds with 1NFK and binding energy were given in **Table 11**. The values obtained were compared with the values of hepatoprotective compound silymarin. 3D image of silymarin and ligand (of best docking score) are presented in **Figure 27 & 28**.

A.



B.

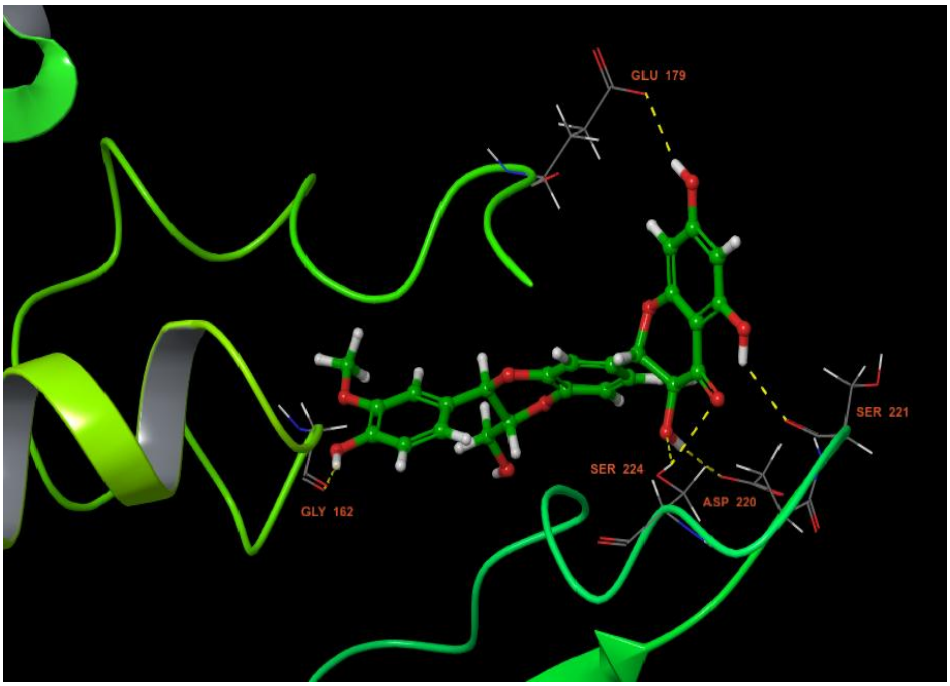
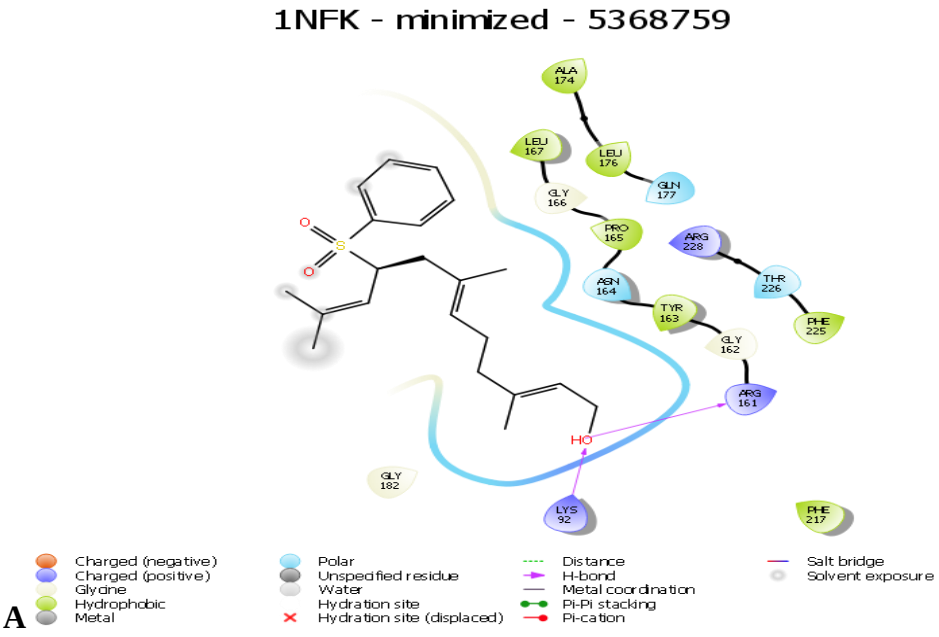
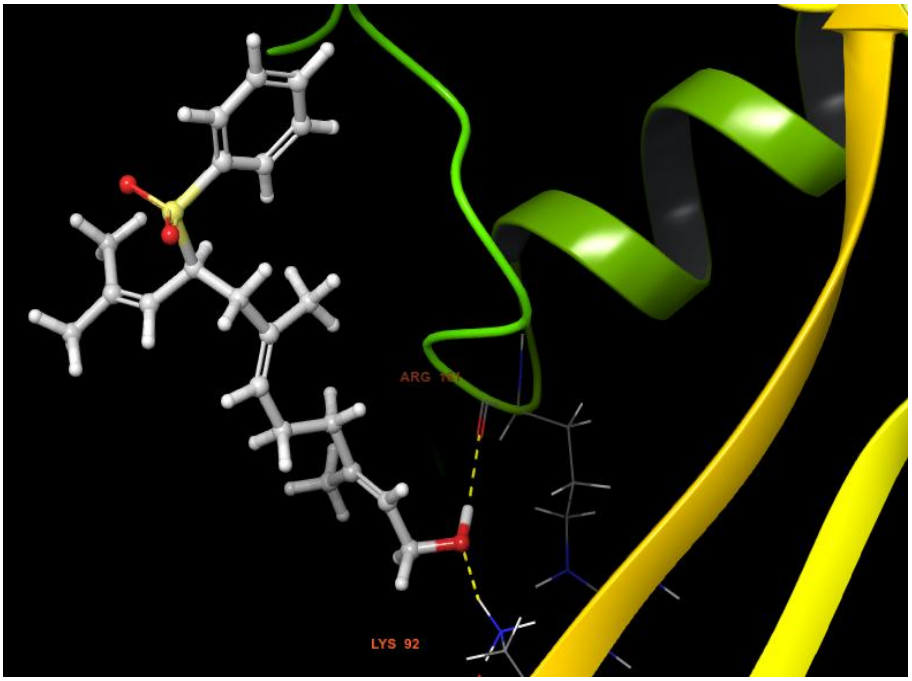


Fig 27: A. 2D & B. 3D image of silymarin binding with 1NFK site-4.



A



B

Fig 28: A. 2D & B. 3D image of ligand CID: 5368759 [2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)] binding with 1NFK site-4.

Results obtained from the docking studies showed that silymarin have highest docking score of -5.956 and highest binding energy (ΔG) -54.79 and showed five hydrogen bonding with the Gly 162, Glu 179, Asp 220, Ser 221 and Ser 224 residues of 1NFK. 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)- [CID: 5368759] showed best docking result among the ligands selected having docking score of -4.958 and binding energy (ΔG) of -45.35, the 3D image shows two hydrogen bonding with the Lys 92, and Arg 161 residues of active site (4) of 1NFK.

5.18 Docking analysis of COX-2 (3LN1) protein

2D and 3D image of ligand (5368759) docking with 3LN1 protein is presented in **Figure 30** (A & B). The 3D image of protein 3LN1 is presented in the **Figure 29**.

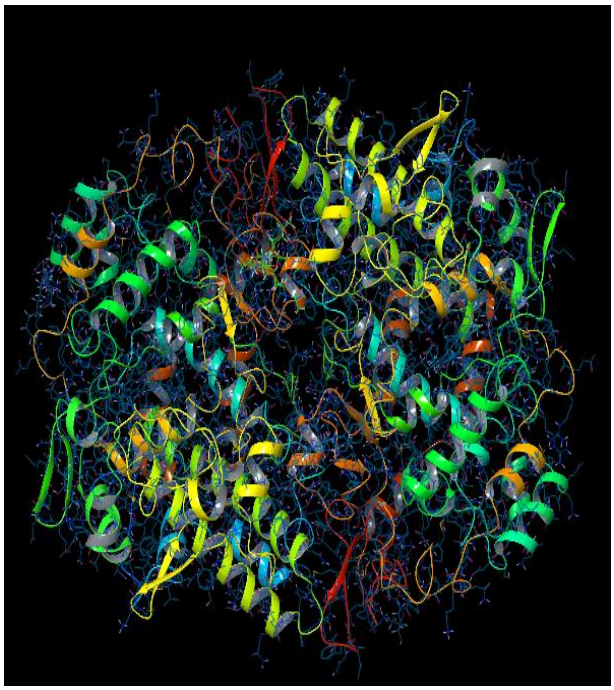
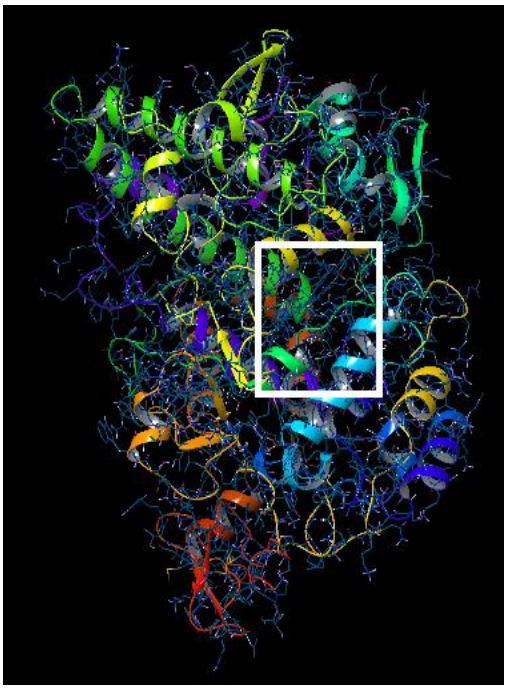
A.  **B.** 

Fig 29: A. 3D image of 3LN1 (COX-2 protein) & B. 3D image of 3LN1 (β subunit).

A.

