EFFECTS OF MEDICINAL PLANT *SANGUISORBA MINOR* SUBSP. *BALEARICA* ON SELECTED CYP450 ISOZYMES AND PROINFLAMMATORY CYTOKINES ACTIVITY IN INFLAMMATION INDUCED RAT MODEL: AN IMMUNOCHEMICAL APPROACH

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ABSTRACT

EFFECTS OF MEDICINAL PLANT *SANGUISORBA MINOR* SUBSP. *BALEARICA* ON SELECTED CYP450 ISOZYMES AND PROINFLAMMATORY CYTOKINES ACTIVITY IN INFLAMMATION INDUCED RAT MODEL: AN IMMUNOCHEMICAL APPROACH

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Medicinal uses of plant extracts have been based on traditional knowledge without enough scientific evidence. *Sanguisorba minor* subsp. *balearica* was used traditionally to treat various diseases. This study was performed to evaluate the anti-inflammatory effect of *Smb*. Experimental groups are; healthy control, CFA control, diclofenac sodium, quercetin, and 34-70mg/kg *Smb* treated groups. Anti-inflammatory effect was examined on Complete Freund's Adjuvant model, using biochemical, immunohistopathological, immunohistochemical, and immunoblotting methods on the CYP1A2, CYP3A4, CYP2C9, CYP2E1 enzymes as well as TNF-α, IL-1 beta, and IL-12. CFA group shows an increase in both TNF-α and IL-1beta. The remaining groups show a decrease in both cytokines. CFA group exhibited lots of inflammatory signs, diclofenac sodium has a recovery effect on inflammation. 34 and 70 mg/kg *Smb* and quercetin ameliorated the inflammation. CFA group has the highest TNF-α and IL-12. 34-70mg/kg *Smb* and diclofenac sodium groups have

lower TNF-α and IL-12. Quercetin group has the lowest TNF-α and IL-12. In all treated groups, CYP1A2 levels were lower than the healthy controls. CYP2C9 levels in the positive control and 34-70mg/kg *Smb* groups were higher than the healthy controls. CYP2C9 levels in negative control and quercetin groups were lower levels. CYP3A4 and CYP2E1 levels in all treated groups were higher than in the healthy controls.

There isn't enough data to clarify the possible effects of *Smb* on CYP450s and their relation with the anti-inflammatory response. This current study aims to clarify the possible anti-inflammatory effects of *Smb* on proinflammatory cytokines and on targeted CYP450 on the CFA model.

Keywords: *Sanguisorba minor* subsp. *balearica,* CYP450, Cytokines, Inflammation, Complete Freund's Adjuvant model

ÖZ

TIBBİ BİTKİ *SANGUISORBA MINOR* SUBSP. *BALEARICA*'NIN ENFLAMASYON OLUŞTURULMUŞ RAT MODELİNDE SEÇİLMİŞ CYP450 IZOZİMLERİ VE PROENFLAMATUVAR SİTOKİN AKTİVİTELERİ ÜZERİNE OLAN ETKİLERİ: İMMUNOKİMYASAL YAKLAŞIM

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Bitki özlerinin tıbbi kullanımları, yeterli bilimsel kanıt olmadan geleneksel bilgilere dayanmaktadır. *Sanguisorba minor* subsp. *balearica*, geleneksel olarak çeşitli hastalıkların tedavisinde kullanılmıştır. Bu çalışma, *Smb*'nin anti-inflamatuar etkinliğini değerlendirmek amacıyla yapılmıştır. Complete Freund's Adjuvant rat modelinde; sağlıklı kontrol, CFA kontrol, diklofenak sodyum, kuersetin, 34 ve 70 mg/kg *Smb* uygulanmış gruplarda; CYP1A2, CYP3A4, CYP2C9, CYP2E1 enzimleri ve ayrıca TNF-α, IL-1 beta ve IL-12 sitokinleri üzerinde biyokimyasal, immünhistopatolojik, immünhistokimyasal ve immünoblotlama yöntemleri kullanılarak anti-inflamatuar etki incelenmiştir. CFA grubu, TNF-α ve IL-1beta seviyelerinde artış göstermiştir. Geri kalan tüm gruplarda TNF-α ve IL-1 beta seviyeleri düşüktür. CFA grubu pek çok inflamatuar belirti göstermiştir, diklofenak sodyum artritik belirtiler üzerinde bir iyileştirme etkisine sahiptir. 34, 70 mg/kg *Smb* ve kuersetin uygulanması ise artritin şiddetini iyileştirmiştir. CFA grubunda, yüksek TNF-α ve IL-12 seviyeleri görülmüştür. Geri kalan tüm gruplarda TNF-α ve IL-12 düzeyleri azalmıştır. Kuversetin uygulanan grup en düşük TNF-α ve IL-12'ye

sahiptir. Western blot analizlerine göre tüm gruplarda CYP1A2 seviyeleri, sağlıklı kontrol grubundan daha düşüktür. Uygulama yapılan tüm gruplarda CYP3A4 seviyeleri, sağlıklı kontrol grubundan daha yüksektir. Pozitif kontrol, 34 ve 70 mg/kg *Smb* uygulanan gruplarda CYP2C9 seviyelerinin, sağlıklı kontrol grubundan daha yüksek olduğu belirlenmiştir. CYP2C9, negatif kontrol ve kuersetin gruplarında azalmış, 34 ve 70 mg/kg *Smb* gruplarındaysa artmıştır. Uygulama yapılan tüm gruplarda CYP2E1 seviyeleri sağlıklı kontrol grubundan daha yüksek bulunmuştur.

Literatürde *Smb*'nin CYP450'ler üzerindeki olası etkilerini ve anti-inflamatuar yanıtla ilişkisini netleştirecek yeterli veri bulunmamaktadır. Bu çalışma, *Smb*'nin olası anti-inflamatuar etkisini CFA modelinde; proinflamatuar sitokin aktivitelerini ve hedef CYP450 ekspresyonunu modüle eden mekanizmalar üzerinden netleştirmeyi amaçlamaktadır.

Anahtar Kelimeler: *Sanguisorba minor* subsp. *balearica*, CYP450, Sitokinler, Enflamasyon, Complete Freund's Adjuvant model

To my Family,

For their endless love and support

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ANOVA	Analysis of variance
APS	Ammonium per sulfate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
COX	Cyclooxygenase
CYP450	Cytochrome P450 enzymes
EET	Epoxyeicosatrienoic acids
ELISA	Enzyme-Linked immunosorbent assay
GST	Glutathione S-Transferase
H&E	Haemotoxylin-Eosin
HETE	Hydroxyeicosatetraenoic acid
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IPNI	International plant naming index
JAK	Janus kinase
HPLC	Liquid chromatography mass spectrometry
LOX	Lipoxygenase
LPS	Lipopolisaccharide

MAPK	Mitogen-activated protein kinase
MAPKK	MAPKinase
MAPKKK	MAPKinase kinase
NF-kB	Nuclear factor kappa-B
NK	Natural Killer
NSAID	Nonsteroidal anti-inflammatory drug
o.p.	Orally per
PLA_2	Phospholipase A_2
PUFA	Polyunsaturated fatty acid
SDS-PAGE	Sodium dedocyle sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Smb	*Sanguisorba minor* subsp. *Balearica*
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptors
TNF- alpha	Tumour necrosis factor-alpha

CHAPTER 1

INTRODUCTION

1.1 Introduction

The relationship between humans and plants has been going on since the existence of humankind. Ethnobotanical studies have shown that these relationships take place both for agricultural and medicinal purposes and have continued throughout history. According to the International Plant Naming Index (IPNI), it was estimated that there are approximately 400.000 species of vascular plants worldwide (IPNI, 2020). Around 2.000 new plant species are registered by the IPNI index each year.

The usage areas of 31.000 plant species with economic value are examined, and it is obtained that; 17.810 plant species are used for medical purposes, 11.365 plant species are used in the textile industry, 8.140 plant species are used for environmental purposes, and 5.538 plant species are used in the food industry ("Royal Botanic Gardens, Kew | Kew," n.d.). As seen, the most intensive usage area of these plant species is for "medical purposes."

Turkey has a substantial flora due to its geographical and climatic conditions. International Indexes have confirmed this potential also. It was reported that Turkey is included in the worldwide "Important Vegetative Areas" category (Anderson, 2002). Approximately 12.000 taxa have been defined in the flora of Turkey so far. Considering that the entire European continent has about 12.000 plant taxa, this shows how rich our country is in terms of plant diversity.

According to the IPA records, 8.897 native plant species were identified in Turkey, and 3.022 are classified as endemic species. The number of medicinal plants in Turkey has been reported to be around 500. This number represents only the registered ones, and it is known that the number of existing medicinal plants is much higher than this (Byfield, 2010).

Medicinal plants with their therapeutic properties have been used worldwide for centuries. Phytochemical compounds are responsible for the therapeutic effects of medical plants. Phytochemicals or secondary metabolites produced by plants are classified into different groups. Polyphenols, flavonoids, alkaloids, and terpenoids are the main ones.

To understand their effectiveness and safety potential, medicinal plants should be investigated pharmacologically. Their phytochemical contents and the concentrations of these phytochemicals must be analyzed firstly. Then, the efficacy and safety profiles of the phytochemicals should be clarified through long-term experimental and clinical studies. Finally, those who successfully passed these stages will be able to achieve pharmaceutical quality standards.

Phytochemicals, therapeutical agents, and other foreign substances entering the human body must undergo "xenobiotic metabolism" to show their pharmacological, toxicological, or biological effects (S. Zhou et al., 2003). Cytochrome P450 enzymes (CYP450) are an essential xenobiotic-metabolizing enzyme system, and they play crucial roles in the metabolism of thousands of endogenous and exogenous chemical compounds in biological systems. More importantly, this enzyme system has an essential role in the proper functioning of cellular metabolism and homeostasis (Hasler et al., 1999).

Cytochrome P450 enzyme system is found in all living organisms, from bacteria to mammals. It is attached to the cell membrane in different tissues such as

the kidney, lung, skin, intestine, adrenal cortex, testis, and placenta, but especially liver tissue contains intense CYP450 enzymes. Microsomes and mitochondria are the regions where CYP450 enzymes are localized in the liver (Nebert and Gonzalez 1987).

Mammalian CYP450s play a role in the metabolism of xenobiotics such as drugs, carcinogens, organic solvents, pesticides, alcohols, and environmental pollutants. A xenobiotic molecule that enters the CYP450 enzyme system is subjected to various oxidation, reduction, and hydrolysis reactions, which eventually turns into a water-soluble (hydrophilic) form and is easily excreted metabolism. This whole process is called "Phase I Xenobiotic Metabolism" (Meunier, de Visser, & Shaik, 2004).

Human CYP450s also play essential roles in the overall metabolism and regulation of endogen molecules, including; steroid hormones, vitamin D, bilirubin, cholesterol and fatty acids (Nebbia, Dacasto, & Carletti, 2004).

Intermediate compounds formed by the CYP450 enzyme system may have toxic, carcinogenic or mutagenic characteristics. Therefore, these intermediate compounds should be subjected to a second reaction pathway called "Phase II Xenobiotic Metabolism." Intermediate molecules entered the Phase II metabolism undergo conjugation reactions through conjugation enzymes such as Glutathione S-Transferase (GST), glucuronosyl transferases (UGTs) so that they can be transformed into more harmless forms and excreted from the body (Oleksiak et al., 2003; Zanger & Schwab, 2013).

CYP450 enzymes are also involved in many pathophysiological processes of the diseases, given the emerging importance of these enzyme systems, both physiological and pathophysiological aspects. Stimulation of CYP450 enzyme activity occurs at the gene transcription level. Many factors affect the expression

levels and function of CYP450s, such as genetic polymorphism, xenobiotic stimuli, regulation through cytokines, and particular disease states (Denisov, Makris, Sligar, & Schlichting, 2005; Walsky & Obach, 2004).

The data obtained from the number of studies show that Cytochrome P450 enzymes play a crucial role in the regulation of inflammation through the biosynthesis of endogenous bioactive lipid mediators, such as the arachidonic acid (AA) derivatives and epoxyeicosatrienoic acids (EETs) (Shahabi, Siest, Meyer, & Visvikis-Siest, 2014; Theken et al., 2011).

Inflammation and inflammation-related disorders are an area of interest and studied extensively. Inflammation and inflammatory processes are usually defense mechanisms of the body, but it is considered as a pathological condition when the inflammation takes a long time and becomes chronic (Wellen & Hotamisligil, 2005; Y. Zhang et al., 2016). It is a known fact today that chronic inflammation plays a role in the pathogenesis of many diseases such as cancer, diabetes, obesity, and cardiovascular diseases (Chiurchiù & MacCarrone, 2011; Frey, Hehlgans, Rödel, & Gaipl, 2015). The treatment costs of these diseases have reached a very high level worldwide. A number of studies have proved these destructive effects of chronic inflammation in biological systems, and therapeutic approaches to these pathological processes have been intensively studied and investigated (Al-Harbi et al., 2016).

Anti-inflammatory effects of medicinal plant *Sanguisorba minor* subsp. *balearica* has been investigated in a number of studies. It is thought that this anti-inflammatory effect is mainly due to its flavonoids content (Arıhan, Özbek, & Özkan, 2015; J. feng Chen et al., 2020; Shin, Lee, & Kim, 2002; Yang, Hwang, Gu, Cho, & Ma, 2015; Yu et al., 2011). Non-steroidal Anti-inflammatory drugs are the main therapeutical agents used in the treatment of inflammation. Still, there is a lot of information in the literature about the side effects of these drugs on the

gastrointestinal and cardiovascular systems (Scarpignato et al., 2015; Vonkeman & van de Laar, 2010). These side effects have reached such a severe extent that some of these drugs are withdrawn from the market (Blumenthal et al., 2017; Tabas Ira & Christopher, 2013).

Therefore, safe therapeutical agents with low side effects are needed in this area, which means "High Bioavailability" and "Low Toxicity." Actually, bioavailability and toxicity are the two most significant barriers in drug development research. Targeted therapeutical molecules should be evaluated firstly from these two aspects. At this point, the CYP450 enzyme system is the main targeted system for toxicity and bioavailability studies.

1.2 Taxonomy of *Sanguisorba minor* subsp. *balearica*

Division: Tracheophyta – vascular plants, tracheophytes

Subdivision: Spermatophytina – spermatophytes

Class: Magnoliopsida

Order: Rosales

Family: Rosaceae

Genus: *Sanguisorba* L.

Species: *Sanguisorba minor* Scop.

Subspecies: *Sanguisorba minor* subsp. *balearica*

Common names: Small burnet, Salad burnet (Europe, North America) Küçük çayır düğmesi, yanık otu, kelebek ayağı, mide otu (Turkey)

Sanguisorba minor subsp. *balearica* is a flowering plant belonging to the Rosaceae family Sanguisorba genus and consists of approximately 27 species and 4 subspecies. *Sanguisorba minor* is an edible, perennial herb with pinnate leaves and reddish-green flowers. It is an herbaceous plant growing to 40–90 cm tall. Sanguisorba plants originated from seeds. Seeds of Sanguisorba spp. germinated at a constant temperature of 24 to 25° C, followed by six months of dry storage at 4 ° C (KARKANIS et al., 2014). It is drought-tolerant, typically found in dry grassy meadows. It is widely distributed throughout Europe, West Asia, and North America. In Turkey, its distribution is extensive. It is known as "small burnet" or "salad burnet" in Europe and North America, and in Turkey it is known by many different names which are "küçük çayır düğmesi", "yanık otu", "kelebek ayağı" and "mide otu" (Baytop, 1994; Baytop,1999). A representative morphological image of the *Sanguisorba minor* subsp. *balearica* is given in Figure 1.1.

Figure 1.1 Morphology of *Sanguisorba minor* subsp. *balearica* (*Gençler Ozkan A.M., July 2006)*.

1.3 Therapeutic Properties of *Sanguisorba minor* subsp. *balearica*

Sanguisorba officinalis L. (great burnet) and Sansguisorba minor Scop. (small burnet) are the most widespread and well-known Sanguisorba species in the literature. Medicinal properties of Sanguisorba species have been known since the 16th century, and they have been used in folk medicine since then. *Sanguisorba minor*, known as a medicinal plant, is traditionally used in the treatment of diseases such as diarrhea, hemorrhoids, goiter in Anatolia. It has been determined that the therapeutic properties of *Sanguisorba minor* plants are originated from its high phytochemical content (Bedoya, Sanchez-Palomino, Abad, Bermejo, & Alcami, 2001; Ferreira, Proença, Serralheiro, & Araújo, 2006; Gürbüz, Özkan, Yesilada, & Kutsal, 2005; Ranfa, Maurizi, Romano, & Bodesmo, 2014).

