STUDIES ON PROSPECTING OF *CITRUS MACROPTERA* MONTRUZ. FROM MANIPUR FOR THERAPEUTIC AGENTS

Thesis submitted to the Department of Botany, Nagaland University, Lumami, Nagaland in partial fulfillment for the requirement of Degree of Doctor of Philosophy in Botany

By

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on prospecting of *Citrus macroptera* Montruz. from Manipur for therapeutic agents" s**ubmitted to Nagaland University, Lumami in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Botany is an original research work carried out by **Ms. Khumukcham Nongalleima**, **681/2015 dated 27.05.2015** under our joint supervision with **Dr. Ch. Brajakishor**, IBSD, Imphal..

Further, certified that no part of this thesis has been submitted anywhere for any other research degree.

Place: Lumami (Talijungla)

Date: 9.5.2018 Supervisor

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DECLARATION

I, Ms. Khumukcham Nongalleima, bearing registration number no.**681/2015 dated 27.05.2015, hereby declare that the thesis entitled "Studies on prospecting of *Citrus macroptera* Montruz. from Manipur for therapeutic agents"** being submitted to Nagaland University, Lumami, for the degree of doctor of Philosophy in Botany is the record of original and independent research work carried out by me under the joint supervision of Dr. Talijungla, Associate professor, Department of Botany, Nagaland University, Lumami and Dr. Ch. Brajakishor Singh, Scientist-C, Institute of Bioresources and Sustainable Development, Takyelpat, Imphal.

I further declare that this thesis has not previously been submitted for award of any other degree or diploma to any University or other tertiary institutions. My declaration/thesis is hereby forwarded by my Supervisors and head of department.

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CONTENTS

List of abbreviations and symbol used

AST	- Aspartate aminotransferase
ALT	- Alanine aminotransferase
AP-1	- activator protein-1
CAT	- catalase
CCl_4	- carbon tetra chloride
COSY	- correlation spectroscopy
COX	- Cyclooxygenase
Cys	- cysteine
CySS	- disulfide cysteine
DPPH	- 1, 1-Diphenyl-2-picryl hydrazil
EDTA	- ethylene diamine tetra acetic acid
ELISA	- enzyme linked immunosorbent assay
FDA	- Food and drug administration
FGF/FGFR	- fibroblast growth factor/receptor
F-C reagent	- Follin- Ciocalteu reagent
FT-IR	- Fourier transform infra red

g	- Gram
GPx	- glutathione peroxidase
GR	- glutathione reductase
GS-SG	- Glutathione disulfide
HCl	- hydrochloric acid
H_2SO_4	- sulphuric acid
HPLC	- high performance liquid chromatography
hr	- hour
IC_{50}	- Inhibition Concentration 50
iNOS	- nitric oxide synthase
IL-1β	- interleukin-1β
IL-10	- interleukin 10
IFN-γ	- interferon-γ
Kg	- Kilogram
mg	- milligram
mL	- milliliter
LC-MS	- Liquid chromatography mass spectrophotometry
LOD	- Limit of Detection

LOQ - Limit of Quantitation

M - molar

MAP kinases - mitogen-activated protein kinases

MIC - minimum inhibitory concentration

Min. - minute

MTT - (3-(4, 5- dimethylthiazol-2-yl)-2,5 dimethyltetrazolium bromide

MVD - Molegro Virtual Docker

Na_2CO_3 - sodium carbonate

ng - nanogram

nm - nanometer

NADPH - nicotinamide adenine dinucleotide phosphate

NF-kB - nuclear factor-kB

NMR - nuclear magnetic resonance

NSAIDs - non-steroidal anti-inflammatory drugs

NO - nitric oxide

% - percent

PBST - Phosphate buffered saline Tween

PDB - Protein data bank

pg - picogram

PLP - piecewise linear potential

p.o. - per organism

PVDF - Polyvinylidene difluoride

RBC - red blood cells

ROS - reactive oxygen species

ROI - Reactive oxygen intermediates

rpm - revolutions per minute

RSD - relative standard deviation

RNI - reactive nitrogen intermediates

SOD - superoxide dismutase

TFC - total flavonoid content

TFoC - total flavonol content

TNF-α - Tumor necrosis factor-α

TPC - total phenolic content

UCSF - University of California San Fransisco

µg - microgram

µl - microliter

v/v	- volume/volume
w/w	- weight/weight
^{0}C	- degree Celsius

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CHAPTER-1

INTRODUCTION & REVIEW OF LITERATURE

1.1 Anti-oxidant and Oxidative stress

Biochemists and nutritionists used the term "anti-oxidant" which often suggests a compound capable of quenching metabolically generated reactive oxygen species (ROS) (Finley *et al*., 2011). However, some food scientists used the term that implies a substance used for functional characteristics (e.g., retard oxidation), whereas others may understand the term as describing foods or substances with high values for measures of radical quenching ability, such as the oxidative radical absorbance capacity test (Prior *et al*., 2005). Many dietary compounds are capable of negating the danger of ROS e.g., vitamin C, tocopherols (vitamin E), carotenoids, polyphenolics etc. It has been suggested that inclusion of these compounds in food will enhance their capacities to support protection against ROS damage and reduce the risk of chronic disease (Mayes and Botham 2003).

In particular, concerning the corticosteroids of the adrenal cortex, the term "stress" was first used in the biomedical literature as a description of hyperactivity in the hormone system (Selye 1936). The term redox imbalance is also used for oxidative stress which is based on the Nernst equation taking into account all the redox couples present in the cell or in the different cellular sub-compartments (Aung-Htut *et al*., 2012). According to Lushchak 2014, oxidative stress is a situation when steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents. Oxidative stress is encountered by cells after bacterial infection and in a state of inflammation (Salzano *et al*., 2014) and

is, in fact, part of the primary innate immune defense of the body, including the well-known oxidative burst of macrophages and monocytes (Breitenbach and Eckl 2015). Among many diseases; cancer and heart disease are the most common disease associated with oxidative damage caused by free radicals (Azad *et al*., 2008; Heitzer *et al*., 2001; and Madamanchi *et al*., 2005).

Chemically, oxidation is defined as the removal of electrons and reduction as the gain of electrons (Mayes and Botham 2003). In reaction, a free radical may act as an oxidizing agent by taking a single electron from other species or as a reducing agent by donating a single electron to other species (Halliwell 2006). A pro-oxidant is another term that is used for any substance which generates the reactive species thereby inducing oxidative stress. However, an anti-oxidant is defined as any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Halliwell and Whiteman 2004). Oxidative stress is conventionally defined as an imbalance between pro-oxidant stress and anti-oxidant defense (Biswas 2016). However, latest evidence points out that the disruption of redox signaling is an important aspect of oxidative stress, sometimes more important than the pro-oxidant-anti-oxidant imbalance or the tissue damage induced by such imbalance (Jones 2006).

Anti-oxidant such as glutathione, ubiquinone, uric acid and the anti-oxidant enzymes such as glutathione peroxides, superoxide dismutase, and catalase can be generated in the body; however, under circumstances of oxidative stress or inflammation when free radicals production increases, the amount of this anti-oxidant generated in the body might be insufficient (Gope *et al*., 2004). Natural anti-oxidant may act as free radical scavengers, chain breakers, chelators of pro-oxidant metal ions, and quenchers of singlet oxygen present in the environment (Amarowicz *et al*., 2004).

Plant extracts having anti-oxidant activities have health-promoting effects, anti-aging effects and used for various metabolic and chronic disease like cancer, liver diseases, inflammation, diabetic, arthritis, stroke (Fusco *et al*., 2007, Willcox 2004). Impairment caused by reactive oxygen species, including superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide can be prevented by anti-oxidant, a bioactive reducing agent (Paul *et al*., 2015). The anti-oxidants have defensive properties against various chronic diseases, diabetes, sclerosis, atherosclerosis, cataracts and chronic inflammation (Willcox *et al*., 2004).

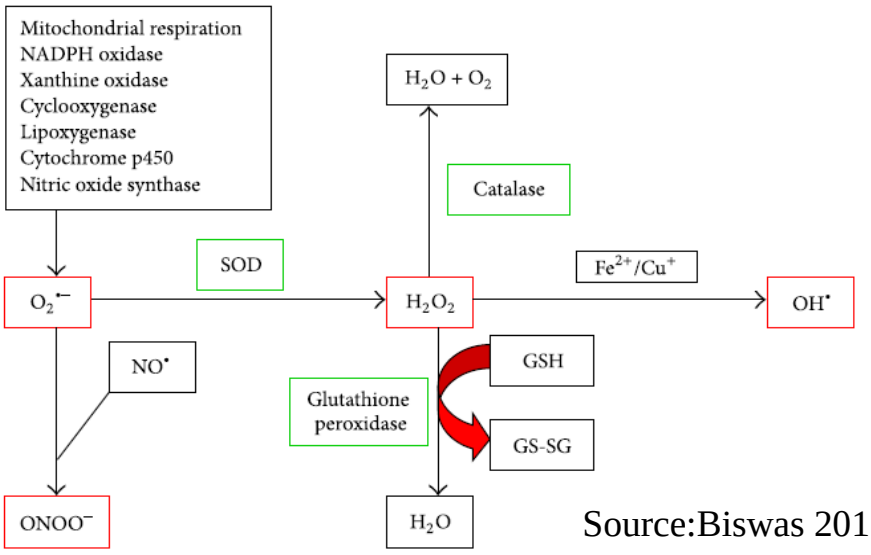


Fig. 1: Major prooxidant-anti-oxidant reactions relevant in a biological system. Superoxide (O_2^-) produced from a number of sources acts as a primary reactive species. peroxynitrite ($ONOO^-$) is produced by O_2^- rapidly reacting with nitric oxide (NO^-) or produce hydrogen peroxide (H_2O_2) catalyzed by superoxide dismutase (SOD). Catalase or glutathione peroxidase neutralized H_2O_2..

However, highly toxic hydroxyl free radicals ($OH^{\cdot}$) can be produced from H_2O_2 via the Fenton reaction in presence of transition metal ions, like iron (Fe^{2+}) and copper (Cu^{+}).

Reactive species are shown in red and anti-oxidant enzymes are shown in green boxes. GSH, reduced glutathione; GS-SG, oxidized glutathione (Biswas 2016).

Reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) constantly producing under physiological conditions (Nathan 2003; Kroncke 2003), is the crucial event in living organisms, therefore, "oxidative stress" should also include the pathways related to the "nitrosative stress" and, for their implication in cellular and extracellular metabolic events, to the "metabolic stress" (Rahman *et al*., 2012). At the moment, the concept of oxidative stress confined to ROI such as hydroxyl, superoxide radicals, whereas hydrogen peroxide and singlet oxygen have been extended to RNI such as nitric oxide (NO), peroxynitrite and S-nitrosothiols (Kroncke 2003). Thus, ROI and RNI react with proteins, carbohydrates, and lipids, with consequent alteration both in the intracellular and intercellular homeostasis, leading to possible cell death and regeneration (Fig.2) (Garrido *et al*., 2004). To manage the oxidative stress elicited by aerobic metabolism, animal and human cells have developed a universal anti-oxidant defense system, which consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase together with a number of low molecular weight anti-oxidant such as ascorbate, α-tocopherol and glutathione, cysteine, thioredoxin, vitamins, etc (Fridovich 1997; Halliwell and Gutteridge 1999). Superoxide is converted to a far less reactive product, hydrogen peroxide (H_2O_2), by a family of metallo enzymes known as superoxide dismutase (SOD) (Vaziri *et al*., 2003). However, this anti-oxidant defense system may be overwhelmed by various pathological or environmental factors so that a fraction of ROS may escape the destruction and form the far more reactive hydroxyl radicals (Fridovich 1997; Halliwell and Gutteridge 1999). An increase in ROS stimulated oxidative damage to DNA and other biomolecules which result in abnormal functions of tissue cells leading to human

aging and disease (Beckman and Ames 1999; and Wei *et al*., 2001). It is now nearly universally accepted that oxidative stress is not only associated with but also plays a major role in the aging processes of all cells (Jones 2015). Many biological processes such as apoptosis, viral proliferation, and inflammatory reactions are influenced by oxidative stress (Yoshikawa and Naito 2002). In these processes, gene transcription factors such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) act as oxidative stress sensors through their own oxidation and reduction cycling (Yoshikawa and Naito 2002).

1.2 Inflammation

Inflammation is a complex process, which is recurrently associated with pain and involves events such as the increase of vascular permeability, increase of protein denaturation and membrane alteration (Anup and Bindu 2015). It is a part of the host defense mechanisms that are known to be involved in the inflammatory reactions such as the release of histamine, bradykinin, and prostaglandins (Tailor *et al*., 2010).

Inflammation is of two types: acute inflammation and chronic inflammation. Acute inflammation may be an initial response of the body to harmful stimuli (Murugesan and Deviponnuswamy 2014). In chronic inflammation, the inflammatory response is out of proportion resulting in damage to the body (Murugesan and Deviponnuswamy 2014). The synthesis of prostaglandins, prostacyclins, and thromboxanes which are involved in inflammation, pain and platelet aggregation is associated with a key enzyme called Cyclooxygenase (COX) (Pilotto *et al*., 2010). The inflammatory process involves the activity of inflammatory mediators such as neutrophil-derived free radical, reactive oxygen species (ROS), nitric oxide (NO), prostaglandins and cytokines (Udegbunam *et al*., 2010). This overproduction leads to

tissue injury by damaging macromolecules, lipid peroxidation of the membrane where the tissue damage show an important part in pathogenesis of numerous inflammatory diseases (Anoop and Bindu 2015).

During the course of inflammatory response, a large amount of NO is produced by nitric oxide synthase (iNOS) in activated macrophages which exceed the physiological amount of NO (Anoop and Bindu 2015). It also causes increased vascular permeability, vasodilation, and endothelial tissue damage leading to inflammation (Anoop and Bindu 2015). An increase in cytokine production such as Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) have important roles in the maintenance of the inflammatory profile (Parodi *et al*., 2010; Moermans *et al*., 2011). Numerous inflammatory mediators are synthesized and secreted during inflammatory responses of different types (Azab *et al*., 2016).

Inflammatory substances are usually divided into two main categories: pro- and anti-inflammatory intermediaries (Azab *et al*., 2016). Nevertheless, some mediators such as interleukin (IL)-12 possess both pro- and anti-inflammatory properties (Vignali *et al*., 2012). Cytokines (e.g., interleukins, tumor necrosis factor α and interferons), chemokines (monocyte chemo-attractant protein 1), eicosanoids (e.g. leukotrienes and prostaglandins) and (NF*k*B), the potent inflammation-modulating transcription factor nuclear factor κ B are among the inflammatory mediators and cellular pathways that have been extensively studied in association with human pathological conditions are (Azab *et al*., 2016). Tumor necrosis factor (TNF-α) is an important pro-inflammatory cytokine which is secreted from various cells and exerts many cellular effects (Montgomery *et al*., 2012; Zelova and Hosek 2013). TNF-α has been connected with multiple illnesses in human, including immune and inflammatory diseases, cancer, psychiatric disorders. Another cytokine which mostly exerts a pro-inflammatory

activity is IL-1α (Fenton 1992; Rider *et al*., 2013). It stimulates the release of pro-inflammatory cytokines such as IL-1β and TNF-α (Fenton 1992; Rider *et al*., 2013). On the other hand, IL-10 is a strong anti-inflammatory cytokine the activity of which hampers the action of many pro-inflammatory mediators (Sabat 2010; Ng *et al*., 2013; Kwilasz 2015). IL-10 helps in maintaining the tissue homeostasis and lessen the damages that may result from an exaggerated inflammatory response by weakening and controlling the inflammatory response (Sabat 2010; Ng *et al*., 2013; Kwilasz 2015).

1.3 Anti-oxidant and anti-inflammation: relationship

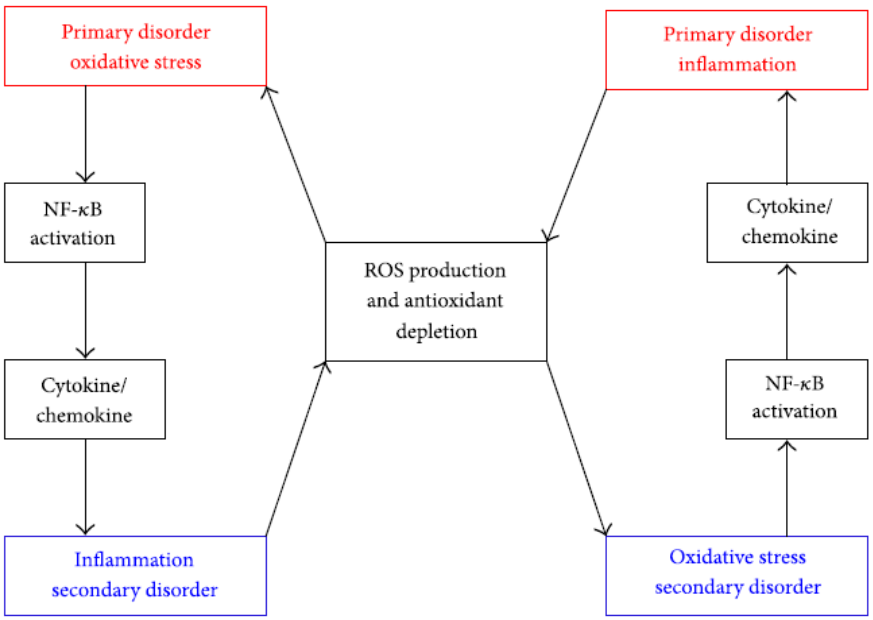
Premature aging of cells is associated with oxidative stress which can lead to tissue inflammation, damaged cell membranes, auto-immunity, and cell death (Klein and Ackerman 2003). If the accumulation of intracellular triglycerides or tissue adiposity promotes increased oxidative stress, the reduction of total body fat through diet and/or exercise may be an effective means of reducing systemic inflammation and oxidative species (Rahman *et al*., 2012). In the airway, losing control of oxidant may generate initiation of Th2-dominant immunity rather than inducing immune tolerance in the initial phase of development of airway allergic inflammation (Peterson *et al*., 1998; Kim *et al*., 2007; Murata *et al*., 2002). Furthermore, enhanced oxidative stress may contribute to the progression or perpetuation of existing airway inflammation through enhanced airway hyperresponsiveness, stimulation of mucin secretion, and induction of various pro-inflammatory chemical mediators (Rahman 2003).

Persistent inflammation results in the destruction of cartilage and bone which occurs through a number of mechanisms, including an oxidative and proteolytic breakdown of collagen and proteoglycans (Wright *et al*., 2010; Nissim *et al*., 2005; Eaggleton *et al*., 2008). Peroxides can also be formed by aldehyde compounds, causing

inflammation and organ fibrosis, as in the case of alcoholic liver disease (Stehbens 2004). As free radicals provoke or sustain inflammatory processes, their neutralization by anti-oxidant and radical scavengers can reduce inflammation (Confortia *et al*., 2008). Inflammatory processes also involve reactive oxygen species initiated by leukocyte activation (Anup and Bindu 2015). Screening of anti-oxidant properties may provide important information about the potential activity of a drug on inflammatory processes (Akula and Odhav 2005).

Inflammatory cells release a number of reactive species at the site of inflammation leading to exaggerated oxidative stress (Collins 1999). On the other hand, a number of reactive oxygen/nitrogen species can initiate an intracellular signaling cascade that enhances pro-inflammatory gene expression (Anderson *et al*., 1994; Flohe *et al*., 1997). To kill the invading agents, the activated phagocytic cells like neutrophils and macrophages produce large amounts of ROS, reactive nitrogen and chlorine species including superoxide, hydrogen peroxide, hydroxyl free radical, nitric oxide, peroxy-nitrite, and hypochlorous acid during the inflammatory process (Fialkow *et al*., 2007). Pathological inflammatory conditions can induce localized oxidative stress and tissue injury which was due to the exaggerated generation of reactive species; and diffusion of those reactive species out of the phagocytic cells (Fialkow *et al*., 2007). However, apart from the direct production of reactive species by the professional phagocytic cells, the non-phagocytic cells can also produce reactive species in response to pro-inflammatory cytokines (Wu *et al*., 2013; Li *et al*., 2015). The pro-inflammatory cytokine interferon-γ and the pro-inflammatory component of bacterial cell wall lipopolysaccharide have been found to synergistically increase ROS production in human pancreatic cancer cell lines and in human pancreatitis through toll like receptor (TLR)-4-NF-κB-dependent expression of Duox2, a member of NADPH oxidase family

(Wu *et al*., 2013). Recent finding also showed that the co-stimulation of TLR produces oxidative stress with imbalance of pro-inflammatory and anti-inflammatory cytokine production, as mentioned above (Lavier *et al*., 2014). Furthermore, the inflammatory cytokine IL-6 has been found to produce ROS through increased expression of NADPH oxidase 4 (NOX4) in non-small cell lung cancer (Li *et al*., 2015).



Source: Biswas 2016.

Fig. 2: Overview of the interdependence between inflammation and oxidative stress. When oxidative stress appears as a primary disorder, inflammation develops as a secondary disorder and further enhances oxidative stress. On the other hand oxidative stress (as secondary disorder), can be induced by inflammation as a primary disorder, which can further enhance inflammation.

As the inflammatory process can induce oxidative stress, the oxidative stress can also induce inflammation through activation of multiple pathways (Biswas 2016). The reactive species hydrogen peroxide can induce inflammation through activation of

transcription factor NF-κB, as mentioned above (Anderson 1994, Flohe *et al*., 1997). Furthermore, oxidative stress plays an important role in the stimulation of NOD-like receptor protein 3 (NLRP3) inflammasome (Zhou *et al*., 2011; Shimada *et al*., 2012, Zhou *et al*., 2010). The NLRP3 inflammasome is an oligomeric molecular complex that activates innate immune defenses through the maturation of pro-inflammatory cytokines like IL-1β and IL-18 (Schroder and Tschopp 2010). Several mechanisms of ROS-mediated activation of NLRP3 inflammasome have recently been shown (Zhou *et al*., 2011; Shimada *et al*., 2012; Zhou *et al*., 2010). The ROS released from damaged mitochondria has been shown to activate NLRP3 inflammasomes leading to IL-1β secretion and localized inflammation (Zhou *et al*., 2011). Oxidized mitochondrial DNA has also been found to activate NLRP3 inflammasomes during programmed cell death (Shimada *et al*., 2012). Furthermore, in conditions of oxidative stress the ROS causes the thioredoxin-interacting protein, an inhibitor of endogenous anti-oxidant thioredoxin, to dissociate from thioredoxin and to bind with NLRP3 leading to activation of NLRP3 inflammasome (Zhou *et al*., 2010).

The ROS-induced DNA base modification has also been shown to induce inflammation (Biswas 2016). The base excision repair of oxidatively damaged/modified DNA base (7, 8-dihydro-8- oxoguanine) by 8-oxoguanine-DNA glyoxalase-1 induces a signaling cascade that culminates in the activation of NF- κB pathway resulting in pro-inflammatory gene expression and inflammatory cell accumulation (Aguilera-Aguirre *et al*., 2014). The 8-isoprostane, an end product of arachidonic acid belonging to the F2-isoprostanes and an indicator of oxidative stress, has been found to escalate the expression of inflammatory chemokine IL-8 in human macrophages through activation of mitogen-activated protein kinases (MAP kinases) (Scholz *et al*., 2003). Furthermore, the oxidative stress induced oxidation of the extracellular redox potential of plasma

cysteine (Cys) and its disulfide cystine (CySS) has been shown to trigger monocyte adhesion to vascular endothelial cells, activate NF-κB, and increase the expression of pro-inflammatory cytokine IL-1β (Iyer *et al*., 2009; Go and Johnes 2005). The above discussion indicates that the inflammation and oxidative stress are closely inter-related and tightly linked interdependent of their pathophysiological processes (Biswas 2016). Either one of them may appear before or after the other, but when one of them appears the other one is most likely to appear; and then both of them take part in the pathogenesis of many chronic diseases (Biswas 2016).

1.4 Anti-Inflammation and anticancer: relationship

Exemplary studies have indicated that there is an approximately 14% increase in prostate cancer risk due to prostatitis (Rothman *et al*., 2004; Nelson *et al*., 2004; Rosenblatt 2001), a 25% increase in colorectal cancer risk owing to ulcerative colitis (Adelstein 1979; Loftus 2006) and a 10-20-fold increase in the risk of pancreatic cancer for patients who have experienced pancreatitis (Farrow 2004; Otsuki 2003; Ammann 1984). Initiation of cancer may be caused by inflammation and is reasonable considering that chronic inflammation is characterized by infiltration of mononuclear immune cells (including macrophages, lymphocytes, and plasma cells), tissue destruction, fibrosis, and increased angiogenesis (Strukov *et al*., 1984; Williams and Williams 1983). Chronic inflammation is associated with increased genomic damage, increased DNA synthesis, cellular proliferation, disruption of DNA repair pathways, inhibition of apoptosis, and the promotion of angiogenesis (Hofseth and Ying 2006). As a tumor develops, it expresses phenotypes similar to inflammatory cells (Arias 2007). The most frequently evaluated anti-cancer anti-inflammatory target is COX-2 (cyclooxygenase 2, PTGS2), although various other targets, such as FGF/FGFR (fibroblast growth factor/receptor), NF-kB, cytokines/cytokine receptors, chemokines/chemokine

receptors, and VEGF have also been examined (Rose-John *et al*., 2007; Galliera *et al*.,2008; Van Waes 2007; and Knowles 2008). More than two decades ago, it was demonstrated that NSAIDs (non-steroidal anti-inflammatory drugs) have anti-colon cancer effects (Waddell and Loughry 1983; Kune *et al.*, 1988). Tumors themselves or the tumor microenvironment can be altered by many anti-inflammatory agents, including the NSAIDs, potentially by decreasing migration (Zlotnik 2006), increasing apoptosis (Jana 2008), and increasing sensitivity to other therapies (de Groot *et al*., 2007); thus, the agents still have immense promise against cancer.

Decontrolled inflammation plays a major role in chronic illnesses, including diabetes, cardiovascular disease, arthritis, psoriasis, and cancer (Pari *et al*., 2008). Although they were designed to decrease or prevent inflammation, many of these agents exhibit other properties such as anti-emetic, anti-thrombotic (Salman and Ayhan 2006), anti-angiogenic (Monnier *et al*., 2005), pro-apoptotic and anti-proliferative activities Gridelli *et al*., 2007, rendering them potential source for cancer treatment. Moreover, colorectal cancer patients who were long-term NSAID users had dramatically lesser mortality rates than non-NSAID users (Smalley and DuBois 1997). Celecoxib remains the only NSAID with FDA-approval for patients with familial adenomatous polyposis (FAP) (Casanova *et al*., 2006) and is currently being investigated in a number of Phase II and III clinical trials both alone and in combination with other standard chemotherapy regimens (www.clinicaltrials.gov). These agents, particularly the NSAIDs, have been documented to decrease migration (Zlotnik 2006), increase apoptosis (Jana 2008; Fecker *et al*., 2007), and decrease angiogenesis (Albini *et al*., 2005; Sawaoka H, *et al*. 1999) of tumors. Another NSAID, diclofenac, inhibited the metabolism of the novel chemotherapeutic agent 5, 6-dimethylxanthenone-4-acetic acid, DMXAA in mice by preventing its glucuronidation, leading to increased plasma AUC and decreased

clearance (Wang *et al*. 2008). Many studies have suggested that the anti-cancer activity of a number of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, sulindac, and indomethacin, may be dependent upon apoptosis brought about by their effects on NF-kB (RelA) signaling (Stark *et al*., 2001; Loveridge *et al*., 2008). Novel drugs based on the NSAIDs that lack their conventional COX-1/2 inhibitory effects, but still retain anti-cancer activity are being developed by various investigators (Schonthal 2007; Chuang *et al*., 2008). When carcinogen-dosed rats were fed a diet containing both curcumin and celecoxib, they developed fewer aberrant crypt foci compared to rats treated with either agent alone, and dramatically fewer foci compared to rats fed a control diet (Shpitz *et al*., 2006). A large number of foods and natural compounds have been found to have anti-inflammatory properties, including chili peppers (capsaicin), grapes/red wine (resveratrol), garlic (various compounds), curry powder (curcumin), ginseng (ginsenosides), as well as numerous other fruits, vegetables and herbs (Aggarwal and Shishodia 2006; Das and Das 2007; Anand *et al*., 2008; Hofseth and Wargovich 2007). Natural products have been shown to prevent or decrease inflammation through a variety of mechanisms, including inhibition of NF-kB, COX-1 and -2, and MAPK, JNK and ERK1/2 signaling as well as decreasing VEGF and iNOS (Aggarwal and Shishodia 2006; Das and Das 2007; Anand *et al*., 2008; Hofseth and Wargovich 2007). Many of these agents also exert anti-proliferative, pro-apoptotic or cell cycle inhibitory activities (Aggarwal and Shishodia 2006). Many compounds derived from natural products have potent activity against xenograft tumors, cancer cells, and also they can prevent carcinogenesis or metastasis of existing tumors and these were recommended by Pre-clinical studies (Strimpakos and Sharma 2008). For example, studies have shown that combining curcumin with vinorelbine or 5-fluorouracil leads to synergistic effects against cancer cells, while pre-treatment of rats

with curcumin avoids doxorubicin-associated cardiotoxicity (Strimpakos and Sharma 2008).

Inflammation may provide both the key mutations and the proper environment to foster tumor growth (Elizabeth *et al*., 2009). These effects and epidemiological evidence of decreased cancer incidence and aggressiveness prompted the numerous investigations of the anti-inflammatory agents' potential for cancer prevention and therapy (Elizabeth *et al*., 2009). The precise mechanism by which NSAIDs restore apoptosis remains controversial (Marx 2001), although it clearly affects factors related to APC deficiency or the induction of COX-2 or both. Apoptosis can be suppressed in normal human or rodent intestinal epithelial cells by manipulating these cells to overexpress COX-2 (Tsujii *et al*., 1995; DuBois *et al*., 1996). Other anti-apoptotic and anti-proliferative effects have been observed for the different anti-inflammatory agents (Elizabeth *et al*., 2009). In human HT-29 colon cancer cells, apoptosis can be restored by treatment with selective (Elder *et al*., 1997) or nonselective (Shiff *et al*., 1996; Shiff *et al*., 1995) COX inhibitors or by restoring Adenomatous polyposis coli (APC) gene function (Morin *et al*., 1996). COX-2 expression in human colorectal carcinomas is associated with larger tumor size and deeper invasion, although not with metastases (Fujita *et al*., 1998). A second cellular process by which COX-2 inhibitors may inhibit tumor growth is through inhibition of angiogenesis and neovascularization (Tsujii *et al*., 1998; Jones *et al*., 1999). The cox-2 expression is widely induced in the angiogenic vasculature of colorectal adenomatous polyps and in carcinomas of the colon, lung, breast, esophagus, and prostate (Holash *et al*., 1999; Masferrer *et al*., 2000). The growth of corneal capillary blood vessels in rats exposed to basic fibroblast growth factor is suppressed by selective COX-2 inhibitors (Masferrer *et al*., 2000) and inhibit the growth of several human tumors transplanted into mice (Masferrer *et al*., 2000; Williams 2000).

Anti-inflammatory agents seem to have promise for the prevention or treatment of various human cancers (Elizabeth *et al*., 2009). Anti-inflammatory agents may also protect against neurotoxicity by decreasing or inhibiting the disruption of the blood-brain barrier (Elizabeth *et al*., 2009). The use of various plants and plant extracts is now being investigated as a complementary or alternative approach to conventional therapies in westernized countries while they have been used for millennia in Ayurvedic and Chinese medicine (Elizabeth *et al*., 2009). It is therefore not surprising that natural products are now being used for cancer prevention and/or therapy, and as adjuvants for conventional therapies (Elizabeth *et al*., 2009). Although the results of most of the recent clinical studies have been disappointing, accumulating evidence suggests that combining these natural anti-inflammatory compounds with conventional therapies can lead to improved effects for patients (Elizabeth *et al*., 2009). By sensitizing cancer cells to conventional cancer therapies or protecting host cells from such treatment, the natural products can exert activities similar to conventional anti-inflammatory agents and may have applications in preventing or treating human cancers (Elizabeth *et al*., 2009). There are numerous other anti-cancer approaches that seek to modify the host immune response, decreasing energy toward the tumor or decreasing the inflammatory microenvironment of the tumor (Elizabeth *et al*., 2009).

1.5 Prospecting of new therapeutic drugs

Medicinal plants have been known to be a rich source of therapeutic agents for curing different diseases. Potential of natural products of medicinal plant is very diverse, widespread and known to us since early civilization. Prospecting of medicinal plants including selective plant parts or whole plant for therapeutic agents involves exploration, isolation, and screening for the search of natural products/new compounds with the prospect of product development. Natural products sources can be biologicals

including cells, tissues, body fluids, secretions of microbes, plants, and animals. The search or hunt of the natural resources for natural products to produce medicinally important drugs or commercially valuable compounds is generally referred as a prospecting of natural products.

Natural products being prospected for therapeutic resources are produced by plants as secondary metabolites. The secondary metabolite, by the name itself, hints secondary function to the plants i.e., they are not required by plants for their normal growth and function, unlike primary metabolites. These metabolites are organic compounds which serve as a means of providing protection to plants from herbivores, insects and other organisms aggressive of preying them. Though plants produce various classes of metabolites, some of them represent a marker metabolite for certain group of plant family or genus. Usually, secondary metabolites are specific to an individual species (Pichersky and Gang 2000). Secondary metabolites are mainly produced by three pathways: acetate pathway, shikimic acid pathway, mevalonate pathway and methylerythritol pathway. These metabolites fall into major classes of alkaloid, polyketide, polyphenols, phenylpropanoids, terpenoids, sesquiterpenes. It is due to their presence that imparts medicinal properties to the plants. They have a wide range of applications in therapeutic uses. Penicillin, a well-known antibiotic is a metabolite isolated from a fungus. It is often not fully appreciated that the major hurdle in bringing a natural-product-based complex molecule to market is not the isolation, basic semi synthesis, or total synthesis, but the immense supply problems faced by chemists in translating research laboratory discoveries to commercial items (Newman and Crag 2016).

Sources of natural products may be from microbes, endophytes, terrestrial plants, animals; also from marine organisms. Newman and Crag 2016 divided natural

product sources into 8 major categories and they are listed as 1. “B” sources; they are Biological, usually a large (>50 residues) peptide or protein either isolated from an organism/cell line or produced by biotechnological means in a surrogate host, 2. “N” sources; they are natural product, unmodified in structure, though might be semi- or totally synthetic 3. “NB” sources; they are natural product “botanical drug” (in general these have been recently approved) 4. “ND” sources; derived from a natural product and is usually a semisynthetic modification, 5. “S” sources, they are a totally synthetic drug, often found by random screening/ modification of an existing agent, 6. “S*” sources; they are made by total synthesis, but the pharmacophore is a natural product, 7. “V” sources, they are Vaccine, and 8. “NM” sources; they are natural product mimic (Newman and Crag 2016). Since 1981-2014, there were 174 new chemical entities (NCE) for indication of the anticancer compound, out of which 33 were from B source, 17 were from N source, 1 was from NB, 38 from ND, 23 from S source, 13 from S*. And there were 51 NCE for anti-inflammatory indication out of which 1 was from B sources, 13 from ND sources, 37 from S source. During 1981 to 2014, 11 drugs from N sources, 71 drugs from ND sources were introduced under different generic and trade names. Likewise, for antifungal, 3 drugs from ND source were introduced in 2001-06. For antiviral, 4 drugs from ND source, 24 drugs from S* source were introduced, 17 from S*/NM source were introduced. 2 drugs from N source, 5 drugs from ND source and 2 from S* source were introduced as anti-parasitic drugs. As an anti-diabetic, 1 drug from ND source, 11 from S/NM source, 6 drugs from S*/NM source were introduced (Newman and Crag 2016). In the area of cancer, over the time frame from around the 1940s to the end of 2014, of the 175 small molecules approved, 131, or 75%, are other than “S ”(synthetic), with 85, or 49%, actually being either natural products or directly derived therefrom (Newman and Crag 2016). This show the importance of natural

products and its derivatives as a rich source for production of therapeutic drugs for the treatment of various diseases. The award of half of the 2015 Nobel Prize in Physiology or Medicine to Drs. Omura and Campbell for their discovery and development of the avermectin/ivermectin complexes, with the other half being awarded to Prof. Tu for her discovery and development of artemisinin, is truly excellent news for the general public, as they may now begin to understand where these significant drugs were sourced (Newman and Crag 2016).

Development or the search for new drugs involves various steps which need to be followed sequentially and systematically. After collection of plants from their natural habitat, they are processed for extraction using various organic solvents. Prior to extraction plant samples are allowed to dry; drying process may be shed drying, air drying, microwave drying, oven drying or freeze drying. Sometimes fresh samples without drying them are also used.

1.6 *In vitro-in vivo* and toxicological studies

Flavonoids, phenolic acids, and triterpenoid possessed anti-nociceptive and anti-inflammatory effects in animal models (Arslan *et al*., 2010). There are also reports that flavonoids such as rutin, quercetin, luteolin produced substantial amount of anti-nociceptive and anti-inflammatory activities (Deliorman *et al*., 2007). An existing cross-talk between the release of NO and PGs in the modulation of inflammation have been indicated by *et al* and *et al* studies (Salvemini *et al*., 1995). The carrageenan-induced paw edema is a well-known acute model of inflammation that is widely used for screening novel anti-inflammatory compounds (Mansouri *et al*., 2015). The carrageenan-induced paw edema is a well-defined model of acute inflammation that a variety of inflammatory mediators involved in its development and has wildly been used

to evaluate the anti-edematous effect of natural products (Mansouri *et al*., 2015). The early phase observed around 1 h is related to the release of histamine, serotonin, bradykinin, and to a less extent prostaglandins produced by cyclooxygenase enzymes (COX), whereas the delayed phase (after 1 h) is attributed to neutrophil infiltration, and the continuing of the prostaglandin generation (Gilligan *et al*., 1994; Halici *et al*., 2007). The delayed phase of carrageenan-induced acute inflammation also involves the release of the neutrophil-derived free radicals, nitric oxide (NO) and pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), and interleukin-1 β (IL-1 β) (Halici *et al*., 2007). On the other hand, the release of some important pro-inflammatory cytokines such as TNF-α and IL-1 β are provoked by carrageenan injection (Nacife *et al*., 2004). Different observations suggest that drugs targeting COX enzyme, free radical formation and pro-inflammatory protein expression (e.g. inducible nitric oxide synthase; iNOS) might provide a better control over inflammatory states than the therapeutic agents currently available (Ronchetti *et al*., 2009).

For studies involving *in-vivo* experiment, the animals should be allowed to fast overnight prior to the experiment. Acute oral toxicity – acute toxic class method (OCED Guideline no. 423, Annexure – 2d) adopted by CPCSEA, Government of India should be followed for toxicity studies.

1.7 Review of Literature

C. macroptera Montrouz. were reported to occur in the subtropical forests of North-east India and the foothills of the East Himalayas (Tanaka, 1937; Bhattacharya and Dutta, 1956). *Citrus macroptera* (Var annamensis) belongs to the family of Rutaceae and it is native to the regions of South East Asia mainly Myanmar, Thailand, Indonesia Malaysia, Papua New Guinea, Sylhet Division of northeastern Bangladesh

and northeastern India mainly Manipur and Assam; in Bengali it is called as "hatkora" or "shatkora" and in English known as Wild orange (Carpenter and Reece 1969; Dreyer and Huey 1973). It is known as heiribob in Manipuri. It is a semi-wild species and used as medicine by local tribes of Assam, India (Ghosh, 1990). Nair and Nayar, (1997); Sharma *et al*., (2004) reported that *C. macroptera* Montr. occur in the subtropical forests of North-east India and the foothills of the Eastern Himalayas.