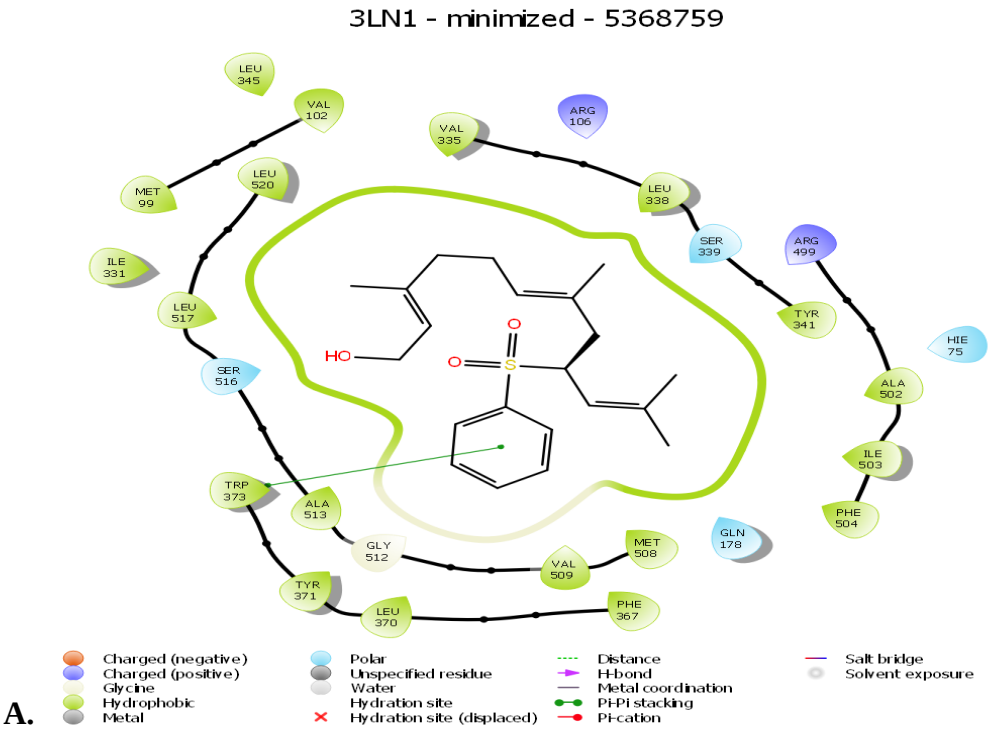


Fig 30: A. 2D image of CID-5368759 (2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)) binding with 3LN1 (β subunit) showing pi-pi interaction.

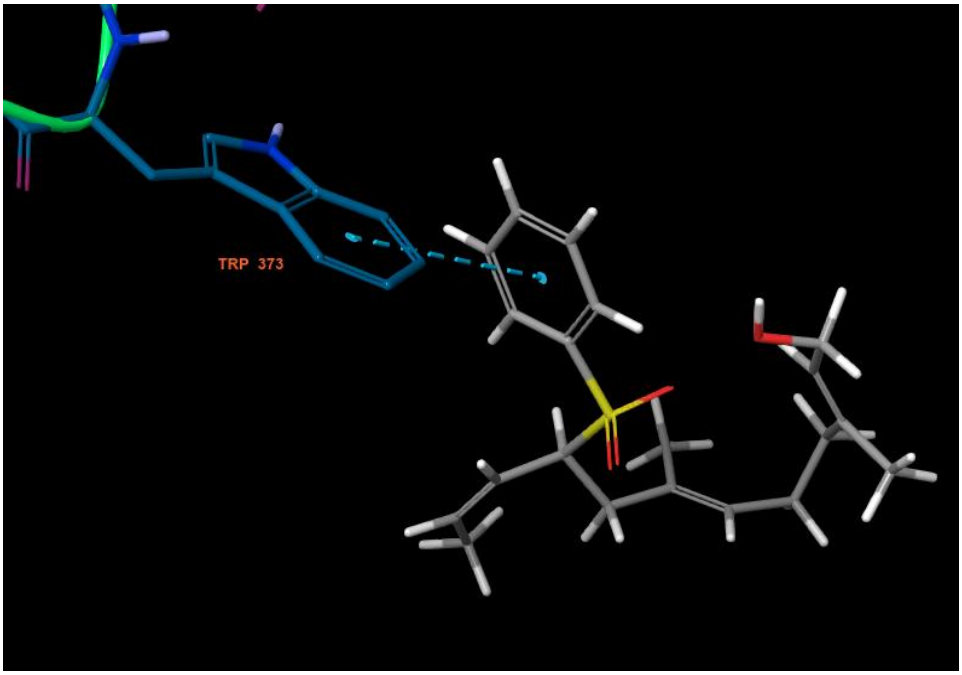
B. 

Fig 30: B. 3D image of CID-5368759 (2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)) binding with 3LN1 (β subunit) showing pi-pi interaction.

The docking study with 3LN1 crystallized protein shows that the compound: 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E) showed the Pi-Pi stacking interaction between the aromatic ring of the ligand with another aromatic ring of Tryptophan (TRP 373) residue of the target receptor. This interaction also was the best interaction among the selected ligands which showed best docking score -9.78 and ΔG binding affinity of -27.8173 kcal/mol, following by the compound 9,12-Octadecadienoic Acid (Z,Z)- (CID: 3931), which showed second best docking score of -8.047 and ΔG binding affinity of -21.081 kcal/mol. The compound 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E), also bound to 1NFK protein with best docking score and highest ΔG binding affinity among the selected ligands. However, the silymarin which showed best docking score with 1NFK, did not show any binding affinity to the 3LN1 protein.

The docking score of other ligands were in order of CID: 3931 (-8.047) > 550196 (-7.569) > 610038 (-7.559) > 519794 (-6.472) > 13849 (-5.995) > 69425 (-5.496) > 445631 (-5.355) > 8122 (-5.028) > 985 (-4.838). The values of docking score of all compounds (ligands) with 1NFK and 3LN1 protein with their binding energy were given in **Table 11**. The compounds CID: 6452096 and 73170 did not show any interaction with the 3LN1 protein, whereas the compounds CID: 3931, 25771, 13849, 69425, 445631 and 985 did not show any interaction with the 1NFK protein.

Table 11: Presenting best docking NFκB (1NFK) and COX-2 (3LN1) score (below -1.5), and MMGBSA ΔG binding affinity of the molecules.

Sl no.	PubChem CID	Compound Name	NFκB docking (1NFK)		COX-2 docking (3LN1)	
			Docking Score	MMGBSA ΔG binding	Docking Score	MMGBSA ΔG binding
1.	6610285	Silymarin	-5.956	-54.79	---	---
2.	5368759	2,6,10-Dodecatrien-1-Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)	-4.958	-45.35	-9.78	-27.8173
3.	8122	Octadecanoic acid, ethyl ester	-4.528	-33.85	-5.028	-1.44194
4.	6452096	Ethyl Iso-Allocholate-	-3.679	-39.5	---	---
5.	550196	1-Methylene-2b-Hydroxy methyl-3,3-Dimethyl-4b- (3-Methylbut-2-Enyl)-C	-3.277	-5.67	-7.569	-10.189
6.	519794	1,2-Bis(Trimethylsilyl) Benzene	-3.001	-29.05	-6.472	2.080533
7.	73170	Alpha.-Amyrin	-1.629	-27.59	---	---
8.	610038	2,4,6-Cycloheptatrien-1-One, 3,5-Bis-Trimethylsilyl-	-1.595	-26.24	-7.559	-10.7121
9.	3931	9,12-Octadecadienoic Acid (Z,Z)-	---	---	-8.047	-21.081
10.	25771	Silane, 1,4-Phenylenebis [Trimethyl-	---	---	-7.025	-3.6666
11.	13849	Pentadecanoic Acid-	---	---	-5.995	-18.4921
12.	69425	1-Octadecyne	---	---	-5.496	-19.8241
13.	445631	Oleic Acid-	---	---	-5.355	-1.51702
14.	985	N-Hexadecanoic Acid	---	---	-4.838	11.81801

5.19 ADME property of ligands

Physically significant and pharmaceutically significant properties of all the lead molecules were analysed by using “molsoft” prediction tool. Molecular weight, H-bond donors, log P Octanol/water partition coefficient, H-bond acceptors, Mol Log S and their

positions according to Lipinski's rule of five were presented in **Table 12**. Almost all the compounds were in the acceptable range of Lipinski's rule of five, indicating their potential for use as drug-like molecules.

Table 12: Physical properties of molecules calculated by Lipinski's rule of five.

Sl no.	PubChem CID	Molecular weight[a]	Number of HBA[b]	Number of HBD[c]	Mol Log P[d]	Mol Log S[e]
1.	6610285	482.12	10	5	2.59	-6.19
2.	5368759	362.19	3	1	5.61	-5.37
3.	*8122*	*312.3*	*2*	*0*	*8.45*	*-7.21*
4.	6452096	436.32	5	3	4.67	-5.06
5.	550196	222.2	1	1	4.77	-4.16
6.	519794	222.13	0	0	2.6	-3.96
7.	*73170*	*426.39*	*1*	*1*	*9.21*	*-8.11*
8.	610038	250.12	1	0	1.73	-2.73
9.	*3931*	*280.24*	*2*	*1*	*6.75*	*-5.44*
10.	25771	222.13	0	0	2.72	-2.77
11.	13849	242.22	2	1	6.17	-5.24
12.	*69425*	*250.27*	*0*	*0*	*7.69*	*-7.34*
13.	*445639*	*282.26*	*2*	*1*	*7.15*	*-5.97*
14.	*985*	*256.24*	*2*	*1*	*6.65*	*-5.66*

a. Molecular weight of the molecule (160 to 500)

b. Hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution (not more than 10).

c. Hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution (not more than 5).

d. Log P for octanol/water (−2.0 to 6.5).

e. Water solubility, log S (−6.5 to 0.5).

Most of the ligands were under the acceptable range of molecular weight, hydrogen bond acceptor, hydrogen bond donor and water solubility (other than CID: 8122, 73170 & 69425) of Lipinski rules. The log P value of the ligands (except CID: 8122, 73170, 3931, 69425 and 445639) also suggests that it cannot cross the blood–brain barrier and hence it can be used as drug in other organs or body parts without affecting the brain tissue.

CHAPTER-VI

6. DISCUSSION

Findings of the study

The present survey of traditionally used hepatoprotective medicinal plants, a sum of 40 plants from 26 different families and 40 genus were identified and documented. From the result it was revealed that the tribe uses 29 types of leaves, 10 types of roots, 3 types of whole shoot, 2 types of flowers, one type of bark, fruit and seed respectively. The plants of the family Acanthaceae is found to be the highest (with 4 plants) in use followed by Moraceae, Rubiaceae, Rutaceae with 3 plants among them. The process of medicine preparation/formulation depend on traditional healers, who may use single or combination of different plants or plant parts for treating hepatic related disorders. Out of 40 different medicinal plants collected, various literature review on medicinal plants have revealed that the plants *viz*: *Pogostemon plectranoides* Desf., *Amphineuron opulentum* (Kaulf.), *Hygrophila plomoides* Nees. and *Morinda angustifolia* Roxb., were not reported earlier as hepatoprotective medicinal plants as well as no scientific research were carried out.