In the literature, *Sanguisorba minor* plant is mentioned as a "Promising Medicinal Plant" due to its high bioactive content (Guarrera & Savo, 2013). More than 120 phytochemical compounds belonging to Sanguisorba genus plants, especially *S. officinalis* and *S. minor* have been identified, and the major phytochemical compounds in plant extract of the Sanguisorba genus have been described by different researchers as given in Table 1.1 (Ayoub, 2003; Cuccioloni et al., 2012; Hu, Shi, Chen, & Li, 2012; Liu, Cui, Yu, & Yu, 2005). These identified phytochemicals are grouped as phenolics, flavonoids, neolignans, and terpenoids.

Table 1.1 Bioactive Compounds Isolated from *Sanguisorba* Plants

Compound	Reference	Compound	Reference
Sanguidioside A	Liu et al. (2005)	Gallic acid	Ayoub (2003)
Sanguidioside B	Liu et al. (2005)	Ellagic acid	Ayoub (2003)
Sanguidioside C	Liu et al. (2005)	quercetin-3-O-(600 galloylglucose)	Ayoub (2003)
Sanguidioside D	Liu et al. (2005)	b-glucogallin	Ayoub (2003)
Quercetin	Ayoub (2003)	quercetin-3-glucuronide	Cuccioloni (2012)
2,3-hexahydroxydiphenol -(a/b)-glucose	Ayoub (2003)	1-gallory-2,3-hexahydroxydroxyediphenol-a-glucose	Ayoub (2003)
2-(4-carboxy-3 methoxystyryl)-2 methoxysuccinic acid	Ayoub (2003)	4,8-dimethoxy-7 hydroxy-2-oxo-2H-1-benzopyran-5,6-dicarboxylic acid	Ayoub (2003)
(7S,8R)-4,9,5',9'-tetrahydroxy-3,3'-dimethoxy-8-O-4'neolignan-7-O-α-1-rhamnopyranoside	Hu et al. (2012)	3b[(a-L-arabinopyranosyl)oxy]-19b-hydroxyurs-12,20(30)-dien-28-oic acid	Liu et al.(2004)
(7S,8R)-4,7,9,9'-tetrahydroxy-3,3'-dimethoxy-8-O-4'-neolignan	Hu et al. (2012)	(7S,8R)-4,9,9'-thrihydroxy-3,3',5'-trimethoxy-8-O-4'-neolignan-7-O-α-1-rhamnopyranoside	Hu et al.(2012)

The pharmacological effects of *Sanguisorba* plants are supported by numerous in vivo and in vitro studies. Some of their therapeutic effects are anti-viral, anti-ulcerogenic, anti-cancer, anti-acetylcholinesterase, radioprotective, antiallergic, and immunomodulatory (Cai et al., 2012; Ferreira et al., 2006; Ranfa et al., 2014; Ravipati et al., 2012; Shin et al., 2002; Yu et al., 2011).

It was shown that in a study, methanolic extract of *Sanguisorba officinalis* showed inhibitory effects on mouse leukemia L1210 cells. This result is a possible sign of its anticancer properties (Goun et al., 2002). In another study, the crude polysaccharide fractions of the Sanguisorba plants were evaluated on a mouse monocyte-macrophage cell line. It was found that fractions have stimulated nitric oxide and tumor necrosis factor production (TNF- alpha), supporting its possible antioxidant activity (L. Zhang et al., 2012). All these results can be interpreted into possible anticarcinogenic effects of Sanguisorba plants.

According to a case study, flowering parts of *Sanguisorba officinalis L*. have been investigated about their protective effects on proteins and lipids of human plasma against γradiation-induced oxidative damage. It was found that radiation-induced oxidation of protein thiols and lipid peroxidation were inhibited after *Sanguisorba officinalis L.* extract treatment (Zbikowska et al., 2016).

It was asserted from two independent studies that Sanguisorba plants have antiviral and antimicrobial effects also. Its inhibitory effects against Human Immuno Deficiency Virus Type-I (HIV-1) and Herpes Simplex Virus Type-1 (HSV-1) were demonstrated by different groups (Abad, Guerra, Bermejo, Irurzun, & Carrasco, 2000; Bedoya et al., 2001).

Another significant therapeutic effect of Sanguisorba plants is anti-inflammatory effects. Recently, the anti-inflammatory effects of medicinal plants begin to gain interest from different research groups. Anti-inflammatory effects on peritoneal macrophages (from the C57BL/mice, RAW 264.7 and HEK293 cells lines) was examined and it was asserted that the ethanolic extract of *Sanguisorba officinalis* has a potent anti-inflammatory activity on these targeted cell lines,

mediated by its NF-ƙB, and AP-1 inhibitory properties (Yu et al., 2011). Its anti-inflammatory effects on a skin disorder were also tested using ethanolic extract of Sanguisorba officinalis L. in human keratinocyte HaCaT cells. The results obtained revealed that the ethanolic extract of Sanguisorba officinalis L. exerts its anti-inflammatory effects by suppressing the expression of TNF-α/IFN-γ stimulated chemokines and pro-inflammatory molecules in humans keratinocyte HaCaT cell lines (Yang et al., 2015). In another study with *Sanguisorba minor* subsp. *balearica*, the anti-inflammatory effects of the aqueous extract were examined on an inflammation-induced rat model. Anti-inflammatory effects of the plant were observed to be in a dose-dependent manner on model animals (Arıhan et al., 2015).

It is hypothesized that the therapeutic effects of the Sanguisorba species, which have been studied extensively in the literature, were mainly due to their high polyphenolic content (Hachiya, Kobayashi, Ohuchi, Kitahara, & Takema, 2001; Liu et al., 2005; Shin et al., 2002). In addition, it has also been shown that there is a relationship between the flavonoid content of the plant and its anti-inflammatory effects (Y. H. Kim et al., 2008; Ravipati et al., 2012; Yu et al., 2011).

1.4 Inflammation

Inflammation and inflammatory processes are the defense mechanism of the body. Indeed inflammation is a biological response of the immune system. It can be stimulated by a variety of factors, including pathogens, toxic and allergic compounds, injury, and diseases. These factors induce acute and/or chronic inflammatory responses. The acute inflammatory response is a normal physiological process that maintains the immune defense and cellular homeostasis. On the other hand, chronic inflammation is an abnormal immune response, and it turns into a pathological state (Wellen & Hotamisligil, 2005; Y. Zhang et al., 2016).

During the acute inflammatory processes, all the local, vascular and cellular immune responses work collectively. As a result, the cellular and molecular

interactions initiate the healing process. Three important circulatory events of inflammation are; change in vascular permeability, leukocyte recruitment, and inflammatory mediator release. At the tissue level, tissue redness, tissue swelling, heat, pain, and loss of tissue function might occur.

The molecular mechanism of the inflammatory response is a complex and coordinately regulated process. It can be summarized by the following steps: Cell surface signaling receptors recognize the stimuli first, which activates the inflammatory pathways, and inflammatory mediators are released, followed by the recruiting of inflammatory cells around the injured area.

1.4.1 Intracellular Signaling Pathways of Inflammation

Inflammatory stimuli activate intracellular signaling pathways via a cascade mechanism, and it stimulates the production of inflammatory mediators. The primary inflammatory stimuli such as infectious agents or cytokines (IL-1beta, IL-6, TNF-alpha) stimulate inflammation through their interactions with the TLRs, IL-1 receptor, IL-6 receptor, and TNF-receptor. Receptor activation triggers critical intracellular signaling pathways, including "Mitogen-activated protein kinase (MAPK)," "Nuclear factor kappa-B (NF-kB) and "Janus kinase (JAK)-signal transducer" and "Activator of transcription (STAT)" pathways (Figure 1.2) (Turner, Nedjai, Hurst, & Pennington, 2014).

Activation of the pattern recognition receptors occurs through successive molecular events. Firstly, "Pathogen-associated molecular patterns (PAMPs)" trigger the inflammatory response through activation of "Germline-encoded pattern-recognition receptors (PRRs)." Toll-like receptors (TLRs) are a highly conserved group of mammalian PRRs and have important roles in the activation of the inflammatory response. Signaling through TLRs triggers an intracellular signaling cascade that leads to nuclear translocation of transcription factors such as activator protein-1 (AP-1) and NF-kB (L. Chen et al., 2018; Zbikowska et al., 2016).

NF-kB transcription factor plays an essential role in the inflammatory immune response. Induction of NF-kB activity is induced by infectious agents, intracellular inflammatory cytokines, and biologically active enzymes. NF-kB activity is inhibited by IKB proteins present in the cytoplasm; at this step, PRRs activate IKB kinase (IKK) by signal transduction mechanisms. Then, NF-kB pathway activation occurs through IKB phosphorylation. IKB phosphorylation leads to its degradation by the proteasome and subsequent release of NF-kB. Ultimately, this pathway regulates inflammatory response inducing pro-inflammatory cytokine production and inflammatory cell recruitment (Lawrence, 2009; Moynagh, 2005).

MAPKs are a family of serine/threonine protein kinases that regulate inflammatory response via inflammatory cytokine stimuli (IL-1beta, IL-6, TNF-alpha). Mammalian MAPKs are a complex family composed of extracellular-signal-regulated kinase ERK1/2, p38, MAPKinase, and c-Jun-N-terminal kinases (JNK). Each MAPK signaling pathway comprises three components: MAPK, MAPKinase (MAPKK), and MAPKinase kinase (MAPKKK). When inflammatory stimuli start, it triggers a sequential phosphorylation cascade between these three MAPK of JNK and p38 pathways. After sequential phosphorylation of these two pathways, activated MAPKs phosphorylate various proteins, which have an important role in inflammatory response (E. K. Kim & Choi, 2010; Turner et al., 2014).

JAK/STAT pathway is another signaling pathway that is activated by inflammatory stimuli. JAK/STAT pathway is responsible for the differentiation of cytokine molecules. After subsequent phosphorylation and dimerization steps, JAK/STAT transduces the extracellular signals into a transcriptional response. STAT proteins are translocated into the nucleus, bind to the target gene promoter regions to regulate the transcription of inflammatory genes (O'Shea J.J. et al. 2015).

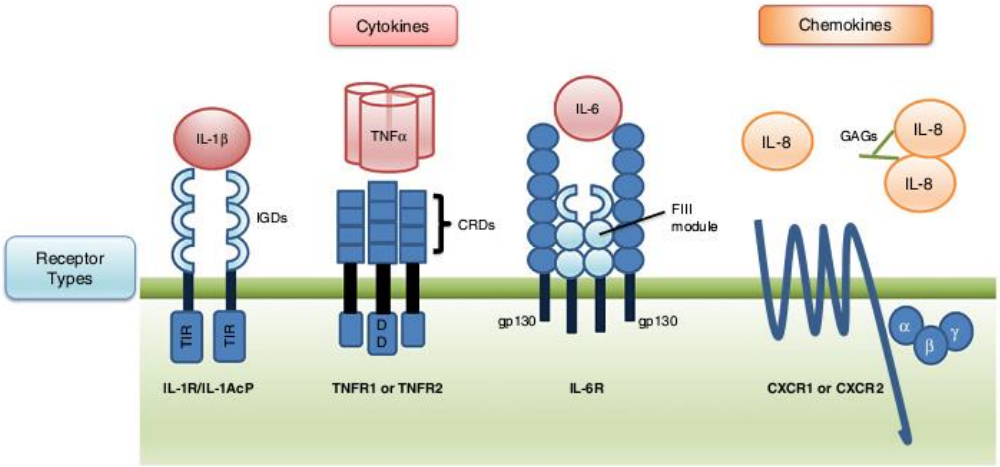


Figure 1.2. Cytokines chemokines and their receptors *(Turner et al. 2014).*

Dysregulation of NF-kB, MAPK, or JAK/STAT signaling pathways is related to abnormal conditions in the metabolism, such as chronic inflammation, autoimmune and/or metabolic diseases. In the normal biological state, signaling through transcription factors leads to the secretion of cytokines and numerous transcription factors through the regulation of inflammatory genes.

1.4.2 Inflammatory Response and Its Mediator Molecules

The inflammatory response is a properly working network controlled by the body's extracellular molecules and regulators. A variety of mediator molecules involved in this process includes cytokines, growth factors, eicosanoids, and prostaglandins. When inflammatory response is concerned, these extracellular events work collectively with the complex intracellular signaling pathways, as given in Figure 1.3 (Headland & Norling, 2015). Primary cells of the immune system with specific roles of the inflammatory response are; monocytes, macrophages, neutrophils, basophils, dendritic cells, mast cells, T-cells, and B-cells.

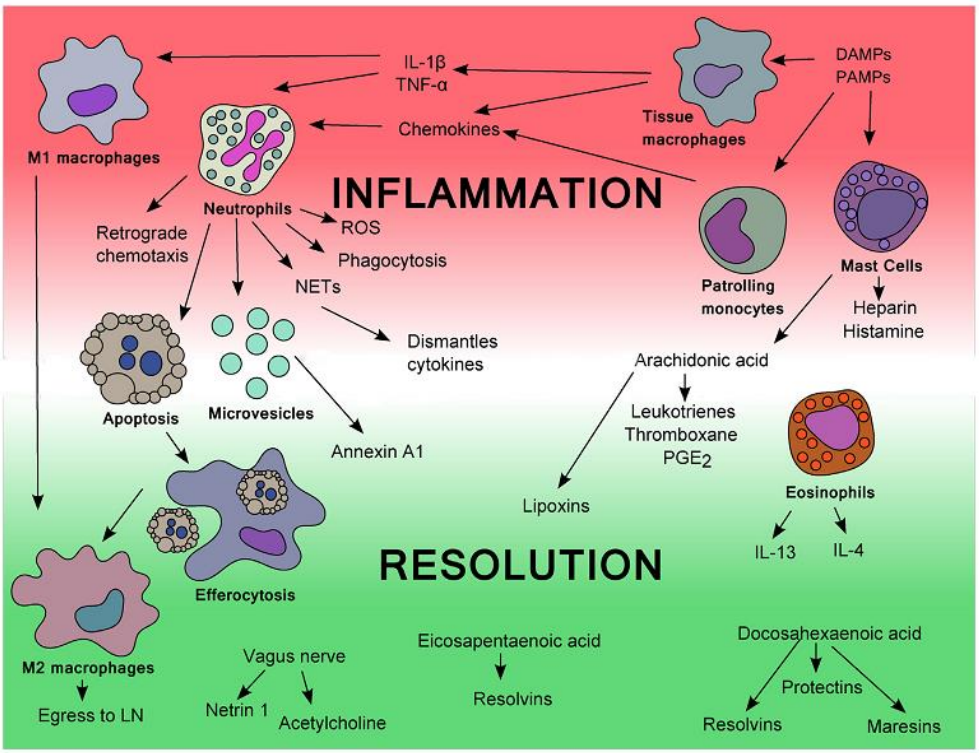


Figure 1.3. Essential cellular components and mediators of inflammation *(Headland & Norking, 2015).*

In case of tissue injury or infection, epithelial and endothelial cells around these target areas release factors that immediately induce the inflammatory cascade and immune response immediately. The first immune cells that arrived at the target injured site are neutrophils, followed by monocytes, lymphocytes (Natural Killer cells [NK-cells]), T-cells, B-cells, and mast cells. A representative illustration of the time course of the inflammatory response was given in Figure 1.4 (Souza-Moreira, Campos-Salinas, Caro, & Gonzalez-Rey, 2011). Inflammation-mediated immune cells can differentiate into specific types of inflammatory cells. Indeed, inflammation-mediated immune cell alterations are associated with the stage of inflammation, affected tissue area, and the nature of the immune response (Libby, 2007; J.-M. Zhang & An, 2009).