1.7.1 Classification

Kingdom	Plantae
Division	Tracheophyta
Subdivision	Spermatophyta
Class	Magnoliopsida
Order	Sapindales
Family	Rutaceae
Genus	*Citrus*
Species	*macroptera*

1.7.2 Morphological characteristics

Sanabam *et al*., 2012 characterized *C. macroptera* Montr. morphologically, and described hereunder:

Leaf :

Division	simple
Lamina Shape	orbicular
Lamina Length	6.19 cm
Lamina Width	4.5 cm
Lamina Attachment	longipetiolate

Margin	entire
Apex	acuminate
Petiole Length	9.22 cm
Petiole Wing	present
Petiole Wing Wide	broad
Petiole Wing Shape	obovate
Junction Between Petiole and Lamina	articulate
Fruit :	
Weight	578.26 gm
Diameter	8.33 cm
Length	9.32 cm
Shape	spheroid
Base Shape	convex
Apex Shape	rounded
Skin Texture	smooth
Segment	17 nos.
Segment Shape Uniformity	present
Rind Colour	white
Rind Thickness	1.08 cm
Pulp Colour	white
Pulp Colour Intensity	light

Axis	solid
Axis Shape	oval
Axis Diameter	0.67 cm
Oil Density (sq.cm)	86.25
Oil Gland Nature	moderately conspicuous
Areola	absent
Areola Diameter	0 cm
Style Scar	absent

Seed :	
Shape	semi-deltoid
Surface	wrinkle
Colour	white
Length	1.57 cm
Width	0.66 cm
Cotyledon Colour	white
Average Seeds	15-23

Although some literature is available and reviewed, there is no report of extensive research on *C. macroptera* about its phytochemistry, pharmacological and therapeutic properties. In Manipur this Wild Orange (*C. macroptera*) grows well with good quality fruiting as location specific crop of Chandel District and Jiribam Sub-Division of Imphal East District, although all Citrus are primarily valued for the fruit, which is either eaten alone (sweet orange, tangerine, grapefruit, etc.) as fresh fruit, processed into juice, or added to dishes and beverages (lemon, lime, etc.) (http://manipursfac.com/wild-orange-

citrus-macroptera/ 1/.); wide range of uses of this wild orange has been reported where the dried rind of the fruit is used in preparation of meat dishes as flavouring spice, the juice of the fruit is used as medicine for treatment of abdominal ailments as well as digestive enzyme, the fruit pulp is used as washing detergent, not the least the most important is the essential oil from the leaves which otherwise wasted (http://manipursfac.com/wild-orange-citrus-macroptera/ 1/.).

1.7.3 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

Chaudhury and Bhat (2013) used RAPD markers to evaluate genetic diversity and inter-relationship among 30 accessions of *C. macroptera* collected from Tripura and Mizoram states. They generated a total of 92 bands in *C. macroptera* based on 12 RAPD primers, of which 91 bands (98.8%) were polymorphic; a pair-wise genetic similarity value between the accessions ranged from 0.26 to 1.00 with an average value of 0.57. Their study indicates the existence of high level of genetic diversity among the accessions of *C. macroptera* collected from different geographical regions; a unweighted pair-group method for the arithmetic mean dendrogram separated all the accessions of *C. macroptera* into two major clusters with a similarity value of 0.53 and various sub-clusters (Chaudhury and Bhat 2013). Genetic similarity among the accessions forms the basis of grouping of accessions rather than geographical distribution. Based on the marker analysis, accessions collected from Mizoram were found to be more diverse as compared to those collected from Tripura (Chaudhury and Bhat 2013). The distinction between close accessions, inter-relationship and capacity to identify genetic variation among the accessions of *C. macroptera*, which has implications for the genetic improvement of citrus germplasm is confirmed by the RAPD markers (Chaudhury and Bhat 2013).

1.7.4 Compounds of *Citrus macroptera Montruz.*

There was a report on the isolation of coumarins like bergamottin, psoralen, margin, severine and geiparvarin (Dreyer and Huey, 1973). Bergamottin and analogues are also constituents of non-commercial citrus fruits such as *Citrus macroptera*, a species native to islands of the S. Pacific (Dreyer and Huey, 1973). The distillation and subsequent extraction of the distillate obtained from the fresh peels of the fruits of *Citrus macroptera* var. *annamensis* afforded 120 mg of an oil (yield 0.12 %). Previous phytochemical investigations resulted in the isolation of alkaloids like (+) ribalinine and isolated desmine, (Gaillard *et al*., 1995) aromatic compounds like cinnamic acid, syringaldehyde, vanillin and methyl vanillate (Gaillard *et al*., 1995). The two alkaloids (Kokusaginine and rybalinine) are of the quinolone type and have not been found in Ruta species, although they have been found in other plants, e.g. *Haplophyllum platinum* and *Citrus macroptera* Montr. (Gaillard *et al*., 1995). The isolation and structure elucidation of the Lupeol and Stigmasterol by using spectroscopic techniques and the preliminary antioxidant activities of the organic extractives are being reported (Chowdhury *et al*., 2008). The leaves and flowers together with those of *Colubrina asiatica* and *Citrus macroptera* were used to make shampoo in Samoa (Thomson and Thaman 2008). Chowdhury *et al*., (2008) reported the presence of Lupeol and Stigmasterol in leaves of *C. macroptera.* Waikedre *et al*., 2010 reported 35 compounds in *C. macroptera* leaves essential oil in GC/MS. According to their GCMS report, the oil was mainly constituted of monoterpenes (96.3%), among which b-pinene (33.3%), a-pinene (25.3%), p-cimene (17.6%), (E)-ocimene (6.7%), sabinene (4.8%), g-terpinene (3.1%), and limonene (2.4%) (Waikedre *et al*., 2010). Moreover, spathulenol (0.6%) and caryophyllene oxide (0.5%) were the major oxygenated sesquiterpenes present in the oil (Waikedre *et al*., 2010). Other sesquiterpenes such as α-cardinal (0.2%),

viridiflorol (0.2%), and a-murolol (0.1%) were also identified (Waikedre *et al*., 2010). Analyses both by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) of the sample resulted in the identification of 25 terpenoids, predominantly monoterpene hydrocarbons, accounting for 97.0 % of the total oil, and limonene (73.5 %) as the major component (Miah *et al*., 2010). Their leaves contain mainly terpenoids like limonene and aromatic hydrocarbons (Waikedre *et al*., 2010; Rana and Blazquez 2012). *Citrus macroptera* essential oil contained 55.3% limonene, 4.7% beta-caryophyllene and 3.5% geranial as main compounds (Rana and Blazquez, 2012). A paste of *C. macroptera* leaves are applied to the navel for children, and pills prepared from leaf paste are taken orally to cure helminthiasis (Nahar *et al*., 2013).

1.7.5 BIOLOGICAL ACTIVITIES

1.7.5.1 Antioxidant activity

It is reported that stem bark of *Citrus macroptera* possesses antioxidant activity (Chowdhury *et al*., 2008). The hot methanol extract of the stem bark of *Citrus macroptera* showed potent antioxidant activity with the IC_{50} value of 178.96 μg/ml whereas the cold methanol and the dichloromethane extracts showed moderate activity with the IC_{50} of 242.78 μg/ml and 255.78 μg/ml respectively (Waikedre *et al*., 2010). The n-hexane extract showed mild activity (IC_{50} 422.94 μg/ml) against DPPH free radical. It is evident that all possess antioxidant activity (Chowdhury *et al*., 2008). Miah *et al*., (2010) reported that the oil did not exhibit any *et al* free-radical-scavenging (DPPH). In the extraction of phenolic contents and their antioxidant properties of *C. marcoptera*, a vital role is played by both time and solvent extractions (Gope *et al*., 2014). Rahman *et al*., (2014a) reported that *Citrus macroptera* fruit peels possess anti-oxidant activities. Rahman *et al*., 2014 reported that ethanolic extract of *Citrus macroptera* fruit peels (EECM) in DPPH scavenging activity with IC_{50} at 281.11 μg/ml,

hydrogen peroxide scavenging activity with IC_{50} at 216.49 µg/mL, and nitric oxide scavenging activity with IC_{50} at 182.89 µg/ml were comparable with standard Ascorbic acid. Further Rahman *et al*., 2014 reported that the total phenolic content was highest in ethanolic extract of *C. macroptera* (142.5±3.29 mg/gm equivalent of Gallic Acid) and total Flavonoid content was highest in ethanolic extract of *C. macroptera* (333.0±36.06 mg/gm equivalent of Quercetin.

1.7.5.2 Hypoglycemic activity

Uddin *et al*., 2014a investigated the therapeutic effects of methanol extract of *Citrus macroptera* fruit in α-amylase inhibitory activity (*in vitro*) and hypoglycemic activity in normal and glucose-induced hyperglycemic rats (*in vivo*), presence of saponin, steroid, terpenoid were identified were also investigated and they found out that the fruit extract showed moderate α-amylase inhibitory activity (IC_{50} value at 3.638 ± 0.190 mg/mL) as compared to acarbose.

1.7.5.3 Antimicrobial activity

According to the literature, many monoterpenoids such as limonene or p-cymene identified in the essential oils of *C. macroptera* and *C. hystrix*, are known as anti-candidal (Pauli, 2006). Essential oil obtained from leaves possess anti-microbial activity and traditionally fruits as appetite stimulant activity (Waikedre *et al*., 2010). It showed considerable anti-bacterial activity against *Bacillus cereus*, *B. subtilis*, *Escherichia coli* and *Staphylococcus aureus* with the MIC values ranging from 1.25 to 5.0 mg/mL (Miah *et al*., 2010). The minimum inhibitory concentration (MIC) values of ketoconazole was 5 mg/mL and that of *C. macroptera* essential oil was 12.5 mg/mL against *T. mentagrophytes* var. interdigitale (filamentous fungus) while ketoconazole and *C.macroptera* essential oil showed MIC at 40, 75 mg/ml, respectively against *Candida albicans* (Waikedre *et al*., 2010). As contrast to the finding of Mia *et al*., 2010,

the essential oils of leaves of *C. macroptera* have no antibacterial activity against all five bacteria viz., *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis*, and two Gram-negative strains, viz., *Escherichia coli* and *Klebsiella pneumoniae* (Waikedre *et al*., 2010).

1.7.5.4 Neuroprotective activity

There is a little published work on the neuroprotective and neuropharmacological effects of herbal extracts or natural phytochemicals having anti-oxidant activities. Phytochemicals containing flavonoid polyphenols and organo-sulfur compounds have neuroprotective effects, as shown experimentally in cell and animal studies (Parihar and Hemnani 2003; Heo and Lee 2005)**.** Ethanolic extract of *Citrus macroptera* fruit peels (EECM) found to possess anti-depressant and anxiolytic activity, it was found to protect from oxidative stress in brain and it was found to protect brain antioxidant enzyme levels in *in-vivo* (Rahman *et al*., 2014_b). It can be concluded that neuroprotective activity of *Citrus macroptera* fruit peels may be due to protection from oxidative stress and it may prove the traditional uses of *Citrus macroptera* fruit peels for anxiety and depression in Assam (Rahman *et al*., 2014_b).

1.7.5.5 Organoprotective activity

A study confirmed that *C. macroptera* fruit does not produce any toxic effects to vital organ structures or serum biochemical parameters, therefore, the use of this fruit as a potential source of natural antioxidants is an attractive option, particularly because it also has the potential to combat many diseases that are induced by oxidative stress (Paul *et al*., 2015). *C. macroptera* also confers strong protection against lipid peroxidation in rat liver and kidney tissues because of its strong antioxidant properties (Paul *et al*., 2016). Treatment with 1000mg/kg ethanolic extract of *C. macroptera* was the most effective based on the biochemical and histological findings, it is plausible that

C. macroptera fruit improves the structural integrity of the cell membrane via inhibition of lipid peroxidation while ameliorating the histopathological changes and biochemical perturbations (Paul *et al*., 2016).

1.7.6 Traditional and local uses

In Guam, Stone (Stone, 1985) noted that the pulp was used for washing clothes and hair. The rinds of ripe citrus fruits can be used in decoction for coughs, colds, indigestion, and diarrhea. A decoction of the leaves is used as a bath to induce sweating in patients with fever. In Guam, Samoa, and Fiji the macerated pulp and leaves of wild orange were used as a shampoo (Walter and Sam, 2002). The seeds are also said to have pain-relieving effects. The fruit of *Citrus macroptera* (var annamensis) is edible and popular among the people of Bangladesh, Meghalaya, and Assam of India as green matured fruits, used in cooking for flavoring curry mainly meat dishes, pickle preparation, and oil is used in perfume production (http://gsl.articlealley.com/an-introduction-of-satkara-1932990.html).

Malik and Choudhury 2006 reported *Shatkara* as an endangered wild species, used by locals in Northeast India as medicine for stomach pain and alimentary disorder. The traditional healers of Manipur give peels of this fruit to the person suffering from epilepsy to smell to recover from the epileptic state. *Citrus macroptera* Montr. was used in remedies against ringworm, complex remedies against sicknesses; epilepsy-like symptoms, antiprotozoal and nematocidal activities of some medicinal plants from New Caledonia (Desrivot *et al*., 2007). In Bangladesh, the Laleng (Patra) historical indigenous community call *C. macroptera* Montruz. as Jamir uses: loc. Kushirgool: and their fruits are edible (Partha, 2014).

1.7.7 Economic importance & toxicity

By exporting *Citrus macroptera* Montruz. fruits especially to UK, USA, and Middle East countries, every year, Bangladesh earns handsome amount of foreign currency; like other citrus species Sat Kara is also propagated conventionally by means of seeds, grafting and budding methods (Miah *et al*., 2002). During the season, per fruit it cost Rs.15-20 but when the fruiting season is over the cost is doubled or sometimes more than double (http://manipursfac.com/wild-orange-citrus-macroptera/ 1/). Due to present demand of this fruit in both local and foreign markets, it is necessary to develop a suitable protocol for mass propagation from existing elite cultivars (Miah *et al*., 2002). Uddin *et al*., 2014_b assessed *Citrus macroptera* via biochemical and hematological evaluation in female Sprague-Dawley rats and the evaluated non-toxic effect suggests a wide margin of safety for therapeutic doses. Use of naturally derived anti-oxidant from herbs, fruits, and vegetables has been an increasing worldwide trend in recent years (Afroz *et al*., 2014). However, studies on biochemical effects of the anti-oxidant potential of natural products on vital organs would be useful (Paul *et al*., 2015).

As there is literature on the antioxidant activities of *C. macroptera* Montruz., it can be studied for bioprospecting the therapeutic resources including the anti-oxidant and anti-inflammatory associated diseases.

AIM AND OBJECTIVE

1.To establish the protocol for evaluation and screening for its therapeutic agents using mice model.

2.To study the Chemo profiling of their components for identification of bioactive compounds by using HPLC, LCMS, IR, NMR.

3.To study the therapeutic efficacy of anticancer activities using animal cell lines.

4.To evaluate Pharmacological and toxicological aspects of the bioactive products.

5. To study the molecular docking of the bioactive compound.

CHAPTER- 2

MATERIALS AND METHOD

2.1 Plant material

Fruits of *Citrus macroptera* Montruz. were collected from Kwatha Village, Chandel District, Manipur, North East India. It was identified by taxonomist of the institute, Imphal, Manipur and faculty of Botany Department, Nagaland University (Fig.3A and B). A voucher specimen was deposited at IBSD with voucher number IBSD/M-1031A.

A **B**

Fig. 3. A. Leaves, seed, and fruit of *Citrus macroptera* Montruz., B. Fruit of *Citrus macroptera* growing in its tree

2.2 Preparation of extract

Rinds of the fruits were peeled off, dried and coarse powder was made using a commercial blender. 60 g powdered sample each was macerated in aqueous (500mL), methanol (300 mL) and 500 mL aqueous methanol (1:1 volume/volume) at room temperature for 2 days with occasional stirring. Aqueous, methanol and aqueous methanol extract are denoted as NC1, NC2, and NC3 extract respectively. After filtration, the filtrate was evaporated at

40^0C under reduced pressure in a rotary evaporator (Buchi, Switzerland). Extract yield and % recovery was calculated (Nongalleima *et al*., 2017_a).

Fig.4. Cut peels of *Citrus macroptera* Montruz.

2.3 PRELIMINARY PHYTOCHEMICAL SCREENING

2.3.1 Test for alkaloid

Dragendorff's test

To 3 mL filtrate of the extracts, 5 drops of Dragendorff's reagent were added. Formation of orange-brown precipitation was observed.

Hager's test- To 3 mL filtrate of the extracts, Hager's reagent gives yellow precipitation.

Wagner's test

To 3 mL filtrate of the extracts, 5 drops of Wagner's reagent was added and observed for color change. Reddish brown precipitation indicated the presence of alkaloid.

2.3.2 Test for flavonoids (Shinoda test)

To dry extract, 5 mL of 95% ethanol, few drops of concentrated Hydrochloric acid (HCl) and 0.5g magnesium turnings were added. Observance of pink color indicates the presence of flavonoids. To a small quantity of residue, lead acetate solution was added.

It was observed for yellow color precipitation. Increasing amount of sodium hydroxide to the residue shows yellow coloration which decolorizes after addition of acid.

2.3.3 Test for phenolic compound

2.3.3 a. Lead acetate test

To the test solution of the extracts, a few drops of 10% lead acetate solution were added. Formation of white precipitate indicated the presence of phenolic compounds.

2.3.3. b. Ferric chloride test

To the test solution of the extracts, a few drops of ferric chloride were added. A dark green color indicated the presence of the phenolic compound.

2.3.4 Test for carbohydrate

Molish's test

To 3 mL of the extracts, few drops of alpha-naphthol solution in alcohol was added. The solution was shaken and added conc.H_2SO_4 from sides of the test tube. A violet ring formed at the junction of two liquids indicated the presence of carbohydrates.

2.3.4 a. Test for reducing sugars

Fehling's test

1 mL of Fehling's A and 1 mL of Fehling's B solution were mixed and boil for 1 minute. An equal volume of test solution was added. It was heated in boiling water bath for 5 min. The appearance of yellow precipitation followed by brick red precipitation indicate the presence of reducing sugars.

2.3.4. b. Test for non-reducing polysaccharides (starch)

Iodine test- 3 mL of test solution was mixed with few drops of dilute iodine solution. Blue color appearance, it disappears on boiling and reappearance on cooling indicated the presence of non-reducing polysaccharides.

2.3.5 Test for gum

The test solution was hydrolyzed using dilute HCl. Fehling's test was performed on it. Development of red color indicated the presence of gum.

2.3.6 Test for mucilage

Powdered drug material showing red color with ruthenium red indicated the presence of mucilage.

2.3.7 Tests for proteins

Biuret test

To 3 mL of the test solution, 4% Sodium hydroxide and few drops of 1% Copper sulfate were added. The appearance of yellow and pink color indicated the presence of proteins.

2.3.8 Test for steroid

Salkowski test-

To 2 mL of extract, 2mL of chloroform and 2 mL of concentrated sulphuric acid was added. It was shaken well, chloroform layer appears red and acid layer shows greenish yellow fluorescence.

2.3.9 Test for cardiac glycosides

Test for deoxy-sugars (Keller-Killiani test)

To 2 mL extract, glacial acetic acid, a 1-2 drop of 5% Ferric chloride and concentrated sulphuric acid was added. The appearance of reddish brown color at the junction of the two liquid layers and bluish green upper layer indicated the presence of deoxysugars.

2.3.10 Test for anthraquinone glycoside

To 3 mL extract, dilute sulphuric acid was added. It was boiled and filtered. To the cold filtrate, an equal volume of benzene or chloroform was added. It was shaken well and separated the organic solvent. Ammonia was added. The appearance of ammonical layer turning pink or red indicated the presence of anthraquinone glycoside.

2.3.11 Test for saponin glycoside

Foam test

The extract was shaken vigorously with water. The appearance of persistent foam indicated the presence of saponin glycosides.

2.3.12 Test for coumarin glycosides

Moistened dry powder was taken in a test tube. The test tube was covered with filter paper soaked in dilute sodium hydroxide. It was kept in water bath. The filter paper was exposed to UV light. Yellowish green fluorescence indicated the presence of coumarin glycosides.

2.4 Estimation of total phenolic content (TPC)

Total Phenolic content of extracts was determined by Folin– Ciocalteu method following Madan *et al.,* 2010 with little modification. 100µl of Extract solution (100µg/mL) was mixed with 500µl 10% (v/v) F–C reagent. 400 µl of 7.5 % Na_2CO_3 was added into each

tube and incubate the assay tubes at room temperature for 1 hr. 300 mL sample or blank was transferred from the assay tube to a clear 96 well microplate and absorbance of each well was taken at 765 nm. Standard curve was calculated from standard Gallic acid at 765 nm in Multiscan Spectrum (ThermoScientific) and total Phenolics was obtained as Gallic acid equivalents using the regression equation.

2.5 Estimation of total flavonoid content (tfc)

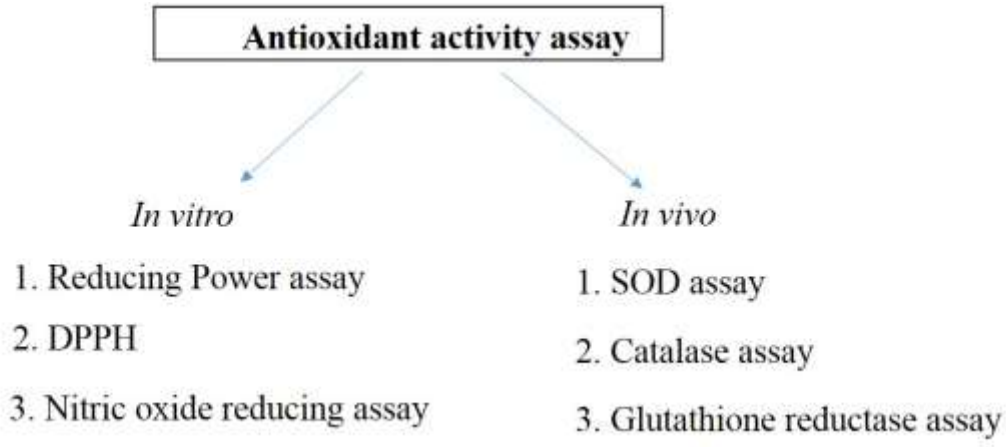
The total flavonoid content of the extracts was determined by aluminium chloride ($AlCl_3$) colorimetric method. 0.5 mL of extract was mixed with 1.5 mL methanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415nm with Thermo Multiscan Spectrum. Using Quercetin as standard, standard curve was prepared and linearity was obtained in the range of 10-100µg/mL using the standard curve the total flavonoid content was expressed as quercetin equivalent in percentage w/w of the extracts (Madan *et al.*, 2010).

2.6 Estimation of total flavonol content (tfoc)

Total flavonol content was estimated following Miliauskas *et al.*, 2004. 250 µl of sample (100µg/mL) was mixed with 250 µl $AlCl_3$ (20g/l), 750 µL of sodium acetate. It was incubated at 25 $^{\circ}$C for 2.5 h. The absorbance was read at 440 nm. Quercetin (10-100 µg/mL) was used to generate standard curve.

2.7 Antioxidant assay

Antioxidant assay was conducted by *in vitro* as well as *in vivo* models. An outline of antioxidant assay is given as follows.



2.7.1 *In vitro* antioxidant assay

2.7.1.1 Reducing power assay

The reducing power assay was done following the method described by Das *et al.*, 2014 with slight changes. 100 µL of 0.2 M sodium phosphate buffer (pH 6.6) were mixed with 100 µL of sample with different concentrations (10-100µg/mL) of the extracts and 100 µL of 1 % Potassium fericyanide was added. The reaction mixture was incubated at 50^0C for 20 minute. After incubation, 100 µL of 10 % (w/v) trichloro acetic acid (Sigma) was added. It was then centrifuged at 5000 rpm for 10 min (Eppendorf centrifuge 5430 R). The upper layer (200 µl) was mixed with 200 µL deionized water and 40µL of 0.1 % ferric chloride. The absorbance was read at 700 nm in a 96 well microplate reader (Thermo Scientific). Higher absorbance indicates higher reducing power. The assays were carried out in triplicate and the results are expressed as mean values ± standard error mean. Ascorbic acid

was used as standard. Percentage inhibition was calculated and this activity was expressed as an inhibition concentration 50 (IC_{50}).

The percent increase in reducing power was calculated using the following equation.

% Reduction= [1- (1-As/Ac)] X 100

As=maximum absorbance of max concentration of standard,

Ac- absorbance of sample

2.7.1.2 Nitric oxide reducing assay

Under aerobic conditions, nitric oxide reacts with oxygen to produce stable products (nitrates and nitrite).The quantities of which can be determined using Griess reagent. The scavenging effect of the plant extract on the nitric oxide was measured according to the modified method of Gangwar *et al.,* 2014. 500 µL of test sample with different concentration (10-100 µg/mL) was mixed with 2 mL of 10 mM Sodium nitroprusside, 500 µL of 50 mM phosphate buffer saline pH 7.4. They were incubated at 25°C for 150 min. Griess reagent (500 µl) was added and incubated at 25°C for 30 minute. The absorbance was read at 540 nm. A phosphate buffer saline served as blank.

2.7.1.3 DPPH free reducing assay

The free radical scavenging activity of the extract were measured by 1, 1-Diphenyl-2-picryl hydrazil (DPPH) purchased from HiMedia laboratories Pvt. Ltd. India using the method of Sakthivel *et al.,* 2013. Briefly, 0.1 mM solution of DPPH in ethanol was prepared. Then, 100 µL of this solution was mixed with 300 µL of extracts solution in a 96 well plate. L Ascorbic acid was used as positive control. Both the control and the extract were tested at different doses (10– 100µg/mL). The mixture were shaken vigorously and allowed to stand

at room temperature for 30 min under dark condition. Then the absorbance was measured at 517 nm in Thermo Multiscan Spectrum. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity

The percentage inhibition was calculated by using the formula. And a linear graph was plotted using % inhibition against concentration, and IC_{50} was then calculated.

$$\% \text{ Inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

2.7.2 *In vivo* studies

2.7.2.1 Animal

Adult female Swiss albino mice (30 g ± 1.2) was used for the study. The animals were purchased from Regional Institute of Medical Sciences, RIMS, Imphal, Manipur (India). They were kept under standardized conditions (temperature 27 ± 2° C, and light/dark cycle of 12 hrs.) and fed a normal laboratory diet. The experiments were performed based on animal ethics guidelines of Institutional Animal Ethics Committee.

2.7.2.2 Acute toxicity

Acute oral toxicity was tested. Female albino mice (n=6) were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose of 5mg/Kg/p.o. and observed for 14 days. Mortality was not observed, the procedure was repeated for higher dose (100, 500, 2000 mg/Kg/p.o.)

2.7.2.3 Experimental design

After 1 week of time, mice were divided into 5 groups, n= 5. The extract was dissolved in 0.3 % Tween 80 to obtain a 15mg/mL solution. Group 1 animal received 1 mL distill water and served as normal control. Using a metal oropharyngeal cannula, the extracts and

standards were administered orally. The following grouping pattern was adopted for the study.

- Group I – Normal Control (1mL distill water p.o.)
- Group II – CCl_4 control
- Group III - CCL4 + L-ascorbic acid 10 mg/Kg
- Group IV- CCL_4 + aqueous extract (NC1) 50mg/Kg/ p.o
- Group IV - CCL_4 + methanol extract (NC2) 50mg/Kg/ p.o
- Group V - CCL_4 + aqueous methanol extract (NC3) 50mg/Kg/ p.o

After 24 hr. of 7 day treatment blood was collected from retro-orbital plexus under ether anesthesia. The blood samples were allowed to stand to get the blood clot which was subjected to centrifugation and the upper serum layer was collected. Certain volume of blood was separately transferred to a tube and treated with EDTA (plasma EDTA). The animals were then sacrificed and dissected. Liver and brain tissues were collected for *in vivo* antioxidant and other studies.

2.7.2.4 Superoxide dismutase (SOD) assay

The assay was done using the SOD assay Kit (Sigma). Briefly, 1 mL of working standard solution (WST) was diluted with 19 mL of buffer solution. The enzyme solution tube was centrifuged for 5 sec. It was mixed by pipetting 15µl of enzyme solution was diluted with 2.5 mL of dilution buffer. 20 µL of sample solution was added to sample well and blank 2 well. 20 µL of double distill H_2O was added to each blank 1 and blank 3 well. 200 µL of WST solution was added to each well, and they were mixed. To the blank 2 and blank 3 wells, 20 µL of dilution buffer was added. To the sample wells and blank 1 well 20 µL of

enzyme working solution was added and mixed thoroughly. The plate was incubated at 37°C for 20 min. It was then read at 450 nm.

SOD activity (% Inhibition) =

$$\frac{100 \text{ X (Absorbance of blank 1- Absorbance of blank 3) – (Absorbance of sample-Absorbance of blank 2)}}{\text{(Absorbance of blank 1- Absorbance of blank 3)}}$$

2.7.2.5 Catalase (CAT) assay

Catalase assay was done using catalase kit (Sigma) following the instructions provided in it. Briefly, 10 µL of serum or EDTA plasma or liver tissues, 25 µL of assay buffer was mixed with 12.5 µL of 200 mM H_2O_2 and incubated for 1-5 min. 450 µL of stop solution was added. 10 µL of reaction mixture from the above was transferred to a microtube and 500 µL of color reagent was added. It was kept at room temperature for color development. OD was taken at 520 nm within 15 min.

2.7.2.6 Glutathione reductase assay

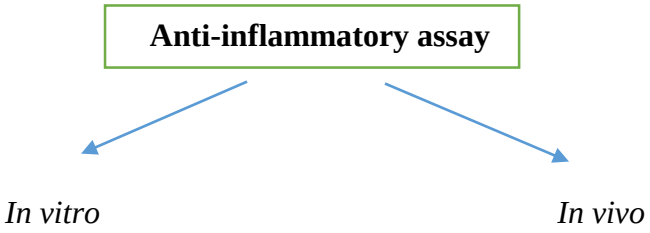
The assay was carried out using glutathione reductase assay kit (Cayman, USA). Briefly, 120 µL of assay buffer and 20 µL of GS-SG was added to non-enzymatic wells. 100 µL of assay buffer, 20 µL of GS-SG and 20 µL of diluted GR control was added to positive control well. 100 µL of assay buffer, 20 µL of GS-SG and 20 µL of sample (serum, EDTA plasma, liver, brain tissue) was added to sample well. 50µL of NADPH was added to all the wells. The plate was shake for a few seconds. The absorbance was read at 340nm once every minute to obtain at least 5 time points. The activity was calculated using the following

$$\blacktriangle A_{340}/\text{min.} = \frac{A_{340}\text{ (Time 2)- }A_{340}\text{ (Time 1)}}{\text{Time 2 (min.) – Time 1 (min.)}}$$

$$\text{GR activity} = \frac{\blacktriangle A_{340}/\text{min}.}{0.00373\ \mu M^{-1}} \times \frac{0.19\ \text{mL}}{0.02} \times \text{sample dilution}.$$

2.8 Anti-inflammatory assay

Like antioxidant assay, anti-inflammatory assays are also done by *in vitro* as well as *in vivo* studies.



1. Protease inhibitory assay
2. Albumin denaturation assay 1. Carrageenan induced paw edema
3. Heat induced haemolysis assay

2.8.1 *In vitro* anti-inflammation

2.8.1.1 Protease inhibitory assay

Protease inhibitory assay was done following the protocol described by Bijina *et al*., 2011 with minor modification. Briefly, 500 µL of trypsin (0.5mg/mL) prepared in 0.1 M phosphate buffer, pH-7 was pre-incubated with 500 µL of sample with different concentration (10-100 ug/mL) at 37 $^{\circ}$C for 15 minute. After incubation 1 mL of 1 % casein prepared in 0.1 M phosphate was added. It was then incubated at 37 $^{\circ}$C for 30 minute. The reaction was terminated by adding 1.25 mL of 0.44 M Trichloroacetic acid. It was transferred to centrifuge tube and centrifuge at 10,000 rpm for 15 minute. Supernatant was taken and absorbance was measured at 280 nm.

2.8.1.2 Heat induced haemolysis assay

Stock solution of 1 mg/mL plant extract was prepared. 500 uL of varying concentrations of this extract solution (10, 20, 40, 80 and 100 ug/mL) was mixed with 500 uL of 10 % RBC suspension. It was incubated at 56°C for 30 min. The reaction mixture was cooled and centrifuged at 2500 rpm for 5 min. The pellet was discarded and absorbance was taken in the supernatant at 560 nm. Saline and Diclofenac sodium was taken as control and standard reference respectively. The assay was done following Gupta *et al*., 2013 with slight modification.

2.8.1.3 Inhibition of albumin denaturation

The assay was done following Sakat *et al*., 2010. Reaction mixture of 1 % aqueous solution of bovine serum albumin (Sigma) and test extract at different concentration (10-100 µg/mL) was taken in a centrifuge tube and pH was adjusted to 6.8 using 1N HCl. It was incubated at 37 °C for 20 min followed by heating at 57 °C for 20 min. The solution was cooled and absorbance was taken at 660 nm.

2.8.2 *In vivo* anti-inflammatory studies

2.8.2.1 Carrageenan - induced paw edema

Experimental design

After 7 days of acclimatization, the animals (Swiss albino mice) were divided into seven groups of 5 animals each. The treatment was done as follows.

Group I - Animals (Control) were administered 1mL distill water p.o., /animal, Group II - Aqueous extract (NC1) 50mg/Kg/ p.o,

Group III - Aqueous extract (NC1) 100mg/Kg/ p.o.,

Group IV - Aqueous extract (NC1) 150mg/Kg/ p.o.,

Group V - methanol extract (NC2) 50mg/Kg/ p.o,

Group VI - methanol extract (NC2) 100mg/Kg/ p.o.,

Group VII- methanol extract (NC2) 150mg/Kg/ p.o.,

Group VIII- aqueous methanol extract (NC3) 50mg/Kg/ p.o.,

Group IX- aqueous methanol (NC3) extract 100mg/Kg/ p.o.,

Group X- aqueous methanol (NC3) extract 150mg/Kg/ p.o.,

Group XI- Diclofenac sodium salt 5 mg/Kg/p.o.

The extracts were prepared in 0.3 % tween 80. The plant extracts NC1, NC2, NC3 in three different doses, 50, 100 and 150 mg/Kg/p.o and vehicle was administered orally. Diclofenac Sodium (Sigma), was used as positive control and administered once at a dose of salt 5 mg/Kg/p.o. 30 minutes after administration of reference and test drugs, paw edema was induced by injecting 0.1 mL of 0.1% w/v carrageenan sodium salt subcutaneously in the sub-plantar region of the left hind limb in each groups. The paw volume was measured a Plethysmometre (Ugo Basile, Italy) before injection and at 0 min, and 1, 2, 3, 4, 6 hours.

2.8.2.2 Measurement of pro-inflammatory mediators (TNF-α, IL-1ß, IL-10) in serum

Blood samples were collected from the heart of the mice, treated with 100 mg/Kg extracts of NC1, NC2 and NC3, after sacrificing. Blood samples were allowed to clot for 30 minutes at room temperature. It was then centrifuged at 1000 g for 10 minutes, serum was then collected and assayed immediately. The levels of TNF-α, IL-1ß, IL-10 were measured using commercial ELISA kit. All the analyses were performed according to manufacturer's instructions.

2.8.2.2.1 TNF- α ELISA assay

TNF- α assay was done following the assay protocol provided in the Mouse TNF- α ELISA Kit (Millipore, California)

2.8.2.2.1. a Reagent preparation

20X Wash buffer: The wash buffer provided was 20X concentrate. It was diluted to 1X strength using Milli-Q water.

Mouse TNF- α standard: The lyophilized Mouse TNF- α standard was reconstituted by adding the volume of assay buffer A indicated on the vial label to make 20ng/mL standard stock solution. The reconstituted standard was allowed to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely. The lyophilized Matrix A was reconstituted by dispensing 2 mL of Milli-Q water into the vial and allowed the matrix to sit at room temperature for 15 minutes, then vortex to mix completely for serum and plasma samples assay.

2.8.2.2.1.b Assay procedure

All the reagents were brought to room temperature prior to use. By diluting 25µL of the standard stock solution in 475 µL of Assay Buffer A 500 µL of the 1000 pg/mL was prepared. By serial dilution with Assay buffer A as diluent, different concentrations 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL and 31.3 pg/mL of TNF-α standard was prepared. Assay buffer A serves as the zero standard (0 pg/mL).

50µL of serum and plasma sample of Swiss albino mice treated with *C. macroptera* extracts (NC1, NC2, NC3) were separately added to the well, normal group of mice without any treatment served as control. 50 µL of standard dilutions were added to the appropriate well marked for it. 50 µL of Assay buffer A was added to the well containing the samples, and

50 µL of matrix A were added to the well containing the standard dilutions. The plate was sealed with a plate sealer and incubated at room temperature for 2 hr while shaking at 200 rpm. The contents of the plate were discarded and washed 4 times with 1X wash buffer. 100µLof mouse TNF-α detection antibody solution was added to each well, the plate was sealed and incubated at room temperature for 1 hr while shaking. Discarded the content of the plate and washed thoroughly 4 times with 1X wash buffer. 100 µL of Avidin-HRP A solution was added, sealed the plate and incubated at room temperature for 30 min. while shaking. After 30 minutes, the contents of the plate were discarded and washed 5 times with 1X wash buffer. To minimize background, wash buffer was soaked in each well for 30 sec to 1 min. for each wash of this final washing step. 100 µL of substrate solution F to each well and the plate was incubated for 15 min. in the dark.

Wells containing mouse TNF-α should turn blue color with intensity proportional to the concentration. The reaction was stop by adding 100 µL of stop solution to each well. The solution color should change from blue to yellow. The absorbance was read at 450 nm within 30 min. Also it was read at 570 nm. Absorbance at 570 nm was subtracted from absorbance at 450 nm. A standard curve was plotted with different concentrations of mouse TNF-α (pg/mL) on X-axis Vs absorbsance value on Y-axis. From this standard curve, concentrations of samples were extrapolated.

2.8.2.2.2 IL-10 ELISA

IL-10 assay was carried out following the protocol provided in the assay kit (In vitrogen, California). The reagents were prepared following the instructions provided. Briefly, background and standard wells were marked in the 96 well plate strip. Serum of Swiss

albino mice treated with *C. macroptera* extracts (NC1, NC2, and NC3) were used for the assay. Normal group of mice without any treatment served as control.

100 µL of background control and standard control was added in the marked well. 50 µL of standard diluent buffer to sample wells followed by 50 µL of serum and control to appropriate wells. 50 µL of Rat IL-10 biotin conjugate solution was added into all the wells except chromogenic blank. The plate was covered with plate sealer provided in the kit, and incubated for 2 hr at room temperature. The solutions were thoroughly aspirated and wash the wells 4 times with diluted wash buffer. 100µL of Streptavidin-HRP was added into each well except chromogenic blank. The plate was covered with plate sealer and incubated for 30 minutes at room temperature. The content of the wells were discarded and wash 4 times with wash buffer. 100 µL of stabilized chromogen (TMB) was added to each well. The substrate was turning to blue color. The plate was covered again and incubated at room temperature in dark. 100 µL of stop solution was added to each well, change of solution color was observed from blue to yellow. The absorbance was read at 450 nm.

2.8.2.2.3 IL-1ß

IL-1ß assay was done following the protocol provided in the IL-1ß assay kit (In vitrogen, California). All the reagents was prepared as per the instruction provided in the kit. IL-1ß standard was prepared in 6 different concentrations (0, 25.6, 64, 160, 400, 1000, 2000) pg/mL using provided sample diluent.