In a different survey, Kpodar *et al.* 2016, Sharma *et al.* 2012 and Thokchom *et al.* 2016, reported 99 plant species (49 families & 88 genera), 40 plants (31 families & 38 genera) & 40 plants (under 22 families) are used as hepatoprotective medicinal plants by the traditional healers of Togo, Africa; Bhoxa, Tharu & nomadic Gujjars community of Sub-Himalayan region of Uttarakhand; and Meitei community of Imphal east & west districts of Manipur, India respectively. Kpodar *et al.* 2016, reported that *Caesalpiniaceae* families were the highest with 8 species, followed by *Euphorbiaceae* with 7 species and leaves followed by root parts were mainly used. As per literature supported by Sharma *et al.* 2012, the most commonly used hepatoprotective plant species in India is *Boerhavia diffusa* followed by *Tinospora cordifolia*, *Saccharum officinarum*, *Phyllanthus amarus*, *Ricinus communis*, *Andrographis paniculata*, *Oroxylum indicum*, *Lawsonia inermis* and *Eclipta prostrata*. Thokchom and co-worker 2016, found out that *Andrographis paniculata* (Burm.f.) Nees was having highest DCI (Disease concensus index) value among studied plants and also revealed that the whole plant (21%) followed by leaves & fruit (16%) were found to be highest in use.

In Bangladesh, Bin Nyeem *et al.* 2017, reported some plants to have hepatoprotective properties such as *Solanum nigrum, Berberis aristata, Rosa damascena, Tinospora cordifolia, Phyllanthus niruri, Foeniculum vulgare, Rubia cordifolia, Mimosa pudica,*

Phyllanthus emblica, Phyllanthus emblica, Nyctanthes arbortritis, Ficus hispida, Aegle marmelos, Capparis spinosa, Cassia fistula, Azadirachta indica, Andrographis paniculata etc., and *Cichorium intybus*, *Herpetospermum caudigerum* in different study by Khalid *et al*. 2018 and Cao *et al*. 2017. These medicinal plants have promising phytochemicals that have already been tested in hepatotoxicity models using modern scientific system.

Though antioxidant properties of different parts (leaves, flower, fruit and bark) of *Morus indica, Averrhoa carambola* and *Phlogacanthus thyrsiflorus* plants were reported earlier, very limited or no scientific study was done on the roots of these plants. Although a study on anti-inflammatory activity of *Morus indica* root was conducted and the results obtained from the study did showed anti-inflammatory activity of the plant.

Root extracts of *M. indica*, *P. thyrsiflorus* and *A. carambola*, showed important phyto-constituents such as phenolics, flavonoids and tannins which are major group of compounds that act as natural antioxidants or free radical scavengers (Jayshree *et al.* 2016; Song *et al*. 2010) which possess antimicrobial, anti-allergic, anti-mutagenic, anti-inflammatory and anti-carcinogenic properties (Yao *et al.* 2004; Najafabad & Jamei 2014; Anupa *et al*. 2016; Ghasemzadeh & Ghasemzadeh 2011) and also showed positive for resins, terpenoids, glycosides and steroids. In another study by Niratker & Singh 2014, phytochemical screening of leaves of *M. indica* L., have shown that methanolic and ethanolic extracts are rich in carbohydrates, saponins, alkaloids, flavonoids, proteins and amino acids, terpenoids, reducing compounds, tannins, phenols and cardiac glycosides. Presence of tannins, flavonoids, saponins, carbohydrates, steriods, alkaloids, reducing sugar, and terpenoids were reported in the methanolic leave extracts of *P. thyrsiflorus* (Das *et al.* 2015). *A. carambola* preliminary phytochemical analysis of fruit extract showed the presence of flavonoids, tannins, saponins and alkaloids (Thomas *et al.* 2008). Whereas the acetone leave extract of *A. carambola* revealed the presence of various phytoconstituents including alkaloids, tannins, reducing sugar and flavonoids in them (Mazumder & Choudhury 2013).

The compounds such as phenolic are rich in plants/plant parts (Maheshwari *et al.* 2011 & Hseih *et al.* 2016). The current study on total phenolic content were in order of RoAc-EE (235.26 ± 11.91) > RoMi-EE (214.71 ± 2.21) > RoAc-AE (213.91 ± 11.18) > RoMi-AE (190.61 ± 2.88) > RoPt-EE (101.26 ± 2.52) > RoPt-AE (84.21 ± 4.82). Phenolics such as phenolic acid, tannins, flavonoids, tocopherols etc., are natural antioxidants that possess antimicrobial, anti-allergic, anti-mutagenic, anti-inflammatory and anti-carcinogenic

properties (Yao *et al.* 2004; Najafabad & Jamei 2014). Higher amount of phenolic and flavonoid content corresponds to their stronger antioxidant capacity. Therefore, phenolics and flavonoids have many essential roles in decreasing the risk of various human diseases (Anderson *et al.* 2001; Alam *et al.* 2013; Florence 1995). Moresco *et al.* 2012, found total phenolic content of 79.07+0.63 mg/g of dry fraction from *A. carambola* leaves ethanolic extract. Antioxidant study on 56 selected Chinese medicinal plants by Song *et al.* 2010, have found highest phenolic content in the plant of *Dioscorea bulbifera* L. with 59.43 ± 1.03 and only 5.34 ± 0.09 mg GAE/g of dried extract from *Morus alba* L. (bark of root). Chanu *et al.* 2012, found total phenolics in the aqueous seed extract of *Parkia javanica* having 51.09 ± 0.78 and 48.75 ± 1.43 mg GAE/g in the *P. thyrsiflorus* methanolic leave extract. Several studies have shown that polyphenols are directly attached with several biological activities such as anti-inflammatory, hepatoprotective activity etc. (Santillan *et al.* 2014; Wu *et al.* 2017).

In the present study higher amount of total flavonoid contents are in order of RoMi-EE (123.39 ± 2.04) > RoMi-AE (113.09 ± 7.25) > RoAc-EE (101.96 ± 6.87) > RoPt-EE (99.92 ± 5.49) > RoAc-AE (82.81 ± 5.94) > RoPt-AE (68.22 ± 4.82). Hsieh *et al.* 2016 has found the maximum yield of total flavonoid in *Ajuga nipponensis* with 7.87 ± 0.10 mg/g was obtained in 70% ethanol extract when the extraction time was 50 minutes and the extraction temperature was at 60ºC. In another study by Raman *et al.* 2016, have found flavonoid content of 187.23 mg/g from the 70% ethanol flavonoid extracted from that of mulberry fruit (*Morus alba* L.). From the current study, it was found that total flavonoids in RoMi-EE is 16 folds higher than that of *A. nipponensis* ethanolic extract but was only 0.6 folds lower than the *M. alba* fruit ethanolic extract.

The reducing power activity increases with increased concentration of the extract which was comparable to that of standard. Reducing power assay acts by reducing ions or by donating electron and the antioxidants present in the plant extracts causes the reduction of Fe3+/ ferricyanide complex to the ferrous form (Ahmed *et al.* 2015; Najafabad & Jamei 2014). The activity of extracts might be due to the occurrence of flavones hydroxyl, phenolic hydroxyl or methoxyl groups, free carboxylic groups, keto groups and others such as triterpenes and their derivative (Najafabad & Jamei 2014). In the current study, the increased absorbance value of reducing power activity of the extracts were significantly lower than the standard in order of BHA (2.928 at 160 µg/mL) > RoAc-EE (0.907 ± 0.015) > RoMi-EE (0.878 ± 0.035) > RoAc-AE (0.732 ± 0.014) > RoMi-AE (0.498 ± 0.03) > RoPt-EE (0.421 ±

0.013) > RoPt-AE (0.395 ± 0.015) at 200 µg/mL concentration of dried extract. The reducing power ability of extract was compared with standard BHA at a concentration of 10-160 µg/mL at 0.278- 2.928 OD respectively. Study on *Ajuga nipponensis* reducing power activity by Hsieh *et al.* 2016, indicated that when the standard (BHA) as well as extract concentration was 5 mg/mL, the absorbance values of reducing power were (3.00 ± 0.09) mg/mL and (2.43 ± 0.04) mg/mL of BHA/ extract respectively.

Total antioxidant assay follows the principal that chemistry of conversion of Mo (IV) to Mo (V) compounds in presence of reducing agents (antioxidants) which results in formation of green phosphate/Mo (V) complex which can provide maximum absorbance at 765 nm (Ahmed *et al*. 2015; Pisoschi *et al*. 2016). In present study, highest concentration of ascorbic acid equivalent total antioxidant capacity were found to be in order of RoMi-EE (584.98 ± 22.28) > RoAc-EE (512.87 ± 29.72) > RoAc-AE (478.57 ± 24.99) > RoMi-AE (287.3 ± 17.3) > RoPt-EE (198.35 ± 18.25) > RoPt-AE (189.94 ± 16.72) mg AAE/g of the dried extracts. The data obtained are presented in the **Figure 8**. Shah *et al.* 2013, have found the highest concentration in *Sida cordata* ethanolic extract (antioxidant value of 1.129 ± 0.01) with 200 µg/mL. Sasikumar and Kalaisezhiyen 2014, have studied the total antioxidant activity of leaves of *Kedrostis foetidissima* was found to be higher in methanolic extract with (60.88 ± 1) than other extracts and lowest activity was found in the petroleum ether extract with 17.99 ± 0.66 mg of AAE/g of extract. The total antioxidant activity of the five extracts, was found to decrease in decreasing order of *K. foetidissima* Methanolic extract > *K. foetidissima* Chloroform extract > *K. foetidissima* Aqueous extract > *K. foetidissima* acetone extract > *K. foetidissima* petroleum ether extract.

DPPH possesses proton free radicals with property of absorption that decreases on exposure of proton radical scavengers (Shah *et al.* 2013; Sravanthi & Rao 2015). The results of DPPH radical scavenging activities were expressed in IC_{50} value. The IC_{50} is the inhibitory concentration of the sample having the potential to scavenge 50% reactive oxygen species (ROS) or inhibit oxidation process by 50%. IC_{50} values are inversely related to the scavenging activity, higher the IC_{50} value means lower antioxidant activity and lower the value means higher antioxidant activity. In the current study, extracts showed high DPPH radical scavenging activity with increased concentration of extracts and ascorbic acid. The ethanolic root extract of *M. indica* showed highest percent inhibition of 54.36 ± 2.15% and acetone extract of *P. thyrsiflorus* roots showed lowest inhibition with only 30.05 ± 3.56% at only 160 µg/mL concentration. The IC_{50} values were found to be in order of Ascorbic acid

(48.93 µg/mL) > RoMi-EE (130.57 ± 12.46) > RoAc-EE (138.66 ± 11.41) > RoAc-AE (174.1 ± 21.18) > RoMi-AE (233.92 ± 14.46) > RoPt-EE (265.87 ± 17.58) > RoPt-AE (302.55 ± 35.68). The radical scavenging activity of *M. indica* roots ethanolic extract was better than that of *Lippia javanica* methanol leave extract that showed best IC_{50} value of 135.00 ± 1.49 µg/mL among other plant extracts (Narzary *et al.* 2016) and was also 3.5 folds better than the water extract of *Reinwardtia indica* leaves having IC_{50} value of 450 ± 0.07 µg/mL (Shukla *et al.* 2016) and that of seed extracts of *Parkia javanica* and leave extracts of *Phlogacanthus thyrsiflorus* reported by Chanu *et al.* 2012.