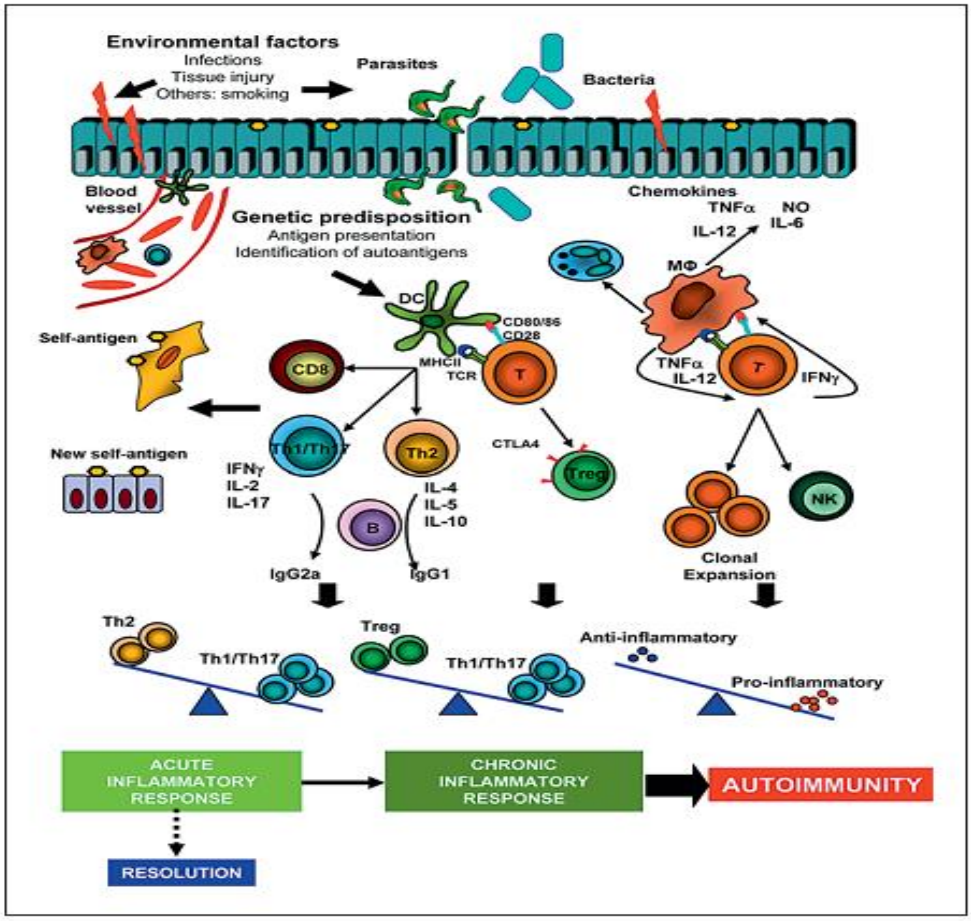


Figure 1.4. Time course of the inflammatory response *(Moreira et al.2011).*

Cytokines are key inflammatory signaling molecules and have important roles in the inflammatory response. Cytokines are originated into lymphocytes, monocytes, leukocytes, and chemotactic activities. They can be classified according to their originated immune cells. In terms of the inflammatory response, they can be classified into two groups: pro-inflammatory cytokines and anti-inflammatory cytokines. Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the upregulation of the inflammation. The main pro-inflammatory cytokines are; interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α).

IL-1 family includes 11 members; IL-1α, IL-1β, IL-1 receptor antagonist [IL-1Ra], IL-18, IL-33, IL-36α, IL-36β, IL-36ɣ, IL-36Ra, IL-37, and IL-1Hy2. IL-1 family is primarily expressed by monocytes and macrophages as well as by fibroblasts and endothelial cells. Some IL-1 family members also have anti-inflammatory effects. Among them, IL-1β is a potent pro-inflammatory cytokine.

IL-6 family includes the members IL-6 themselves and IL-11. IL-6 is expressed by mononuclear phagocytes, T-cells, B-cells, fibroblasts, endothelial cells, and hepatocytes. It has a vital role in the final maturation of B-cells, T-cell activation, and differentiation and regulation of Th2 cells.

TNF-α is the only member of the TNF superfamily. It is one of the most crucial cytokines. It is a potent inflammatory mediator, regulating the inflammatory response of the innate immune system. TNF-α stimulates cytokine production and activation as well as expression of adhesion molecules. It is primarily secreted from activated macrophages and also from by monocytes, T-cells, mast cells, NK-cells, and fibroblasts (L. Chen et al., 2018; Headland & Norling, 2015; Jabbour, Sales, Catalano, & Norman, 2009; Nathan & Ding, 2010; Wojdasiewicz, Poniatowski, & Szukiewicz, 2014; J.-M. Zhang & An, 2009). Anti-inflammatory cytokines are a group of cytokines that control the proinflammatory cytokine response and help the resolution of inflammation. Major anti-inflammatory cytokines include interleukin-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13. Summary of selected cytokines molecules and their specific function were given in Table 1.2.

Table 1.2 Summary of Cytokines and Their Functions

Cytokine	Family	Main Sources	Function
IL-1β	IL-1	Macrophages, monocytes	Pro-inflammation, proliferation, apoptosis, differentiation
IL-4	IL-4	Th-cells	Anti-inflammation, T-cell and B-cell proliferation, B cell differentiation
IL-6	IL-6	Macrophages, T-cells, adipocyte	Pro-inflammation, differentiation, cytokine production
IL-8	CXC	Macrophages, epithelial cells, endothelial cells	Pro-inflammation, chemotaxis, angiogenesis
IL-10	IL-10	Monocytes, T-cells, B-cells	Anti-inflammation, inhibition of the pro-inflammatory cytokines
IL-12	IL-12	Dendritic cells, macrophages, neutrophils	Pro-inflammation, cell differentiation, activates NK cells
IL-11	IL-6	Fibroblasts, neurons, epithelial cells	Anti-inflammation differentiation induces acute-phase protein
TNF-α	TNF	Macrophages, NK cells, $CD4^+$, lymphocytes, adipocyte	Pro-inflammation, cytokine production, cell proliferation, apoptosis, anti-infection
INF-γ	INF	T-cells, NK cells, NKT cells	Pro-inflammation, innate, adaptive immunity anti-viral
GM-CSF	IL-4	T-cells, macrophages, fibroblasts	Pro-inflammation, macrophage activation, increase neutrophil and monocyte function
TGF-β	TGF	Macrophages, T cells	Anti-inflammation, inhibition of the pro-inflammatory cytokines

1.5 Cytochrome P450 Isozymes

Cytochrome P450 enzymes (CYP450s) are intracellular heme proteins belonging to the ubiquitous superfamily. This enzyme system is found in animals, plants, and fungi to bacteria. In eukaryotic cells, cytochrome P450s exist as membrane-bound heme proteins, with the iron-protoporphyrin IX prosthetic group (Figure 1.5). They are found on the cell membrane of different tissues such as kidney, lung, skin, intestine, adrenal cortex, testis, placenta, but especially liver tissue contains intense amounts of CYP450 enzymes.

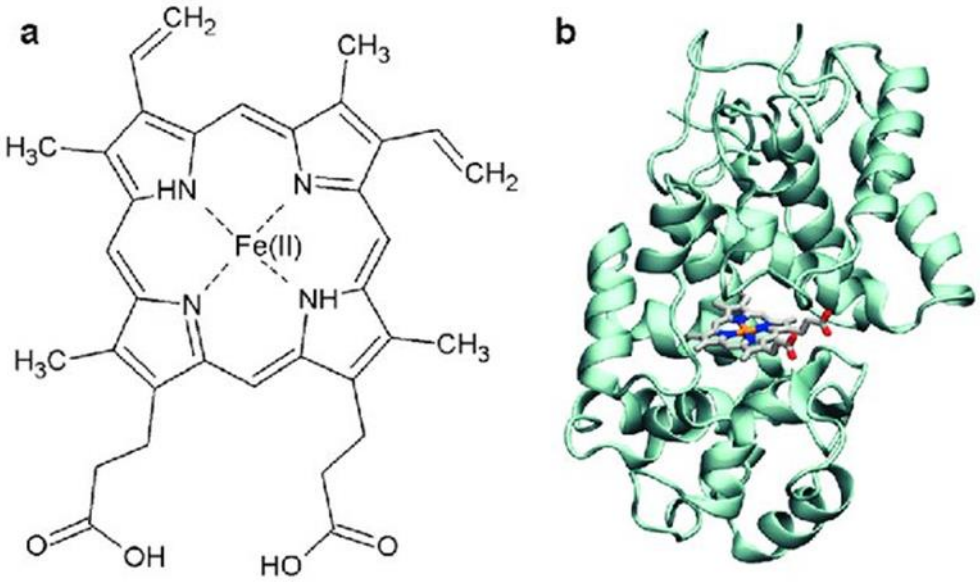


Figure 1.5. (a) Structure of iron protoporphyrin IX (b) Protein structure with iron protoporphyrin IX cofactor side highlighted.

Cytochrome P450 enzymes were firstly described by Martin Klingenberg, who was investigating the spectrophotometric characteristics of pigments in rat microsomal fractions. The name P450 is derived from this invention which refers to

the characteristic signature of this pigment with an absorbance band maximum at 450 nm. (Klingenberg 1958).

After his discovery, Omura and Sato characterized these “spectrometrically characteristics pigments” as “hemeproteins” (1962). Cooper and his coworkers described the location and biological roles of these “Hemeprotein.” They announced that these “characterized hemeproteins” are located on liver microsomes and have crucial functions in the biotransformation of drugs and other xenobiotic molecules in biological systems (Cooper et al. 1965).

Today, many things have become clear about the CYP450 enzyme system, and new discoveries are still ongoing in this area. Now, CYP P450 enzymes are defined as intracellular hemeproteins with a role in oxidative metabolism of lipophilic organic chemicals by activating molecular oxygen (Figure 1.6).

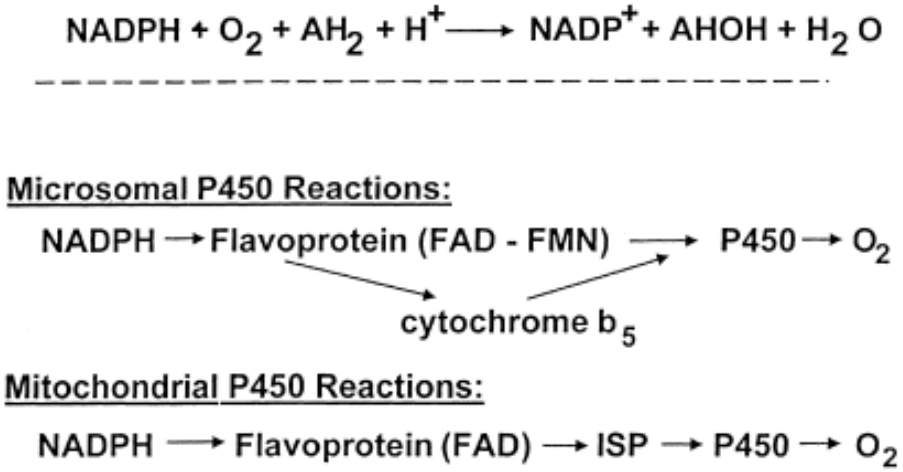


Figure 1.6. CYP450 dependent oxygenation reaction in the different cellular compartments *(Hasler et al. 1999).*

CYP450s can activate the molecular oxygen in organic chemicals via oxidative metabolism. Most heme proteins in mammals (e.g., hemoglobin, peroxidases) have nitrogen from the imidazole group of their histidine residues;

conversely, CYP450s have a characteristic thiol-group in a cysteine amino acid which serves as a ligand to the heme-iron. This characteristic thiol-group works as a ligand, which alters the electron density of the resonant porphyrin ring of the heme and "activation of molecular oxygen" (Hasler et al., 1999).

Especially, hepatic microsomal CYP450s catalyze a variety of monooxygenase reactions which are; aliphatic and aromatic hydroxylations, N-hydroxylations, dealkylations, dehydrogenations, oxygenations of heteroatoms, dehalogenations, alkene, and arene epoxidations. Examples of the types of CYP P450 reactions catalyzed in humans with their tissue localization were given in Table 1.3.

Table 1.3 CYP450 Reactions Catalyzed in Human and Their Tissue Localization

TISSUE	MITOCHONDRIA	MICROSOMES
Adrenal	Cholesterol	21 -OH of Progesterone
Placenta	Side Chain Cleavage	17α-OH of Progesterone
Ovary Testis	11β-Hydroxylation of DOC	Aromatase
Kidney	1α-OH of Vitamin D	Omega hydroxylations of fatty acids
Liver and Intestine	27-OH of Cholesterol	Bile Acid Formation Polyunsaturated Fatty Acid Epoxidation Xenobiotic Metabolism N- & O- dealkylations Alcohol oxidation Alkane & Arene Oxygenation Aromatic hydroxylations

CYP450 enzymes belong to a complex superfamily of genes with a common evolutionary origin. The nomenclature of the CYP450 gene superfamily was first described by Nebert et al. (1987). Based on this nomenclature, CYP450s were firstly categorized by families and sub-families. The basic principle of this categorization involves the minimum 40% protein sequence similarities between two gene families and 60% protein sequence similarities between sub-families. Approximately 115

CYP genes, including 57 putatively active genes and 58 pseudogenes, have been identified in humans, grouped according to their sequence similarity into 18 families and 44 subfamilies. It was estimated that more than 11,000 genes and 1000 CYP families had been found naturally in total (Shahabi et al., 2014; Werck-Reichhart & Feyereisen, 2000).

Most of the CYP enzymes belonging to CYP1, 2, 3, and 4 families are responsible for the metabolism of endogenous substrates and the majority of xenobiotics. Some CYP450 enzymes are classified based on their expression as "constitutive" and "inducible" ways. In fact, the main reason for this difference is their role in the metabolism of endogenous substrates or xenobiotic molecules.

Constitutive CYP enzymes catalyze the reactions involving "endogenous substrates" and play vital roles in the synthesis, activation, and degradation of steroid hormones, prostaglandins, vitamin D, and bile acids. On the other hand, inducible CYP enzymes often have "various substrate specificities" (except endogenous substrates) and have a role in the biotransformation of 75% of xenobiotics and pharmaceuticals in human metabolism (Tesfaigzi, Kluger, & Kozak, 2001; Zanger & Schwab, 2013).

Both constitutive and inducible CYP450 enzymes play a vital role in cellular metabolism and the maintenance of cellular homeostasis. Selected human CYP families and their main functions were given in Table 1.4.

Table 1.4 Selected Human CYP Families and Their Functions *(Hukkanen, 2000)*

Human CYP Families	Function
CYP1A&B	Polycyclic Hydrocarbons, Nitrosamines
CYP2A	Drugs, alcohol, and steroid metabolism
CYP3	Drugs, antibiotics, flavonoids
CYP4	ω-oxidation fatty acids
CYP5	Thromboxane synthesis
CYP7	7-α hydroxylase, bile acid metabolism
CYP8A&B	Prostacyclin and bile acid synthesis
CYP11A&B*	Cholesterol side-chain cleavage, aldosterone synthesis
CYP17	Steroid 17-α hydroxylase, steroid C17/21 lyase
CYP19	Estrogen synthesis, aromatase
CYP20	Drug metabolism and cholesterol synthesis
CYP21	Progesteron 21-hydroxylase
CYP24*	Vitamin D degradation
CYP26	Retinoic acid hydroxylase
CYP27*	Bile acid synthesis and vitamin D_3 activation
CYP39	Cholesterol synthesis
CYP40*	Vitamin D_3 1-α hydroxylase
CYP51	Cholesterol biosynthesis, 14-demethylase

* Mitochondrial Enzyme

Both constitutive and inducible CYP450 genes are regulated via the interactions of a complex network. Important components of this network are; ligand-dependent nuclear receptors (NRs), transcriptional factors (TFs), nuclear coactivators, and corepressors on the specific region of CYP genes (Chun et al., 2001).

Expression of CYP450 enzymes are influenced by a unique combination of mechanisms and factors; including genetic polymorphism, induction by xenobiotics, regulation by cytokines, induction by hormones and disease states; as well as sex, age, and pregnancy conditions (Nebbia et al., 2004; Oleksiak et al., 2003).

1.5.1 Inter-Species Differences of CYP450 Monooxygenases

In mammals, CYP450 monooxygenases are located on the microsomes and inner membrane of the mitochondria. The expression levels of CYP450 enzymes are affected by inter-species, inter-individual, and inter-tissue variations as well as environmental and physiological factors (Williams, Cosme, Sridhar, Johnson, & McRee, 2000). For in vivo studies concerning species-species comparison, it is important to know which similarities, differences, and expression levels exist between the CYP450 enzymes.

Three families of CYP450 monooxygenases, CYP1, CYP2, and CYP3 mainly involved in the oxidative metabolism of xenobiotics and endogenic substrates in mammals. It was studied in the literature that whether there are differences or not in terms of the activity and substrate specificity of CYP450 isoforms across species. It was found that minor differences in the aminoacid sequences cause radical changes in substrate specificity and catalytic activity of CYP450 across different species. Human, rat, and mouse CYP450 isoforms are frequently studied groups in the literature, and differences between the inter-species CYP orthologues were proven (Kobayashi, Urashima, Shimada, & Chiba, 2002). Selected CYP isoforms in

humans, rats, and mice can be seen in Table 1.5 (Hammer, Schmidt, Marx-Stoelting, Pötz, & Braeuning, 2021).