1. 50 µL of serum was added to wells marked for it.
2. 50 µL of sample diluent was added to each well. The plate was covered and incubated at room temperature (20-25° C) for 2 hrs.
3. The plate was washed five times with wash buffer.

4. 100 μL of biotinylated antibody reagent was added to each well. The plate was covered and incubated at room temperature for 30 minutes.
5. The plate was washed five times with wash buffer.
6. 100 μL of prepared Streptavidin- HRP solution was added to each well. The plate was covered and incubated at room temperature for 30 min.
7. The plate was washed five times.
8. 100 μL of TMB substrate was added to each well, and the plate was developed in the dark at room temperature for 30 min.
9. The reaction was stop by adding 100 μL of stop solution. Absorbance was read at 450 nm and 550 nm.
10. Absorbance at 550 nm was subtracted from absorbance at 450 nm. Standard curve was plotted with concentration Vs absorbance of IL-1ß standard. The result was then calculated.

2.8.3 Hepatic assessment of Aspartate aminotransferase (AST) and Alanine Aminotransferase (ALT) Levels

Enzyme such as AST and ALT are main liver transaminases and they have been used for the assessment of liver damage (Howell *et al*., 2014). As anti-inflammation and anti-oxidant are related and dependent to each other, content of AST and ALT enzymes were measured to determine the antioxidant driven anti-inflammatory effect of extracts of *C. macroptera* Montruz. and also as an indicator of liver function parameters or hepatoprotective efficacy. The assay was carried out using commercial diagnostic assay kits.

2.8.3.1 Aspartate aminotransferase (AST)

The assay was carried out following the protocol provided in the AST activity assay kit (Sigma-Aldrich, USA). ALT is also known as serum glutamic pyruvic transaminase (SGPT).

2.8.3.1a. **Sample Preparation:**

50 mg Tissue (brain, liver) treated with NC1, NC2 and NC3 extracts was homogenized in 200 μL of ice-cold AST Assay Buffer. The samples were centrifuged at 13,000 × g for 10 minutes to remove insoluble material. Serum samples was directly added to wells.

2.8.3.1b. **Glutamate Standards for Colorimetric Detection:**

10 μL of the 0.1 M Glutamate Standard solution was diluted with 990 μl of the AST Assay Buffer to prepare a 1 mM standard solution. 0, 2, 4, 6, 8, and 10 μL of the 1 mM standard solution was added into a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. AST Assay Buffer was added to each well to bring the volume to 50 μL.

Procedure

1. Master Reaction Mix was prepared by adding 80 μL AST Assay Buffer, 2 μL AST Enzyme Mix , 8 μL AST Developer, 10 μL AST Substrate. 100 μL of the reaction mix was required for each well.

2. 50 μL of the liver, brain, serum and plasma samples were added to wells marked for it. Reaction Mix was added to each of the wells, and mix well using a horizontal shaker. The plate was protected from light during the incubation

3. The plate was incubated at 37° C. After 2–3 minutes, take the initial measurement (Ti_{nitial}). Measure the absorbance at 450 nm at the initial time (A450) initial.

4. The plate was continued to incubate at 37° C taking measurements (A450) every 5 minutes. The plate was incubated under dark.

5. Measurements were taken until the value of the most active sample was greater than the value of the highest standard (10 nmole/well). At this time the most active sample is near or exceeds the end of the linear range of the standard curve.

6. The final measurement [(A450) final] for calculating the enzyme activity would be penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve. The time of the penultimate reading is T_{final}.

The AST activity of a sample may be determined by the following equation:

$$\text{AST Activity} = \frac{B \times \text{Sample Dilution Factor}}{(\text{Reaction Time}) \times V}$$

B = Amount (nmole) of glutamate generated between $T_{initial}$ and T_{final}.

Reaction Time = $T_{final} - T_{initial}$ (minutes)

V = sample volume (mL) added to well

AST activity is reported as nmole/min/mL = milliunit/mL One unit of AST is the amount of enzyme that will generate 1.0 μmole of glutamate per minute at pH 8.0 at 37° C.

2.8.3.2 Alanine Aminotransferase (ALT) activity

ALT assay was done using ALT activity assay kit (Sigma-Aldrich, USA). The reagents were prepared as per the instruction.

2.8.3.2a Sample Preparation

50 mg Tissue (liver tissue) and serum treated with *C. macroptera* extracts (NC1, NC2, and NC3) was rapidly homogenized with 200 μL of ALT Assay Buffer. Centrifuge at 15,000 × g for 10 minutes to remove insoluble materials. Serum samples were directly added to

wells. Add 1–20 μL samples into wells of a 96 well plate. Samples were brought to a final volume of 20 μL with ALT Assay Buffer as 20 μL of sample for each reaction (well) was required.

2.8.3.2b Pyruvate Standards for Colorimetric Detection

10 μL of the 100 nmole/μL Pyruvate Standard was diluted with 990 μl of ALT Assay Buffer to prepare a 1 nmole/μL standard solution. 0, 2, 4, 6, 8, and 10 μL of the 1 nmole/μL standard solution was added into a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. ALT Assay Buffer was added to each well to bring the volume to 20 μL.

2.8.3.2c Procedure:

1. 100 μL of master reaction mix was required for each reaction well. It was prepared by mixing 86 μL ALT Assay Buffer, 2 μL Fluorescent Peroxidase Substrate, 2 μL ALT Enzyme Mix and 10 μL ALT Substrate.

2. 100 μL of the Master Reaction Mix was added to each of the standard, positive control, and test wells. 5 μL of the AST positive control solution was added to wells and adjust to 50 μL with the AST Assay Buffer. The well was mixed using a horizontal shaker or by pipetting.

3. After 2–3 minutes, the initial measurement ($T_{initial}$) was taken. For colorimetric assays, measure the absorbance at 570 nm (A570).The plate was incubated under dark condition at 37 °C taking measurements every 5 minutes

4. Measurements were taken continuously until the value of the most active sample was greater than the value of the highest standard. At this time the most active sample is near or exceeds the end of the linear range of the standard curve.

5. The final measurement for calculating the enzyme activity would be the penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve. The time of the penultimate reading is T_{final}.

6. The change in measurement from $T_{initial}$ to T_{final} for the samples and positive control was calculated.

$$\Delta A570 = (A570)_{final} - (A570)_{initial})$$

The ALT activity of a sample may be determined by the following equation:

$$\text{ALT Activity} = \frac{B \times \text{Sample Dilution Factor}}{(T_{final} - T_{initial}) \times V}$$

B = Amount (nmole) of pyruvate generated between Tinitial and Tfinal

$T_{initial}$ = Time of first reading in minutes.

T_{final} = Time of penultimate reading in minutes.

V = sample volume (mL) added to well.

ALT activity reported as nmole/min/mL = milliunit/mL, where one milliunit (mU) of ALT is defined as the amount of enzyme that generates 1.0 nmole of pyruvate per minute at 37 °C.

2.9 Cytotoxicity study

2.9.1. Cell culture

HeLa cells, a human cervical cancer cells were obtained from American Type Culture Collection (ATCC). They were cultured in DMEM (Dulbecco's modified essential media) fortified with 10 % (v/v) fetal bovine serum, 100µg/mL streptomycin and 100µg/mL penicillin. The cells were incubated in a humidified $C0_2$ incubator (Thermo Scientific, USA) at 37° C with 5% $C0_2$ supply.

2.9.2 MTT assay

Cell proliferation was determined by MTT assay. Cells (HeLa) were plated at 5000/well/0.1 mL in complete medium.1 mL different concentration of the aqueous extract and methanol extract of *C. macroptera* Montruz. was added and incubated at 37°C at 95 % humidity, 5 % CO_2 ± for 72 h. At the end of 72h, 20µLof MTT, 3-(4, 5- Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide solution (5mg/mL) per well were added and incubated at 37°C for 2 h. At the end of 2h, 0.1 mL of lysis buffer (20% W/V of SDS dissolved at 37°C in a solution of 50 % N,N-dimethyl formamide in distilled water) was added per well and incubated at 37^0C for 4 h-overnight. The plate was read at 590 nm with just the lysis buffer as control. Cisplatin was used as positive control.

2.9.3 Clonogenic assay

Cell survival was assessed using clonogenic assay of Puck and Marcus, 1955. 100-200 cells were inoculated in 5 cm^2 petridishes containing 5 mL media. The cells were allowed to attach for 12-16 hr prior to addition of plant extracts. After 16 hr the cultures were exposed to 100 µg/ mL concentration of aqueous extract. Medium containing the extract were removed, and washed with phosphate buffer saline twice. Fresh medium without any extract or drug was added and allowed the culture to grow for another 10 -11 days. After that, the colonies were stained in 1 % crystal violet in methanol for 15-20 minutes. Colonies were fixed in 3.7% Paraformaldehyde (PFA) or 10 % formalin. The culture dishes were washed in running water until the blue background of the dishes become clear. The clusters containing 50 or more cells were scored as a colony, and they were counted.

2.10 Statistical Analysis

The results were expressed as the mean ± SEM for three replicates. Linear regression was used to calculate IC50. Results were considered significant at ***P<0.001, or **P < 0.01 or * P<0.05 when compared test groups v/s control group. For numerical results, one-way analysis of variance (ANOVA) with Tukey- Kramer Multiple Comparisons post tests were performed using GraphPad InStat Version 3 (GraphPad Software). All the graphs and figures were drawn using GraphPad Prism.

2.11 Oil Extraction and GCMS analysis

2.11. 1 Oil extraction

The peel of the collected fresh fruit was freshly peeled off. Leaves were washed to remove dust and dirt. 560 g of the finely grated peel and 1 Kg leaves were separately extracted by hydro distillation with the help of Clevenger type apparatus for 3-4 hrs. The collected oil droplets along with water was transferred to a conical flask and dried over anhydrous sodium sulfate (approx. 1g). The resulting solution was filtered through a funnel containing a cotton plug to enable complete removal of sodium sulfate. The essential oil yield were measured. The extracted oils were stored at 4°C until gas chromatographic determination of its components and bioactivity assays were done.

2.11.2 Gas chromatographic analysis of essential oil extract

Gas chromatographic mass spectrometry analysis was carried out at Advanced Instrumentation Research Facility (AIRF), JNU, New Delhi on a Shimadzu GCMS-QP_{2010} equiped with an AOC-2oi auto-injector and AOC-2os autosampler units, column Rtx-5 MS (Restek Corporation). The column length was 30m, 0.25 mm diameter and 0.25µm film thickness). Column temperature was initially kept at 50^0 C, then gradually increased to

280°C at 5^0 C/min rate, column pressure was 69.0 KPa. Detector interface temperature was 270^0C, Carrier gas was helium. Purge flow was 3.0 mL/min. Injection temperature was 260 ^{0} C. In split mode in a 1:10 split ratio, 1 microlitre of sample was injected by autosampler attached to the instrument. Flow rate was 1.21 mL/min.

In mass spectrophotometry, ion source temperature was 230°C, interface temperature was kept at 270 °C, solvent cut time at 2.50 min. thresshold at 1000. Temperature program used was 2 min. hold at 50^0 C followed by 3^0C/min ramp to reach temperature of 210°C held for 0 min., and final ramp of 8 °C /min. to reach the final temperature of 280°C held for 8 min. The total length of run was 72.07 min. with scan range of 40-650 m/z and scan speed 333 amu/sec.

2.11.3 Data acquisition and processing

Chromatograms and mass spectra recorded were acquired by GCMS 2010QP-PLUS (Shimadzu) and processed by GCMS Solution post run analysis software (ver. 2.5) provided with the instrument. The components were identified based on the comparison of their retention indices relative to n-alkanes series and mass spectra with those of authentic samples using commercially available mass spectral libraries NIST 05, NIST 08 with a similarity index (SI) higher than 75%.

2.11.4 Comparative *in vitro* antioxidant and anti-inflammatory activity of leaves and peel essential oil.

In vitro anti-oxidant and anti-inflammatory activity of leaves as well as peel of *Citrus macroptera* Montruz. essential oil was determined following the methods employed for determining the said activities of the extracts.

2.12 High Performance Liquid Chromatography (HPLC) analysis

2.12.1 Preparation of sample

2.12.1a Rind/Peel

Rinds of the fruits were peeled off; dried and coarse powder was made using a commercial blender. Rinds of the fruits were peeled off; dried and coarse powder was made using a commercial blender. 60 g powdered sample each was macerated in aqueous (500mL), methanol (300mL) and 500 mL aqueous methanol (1:1 volume/volume) at room temperature for 2 days with occasional stirring. After filtration, the filtrate was evaporated at 40^0C under reduced pressure in a rotary evaporator (Buchi, Switzerland).

2.12.1b Seeds

Seeds of C. macroptera were removed, washed and shade dried. Dried seeds (5g) was ground with methanol (50 mL) at 60 $^{\circ}$C for 6 hrs. The extract was cooled, filtered and concentrated.

2.12.1c Juice

The pulp of *C. macroptera* were taken out and blended using a commercial grinder. The juice was extracted, filtered and freeze dried using a freeze drier (LabConco, Kansas).The dried powder (100 mg) was extracted with 100 mL of methanol twice for 30 minute. All the extracts were pooled separately, concentrated in *in vacuo* and stored at 4 $^{\circ}$C till use. The samples were prepared as 1 mg/mL and were then filtered through 0.45 µm filter.

2.12. 2 HPLC instrument, reagents and standard preparation

The analytical separations were carried out on UFLC Shimadzu, CBM-20A communication bus module, 2 solvent delivery system, pump LC-20 AD (A), LC -20 (B), a SPD-M20 photodiode array detector, a 2 chamber in-line degasser. The analysis was carried on a Purospher STAR RP-18 endcapped column (Milipore), 5µm particle size, 250mm X 4.6 mm. Naringin, Coumarin and Naringenin was purchased from Sigma. Acetonitrile, water and acetic acid used were all HPLC grade.

Stock solution of standards (Naringin, Coumarin and Naringenin) were prepared by dissolving 1 mg of each compound in 1 mL of methanol or methanol water (1:1 v/v). The stock solutions were diluted to five different concentrations *viz*. 25, 50, 100, 200, 400 µg/mL with binary system of methanol and water for Naringin, water for coumarin, and methanol for Naringenin.

2.12.3 HPLC method validation and simultaneous quantification of Naringin, Naringenin and Coumarin

2.12.3a Calibration curve

Each of the standard solutions with five different concentration containing 25-400 µg/mL was injected onto the HPLC and elution was carried out following the above chromatographic conditions. The analysis was done in triplicate with an injection volume of 10 µl. The calibration curve was prepared by plotting peak area (average of three runs) versus concentration of the standard analyte.

2.12.3b Limit of Detection and Quantitation

The Limit of Detection (LOD) is the minimum amount of concentration of component or substance that can be detected with a given analytical method. Limit of Quantification (LOQ) is the minimum amount of concentration of compound that can be detected. LOD and LOQ were determined from the calibration curve. A signal to noise ratio of 3 and 10 was considered acceptable for estimating the LOD and LOQ respectively. Different injection volumes were used and it varied from 5 to 20 µL. They can be calculated using the equations,

LOD (µg/mL) = 3.3 * standard deviation of *y*-intercept of regression life/ Slope

LOQ (µg/mL) = 10 * standard deviation of *y*-intercept of regression life/ Slope.

However, it can be easily calculated using Microsoft Excel by giving the command

= STEYX (known value of x, known value of y)

LOD = STEYX/Slope * 3.3

LOQ = STEYX/ Slope * 10

2.12.3c Precision and accuracy

Intraday precision was determined by calculating the % relative standard deviation (RSD) for five determinations at each three concentration of samples (100, 200, and 400 µg/mL) on the same day. Inter-day precision was determined by assaying three samples in triplicate on three separate occasions. Precision was calculated by calculating the mean, standard deviation, and coefficient of variation (RSD %) of these values. Accuracy was determined by calculating the RSD % between estimated concentrations and nominal concentrations.

2.12.3d Robustness

The robustness of the method was performed by evaluating small variations (±2 %) in mobile phase composition- 37% Acetonitrile, MeCN as solvent B and water as solvent A. Variation of the MeCN content in the mobile phase in the 35-39 % range significantly affects the elution behavior. The retention time for all analytes decreased with increasing the MeCN content in the mobile phase. No loss of the column performance was noted.

Three different concentrations (100, 200 and 400 µg/mL) of Naringin, Coumarin and Naringenin were added to the known amount of aqueous, methanol, aqueous-methanol, seed and juice solutions. The spiked samples were analyzed three times (*n*= 5).

2.12.3e Stability

The stability of the standard stock solutions was determined by comparing the peak areas of aliquots which are kept undisturbed for more than 8 hours with the peak areas of the standard aliquots prepared freshly. The peak areas and concentration of the prepared standard aliquots stored at -20^0C for 20 days were calculated and its stability was determined.

2.12.3f Statistical analysis

The results were expressed as the mean ± SEM for three replicates. Linear regression was used to calculate IC_{50}. The analysis was done in GraphPad inStat 3.

2.12.4 Simultaneous RP-HPLC determination of phenolics

2.12.4.1 Preparation of standard

A stock solution of standards (Quercetin, Rutin, and Kaempferol) were prepared by dissolving 1 mg of each compound in 1 mL of methanol or methanol-water (1:1 v/v).

The stock solutions of each standard were diluted to five different concentrations *viz*. 31.25, 62.5, 125, 250, 500 µg/mL with methanol.

2.12.4.2 Chromatographic condition

The solvent system used was water with 0.2 % acetic acid (Solvent A) and methanol (solvent B). The elution was conducted at 37% solvent B. The flow rate was kept at 0.75 mL^{-1} minute. All standards and samples were filtered through 0.25 µm Axiva syringe filter and 20 µL loop was used in the study.

2.12.4.3 Quantification of Quercetin, Rutin, and Kaempferol by RP-HPLC

Kaempferol, Rutin and Quercetin quantification determined by RP-HPLC was performed for each extract of *Citrus macroptera* Montruz as per the above optimized chromatographic conditions. The retention time of the peaks of the samples was compared with a retention time of the tested flavonoids' peak. Each of the standard solutions with five different concentration containing 31.25- 500 µg/mL were injected onto the HPLC and elution was carried out following the above chromatographic conditions. The analyses were done in triplicate with an injection volume of 10 µL. The calibration curve was prepared by plotting peak area (average of three runs) versus concentration of the standard analyte. The amount of quercetin, Rutin, kaempferol were quantified from the linear regression equation of the calibration curve. Limit of detection and limit of quantification was also calculated. LOD and LOQ of the system were also calculated.

2.13 Bioactive guided fractionation, isolation and purification of bioactive compounds

The extracts of *C. macroptera* showing potent activity was chosen and fractionation was done. The bioactive guided fractionation of the extracts was followed (Fig.19). DPPH radical scavenging, nitric oxide assay, reducing power assay and protease inhibitory assay of the fractions of aqueous and methanol extract was done following the method described above. Aqueous extract thus has ethyl acetate (NC4), n-butanol (NC5) and an aqueous (NC6) fraction. Similarly, methanol extract was fractionated into ethyl acetate (NC7), n-butanol (NC8) and an aqueous (NC9) fraction. n-butanol fraction (NC8) of methanol extract was further concentrated in vacuo under reduced pressure. A slurry of silica gel (60-120 mesh) was made by mixing with hexane and loaded in a glass column. They were allowed to settle and kept undisturbed overnight with its opening valve close. 25g of the NC8 fraction was mixed with silica and loaded in the glass column packed with silica. Vacuum Liquid Chromatography was done with chloroform and methanol as eluting solvent. Thirteen nos. of fractions were collected. Care should be taken while packing the column with silica and hexane to avoid bubble formation and breakage of the column. The percentage of chloroform-methanol ratio used for eluting the fractions were started from 100% $CHCl_3$+ 0% MeOH with gradual increase of 5% MeOH in each step of elution till 100% MeOH+ 0% $CHCl_3$.

With varying concentration/percentage of eluting solvent, eluted subfractions were collected and Thin Layer Chromatography (TLC) was performed on TLC Silica gel 60 F_{254} Aluminium sheets (Merck Millipore, USA) using solvent system (Ethyl acetate: Methanol: Water: Formic acid: 8.5:1.0:0.5:0.1). The TLC plate was observed under UV lamp (Genei, Bangalore). Some spots or compounds though present may not be detected in UV radiation.

Hence, the plates were developed by spraying with 10% H_2SO_4, charred at 110°C for 2-4 minutes and observed under UV light at a short wavelength (254 nm) as well as the long wavelength (365 nm).

Spots on TLC were marked and eluted separately using preparative TLC. Silica gel was separated out, concentrated the solvent containing the dissolved spot/ compound. It was repeatedly done to increase the volume of the collected compound. 4 different compounds were collected and marked as BK1, BK2, BK3, and BK4. It was purified, dried and stored at 4°C till further use. Its melting point was checked using Melting Point M-560 (Buchi, Switzerland).

2.14 Identification of compound

The isolated compounds were sent to SAIF center at NEHU, Shillong to take MS and NMR spectra. FTIR spectra were taken at Chemistry Department, Manipur University. Structure elucidation was done to identify the compounds.

2.15 Study on the effect of BK1, BK2, BK3, and BK4 on carrageenan-induced inflammation

Following the same method of *in vivo* model of inflammation described in 3.8.2.1 section, BK1, BK2, BK3, and BK4 were tested for its anti-inflammatory activity against carrageenan-induced paw edema. 15mg/Kg.p.o. BK1, BK2, BK3, and BK4 was studied. After the assay, the inflamed lesions of paw tissues were dissected out and histopathological studies were done. The paw tissues were washed with ice-cold phosphate buffer and stored at -20^0C in RIPA buffer till further use.

2.15.1 Protein isolation

Paw tissues treated with the isolated compounds (BK1, BK2, BK3, and BK4) were taken out and thawed. Following the standardized extraction procedures, the protein was isolated. Whole cell lysis buffer with the composition listed was prepared.

Whole cell lysis buffer (Kinase buffer)

Distilled water	- 1180 µl
10% Nonidet P-40	- 150 µl
5 M NaCl	- 75 µl
1 M HEPES	- 30 µl
0.1 M EGTA	- 7.5 µl
0.5 M EDTA	- 6 µl

Paw tissues were treated with 980 µL of lysis buffer having 20 µL of 2% protease inhibitor cocktail (Sigma) and homogenized using homogenizer. The homogenized tissues were vortexed for the 2-minute interval for 3-4 times. It was then centrifuged at 14000 rpm for 30 min. The supernatant was taken and discarded the pellet. It was then centrifuged at 14000 rpm for 30 minutes. Protein is thus ready for estimation.

2.15.2 Protein estimation

Dye was prepared by mixing 4.5 mL water with 1 mL of quickstart Bradford reagent. 200 µL of diluted dye was added to each well of the 96 well plate. The 50µlof protein sample was added and read absorbance at 590 nm in a plate reader. Different concentrations of Bovine Serum Albumin (BSA) 0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 mg/

mL was used as standard. The protein content of the samples was estimated from the standard curve of BSA. Different tissue samples were estimated to have a different amount of protein. For loading equal quantity (50 µg) of 15 µL protein in gel electrophoresis, the isolated proteins were diluted with distilled water.

2.15.3 SDS-PAGE electrophoresis

2.15.4 Separating gel

1. Sterile water - 4.0 mL
2. 1.5 M Tris - 2.5 mL
3. 30 % acrylamide - 3.3 mL
4. 10% SDS - 100 µL
5. 10% APS - 100 µL
6. TEMED - 15 µL

2.15.5 Stacking gel

1. Sterile water - 2.8 mL
2. 0.5 M Tris pH 6.8 - 1.25 mL
3. 30 % Acrylamide - 0.85 mL
4. 10 % SDS - 50 µL
5. 10 % APS - 50 µL
6. TEMED - 7.5 µL

2.15.6 Electrode buffer

15 µL protein samples were mixed with 5 µL loading dye. The separating gel was cast first, allowed to set and then the stacking gel was stacked above the separating gel. The comb was kept in stacking gel portion. After the complete casting of gel, the gel was run in an electrophoretic chamber having electrode buffer at 100V, 400 mA for 100 min.

1.15.7 Wash buffer (PBST)

For making 1L of wash buffer, 100 mL of 10X PBS was taken, added 900 mL of distilled water to it. 0.5% (5 mL) of tween 20 was added. For making 10X Phosphate Buffer Saline (PBS), 80g NaCl, 2g KCl, 14.4g Na_2HPO_4, and 2.4g KH_2PO_4 in 800 mL of distilled water. The pH was adjusted to 7.4 with HCl or NH_4OH. The volume was made up to 1 liter of distilled water.

2.15.8 Transfer buffer

For making 4000 mL of transfer buffer, 56.56 g of glycine and 12.12 g of Tris in 3000 mL of distilled water. It was stored at $4^{\circ}C$.

2.15.9 Blocking buffer

5 g of non-fat milk was weighed and dissolved in 100 mL of PBST. One-half can be used for blocking and another half for diluting antibodies and washing.

2.15.10 Western blotting

50 µg of protein samples were separated on 8% SDS polyacrylamide gel by electrophoresis at 100 Volt for 90 min. The separated proteins were then transferred to a PVDF (Polyvinylidene difluoride) membrane purchased from Millipore, USA. The membrane was washed thrice with PBST with 0.5 % Tween 20. Blocking buffer containing 5% non-fat dry milk was used for blocking the membrane for 1 hr. Afterwards, the blocked

membrane was washed with PBST three times. Primary antibody TNF-α was diluted at 1:1000 in 2.5% BSA-PBST buffer and incubated overnight at 4°C. After incubation membrane was washed 4 times with PBST for 5 min each. The membrane was incubated for 3 h at room temperature with a corresponding HRP-conjugated secondary antibody specific to the primary TNF- α diluted at 1:1500. The membrane was washed 4 times for 5 min each and treated with BCIP/NBT western blotting detection reagent. The membrane was then visualized under trans-illuminator.

2.16 *In Silico* Anti-Oxidant Activity tested against Tyrosine Kinase (PDB ID 2HCK)

2.16.1 Molecular docking Study

Molecular docking predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. The knowledge of the preferred orientation, in turn, predict the strength of association or binding affinity between two molecules based on the scoring functions such as the docking scores.

Docking can be consider as a problem of "lock-and-key", in which one wants to find the correct relative orientation of the "key" which will open up the "lock" where on the surface of the lock is the key hole, which direction to turn the key after it is inserted (Fig. 5). The protein can be thought of as the "lock" and the ligand as a "key". Molecular docking may be described as the "best-fit" orientation of a ligand that binds to a particular protein of interest. It usually focuses on computationally simulating the molecular recognition process.

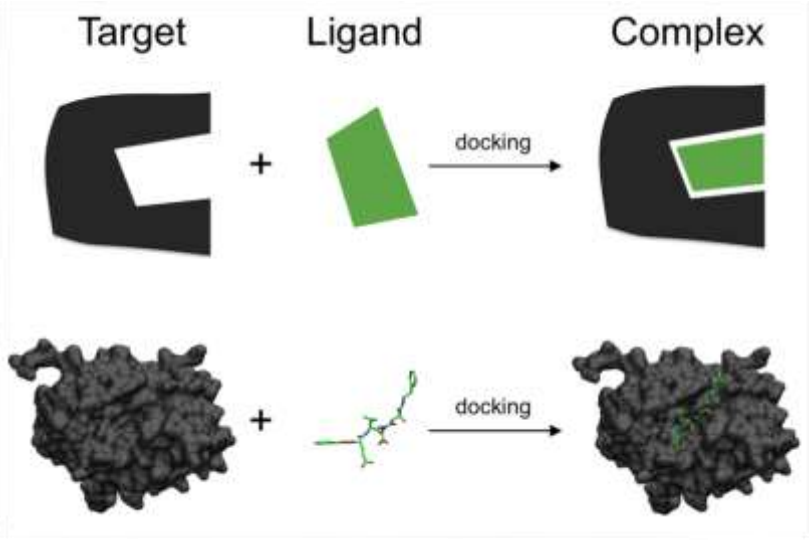


Fig. 5. Docking illustration of a small molecule ligand (grey) to a protein target (black) producing a stable complex (courtesy: wikipedia)

2.16.2 Chemical structure generation

For molecular docking simulation, the compounds namely, Vitamin E, Naringin, Coumarin, Vitamin C and ß-Pinene were sketched using ChemOffice 2010 (Cambridge Soft, Massachusetts, USA). The compounds were further optimized until stable energy conformations and converted to a 3D format for docking simulations.

2.16.3 Docking computation

Molecular docking was carried out against these mentioned compounds to understand the molecular interaction that might be responsible for inhibiting the Tyrosine Kinase enzyme computationally. Molecular docking studies were carried out using Molegro Virtual Docker 6.01 (MVD). The MVD is based on a differential evolution algorithm; the solution of the algorithm considers the sum of the inter-molecular interaction energy between the ligand and the protein and the intra-molecular interaction energy of the ligand. The energy scoring function is based on the modified piecewise linear potential (PLP) with new hydrogen bonding and electrostatic terms. Docking programs tend to predict possible

orientation (poses) of ligand in the receptor binding site. A scoring function is used to rank the poses based on inter-molecular binding affinity or the molecular docking scores

2.16.4 Cavity prediction

The potential ligand binding site of the metagenomic alkane hydroxylase protein was predicted using MVD. The binding cavity was set at the site X: 13.44; Y: 24.14; Z: 24.58 within a constraint of radius 15 Å with a volume of 56.32 $A^{\circ 3}$and a surface area of 175.36 $A^{\circ 2}$. The methodology adopted to find out the potential binding sites is a grid-based cavity prediction algorithm.

2.16.5 Docking computation

Molecular docking simulation was carried out using Molegro Virtual Docker (MVD). MVD requires the 3D structure of both protein and ligand, usually derived from X-Ray/NMR experiments or homology modeling. For such docking approaches, the n-alkanes, n-alkenes, and n-alkynes were imported in the MVD. The bond flexibility of the compounds and the side chain flexibility of the amino acids at the active site (Val281, Phe340, Gly344, Thr338 and Met 341) were set with a tolerance of 1.6 and strength of 0.8 for docking simulations. The root mean squared deviation (RMSD) threshold for the multiple cluster poses were set at 2.00 Å. The docking algorithm was set at a maximum iteration of 1,500 with a simplex evolution size of 50 and a minimum of 100 runs was performed for each compound to ensure conformations to the lowest energy state. Five poses of each molecule in a complex with the protein were returned, which were finally ranked on the basis of MolDock score and Rerank score. The resulting poses were visualized in the Pose Organiser and the best pose of each compound of the top docking hits was selected for the ligand-protein interaction analysis.

2.17 Binding mode prediction of Naringin and Naringenin against TNF-α, IL-10, IL-1ß

To obtain the conformational binding site of the 2 natural products (NPs); Naringin and Naringenin against the 3 known cancer targets namely interleukin-1 beta (PDB id: 1HIB), interleukin-10 (PDB id: 1ILK) and tumor necrosis factor (PDB id: 1A8M) and we have carried out flexible ligand docking using Autodock Vina (Trott & Olson, 2010). The starting atom coordinates for the 3 targets were taken from protein data bank (https://www.rcsb.org) and the cleaned structure was provided as a target (receptor) input structure in Autodock Vina. Autodock Vina has significant improvements and average accuracy of the binding mode prediction compared to other docking algorithms and also allows flexibility in the ligand binding. The docking parameters include ligand and receptor (target) preparation involving the addition of hydrogen atoms, merging non-polar hydrogen atoms, rotatable torsion bond followed by grid box construction of each target protein. The hydrogen-bond interaction between 2 NPs and each of the target structure was analyzed using UCSF Chimera (Pettersen *et al*., 2004).

CHAPTER-3

RESULTS

3.1 Preliminary phytochemical screening

Extract yield and percentage recovery of *Citrus macroptera* Montruz. peel organic extraction are presented in Table 1. Peels of *Citrus macroptera* extracted with methanol yielded higher extract and the recovery percentage was also found higher. The phenolic, flavonoid, alkaloid, and glycosides are present in all the extracts. Protein, iodine and steroid were not present in any of the extracts. *Citrus macroptera* peels extract revealed the presence of phytochemicals responsible for medicinal bioactivities. The studied chemical test in the extracts are presented in Table 2. The presence of alkaloid, phenolic, flavonoid, coumarin glycosides, saponin glycoside, and mucilage were demonstrated by the various test employed. However gum and reducing sugar were found to be present in aqueous-methanol extract only. Resin, anthraquinone glycosides, steroid, protein and Iodine were absent in all the tested extracts.

Table 1. Extract yield and % recovery of *Citrus macroptera* extraction

Sl. No.	Sample	Weight of sample (g)	Extract Yield (g)	% Recovery
1	NC1	60	52.2	40.0
2	NC2	60	48.8	66.6
3	NC3	60	46.1	60.0

Ta**ble 2. Phytochemical screening of NC1, NC2 and NC3 extracts of *Citrus macroptera* Montruz.**

Phytochemical constituent	NC1	NC2	NC3
Alkaloid	+	+	+
Dragendorff's test	-	-	-
Hager's test	+	+	-
Wagner's test	+	-	-
Phenolic	+	+	+
Flavonoid	+	+	+
Resin	-	-	-
Coumarin glycosides	+	+	+
Cardiac glycoside (Keller Keliani test)	+	+	+
Anthraquinone glycoside	-	-	-
Saponin glycoside	+	+	+
Steroid	-	-	-
Mucilage	+	+	+
Protein	-	-	-
Iodine	-	-	-
Gum	-	-	+
Reducing sugar	-	-	+

'+'- presence. '-' - absence of tested phytochemicals

3.2 Total phenolic, total flavonoid and total flavonol content

Total phenolic, total flavonoid and total flavonol content varies with the varying solvent used for extraction. Among the tested samples, methanol extract (NC2) showed highest phenolic content (7.47 ± 0.01 µg/mL Gallic acid equivalent). Methanolic extract also showed highest flavonoid content (7.28 ± 0.12 µg/mL Quercetin equivalent) whereas total flavonol content was highest in aqueous methanol extract (Table 3). Standard curve of quercetin and gallic acid are presented in Fig.6 A and B. Total phenolic, flavonoid and flavonol content of *C.macroptera* is given in Fig.7.

Table 3. Quantification of Total phenolic, total flavonoid and total flavonol content of three extract (NC1-NC3) of *Citrus macroptera* Montruz. (Nongalleima *et al*., 2017[a])

Sample	Total Phenolic content µg/mL GAE* ± SEM	Total flavonoid content µg/mL QE** ± SEM	Total flavonol content µg/mL QE ± SEM
Aqueous (NC1)	4.26 ± 0.01	5.49 ± 0.01	0.89 ± 0.2
Methanol (NC2)	7.47 ±0.01	7.28 ± 0.12	0.53 ± 0.02
Aqueous methanol (NC3)	6.14 ±0.001	6.58 ± 1.0	1.07 ± 0.19

*expressed as GAE (Gallic acid equivalent) µg /mL, ** expressed as QE (Quercetin equivalent) µg/mL, n=3, p<0.005

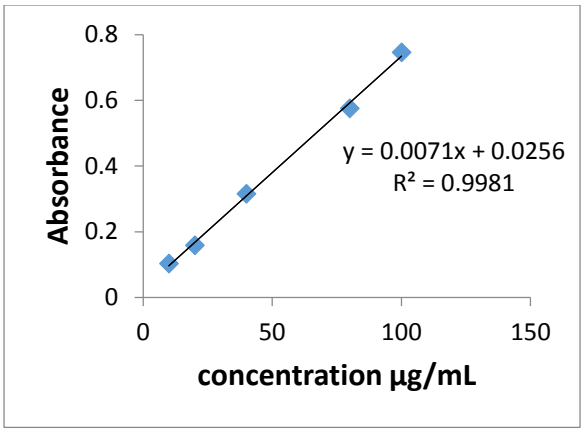


Fig.6 A: Standard Curve of Quercetin

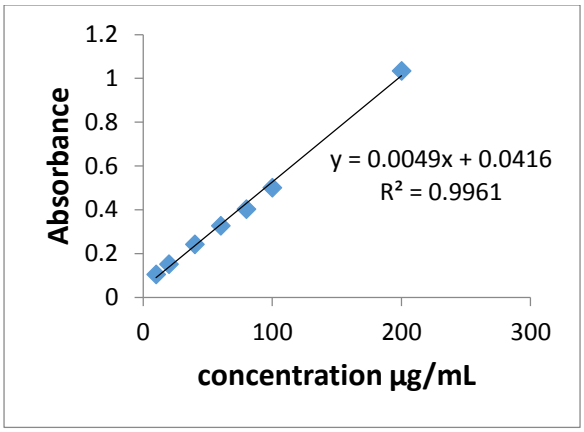


Fig.6 B: Standard Curve of Gallic Acid

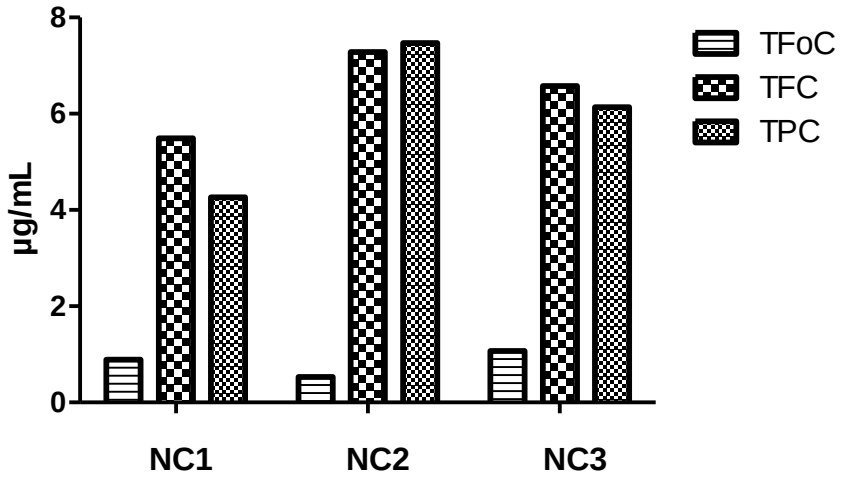


Fig.7. TPC: Total phenolic content (µg of GAE/mg of DW), TFC: Total Flavonoid content (µg of QE/mg of DW) and TFoC: Total Flavonol content (µg of QE/mg of DW), NC1- aqueous extract, NC2- methanol, NC3- aqueous methanol extract of *C. macroptera* Montruz., *n*= 3 analyses, $p<0.05$.