The method of ABTS radical scavenging activity depends on inhibition of the absorbance of ABTS radical cation. Decolonization of ABTS reflects the capacity of the antioxidant species to donate electrons or hydrogen atoms to inactivate these free radical chain reaction or oxidation of other molecules (Pellegrini *et al.* 2003). In the presence of antioxidant molecule, the coloured radicals were converted back to colourless ABTS. The assay is widely used to evaluate the antioxidant properties of plant extracts. In the present study, RoAc-EE showed lowest IC_{50} values having percent inhibition of 97.2 ± 2.28% and RoPt-AE showed highest IC_{50} values among the extracts having inhibition of only 58.97 ± 2.19% at 32 µg/mL concentration. The IC_{50} values of the standard is comparable to that of extracts and are in order of BHT (7.04) > RoAc-EE (7.94 ± 1.33) > RoAc-AE (8.81 ± 1.66) > RoMi-EE (8.82 ± 1.42) > RoMi-AE (12.75 ± 1.61) > RoPt-EE (17.89 ± 1.18) > RoPt-AE (24 ± 1.61). Shah *et al.* 2013, reported the IC_{50} value of 143 ± 0.8 µg/mL in the ethyl acetate fraction of *Sida cordata* whole plant methanolic extract. In another study by Ismail *et al.* 2015, *Nepenthes bicalcarata* methanolic leaves extract showed IC_{50} value of 16.13 ± 0.33 µg/mL. The values of RoAc-EE, RoAc-AE and RoMi-EE were 17 folds lower than the *S. cordata* and 2 folds better than that of *N. bicalcarata*. The results were also better than ethanolic leaf extract of *Celtis toka* (48.6 ± 6.8) Fall *et al.* 2017, rhizome methanol (137.3) and ethanol extract (125.01) of *Helicoria rostrata* (Moonmun *et al.* 2017) and the ethanol fruits extract of *Artemisia nilagirica* which showed the IC_{50} value of 300 µg/mL reported earlier by Suseela *et al.* 2010.

For the oxygen transport, respiration and for many enzyme activity, the Iron (II) is very much important. Chelating agents inhibits the process of lipid peroxidation by stabilizing the transition metals (Sasikumar & Kalaisezhiyen 2014; Pavithra & Vadivukkarasi 2015; Amaral *et al*. 2018). It is reported that metal chelating ability of leaf extracts of *K.*

foetidissima, the methanolic extract was most effective having EC_{50} value 1000 µg/mL and least effective was found in petroleum ether extract with EC_{50} value 7600 µg/mL (Sasikumar & Kalaisezhiyen 2014). This might be due to increase in secondary metabolite of the extracts. The chelating agents act as secondary metabolites which reduces the redox potential by stabilizing the oxidized form of metal ion (Shukla *et al.* 2016; Pavithra & Vadivukkarasi 2015). Study from the *Morus alba* fruits flavonoid extract, Raman *et al.* 2016, found little Fe^{2+} chelating capacity at low concentration but at 6000 µg/mL FEM reached 72.6% chelating activity. The results of the present study showed concentration dependent activity. At 1000 µg/mL concentration the RoPt-EE showed 72.06 ± 6.69% highest chelating activity followed by RoMi-EE with 50.06 ± 6.08% which is far better than the results obtained by Raman *et al.* 2016. The EC_{50} value of the samples/standard were in order of EDTA (63.33) > RoPt-EE (535.16 ± 121.56) > RoMi-EE (1038.6 ± 143.97) > RoPt-AE (1471.32 ± 91.7) > RoAc-EE (1500.43 ± 130.1) > RoAc-AE (1817.3 ± 183.26) > RoMi-AE (2006.9 ± 170.4). The EC_{50} value of RoPt-EE was showing 2 times better result than the *K. foetidissima*, whereas the RoMi-EE was comparable to that of *K. foetidissima* but were higher than the *Hyoscyamus squarrosus* fruits extract reported earlier by Ebrahimzadeh *et al.* 2009.

The conversion of H_2O_2 to hydroxyl radical might be toxic to the cells which is extremely reactive free radical formed naturally in the biological system and known to implicate highly destructive among the free radical species, and therefore, its inhibition is very much important in order to protect the body cells/tissues (Oyedemi & Afolayan 2011; Ozcan & Ogun 2015; Nimse & Pal 2015). The result indicated concentration dependent activity with highest percent inhibition observed in RoAc-EE with 44.8 ± 2.93 followed by RoMi-EE of 37.85 ± 6.23 which was depicted IC_{50} value of 12.67 ± 1.58 and 12.88 ± 1.54 µg/mL with slightly lower activity than BHA having inhibition of 57.23% and IC_{50} value of 7.59 µg/mL. The results were convincingly better than *B. lanceolaria* methanolic leave extract (Narzary *et al.* 2016) having highest percent inhibition of 73.52±0.04 % with IC_{50} value of 20.37 ± 0.01 µg/mL and was also better than that of Triphala reported earlier using the same methodology by Babu *et al.* 2013, having IC_{50} value of 16.63 ± 2.01 µg/mL. Higher scavenging activity of the extracts may be attributed to phenols and tannins presence which can donate electrons and thereby converting it into water Babu *et al.* 2013. Oyedemi and Afolayan 2011, found out *Schotia latifolia* hydroalcoholic stem bark extract could scavenge the 4mM H_2O_2 radicals with inhibition up to 86.48 % at 500µg/mL concentration which was depicted to IC_{50} value of 66 µg/mL. The IC_{50} value of the samples/standard were in order of

BHA (7.59) > RoAc-EE (12.67 ± 1.58) > RoMi-EE (12.88 ± 1.54) > RoAc-AE (15.82 ± 2.13) > RoMi-AE (16.9 ± 1.8) > RoPt-EE (17.89 ± 1.05) > RoPt-AE (18 ± 1.87).

Originally, Benzie and Strain developed the FRAP assay which measures the reducing power of the plasma (Benzie & Strain 1996). The method is based on the capacity of antioxidants that has the potentials to reduce the ferric complex (Fe^{3+}/ TPTZ) to the coloured ferrous complex (Fe^{2+} /TPTZ) at pH 3.6 (Apak *et al*. 2016; Adebiyi *et al*. 2017). The result interpretation is based on the assumption that the capability of extract/antioxidants which reduces ferric ions has the ability to reduce reactive oxygen species (ROS) (Pinchuk *et al.* 2012). *Dioscorea bulbifera* showed highest antioxidant capacity of 856.92 µmol Fe^{2+}/g, followed by *Tussilago farfara* with 455.64 µmol Fe^{2+}/g and least antioxidant property in the plant *Sargassum fusiforme* with 0.15 µmol Fe^{2+}/g (Song *et al.* 2010). According to the present result, highest antioxidant activity was in order of RoAc-AE> RoAc-EE> RoMi-EE> RoMi-AE> RoPt-AE> RoPt-EE having better ferrous ion concentration with 2512.7 ± 157.37, 2484.27 ± 135.3, 1116.4 ± 98.56, 1027.9 ± 115.03, 820 ± 110.63 and 751.67 ± 85.48 µmol Fe^{2+}/g respectively and are far better than the *D. bulbifera, T. farfara, Eriobotrya japonica* (437.40 µmol Fe^{2+}/g), *Ephedra sinica* (388.68 µmol Fe^{2+}/g) and *Arctium lappa* (223.68 µmol Fe^{2+}/g) and *S. fusiforme* except RoPt-AE and RoPt-EE which is slightly lower than the *D. bulbifera.* In an antioxidant study of methanolic extracts of 50 different medicinal plants by Gan *et al.* 2010, the highest Fe (II) concentration was found in methanolic extracts of *Loranthus parasiticus* (580.02 ± 31.32) followed by *Geranium wilfordii* (347.33 ± 7.99) and the least concentration was observed in *Poria cocos* (3.88 ± 0.15) µmol Fe(II)/g and were lower than the present results. The current results also showed better Fe(II) concentration than the ethanolic extract of Brazilian native fruits reported earlier by Denardin *et al.* 2015.

GC-MS profile indicated the presence of 1,2-Bis(Trimethylsilyl) Benzene; Silane, 1,4-Phenylenebis (Trimethyl-; Alpha.-Amyrin; 2,4,6-Cycloheptatriene-1-One, 3,5-Bis-Trimethylsilyl; Octadecanoic Acid, Ethyl Ester ; N-Hexadecanoic Acid; Eicosanoic Acid; 9,12-Octadecadienoic Acid (Z,Z)-; 1-Octadecyne; Pentadecanoic Acid-; Oleic Acid-; Urs-12-En-28-Ol -25.918-; Ethyl Iso-Allocholate-; 7-Dehydrocholesteryl Isocaproate-; 1-Methylene-2b-Hydroxymethyl-3,3-Dimethyl-4b-(3-Methylbut-2-Enyl)-C; 2,6,10-Dodecatrien-1-Ol, 3,7, 11-Trimethyl-9-(Phenylsulfonyl)-, (E,E); Lanosterol; 2,2-Dibromocholestanone; 3-O-Acetyl-6-Methoxy-Cycloartenol- and 2-Isopropyl-5-Methylcyclohexyl 3-(1-(4-Chlorophenyl) -3-Oxobutyl)-C, were found to be novel and first time reported from ethanolic root extracts of

Morus indica. Some of the compounds that were detected by the GC-MS analysis were having good biological activities (**Table 13**) that were reported earlier by various researchers.

Table 13: Some compounds with possible biological activity:

Compound name	Biological activity	References
Alpha-amyrin	Anti-inflammatory, anti-diabetic, anti-cancer, anti-arthritic, three times more potent than aspirin	Raman *et al.* 2012.
Octadecanoic acid, ethyl ester	Anti-bacterial and anti-fungal	Elela *et al.* 2009.
N-hexadacanoic acid	Block HIV-1 entry and infection. Anti-inflammatory, antioxidant, hypocholesterolemic nematicide, 5-alpha reductase inhibitor.	Lee *et al.* 2009. Abubakar and Majinda 2016.
Oleic acid	Antibacterial.	Abubakar and Majinda 2016.
Pentadecanoic acid	Lubricant and adhesive agents.	Arora and Kumar 2017.
Urs-12-En-28-Ol	Antimicrobial and anti-inflammatory activity.	Deepa and Selvakumar 2014.
Ethyl-iso-allocholate	Antimicrobial, diuretic, anti-inflammatory, Anti-asthma. Anticancer.	Muthulakshmi *et al.* 2012. Zekeya *et al.* 2014.
9, 12- Octadecadienoic acid (Z,Z)-	Anti-inflammatory, Hepatoprotective, Antiacne, Anticancer, Antiarthritic, Hypocholesterolemic, Nematicide, Anti-coronary, Alpha reductase inhibitor, Insectifuge, Antihistaminic, Antieczemic, Antiandrogenic.	Rajeswari *et al.* 2012.
1-Methylene-2b-hydroxymethyl-3,3 -dimethyl-4b-(3-methylbut -2-enyl)-cyclohexane.	Anti-inflammatory, Anti-hyperlipidemic, Antimicrobial.	Jasmine *et al.* 2013.