Detailed analyses have been done on CYP1, CYP2, and CYP3 families especially. For example, the CYP1A subfamily has two highly conserved members; CYP1A1 and CYP1A2 in humans, rats, and mice. CYP1A1 and CYP1A2 have substrate specificity to polycyclic aromatic hydrocarbons and arylamines. CYP2A subfamily has different isoforms in different species; three isoforms in humans, three in rats, and four in mice. Rat and mouse CYP2A isoforms have substrate specificity to hydroxylation of steroids, while human CYP2A6 catalyze oxidation reactions of specific substrates such as aflatoxin B1 and nicotine. CYP2C subfamily has several isoforms; four in humans, seven in rats, and nine in mice. The substrate specificity of CYP2C isoforms differs significantly (Kobayashi et al., 2002; Mekjaruskul, Jay, & Sripanidkulchai, 2012; Taneja et al., 2018).

The subfamily of CYP3A recognizes a very broad range of substrates and is responsible for 70-80% of the biotransformation of drugs and xenobiotics. Humans have four rats, and mouse species have six CYP3A isoforms (Hammer et al., 2021; Sutton, Sutherland, Shnyder, & Patterson, 2010).

For nearly 40 years, it has been known that CYP450 enzymes catalyze oxidation and hydroxylation reactions in biological systems. Since then, a lot of studies related to CYP monooxygenase pathways have been conducted on rodents. As a result of these studies, lots of CYP450 monooxygenase isoforms were detected in rats which are; CYP1A1, CYP1A2, CYP4A1, CYP2B1, CYP2B4, CYP2C9, CYP1A1, CYP2C11, CYP2C23, CYP2E1, CYP2G1, and CYP2J3 (Tesfaigzi et al., 2001).

Table 1.5 CYP450 Isoforms in Human, Rat and Mouse Species (*Hammer et al.2021)*

Human	Rat	Mouse
CYP1A1	CYP1A1	(CYP1A1)
CYP1A2	CYP1A2	(CYP1A2)
CYP2B6	CYP2B1 and CYP2B2	CYP2B10
CYP2C8	CYP2C11	CYP2C29
CYP2C12	CYP2C9	CYP2C38
CYP2C19	CYP2C13	CYP2C39
	CYP2C55	CYP2C55
CYP2D6	CYP2D3	CYP2D9
		CYP2D10
CYP2E1	CYP2E1	CYP2E1
CYP3A4	CYP3A4	
CYP3A5	CYP3A18	CYP3A25

1.5.2 Cytochrome P450 Epoxygenases and Arachidonic Acid Pathway

Arachidonic acid (AA) is a 20-carbon omega-6 polyunsaturated fatty acid (PUFA) and the vital precursor molecule in biological systems. AA is one of the essential fatty acids required to maintain the normal biological functions of the majority of mammals. It is embedded into the cell membrane phospholipids and released by the stimulation of the phospholipase A_2 (PLA_2) enzyme. AA synthesis is triggered by some biological inducers such as; inflammatory signals, cytokines, growth factors, and hormones. The free intracellular AA is converted to a series of biologically active metabolites referred to as “eicosanoids.” Eicosanoids embrace

several lipid signaling mediators that play a central role in cellular signaling cascades of physiological and pathophysiological processes.

Arachidonic acid (AA) is converted into biologically active eicosanoids enzymatically or non-enzymatically. It is known that AA is converted to eicosanoid mediators by three independent pathways; these are Cyclooxygenase (COX), Lipoxygenase (LOX), and Cytochrome P450 (CYP450) monooxygenase pathways. Cyclooxygenase (COX) and Lipoxygenase (LOX) are well-known pathways of AA metabolism and produce prostaglandin and leukotriene metabolites, respectively. These biologically active metabolites have critical roles in numerous metabolic processes (Jarrar, Jarrar, abed, & Abu-Shalhoob, 2019; Sausville, Williams, & Pozzi, 2019; Xu, Zhang, & Wang, 2011). Schematic representation of the arachidonic acid metabolism via the cyclooxygenases, lipoxygenases, and epoxygenases pathways were given in Figure 1.7.

In addition, Cyclooxygenase (COX) and Lipoxygenase (LOX) pathways; CYP monooxygenase pathway also plays a specific role in arachidonic acid metabolism. Arachidonic acid metabolism by the CYP pathway produces two types of eicosanoid products; epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs) metabolites. “CYP epoxygenases” and “ω-hydroxylase“ are the main enzymes of this pathway. CYP epoxygenases have a critical role in arachidonic acid metabolism. This specific group of CYP450 monooxygenases belongs to the several CYP450 subfamilies, including; CYP1A, CYP2B, CYP2C, CYP2E, and CYP2J and have been classified as “CYP epoxygenases” (Konkel & Schunck, 2011; Panigrahy, Kaipainen, Greene, & Huang, 2010; Shahabi et al., 2014).

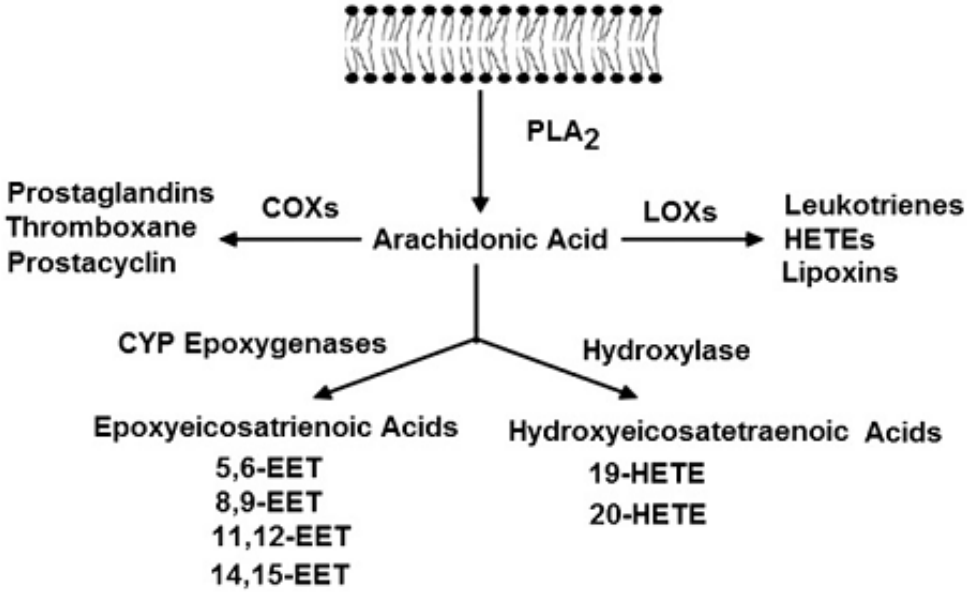


Figure 1.7. Arachidonic acid pathway via cyclooxygenases, lipoxygenases, and epoxygenases *(Xu et al.2011).*

Cytochrome P450 (CYP450) monooxygenase-dependent arachidonic acid (AA) metabolism occurs in several tissues in mammals, including the liver, kidney, and cardiovascular system. The epoxygenase pathway of AA is carried out predominantly by the CYP2C and CYP2J isoforms and generates EETs. Conversely, the ω-hydroxylases of AA are carried out by the CYP4A and CYP4F subfamilies and produce HETEs. EETs converted into four epoxidation regioisomer products; 5,6 -, 8,9 -, 11,12- and 14,15- EETs. Moreover, HETEs converted into two hydroxylation regioisomer products; 19- and 20- HETEs (Panigrahy et al., 2010; Tesfaigzi et al., 2001; Theken et al., 2011). CYP epoxygenase pathway and its products were shown in Figure 1.8.

Both EETs and HETEs regulate numerous physiological and pathophysiological processes in biological systems. These are; angiogenetic and

apoptotic processes, vasomotor functions, cardiac regulatory functions, and "inflammatory progress." It is known that EETs work via various mechanisms in different tissues and different physiological and/or pathological conditions. They reveal these specific biological functions in either a stimulatory or inhibitory way. For example, they have stimulatory effects on angiogenesis and vasodilatation. On the other hand, they have inhibitory effects on apoptosis, cell migration, ischemic injury, cardiac hypertrophy, and "inflammation" (Fan & Roman, 2017; Fava & Bonafini, 2018; Sausville et al., 2019).

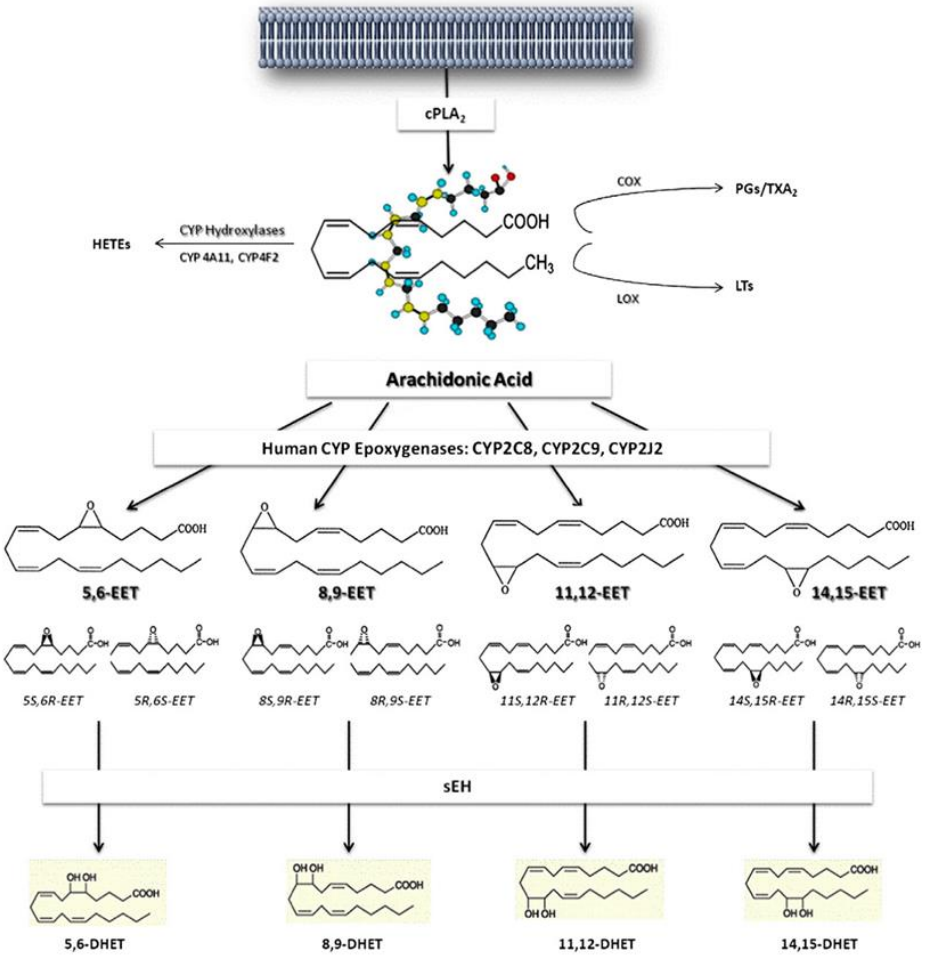


Figure 1.8. CYP epoxygenase pathway and their products *(Shahabi et al. 2014).*

1.5.3 CYP450 Epoxygenase Expression and Metabolic Activity in the Presence of Inflammation

Accumulating evidence suggests that the Cytochrome P450 monooxygenases play an indispensable role in the regulation of inflammation. CYP450 monooxygenase enzymes execute their role through the biosynthesis of endogenous bioactive arachidonic acid (AA) derivatives; epoxyeicosatrienoic acids (EETs) produced by "CYP epoxygenase" and hydroxyeicosatetraenoic acids (HETEs) produced by "ω-hydroxylase" (Theken et al., 2011; Xu et al., 2011).

When it comes to regulation of inflammation, CYP epoxygenase and ω-hydroxylase pathways work in opposite ways. EETs modulate inflammation by their anti-inflammatory properties, which include inhibition of pro-inflammatory mediators and inhibition of cell adhesion molecules. Conversely, HETEs show their pro-inflammatory properties by activating cellular adhesion molecules and cytokines, supporting inflammation (Thomson, Askari, & Bishop-Bailey, 2012).

There is a strong relationship between inflammation and CYP450 monooxygenase expression. It has been revealed that acute inflammatory stimuli alter hepatic CYP expression. Pro-inflammatory cytokines suppress hepatic CYP expression via a translational mechanism. In detail, CYP-derived EETs possess anti-inflammatory properties via inhibition of cytokine-induced nuclear factor NF-kB activation; contrary, 20-HETE activates cytokine-induced nuclear factor NF-kB signaling and reveals pro-inflammatory effects. These opposing effects on the regulation of inflammation suggest that inflammation-induced alterations in the functional balance between these parallel pathways may contribute to the pathologic consequences of the inflammatory response (Aitken, Richardson, & Morgan, 2006; Kalsotra et al., 2007; Theken et al., 2011). Activation and inactivation of the inflammatory response by CYP epoxygenase and ω-hydroxylase pathways were shown in Figure 1.9.

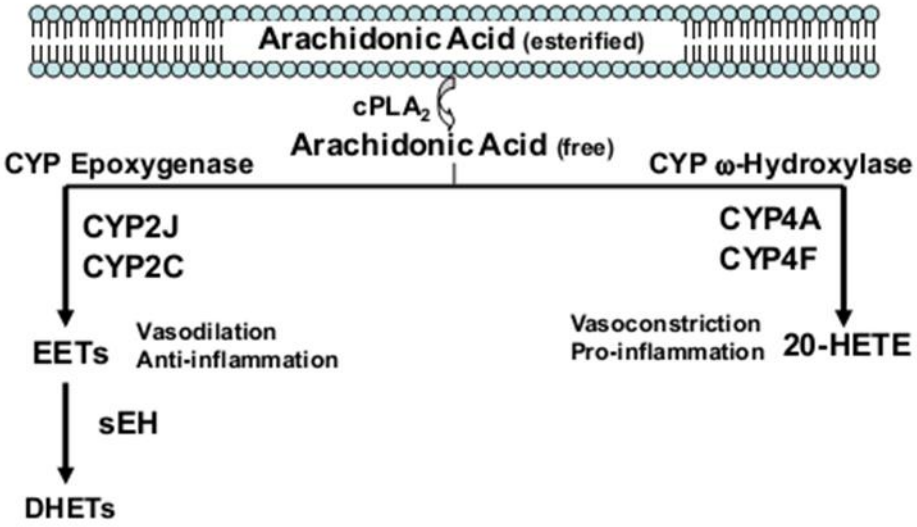


Figure 1.9. Activation and inactivation of the inflammatory response by CYP epoxygenase and ω-hydroxylase pathways *(Theken, 2011).*

1.6 Aim of The Study

Inflammation and inflammation-derived disorders are an area of interests and studied extensively. Inflammation and the inflammatory processes are defense mechanisms of the body under normal conditions, but it turns into a pathological state if the inflammation continues for a long time. It was known that inflammation plays a central role in the pathogenesis of various diseases such as rheumatoid arthritis, cancer, diabetes, obesity, and cardiovascular complications. For this reason, it has been studied universally to combat its deleterious effects on the human body and to find therapeutic approaches to it. Today, non-steroidal anti-inflammatory drugs are the main therapeutical agents used in the treatment of inflammation. Still, there is a lot of information in the literature about the side effects of these groups of drugs on the gastrointestinal and cardiovascular systems (Scarpignato et al., 2015; Vonkeman & van de Laar, 2010). These side effects have reached such a serious extent that some of these drugs are withdrawn from the market (Blumenthal et al., 2017; Tabas Ira & Christopher, 2013).

Sanguisorba minor subsp. *balearica* is known as a medicinal plant in Anatolia and has been used traditionally for the treatment of different diseases such as diarrhea, hemorrhoids, goiter, and gastric problems. Apart from these traditional uses, it is noteworthy to state its ability to relieve inflammation. Its high polyphenolic and flavonoid content could explain the therapeutic effects of *Smb*. Scientific investigations of the *Sanguisorba* genus have been carried out, and these studies demonstrated their high quantity of phytochemical content and their therapeutical potential. Anti-inflammatory effects of medicinal plant *Sanguisorba minor* subsp. *balearica* has also been shown in a small number of studies. It is thought that these anti-inflammatory effects are mainly due to its flavonoids content (Arıhan et al., 2015; J. feng Chen et al., 2020; Yang et al., 2015; Yu et al., 2011), (Guo et al., 2019).