3.3 Anti-oxidant assay

3.3.1 *In vitro* anti-oxidant assay

Antioxidant activity were found varied over the organic solvent used for extraction of the plant and also the method employed for determination. Antioxidant activity (IC_{50}) of all the three extracts are concisely presented in Table 4, Fig.8. In reducing power assay NC1, NC2, NC3 showed 36.71 ± 0.01 µg/g, 59.4 ± 0.05 µg/g, and 926 ± 0.333µg/g respectively as their $IC50_s$. Reducing power assay measures the capacity of the extracts in oxidizing Fe^{3+} in ferric chloride to Fe^{2+}. In Nitric oxide assay NC1, NC2, NC3 showed 94.35 ± 0.008, 78.11 ± 0.101, and 95.82 ± 0.090 µg/g respectively as their IC50s. Aqueous extract (NC1), Methanol (NC2), aqueous methanol (NC3) showed IC50 of 87.83 ± 0.012, 237.95 ± 0.005, 276.11 ± 0.101 µg/g respectively in DPPH assay. Ability of the extracts and the standard antioxidant in reducing the absorbance at 517 nm in dose dependent manner is measured by DPPH method. In nitric oxide assay, the scavenging capacity of the extract and ascorbic acid against nitric oxide radical and in prevention of nitrite formation was measured. Ascorbic acid, standard antioxidant showed IC50 7.64 ± 0.005 µg/g in DPPH assay, 8.43 ± 0.01 µg/g in reducing power assay, and 7.6 ± 0.7 µg/g in Nitric oxide assay

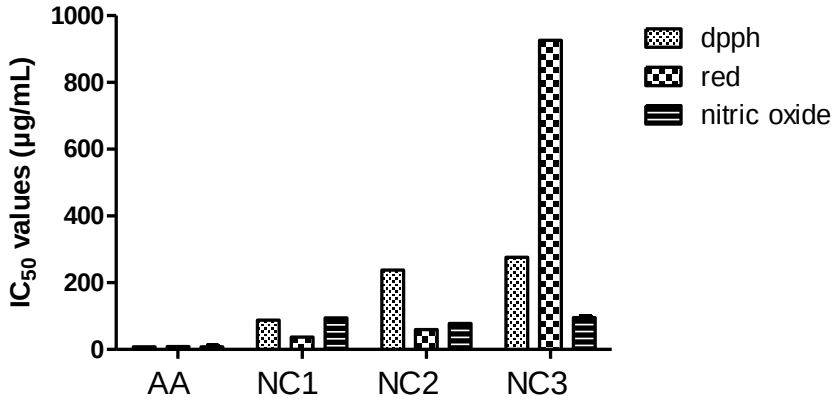


Fig. 8. IC_{50} of AA: ascorbic acid, NC1: aqueous extract, NC2: methanol extract, NC3: aqueous methanol extract of *Citrus macroptera* Montruz. in DPPH, red: reducing power assay, nitric oxide assay

Antioxidant activity of three fractions each of aqueous extract and methanol extract was done and presented in Table 5. On the average N-butanol fraction (NC8) of methanol extract was found to have good activity in all the assays as compared to all the fractions thus obtained. IC50 of NC8 was 21.69 µg/mL, 25.76 µg/mL, 24.17 µg/mL respectively in DPPH, reducing power and nitric oxide reducing assay. Aqueous fraction of aqueous extract was having the best activity in DPPH (7.9 µg/mL) comparable to ascorbic acid. While the aqueous fraction of methanol extract showed the least activity.

Table 4. *In vitro* antioxidant assay of extracts (NC1-NC3) of *Citrus macroptera* Montruz.

Sample	DPPH assay IC50 µg/g ± SEM*	Reducing power assay IC50 µg/g ± SEM*	Nitric oxide assay IC50 µg/g ± SEM*
Ascorbic acid	7.64 ± 0.005	8.43 ± 0.01	7.6 ± 0.7
Aqueous (NC1)	87.83± 0.012	36.71 ± 0.01	94.35 ± 0.008
Methanol (NC2)	237.95±0.005	59.4 ± 0.05	78.11 ± 0.101
Aqueous methanol (NC3)	276.11±0.101	926 ± 0.33	95.82 ± 0.090

*- mean ± standard error mean, µg Ascorbic acid equivalent per gram of DW (dry weight), $p < 0.05$, $n=5$ analyses

Table 5. *In vitro* antioxidant assay of fractions of aqueous and methanol extract of *Citrus macroptera* Montruz.

Samples	DPPH assay IC_{50} ± SEM	Reducing power assay IC_{50} ± SEM	Nitric oxide assay IC_{50} ± SEM	Protease Inhibitory activity IC_{50} ± SEM
Fractions of Aqueous extract				
Ethyl acetate fraction (NC4)	108.84 ± 0.01	4.20 ± 0.1	220.74 ± 0.01	851.9 ± 0.001
n-butanol fraction (NC5)	87.75± 0.05	12.82 ± 0.01	286.64 ± 0.21	811.66 ± 0.05
Aqueous fraction (NC6)	7.9± 0.001	69.38 ± 0.07	191.14 ± 1.01	496 ± 2.09
Fractions of Methanol extract				
Ethyl acetate fraction (NC7)	66.31± 0.001	110.89 ± 0.001	230.06 ± 0.001	561.18 ± 1.14
n-butanol fraction (NC8)	21.69± 0.02	25.76 ± 0.010	24.17 ± 0.005	15.54 ± 0.990
Aqueous fraction (NC9)	284.5± 0.005	299.87 ± 0.005	248.6 ± 0.010	132.43 ± 0.01

Results are expressed as mean ± SEM: standard error mean, n=3, p<0.005

3.3.2 *In vivo* anti-oxidant assay

Reduced activities of enzymic Superoxide dismutase (SOD), catalase CAT) and non-enzymic (Glutathione reductase) were summarized in Table 6. SOD, CAT and reduced glutathione are endogenous oxidative enzymes widely found in cells and tissues that protect cells against oxidative stress. Their level is high in normal groups of treatment. The results of SOD, CAT, reduced glutathione assays which were done using serum, plasma EDTA, liver and brain tissues were presented in Table 6a. The level of SOD, CAT and reduced glutathione were increased. When oxidative stress is high, there is reduced anti-oxidative enzymes. Increase in level of such enzymes suggest the antioxidant property of the extracts.

Serum of normal group of animal showed 68.93 U/mL, 1.03 U/mL, 1. 743 U/mL in SOD, catalase and glutathione reductase activity. Serum of animal group under high oxidative stress condition being treated with Carbon tetrachloride showed reduced level of 53.4 U/mL, 0.55 U/mL and 0.55 U/mL in In SOD, catalase and glutathione reductase activity assay respectively. This reduced levels were increased in the serum of animal treated with ascorbic acid (67.4 U/mL, 0.99 U/mL, 1. 21 U/mL in SOD, Cat, and GR). Animal groups treated with NC1, NC2 and NC3 extracts showed increased level from the CCL4 treated group. NC1 treated group showed the most potent activity (60.85, 0.83 and 1.31 in SOD, Cat and GR activity).

Likewise, plasma EDTA, liver and brain tissues of normal group of animals showed high amount of SOD, Cat and GR enzymes. Their level decreases in CCL4 treated group of animal which are having high oxidative stress. The potency of the extracts showed their effectiveness in increasing the levels of this enzymes showing that they have good antioxidant properties. In all the tissue samples treated with extracts, aqueous extract (NC1)

showed the highest antioxidant activity. Plasma EDTA treated with NC1 showed 50.63 U/mL, 0.4 U/mL, and 0.527 U/mL respectively in SOD, Cat and GR activity assay. Liver tissues of group treated with NC1 showed 98.29 U/mL, 1.76 U/mL, and 0.89 U/mL respectively in SOD, Cat and GR activity assay. Brain tissues treated with NC1 showed 99.57 U/mL, 1.24 U/mL and 0.61 U/mL respectively in SOD, Cat and Gr activity assay. Plasma EDTA of group treated with ascorbic acid showed 95.6 U/mL, 1.34 U/mL, 1. 37 U/mL in SOD, Cat and GR assay. In liver tissue treated with ascorbic acid, SOD, Cat and GR assay showed 98.2 U/mL, 1.6 U/mL and 10.07 U/mL respectively. In brain tissue treated with ascorbic acid, SOD, Cat and GR assay showed 95.9 U/mL, 1.14 U/mL and 1.07 U/mL respectively.

3.4 Anti-inflammatory assay

3.4.1 *In vitro* inflammatory assay

Protease inhibitory assay, albumin denaturation and heat induced haemolysis of the three extracts (NC1-NC3) were done to assess the preliminary anti-inflammatory activity. In protease inhibitory assay NC1, NC2, NC3 showed 20.44 ± 0.0057 µg/mL, 59.66 ± 0.0057 µg/mL, and 57.76 ± 0.005 µg/mL respectively as their IC_{50s}. In protease inhibition assay, protease inhibitor cocktail (Sigma) showed 11.49 ± 0.008 µg/mL IC_{50}. Solvents used for extraction also influence the bioactivity of the plant extracts and their subsequent fractions. Diclofenac sodium showed IC_{50} at 55.8 and 11.79 µg/g respectively in albumin denaturation and heat induced haemolysis assay. The highest protease inhibitory activity was observed in aqueous extract with IC_{50} 20.44 ± 0.0057 ug/mL, and methanol extract showed highest activity with 100.36 µg/mL IC_{50} in heat induced hemolysis (Table 7). n- butanol fraction of the methanol extract showed IC_{50} of 15.54 µg/mL in Protease inhibitory.

Table 6. *In vivo* antioxidant assay (SOD, CAT, and reduced glutathione) of three extracts of *Citrus macroptera* Montruz. observed in serum, plasma EDTA and liver

Sample tissue treated with extract	SOD activity U/mL	Catalase activity U/mL	Glutathione reductase (GR) activity µmole/min./mL
Serum (S)			
Normal/control	68.93	1.03	1.743
CCl_4	53.4	0.55	0.55
Ascorbic acid	67.4	0.99	1.21
CCl_4 + NC1S	60.85	0.83	1.31
CCl_4 + NC2S	55.54	0.76	0.61
CCl_4 + NC3S	58.51	0.92	0.54
Plasma EDTA (P)			
Normal/ control	94.89	1.62	1.36
CCl_4	44.2	0.01	0.39
Ascorbic acid	95.6	1.34	1.37
CCl_4 + NC1P	50.63	0.4	0.527
CCl4 +NC2P	42.97	0.006	0.438
CCl_4 +NC3P	43.40	0.866	0.570
Liver (L)			
Normal/control	99.7	1.93	11.14
CCl_4	95.6	0.98	0.95
Ascorbic acid	98.2	1.6	10.07
CCl4 + NC1	98.29	1.76	0.89
CCl4 + NC2	96.59	1.60	1.14
CCl4 + NC3	95.78	1.52	0.88

Table 6a. *In vivo* antioxidant assay (SOD, CAT, and reduced glutathione) of three extracts of *Citrus macroptera* Montruz observed in brain tissue.

Sample tissue treated with extract	SOD activity U/mL	Catalase activity U/mL	Glutathione reductase (GR) activity µmole/min./mL
	Brain (B)		
Normal/ control	95.3	1.72	0.66
CCl_4	74.2	0.09	0.30
Ascorbic acid	95.9	1.14	1.07
CCl_4 + NC1B	99.57	1.24	0.61
CCl4 +NC2B	91.53	0.74	0.53
CCl_4 +NC3B	98.31	0.72	0.52

3.4.2 *In vivo* anti-inflammatory activity

When compared to the control group the effect was almost comparable with standard drug diclofenac sodium at 4^{th} and 6^{th} hr after administration. Inflamed mice paw and extract treated mice, paw volume measurement taken in Plethysmometer. There was a gradual increase in the paw volume of mice in normal control group. The anti-inflammatory effect of aqueous, methanol and aqueous methanolic extract of *Citrus macroptera* Montruz. on Carrageenan induced mice paw edema are presented in Table 8, Fig.9. It is observed that the extracts at the dose level of 100 mg/Kg/p.o. exhibited more degree of reduction in mice edema significantly. Methanolic extract (NC2) exhibited more amount of anti-inflammatory activity than the aqueous and aqueous methanolic extract

Table 7. *In vitro* anti-inflammatory activity of three extract (NC1-NC3) of *Citrus macroptera* Montruz.

sample	Protease Inhibitory activity IC50 µg/mL^{+} ± SEM*	Albumin denaturation IC50 µg/g^{++} ± SEM*	Heat induced haemolysis IC50 ± SEM*
Protease inhibitor cocktail (Sigma)	11.49 ± 0.008	---	---
Diclofenac Sodium	---	55.8± 0.16	11.79± 0.01
Aqueous (NC1)	20.44 ± 0.008	312.31± 0.2	115.4 ±0.05
Methanol (NC2)	59.66 ± 0.00	104.75± 0.00	100.36±0.008
Aqueous methanol (NC3)	57.76± 0.01	163.98± 0.01	208.32± 0.045

+ : µg/mL equivalent of Protease inhibitor cocktail, ++ : µg/g equivalent of Diclofenac sodium, *:Standard error mean, n=5, p< 0.005

Table 8. Effect of NC1, NC2 and NC3 extracts of *Citrus macroptera* Montruz. on carrageenan induced paw edema

Name of group mg/kg/ p.o.	Before injection 0 hr	After injection (Paw volume, mean ± SEM)					
		0 hr	1 hr	2 hr	3 hr	4 hr	6 hr
Normal Control (1 mL dH_20)							
	0.29±1.0	0.36±13	0.49±19	0.65±1.3	0.77±0.6	0.79±0.01	0.88±.67
NC1							
50	0.21±0.9	0.24±1	0.29±0.7	0.33±0.0	0.48±1.3	0.34±.76	0.31±3.7
100	0.23±0.8	0.31±1	0.33±0.4	0.34±0.8	0.37±1.6	0.37±.32	0.30±0.99
150	0.30±0.6	0.45±.7	0.40±0.2	0.34±1.4	0.45±.78	0.33±1.78	0.29±.78
NC2							
50	0.27±2.8	0.29±.5	0.35±3.4	0.34±5.6	0.37±.89	0.34±4.2	0.24±0.69
100	0.29±1.3	0.40±.1	0.43±0.8	0.54±0.0	0.57±2.9	0.46±1.2	0.25±2.43
150	0.19±1.9	0.41±.8	0.54±1.3	0.55±1.4	0.59±2.6	0.45±0.91	0.24±0.44
NC3							
50	0.27±0.9	0.46±11	0.48±0.3	0.57±0.0	0.63±4.6	0.23±0.11	0.28±1.4
100	0.23±0.3	0.35±13	0.36±1.7	0.39±0.0	0.53±.78	0.46±0.78	0.32±1.9
150	0.14±1.3	0.31±.6	0.49±0.2	0.55±1.9	0.57±1.4	0.23±0.44	0.39±0.88
Diclofenac sodium							
50	0.22 ±0.1	0.49 ±1	0.51± 0.0	0.52± 1.5	0.54± 3.8	0.28±0 .89	0.23±1 .55
100	0.24 ±0.4	0.39 ±1	0.45± 0.0	0.46± 0.0	0.48± 1.4	0.29±1 .45	0.20±. 88
150	0.24 ±1.0	0.39 ±.9	0.45± 0.8	0.39± 0.8	0.33± 0.6	0.29±0 .67	0.24±0 .23

Results are expressed as mean± Standard error mean, n=3, $p<0.05$

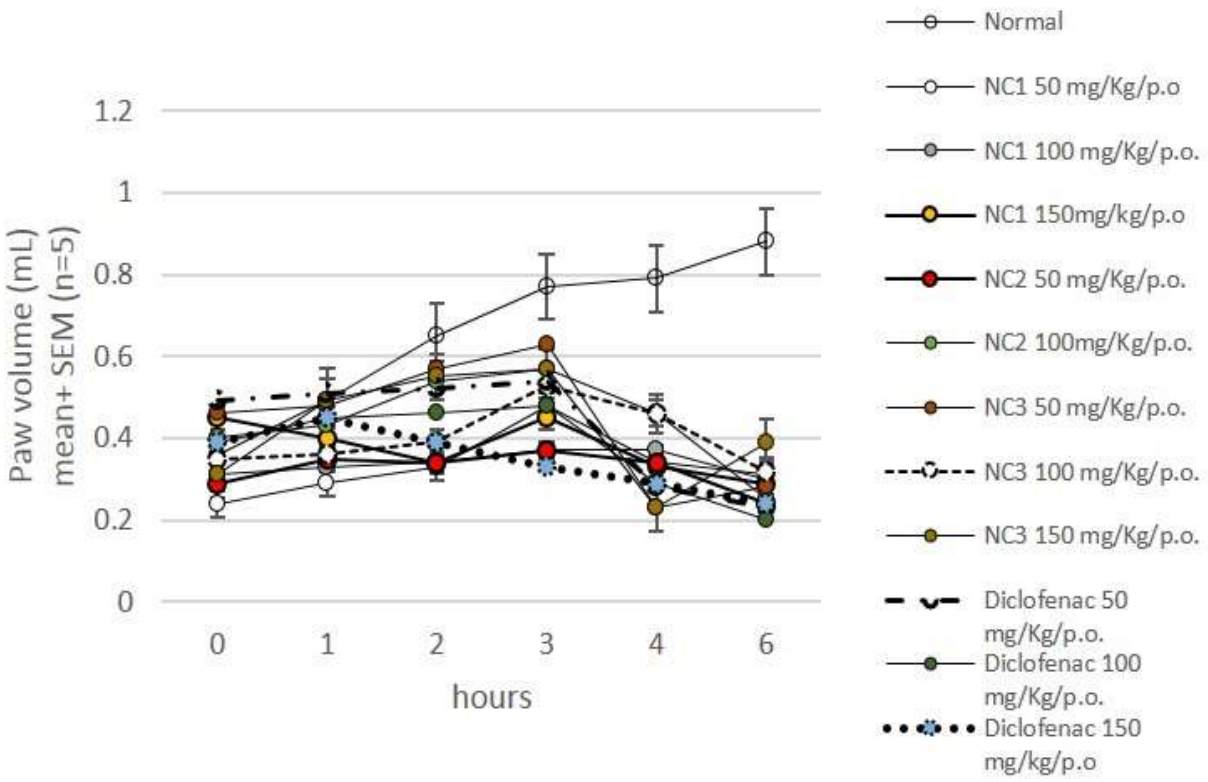


Fig.9. Effect of NC1, NC2, NC3 extract on mice paw edema with reference to Diclofenac sodium

3.5 Measurement of pro-inflammatory mediators (TNF-α, IL-1ß, IL-10) in serum of mice treated with extracts of *Citrus macroptera* Montruz.

Normal group of animals (Carrageenan induced) fed with distilled water have highest level of tested pro-inflammatory mediators (TNF-α, IL-10, IL-1ß). Level of NC1 treated mice release 18.49 pg/mL, 137.89 pg/mL and 112.5 pg/mL TNF-α, IL-10, IL-1ß respectively (Table 9). This level was decreased when the animals were treated with extracts of *C. macroptera* Montruz. Under 100 mg/Kg/p.o. NC1 treatment, the level of TNF-α and IL-10 release was found to be 15.36 pg/mL, 58.89 pg/mL respectively while there was negative result in IL-1ß assay. NC2 treated cells decreases the level of TNF- α, IL10 and IL-1ß upto

14.98 pg/mL, 67.78 pg/mL and 52.5 pg/mL respectively. Likewise, NC3 treated cells decreases the TNF- α upto 18.32 pg/mL, and IL-1ß upto 52.5 pg/mL. There was negative result in IL-10 assay. Diclofenac sodium treated animal groups decreases the level of TNF-α, IL10 and IL-1ß upto 10.23 pg/mL, 45.67pg/mL and 20.78 pg/mL

Table 9. Effect of NC1, NC2 and NC3 extract of *Citrus macroptera* Montruz. on release of pro-inflammatory cytokines

Treatment group	Pro-inflammatory mediators		
	TNF- α (pg/mL)	IL10 (pg/mL)	IL-1ß (pg/mL)
Normal Control	18.49 ± 0.01	137.89 ± .00	112.5 ± .089
Diclofenac sodium (5mg/Kg.p.o.)	10.23 ± 0.00	45.67 ± .008	20.78 ± 2.09
Serum+NC1 100mg/Kg.p.o	15.36 ± .089	58.89 ± 1.00	-
Serum+NC2 100mg/Kg.p.o	14.98 ± 0.54	67.78 ± 0.09	52.5 ± 2.56
Serum+NC3 100mg/Kg.p.o	18.32 ± 1.29	-	22.5 ± 0.99

values are given in mean ± SEM: standard error mean, *n*=3, -: showed no activity

3.6 Hepatic assessment of Aspartate aminotransferase (AST) and Alanine Aminotransferase (ALT) Levels

AST and ALT enzymes are normally present in the serum and liver tissues of animals. Their concentration increases when there is damaged or oxidative stress in the liver tissues. They are thus used as marker for detecting the hepatocellular damages. Carrageenan injected in mice paw induced elevated levels of AST and ALT enzymes after 6 hr injection as compared to the control groups of samples. The increased level got reduced when the animals were fed with extracts (NC1, NC2 and NC3) of *Citrus macroptera*. Montruz. Effect of the extracts on AST and ALT present in serum of carrageenan induced mice treated with NC1, NC2 and NC3 extracts is presented in Table 10. Likewise, their effect on these enzyme levels in liver of CCL_4 treated mice is presented in Table 11.

Table 10. Effect of Citrus macroptera on ALT and AST enzymes in mice serum

	ALT ± SEM µmole/min./mL	AST ± SEM µmole/min./mL
Control	1.60 ± 0.001	0.099 ± 0.01
Carageenan	3.11 ± 0.01	1.34 ± 0.01
*Serum+NC1	1.37 ± 0.1	0.079 ± 0.01
**Serum+NC2	1.54 ± 0.01	0.156 ± 0.001
***Serum+NC3	1.05 ± 0.23	0.099 ± 1.1

*,**,***- serum of mice treated with NC1, NC2 and NC3 extract respectively on carrageenan induced mice, values are given in mean ± SEM: standard error mean, *n*=3.

Table 11. Effect of *Citrus macroptera* Montruz extract on ALT and AST liver enzymes in mice liver

	ALT µmole/min./mL	AST µmole/min./mL
Control	2.10 ± 2.01	0.216 ± 3.6
CCl_4	3.40 ± 4.31	1.94 ± 0.77
NC1	1.33 ± 7.01	0.260 ± 6.89
NC2	1.54 ± 0.67	0.250 ± 1.14
NC3	2.16 ± 0.0074	0.274 ± 1.09

values are given in mean ± SEM: standard error mean, *n*=3.

3.7 **Cytotoxicity assays**

3.7.1 MTT assay

In MTT assay the aqueous extract showed IC_{50} at 148.44 µg/mL as Methanol extract however showed IC_{50} 87 µg/mL at Cisplatin showed IC50 at 23 µM. As the concentration of the tested extract increases, % control of cell proliferation also increases (Fig. 10A).

3.7.2 Clonogenic assay

Significant inhibition of colony formation was also observed. Different concentrations (5, 20, 40, 80, 160 and 320 µg/mL) of test extract, NC1, when treated with the colonies of HeLa cells, they show their colony inhibition potential. The number of colonies decreases as the concentration of the test extract increases (Fig.10B)

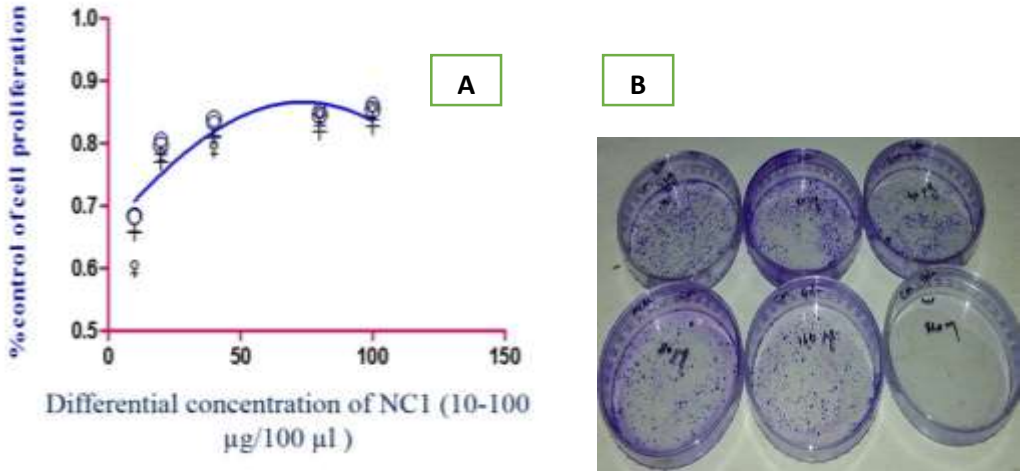


Fig. 10. A. Cytotoxicity assay (MTT) against Hela cells. The extract dose- response inhibit the proliferation of Hela cells. Each point represents the mean ± SD of 3 determations. *p<0.05 compared with the control (medium), B. Dose response colony inhibition shown by 5-320 µg/mL of NC1 extract of *C. macroptera* in Clonogenic assay against Hela cells.

3.8 Oil Extraction and Gas chromatographic analysis of essential oil extract

Hydrodistillation of peel and leaves of *C. macroptera* yielded 8 mL for peel and 4.54 mL for leaves which represent 1.43 % and 0.46 % respectively. Composition negating the trace amount (< 0.1%), and that of peels accounting to 99.18% of total composition. In both the essential oil, there are total of 10 compounds which were present in both the essential oil of leaves and peel (Table 12). Relative percentage composition of essential oil of the leaves (Table 13) and peel (Table 14) are presented. However, the percentage oils components calculated as the percent peak area were different. GCMS Chromatogram of leaves and peel essential oil were presented in Annexure

57 compounds each were identified from leaves as well as peel of *C. macroptera* Montruz. Compounds identified in leaves are presented accounting to 96.79 % of total,

presenting only 37 compounds negating the compounds present in minor traces (Table 13). The major volatile component present in peels were Bicyclo [4.1.0] heptane, 7-(1-methylethylidene)- (60.03 %), Mentha-2, 8-dien-1-ol <trans-,para-> (4.0%), Limonene oxide (3.53%), trans Carveol (2.67%) whereas in leaves essential oil major components were 2-methylaminobenzoic acid methyl ester (57.16 %), bicyclo [4.1.0] heptane, 7-(1-methylethylidene)- (23.23 %), β- pinene (8.79 %), ocimene <(E)-BETA-> DB5-519 (3.29 %) (Nongalleima *et al*., 2017$_b$). *Citrus macroptera* has been reported to contain lupeol, stigmasterol, beta-pinene, limonene, beta-caryophyllene, geranial edulinine, ribalinine and isoplatydesmine (Gaillard *et al*., 1995; Rana *et al*., 2012; Waikedre *et al*., 2010)

Table 12. Common compound found in essential oil of leaves and rind of *Citrus macroptera* Montruz with their area %.

Component	Area % Leaves essential oil	Area % Peels essential oil
BICYCLO[3.1.1]HEPT-2-ENE, 2,6,6-TRIMETHYL-	0.64	0.46
Bicyclo[4.1.0]heptane, 7-(1-methylethylidene)-	23.23	60.03
NONANAL	tr	1.45
Limonene oxide <cis->	tr	3.53
P-MENTHA-E-2,8(9)-DIEN-1-OL	tr	1.49
Carveol <trans->	tr	2.67
Copaene <alpha->	tr	0.52
1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)-	0.87	0.30
T-Muurolol	tr	tr
.alpha.-Cadinol	0.10	0.14

tr- trace (<0.1%).

Table 13: Chemical composition of essential oil of leaves of *citrus macroptera* Montruz with area % and retention time

Pea	Compound	R.Time	Area%
1	Nonane <n->	8.062	tr
2	Thujene <alpha->	9.141	tr
3	BICYCLO[3.1.1]HEPT-2-ENE, 2,6,6-	9.429	tr
4	BICYCLO[2.2.1]HEPTANE, 2,2-DIMETHYL-3-	10.013	tr
5	BICYCLO[3.1.1]HEPTANE, 6,6-DIMETHYL-2-	11.420	8.79
6	1,6-OCTADIENE, 7-METHYL-3-METHYLENE-	11.895	tr
7	OCTANAL	12.423	tr
8	Bicyclo[4.1.0]heptane, 7-(1-methylethylidene)-	14.022	23.23
9	1,3,7-OCTATRIENE, 3,7-DIMETHYL-, (E)-	14.149	0.17
10	OCIMENE <(E)-BETA-> DB5-519	14.675	3.29
11	1,4-CYCLOHEXADIENE, 1-METHYL-4-(1-	15.040	0.28
12	BENZENAMINE, N-METHYL-	15.348	0.36
13	2-FURANMETHANOL, 5-	15.666	tr
14	2,7,7-TRIMETHYL-3-	15.880	tr
15	CYCLOHEXENE, 1-METHYL-4-(1-	16.339	tr
16	1,6-OCTADIEN-3-OL, 3,7-DIMETHYL-	16.987	0.76
17	NONANAL	17.109	tr
18	1H-PYRAZOLE, 3-METHYL-	17.531	tr
19	Mentha-2,8-dien-1-ol <trans-, p->	17.897	tr
20	Limonene oxide <cis->	18.468	tr
21	P-MENTHA-E-2,8(9)-DIEN-1-OL	18.575	tr
22	(Z)-2,2-Dimethyl-3-(3-methylpenta-2,4-dien-1-	18.892	tr
23	Pinocarvone	19.848	tr
24	1-ISOPROPYL-4-METHYL-3-CYCLOHEXEN-1-	20.612	0.56
25	Heptanedinitrile, 4-acetyl-4-methyl-	20.990	tr
26	(+)-ALPHA-TERPINEOL (P-MENTH-1-EN-8-	21.257	tr
27	2,4,6-Trimethyl-1,3,6-heptatriene	21.442	tr
28	DECANAL	21.853	tr
29	2,6-Dimethyl-3,5,7-octatriene-2-ol, ,E,E-	22.034	tr
30	Carveol <trans->	22.546	tr
31	2-CYCLOHEXEN-1-OL, 2-METHYL-5-(1-	23.078	tr
32	2-CYCLOHEXEN-1-ONE, 2-METHYL-5-(1-	23.659	tr
33	Dihydro carveol<neoiso->	25.061	tr
34	Formamide, N-methyl-N-phenyl-	25.486	0.11
35	2-METHYLAMINOBENZOIC ACID METHYL	32.222	57.16
36	Bicyclogermacrene	34.881	0.12
37	Methyl anthranilate		0.17

Tr- trace (<0.1%), R.time- retention time (published in Nongalleima *et al*., 2017$_b$

Table 14: Chemical composition of essential oil of peels of *Citrus macroptera* Montruz. with area % and retention time

Pea	Compound	R.Time	Area%
1	HEPTANAL	8.166	tr
2	BICYCLO[3.1.1]HEPT-2-ENE, 2,6,6-	9.405	0.46
3	Pinene oxide <beta->	11.179	0.36
4	1,6-OCTADIENE, 7-METHYL-3-	11.843	0.75
5	OCTANAL	12.505	0.82
6	Bicyclo[4.1.0]heptane, 7-(1-	14.115	60.03
7	1-OCTANOL	15.791	1.43
8	2-FURANMETHANOL, 5-	16.500	0.31
9	NONANAL	17.227	1.45
10	Mentha-2,8-dien-1-ol <trans-, para->	18.219	1.49
11	Limonene oxide <cis->	18.690	3.53
12	Mentha-2,8-dien-1-ol <cis-, para->	18.919	4.00
13	3-METHYL-3,3A,4,6A-	20.227	0.11
14	2-ISOPROPENYL-5-METHYL-HEX-	20.651	1.26
15	3-CYCLOHEXENE-1-METHANOL,	21.425	0.45
16	1,3,6-Heptatriene, 2,5,5-trimethyl-	21.540	0.34
17	1,3,6-Heptatriene, 2,5,6-trimethyl-	21.709	0.45
18	DECANAL	21.927	0.92
19	Carveol <trans->	22.904	2.67
20	2-CYCLOHEXEN-1-OL, 2-METHYL-5-	23.473	1.79
21	2-CYCLOHEXEN-1-ONE, 2-	23.929	2.07
22	2-CYCLOHEXEN-1-ONE, 3-	25.081	0.28
23	Limonen-10-ol	26.014	0.47
24	Undecanal	26.525	0.53
25	(3R,6R)-3-Hydroperoxy-3-methyl-6-	26.824	1.71
26	(3R,6R)-3-Hydroperoxy-3-methyl-6-	27.411	1.14
27	P-MENTHA-1,8-DIEN-4-	27.851	0.79
28	1,2-Cyclohexanediol, 1-methyl-4-(1-	28.481	1.23
29	3-Heptadecen-5-yne, (Z)-	28.927	0.24
30	2(5H)-Furanone, 4-methyl-3-(2-methyl-	29.333	1.71
31	Copaene <alpha->	29.621	0.52
32	Sinensal <alpha->	29.951	1.03
33	.beta.-copaene	30.222	0.29
34	Carvone oxide<cis->	30.628	tr
35	Limonene oxide <cis->	31.023	0.58
36	1-CYCLOHEXENE-1-METHANOL, 4-	31.650	tr
37	.beta.-copaene	31.854	tr
38	2,6,10-DODECATRIEN-1-OL, 3,7,11-	32.191	tr
39	Limonene oxide <cis->	32.636	0.52

40	Heptan-2-one	33.642	tr
41	2,7-Octadiene-1,6-diol, 2,6-dimethyl-	34.489	0.13
42	Thujyl acetate	35.117	tr
43	CADINENE <DELTA-> DB5-1700	35.712	tr
44	1-ISOPROPYL-4,7-DIMETHYL-1,2-	36.503	tr
45	Hedycaryol	36.863	0.27
46	1,6,10-Dodecatrien-3-ol, 3,7,11-	37.361	0.30
47	(-)-5-	38.259	1.63
48	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-	39.195	0.22
49	Eudesmol <epi-gamma->	40.017	0.16
50	T-Muurolol	40.393	tr
51	.alpha.-Cadinol	40.877	0.14
52	Caryophyllene oxide	41.552	tr
53	2-Propenoic acid, tridecyl ester	42.157	0.12
54	2(3H)-NAPHTHALENONE,	46.351	0.21
55	3-Methyl-hepta-1,6-dien-3-ol	51.255	0.14
56	Isophytol	54.485	tr
57	4,8-DIMETHYLNONA-3,7-DIEN-2-	58.257	0.13

tr- trace (<0.1%), R.time- retention time (published in Nongalleima *et al*., 2017_b)

3.9 Comparative *in vitro* antioxidant anti-inflammatory activity of leaves and peel essential oil.

The essential oil were extracted from both the peel and leaves of *Citrus macroptera* Montruz. and their comparative antioxidant activity was determined. Essential oil of *C. macroptera* leaves showed IC_{50} at 252.93 µg/mL while the essential oil of peels showed IC_{50} at 118.07 µg/mL in DPPH (1, 1- diphenyl-2-picrylhydrazyl) (Table 15). In reducing assay, peel and leaves oil showed IC_{50} at 122.5 µg/mL and 208.24 µg/mL respectively; in nitric oxide assay essential oil of peel showed 236.71 µg/mL and leaves showed 135.43 µg/mL as their respective IC_{50s} . Analyzing the accumulated IC_{50} of all the three antioxidant assays, the peels essential oil demonstrated to be more potential candidate of antioxidant as compared to leaves essential oil. Although there was a report that the oil did not exhibit any

in vitro free-radical-scavenging (DPPH) (Miah *et al*., 2002), we are reporting the antioxidant potential of the essential oil of *Citrus macroptera* Montruz (Nongalleima *et al*., 2017_b).

The IC50 of the *in vitro* anti-inflammatory assay (protease inhibitory, heat induced haemolysis and albumin denaturation) of the essential oil sample are presented in (Table 16). In albumin denaturation assay, leaves showed IC_{50} at 87.48 µg/mL and the peels showed IC_{50} at 73.91 µg/mL. The IC_{50} of the other assay are also presented in the Table 15. Essential oil of peel showed more anti-oxidant and anti-inflammatory activity than leaves essential oil.

Table 15. *In vitro* antioxidant activity of essential oil of peels and leaves of *Citrus macroptera* Montruz.

Sample/ standard	Oil yield %	DPPH (IC_{50}) µg/mL	Reducing power assay (IC_{50}) µg/mL	Nitric oxide reducing assay (IC_{50}) µg/mL
leaves	0.46 %	252.93 ± .004	208.24 ±0.08	236.71± 0.01
Rind	1.43 %	118.07 ± .007	122.5 ±0.12	135.43± 0.09
Ascorbic acid	-	5.62 ± 0.001	8.1 ± 0.04	7.6 ± 0.67

Results are mean ± SD- standard deviation, DPPH- 1, 1-Diphenyl-2-picryl hydrazil

Table 16. *In vitro* anti-inflammatory activity of peels and leaves of *Citrus macroptera* Montruz.

Essential oil sample/ standard	Protease inhibitory assay (IC_{50}) µg/mL ± SD	Heat induced haemolysis (IC_{50}) µg/mL ± SD	Albumin denaturation assay (IC_{50}) µg/mL ± SD
C. macroptera leaves	106.71 ± 0.11	187 ± 0.33	87.48 ± 0.32
C. macroptera rind	96.4 ± 0.25	124.89 ± 0.07	73. 91 ± 0.05
Diclofenac sodium	-	11.79± 0.01	55.8 ± 0.16
Protease inhibitor cocktail	11.49 ± 0.008	-	-

Activity of essential oil was tested using Protease inhibitor cocktail (Sigma) and Diclofenac as standard. Results are expressed as mean ± standard deviation.

3.10 HPLC method validation and simultaneous quantification of Naringin, Naringenin and Coumarin

3.10.1 Optimization of chromatographic performance

A preliminary chromatographic study was done with different mobile solvents with varying concentrations, different flow rate and varying temperature of the column. Among the tested solvent system and method, 37% Acetonitrile (Solvent B) and Water (Solvent A) with flow rate 0.55 mL^{-1} min. cell temperature 30°C was used for the simultaneous determination of flavanone glycosides (Naringin, coumarin and Naringenin). This method was used for determination of marker compounds (Naringin, Naringenin and Coumarin) in

the extracts (NC1, NC2 and NC3), seed and juice of *Citrus macroptera* Montruz. Chromatogram of marker compounds and the tested samples are presented in Fig. 11-14.

3.10.2 Calibration curve, Limit of Detection and Quantitation

A standard curve of marker compound was ploted, and a regression equation was generated. In the regression equation y= ax+b, y refers to the peak area, a is the slope rate of the line and b is intercept of the straight line with y axis, x is the concentration of standard compound. Over the tested concentration range, standard compounds showed linearity range of 25-400 µg/mL. Naringin and coumarin showed correlation co-efficient linearity of R^2= 0.9906 while Naringenin showed R^2 value of 0.9943 (Table 17). LOD and LOQ were calculated for compounds using the linear regression equation. LOD and LOQ of Naringin was found to be 57.06 ng/mL, 171.3 ng/mL respectively. LOD of coumarin and Naringenin was found to be 485.1 ng/mL and 155.5 ng/mL respectively. While LOQ of coumarin and Naringenin was found to be 1456.9 ng/mL and 467.07 ng/mL (Table 17).

3.10.3 Retention time, retention factor and separation factor

The retention time of Naringin, Coumarin and Naringenin was 6.122 ± 0.056 min., 18.17 ± 0.089 and 21.91 ± 0.204 min (Table 18). K^a (Retension factor) and α^b (Seperation factor) were determined. K^a was calculated by $(t_R-t_0)/t_0$, where, t_R and t_0 are retention times of sample components and sample solvent respectively. α^b was calculated by $t_{R2} – T_0)/(t_{R1}-t_0)$, where t_{R1} and t_{R2} are retention times of two neighboring peaks

3.10.4 Robustness and stability

By spiking the samples with low, medium and high concentration of marker compounds, recovery of the assay was determined (Table 19). The robustness, a measure of method's ability to remain unaffected by small variations in certain method parameters, is shown in table. Mobile phase composition, flow rate and column temperature were the parameters studied. The elution behavior, retention time was affected by the variation of the acetonitrile concentration in the mobile phase. Concentration of the marker compounds were affected but not significantly affected. The stability of stock solutions of marker compound was determined by comparing the peak of aliquots kept for 12 hrs and the one which were freshly prepared. The same aliquots stored at -20^0C for 30 days were evaluated for stability.

3.10.5 Precision and accuracy

Inter-day precision and intra-day precision was determined (Table 20). Relative mean Standard Deviation (RSD) % of the inter-day precision test range from 0.069% to 1.17% and that of intra-day precision range from 0.72% to 13.2%. Inter-day accuracy % and intra-day accuracy % is presented in Table. Inter-day accuracy range from 95.16% to 136.89%, and intra-day accuracy range from 95.45% to 137.64%. Precisions were used to study the variability of the method.