Oxidative stress and inflammation are not always harmful, they help phagocytes to kill microorganisms and modulate signaling events through redox regulation (Gordillo *et al.*

2017; Biswas 2016; Kasote *et al*. 2015). However, unregulated and prolonged imbalance in the liver between the production of free radicals and/or reactive oxygen species (ROS) and their elimination by protective mechanisms (antioxidants) leads to damage of important biomolecules and cells, with potential impact on whole organism causing many chronic diseases (Ksouri *et al.* 2015). Various studies have also shown that *M. indica* is having anti-inflammatory activity (Chatterjee *et al.* 1983; Balasubramanian 2005; Oh *et al.* 2010).

Numerous studies have demonstrated the effects of CCl_4 and its interventions on the liver. CCl_4 is a classic compound commonly used for xenobiotic-induced hepatic injury to explain the pathogenesis of hepatic steatosis in the experimental animal model (Johnston & Kroening 1998; Chen *et al.* 2012; Ma *et al.* 2015; Lee *et al.* 2019). The liver injury is due to the ROS-induced oxidative stress that can generate toxic lipid intermediates (Ksouri *et al.* 2015). Weber *et al.* 2003, understood in their study that CCl_4 ingestion activates cytochrome system (i.e., CYP2E1) and forms trichloromethyl radicals (CCl_3). It becomes toxic because of a reactive intermediate generated by its reductive metabolism, and this highly reactive intermediate is known to induce leakage of serum enzymes (AST, ALT, ALP, and GGT), lipid peroxidation, depletion of antioxidant capacity and hepatic necrosis around the central vein. There are other important indices to evaluate the hepatic function, such as TC, TG, HDL, LDL, VHDL, total protein and albumin level in serum.

The current *in-vivo* study demonstrated that the CCl_4-induced control group had a significant increase in the activities of liver indices suggesting acute cellular damage which signifies elevated levels of serum enzymes activities and other indices (Karakus *et al.* 2011; Li *et al.* 2016; Lee *et al.* 2019; Srivastava & Shivanandappa 2010). On the other hand, daily administration of RoMi-EE to CCl_4-induced hepatotoxic rats attenuated the increased activity of liver marker enzymes and alleviated the loss of functional integrity of the cell membrane, indicating its hepatoprotective activity. RoMi-EE at 200 mg/kg concentration was able to pull down the levels of these serum enzymes compared to standard drug silymarin, which showed a better index. Several study, (Wills & Asha 2006; Mani *et al*. 2016) signify that after liver injury, AST and ALT progresses from the cytoplasm to circulatory system because of the toxicity mediated transformed permeability of the cellular membrane.

Creatinine is an important parameter indicating the health of both the liver as well as the kidneys. It was evident from the previous studies that the administration of CCl_4 induced a renal failure indicated by elevation of creatinine level (Abdel Monein & El-Deib 2012; Al-

Yahya *et al.* 2013; Abdulhameed *et al.* 2017). Creatinine is a by-product of muscle metabolism that is excreted unchanged by the kidneys, hence making it an important indicator of renal health. Upon treatment with RoMi-EE, higher concentration (200 mg) showed 0.71 ± 0.04 mg/dL creatinine level which was comparable with that of silymarin (0.70 ± 0.01 mg/dL) with no significant change.

Furthermore, the histopathological examination of the liver provided evidence of the effects of investigated components against acute CCl_4-induced liver injury and also substantiated the biochemical analysis. The histology showed that CCl_4 administration caused serious oxidative liver damage to characterize by severe necrosis, inflammation, hepatocellular degeneration, cytoplasmic vacuolation and loss of cellular boundaries, which confirmed with the previous studies for the live injury (Lee *et al*. 2019; Maheshwari *et al.* 2011; Vuda *et al.* 2012; Nwidu *et al*. 2017). Treatment with RoMi-EE was noteworthy in a dose-dependent manner as it reduced the severity caused due to oxidative damage. This was evidenced by a decrease in necrosis and hemorrhage. Hence, it clearly indicates the protection provided by the administration of RoMi-EE. The high dose of RoMi-EE (**Fig 24**) induced an effect close to normal emergence, recommending that the high dose of 200 mg/kg was more effectual than the lower dose. CCl_4 group wistar rat kidney (cortex) cross section showed vacuolation, glomerular atrophy, widening of capsule space, cell layer thickening and degeneration of cells which were also reported earlier by Abdulhameed *et al.* 2017; Jan & Khan 2016; Yoshioka *et al.* 2016; Sukandar *et al.* 2013. After administration of 100 mg RoMi-EE and 200 mg RoMi-EE experimental drug, the recovery of glomerular atrophy, capsule space decrease and less degeneration of cell was observed. However the 200 mg RoMi-EE experimental drug were better than the low concentration of RoMi-EE and was comparable to that of silymarin having identical structure in kidney histopathology (**Fig 25**) and was also similar to that of normal group.

Antioxidant enzymes, such as SOD, CAT, and GPx are the endogenous enzymes which form an imperative part of the antioxidant defense system. They detoxify the free radicals and thus protect the hepatic cells against oxidant-mediated injury. Treatment with CCl_4 alone can deplete the activity of these enzymes. It also depletes the hepatic GSH system, which is a key component of the overall antioxidant defense system and can also cause lipid peroxidation resulting in liver cirrhosis (Lin *et al.* 2008; Khan *et al.* 2012; Kasote *et al*. 2015). As shown in **Fig 19-21**, the decline in the levels of antioxidant enzymes were observed in CCl_4 treated rats which are clear indicator of excessive formation of hepatic lipid

peroxidation in comparison to the normal group. This has also been reported in previous studies (Tung *et al.* 2009; Maheshwari *et al.* 2011; Lee *et al.* 2019; Ogaly *et al*. 2018; Nwidu *et al*. 2017). On the contrary, the groups treated with RoMi-EE at two different doses significantly increased the levels of SOD, CAT and GPx activities, increased GSH contents and reduced the lipid peroxidation (MDA) level in liver. The preventive effect of RoMi-EE at 200 mg/kg was similar to that of silymarin treatment.

Identification and characterization of binding sites is the key in the process of structure-based drug design (Halgren 2009; Ferreira *et al*. 2015). In some cases there may not be any information about the binding site for a target of interest. In other cases, a putative binding site has been identified by computational or experimental means, but the druggability of the target is not known. Even when a site for a given target is known, it may be desirable to find additional sites whose targeting could produce a desired biological response (Halgren 2009). A new program, called Site Map, is presented for identifying and analyzing binding sites and for predicting target druggability and the Site Score can be used as one criterion for deciding whether to target a given site (Halgren 2009; Patschull *et al.* 2012), but they do not show that a site that scores well is a drug-binding site, sometime it could be (Halgren 2009). Hence, druggability predictions are important to avoid intractable targets and to focus drug discovery efforts on sites offering better prospects (Schmidtke & Barril 2010).

As previously noted, many drug-design projects fail because the target proves not to be druggable (Liu & Altman 2014). Accurate determination at an early stage of whether a given protein is or is not druggable therefore has the potential of saving considerable time and expense (Halgren 2009). Druggability predictions are important to avoid intractable targets and to focus drug discovery efforts on sites offering better prospects and is directly associated to a cavity detection method, screening for druggable cavities in large structural data sets is straight forward (Schmidtke & Barril 2010).

In the current docking study with that of 1NFK protein, the silymarin (CID: 6610285) showed 5 hydrogen bonding with the residues of Gly 162, Glu 179, Asp 220, Ser 221 and Ser 224 of 1NFK site-4, which showed the highest docking score of -5.956. Following the silymarin the compound 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)- (CID: 5368759) showed best docking score among the ligands with -4.958 and also showed two hydrogen bonding with residues of Lys 95 and Arg 161. The molecular mechanics/ generalized born suface area (MMGBSA) ΔG binding affinity were in order of

silymarin (-54.79) > 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)- (-45.35) > Ethyl Iso-Allocholate- (-39.5) > octadecanoic acid, ethyl ester (-33.85) > 1,2-Bis(Trimethylsilyl) Benzene (-29.05) > Alpha.-amyrin (-27.59) > 2,4,6-Cycloheptatrien-1-One, 3,5-Bis-Trimethylsilyl- (-26.24) > 1-Methylene-2b-Hydroxy methyl-3,3-Dimethyl-4b-(3-Methylbut-2-Enyl)-C (-5.67).

Glushchenko *et al.* 2015, studied flexible molecular docking of chemical compositions of *Bupleurum aureum* plant with NFκB protein (PDB: 1VKX) with "dock into active site" function using Scigress software and found highest docking score in rutin with -3.26. Other notable outcomes are Eicosanoic Acid [CID: 8122]- (Docking score -4.528 & ΔG of -33.85); Ethyl Iso-Allocholate [CID: 6452096]- (Docking score -3.679 & ΔG of -39.5); 1-Methylene-2b-Hydroxy methyl-3,3-Dimethyl-4b-(3-Methylbut-2-Enyl)-C [CID: 550196]- (Docking score -3.277 & ΔG of -5.67); 1,2-Bis(Trimethylsilyl)-benzene [CID: 519794]- (Docking score -3.001 & ΔG of -29.05).

Several studies have also revealed that CCl_4 induction causes increase in concentration of pro-inflammatory markers (*viz*; cytokines, TNF-α, PGE-2, IL-6) due to which elevation in the activity of COX-2 enzymes can be observed which is one of the major inflammatory protein and are also involve in liver injury (Shah *et al*. 2017, Zhang *et al*. 2004, Gunalan *et al.* 2014; Oh *et al.* 2010).

Docking study with that of 3LN1 revealed that the compound: 2,6,10-Dodecatrien-1-Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E), showed best docking score (without violating Lipinski *et al*. 1997, rule of five) among ligands in both docking with 1NFK and 3LN1 proteins. Although the silymarin had shown best docking score in 1NFK receptor, but it didn't show any type of interaction with that of 3LN1 receptor, which clearly indicates the silymarin compound as target specific activity. Amaravani *et al*. 2012, showed that COX-2 has the active binding site of Ala 185, Phe 186, Phe 187, Ala 188, Gln 189, His 190, Thr 192, His 193, Gln 194, Phe 196, Thr 198, Asn 368, Leu 370, Tyr 371, His 372, Trp 373, His 374, Leu 376, Leu 377, Val 433, Ser 437, Gln 440, Tyr 490, Leu 493, Leu 494 amino acids at the active binding pocket. The ligand 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E), have showed Pi-Pi interaction with that of aromatic ring of Trp 373 of the target protein and also showing the best ΔG binding affinity of -27.8173 kcal/mol and was better than the Celecoxib (standard ligand with -17.27 kcal/mol) reported earlier by Lamie *et al.* 2015. The other ligands which showed interaction with both 1NFK and 3LN1

proteins without violating Lipinski rule of five (Lipinski *et al.* 1997) are CID: 550196 (1-Methylene-2b-Hydroxy methyl-3,3-Dimethyl-4b- (3-Methylbut-2-Enyl)-C); CID: 519794 (1,2-Bis(Trimethylsilyl) Benzene) and CID: 610038 (2,4,6-Cycloheptatrien-1-One, 3,5-Bis-Trimethylsilyl-).