In this study, our main aim is to reveal the possible anti-inflammatory effects of *Sanguisorba minor* subsp. *balearica* and to understand the underlying mechanisms of this effect on inflammation-induced rats. As a result of a detailed literature survey, we understand that there is a strong relationship between inflammation and the CYP450 epoxygenase enzyme system. During the inflammatory state, there are some of the specific molecules involved in the process called "cytokines." Based on this information, in the present study, *Smb* was chosen as a medicinal plant for its anti-inflammatory properties. To mimic inflammatory conditions, CFA-induced inflammatory rats were used as model animals in this study. To clarify the underlying mechanism of the anti-inflammatory of *Smb,* selected CYP50s (CYP1A2, CYP3A4, CYP2C9, CYP2E1) and proinflammatory cytokines (TNF-alpha, IL-1 beta, and IL-12) were examined by using different experimental methods.

To obtain valuable data, firstly, the polyphenolic content of *Smb* was analyzed by the HPLC method, then the inflammation-induced rat model was created and tested. After these initial stages, animal studies were conducted with the treatments of six different groups; healthy control, CFA control, CFA+Diclofenac sodium treated, CFA+quercetin treated, CFA+34mg/kg *Smb* treated, and CFA+70mg/kg *Smb* treated. In the last stage of this work, the biochemical,

immunohistopathological, immunohistochemical, and protein immunoblotting analysis on selected liver microsomal CYP450 enzymes and proinflammatory cytokine molecules were conducted.

Based on our knowledge obtained from the literature, *Sanguisorba minor* subsp. *balearica* has an anti-inflammatory effect on inflammatory disorders. However, there is no data available in the literature to clarify the possible impact of *Smb* on CYP450s and proinflammatory cytokines related to the inflammatory state and anti-inflammatory response. Therefore, this current study is a novel study that clarifies the *in vivo* possible anti-inflammatory effects of medicinal plant *Smb* on targeted CYP450s enzymes and cytokine molecules using an inflammation-induced rat model. If this current study confirms our hypothesis and we understand the underlying mechanism of this effect, *Smb* extract can be further evaluated for its use as an effective and safe alternative for the conventional anti-inflammatory therapeutical agents in terms of the treatment of inflammation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Boric acid (A949265), chloroform (1.02431.2500), copper (II) sulfate pentahydrate, ($CuSO4.5H_2O$; A894987 605), dipotassium phosphate (K_2HPO_4; A611101 528), folin-phenol reagent (1.09001.0500), hydrochloric acid (HCl; 1.00314), magnesium chloride ($MgCl_2$; Art.5833), potassium chloride (KCl; 4935), potassium dihydrogen phosphate (KH_2PO_4; A319173-204), sodium carbonate (Na_2CO_3; 1.06392), sodium chloride (NaCl; 1.06400), sodium hydroxide (NaOH; 06462), Triton X-100 (11869.1), zinc chloride ($ZnCl_2$; 108815) were purchased from Merck KGaA, Darmstadt, Germany.

Acrylamide (A-8887), ammonium persulfate (APS; A-3678), N'-N'-Bis-methylene-acrylamide (M7256), bovine serum albumin (BSA; A788), dimethyl sulfoxide (DMSO; 485519), glycerol (G5516), glycine (G-7126), ß-mercaptoethanol (M6250), methanol (34885), sodium dodecyl sulfate (SDS; L4390), sodium-potassium tartrate (S-2377), Trisma base (T1503), Tween20 (P1379) were the products of Sigma-Aldrich, Germany. Ethylenediaminetetraacetic acid (EDTA; A5097) was obtained from Fermentas, USA.

Non-fat dry milk (170-6404) and tetramethylethylenediamine (TEMED; 161-0801) were purchased from Bio-Rad Laboratories, Richmond, CA, USA.

TRIzol® (12183-555) was purchased from Invitrogen, Thermo Fisher Scientific, Massachusetts, USA.

Isopropanol (AS040-L50) was purchased from Atabay, Turkey, and absolute ethanol (32221) from Honeywell-Riedel, Wabash, Indiana, USA.

Complete Freund's Adjuvant (CFA;100 mg heat-killed *Mycobacterium butyricum*) was purchased from Difco Laboratories, Detroit, MI, USA.

Rat-TNF (Tumor Necrosis Factor Alpha) ELISA kit, 96 tests (E-EL-R0019) and Rat IL-1 (Interleukin1-beta) ELISA kit, 96 tests (E-EL-R0012) were purchased from Elabscience, USA.

Immunoperoxidase staining diagnostic kit was purchased from Thermo Fisher Scientific, Massachusetts, USA.

Pierce BCA Protein Assay kit was purchased from Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA.

Anti CYP450 1A2 (cat no: ab22717), anti CYP450 3A4 (cat no: ab3572), anti CYP450 2C9 (cat no: ab4236) and anti CYP450 2E1 (cat no: ab84598) primary antibodies were purchased from Abcam scientific, Cambridge, MA USA.

Anti beta-actin antibody (SP124) (cat no: ab115777) was purchased from Abcam Scientific, Cambridge, MA USA.

Anti-Mouse IgG AP Conj. and Anti-Rabbit IgG AP Conj. secondary antibodies were purchased from Santa Cruz Biotechnology Inc., Dallas, Texas, USA.

Fisher BioReagents EZ-run prestained rec protein ladder was purchased from Thermo Fischer Scientific Inc., USA.

Pierce ECL Western Blotting HRP substrate solution was purchased from Thermo Fischer Scientific Inc., USA.

All other chemicals that were used in this study were of analytical grade and purchased from commercial sources at the highest grade of purity.

2.2 Methods

2.2.1 Plant Collection, Authentication and Extraction

Within the scope of the field studies, the flowering periods of *Sanguisorba minor* subsp. *balearica (Bourg. ex Nyman), (Muñoz Garm. & C.Navarro)*; localities, habitats, and altitudes; where it is spread have been determined by scanning related resources and herbarium samples (Davis 1978; Davis 1984), (Gürbüz et al., 2005).

According to the information obtained from the fieldwork, the plant samples were collected on July 8, 2016, from the region given below, which is free from environmental pollution.

- Kayseri, Pınarbaşı (Turkey); between Eğrisöğüt village and Aşağı Beyçayır village Kumuk Ali Çeşmesi locality, step, c. at an altitude of 1750 m.

After plants were collected from the field, they were pressed and dried in accordance with the herbarium rules without losing much of their morphological properties. The collected plant samples were identified as *Sanguisorba minor* subsp. *balearica* (Bourg. ex Nyman), (Muñoz Garm. & C.Navarro) under the guidance of " Flora of Turkey" (Davis 1972), (Davis 1982) and by comparison with Ankara University Faculty of Pharmacy Herbarium (AEF) samples.

The press-dried plant was labeled as a voucher specimen and registered with a herbarium number (AEF 26985). Then it was deposited in Ankara University Faculty of Pharmacy Herbarium for future references. Samples collected for bioactivity and chemical studies were dried on a large sieve in a shade and airy place. Afterward, the dried plant samples were powdered in the laboratory scale mill.

Air-dried and powdered 50 g of plant material has been subjected to an active maceration process in sterile distilled water for 8 hours by using a mechanical shaker (Heidolph Instruments) at 300 rpm at room temperature. The extract obtained was filtered through Whatman filter paper, lyophilized (Christ Gamma 2-16 LSC), and

weighed. The yields were calculated (15% for *Smb* weight/weight), and the plant extracts were stored at -20ºC in the absence of oxygen until used in further experiments. The powder extract obtained was dissolved in distilled water or related buffers when needed. This part of the experiments was performed with the collaboration of the Pharmaceutical Botany Department, Faculty of Pharmacy at Ankara University.

Although the general phytochemical content of a plant is known, the content of specific polyphenolic compounds may vary according to the region where the plant-derived from. Therefore, quantitative determination of the active constituents of the plant had to be performed. To determine the phytochemical content, phytochemicals [Flavonoids (quercetin, catechin, kaempferol), and other phenolics (ellagic acid, gallic acid, cumaric acid, p-cumaric acid) found in the plant extract were analyzed by the HPLC method. Analyzes were carried out at METU Biotechnology and R&D Center Laboratories with the purchase of services.

2.2.2 **Animal Studies**

2.2.2.1 Experimental Design and Research Protocol

Animal studies were performed at "Gülhane Experimental Animal Production and Research Center" with the Ethical Committee Permission number of 17/13 on 28.03.2017.

Healthy adult Sprague- Dawley male rats (12 weeks old) weighing 200-250 g. were used in experimental studies. Animals were housed in polypropylene cages (3 animals per cage), maintained under standard conditions (12 hour light, 12-hour dark cycle; 27 ± 3˚C, 35-50 % humidity), fed with standard pellet diet, free from access to water (ad libitium), and allowed to acclimatize in the course of the experiment.

Animals were randomly divided into different treatment groups, each consist of six animals. Testing took place in the middle of the light period of a 12 h/12 h light/dark cycle. All animal experiments were carried out in accordance with the "National Institutes of Health Guide for Care and Use of Laboratory Animals." The Institutional Animal Ethical Committee of "Gülhane Experimental Animal Production and Research Center" approved the experimental protocols. Experimental groups were randomly assorted into six groups before the onset of Complete Freund's Adjuvant (CFA) injection, as given in Table 2.1.

Table 2.1 Experimental Groups

Experimental Groups	Number of animals	Number of Repetition	Total number of animals
Healthy control	6	1	6
Negative control	6	1	6
Positive control (Diclofenac sodium 5mg/kg)	6	1	6
Antioxidant treated (25mg/kg quercetin)	6	1	6
Smb treated (34mg/kg)	6	1	6
Smb treated (70mg/kg)	6	1	6

After a certain time period, each group was treated with their administered reagent listed below. The dosages of the administered reagents were determined based on similar studies in the literature, which were conducted under the same experimental protocols (same experimental animal, same administration route, same administration frequency, and time period). (Arıhan et al., 2015; Jagadish, Latha, Mudgal, & Nampurath, 2016; Roslan, Giribabu, Karim, & Salleh, 2017; Ruckmani et al., 2018; Shi, Zhou, Ji, Xu, & Yang, 2015).

Group 1 (healthy control group): Healthy rats were administered with saline orally (p.o.) daily for three weeks.

Group 2 (negative control (CFA) group): CFA injected rats were administered with saline orally (p.o.) daily for three weeks.

Group 3 (NSAID treated CFA group): CFA injected rats were administered with Diclofenac sodium (5 mg/kg, p.o.) daily for three weeks.

Group 4 (antioxidant treated CFA group): CFA injected rats were administered with Quercetin (25 mg/kg, p.o.) daily for three weeks.

Group 5 (34 mg/kg *Smb* treated CFA group): CFA injected rats were administered with *Smb* (34 mg/kg, p.o.) daily for three weeks.

Group 6 (70mg/kg *Smb* treated CFA group): CFA injected rats were administered with *Smb* (70 mg/kg, p.o.) daily for three weeks.

The research protocol was also given in Table 2.2.

Table 2.2 Research Protocol

Administered chemicals	**Dosage**	**Injection path***	**Injection frequency***	**Injection period (day)***
Complete Freund's Adjuvant (CFA)	0.1 ml.	Intraplantar (hind paw)	Only once	1
Saline	0.1 ml.	p.o.	daily	21
Diclofenac sodium	5 mg/kg***	p.o.	daily	21
Quercetin	25mg/kg***	p.o.	daily	21
Smb treated	34 mg/kg**	p.o.	daily	21
Smb treated	70 mg/kg**	p.o.	daily	21

* Reagents were administered orally (p.o.) from day 8 to 28 (post-CFA injection)

** Both *Smb* 34 and 70 mg/kg were dissolved in dH_2O. The concentration of plant extract solution is kept at about 0.1 g. of extract per milliliter.

*** Diclofenac sodium and quercetin were dissolved in the molecular grade of DMSO and freshly diluted in saline before the treatments. The working concentration of DMSO in all treatments was adjusted to be less than 1%. The final DMSO concentration in the solution is never exceeded 1%.

2.2.2.2 Complete Freund's Adjuvant (CFA) induced Chronic Inflammation Model

Complete Freund's Adjuvant has been the most commonly used immunization stimulant method in the literature. It is used in many experimental animal models to mimic chronic inflammatory diseases. Moreover "CFA-induced chronic inflammation" model is a scientifically justified experimental protocol in laboratory animals (Escobedo-Martínez et al., 2017; Ruckmani et al., 2018; Shi et al., 2015; Sun et al., 2016; Tawfik, 2014).

CFA consists of a solution in the form of water in oil emulsion, containing pulverized heat-killed mycobacteria, which is emulsified together with a solution of the antigen of interest. Adjuvant activity is the result of sustained release of antigens from the emulsion to injected side, and it leads to a local innate immune response. After a while, this "innate immune response" is converted into "enhanced adaptive immunity" (Grumstrup-Scott, 1989). Chronic inflammation model was formed via intraplantar injection of 0.1 ml of 1% w/v complete Freund's adjuvant reagent (CFA) (Difco Laboratories, Detroit, MI, USA) reagent to the left hind paw of animals.

Except for the healthy control group, all the animal groups received CFA intraplantar. Before the injection, the hind limbs of all groups were shaved and sterilized with 70% alcohol. Then 0.1 ml. of CFA containing 10mg/ml heat-killed *Mycobacterium butyricum* was injected in sub-plantar of the left hind paw of rats. Adjuvant was prepared from 100 mg heat-killed *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI, USA) suspended in 10 mL of Bayol F oil. Recommended volume of CFA-Antigen emulsion (CFA-AE) per side and the route of administration for different species were given in Table 2.3.

Table 2.3 Recommended Volume of CFA-Antigen Emulsion (CFA-AE) Per side and Route of Administration on Different Species

SPECIES	SUBQ. (ml.)	INTRADERM. (ml.)	INTRAPERIT. (ml.)	FOOTPAD (ml.)	INTRAMUSC. (ml.)
Mouse	< 0.1	*	< 0.2	< 0.05**	< 0.05**
Rat	< 0.1	< 0.05**	< 0.5	< 0.1**	< 0.1**
Rabbit	< 0.25	< 0.05**	*	*	< 0.25**
Non-Human Primate	Freund's Adjuvant is not generally recommended for use in Non-Human Primates as it may interfere with TB testing results and cause excessive inflammation. * Not recommended ** Only when justified				

The time of adjuvant injection was referred to as day 0. An interval of 7 days was given for chronic inflammation to develop. On the 7th day, development of chronic inflammation, treatment was started orally, which continued for consecutive 21 days in respective treatment groups. Control groups received vehicles orally. Injection times and doses used in the study were determined by considering the doses and times applied for similar studies in the literature (Arıhan et al., 2015; Bais, Abrol, Prashar, & kumari, 2017; Cicala et al., 2000; Houshmand, Mansouri, Naghizadeh,

Hemmati, & Hashemitabar, 2016; Impellizzeri et al., 2016; Jagadish et al., 2016; Tawfik, 2014).

2.2.2.3 Administration of Paw Edema on CFA-induced Chronic Inflammation“ Complete Freund’s Adjuvant ”

Before adjuvant injection, the basal paw thickness of all the experimental groups was measured by plethysmometer. The time of adjuvant injection was referred to as day 0. After post-CFA injection, hind paw measurements were taken in the acute and subacute periods of inflammation on the 1st, 5^{th,} and 7th day respectively. Development and severity of induced chronic inflammation were evaluated by a visual scoring system of the clinical signs and symptoms on a scale of 0-4 per hind paw and limb (A et al., 2015), (Vijayalaxmi et al., 2015). Where 0: no change, 1: slight swelling and erythema of the paw, 2: mild swelling and erythema of the paw and/or limb, 3: severe swelling and erythema of the paw and/or limb, 4: deformity and inability of the paw and/or limb. A score of both hind paw and limb were counted, and a score of more than 1 considered exhibiting chronic inflammation.

On the 7th day, severe inflammation was observed on both local and systemic sites, which is called the “chronic phase of inflammation.” After this stage, treatments were started orally (p.o.) and continued for consecutive 21 days for all experimental groups. Reduction of chronic inflammation was ascertained by measuring the paw thickness periodically after treatment on the 10th, 18th, 24^{th,} and 28th days. The swelling of the adjuvant-injected hind paw was determined to follow the course of the disease. The thickness (mm) of the rat's hind paws was measured using Vernier Caliper (opt lab, Berlin, Germany). Percentage inhibition of chronic inflammation (edema) was calculated among all experimental groups.