3.10.6 Quantitative determination of three flavanone glycoside in extracts (NC1, NC2 and NC3), seed and juice of *Citrus macroptera* Montruz.

The proposed HPLC-DAD method was used for the simultaneous determination of flavanone glycosides (Naringin, coumarin and Naringenin). Retention time and area % of the marker compounds were identified. A standard curve was plotted with tested range of

concentration Vs peak area of the tested flavanone glycoside. The content of tested flavanone glycoside in extracts (NC1, NC2 and NC3), seed and juice of *Citrus macroptera* Montruz are summarized in Table 21. Naringin was found highest in NC2 extract (200 µg/mL) and naringenin was also found highest in NC2 extract (37µg/mL). The detection wavelength was set at 254 nm in the PDA detection where all the compounds could be detected and had satisfactory absorption.

Table 17. Linear regression equation, correlation co-efficient and linearity of naringin, coumarin and naringenin

Compound	Regression equation	Co-relation co-efficient R^2	Linearity range (µg/mL)	LOD ng/mL	LOQ ng/mL
Naringin	y = 3378x + 170555	0.9906	25-400	57.06	171.3
Coumarin	y = 5118.3x + 2E+06	0.9906	25-400	485.1	1456.9
Naringenin	y = 6104x + 229394	0.9943	25-400	155.5	467.07

LOD: Limit of Detection, LOQ: Limit of Quantification

Table 18. System suitability test for three flavanone obtained during test analysis

	Retentime time (mean ±SD)	K^a (mean ±SD)	α^b (mean ±SD)
Naringin	6.122 ± 0.056	0.749 ± 0.016	5.57 ± 0.018
Coumarin	18.17 ± 0.089	4.34 ± 0.355	1.25 ± 0.010
Naringenin	21.91 ± 0.204	5.26 ± 0.058	1.04 ± 0.000

K^a (Retension factor) = $(t_R-t_0)/t_0$, where, t_R and t_0 are retention times of sample components and sample solvent respectively .

α^b (Seperation factor) = $(t_{R2} – T_0)/(t_{R1}-t_0)$, where t_{R1} and t_{R2} are retention times of two neighboring peaks. Results are expressed as mean ±SD, n = 5.

Table 19. Mean concentration of analyte by varying the MeCN content in mobile phase (± 2%)

Analyte	Nominal concentration (µg/mL)	Mean concentration found (µg/mL) normal mobile phase 37 % MeCN	Mean concentration found (µg/mL) mobile phase 39 % MeCN	Mean concentration found (µg/mL) mobile phase 35 % MeCN
Naringin		RT= 6.33	RT= 6.20	RT= 6.27
	100	136.0	94.4	96.00
	200	262	103	163.0
	400	402	155	243.0
Coumarin		RT= 18.29	RT= 16.8	RT= 19.35
	100	127	176.0	134.90
	200	208	274	216.5
	400	407	310	391.0
Naringenin		RT= 22.13	RT= 19.18	RT= 25.29
	100	102	131	130.00
	200	255	180	191.0
	400	320	225	399.0

Table 20. Precission and accuracy study of three marker glavanone glycosides.

Analyte	Nominal concent-ration (µg/mL)	Inter day Mean found concentration (µg/mL)	Inter-day accuracy %	Inter-day precission (RSD %)	Intra day Mean found concentration (µg/m)	Intra-day accuracy %	Intra-day precission (RSD %)
Naringin	100	136.89	136.89	1.17	137.64	137.64	2.08
	200	266.10	133	1.11	266.39	133.19	3.76
	400	421.86	105.47	1.09	421.12	105.28	13.2
Coumarin	100	126.73	126.73	0.79	127.50	127.50	2.79
	200	197.21	98.61	0.69	195.08	97.50	0.72
	400	429.50	107.37	1.09	431.60	107.9	13.0
Naringenin	100	104.56	104.56	0.71	105.13	105.13	1.25
	200	244.00	122	0.87	243.00	121.5	2.46

Table 21. Amount of three flavanone glycoside in *Citrus macroptera* Montruz.

Analyte	Contents (µg/mg) (mean ± S.D.)		
	Naringin	Coumarin	Naringenin
NC1 peel	2.20 ± 0.02	0. 085 ± 0.00	37.44 ± 2.09
NC2 peel	200.37 ± 1.08	13.41 ± 1.08	37.04 ± 1.00
NC3 peel	171.00 ± 2.90	4.41 ± 0.99	36.8 ±10.6
Seed	57.08 ± 0.08	4.12 ± 1.23	33.96 ± 2.37
Juice	57.08 ± 1.57	9.43 ± 0.00	36.90 ± 0.98

contents are expressed in µg/mg as mean ± S.D., *n*=5.

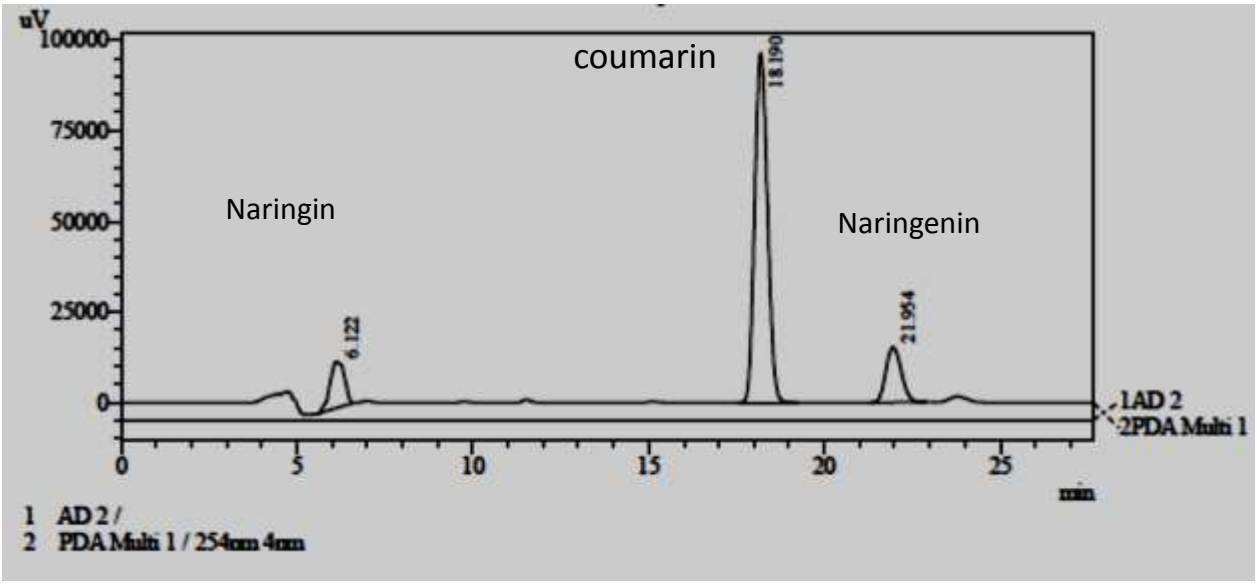


Fig.11. HPLC chromatogram for determination of standard flavanone glycosides (naringin, coumarin and naringenin)

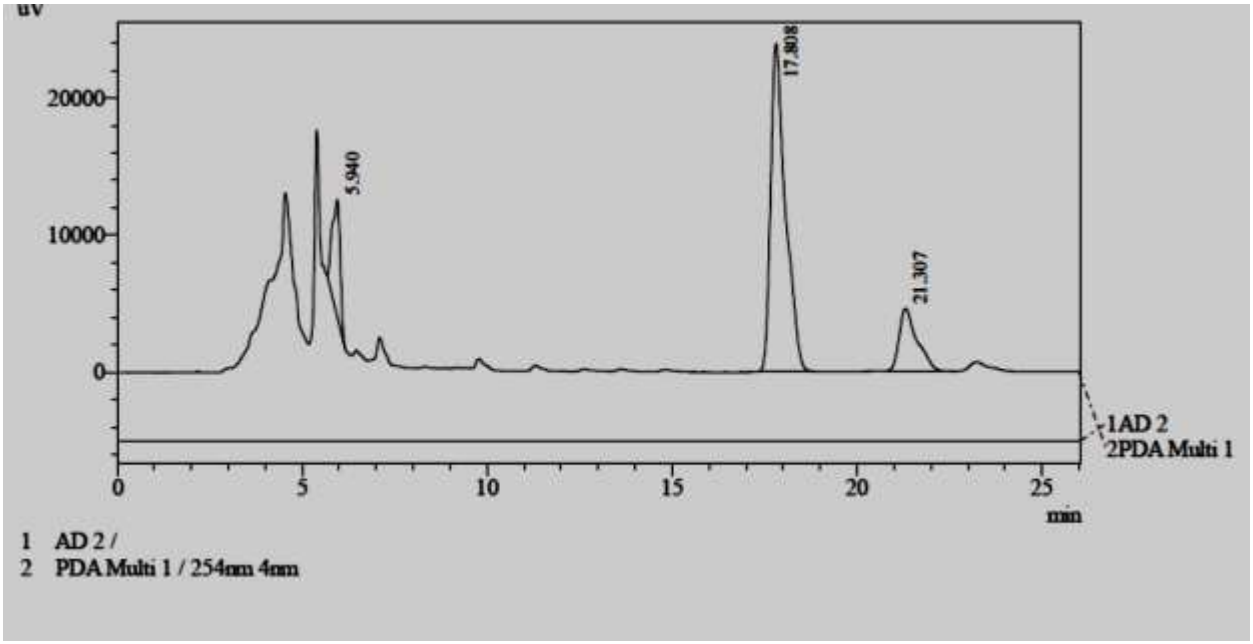


Fig.12. HPLC chromatogram for determination of flavanone glycosides in aqueous extract of *Citrus macroptera* Montruz.

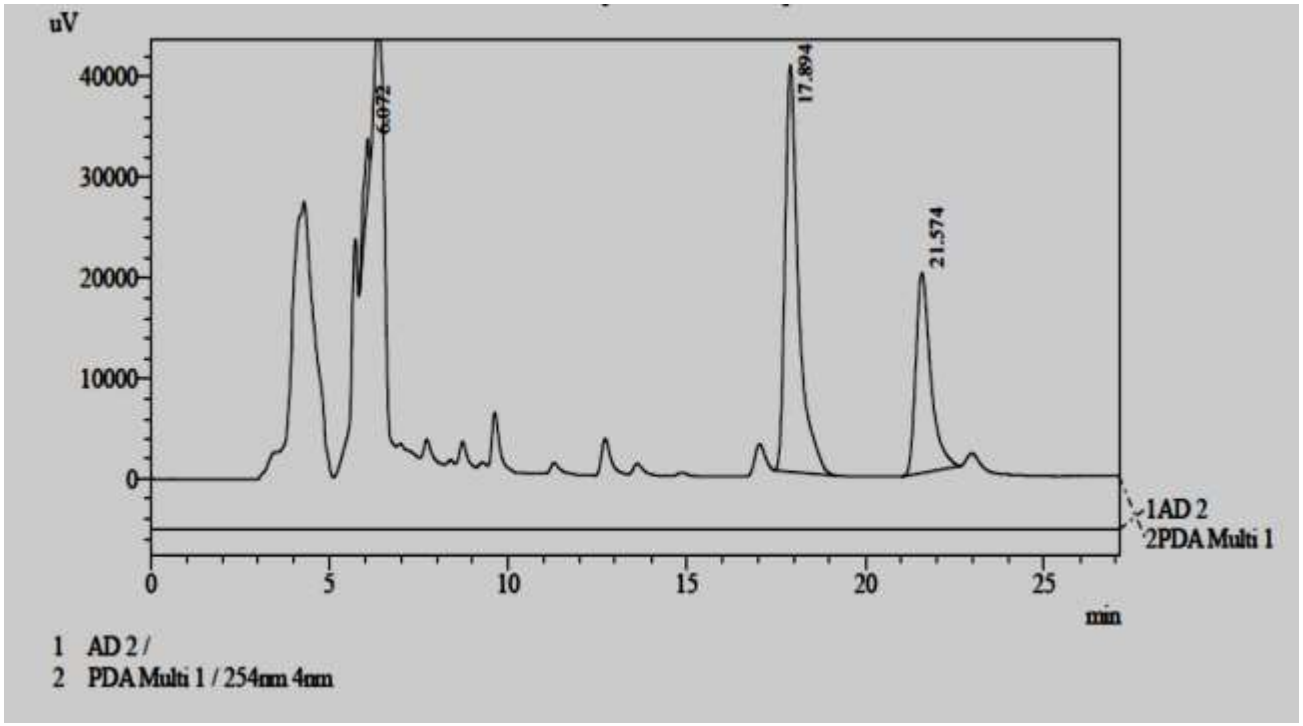


Fig.13. HPLC chromatogram for determination of flavanone glycoside in methanol extract of *Citrus macroptera* extract

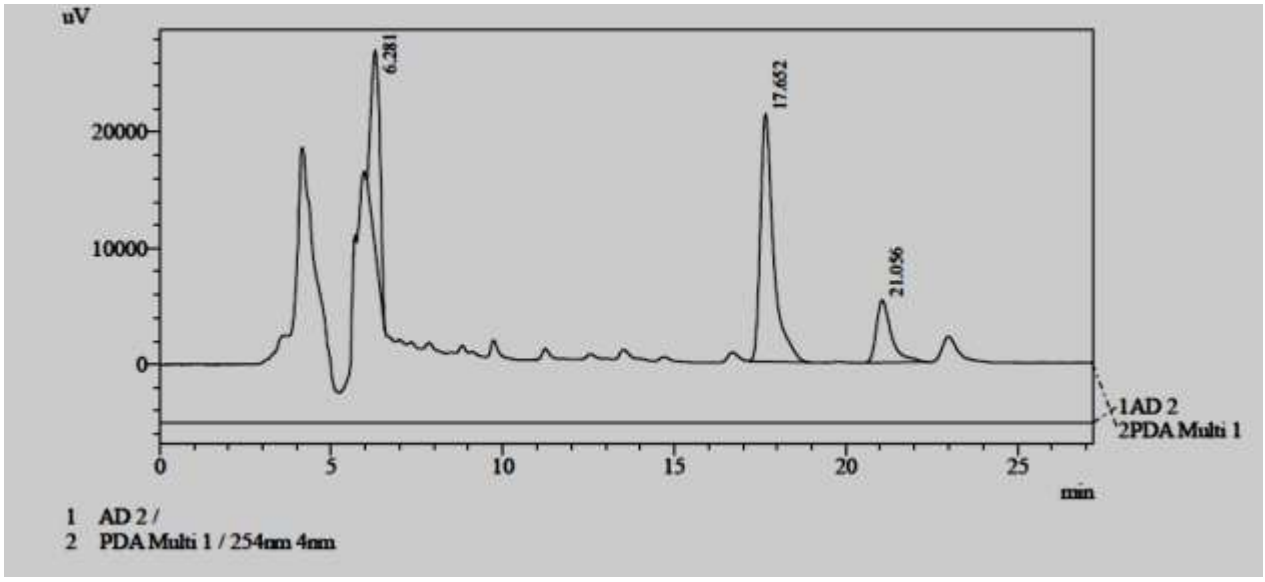


Fig.14. HPLC chromatogram for determination of flavanone glycoside in aqueous methanol extract of *Citrus macroptera* Montruz.

3.11 Simultaneous RP-HPLC determination of phenolics (Quercetin, Rutin and Kaempferol) by RP-HPLC

Kaempferol, Rutin and Quercetin HPLC profile is presented in Fig. 15-18. Their content were determined in different extracts of *Citrus macroptera* using linear regression. Linearity range, LOD and LOD are presented in Table 22. Quercetin content was found to be 17.69 µg/mL, 431.1 µg/mL, and 232.03 µg/mL respectively in NC1, NC2 and NC3 extracts. Rutin content found in NC1, NC2 and NC3 extracts were 67.91, 108.35, 260.38 µg/mL respectively. Kaempferol content was found to be 31.65, 59.50 and 17.66 µg/mL in NC1, NC2 and NC3 extracts respectively (Table 23).

Table 22: Chromatographic parameter of the method used for quantification of compounds in extracts of *C. macroptera* Montruz.

Compounds	Linearity range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Quercetin	31.25 – 500	0.011	0.032
Rutin	31.25 – 500	0.007	0.020
Kaempferol	31.25 – 500	0.0148	0.045

Table 23: Quercetin, rutin and kaempferol content in three extracts of *C. macroptera* Montruz.

Compounds	Aqueous extract (µg/mL)	Methanol extract (µg/mL)	Aqueous methanol (µg/mL)
Quercetin	17.69	431.1	232.03
Rutin	67.91	108.35	260.38
Kaempferol	31.65	59.50	17.66

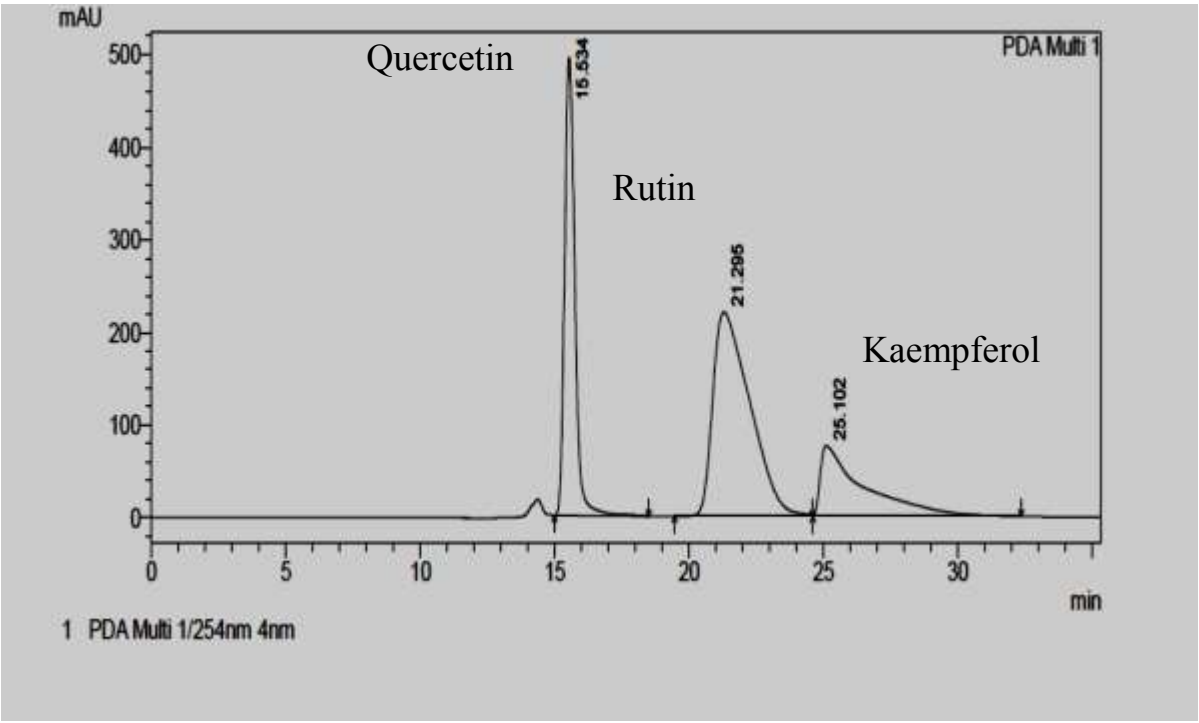


Fig.15. HPLC chromatogram of standard aglycone (quercetin, rutin, kaempferol)

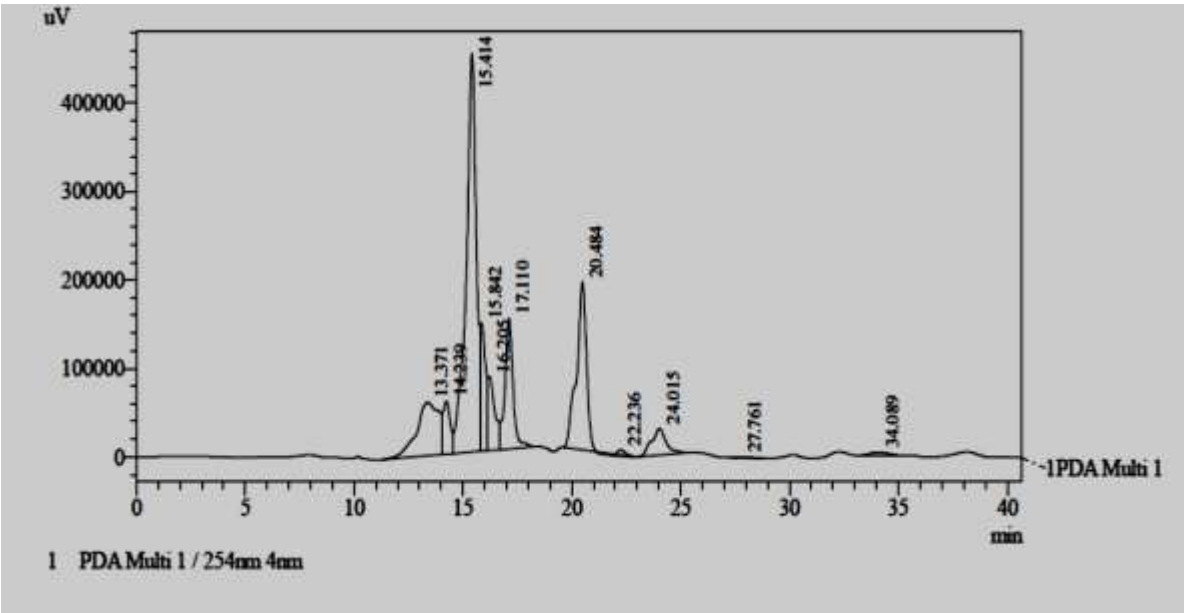


Fig. 16. HPLC chromatogram for determination of standard aglycone (quercetin, rutin, kaempferol) in aqueous extract of *Citrus macroptera* Montruz.

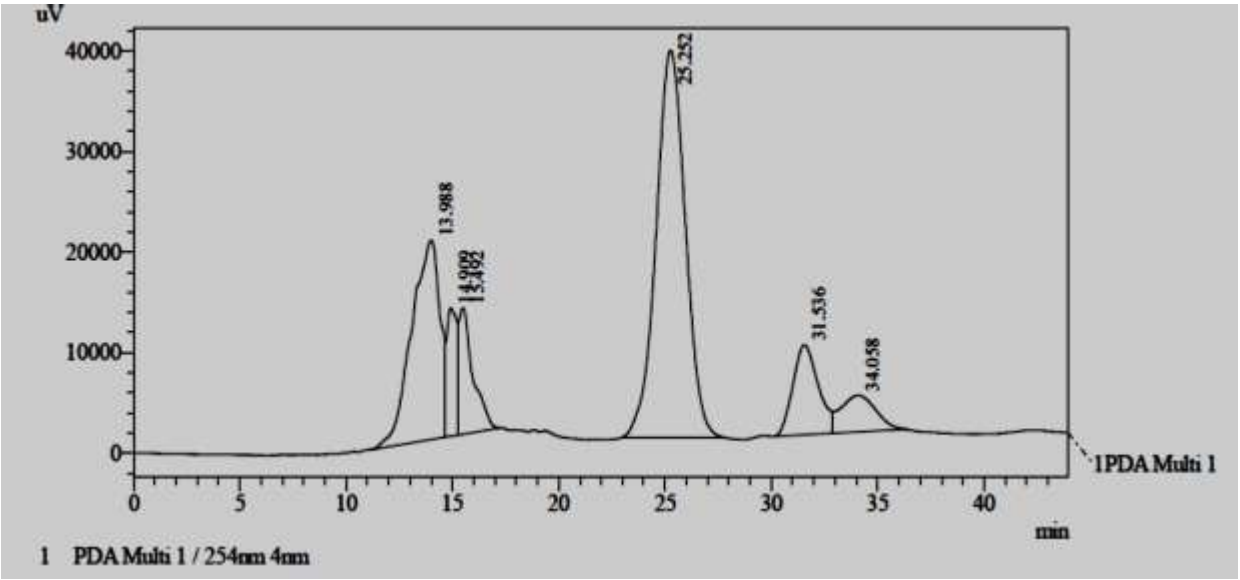


Fig. 17. HPLC chromatogram for determination of standard aglycone (quercetin, rutin, kaempferol) in methanol extract of *Citrus macroptera* Montruz.

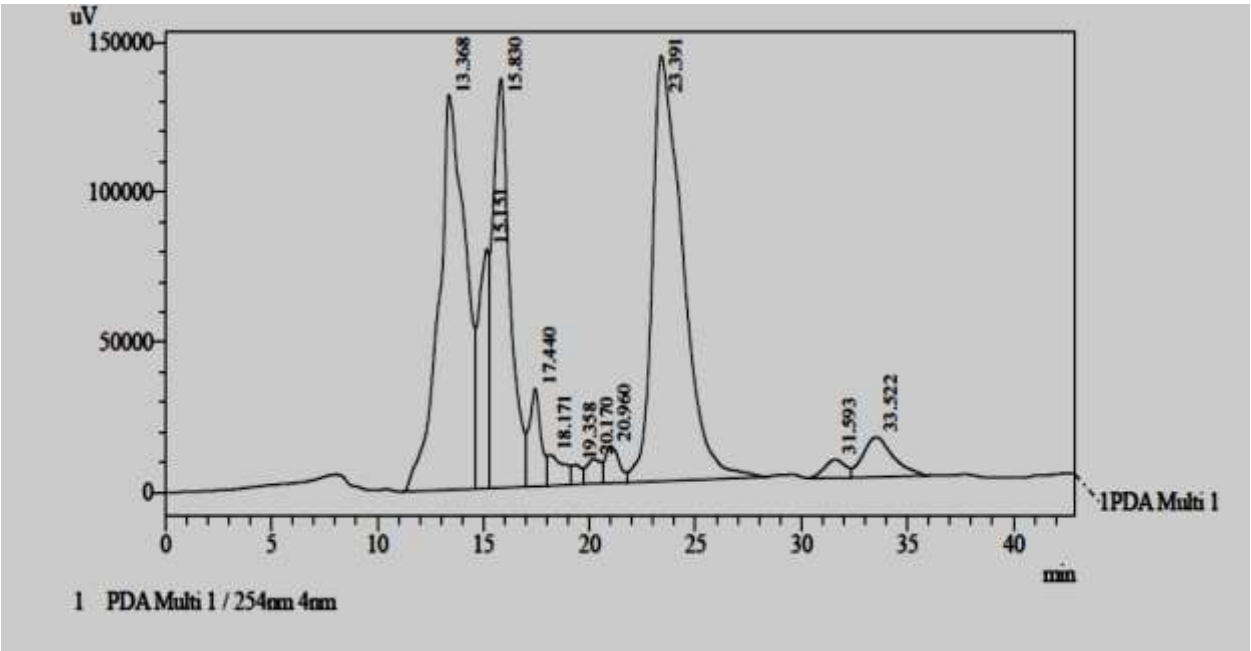


Fig. 18. HPLC chromatogram for determination of standard aglycone (quercetin, rutin, kaempferol) in aqueous methanol extract of *Citrus macroptera* Montruz.

3.12 Bioactive guided fractionation, isolation and purification of bioactive compounds

Methanol extract was fractionated into ethyl acetate, n-butanol and aqueous fraction (Fig.19). Fractionation results in different yield of fractions as per the nature of solvent used for it. 82g of methanol extract was taken for fractionation. It was defatted with n-hexane. Ethyl acetate and water was added to the extract in equal ratio, mixed them and allowed to settle in two different layers. Ethyl acetate fraction was separated out. To the remaining water fraction, an equal proportion of n-butanol was added, mixed them and separated into two separate fractions of n-butanol and aqueous fractions.

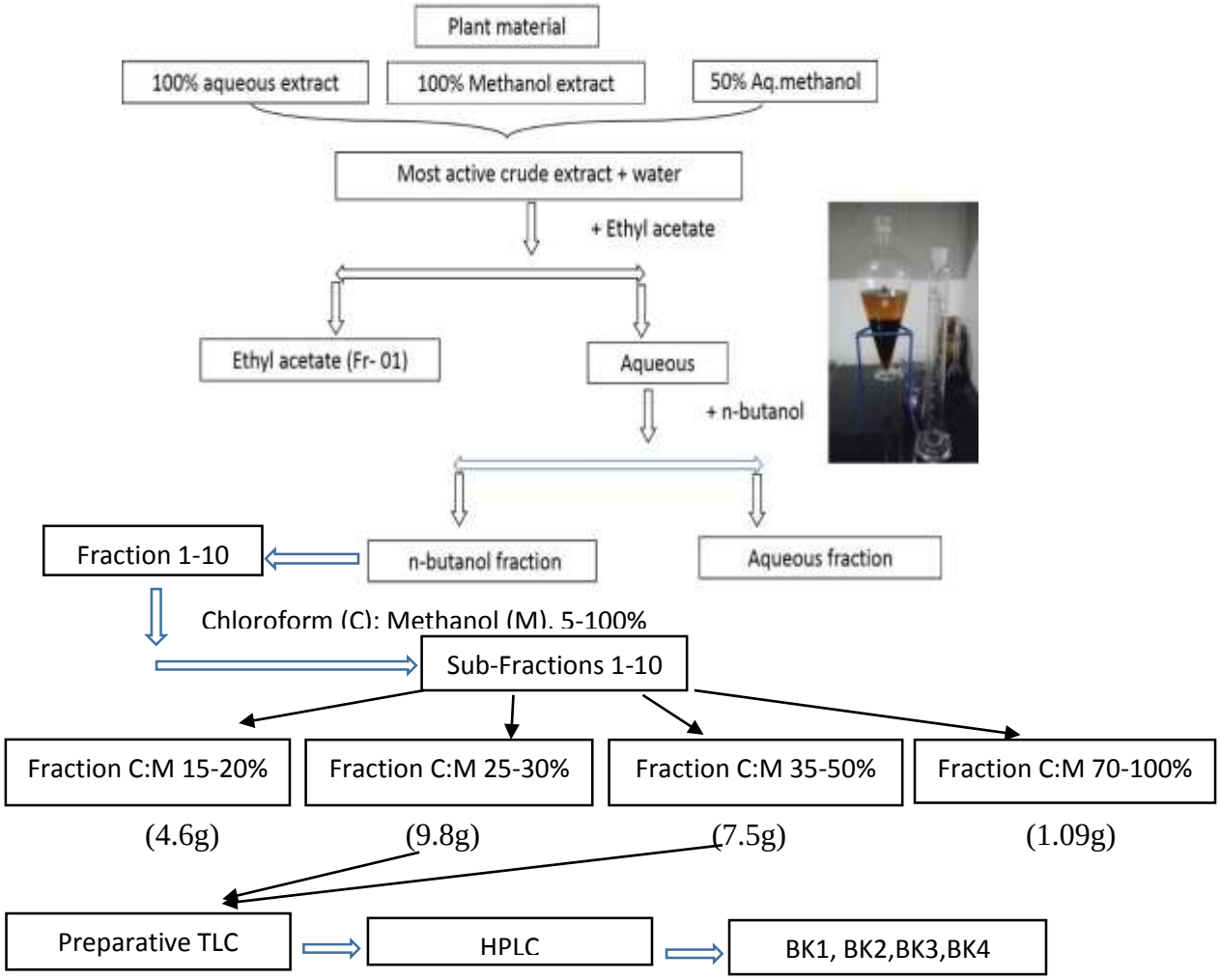


Fig.19. Flowchart of bioactive guided fractionation employed in fractionating the extracts of *Citrus macroptera* Montruz.

Out of the 82 g of methanol extract, 56 g of n-butanol, 12 g of ethyl-acetate and 6 g of n-hexane fractions were obtained. When the n-butanol fraction was subjected to column chromatography, 10 different sub-fractions were collected based on the polarity/ratio of chloroform-methanol used. Sub-fractions collected when eluted with 15 % and 20 % methanol were grouped together and it yielded 4.6g. Likewise, sub-fractions eluted with 25% and 30% were grouped and it yielded 9.8g, 40%, 45%, 50% were grouped and yielded 7.5g. Finally, 70% and 100% methanol were grouped and it yielded 1.09 g.

Using the preparative TLC (Fig.20) and HPLC four different compounds marked as BK1, BK2, BK3 and BK4 were isolated from Methanol extract.

3.13 Identification of compound

The structure of four compound BK1, BK2, BK3 and BK4 was identified by NMR analysis. Mass was determined from MS spectra. FT-IR was also taken for the four compounds. The molecular structure of all the four compounds are presented in Fig.21. Compound BK1 was identified as 4', 5, 7-trihydroxyflavanone 7-rhamnoglucoside or Naringin ($C_{27}H_{32}O_{14}$), 580.59 g/mol molecular weight. Compound BK2 was identified as Naringenin ($C_{15}H_{12}O_5$), 272. 25 g/mol molecular weight. Compound BK3 was identified as Coumarin. Molecular formula $C_9H_6O_2$ was assigned to coumarin having molecular weight of 146.14 g/mol. FTIR, NMR and MS spectra of compound BK1 and BK3 are presented in Annexures

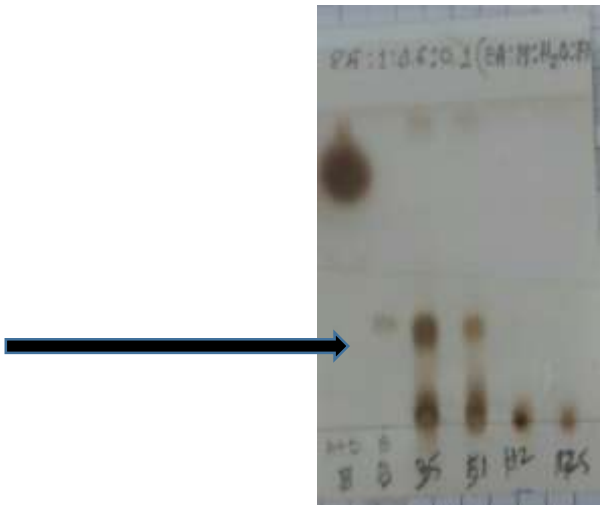
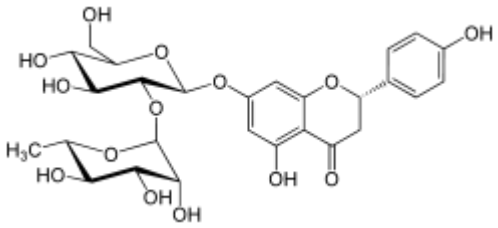
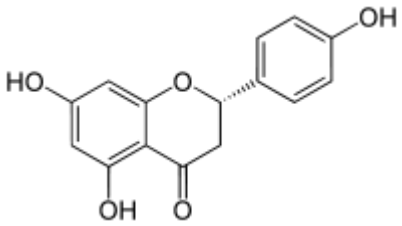


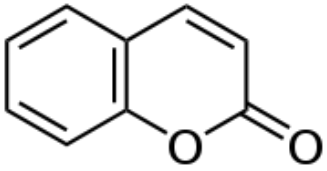
Fig.20. TLC profile of n-butanol fraction of methanol extract, Naringin spotted in 35-51 sub-fractions of n-butanol fraction of methanol extract developed in 10% Sulphuric acid (Charred at 110^{o}C for 3 minutes).



BK1



BK2



BK3

Fig. 21. Chemical structure of BK1: Naringin, BK2: Naringenin, BK3: Coumarin

3.14 Study on the effect of BK1, BK2, BK3 and BK4 on carrageenan induced inflammation

Histopathological study report is presented in Table 24. Till 3 hrs, there was an increase in edema, however, there was a gradual decrease in paw edema volume of mice treated with BK1, BK2, BK3 and BK4 (Fig. 23). Gradual decrease in the paw volume indicated the anti-inflammatory potential of the isolated compounds. There was complete disappearance of edema after 24 hours of treatment. BK1 showed most protective effect against carrageenan induced paw edema. Histopathological study reports of the paw tissue are presented in Table 24.

3.15 Western blotting studies

Treatment with naringin, naringenin, coumarin and ß pinene after carrageenan injection reduced TNF-α expression in mice paw edema (Figure 22). Protein expression showed a reduction of TNF-α expression after treatment with Diclofenac sodium at 5 mg/Kg.p.o. compared with the carrageenan induced alone.

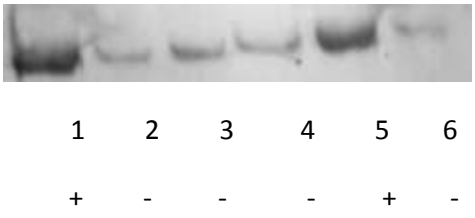


Fig.22. Tnf-α protein expression inhibited by BK1, BK2, BK3 and BK4 on carrageenan induced mice paw edema. 1: Carrageenan, 2: Diclofenac sodium (5mg/Kg), 3: BK1 (15mg/Kg), 4: BK2 (15mg/Kg), 5: BK3 (15mg/Kg), 6: BK4 (10mg/Kg)

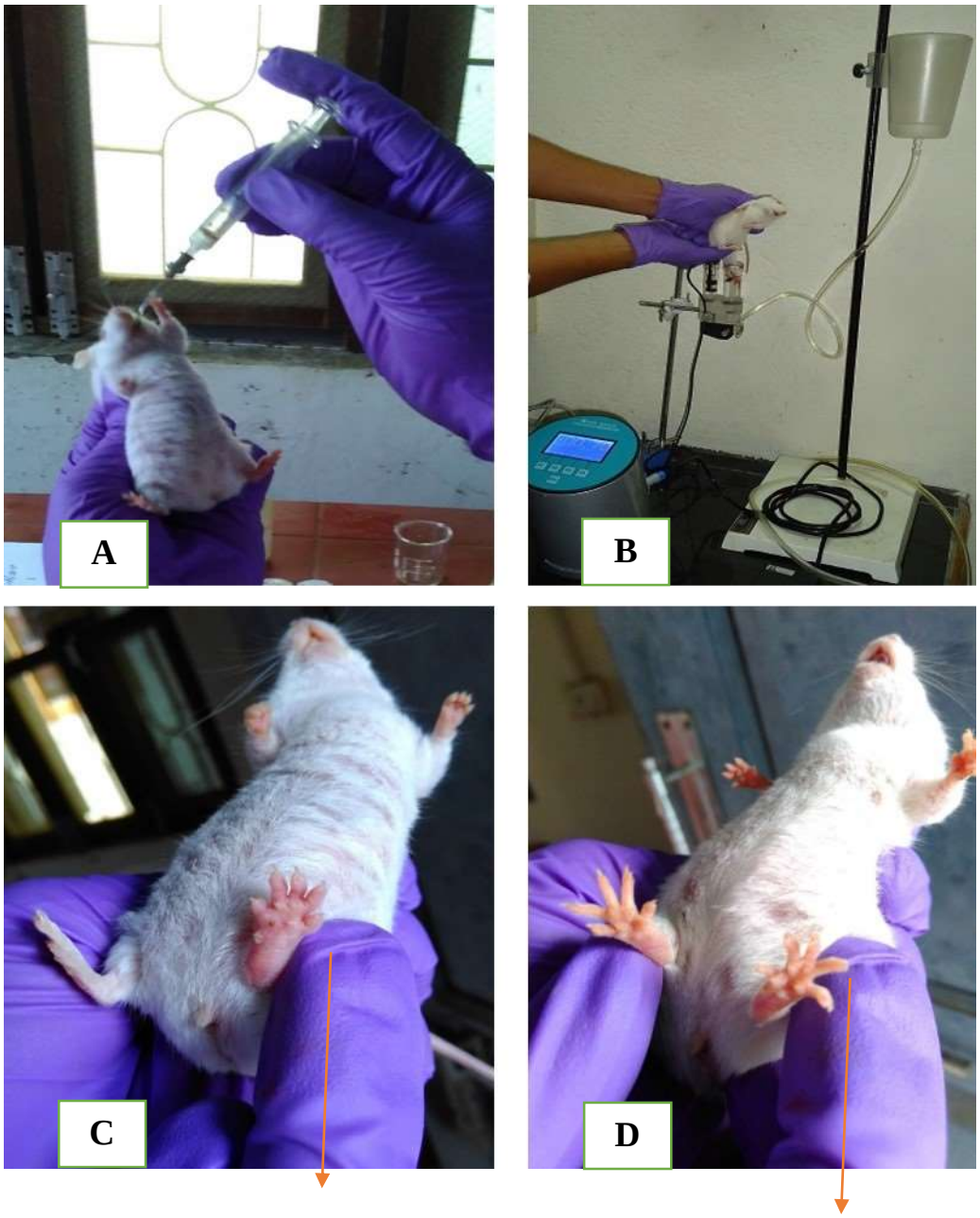


Fig. 23. A: oral administration of Naringin (15mg/Kg body weight), B: measurement of paw volume in digital Plethysmometer, C: Inflammed paw edema 1 min.after 0.1% carrageenan injection, D: reduced paw edema post naringin treatment at 24 hr.