The adsorption distribution metabolism and excretion (ADME) properties of ligands revealed that, except for the ligands Octadecanoic acid, ethyl ester (CID: 8122), alpha.-amyrin (CID: 73170), 9,12-Octadecadienoic Acid (Z,Z)-(3931), 1-Octadecyne (69425), Oleic Acid- (445639) and N-Hexadecanoic Acid (985), all the other ligands were in the acceptable range of Lipinski's rule of five (Lipinski *et al.* 1997) and the log P value of the ligands (except CID: 8122, 73170, 3931, 69425 and 445639) also suggests that it cannot cross the blood–brain barrier and hence it can be used as drug in other organs or body parts without affecting the brain tissue (Raghavan *et al.* 2012), indicating their potential for use as drug-like molecules.

Based on the docking study it was revealed that the ligand 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)- is comparable with that of silymarin while docking with 1NFK protein and also showed high docking score as well as binding affinity with 3LN1 docking study. From the above outcome it can be proposed that the compound can be utilized for inhibiting NFκB as well as COX-2 inflammatory proteins.

CHAPTER-VII

7. CONCLUSION

Significant number of individuals around the world prefer herbal medicines rather than conventional medicines. The present survey it was concluded that a total of 40 plants from 26 families and 40 genus hepatic inducing medicinal plants have been in constant use. Leaves recorded to be highest in use (29 types) followed by roots (10 types). Indigenous communities/ healers have their own conventional medicine system which may use single or combination of different plant parts.

Presence of various phytochemicals viz: phenols, flavonoids, tannins, resins, terpenoids, glycosides and steroids have been detected from the selected plants in qualitative test. In the quantitative test, higher contents of phenolics, flavonoids, total antioxidant capacity and higher activity in total reducing power assay were obtained especially in the RoMi-EE and RoAc-EE. Increase in radical scavenging activity was observed with increase in concentration in the *in-vitro* antioxidant tests of DPPH, ABTS and H_2O_2 having lowest IC_{50} values observed in the RoMi-EE, RoAc-EE and RoAc-EE respectively. In case of ICC and FRAP assay, the lowest EC_{50} value and highest $FeSO_4.7H_2O$ concentration was observed in RoPt-EE (535.16 ± 121.56 µg/mL) and RoAc-AE (2512.7 ± 157.37 µM/mg) respectively.

The GC-MS analysis have revealed the presence of various bioactive compounds that were reported for the first time from the root extracts of *M. indica*, and were reported to have various biological activities such as antibacterial, anti-inflammatory, anti-diabetic, anticancer, anti-arthritic, hepatoprotective, block HIV-1 entry and infection, anti-asthma, etc.

In an *in-vivo* study, the elevated levels of liver serum enzymes (ALT, AST and ALP) induced by CCl_4 as compared to normal group was markedly decreased after treatment with RoMi-EE. After the CCl_4 administration, there was significant increase in the concentration of TC, TG, LDL, VLDL and decrease in the HDL level. However after treatment with test drug RoMi-EE, decrease in the TC, TG, LDL, VLDL and increase of HDL levels were observed.

The serum bilirubin, GGT and creatinine level in the CCl_4 treatment was significantly increased as compared to normal group. After the treatment with RoMi-EE, there was decrease in the levels of bilirubin, GGT and creatinine concentration. In the silymarin group, creatinine level was almost back to the normal. Protein and albumin levels were found to be

low in CCl_4 group. However, the albumin level showed significant increase in RoMi-EE and silymarin group, whereas no significant change was observed in the total protein level.

Meanwhile, significant increase in the antioxidant enzymes viz: SOD, CAT and GPx was noted in the RoMi-EE and silymarin treated groups which was decreased due to CCl_4 treatment. The GSH activity was also improved by 25% upon treatment with two dose of RoMi-EE and were comparable with that of silymarin group. The activity of RoMi-EE and silymarin also reduced the MDA content significantly, which was elevated by 48% upon treatment with CCl_4.

The histopathological reports also revealed that CCl_4 induced severe hepatocyte necrosis, inflammation, biliary cirrhosis, vacuolation, microvesicular steatosis and infiltration of kupffer cells around the central vein than the normal liver architecture observed in healthy rats. CCl_4 group kidney cross section also showed vacuolation, glomerular atrophy, widening of capsule space, cell layer thickening and degeneration of cells. After treatment with different concentrations of RoMi-EE, the severity of CCl_4 induced liver and kidney intoxication was reduced in a dose-dependent manner, although the treatment with silymarin showed much better result.

The *in-silico* molecular docking results (also support *in-vivo* and histopathological study) conducted on 1NFK and 3LN1 proteins have revealed that the compound 2, 6, 10-Dodecatrien-1-Ol, 3, 7, 11- trimethyl-9- (phenyl sulfonyl) –(E,E)- showed best docking score among the selected ligands with -4.958 & ΔG binding affinity of -45.35 kcal/mol in 1NFK and docking score of -9.78 & ΔG binding affinity of -27.8173 kcal/mol from that of 3LN1. However, silymarin showed -5.956 better docking score than the ligands with ΔG binding affinity of -54.79 kcal/mol in 1NFK protein, but didn't show any binding affinity with that of 3LN1 protein. The ligand 2, 6, 10-Dodecatrien-1-Ol, 3, 7, 11- trimethyl-9- (phenyl sulfonyl) –(E,E)- was also in acceptable range of Lipinski's rule of five, indicating their potential for use as drug-like molecule.

From the finding, it was concluded that the *Morus indica* root, which is used traditionally by the local tribe of BTAD, Assam, have shown high contents of antioxidant and *in-vivo* activity can be an alternative for treating liver disorders.

CHAPTER-VIII

8. SUMMARY

From the study it is evident that people prefer herbal medicines rather than conventional medicines. Many indigenous communities/ traditional healers have their own conventional medicine system with diverse medicinal plants for treating many untreatable diseases and may use single or combination of different plant parts.

The present survey of hepatic inducing medicinal plants traditionally utilized by local tribe, a total of 40 plants from 26 families and 40 genus have been documented. The tribe uses 29 types of leaves, 10 types of roots, 2 types of flowers and fruits, and one each type of barks and seed. The Moraceae and Rutaceae were reported highest with 3 plants each.

Phytochemical screening from the selected plants for the study showed presence of various phytochemicals that include phenols, flavonoids, tannins, resins, terpenoids, glycosides and steroids. The extract also showed high contents of total phenolics and flavonoids especially in the root extracts of RoMi-EE and RoAc-EE. The same extract also indicated higher activity in total reducing power and total antioxidant capacity. In the *in-vitro* antioxidant test, increase in radical scavenging activity was observed with increase in concentration. In the radical scavenging activity of DPPH, ABTS and H_2O_2, the lowest IC_{50} values were observed in the extract of RoMi-EE, RoAc-EE and RoAc-EE respectively. In case of ICC, the extract that showed lowest EC_{50} value was observed in RoPt-EE, whereas in FRAP method, highest concentration of $FeSO_4.7H_2O$ µM/mg in extract was observed in RoAc-AE. Apart from the DPPH and ICC, the IC_{50} values of the extracts were comparable with that of the standard used in ABTS (BHT) and H_2O_2 (BHA) radical scavenging activity.

The GC-MS analysis also showed the presence of various bioactive compounds that were reported for the first time from the root extracts of *Morus indica,* and were reported to have various biological activities such as antibacterial, anti-inflammatory, anti-diabetic, anticancer, anti-arthritic, hepatoprotective, block HIV-1 entry and infection, anti-asthma, hypocholesterolemic etc,.

In the present *in-vivo* study conducted on the CCl_4 induced acute liver damage, the RoMi-EE markedly decreased the elevated levels of liver serum enzymes such as ALT, AST and ALP in the control group as compared with the normal group. After the CCl_4 administration, there was significant increase in the concentration of TC, TG, LDL, VLDL

and decrease in the HDL level. However after treatment with test drug RoMi-EE, decrease in the TC, TG, LDL, VLDL and increase of HDL levels were observed.

The creatinine level in the CCl_4 treatment was significantly increased as compared to normal group. But, when the experimental rats were treated with different dosage of RoMi-EE, the creatinine level was decreased and was almost back to the normal in the silymarin treated group.

The protein and albumin levels were found to be low in CCl_4 group. After treatment with RoMi-EE and silymarin, the albumin level showed significant increase whereas the total protein level in the treatment group saw no significant changes. In case of serum bilirubin and GGT level the elevation was observed in CCl_4 treated group as compared to normal group. After the treatment with RoMi-EE and silymarin, there was decrease in the levels of GGT and bilirubin concentration.

While the antioxidant enzyme such as SOD, CAT and GPx were markedly decreased after CCl_4 administration. The significant increase in the antioxidant enzymes was noted in the RoMi-EE and silymarin treated groups. The activity of GSH was also reduced by 25% in the CCl_4 treated group as compared to normal group. Upon treatment with two dose of RoMi-EE, the activity was ameliorated and were comparable with that of silymarin group. The activity of RoMi-EE and silymarin also reduced the MDA content significantly, which was elevated by 48% upon treatment with CCl_4.

The results of *in-vivo* study showed that the RoMi-EE high dose treatment (200 mg) was comparable with the standard drug silymain which was effective in reverting the biochemical parameters in diseased animals.

The histopathological reports also revealed that CCl_4 induced severe hepatocyte necrosis, inflammation, biliary cirrhosis, vacuolation, microvesicular steatosis and infiltration of kupffer cells around the central vein than the normal architecture observed in healthy rats. After treatment with RoMi-EE, the severity of CCl_4 induced liver intoxication was reduced in a dose-dependent manner, although the treatment with silymarin showed much better result. The cross section through cortex kidney of CCl_4 group showed vacuolation, glomerular atrophy, widening of capsule space, cell layer thickening and degeneration of cells. The recovery of glomerular atrophy, decrease in capsule space and less degeneration of cell was

observed in 100 mg RoMi-EE experimental drug (100 mg & 200 mg RoMi-EE). Whereas silymarin showed identical structure and was similar to that of normal group.