Percentage inhibition of paw edema was calculated using the formula given below:

$(1-T_0/T_t)\times100$, where [T_0 is the mean of paw thickness at day 0; Tt is the mean of paw thickness at a particular time].

2.2.2.4 Determination of Serum TNF-alpha and IL-1 beta Levels

TNF-alpha and IL-1 beta were targeted serum inflammatory markers in this study, and their concentration levels were analyzed in all experimental groups. The degree of recovery of chronic inflammation can be detected as the concentration of specific cytokine markers in the serum samples.

Sixteen hours later, after the end of the experimental period on the 28th day, blood samples were collected via the cardiac puncture method from anesthetized animals. Animals were anesthetized with 10mg/kg Xylasin + 60mg/kg Ketamin cocktail. The cardiac puncture method allows the collection of large amounts of blood samples (up to 10 ml of blood can be drawn from a 150 g rat) from a single animal (Luzzi et al. 2005). Serum was separated immediately from the blood samples by centrifugation (3000 rpm for 10 min) and stored at -80 °C.

TNF-alpha and IL-1 beta levels were determined from the centrifugated serum samples by the Enzyme-Linked Immunosorbent Assay (ELISA) method (Engvall and Perlmann, 1971). Assessment of TNF-alpha and IL-1 levels were performed by using diagnostic kits (Elabscience, USA) according to the manufacturer's protocol. The ELISA kit used is working by the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to rat TNF-α and IL-1 beta. All samples and standards were assayed in duplicates.

Standards or samples were added to the micro ELISA plate wells (100 µL for each well) and combined with the specific antibody. The microplate was covered with sealer then incubated for 90 min at 37°C. After the incubation period, the liquid was removed from each well, and 100 µL of Biotinylated detection Ab/Ag-specific

for rat TNF-α and IL-1 beta were added. The microplate was covered with the plate sealer, gently mixed up, and incubated for 1 hour at 37°C.

After that, 350 µl of wash buffer was added to the solution in each well. Free components were washed away from each well. The wash step was repeated three times. After this step, 100 µl of HRP conjugate working solution was added, the micro plate was covered with the plate sealer and incubated for 30 min at 37°C. In the end, the incubation solution was aspirated from each well, and the wash processes were repeated five times. Later, 90 µl of substrate reagent was added to each well and covered with a new plate sealer, and incubated for about 15 min at 37°C. When the incubation period was over, only those wells that contain rat TNF-α, IL-1 beta biotinylated detection antibody, and Avidin-HRP conjugate were appeared blue in color. The enzyme-substrate reaction was terminated by the addition 50 µL of stop solution, and the color turned yellow.

Finally, the optical density (OD) was measured spectrophotometrically by Thermo Scientific ELISA reader at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of rat TNF-α, IL-1 beta. The concentrations of rat TNF-α, IL-1 beta in the samples were calculated by comparing the OD of the samples to the standard curve and expressed as pg/ml of serum samples.

2.2.2.5 Histopathological and Immunohistochemical Examinations of Joint Tissues

This part of the study was carried out with the collaboration of Kırıkkale University, Faculty of Veterinary Medicine. After blood samples were collected from all experimental groups, animals were sacrificed by administering a high dose of Xylasin + Ketamin cocktail. Before organ and tissue removal, hind paws were dissected for histological studies. Lesioned hind paw tissues were cut at the metacarpal joint and fixed in 10 % buffered formalin for 48-72 hours. Subsequently, fixed food tissues were decalcified for 72 hours in decalcification solution (Biodec,

Biooptica 05-03004Q, Germany). The routine tissues were then treated with graded alcohol (70°, 80°, 90°, 96°, and 99.5°) and xylol series.

After this step, processed tissues were embedded in paraffin at 56°C- 58°C for paraffin blocking. Three sections of 4-5 mm thickness were taken from the prepared paraffin blocks, and one of them was stained with Haemotoxylin-Eosin (HE) and evaluated histopathologically under the light microscope. Microphotographs were taken and focusing on the synovium, cartilage, and joint space with Olympus BX51 trinocular microscope and DP25 digital camera. Histopathologically examined tissues were evaluated by a visual scoring system on a scale of 0-4 for each examined and stained tissue area. Scoring was done according to four parameters which are; edema, bleeding, inflammation, and necrosis.

For Immunohistochemical analysis, the immunoperoxidase staining method was used. TNF-alpha and IL-12 were chosen as targeted inflammatory cytokines. According to the manufacturer's protocol, all immunoperoxidase staining assessments were done using diagnostic kits (Thermo Fisher Scientific, Massachusetts, USA).

The immunoperoxidase method was performed mainly in three steps. Firstly, paraffin sections of rat paw tissues were dewaxed, then processed tissues were rehydrated, and finally subjected to antigen retrieval step. 4-5 mm thickness paraffin sections of routine tissues were dewaxed in xylol series for 5 minutes and then treated with graded alcohol (70°, 80°, 90°, 96° and 99.5°) series for rehydration process.

During the antigen retrieval process, tissues were boiled in citrate solution (pH 6.0) for 30 minutes, then treated in 1% hydrogen peroxide solution for 15 minutes to inhibit endogenous peroxidase activity. These processed sections were incubated by protein blocking serum (Thermo Fisher Scientific, Massachusetts, USA) for 10 minutes. After blocking with 5% normal goat serum, sections were incubated with anti-TNF-alpha and anti-IL-12 primary antibodies for 1hr at 4°C. Then incubated again for 30 sec. with secondary antibodies with streptavidin peroxidase enzyme. Sections washed with PBS were stained with AEC chromogen

and Mayer's hematoxylin and finally closed with water-based adhesive. As a positive control for each test, mouse brain tissue previously created by experimental *Toxoplasma gondii* infection was used. Instead of a primary antibody for the negative control, non-immunized normal mouse serum was used.

Signals were quantified using Leica DFC 420 camera with plug-in Olympus BX51 microscope (Japan), and micro photos were taken. Finally, % immunopositive stained areas were evaluated by Leica QWin image analysis software. All obtained data were interpreted with % immunopositive stained areas.

2.2.2.6 Preparation of Rat Liver S.9 Microsomal Fractions

The liver was removed from the animals under high dosage anesthesia. Connective and fatty tissues were removed immediately. Isolated liver samples were washed with cold distilled water and 1.15% KCl solution several times to remove the excess blood. The washed liver sample was undergone flash freezing in liquid nitrogen, and each sample was preserved at -80 ˚C until further experimental studies.

Rat liver microsomes were prepared according to S.9 fraction method (Schenkman and Cinti, 1978; Sen and Kirikbakan, 2004) optimized by (C.F.Arlett & J.M.Perry, 1985; J. Richardson, Bai, A. Kulkarni, & F. Moghaddam, 2016). After blotting the tissues with the help of a filter paper, they were weighed and chopped on the ice with scissors. The resulting minced tissues were transferred into the Eppendorf tubes, and equal volumes of stainless steel beads and homogenization solution (3 times the volume of liver tissue) were added. Homogenization solution contained 3 mM EDTA pH 7.8, 50 mM Tris-HCl, pH7.5, 0.3 mM ε-ACA,0.5 mM PMSF, 0.15mM BTH, %10 glycerol and % 0.15 Triton X-100. The homogenization process was done by the "Bullet Type Tissue Homogenizer" (BenchmarkScientific, Bead Bug 6 Microtube Homogenizer, Edison NJ, USA), which adjusted speed 4/1 minutes with the intervals of 30 sec. for four sets. All subsequent steps were carried out at 0-4 °C.

The homogenate was centrifuged at 10800 g (Rcf) for 25 min in order to remove cell debris, nuclei, and mitochondria. The supernatant was filtered with a cheese cloth and further centrifuged at 105.000 g for 50 min. Obtained supernatant contains cytosol. The remaining pellet part is called "microsomal pellet." The microsomal pellet was washed and resuspended in 3 mL of 1.15% KCl solution. The resuspended homogenate was centrifuged at 105.000 g for 50 min. again, in order to remove excess hemoglobin. The final microsomal pellet was resuspended in 1.5 mL of 1.15% KCl solution and stored at -80 ˚C for further experimental study. All procedures were carried out at 4 ˚C.

2.2.2.7 Determination of Protein Concentration of Microsomal Fractions

Protein concentrations of rat liver S9 microsomal fraction were determined by the BCA (Bicinchoninic Acid) method described by Smith et al., (1985). This method is a colorimetric method based on the reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline solution. Colorimetric detection of the cuprous cation (Cu^{+1}) can be obtained by the bicinchoninic acid reagent. The purple-colored reaction has appeared when the chelation of two molecules of bicinchoninic acid with one cuprous (Cu^{+1}) ion. The reduction of copper is mainly caused by four amino acid residues, including cysteine or cystine, tyrosine, and tryptophan, that are present in protein molecules. This purple-colored reaction complex exhibits a strong absorbance at 562 nm. Crystalline bovine serum albumin (BSA) is used as a standard BCA method.

Protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). Protein Assay was performed as following steps:

- 25 µL of each standard and diluted protein sample replicates were pipetted into a 96-microplate well (working range = 20-2000µg/mL).

- 200 µL of the "working reagent" was added to each well, and the plate was mixed thoroughly on a plate shaker for 30 seconds.
- The plate was covered and incubated at 37°C for 30 minutes.
- The plate was cooled to room temperature. Then absorbances were measured by Thermo Scientific ELISA reader at 562 nm.
- The concentrations of proteins were calculated for Western Blot analysis.

2.2.2.8 Determination of Microsomal CYP450 Protein Expressions by Western Blotting Technique

After calculating protein concentrations of the microsomal fraction of the animals with the BCA method, Western Blotting analysis can be started. Effects of *Sanguisorba minor* subsp. *balearica* extract, quercetin, and diclofenac sodium on rat microsomal CYP450 protein expressions were analyzed by Western Blot analysis as described by Towbin et al. (1979).

Before Western Blotting was applied, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using 4 % stacking gel and 8.5 % separating gel (depending on the molecular weight of proteins) in a discontinuous buffer system as described by Laemmli (1970).

Reagents:

Gel Solution: Acrylamide and N'-N'-Bis-Methylene-Acrylamide (Stock)

14.6 g of Acrylamide and 0.4 g of N'-N'-Bis-Methylene-Acrylamide were dissolved separately and then mixed, followed by filtering through filter paper. The volume was completed to 50 mL with distilled water.

Separating Buffer: 1.5 M Tris-HCl, pH 8.8 (Stock)

18.15 g of Tris-base was dissolved with 50 mL water and then titrated with 10 M HCl till pH 8.8. The volume was completed to 100 mL with distilled water. The pH of the solution was controlled at the end.

Stacking Buffer: 0.5 M Tris-HCl, pH 6.8 (Stock)

6 g of Tris-base was dissolved with 60 mL distilled water and then titrated with 10 M HCl till pH 6.8. The volume was completed to 100 mL with distilled water. The pH was controlled at the end.

Sodium Dodecyl Sulfate SDS: 10% (Stock)

1 g of SDS was dissolved in water, and then the volume was completed to 10 mL.

Ammonium persulfate APS: 10% (Fresh)

12.5 g of APS per gel was dissolved in 125 µL of distilled water.

Tetramethylethylenediamine (TEMED): (Commercial)

Sample Dilution Buffer (SDB): 4x (stock)

2.5 mL of 1 M Tris-HCl buffer (pH 6.8) and 4 mL of glycerol were taken; 0.8 g of SDS was added, 2 mL of ß-Mercaptoethanol and 0.001 g bromophenol blue were added. The volume was completed to 10 mL with distilled water. After several uses, 1 mL of ß-Mercaptoethanol was added.

Electrophoretic Running Buffer (ERB): 0.25 M Tris, 1.92 M glycine (10x Stock, diluted to 1x before use by adding 0.1% SDS)

15 g of Tris-Base was dissolved in 350 mL distilled water, and then 72 g of glycine was added. They were mixed well, followed by completion of the volume to 500 mL with distilled water.1 g of SDS was added per liter of 1x buffer before use.

Mini-PROTEAN Tetra cell 165-8033 equipment (Bio-Rad Laboratories, Richmond, CA, USA) was used for gel preparation and electrophoresis steps. The separating (8.5%) and stacking gel (4%) solutions were prepared following a procedure below in a given order in Table 2.4.

Table 2.4 Components of Separating and Stacking Gel Solutions

Constituents	Separating Gel 0.375 M Tris, pH 8.8		Stacking Gel 0.125 M Tris, pH 6.8
Monomer Conc.	8.5 %	12 %	4 %
Gel solution (ml)	8.5	12	1.3
dH_20 (ml)	13.55	10.035	6.1
Separating gel buffer (ml)	7.5	7.5	-
Stacking gel buffer (ml)	-	-	2.5
10 % SDS (ml)	0.3	0.3	0.1
10 % ammonium persulfate (ml)	0.15	0.15	0.05
TEMED (ml)	0.015	0.015	0.01
Total volume (ml)	30	30	10

Transfer Buffer: 25 mM Tris, 192 mM glycine (Stock)

3.03 g of Trisma-base and 14.4 g of glycine were dissolved in 200 mL of methanol (20% v/v), and the volume was completed to 1 L with distilled water.

Tris-Buffered Saline and Tween 20 (TBST): 20 mM Tris-HCl pH 7.4, 500 mM NaCl, and 0.05% Tween 20 (Fresh).

9.5 g of NaCl was dissolved in water; 6.5 mL of 1 M Tris-HCl Buffer pH 7.4 was added. 165 µL of Tween 20 was added, and finally, the volume was completed to 350 mL with distilled water.

Blocking Solution: 5% Non-Fat Dry Milk (Stock)

Substrate Solution: Pierce ECL Western Blotting HRP substrate solution, Commercial (Fresh, Light Sensitive).

Primary and secondary antibodies used in the Western Blot analysis with their brand names and optimum dilutions were given in Table 2.5. Beta-actin protein standard was used as the loading control.

Table 2.5 Antibodies Used in the Western Blot Analysis with Their Brand Names and Optimum Dilutions

	Primary AB	**Primary AB Dilution**	**Brand Name**	**Secondary AB**	**Secondary AB Dilution**	**Brand Name**
CYP1A2	Mouse Anti-CYP1A2	1:1000	Abcam cat no: ab22717	Anti-Mouse IgG AP Conj.	1:5000	Santa Cruz
CYP3A4	Rabbit Anti-CYP3A4	1:1000	Abcam cat no: ab3572	Anti-Rabbit IgG AP Conj.	1:5000	Santa Cruz
CYP2C9	Rabbit Anti-CYP2C9	1:500	Abcam cat no: ab4236	Anti-Rabbit IgG AP Conj	1:2000	Santa Cruz

* Beta-actin protein standard was used for loading control

Table 2.5 (continued) Antibodies Used in the Western Blot Analysis with Their Brand Names and Optimum Dilutions

CYP2E1	Rabbit Anti-CYP2E1	1:1000	Abcam cat no: ab84598	Anti-Rabbit IgG AP Conj	1:5000	Santa Cruz
Beta-Actin *	Mouse Anti- β-Actin	1:500	Abcam cat no: SP124	Anti-Mouse IgG AP Conj.	1:5000	Santa Cruz

* Beta-actin protein standard was used for loading control

The gel sandwich made between two glass plates was used to prepare polyacrylamide slab gels. First, separating gel solution was added to the gel sandwich till the desired height of the solution was obtained between the glass plates. The top of the polymerizing gel was covered with a layer of isobutanol to obtain a smooth gel surface. Then, the gel was incubated for polymerization for about 30 min at room temperature. After polymerization, the alcohol layer was poured totally. The stacking gel polymerization solution was prepared and added to the gel sandwich till the sandwich was filled completely. Following the stacking gel addition, a 1.0 mm teflon comb containing 15 wells was inserted into the layer of polymerizing stacking gel solution. The attention was given not to get any bubbles at the edges of the comb while placing the comb. Polymerization was completed in about 30 min at room temperature. The teflon comb was carefully removed without disrupting the wells. Then, wells were filled with electrode running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) with a fine needle syringe for removal of any formed air bubbles or unpolymerized chain particles. Finally, the gel running module was filled with a sufficient volume of electrode running buffer.