Table 24. Histopathological result of carrageenan induced mice paw tissue under treatment of KB1, BK2, BK3 and BK4

Microscopy	Carr. only	Diclofenac Sodium	BK1	BK2	BK3	BK4
Dermal edema	+	-	-	-	-	-
Dermal congestion	-	-	-	-	+	+
Dermal inflammatory infiltration	+	-	-	-	-	-
Giant cells	-	-	-	-	-	-
Neutrophilic infiltration	-	-	-	-	-	-
Lymphocytic infiltration	+	-	-	-	-	-
Macrophages	-	-	-	-	-	-
Dermal granulation tissue	-	-	-	-	-	-
Dermal fibroblasts alteration	-	-	-	-	-	-
Dermal collagen	+	+	+ +	+	+	+

Carr.: Carrageenan only treated mice paw tissue, -: absence, +: presence of tested parameters.

3.16 *In Silico* Anti-Oxidant Activity tested against Tyrosine Kinase (PDB ID 2HCK)

The potential ligand binding site was predicted using Molegro Virtual Docker [MVD] for Tyrosine Kinase (PDB ID 2HCK). The cavity has a volume of 56.32 $A°^3$, cavity surface of 175.36 $A°^2$ and positioned at X: 13.44; Y: 24.14; Z: 24.58 with a binding site radius of 15 A° which is shown in Fig. 24.

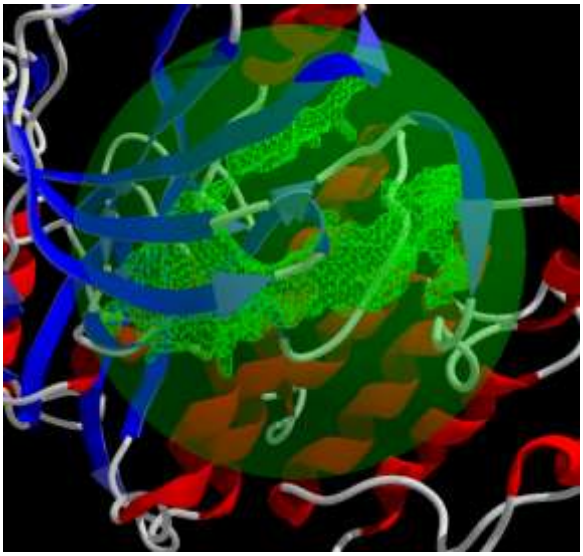


Fig. 24. Predicted binding cavity of Tyrosine Kinase (PDB ID 2HCK).

The values were ranked based on the Rerank score which is shown in Table 25. The details of molecular interactions analysis of Naringin is presented in Table 26. The molecular snapshot of Naringin docked at the active site of Tyrosine Kinase is shown in Fig. 25 A and B. The energy map and electrostatic interaction of the docking hits are shown in Fig. 26 A and B. The surface interaction map and hydrophobic interaction map of Naringin at the active site of Tyrosine Kinase is shown in Fig 27 A and B.

Table 25. Docking score against Tyrosine Kinase (PDB ID 2HCK)

Ligand	MolDock Score[a]	Interaction[b]	HBond[c]	MW[d]	LE1[e]	LE3[f]
Vitamin E	-65.13	-69.31	-2.29	430.706	-2.10	-0.15
Naringin	-64.38	-107.97	-14.11	580.535	-1.57	4.66
Coumarin	-56.67	-72.05	0.00	146.143	-5.15	-4.33
Vitamin C	-48.59	-58.06	-13.26	176.124	-4.05	-3.71
ß-Pinene	-42.82	-56.83	0.00	136.234	-4.28	-2.26
Naringenin	-36.24	-59.24	-8.62	272.253	-1.81	-0.74

a- MolDock Score) used by MVD is derived from the PLP scoring functions (Escore = Einter + Eintra)

b - The total interaction energy between the pose and the protein (kJ mol−1) (more negative means more stable).

c - The internal energy of the pose.(The lesser the better)

d - Hydrogen bonding energy (kJ mol−1). (more negative means more stable)

e - Ligand Efficiency 1: MolDock Score divided by Heavy Atoms count. (more negative means more stable).

f - Ligand Efficiency 3: Rerank Score divided by Heavy Atoms count. (more negative means more stable).

Table 26. Molecular interaction analysis of Naringin

Compound	Protein-Ligand Interaction	Distance	Energy
Naringin	Lys295(NZ)---O(8)	2.60 Å	-2.5
	Lys295(NZ)---O(7)	3.10 Å	-2.5
	Asp404(OD)---O(8)	3.11 Å	-2.44
	Ala275(O)---O(4)	3.33 Å	-1.32
	Arg388(NH)---O(0)	3.52 Å	-0.18
	Arg388(NH)---O(2)	3.10 Å	-2.5
	His384(ND)---O(13)	3.10 Å	-2.15
	Phe405---O(13)	2.36 Å	-0.50

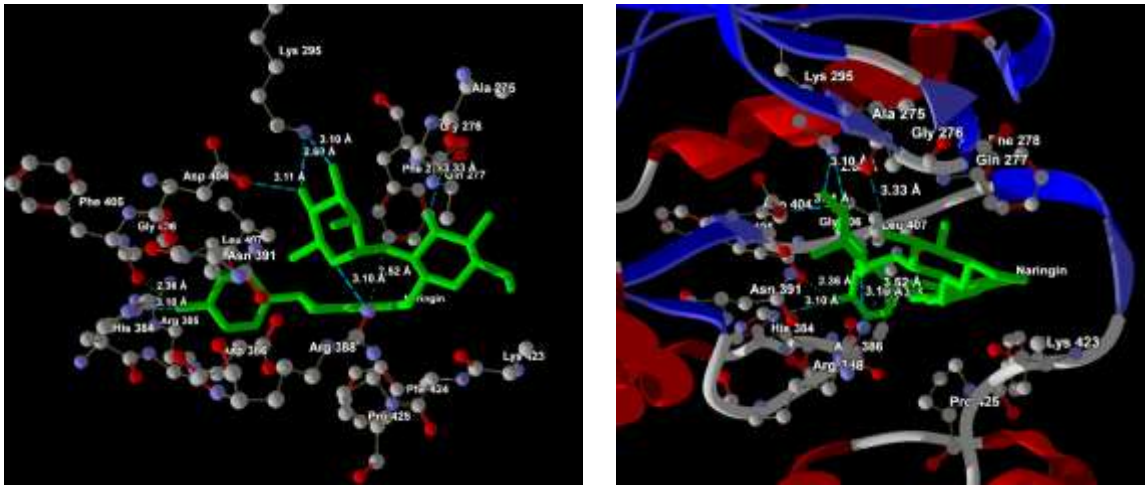


Fig.25. (A) Protein-ligand interactions between Naringin and the active site residues of Tyrosine Kinase enzyme. (B) Binding mode of Naringin at the active site residues of Tyrosine Kinase enzyme.

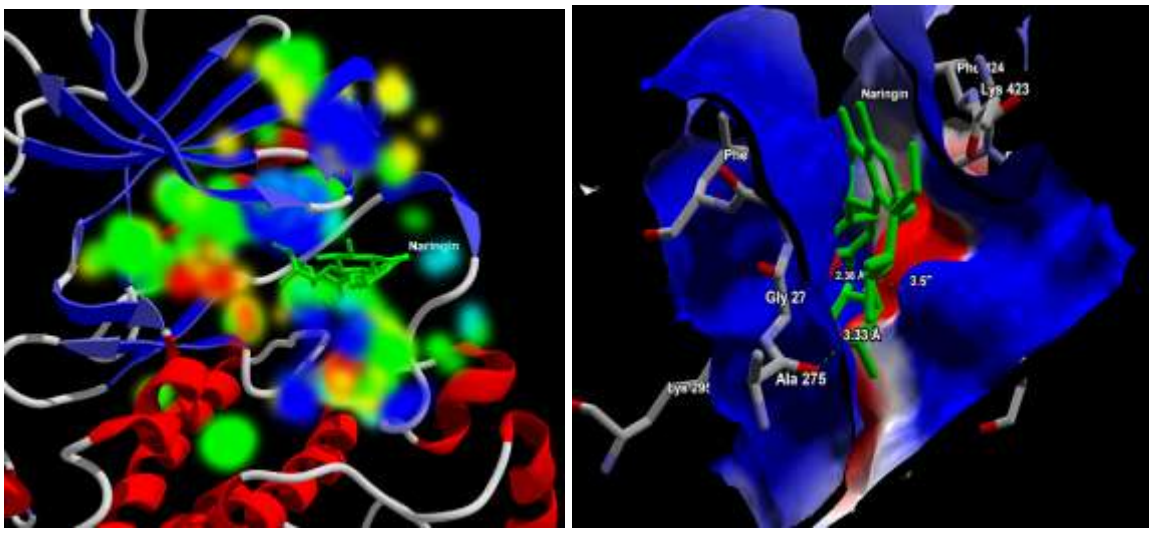


Fig. 26. (A) Energy map of Tyrosine Kinase interacting with Naringin depicting steric interaction favourable (green), hydrogen acceptor favourable (turquoise colour), hydrogen donor favourable (yellow colour) and electrostatic favourable (blue and red colour) regions. (B) Electrostatic interaction of Naringin at the enzyme active site of Tyrosine Kinase enzyme indicating electronegative (blue) and electropositive regions (red)

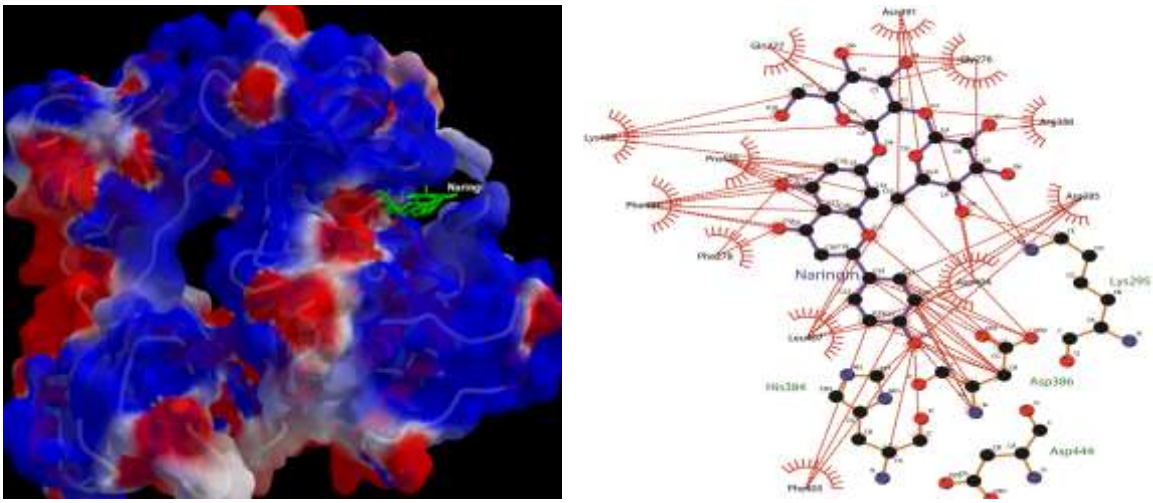


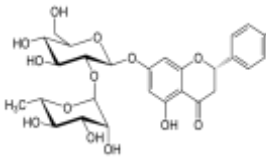
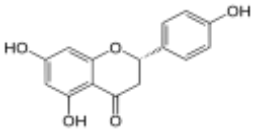
Fig. 27. (A) Surface interaction map and (B) Hydrophobic interaction map of Naringin at the active site of Tyrosine Kinase.

3.17 Binding mode prediction of Naringin and Naringenin against TNF-α, IL-10, IL-1ß

The computational binding free energy of 2 NPs (Naringin and Naringenin) against the 3 known cancer targets namely interleukin-1 beta (PDB id: 1HIB), interleukin-10 (PDB id: 1ILK) and tumor necrosis factor (PDB id: 1A8M) were estimated. The details of molecular docking and their binding affinity was shown in Table 27. It was observed that both the drug compound had the significant estimated binding affinity against all the 3 targets The compound Narigin had a binding affinity of -7.3 (kcal/mol) while Naringenin had a binding affinity of -7.2 (kcal/mol) against the interleukin-1 beta as depicted in Table 27.

The binding conformational mode of each target bound with drug compounds (Narigin and Naringenin) obtained from docking study was shown in Fig. 28-30. As it is evidence of the figure that both the drug compound were bound at the active site region of all the three targets.

Table 27. Details of naringin and naringenin docking against 3 targets (TNF-α, IL-10 and IL-1ß)

SL.No.	Compound name	Pubchem_id	Target name	Binding affinity (kcal/mol)
1.	1. Naringin 	442428	IL-1ß (PDB_id :1HIB)	-7.3
			IL-10 PDB id:1ILK	-7.1
			TNF-α (PDB id:1A8M)	-7.1
2.	2. Naringenin 	932	IL-1ß (PDB_id :1HIB)	-7.2
			IL-10 PDB id:1ILK	-6.8
			TNF-α (PDB id:1A8M)	-6.6

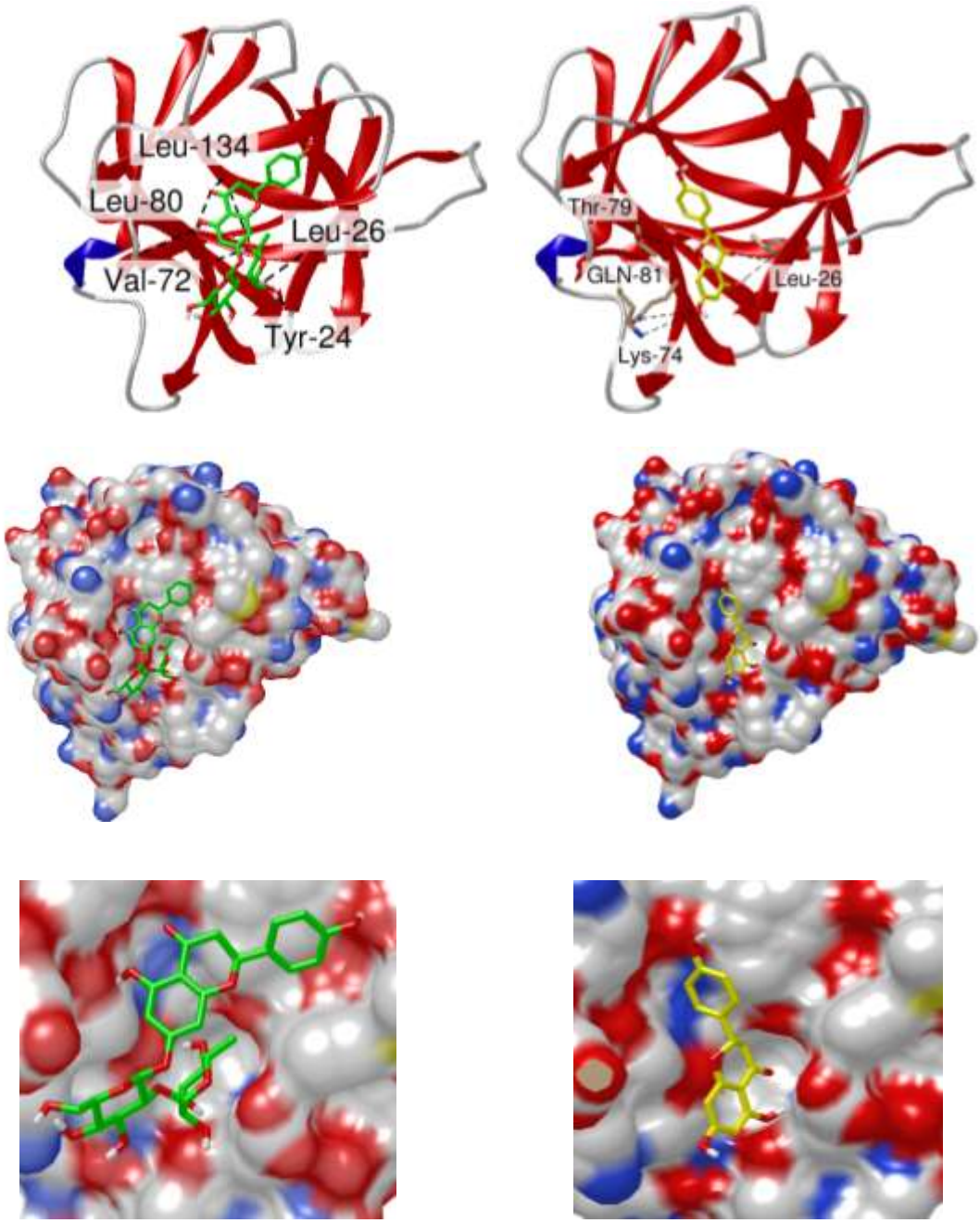


Fig. 28. Docked complex of interleukin-1 beta, (IL1B_HUMAN, PDB id: 1HIB) with 2 different drug compounds represented as Naringin in green and Naringenin in yellow. The protein target was shown in ribbon along with the active site region and NPs compounds were represented in stick model (A, B). The electrostatic surface of the whole protein target and complex (C&D) and close view of the electrostatic surface of drug binding sites (E & F).

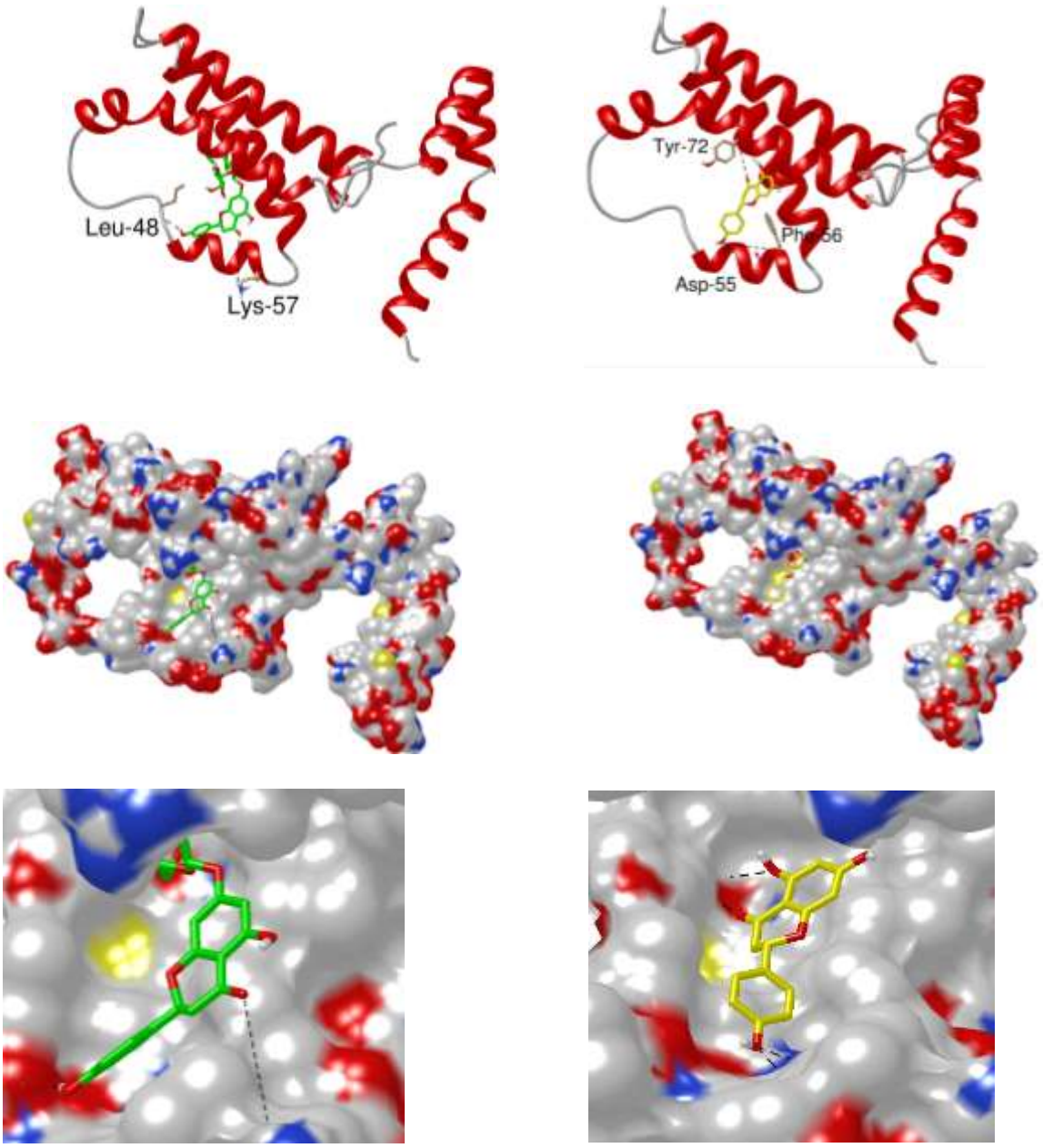


Fig. 29. Docked complex of interleukin-10 (IL10_HUMAN, PDB id: 1ILK) with 2 different drug compounds represented as Naringin in green and Naringenin in yellow. The protein target was shown in ribbon along with the active site region and NPs compounds were represented in stick model (A, B). The electrostatic surface of the whole protein target and complex (C&D) and close view of the electrostatic surface of drug binding sites (E & F).

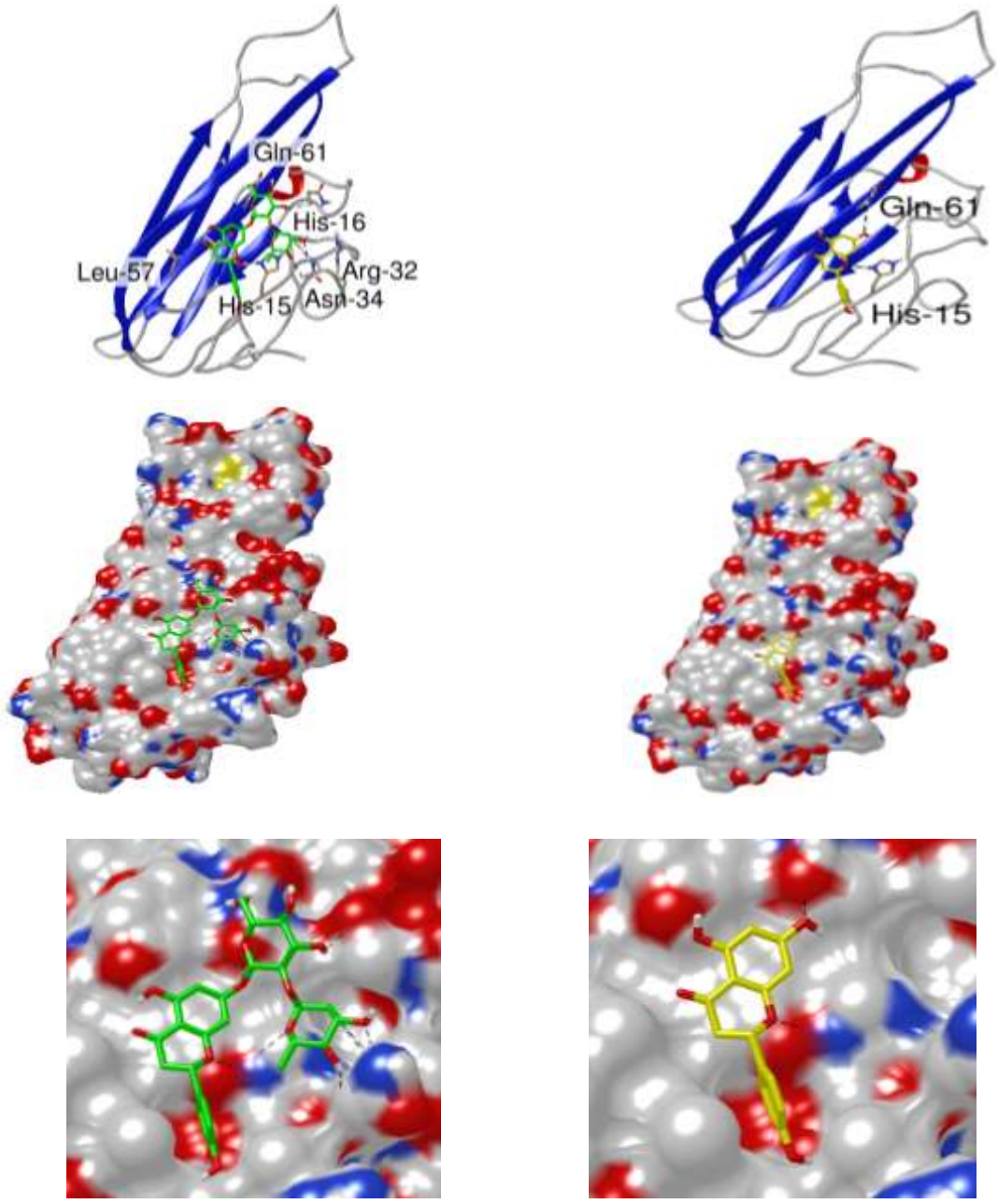


Fig.30. Docked complex of tumor necrosis factor alpha (TNFA_HUMAN, PDB id: 1A8M) with 2 different drug compounds represented as Naringin in green and Naringenin in yellow. The protein target was shown in ribbon along with the active site region and NPs compounds were represented in stick model (A, B). The electrostatic surface of the whole protein target and complex (C&D) and close view of the electrostatic surface of drug binding sites (E & F).

CHAPTER-4

DISCUSSION

4.1 Phytochemical screening, phenolics, flavonoids and flavonol contents in Citrus macroptera Montruz.

The nature of organic solvent used for extraction significantly affect the phytochemicals present in the plant extract i.e., polarity of the solvents affect the content of the phenolics, flavonoids and flavonol. TPC, TFC, TFoC content of the extracts are presented in (Fig. 6, Table 3). The flavonoids in the citrus juice combine with aluminium to form a complex flavonoid-aluminium that could be measured at 430 nm (Quettier *et al*., 2000). Flavonol content was less in all the extract of *Citrus macroptera*, the highest content was however present in aqueous methanol extract (1.07 ± 0.19 µg/mL) Quercetin equivalent. Various experiments have been demonstrated that phenolic compounds such as flavonoids, phenolic acids, tannins, etc. are potential anti-oxidant and anti-oxidant activity of these compounds is due to their ability to scavenge free radicals.

Accumulation of free radicals can cause pathological conditions such as asthma, arthritis, inflammation, neurodegenation, heart disease, aging effect, etc (Cheynier, 2005). Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. The phenolic and flavanoids are widely distributed secondary metabolites in plants having anti-oxidant activity and have wide range of biological activities as anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities (Catherine *et al*., 1996; Bors and Saran 1997). Recent studies have shown that many dietary polyphenolic constituents derived

from plants are more effective anti-oxidants *in-vitro* than vitamins E or C, and thus might contribute significantly to the protective effects *in-vivo* (Catherine *et al*., 1997). Broad spectrum of chemical and biological activities including radical scavenging properties are possessed by this polyphenolic compounds (Ghafar *et al*., 2010). Phenolic compounds are mostly composed of flavonoids, phenolic acids, stilbenes, coumarins and tannins (Islam *et al*., 2015).

Three types of flavonoids which occur in Citrus fruits are flavanones, flavones and flavonols (Calabro *et al.*, 2004). The main flavonoids found in citrus species are hesperidine, narirutin, naringin and eriocitrin (Mouly *et al.,* 1994; Schieber *et al.,* 2001). Epidemiological studies showed that dietary citrus flavonoids improved reduction in risk of coronary heart disease (Majo *et al.,* 2005; Hertog *et al.,* 1993). Because of their lipid anti-peroxidation effects as anti-carcinogenic and anti-inflammatory agents, more and more attention is being drawn by the citrus flavonoids not only due to their anti-oxidant properties (Stavric, 1993; Elangovan *et al.*, 1994; Martin *et al.*, 2002).

4.2 *In vitro* and *in vivo* anti-oxidant activity

Citrus macroptera serves a promising source of natural anti-oxidant, as indicated by its high contents of polyphenols, flavonoids, tannins and proteins and also by its considerable DPPH free radical scavenging activities and ferric reducing anti-oxidant power (FRAP) value (Paul *et al*., 2015). DPPH radicals react with suitable reducing agents and then electrons become paired-off and the solution loses colour stoichimetrically with the number of electrons taken up (Chidambara *et al*., 2003). DPPH method is the most widely used anti-oxidant assay which measures the reduction capabilities of *C. macroptera* extracts on DPPH reagent at 517 nm. Our study showed that the anti-oxidant activity varies with varying the

nature of organic solvent used for extraction. This result go along with the finding of Gope *et al*., 2014 that time and solvent extractions played a vital role in the extraction of phenolic contents and their anti-oxidant properties of *C.macroptera*. Though the extracts showed anti-oxidant activity, they have lower activity than the standard ascorbic acid. The hot methanol extract of the stem bark of *Citrus macroptera* showed potential anti-oxidant activity with the IC_{50} value of 178.96 µg/mL whereas the cold methanol and the dichloromethane extracts showed moderate activity with the IC50 of 242.78 µg/mL and 255.78 µg/mL respectively. The n-hexane extract showed mild activity (IC50: 422.94 µg/mL) against DPPH free radical. It is evident that all possess anti-oxidant activity (Chowdhury *et al*., 2008). Nitric oxide is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities (Karuppagounder *et al*., 2013). Flavonoids are capable of inhibiting the expression and activation of iNOS, and therefore could be used additionally during inflammatory therapy (Anoop and bindu 2015).

Ethanolic extract of *Citrus macroptera* fruit peels (EECM) in DPPH scavenging activity (IC50 281.11 µg/mL), Nitric Oxide scavenging activity (IC50 182.89 µg/mL) were comparable with standard Ascorbic acid (Rahman *et al*., 2014). In our study, the order of anti-oxidant activities was aqueous> methanol> aqueous methanol extracts of *Citrus macroptera* fruit peels. Recently, the anti-oxidant properties of the fruit's peel and pulp were analyzed and reported to contain 620.91 and 291.06 mg of polyphenols (gallic acid), 508.33 and 145.02 mg of flavonoids (catechin), 585.99 and 526.08 mg of tannin (tannic acid), 56.26 and 120.83 mg of ascorbic acids per 100 g, respectively (Paul *et al*., 2015). IC50 of DPPH scavenging assays range from 7.9-276.11 µg/mL which reveal a promising value as

compared to earlier reported value. Out of the three models of anti-oxidant activity, methanol extract showed highest activity in two models i.e. reducing and nitric oxide assay. This may be accredited to the highest content of total phenolic and total flavonoid in the methanol extract of *C. macroptera* Montruz. *C. macroptera* has wide range of uses viz. the dried rind of the fruit as flavoring spice in preparation of meat dishes, the juice of the fruit as medicine for treatment of stomach ailments as well as digestive enzyme. Thus our findings serves to exemplify the potential of this wild orange as natural dietary anti-oxidants.

Superoxide dismutase is one of the chief cellular defense enzymes that dismutase superoxide radicals to water and oxygen. Catalase are heme-containing proteins that protect the cells from toxic effects of reactive oxygen species by converting hydrogen peroxide to water and molecular oxygen (Malomo *et al*., 2011). Catalase activity varies greatly between tissues. Hydrogen peroxide is highly deleterious to the cell and its accumulation causes oxidation of cellular targets such as DNA, proteins, and lipids leading to mutagenesis and cell death (Bai *et al*., 1999). Hence removal of H_2O_2 by catalase enzyme provide protection against oxidative stress related diseases. Endogenous anti-oxidant enzymatic defense plays a key role in neutralizing oxygen free radical-mediated tissue injury (Polidoro *et al*., 1984). SOD, catalase and GSH-Px are the primary free radical- scavenging enzymes Involved in the first line of cellular defense against Oxidative injury, removing both oxygen (O_2 and hydrogen peroxide (H_2O_2) before they can interact to form more reactive hydroxyl radicals (Panda and Naik 2009; Saravanan *et al*., 2013). *C. macroptera* extract pretreatment, however, ameliorated the levels of these enzymes in the heart tissue, suggesting that plant phenolics, flavonoids, tannins and other compounds may function as ROS scavenging compounds and form a cooperative network, inducing a series of redox reactions and interactions between

ascorbic acid, phenolics and glutathione. The oxidative imbalance has been explained as a defeat in the anti-oxidant defense system including; the anti-oxidant enzymes such as SOD, as well as, the anti-oxidant molecules such as α-tocopherol and carotenoids (Chen *et al*., 2003).

4.3 *In vitro* and *in vivo* anti-inflammatory activities

The induced oxidative imbalance provokes the pro-inflammatory mediators that activate reactive oxygen species producing inflammatory cells, thus exaggerating the oxidative stress at the inflammatory lesions (Shaban *et al*., 2017). There is excessive activation of phagocytes, production of O_2^-, OH radicals as well as non-free radicals species (H_2O_2) in many inflammatory disorders, which can harm severely tissues either by activating matrix metello protinase damage seen in various arthritic tissues or by powerful direct oxidizing action (Lewis 1989). Both the steroidal and non-steroidal anti-inflammatory drugs are prone to evoking serious adverse reactions, thus, inflammation has become the focus of global scientific research, more so due to its implication in virtually all human and animal diseases (Dharmasiri *et al*., 2003; Park *et al*., 2004). Inflammation is also caused by the denaturation of protein, anti-inflammatory activities can be checked by testing the inhibition capability of protein denaturation. The present study showed the inhibition of thermally induced bovine serum albumin denaturation under dose dependent manner. The higher inhibition capacity was observed in methanol extract (NC2) than the NC1 and NC3 extract. NC2 showed IC_{50} of 104.75 µg /g in albumin denaturation and 115.4 µg /g in heat induced haemolysis. But the inhibition potential was lesser than Diclofenac sodium. The production of auto-antigens in inflammation disease may be due to *in vivo* denaturation of protein. *C. macroptera* inhibit the denaturation of proteins and its effect was compared with the standard

Protease Inhibitor cocktail. Carrageenan induced edema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1-2 hr) is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotriens, polymorphonuclear cells, and prostaglandin produced by tissue macrophages (Winter *et al*., 1962). The IC50 of anti-oxidant activity and anti-inflammatory activity does not correlate each other, henceforth the *in vitro* and *in vivo* readings. Of course, strong anti-oxidant activity *in vitro* does not necessarily translate to a significant health benefit *in vivo;* indeed, high concentrations of ingested low molecular weight anti-oxidants may be toxic or act as pro-oxidants in humans (Galati *et al*., 2004), or else be rendered inactive by metabolism before they are absorbed into the bloodstream (Williams *et al*., 2004)

4.4 Hepatic assessment of ALT and AST enzymes

Increased levels of activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) have been observed in a variety of conditions like liver metabolic syndrome, atherogenesis (Dunne *et al*., 1990; Roohbakhsh *et al*., 2015) and type I and type II diabetes (Nadeau *et al*., 2005; Vozarova *et al*., 2002; Shafiee-Nick *et al*., 2012). It has been well known that enzyme AST and ALT are markers of hepatocyte damage and the high level of AST and ALT is an important marker for liver injury (McGill and Jaeschke 2013). AST is more highly concentrated in a number of organs e.g., liver, kidney, heart and pancrease as approximately 80% of AST is found in mitochondria, and ALT which is purely a cytoplasmic enzyme is released faster than AST (Paul *et al*., 2015). Aniagu *et al*., 2005 therefore considered ALT to be a more sensitive marker of hepatocellular damage than AST (Aniagu

et al., 2005). The significant reduction in alkaline phosphatase (ALP) levels that is caused by *Citrus macroptera* pulp indicated that cholestasis might not have been occurring at the investigated doses because a rise in plasma ALP levels is usually a characteristic finding of cholestatic liver disease (Aniagu *et al*., 2005). As albumin levels decrease in response to inflammation, there was no evidence of a link with vital organ dysfunction (Ruot *et al*., 2000). Khan and Zafar (2005) stated that changes in blood parameters signify stress, infection and intoxication. The current study also showed that extracts of *Citrus macroptera* Montruz. decreased the ALT and AST enzymes in tissues of CCl_4 treated mice. It also decreased the AST and ALT values in serum of carrageenan induced mice serum i.e bringing the enzyme levels in the range of normal control group. The results hints the hepato-protective potential of *C. macroptera* extract on mice under the conditions of oxidative stress and inflammation. This is in concurrence with report of Paul *et al*., 2016 who reported that *Citrus macroptera* fruit and its anti-oxidant properties has no toxic effect on liver, kidney and pancreas in Wistar rats and it protects against acetaminophen-induced hepatorenal toxicity in rats. *C. macroptera* is a useful inexpensive food product that can be consumed on a daily basis as a prophylaxis because it confers some protection against toxin-induced hepatotoxicity and nephrotoxicity (Paul *et al*., 2016).

Clinically, serum alanine aminotransferase (ALT) has been widely used for evaluating the severity of hepatic inflammation in liver disease (Zhou *et al*., 2017). The accurate assessment and monitoring of the severity of liver inflammation plays an important role not only in the control of disease progression, but in the therapy decision for patients with normal ALT levels (Zhou *et al*., 2017). Higher levels of HBsAg, HBV DNA, anti-HBc, ALT, AST, LP, GGT and Albumin was observed in patients with moderate- to-severe

inflammation than the patients with mild or no inflammation (Zhou *et al*., 2017). In clinical practice, serum ALT is an easily accessible surrogate parameter and is commonly used for monitoring the state of liver inflammation (Kim *et al*., 2008). In clinical trials and in *in vivo* tests, the assessment of adverse drug reaction potential is developed by use of measuring biomarkers alanine transferase (ALT), aspartate transaminase (AST), and bilirubin (Yang *et al*., 2012; Sun *et al*., 2014).

Yano *et al*., 2014 attempted to look at the complexity of this issue by stimulating inflammation in mouse livers. To compare with the current elevations in standard liver biomarkers ALT and AST, they added drugs at known concentrations and measured cytokine levels from mouse models (Roth and Lee 2017). The tissue activities of the transaminase (AST and ALT) enzyme are markers for the functions and integrity of the heart and liver (Adeniyi *et al*., 2010; http://www.ers.usda.gov/Briefing). Elevation of these enzymes in the serum have been reported to indicate cellular damage, tissue necrosis, as well as a calculated risk for cardiovascular diseases, with higher risk of cardiovascular disease and elevated myocardial infarction being attributed to elevation of ALT and AST respectively (Ioannou *et al*., 2006).