The *in-vivo* experiment and the histopathological study were well supported by the *in-silico* molecular docking analysis using Maestro, Schrodinger software. The 1NFK and 3LN1 proteins were selected for docking (against ligands identified in the GC-MS analysis) based on the literature available. The ligands which showed interaction with both 1NFK and 3LN1 proteins without violating Lipinski rule of five are CID: 5368759 (2, 6, 10-Dodecatrien-1-Ol, 3, 7, 11- trimethyl-9- (phenyl sulfonyl) –(E,E)-), CID: 550196 (1-Methylene-2b-Hydroxy methyl-3,3-Dimethyl-4b- (3-Methylbut-2-Enyl)-C); CID: 519794 (1,2-Bis(Trimethylsilyl) Benzene) and CID: 610038 (2,4,6-Cycloheptatrien-1-One, 3,5-Bis-Trimethylsilyl-). However, among the selected ligands, the compound 2, 6, 10-Dodecatrien-1-Ol, 3, 7, 11-trimethyl-9- (phenyl sulfonyl) –(E,E)- showed best docking score of -4.958 having ΔG binding affinity of -45.35 kcal/mol and was comparable with that of silymarin which showed -5.956 docking score with MMGBSA ΔG binding affinity of -54.79 kcal/mol in 1NFK docking. The compound 2, 6, 10-Dodecatrien-1-Ol, 3, 7, 11- trimethyl-9- (phenyl sulfonyl) –(E,E)- also showed best docking score of -9.78 having ΔG binding affinity of -27.8173 kcal/mol in docking with 3LN1 protein, whereas the silymarin didn't show any binding affinity. The ADME properties of ligands revealed that, except for the ligands Octadecanoic acid, ethyl ester (CID: 8122), Alpha.-amyrin (CID: 73170), 9,12-Octadecadienoic Acid (Z,Z)-(3931), 1-Octadecyne (69425), Oleic Acid- (445639) and N-Hexadecanoic Acid (985), all the other ligands were in the acceptable range of Lipinski's rule of five, indicating their potential for use as drug-like molecules.

Overall the study suggest that the *Morus indica* root, which is used traditionally by the local tribe of BTAD, Assam, have shown high contents of antioxidant and *in-vivo* activity can be an alternative for treating liver disorders.

CHAPTER-IX

9. SALIENT FEATURES OF THE STUDY

Thorough study has been conducted during the last five years of research work and based on that it can be concluded that the plant *Morus indica* L., which is utilized by the traditional herbal practitioner of Kokrajhar, Assam is having very good antioxidant properties as well as hepatoprotective activity. Some of the salient features of the study are as follows:

1. From the survey as well as literature review, it was found that 4 plants (*viz. Pogostemon plectranoides* Desf., *Amphineuron opulentum* (Kaulf.), *Hygrophila plomoides* Nees. and *Morinda angustifolia* Roxb.) out of 40 different medicinal plants surveyed for the study, were not reported earlier to have hepatoprotective activity and no significant scientific work were carried out.
2. The roots of *Morus indica* L. is bestowed with excellent antioxidant property and also showed good hepatoprotective activity.
3. Ligands selected from the GC-MS analysis also provided good binding affinity with the NFκB (1NFK) and COX-2 (3LN1) anti-inflammatory proteins and were also comparable with that of proven standard drugs.

CHAPTER-X

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APPENDIX-A

A1: QUESTIONNAIRES USED

Questionnaire that were employed to interview the local population about their knowledge of plants which were used in the treatment of liver disorders.

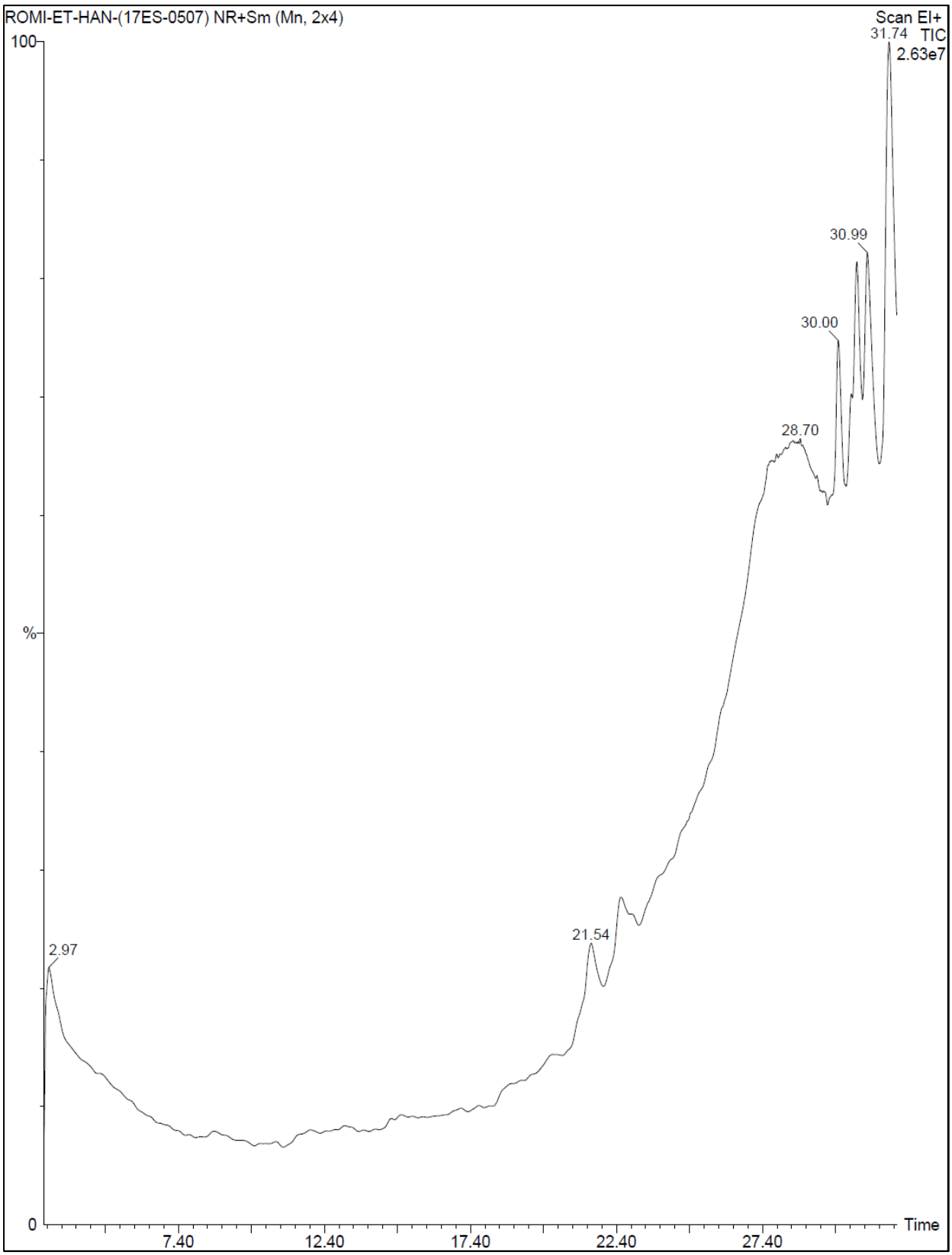
1. Date of interview and location
2. Name of the respondent (optional)
3. Gender of respondents:
4. Age of the respondents:
5. Occupation of the respondents: Traditional medicinal practitioners, Herb seller, Govt. servent, Farmer Others
6. Educational status of the respondent:
7. Religion of respondent:
8. Source of the knowledge: Parental, Training, Divination, Others
9. Name of plants used:
10. Parts of plant used:
11. Nature of plants: Shrub, Herb, Climber, Tree, Sucker
12. Place of plant collection: Cultivated, Wild
13. Do you use single or combination:
14. Mode of preparation (Optional):
15. Mode of administration:

A2: DEMOGRAPHIC CHARACTERISTICS OF THE RESPONDENTS (N = 91):

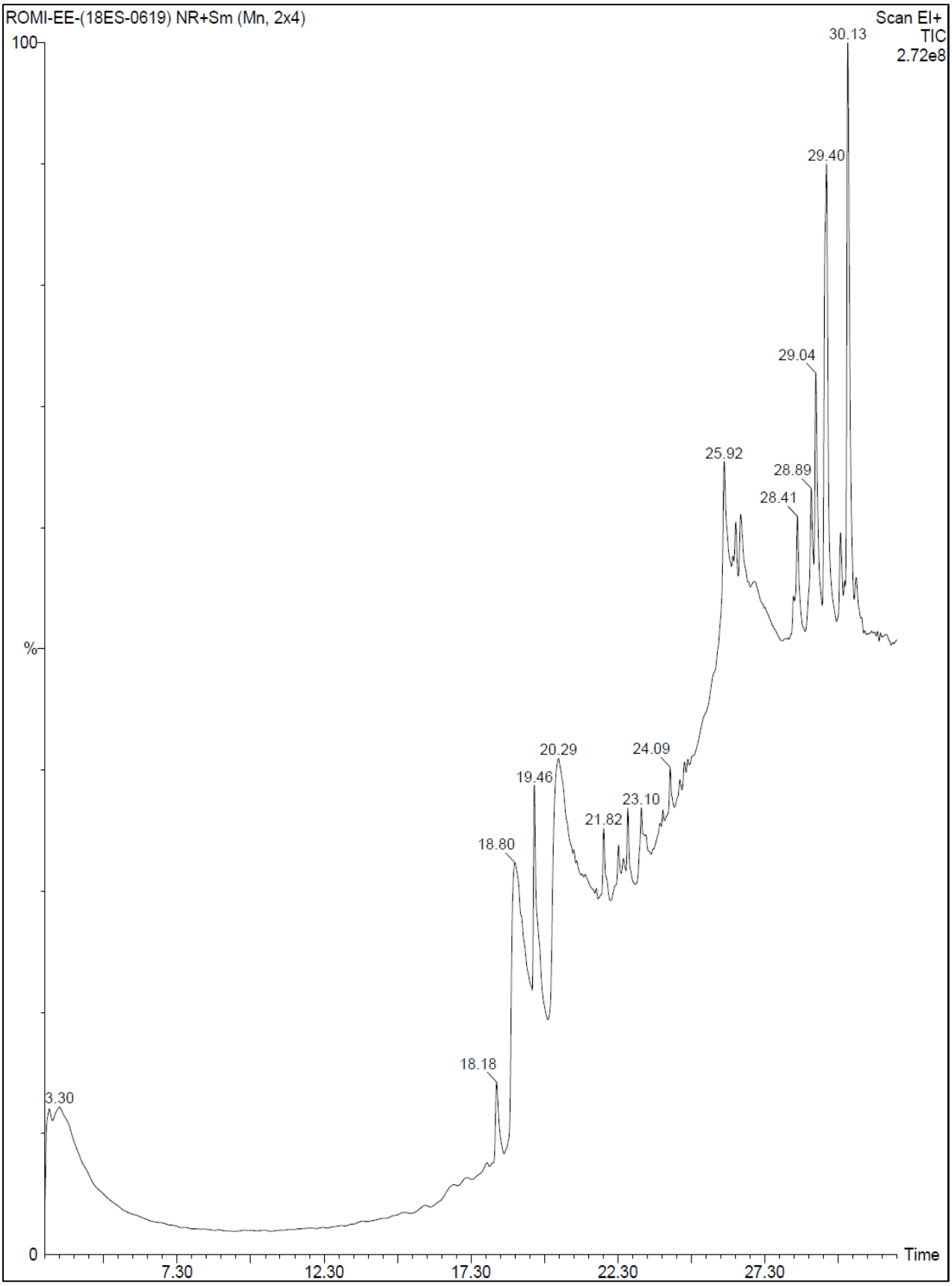
Parameters	Specifications	N (%)
1. Sex	Male	36 (39.56)
	Female	55 (60.44)
2. Age	21-30	7 (7.69)
	31-40	15 (16.48)
	41-50	37 (40.66)
	51-60	21 (23.08)
	61 & above	11 (12.09)
3. Occupation	Traditional herbal practitioner	9 (9.89)
	Herb seller	40 (43.96)
	Farmers	21 (23.08)
	Govt. servant	14 (15.38)
	Others	7 (7.69)
4. Educational status	Primary	26 (28.57)
	Matriculate and above	11 (12.09)
	Graduate and above	9 (9.89)
	No formal education	45 (49.45)
5. Religion	Hindu	69 (75.82)
	Christian	18 (19.78)
	Muslim	4 (4.4)
	Others	0 (0)
6. Source of knowledge	Parental	34 (37.36)
	Training	16 (17.58)
	Divination	0 (0)
	Others	41 (45.05)

APPENDIX-B

B. GC-MS CHROMATOGRAM



Title (B1): GC-MS 1st chromatogram result of 70% ethanolic extracts of *Morus indica* roots.