The protein samples were diluted with 4x sample dilution buffer containing 0.25 M of Tris-HCL, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.01% bromophenol blue to obtain 2 mg/mL in distilled water and boiled in a water bath for 1.5 minutes. Then, 30 µg of samples were applied to different wells by Hamilton syringe. The Gel-running module was placed in the main buffer tank filled with electrode running buffer. The electrophoresis unit was connected to the power supply to run electrophoresis at 150 V and 15 mA for 40 minutes in stacking gel and 300 V and 30 mA for 2 hours in separating gel.

After the electrophoresis was completed, gels were removed from the set-up for Western Blotting. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 15 min with constant shaking to adjust the final size of gel and remove the buffer salts and SDS was coming from the SDS-PAGE step. The nitrocellulose membrane was prepared by cutting 1 cm larger than the dimension of the gel, and two pieces of Whatman paper were cut in dimensions a little bit larger than the membrane.

Nitrocellulose membrane, two filter papers, and fiber pads of the transfer sandwich were placed in transfer buffer and saturated with this solution. Western blot sandwich was prepared as shown in Figure 2.1. A test tube was used to remove any air bubbles between the layers by gently rolling over the sandwich. This was the critical step since any air bubbles formed between layers block the transfer of proteins. Then, the sandwich was put into the Mini Trans-Blot module 165-8033 (Bio-Rad Laboratories, Richmond, CA, USA), and the module was filled with cold transfer buffer. Voltage and current were set to 90 V and 400 mA, respectively. The transfer was carried out 60 minutes with Bio-Rad PowerPac basic power supply (Bio-Rad Laboratories, Richmond, CA, USA).

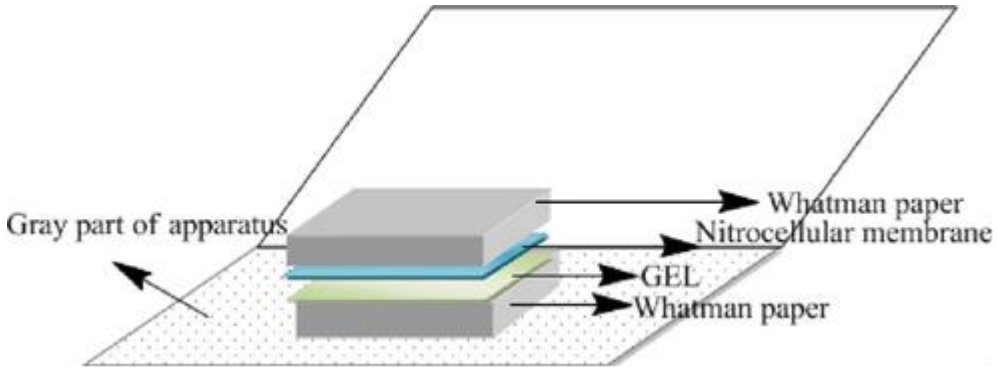


Figure 2.1. Preparation of Western blotting sandwich system

At the end of this process, the nitrocellulose membrane carrying the transferred proteins, i.e., "blot," was obtained and removed from the module. Then, the membrane was transferred to a plastic dish as the protein side facing upwards and washed with TBST (Tris Buffered Saline plus Tween 20: 20 mM Tris-HCL, pH 7.4, 0.5 M NaCl, and 0.05 % Tween 20) for 10 min. This washing step removes the salts and buffers from the transfer medium. Then, the blot was incubated with blocking solution (5% Non-Fat Dry-Milk in TBST) for 60 min so that empty spaces between transferred proteins were filled. This filling inhibits the non-specific binding of antibodies to the membrane.

After that, the blot was incubated with primary antibodies (arranged dilution series of goat, mouse, and rabbit polyclonal CYP1A2, CYP3A4, CYP2C9, and CYP2E1 antibodies in Tris-buffer saline with 5% non-fat dry milk) for 2 hours. Following this step, the blot was washed three times with 50 ml of TBST for 5 min each time to remove excess, unbound antibodies. The blot was then incubated with 5000-times diluted CYP1A2, CYP3A4, and CYP2E1 secondary antibodies and 2000-times diluted CYP2C9 secondary antibodies for (conjugated mouse anti-goat IgG) for 1 h. The blot was washed three times with 50 mL TBST for 5 min each to remove excess antibodies. The complete removal of TBST between each washing step was extremely important since non-specifically bound regions can give reaction with substrate solution.

Finally, substrate solution was prepared while the membranes were being washed with TBST, and then the membranes were incubated with the Pierce ECL Western Blotting HRP Substrate solution, which was prepared by mixing 1 volume of solution A and 1 volume of solution B to visualize the specifically bound antibodies. The final images were photographed by using a computer-based gel-imaging instrument, Infinity 3000 (Vilber Lourmat, Marne-la-Vallee Cedex 1, France) by using Infinity-Capt Version 12.9 software. Immunoreactive protein bands were then quantified by the densitometric scanning method using an Image J software package program developed by NIH.

2.2.3 Statistical Analysis

Statistical analyses were performed by using GraphPad Prism 6.0 statistical software package for Windows (La Jolla, CA). Analysis of the data was applied by One-way-Anova together with Tukey t-test. All obtained results were expressed as means with their "Standard Error of Means (SEM)." The correlation between treated and control animals was tested, and a possibility of 0.05 and 0.005 was stated depending on the statistical significance level.

CHAPTER 3

RESULTS

3.1 Yield Calculation of the Plant Extract

Air-dried and powdered 50 g of plant material has been subjected to an active maceration process in sterile distilled water for 8 hours by using a mechanical shaker (Heidolph Instruments) at 300 rpm at room temperature. The extract obtained was filtered through Whatman filter paper, lyophilized (Christ Gamma 2-16 LSC), and weighed. 7.5 g of lyophilized powder was obtained from 50 g of *Smb* plant extract.

Yield of the plant extract was calculated using the following formula:

$$\text{Yield (\%)} = 7.5 \times 100/50 = 15\%$$

Lyophilized *Smb* plant extract powder was dissolved in dH_2O. This water-soluble plant extract was used for further experimental steps.

3.2 Analysis of the Phenolic and Flavonoid Content of *Sanguisorba minor* subsp. *balearica*

Total phenolic and flavonoid content of *Smb* was analyzed by HPLC method using AGILENT 1200 HPLC series coupled with AGILENT 6460 HPLC spectrometer.

HPLC analysis was carried out in the standardized conditions listed below:

- The spectra of eluted samples were monitored between 200 – 500 nm.
- The flow rate was arranged as 0.3 ml/min.

- Analysis length was 13 minutes with gradual mobile phase flow, and the injection volume was 5 µl.
- Both standard and plant extract samples were dissolved in distilled water.
- Standard curve was drawn based on the serial dilutions (0.15- 0.31- 0.625- 1.25- 2.5- 5- 10 ppm) of reference samples.
- 1 ppm of each standard mixture includes 1 ppm of reference samples.

Seven reference samples were used to identify and quantify the polyphenolic content of *Smb*. These reference samples were; coumarin, p-coumaric acid, gallic acid, kaempferol, catechin hydrate, ellagic acid, and quercetin dihydrate. Representative HPLC chromatograms of the standard mixture and plant extract profile were shown in Figure 3.1 and Figure 3.2. HPLC analysis of *Smb* water extract showed that plant extract includes 4.1288 ppm ellagic acid and 2.5594 ppm gallic acid. There was also a low amount of quercetin hydrate and p-coumaric acid as 0.0657 and 0.0633 ppm, respectively. Catechin hydrate, coumarin, and kaempferol were other detected phenolic compounds, but their concentrations were less than ≤ 0.005ppm. Quantitative results of the phenolic content of *Smb* extract were also given in Table 3.1.

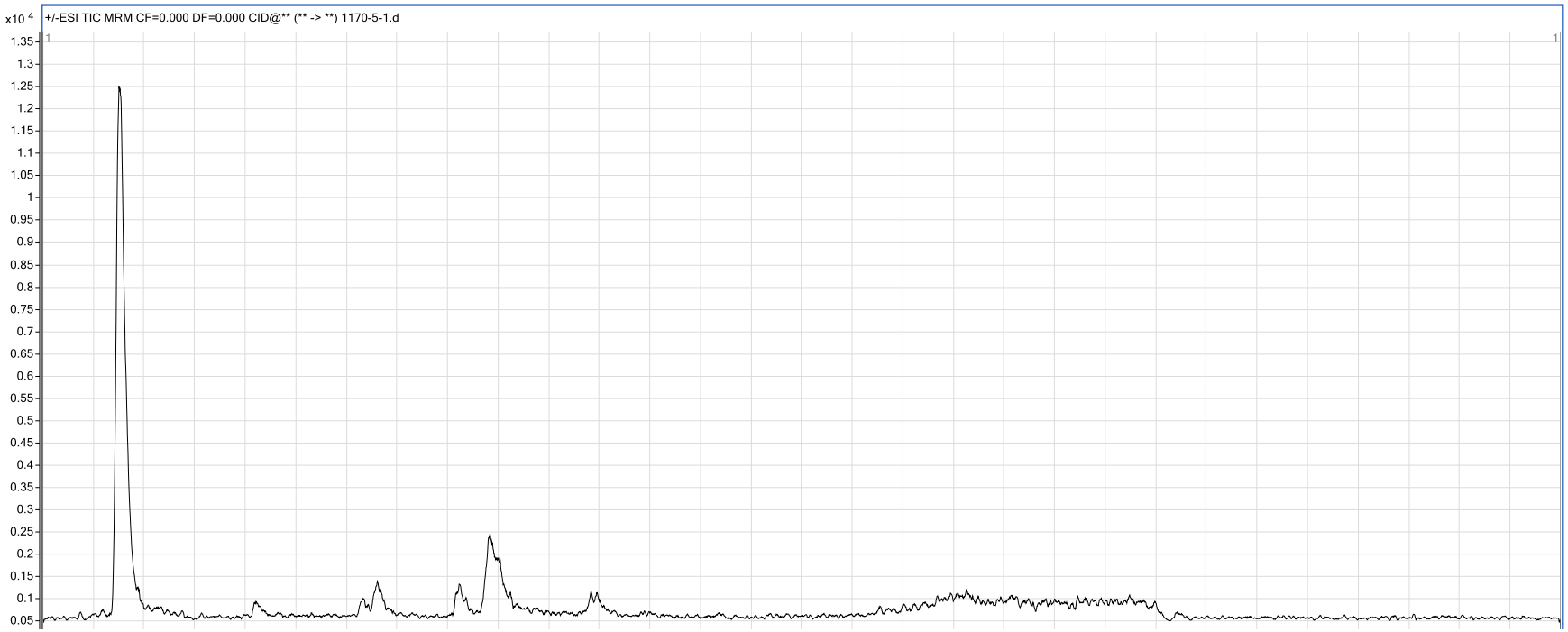


Figure 3.1 . Representative HPLC chromatograms of the standard mixture. Each peak represents the reference sample of the standard mixture (detailed information can be obtained from Appendix B).

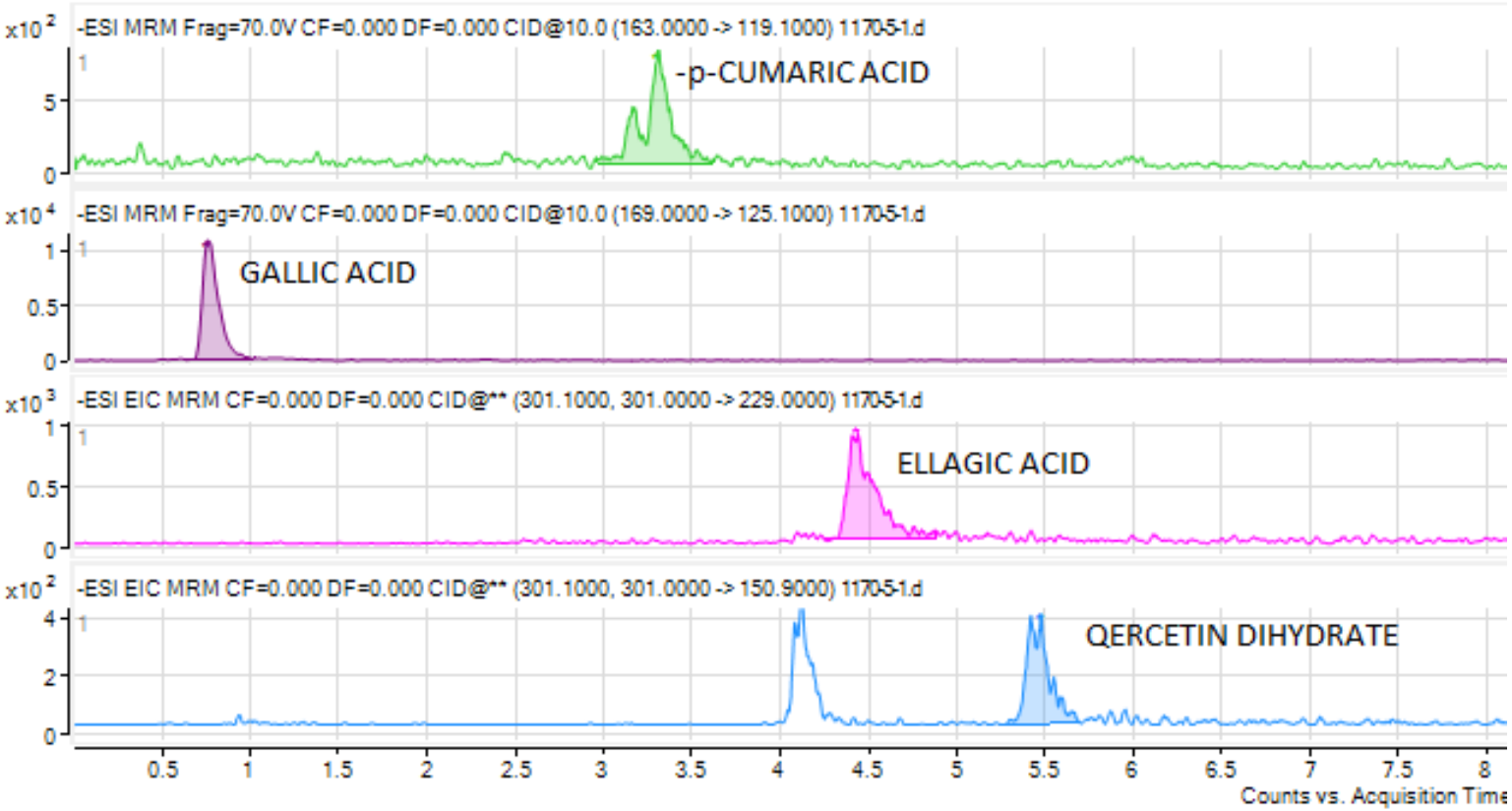


Figure 3.2. Representative HPLC chromatogram profile of *Smb* extract. Each colored peak represents the stated phenolic content of the plant extract.

Table 3.1 Quantitative Phenolic Content Analysis of *Smb* Water Extract by HPLC.

Plant Extract	Ellagic acid (ppm)	Gallic acid (ppm)	Quercetin hydrate (ppm)	p-coumaric acid (ppm)	Coumarin and Kaempferol (ppm)
Sanguisorba minor subsp.*balearica*	4.1288	2.5594	0.0657	0.0633	≤ 0.005

3.3 Assessment of Paw Edema on Inflammation Induced Rats

To produce a similar inflammation degree on the CFA injection side, paw thickness was measured by plethysmometer during the acute and subacute phase of inflammation with an interval of 7 days in all experimental groups. Paw thickness was measured on days 1, 5, 7, 10, 18, 24, and 28. According to the measurements, on day 7, paw thickness increased in all experimental groups after CFA injection except the healthy control (Figure 3.3).