The rats administered with overdose of paracetamol (2 g/kg) caused significant liver damage and necrosis of cells as evidenced by the elevated serum hepatic enzymes (ALT, AST and ALP) and reduced level of protein and increased level of total bilirubin (Abirami *et al*., 2015). An obvious sign of hepatic injury is the leaking of cellular enzymes such as ALT, AST and ALP into plasma due to the disturbance caused in the transport functions of hepatocytes (Abirami *et al*., 2015). ALT is more specific to the liver, and it is a better parameter for analyzing hepatic injury (Abirami *et al*., 2015). High levels of AST indicate

the cellular leakage as well as loss of functional ability of cell membrane in liver (Abirami *et al*., 2015). Oxidative stress causes the rupture of plasma membrane by increasing their permeability which may be result into release of AST and ALT. Treatment with extracts of *Citrus macroptera* increases the activities of the tested marker enzymes.

4.5 Effect of *C. macroptera* on release of pro-inflammatory cytokines (TNF-α, IL-10 and IL-1ß)

Experimental liver injury models were established that resemble fulminant human hepatitis, including TNF-α and IFN-γ- dependent inflammatory liver injury models that allow the evaluation of hepatoprotective interventions, including medicinal plant components (Bai *et al*., 2017). Recently, it has been reported that cholestatic liver injury can result in inflammatory necrosis (Woolbright *et al*., 2013). Plasma biomarkers of pro-inflammatory cytokines such as IL- 2, IL-4, IL-6, TNF-a, and AMPK were significantly reduced by *Citrus aurantium* extract treatment, implying that it has anti-inflammatory activity in cholestasis (Lim *et al*., 2016). Our findings also showed that the levels of TNF-α, IL-10 and IL-1ß were reduced by *Citrus macroptera*. There is no report so far on the study of *C. macroptera* effects on pro-inflammatory cytokines (TNF-α, IL-10 and IL-1ß) release, the present study being the first of its kind. *C. macroptera* extracts ameliorate the anti-inflammatory action by decreasing the level of studied pro-inflammatory cytokines.

4.6 MTT assay

MTT assay determines the cytotoxicity of the extract towards cancer lines, and it give a clue that the tested sample may have anticancer activity. The anticancer effect of *C. macroptera* extract on HeLa, a cervical cancer cell line was studied. There is no report on the anticancer activity of this citrus plants till now. Though the extract showed high IC_{50} value as compared

to standard Cisplatin, it somehow, showed that HeLa cell proliferation is susceptible to aqueous extract.

4.7 GCMS analysis of essential oil

Plant essential oils and their components have been known to have bioactivities e.g., antiparasitic, spasmolytic and anti-oxidant activities (Larousse 2001), antimicrobial (Marzouk *et al*., 2008), antifungal (Bouchra *et al*., 2003) and insecticidal (Pavela 2005). The phytochemical composition of *C. macroptera* was previously reported to contain water (90.40%), β-carotene (22.00 mg/100 g), thiamine (0.08 mg/100 g), riboflavin (0.01 mg/100 g) and (sodium 3.50, potassium 89.00, calcium 25.00, magnesium 10.00, iron 0.15, zinc 0.21, copper 0.07 and phosphorus 12.00 mg/100 g) (Islam *et al*., 2015) . Miah *et al*., 2010 and Waikedre *et al*., 2010 reported identification of 25 and 35 compounds respectively from essential oil *Citrus macroptera*. However, the present study using the sample collected from Manipur showed 57 number of compounds which is higher number compared to the earlier reports. Sakat et al., 2010, however, reported that hydrodistillation of the leaves of *C. macroptera* yielded 1.67 % of essential oil and further reported that GC/MS analysis of the *C. macroptera* essential oil allowed the identification of 35 compounds accounting for 99.1% of the total composition. When compared to the yield and activity of essential oil extracted from leaves and peel of *C. macroptera*, the peels were having higher yield percentage as compared to the leaves. The present study found that the peel essential oil has higher anti-oxidant and anti-inflammatory activity than that of leaves essential oil. Paul *et al*., 2015 also found that the peel extract exhibited a higher level of anti-oxidant activity than the pulp, which supports the findings of previous studies reported by Abu-Amsha *et al*., 1996.

4.8 HPLC method validation and simultaneous determination of flavanone glycosides

The present described HPLC method was finally optimized after experimenting on various parameters. Different solvent systems in different ratios, varying cell temperature, flow rate, injection volume are studied on good eluting power of standard marker compounds to get the best chromatogram with sharp and separated peaks. Methanol-water or acetonitrile –water eluting system were tried with different concentration of acetic acid as mobile phase. Variation in flow rate, mobile phase concentration and injection volume significantly affect the retention time of analyte and efficiency of elution.

The three flavanone glycosides were found in all the three extracts, seed and lyophilized juice. Naringin had regression equation y= 3378x + 170555 (r=0.9906) for Naringin. However, Kanaze *et al*., 2003 found y= -0.00412+0.06206x (r>0.99) in HPLC analysis of Naringin in orange, lemon, tangerine and grapefruits where the mobile phase consisted of tetrahydrofuran/ water/acetic acid (21:77:2, v/v/v). Regression equation obtained for coumarin and naringenin has good r values. Naringin and hesperidin are the main citrus flavonoids with physiological properties present in grapefruit and orange juice (Gorinstein *et al*., 2006; Rapisarda *et al*., 1999). These flavonoids have been detected in human plasma after orange and grapefruit diets (Rapisarda *et al*., 1999). Precision and accuracy study of the developed method was adequate for simultaneous determination of three compounds at a time. LOD was obtained as amount to give signal-to-noise (S/N) ratio of 3. Similarly, LOQ was obtained to give S/N ratio of 10. The content of the tested flavanone glycosides were studied along with the values obtained after spiking with known concentration of standard.

4.9 Western blotting studies

Carrageenan injection also provokes the release of some important pro-inflammatory cytokines such as TNF-α and IL-1 β (Nacife *et al*., 2004). TNF-α is a pro-inflammatory cytokine that is released during the early phase of immunologic response and is known to be involved in modulation of allergic inflammation (Gali *et al*., 2005). TNF-α induces NO synthesis by activating iNOS and augments the responses of neutrophils to inflammatory stimuli (Halici *et al*., 2007). Our study revealed that BK1, BK2, and BK4 considerably reduced the TNF-α expression in carrageenan induced mice paw edema. Whereas, BK3 were not able to reduce the TNF-α expression. Expression of pro-inflammatory mediators are up-regulated in the inflammation process. Down-regulation of its activity demodulates the inflammatory process proving the anti-inflammatory efficacy of the tested of the compounds. Dermal edema, dermal infiltration and lymphocytic infiltration was observed in carrageenan only treated mice group. This were however not observed in paw tissues treated with the isolated compounds.

4.10 Molecular docking simulation study

The molecular docking simulation was performed for Vitamin E, Naringin, Coumarin, Vitamin C, naringenin and ß-Pinene against Tyrosine Kinase using MVD 6.0. The algorithm used in MVD is based on a differential evolution algorithm. The solution of the algorithm considers the sum of the inter-molecular interaction energy between the ligand and the protein and the intra-molecular interaction energy of the ligand. The grid-based scoring function, MolDock Score [GRID] was used to evaluate the docking solutions. Since, the compounds under the study possessed several internal degrees of freedom, MolDock SE was used as an alternative search algorithm.

The molecular docking scores (Table 25) revealed that Naringin could be docked at the active site of the Tyrosine Kinase enzyme with the favourable docking scores alongside Vitamin E based on the MolDock score and Rerank score. The MolDock score employed in the present investigation is derived from the PLP scoring functions originally proposed by Gehlhaar *et al.* and later extended by Yang *et al* (Paul and Choudhury 2010). The docking scoring function, E_{score}, is defined as: $E_{Score} = E_{inter}+E_{intra}$, where, E_{inter} is the ligand-protein interaction energy and E_{intra} is the internal energy of the ligand. While the Rerank score is a linear combination of E-inter (steric, Van der Waals, hydrogen bonding and electrostatic) between the ligand and the protein, and E-intra (torsion, sp2–sp2, hydrogen bonding, Van der Waals and electrostatic) of the ligand weighted by the pre-defined coefficients.

CHAPTER-5

SUMMARY AND CONCLUSION

Citrus fruits are well known for their rich anti-oxidant sources and potent bioactivities. *Citrus macroptera* Montruz., however, is a less studied citrus plant. From the context of a literature survey, the fruit collected from Sylhet division of Bangladesh alone is studied so far. In Manipur, North East India, a biodiversity hotspot region, the livelihood of some villagers entirely depend on the cultivation of this wild orange. The present was undertaken to endorse the medicinal value of *Citrus macroptera*. Fruits were collected and morphological characteristics were studied using the Citrus descriptor of International Plant Genetic Research Institute (IPGRI) and it was further identified by taxonomist of the institute. As a first section of the study, the collected fruits were peeled off, dried and process for organic extraction. There are many ways of organic extraction but in the present study, cold extraction or maceration is employed as it is less time consuming and there is less chance of degrading the compounds present in the plant sample. The preliminary phytochemical screening was done. The various test demonstrated the presence of alkaloid, phenolic, flavonoid, Coumarin glycosides, Saponin glycoside, Mucilage. However, resin, anthraquinone glycosides, steroid, protein, and iodine were absent in all the tested extracts. The content of phenolic, flavonoid and flavonol were quantified for all the extracts and fractions. With varying solvent type, the content of phytochemicals also differ. *Citrus macroptera* peels extract revealed the presence of phytochemicals responsible for medicinal bioactivities.

The present study reveals the anti-oxidant activity, anti-inflammatory activity (heat-induced haemolysis, albumin denaturation and protease inhibitory), cytotoxicity and clonogenic activity in *Citrus macroptera* Montruz. The results of *in vitro* antioxidant and *in vitro* anti-oxidant assays were substantiated further by *in vivo* studies employing Swiss albino mice. The extracts were able to increase the levels of superoxide dismutase, catalase and glutathione reductase enzymes in mice. These enzyme levels were lowered by oxidative stress caused by carbon tetrachloride injection. The increasing level of the enzyme proves the anti-inflammatory activity of *Citrus macroptera*. Montruz. The pro-inflammatory mediators were also found to be inhibited by the extracts of *C. macroptera* Montruz. ALT and AST enzyme levels were also found to be controlled when treated with the plant extract. The protective effect of anti-inflammation and hepatoprotective efficacy may result from antioxidant effect. As a whole, the results illustrated that the extracts exhibited concentration and solvent dependent bioactivities.

Components of essential oil of peel and leaves of *Citrus macroptera* Montruz. were identified. Based on the essential oil yield, peel has a better source of essential oil than that of leaves. Common compounds were detected in the essential oil of both the leaves and plants, but the relative percentage composition of the essential oil was different. Essential oil of peel, as well as leaves, exhibit antioxidant and anti-inflammatory activity. From the calculated activity based on IC_{50} values, essential oil of peel showed more anti-oxidant and anti-inflammatory activity than that of leaves' oil.

High performance liquid chromatographic studies also indicated the presence of naringin, naringenin and coumarin, hence, their contents in the *C. macroptera* extracts was

thus quantified. Further, the concentrations of three aglycones; quercetin, rutin, and kaempferol was determined.

Cytotoxicity study of HeLa cells, a cervical cancer cell lines showed good activity as compared to the standard anticancer marker Cisplatin. Concentration and time-dependent inhibition of cell proliferation were observed. Higher the doses of extracts used for treatment against cultured cancer cells, higher the inhibition of colony formation of the cells were observed. Bioactive compounds are targeted for isolation from the active fractions.

Four compounds named as BK1, BK2, BK3, and BK4 were identified as naringin, naringenin, coumarin, and ß-pinene. Structure of the isolated compounds was elucidated by analyzing the NMR, mass spectrometry and FTIR data. The isolated compounds were found to have anti-inflammatory activity. Western blotting studies on the proteins isolated from paw tissues of mice treated with the isolated compounds showed the inhibition of protein expression of TNF-α, a pro-inflammatory mediator. The interaction between Naringin and tyrosine kinase, a target protein of anti-oxidant activity at the atomic level using molecular docking approach elucidate antioxidant capacity. Its antioxidant activity was higher than vitamin C (standard ascorbic acid) but lower than vitamin E. The radical scavenging potential of Citrus macroptera may be attributed to the presence of naringin. The study is the first of its kind studying the activities of *Citrus macroptera* Montruz growing in Manipur, NorthEast India. The findings serve to exemplify the potential of this wild orange as natural dietary antioxidants, anti-inflammatory and anticancer agents. It can be used for drug development to battle those diseases which are induced by oxidative stress. In conclusion, *Citrus macroptera* Montruz. may be considered as a feasible therapeutic resource to fight antioxidant and inflammatory related diseases.

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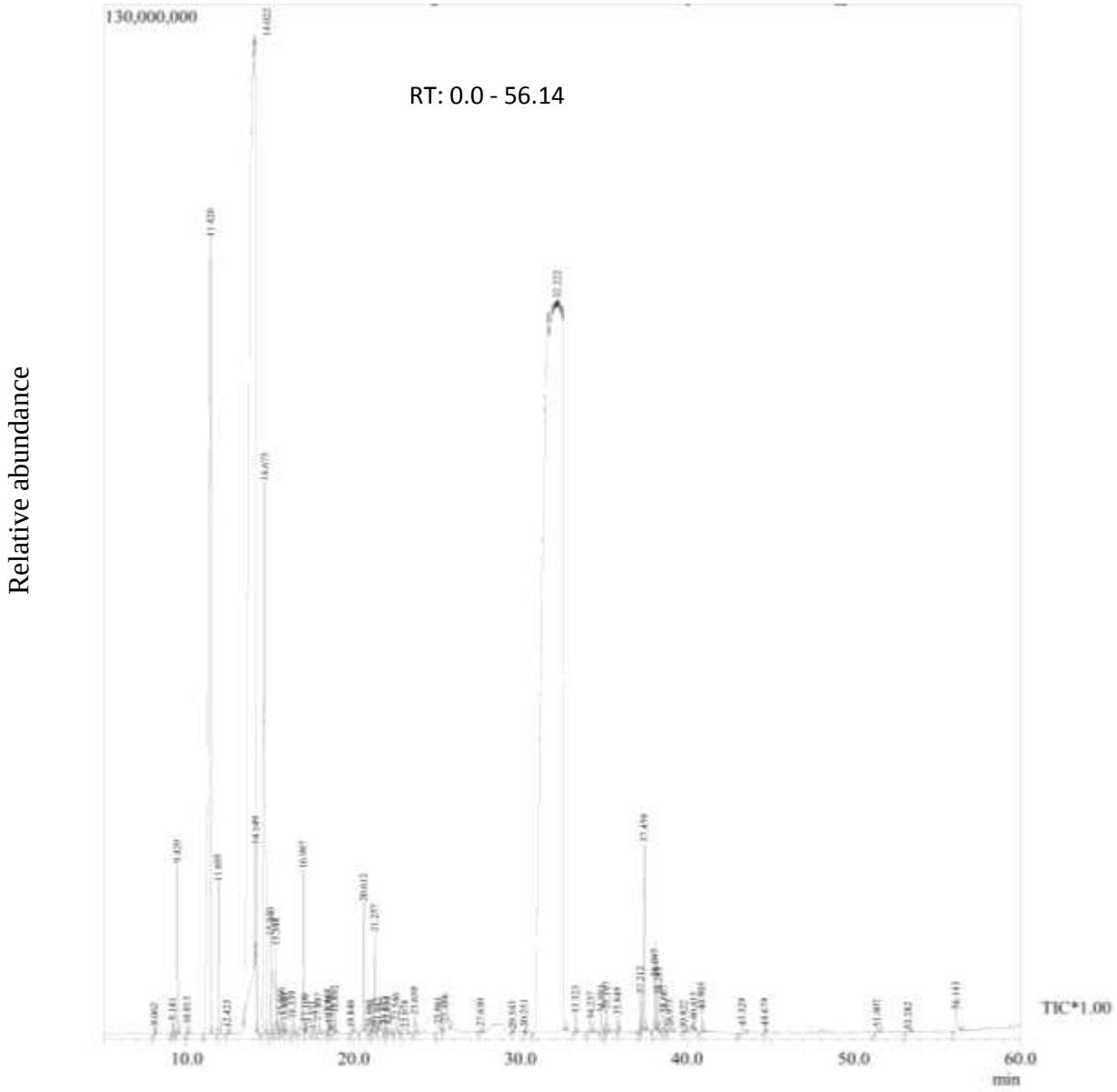
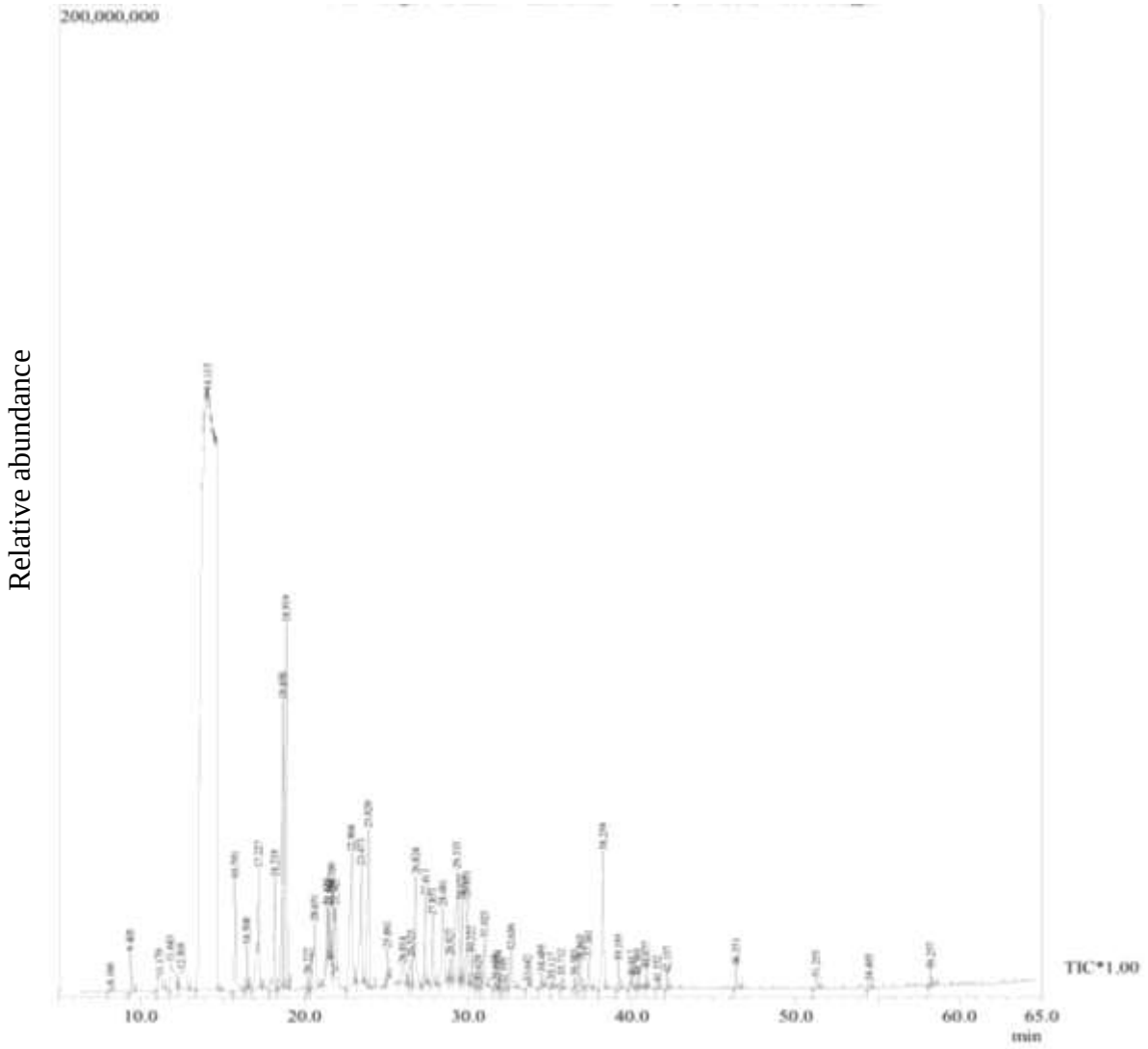
Annexure I



GCMS chromatogram of leaves essential of *Citrus macroptera* Montruz.,

RT- retention time

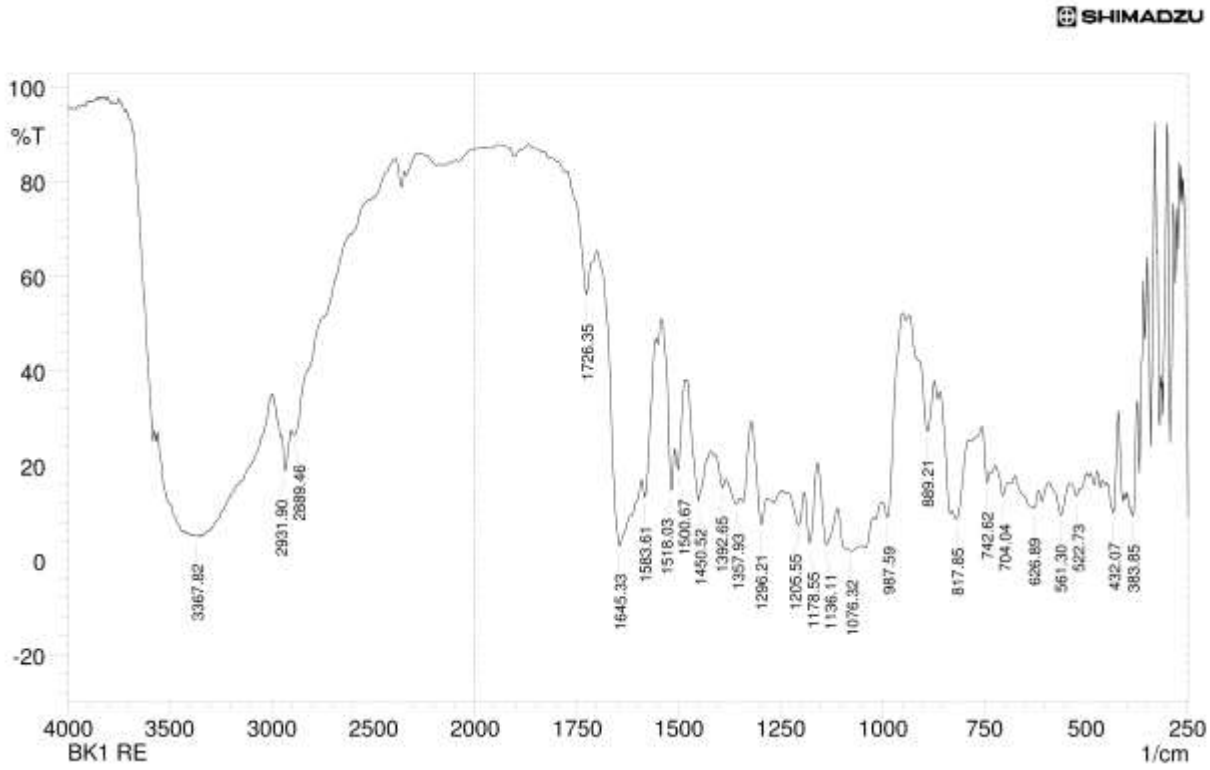
Annexure II



GCMS chromatogram of peel essential oil *Citrus macroptera* Montruz.