Title (B2): 2nd chromatogram GC-MS analysis of 70% ethanolic extracts of *Morus indica* roots.

APPENDIX-C

C. CHEMICALS USED

1, 1, 3, 3- tetramethoxy propane

ABTS

Acetone

Acetic acid

Alanine transaminase

Alkaline copper sulphate

Alkaline phosphatase

Aluminium chloride hexahydrate

Ammonia

Ammonium molybdate

Ascorbic acid

Aspartate transaminase

Benzene

BHA

BHT

Bovine serum albumin

Bradford

Butanol

Chloroform

Carbonate buffer

DPPH

Dragendorff's reagent

DTNB

Di-potassium hydrogen phosphate

EDTA

Eosin

Epinephrine

Ethanol

Fehling's A and B

Ferric chloride

Ferrous chloride

Ferrous sulphate heptahydrate

Ferrozine

Folin-catecholamine reagent

Folin–ciocalteau

Formalin

Gallic acid

Gamma-glutamyl trans-peptidase

Glacial acetic acid

Gluthathione reductase

Hematoxylin

Hydrochloric acid

Hydrogen peroxide

Lead acetate

Methanol

Na-Nitroprusside

NADPH

Nicotinamide

Paraffin

Potassium acetate

Potassium ferricyanide

Potassium di-hydrogen phosphate

Potassium hydroxide

Pyridine

Quercetin

Reduced GSH

Sodium bicarbonate

Sodium carbonate

Sodium chloride

Sodium dodecyl sulphate

Sodium hydroxide

Sodium phosphate

Sulfosalicylic acid

Sulphuric acid

Silymarin

Tert-butyl hydro peroxide

Tetramethoxy propane

Thiobarbituric acid

TPTZ

Trichloroacetic acid

Tripton-x 100

Xylene

APPENDIX-D

D. INSTRUMENTS USED

1. Balance (Denver instrument)
2. Trinocular Microscope (Olympus)
3. PC based double beam spectrophotometer-2202 (Systronics)
4. Spetrometer (Kinetics)
5. Centrifuge (RV/FM, Super Spin, Plastocraft, India)
6. Deep Freeze (White Whale, WF 3046KSS and FORMA 700 Series)
7. Hot Air Oven (NSW India)
8. GCMS (Perkin Elmer Turbo Mass) Spectrophotometer (Norwalk, CTO6859, USA)
9. Incubator (Optics)
10. Lyophiliser (Telstar Lyoquest Freeze Dryer)
11. Micropipette (Eppendorf)
12. Mixer Grinder (Bajaj Rex 500)
13. pH Meter (Elico pH Meter LI617)
14. Rotary Evaporator (Superfit ROTA VAP Model- PBU-6D)
15. Soxhlet Apparatus (Borosil)
16. Ultra Deep Freezer -80°C (Blue Star, CRESCENT)
17. Water Bath Incubator Shaker (Remi Model No. KWBS-2)
18. Fridge (Godrej)
19. Double distillation unit (Borosil)
20. Computer Desktop (HP- 8GB RAM)

APPENDIX-E

E. THESIS RELATED PUBLICATIONS

1. Boro H, Rava M, Ali R and Das S. Phytochemical screening, total phenolic and antioxidant capacity of root extracts of *Morus indica* L. International Journal of Pure and Applied Bioscience, 2017; 5 (5): 1582-1589.

2. Boro H, Mashahary K and Das S. GC-MS analysis, phytochemicals and *in-vitro* antioxidant properties of root extracts of *Phlogacanthus thyrsiflorus* Nees., Western Assam, India. International Journal of Pharmaceutical Sciences and Research, 2019; 10(6): 3012-3021.

3. Boro H, Rava M, Ali R and Das S. Traditional utility of some hepatoprotective plants used by Bodo tribe of Bodoland Territorial Autonomous District, Assam. ECOBIOS, 2014; 7 (1 & 2): 26-46.

APPENDIX-F

F. SEMINAR PRESENTATION

1. Presented a paper in national seminar on the topic **"GC-MS Analysis and Antioxidant Properties of Root of *Morus indica L*. (RoMi),"** on 17th Aug/ 2018, organized by Dept. of Herbal Science and Technology, Anandaram Dhekial Phookan College, Nagaon, Assam.
2. Presented a national seminar on the topic **"Traditionally used ethno botanical study of hepatoprotective medicinal plants utilized by the tribes of BTAD area"** on 28th May/2015, Organized by Centre for Bodo Studies, Bodoland University, Kokrajhar, Assam.

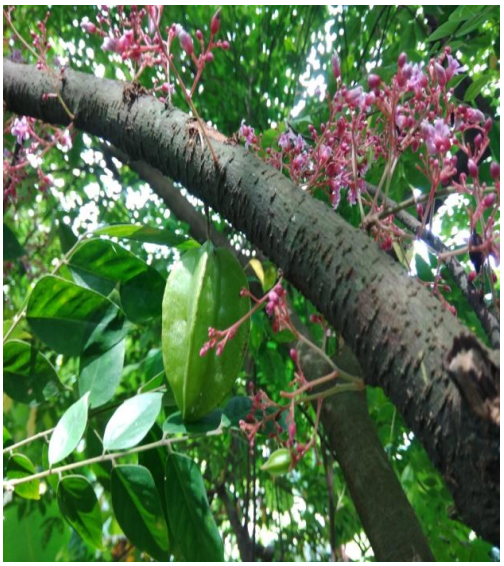
APPENDIX-G

G1. IMAGES OF PLANT ROOTS COLLECTED

Root images of **A.** *Morus indica*, **B.** *Averrhoa carambola* & **C.** *Phlogacanthus thyrsiflorus*.

G2. IMAGES OF PLANTS

1. *Morus indica* L.



2. *Averrhoa carambola* L.

3. *Phlogacanthus thyrsiflorus* Nees.

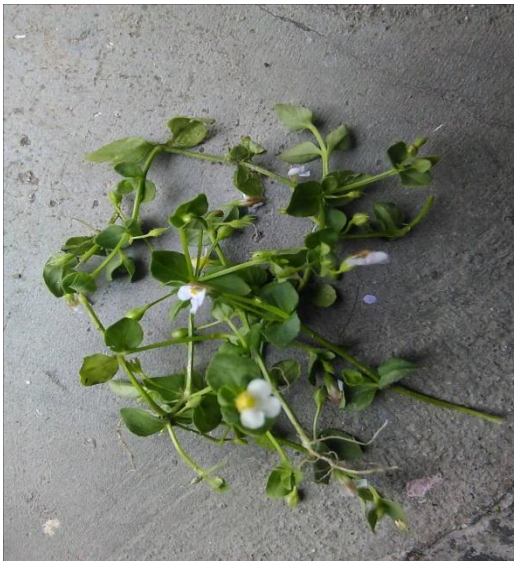
4. *Physalis minima* L.

5. *Stephania japonica* Thunb.

6. *Clerodendrum cordatum* D. Don.

7. *Morinda angustifolia* Roxb.

8. *Costus speciosus* (J. Koenig) Sm.



9. *Stellaria media* L.

10. *Justicia adhatoda* L.

11. *Houttuynia cordata* Thunb.

12. *Mollugo pentaphylla* L.

13. *Murraya koenigii* L.

14. *Premna herbacea* Roxb.

15. *Artocarpus heterophyllus* Lam.

16. *Oldenlandia diffusa* (willd.) Roxb.

17. *Plumbago zeylanica* L.

18. *Hypericum japonicum* Thunb.

19. *Leucas indica* L.

20. *Dillenia indica* L.

21. *Spilanthes paniculata* wall.ex DC.

22. *Citrus medica* L.

23. *Psidium guajava* L.

24. *Scoparia dulcis* L.

25. *Ficus religiosa* L.

26. *Glycosmis pentaphylla* (Rets). DC.

27. *Andrographis paniculata* Nees.

28. *Artemisia vulgaris* L.

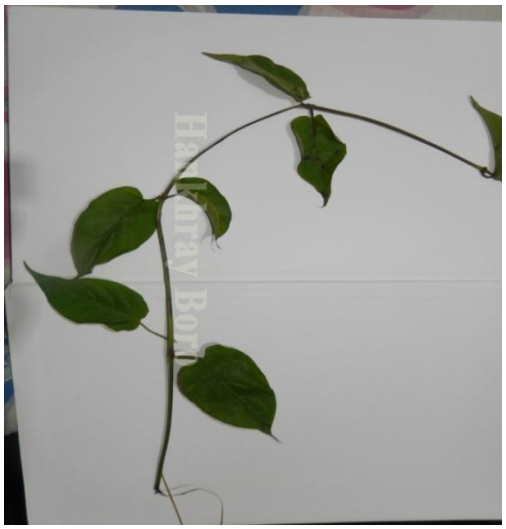
29. *Hygrophila phlomoides* Nees.

30. *Pogostemon plectranoides* Desf.

31. *Hydrocotyle sibthorpioides* Lam.

32. *Centella asiatica* (L.) Urb.

33. *Cuscuta reflexa* L.



34. *Paederia foetida* L.

35. ***Azadiractha indica*** **A.Juss.**

36. ***Amphineuron opulentum*** **(Kaulf.)**

37. ***Solanum indicum*** **L.**

38. ***Cajanus cajan*** **(L.) Millsp.**

39. ***Mangifera indica*** **L.**

40. ***Garcinia cowa*** **Roxb.**