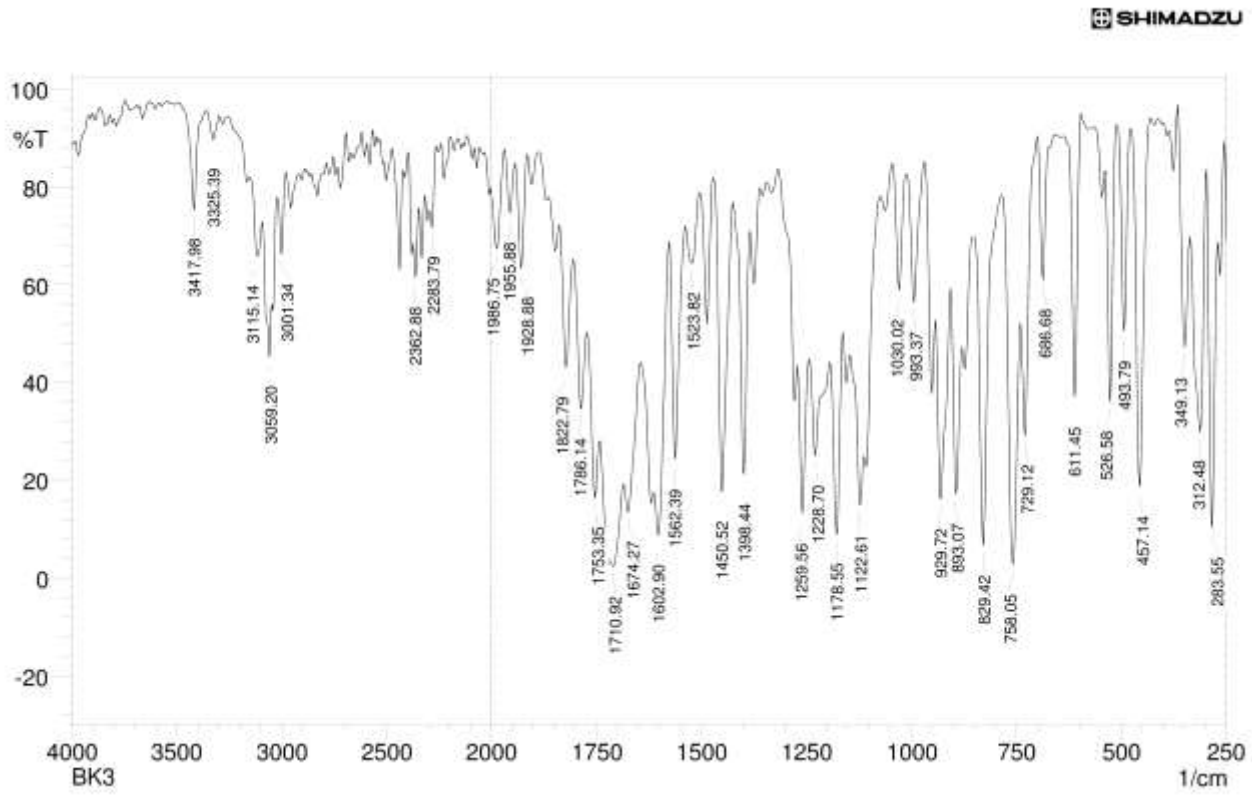
RT-retention tim

Annexure III



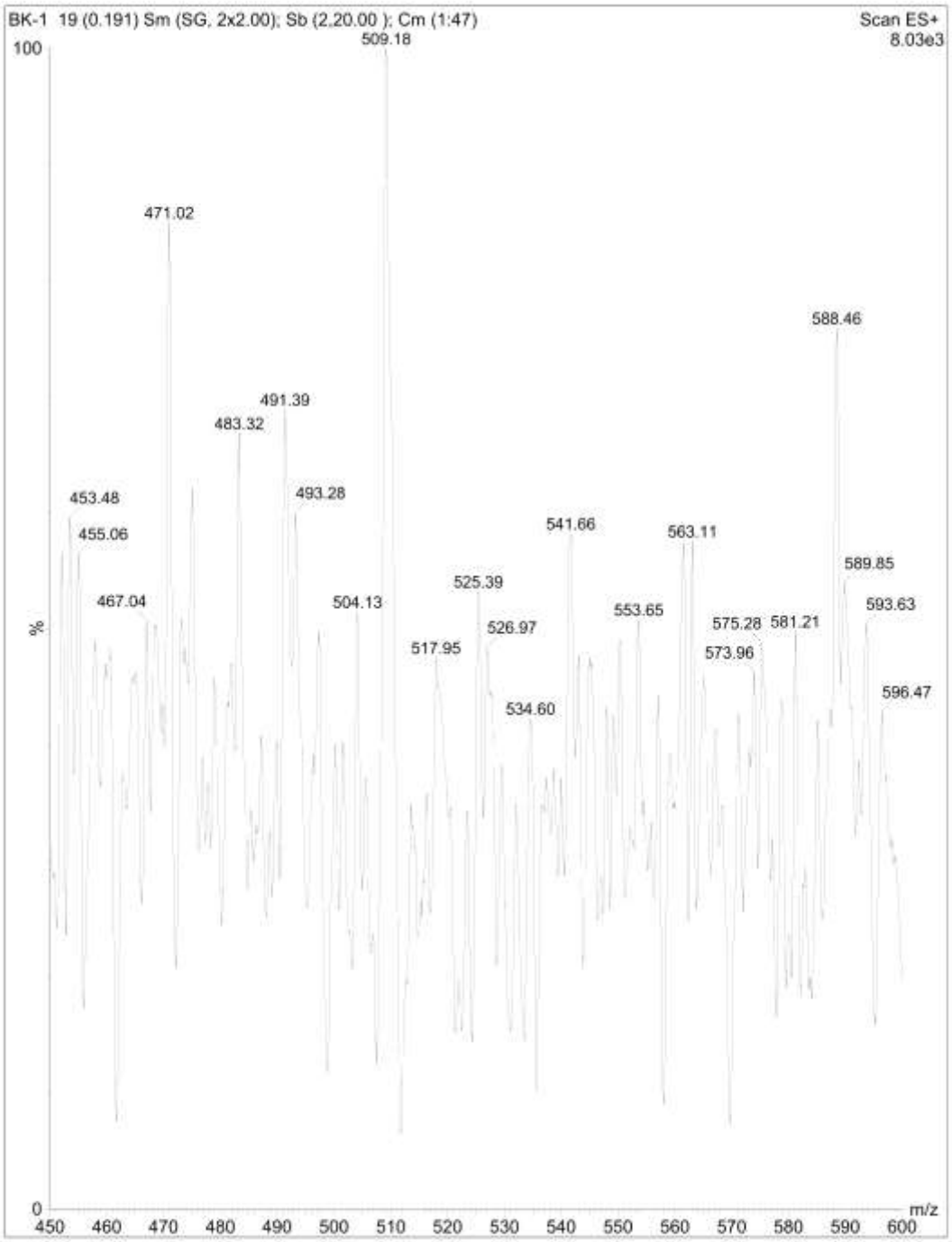
FTIR spectrum of compound BK1

Annexure IV



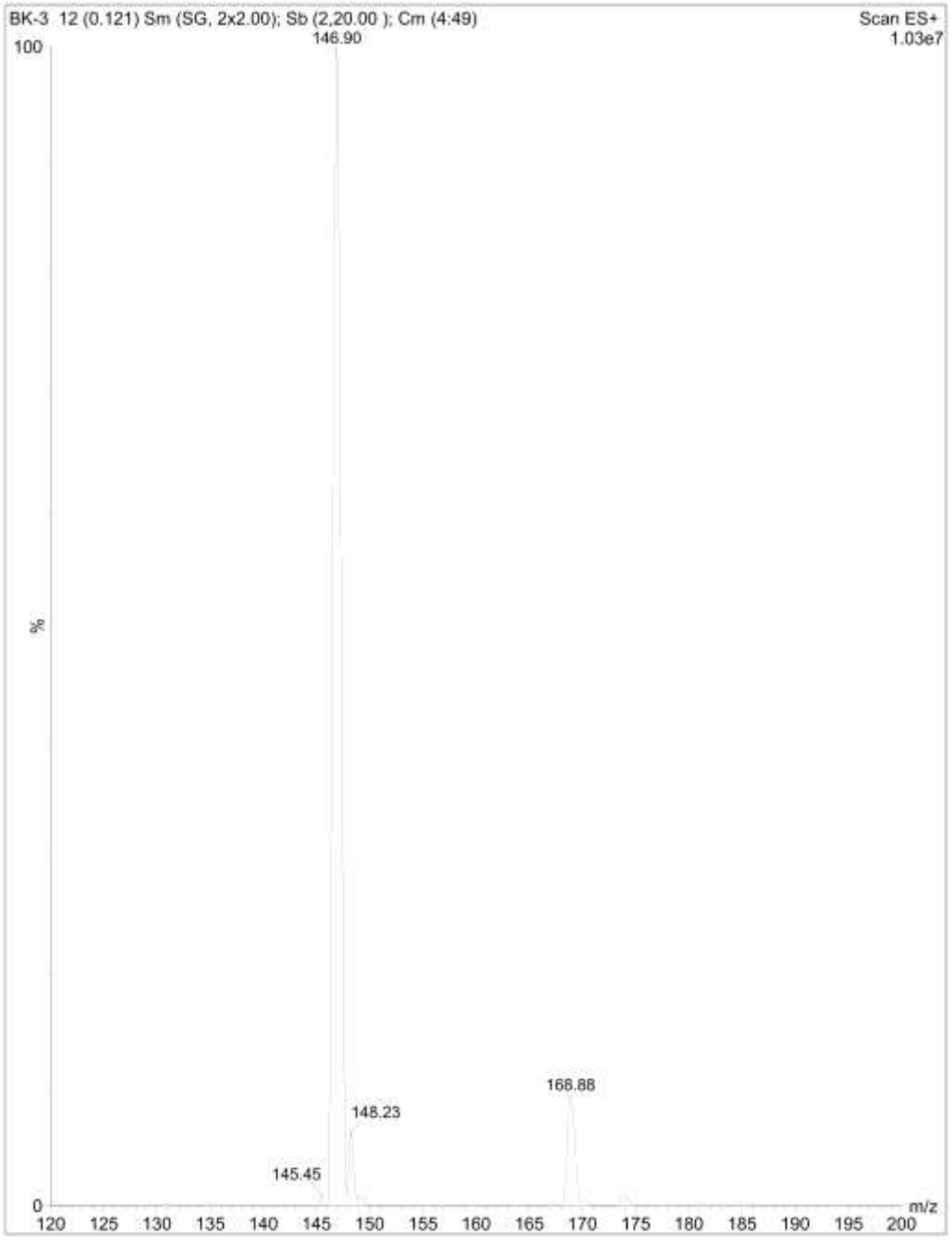
FTIR spectrum of compound BK3

Annexure V



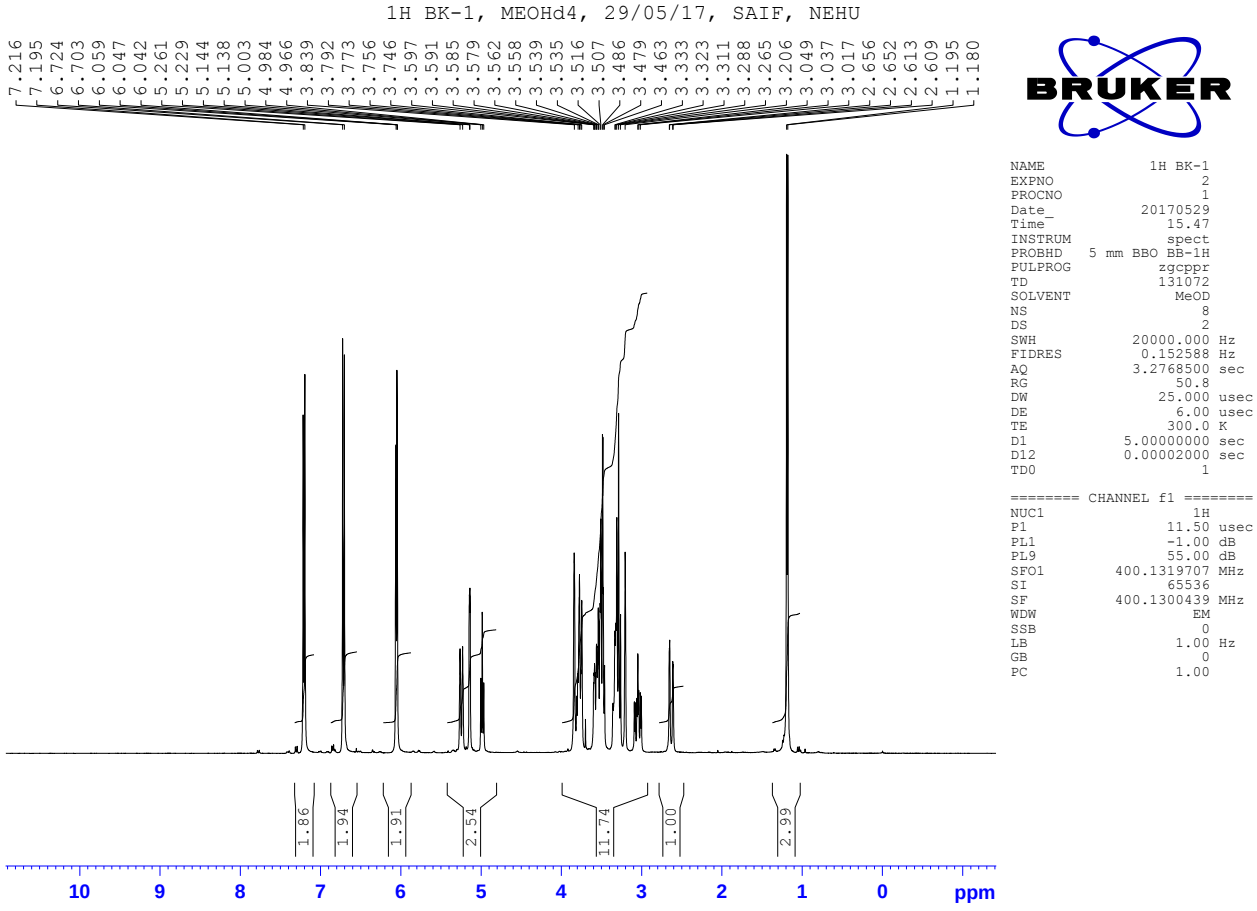
Mass spectrum of compound BK1

Annexure VI



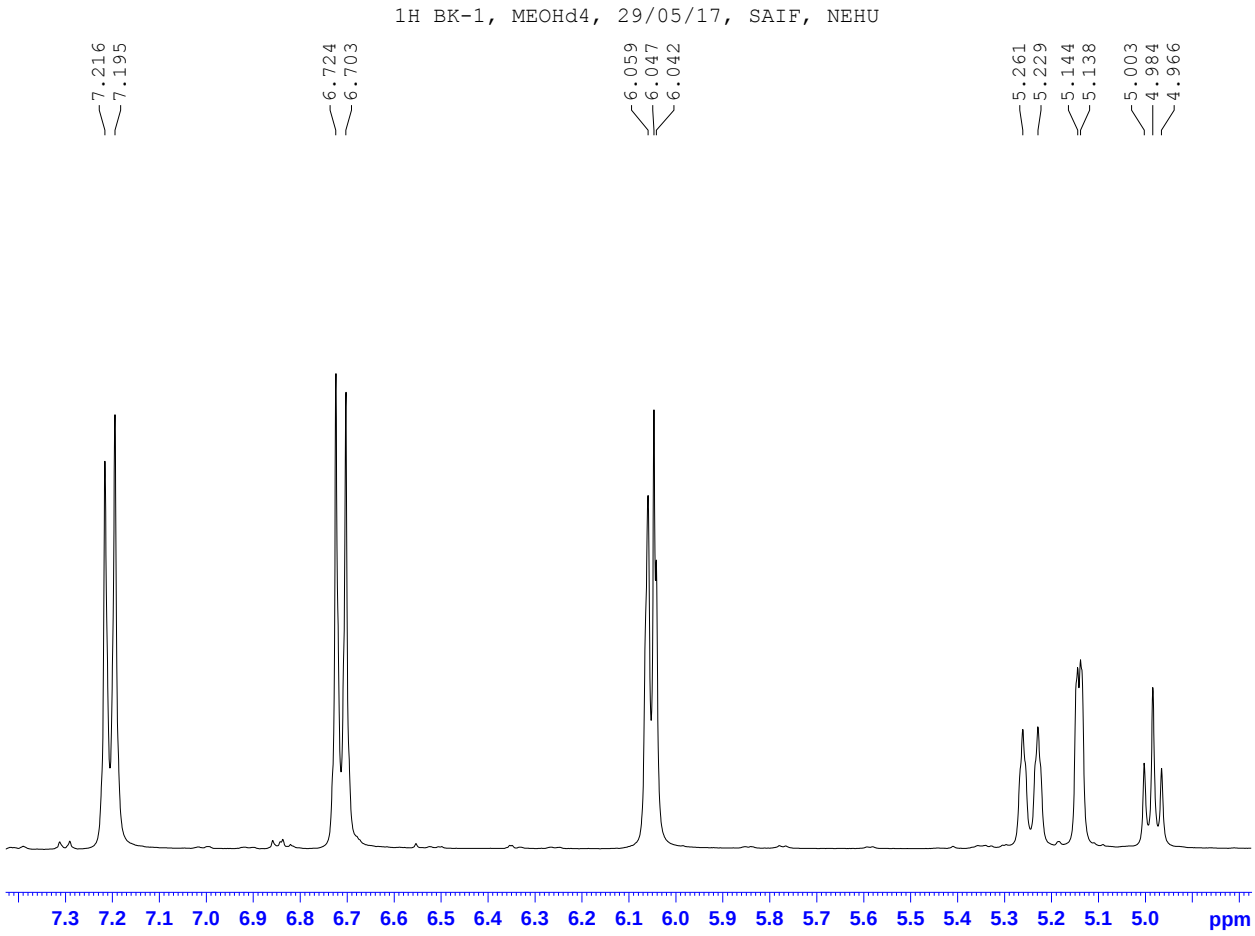
Mass spectrum of compound BK3

Annexure VII



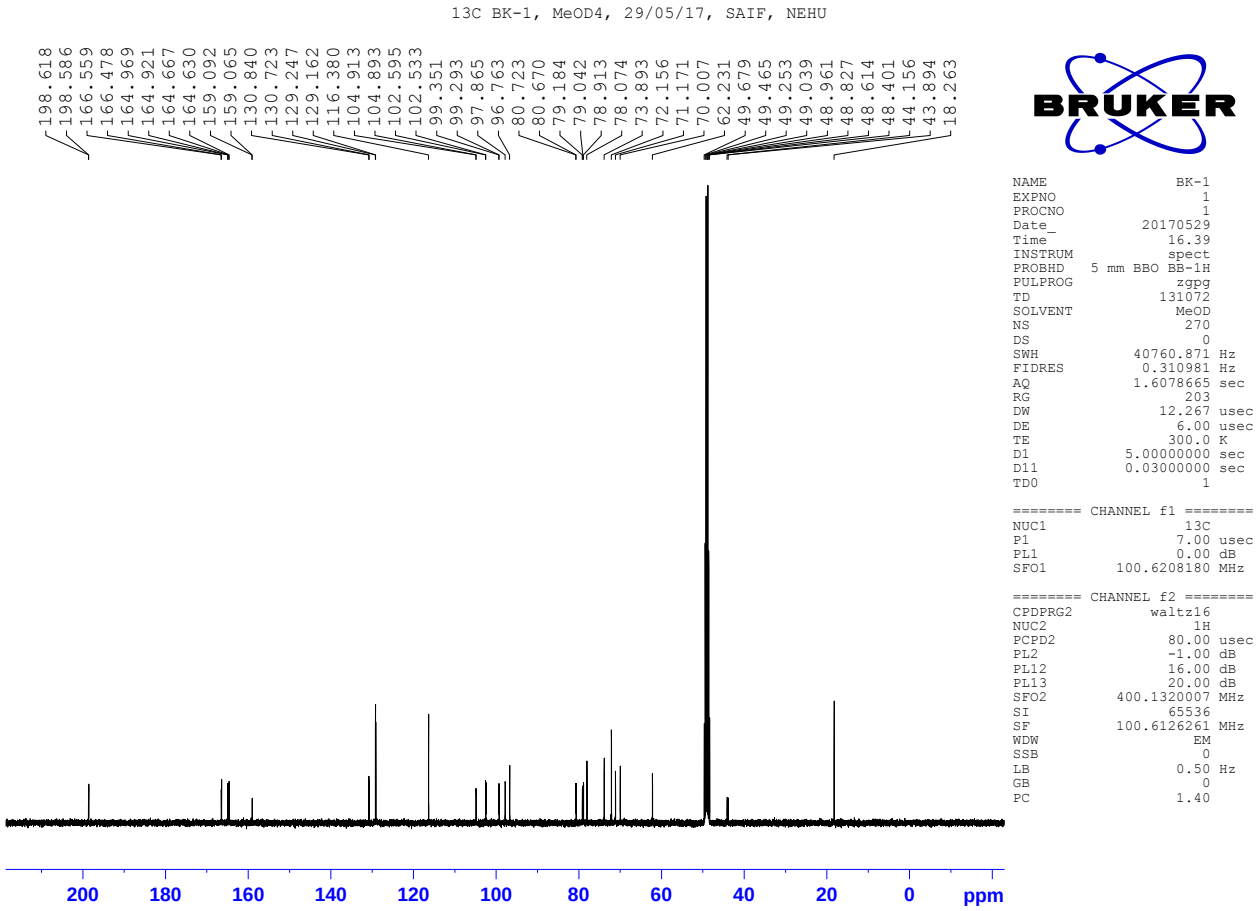
1H NMR spectrum of compound BK1

Annexure VIII



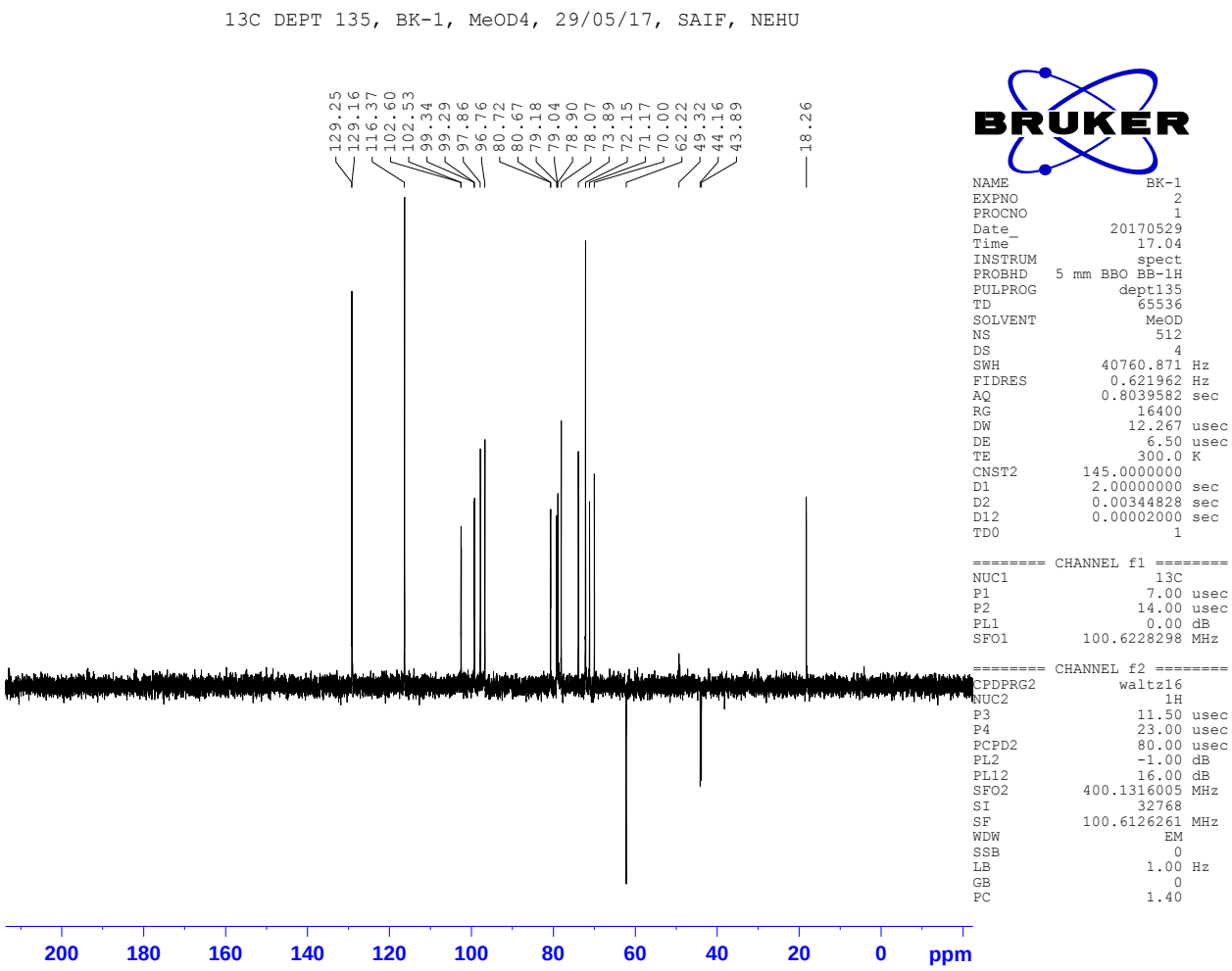
1H NMR spectrum of BK1

Annexure IX



13C NMR spectrum of BK1

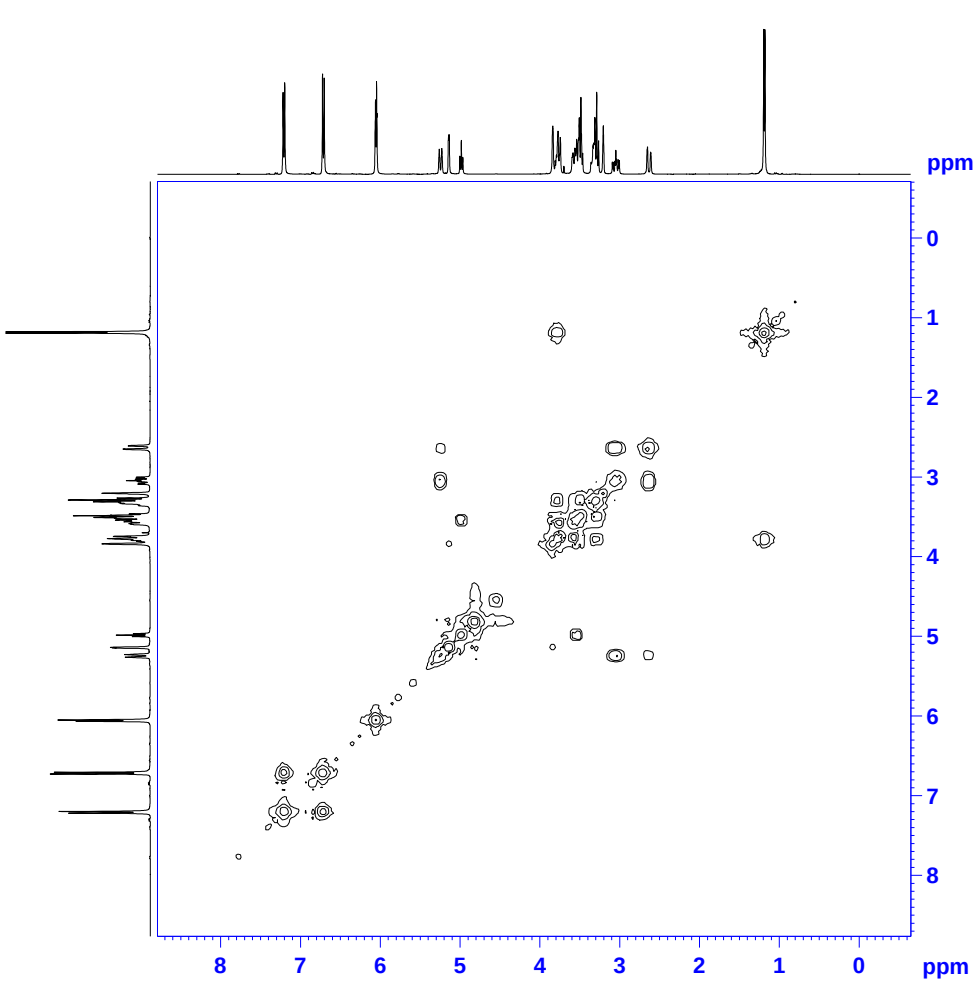
Annexure X



13C NMR spectrum of BK1

Annexure XI

COSY, BK-1, MeOD4, 29/05/17, SAIF, NEHU

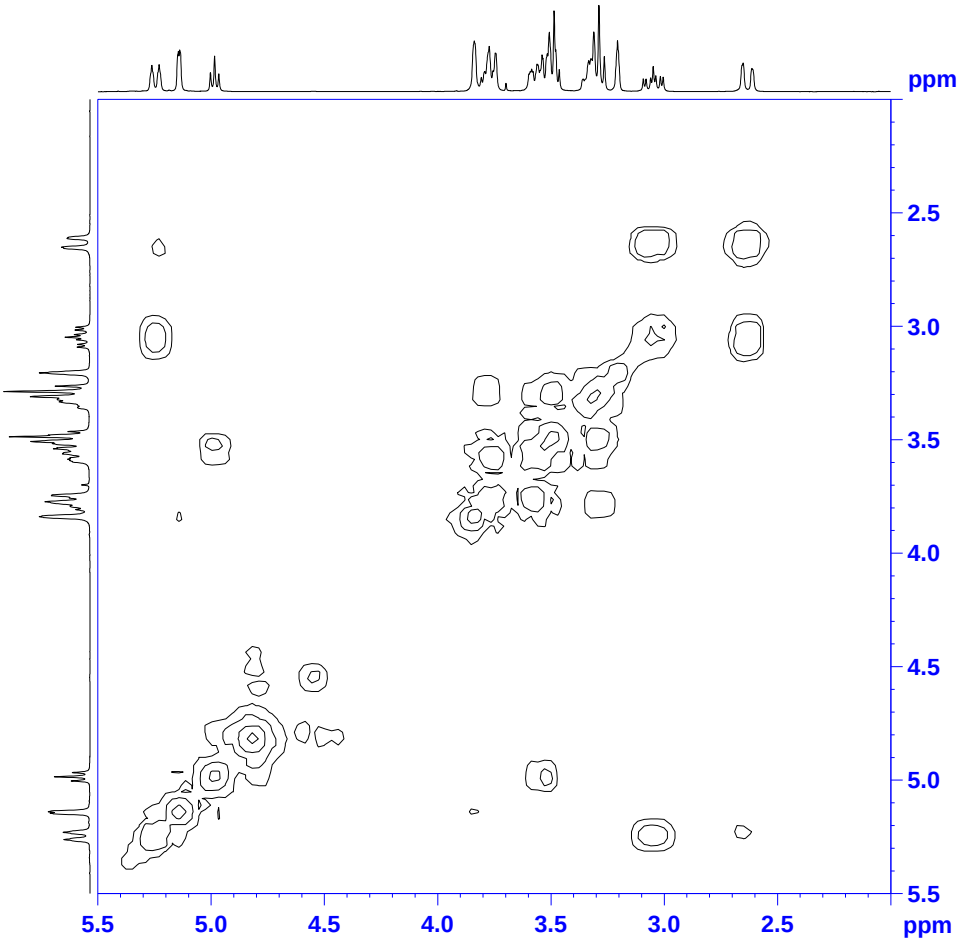



```
NAME                  BK-1
EXPNO                    3
PROCNO                   1
Date_             20170529
Time                 17.05
INSTRUM              spect
PROBHD    5 mm BBO BB-1H
PULPROG          cosygpqf
TD                    2048
SOLVENT               MeOD
NS                       8
DS                       8
SWH              12019.230 Hz
FIDRES               5.868765 Hz
AQ                0.0852468 sec
RG                    45.2
DW                  41.600 usec
DE                   6.50 usec
TE                  300.0 K
D0             0.00000300 sec
D1             1.48689198 sec
D13            0.00000400 sec
D16            0.00020000 sec
IN0            0.00008330 sec

======== CHANNEL f1 ========
NUC1                    1H
P0                   11.50 usec
P1                   11.50 usec
PL1                  -1.00 dB
SFO1          400.1319706 MHz

====== GRADIENT CHANNEL =====
GPNAM1            SINE.100
GPZ1                10.00 %
P16               1000.00 usec
ND0                      1
TD                     256
SFO1              400.132 MHz
FIDRES           46.890465 Hz
SW                  30.000 ppm
FnMODE                  QF
SI                    1024
SF            400.1300366 MHz
WDW                   SINE
SSB                      0
LB                    0.00 Hz
GB                       0
PC                    1.40
SI                    1024
MC2                     QF
SF            400.1300374 MHz
WDW                   SINE
SSB                      0
LB                    0.00 Hz
GB                       0
```

COSY of compound BK1

Annexure XII

COSY, BK-1, MeOD4, 29/05/17, SAIF, NEHU

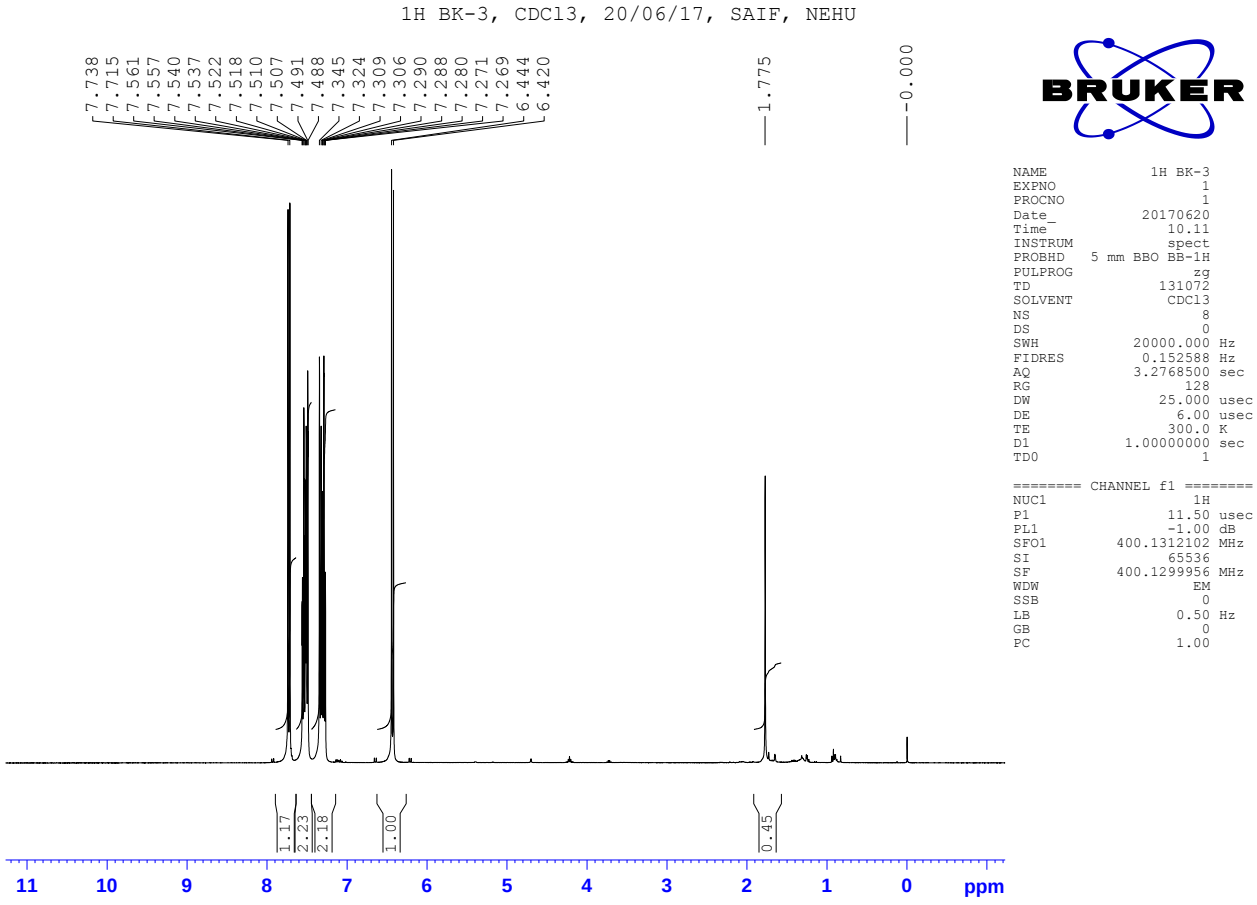


BRUKER

NAME	BK-1
EXPNO	3
PROCNO	1
Date_	20170529
Time	17.05
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	cosygpqf
TD	2048
SOLVENT	MeOD
NS	8
DS	8
SWH	12019.230 Hz
FIDRES	5.868765 Hz
AQ	0.0852468 sec
RG	45.2
DW	41.600 usec
DE	6.50 usec
TE	300.0 K
D0	0.00000300 sec
D1	1.48689198 sec
D13	0.00000400 sec
D16	0.00020000 sec
IN0	0.00008330 sec
======== CHANNEL f1 ========	
NUC1	1H
P0	11.50 usec
P1	11.50 usec
PL1	-1.00 dB
SFO1	400.1319706 MHz
====== GRADIENT CHANNEL =====	
GPNAM1	SINE.100
GPZ1	10.00 %
P16	1000.00 usec
ND0	1
TD	256
SFO1	400.132 MHz
FIDRES	46.890465 Hz
SW	30.000 ppm
FnMODE	QF
SI	1024
SF	400.1300366 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.40
SI	1024
MC2	QF
SF	400.1300374 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

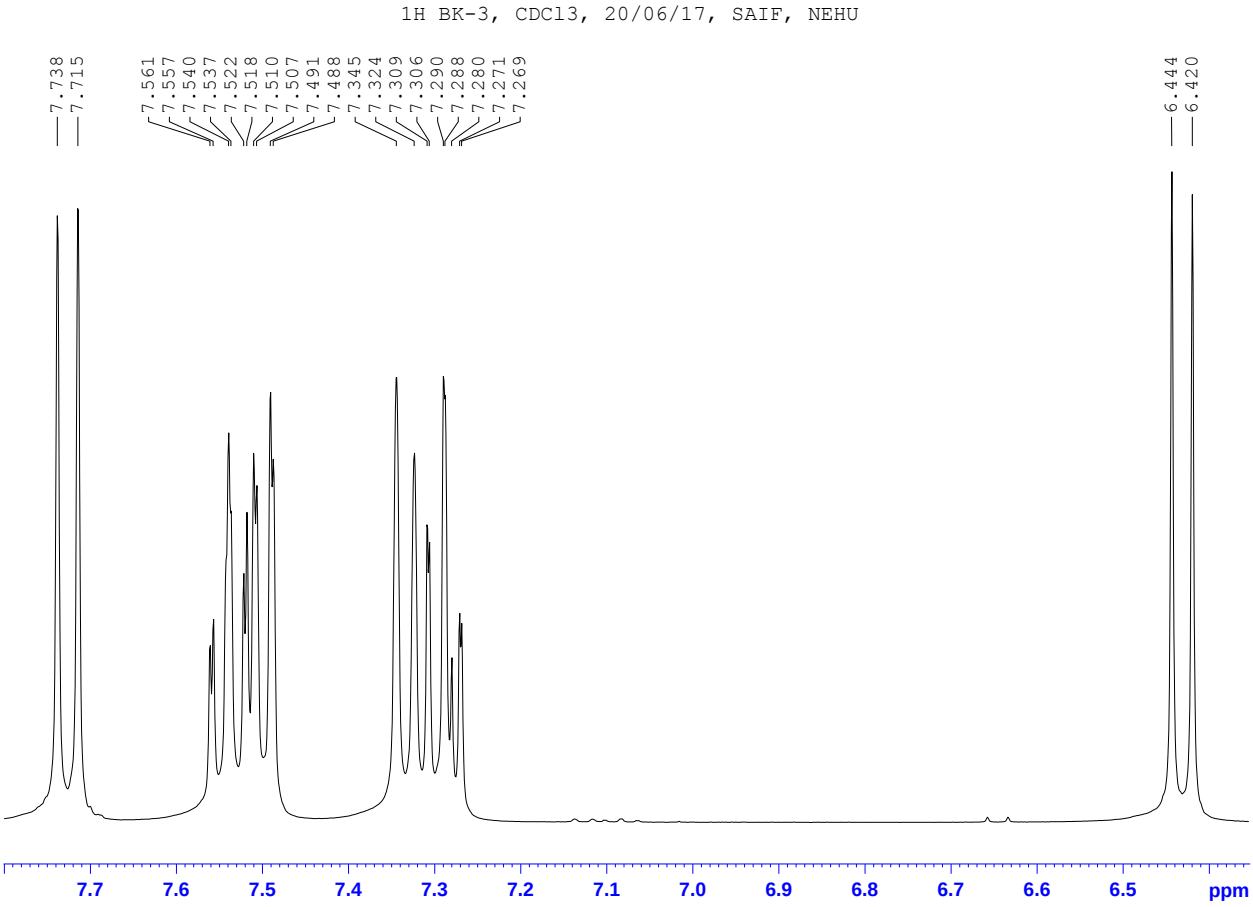
COSY of compound BK1

Annexure XIII



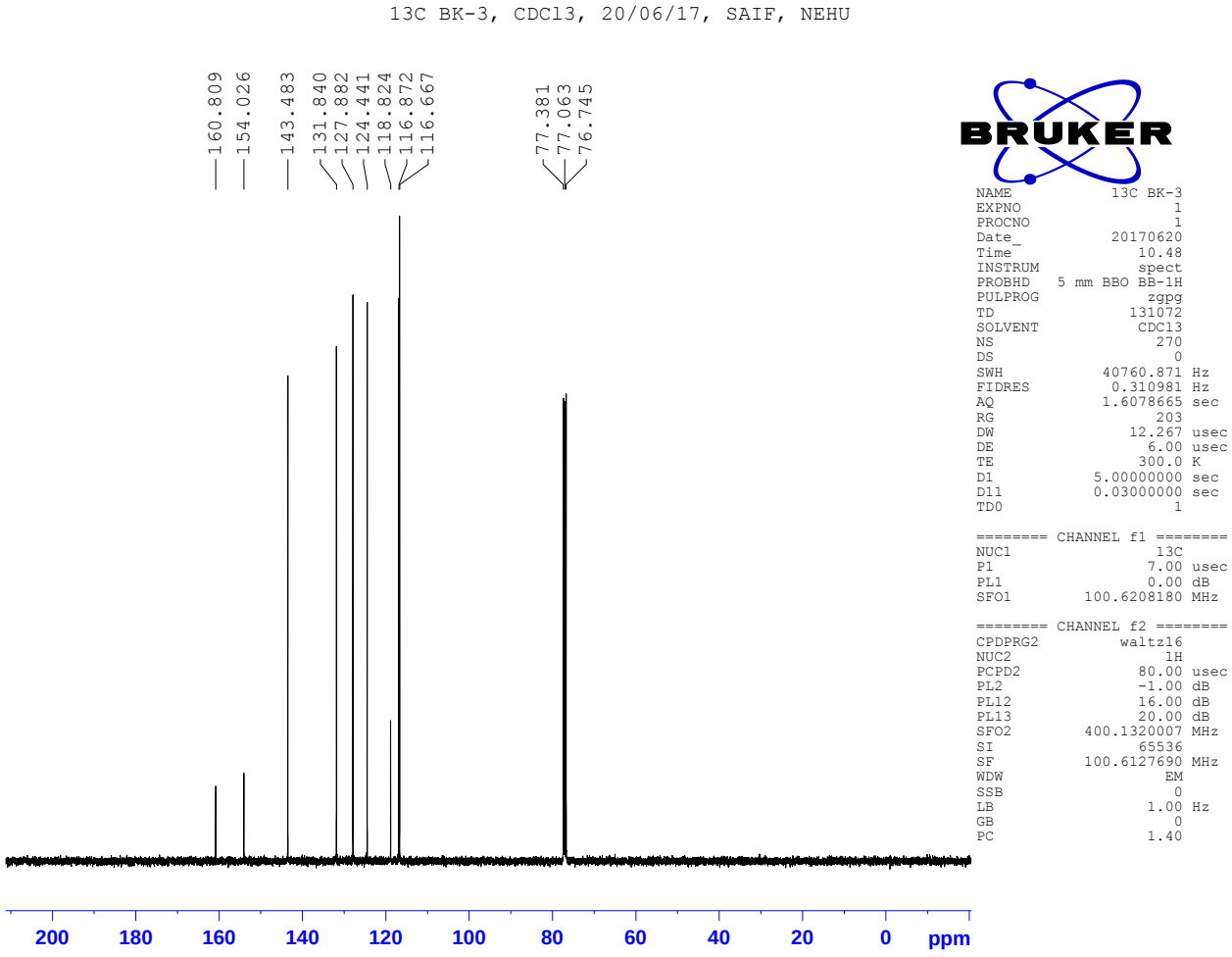
1H NMR spectrum of compound BK3

Annexure XIV



1H NMR spectrum of compound BK3

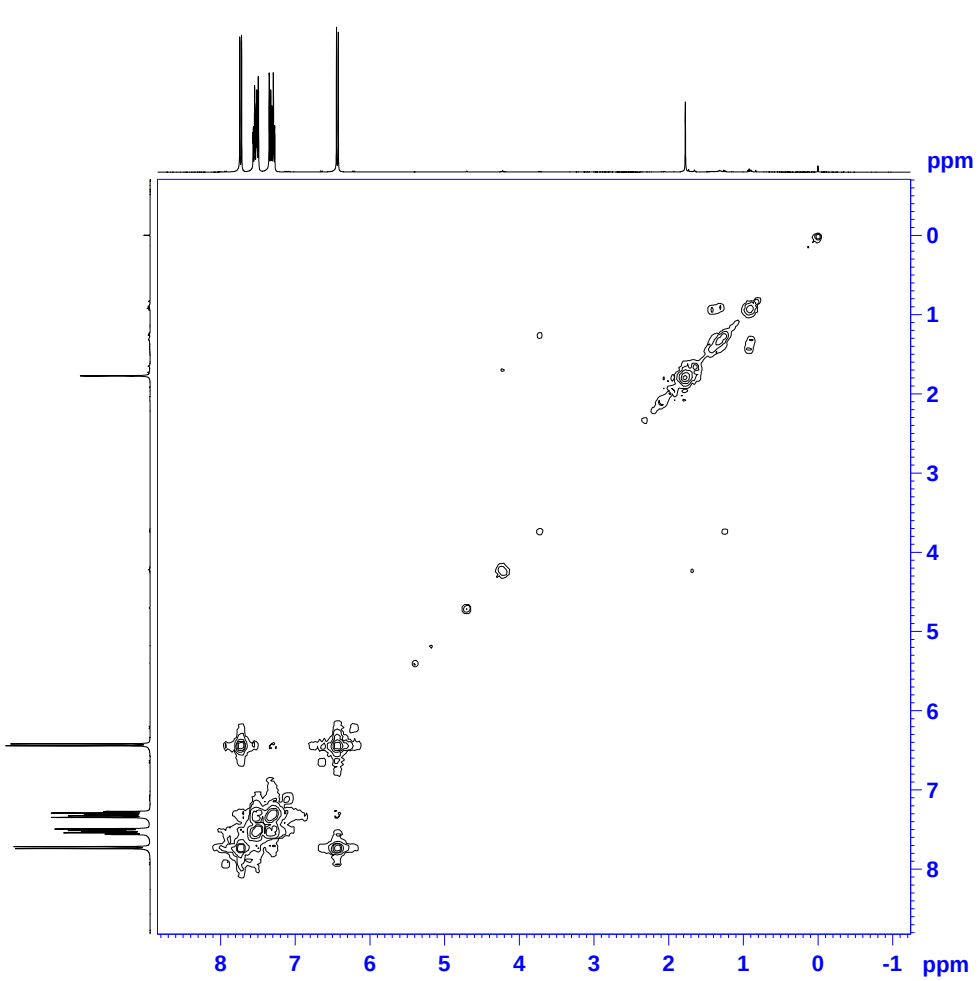
Annexure XV



13C NMR spectrum of compound BK3

Annexure XVI

COSY, BK-3, CDCl3, 20/06/17, SAIF, NEHU



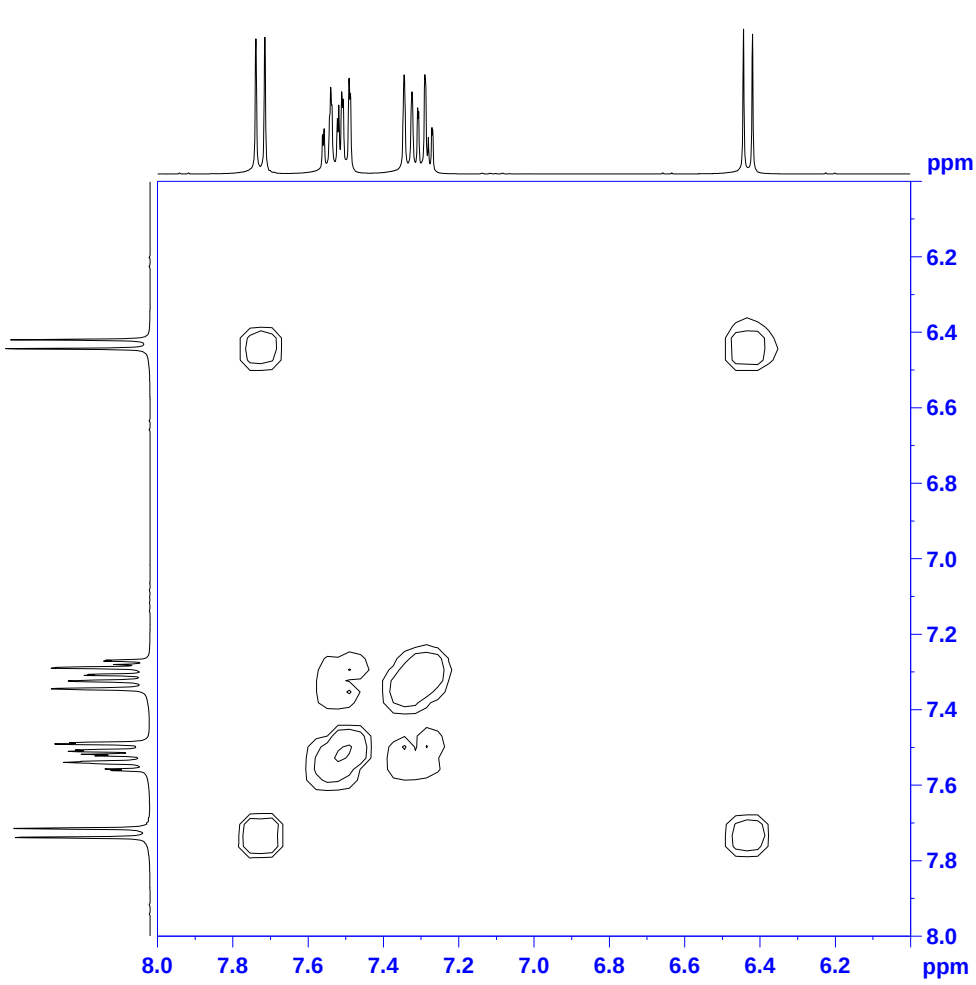
BRUKER

NAME	13C BK-3
EXPNO	3
PROCNO	1
Date_	20170620
Time	11.15
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	cosygpqf
TD	2048
SOLVENT	CDCl3
NS	4
DS	8
SWH	12019.230 Hz
FIDRES	5.868765 Hz
AQ	0.0852468 sec
RG	64
DW	41.600 usec
DE	6.50 usec
TE	300.0 K
D0	0.00000300 sec
D1	1.48689198 sec
D13	0.00000400 sec
D16	0.00020000 sec
IN0	0.00008330 sec
======== CHANNEL f1 ========	
NUC1	1H
P0	11.50 usec
P1	11.50 usec
PL1	-1.00 dB
SFO1	400.1324057 MHz
====== GRADIENT CHANNEL =====	
GPNAM1	SINE.100
GPZ1	10.00 %
P16	1000.00 usec
ND0	1
TD	256
SFO1	400.1324 MHz
FIDRES	46.890518 Hz
SW	30.000 ppm
FnMODE	QF
SI	1024
SF	400.1299898 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.40
SI	1024
MC2	QF
SF	400.1299856 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

COSY of compound BK1

Annexure XVII

COSY, BK-3, CDCl3, 20/06/17, SAIF, NEHU




```
NAME                 13C BK-3
EXPNO                       3
PROCNO                      1
Date_             20170620
Time                   11.15
INSTRUM                spect
PROBHD    5 mm BBO BB-1H
PULPROG             cosygpqf
TD                       2048
SOLVENT                 CDCl3
NS                          4
DS                          8
SWH                12019.230 Hz
FIDRES              5.868765 Hz
AQ                  0.0852468 sec
RG                         64
DW                    41.600 usec
DE                     6.50 usec
TE                     300.0 K
D0                 0.00000300 sec
D1                 1.48689198 sec
D13                0.00000400 sec
D16                0.00020000 sec
IN0                0.00008330 sec

======== CHANNEL f1 ========
NUC1                       1H
P0                     11.50 usec
P1                     11.50 usec
PL1                    -1.00 dB
SFO1           400.1324057 MHz

====== GRADIENT CHANNEL =====
GPNAM1              SINE.100
GPZ1                   10.00 %
P16                  1000.00 usec
ND0                         1
TD                        256
SFO1                400.1324 MHz
FIDRES             46.890518 Hz
SW                    30.000 ppm
FnMODE                     QF
SI                       1024
SF                400.1299898 MHz
WDW                      SINE
SSB                         0
LB                     0.00 Hz
GB                          0
PC                     1.40
SI                       1024
MC2                        QF
SF                400.1299856 MHz
WDW                      SINE
SSB                         0
LB                     0.00 Hz
GB                          0
```

COSY of compound BK3

List of Publications

1. **Kh. Nongalleima, T. Ajungla, C.B. Singh**. Antioxidant, anti-inflammatory and cytotoxic activity of *Citrus macrop*tera Montruz extracts. International Journal of Pharmtech Research,2017, 10 (3): 2455-9563
2. **Kh. Nongalleima, T. Ajungla, C.B. Singh.** Determination of antioxidant activity and simultaneous RP-HPLC analysis of quercetin, rutin and Kaempferol in *Citrus macroptera* Montruz. Journal of Pharmacognosy and Phytochemistry, 2017, 6 (3): 474-478
3. **Kh. Nongalleima, T. Ajungla, C.B. Singh.** Phytochemical, total phenolic, total flavonoid and total flavonol content estimation in *Citrus macroptera* Montruz.Journal of medicinal plant studies, 2017, 5 (3): 114-118
4. **Kh. Nongalleim, C.B. Singh, T. Ajungla.** GCMS based metabolic profiling of essential oil of *Citrus macroptera* Montruz. leaves and peel, assessment of in vitro antioxidant and anti-inflammatory activity. International Journal of Pharmacy and pharmaceutical sciences, 9(9): 107-114

Paper presentation (abstract published) in Conference/ Symposium

1. nternational conference on " Global Biodiversity, climate change and Sustainable Development- 2016", 15th- 18th October, 2016, at Rajiv Gandhi University, Rono Hills, Doimukh, Arunachal Pradesh

2. International Symposium on Plant Biotechnology for Crop Improvement (ISPBCI-2017) from 20-21 January, 2017 in Indian Institute of Technology Guwahati.

Participation in workshop/ training

1. Hands on training on "Molecular profiling and genome analysis", 14-19 March, 2016 at Nagaland University.

2. Workshop on "Computational Modelling Techniques in Structural Biology", 2-3 August, 2017 at Indian Institute of Science, (IISc), Bangalore