Treatment of rats with plant extract and phenolic compounds caused a decrease in paw thickness both in the control and treated groups. The decrease in paw thickness of all experimental groups on day 28 was found to be significant when compared to CFA control. *Smb* 70mg/kg and quercetin groups have a similar and highest decrease in paw thickness compared to diclofenac and *Smb* 34 mg/kg groups. These results were also summarized in

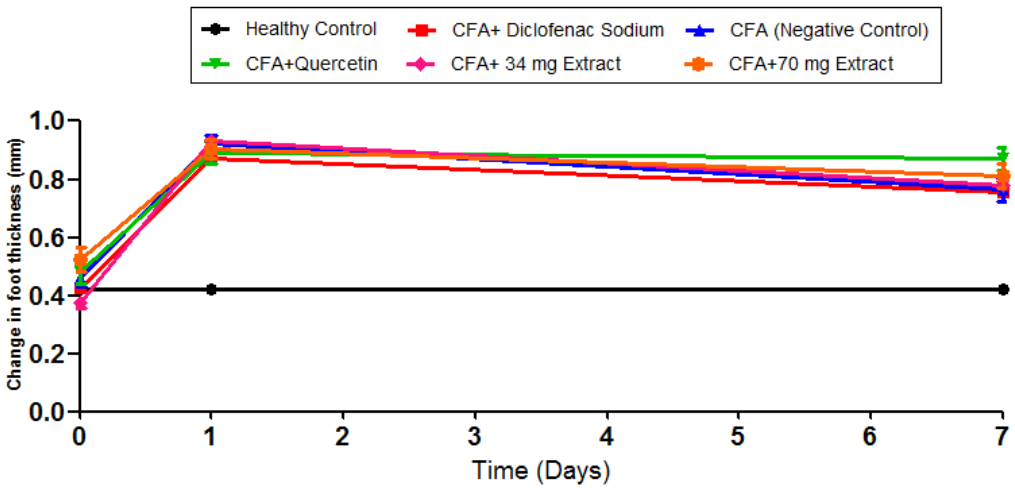


Figure 3.3. Percent increase in paw edema in CFA-induced inflammatory rats before treatment (acute and subacute phase)

Table 3.2 Assessment of paw thickness on inflammation-induced rats in different experimental groups

Groups	**Treatment**	**Paw thickness (mm)**				
		Day 7	Day 10	Day 18	Day 24	Day 28
I	Healthy Control	0.42± 0.006	0.42± 0.006	0.42± 0.006	0.42± 0.006	0.42± 0.006
II	CFA Control	0.70± 0.040	0.78± 0.028	0.71± 0.043	0.64± 0.032	0.62± 0.026
III	Diclofenac sodium	0.75± 0.055	0.78± 0.026	0.61± 0.026	0.63± 0.033	0.59± 0.019
IV	Quercetin	0.76± 0.049	0.83± 0.033	0.56± 0.021	0.56± 0.018	0.52± 0.017
V	*Smb* 34mg/kg	0.808± 0.033	0.85± 0.020	0.68± 0.024	0.64± 0.024	0.60± 0.011
VI	*Smb* 70mg/kg	0.832± 0.033	0.82± 0.019	0.50± 0.034	0.58± 0.039	0.58± 0.066

***** Values expressed as Mean ± SEM, n=6

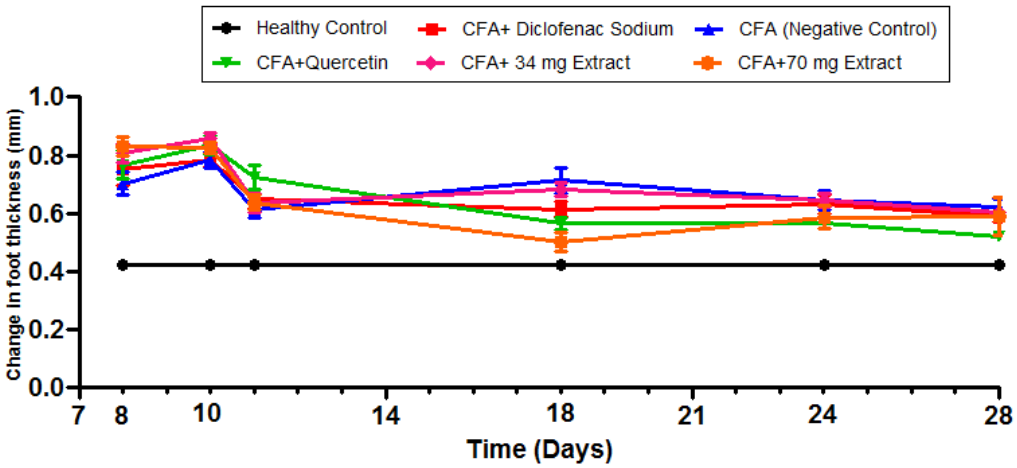


Figure 3.4. Percent decrease in paw edema in CFA-induced inflammatory rats in different experimental groups after treatment (chronic phase)

Percentage inhibition of edema was calculated using the formula given below:

% decrease in foot thickness = $(1-T_0/T_t)\times 100$, where [T_0 is the mean of foot thickness at day 0; Tt is the mean of foot thickness at a particular time].

The highest percent of edema inhibition was expressed by quercetin and *Smb* 70 mg/kg group, especially at chronic phase at (70.08 % and 61.23 %, respectively) and even higher than Diclofenac sodium (49 %). *Smb* 34 mg/kg also shows more percent edema inhibition degree (56 %) than Diclofenac sodium group and followed *Smb* quercetin and 70 mg/kg group respectively. Percent inhibition degree of edema in all experimental groups was given in Table 3.2 and Figure 3.5.

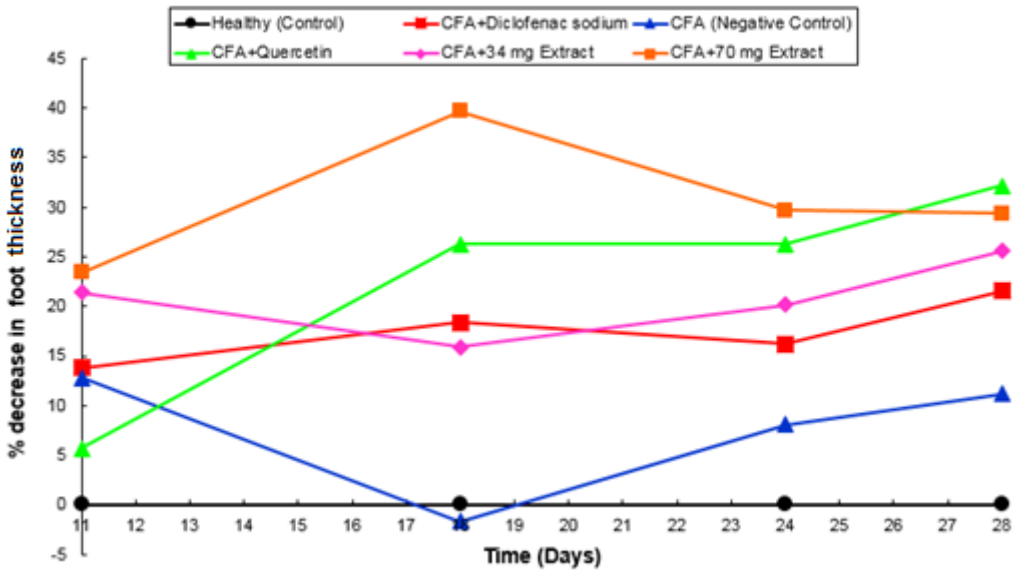


Figure 3.5 Percent decrease in foot thickness from day 10 till day 28 in different experimental groups. Paw thickness was measured on days 10, 18, 24, and 28.

3.4 Enzyme-linked Immunosorbent Assay (ELISA) Analysis of Proinflammatory Cytokines in Serum

To detect systemic effects of *Smb* extract, diclofenac sodium, and quercetin on chronic inflammation, targeted inflammatory cytokines were analyzed in blood serum samples. At the end of the experimental period on the 28th day, cytokines obtained from all experimental groups were measured in serum samples by using the Enzyme-Linked Immunosorbent Assay (ELISA) method. In the present study, TNF-alpha and IL1-beta were chosen as target proinflammatory cytokines. ELISA diagnostic kits (Elabscience, USA) were used for the measurement of TNF-alpha and IL-1beta levels according to the manufacturer's protocol. This type of ELISA kit works by the Sandwich-ELISA principle. Calculated concentrations of TNF-alpha and IL-1 beta were expressed as pg/ml in serum samples of the experimental groups.

To clarify the possible suppressing effects of *Smb* (34 mg/kg and 70 mg/kg daily), diclofenac sodium (5 mg/kg daily), and quercetin (25 mg/kg daily) on CFA induced inflammation of rats ELISA method was used. According to obtained results, all CFA-induced animals show a dramatic increase in both TNF-α and IL-1beta levels compared to the healthy control group ($p < 0.005$). Furthermore, all Smb, quercetin and diclofenac sodium-treated groups show the low level of TNF-alpha and IL-1 beta concentrations ($p < 0.005$, $p < 0.05$) compared to CFA induced negative control group. Very low levels of TNF-alpha and IL-1beta concentrations were observed in the healthy control group as expected. The CFA-induced negative control group has the highest TNF-alpha and IL-1 beta levels ($p < 0.005$) compared to other experimental groups. Smb treatment induced a significant and dose-dependent decrease in both TNF-alpha and IL-1 beta levels as compared to the negative control group ($p < 0.05$). Quercetin treated group also showed a significant decrease in TNF-alpha as compared to Smb treated group ($p < 0.05$); on the other hand, Smb treated group shows a significant decrease in IL-1 beta as compared to quercetin treated group. All the obtained results mentioned above were given in Figure 3.6 and Figure 3.7 with their statistical comparison between each group.

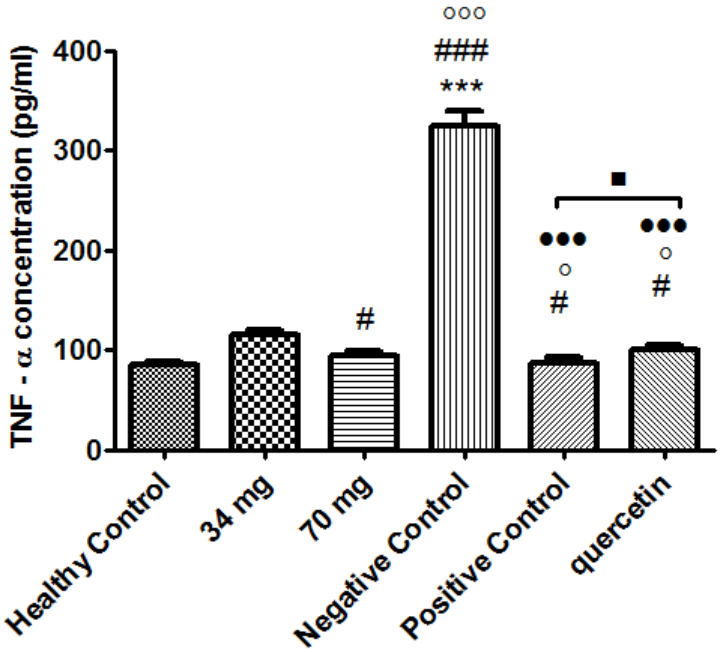


Figure 3.6. Serum TNF-alpha levels in all experimental groups. Values expressed as Mean ± SEM, n = 6, # P <0.05, with negative control, ### P< 0.005, ***P < 0.005 and °°° P < 0.005 negative control compared to all treated groups. ●●● P< 0.005 diclofenac sodium (positive control) and quercetin compared to CFA (negative control) #P < 0.05 with diclofenac sodium, 70 mg *Smb* and quercetin. ■ P < 0.05 diclofenac sodium with quercetin.

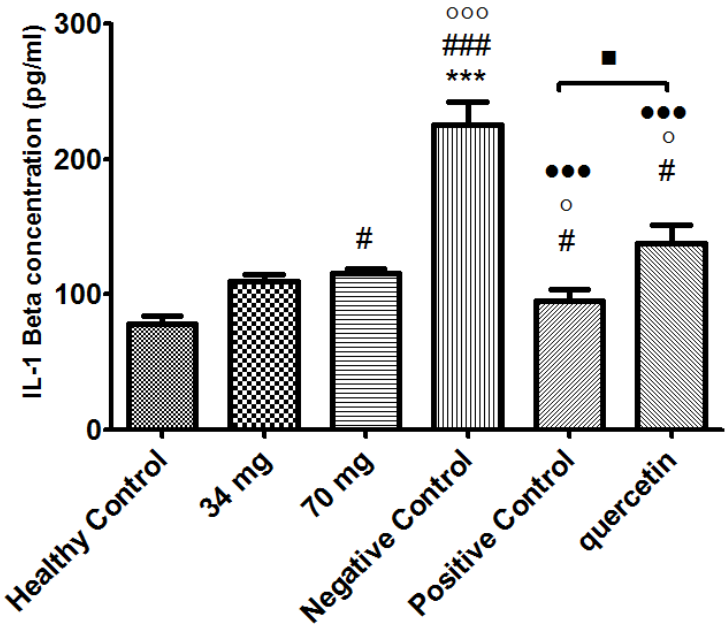


Figure 3.7. Serum IL-1 beta levels in all experimental groups. Values expressed as Mean ± SEM, n = 6, # P <0.05, with negative control, ### P< 0.005, ***P < 0.005 and °°° P < 0.005 negative control compared to all treated groups. ●●● P< 0.005 diclofenac sodium (positive control) and quercetin compared to CFA (negative control) #P < 0.05 with diclofenac sodium, 70 mg *Smb* and quercetin. ■ P < 0.05 diclofenac sodium with quercetin.

3.5 Histopathological Examinations of Joint Tissues

Histopathological changes of the joint tissue microsections were evaluated microscopically. These histopathological changes were defined as the cellular changes in the synovial membrane and in the ankle joints of rat tissues. The histological damage is basically defined as cartilage and bone destruction, mononuclear cell infiltration, and vascularity in synovial tissues.

In the healthy control group, histopathological examination of the ankle joints stained with hematoxylin & eosin revealed a normal histological appearance with structural integrity without inflammation. However, the negative control group (CFA

induced) exhibited thickening of the cartilages plate and synovial membrane with severe necrotic changes and inflammation.

Treatment with diclofenac sodium (5 mg/kg) has a mild recovery effect on arthritic signs. For example, multifocal bordered inflammatory sides were observed around the cartilage and synovial tissues. Treatment with *Smb* 34 mg/kg and *Smb* 70 mg/kg have lessened the severity of arthritis associated with CFA injection. There was a varying degree of reduction in inflammation and also regeneration and repairment area around the joint space with both the doses of plant extract. Treatment with quercetin has ameliorated the severity of arthritis with a significant reduction of "mean histopathological scores" for necrosis and inflammatory infiltration in comparison with the negative control (CFA induced) and the other treated groups. Mean histopathological scores of experimental groups were given in Figure 3.8 Mean histopathological scores of experimental groups. Values expressed as Mean ± SEM, n = 6, * $p < 0.05$ 34 mg Smb and *** $p < 0.005$ negative control compared to healthy control. ° $p < 0.05$ positive control and 70 mg Smb, °° $p < 0.01$ quercetin compared to the negative control

Histopathologically examined, and stained tissues were evaluated by a visual scoring system on a scale of 0-5 in terms of the severity of pathological damage of tissues. Scoring was done based on the four parameters, which are; edema, inflammation, bleeding, and necrosis.

Figure 3.9 Micro sections of histopathological examinations of ankle joints from the experimental groups stained with hematoxylin & eosin. A: Healthy control group B: Negative control group (CFA induced) C: Diclofenac sodium treated

Figure 3.10. Micro sections of histopathological examinations of ankle joints from the experimental groups stained with hematoxylin & eosin D: 34 mg Smb 34 mg treated group, E: 70 mg. Smb treated group, E:

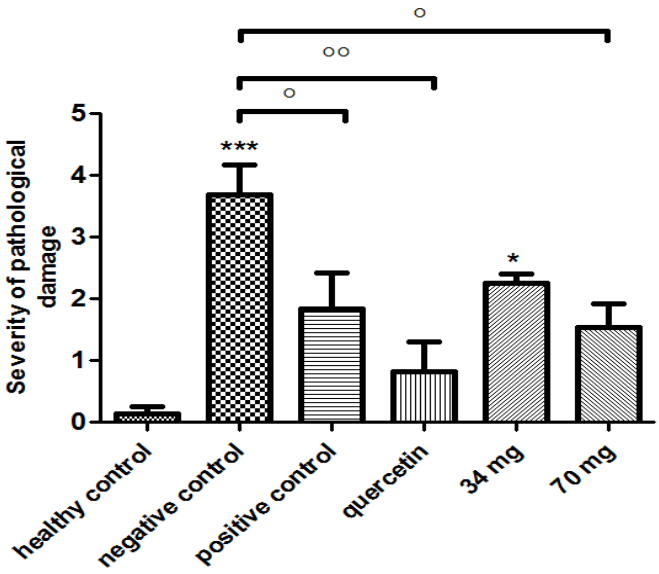


Figure 3.8 Mean histopathological scores of experimental groups. Values expressed as Mean ± SEM, n = 6, * p <0.05 34 mg *Smb* and *** p< 0.005 negative control compared to healthy control. ° p <0.05 positive control and 70 mg *Smb*, °° p <0.01 quercetin compared to the negative control.

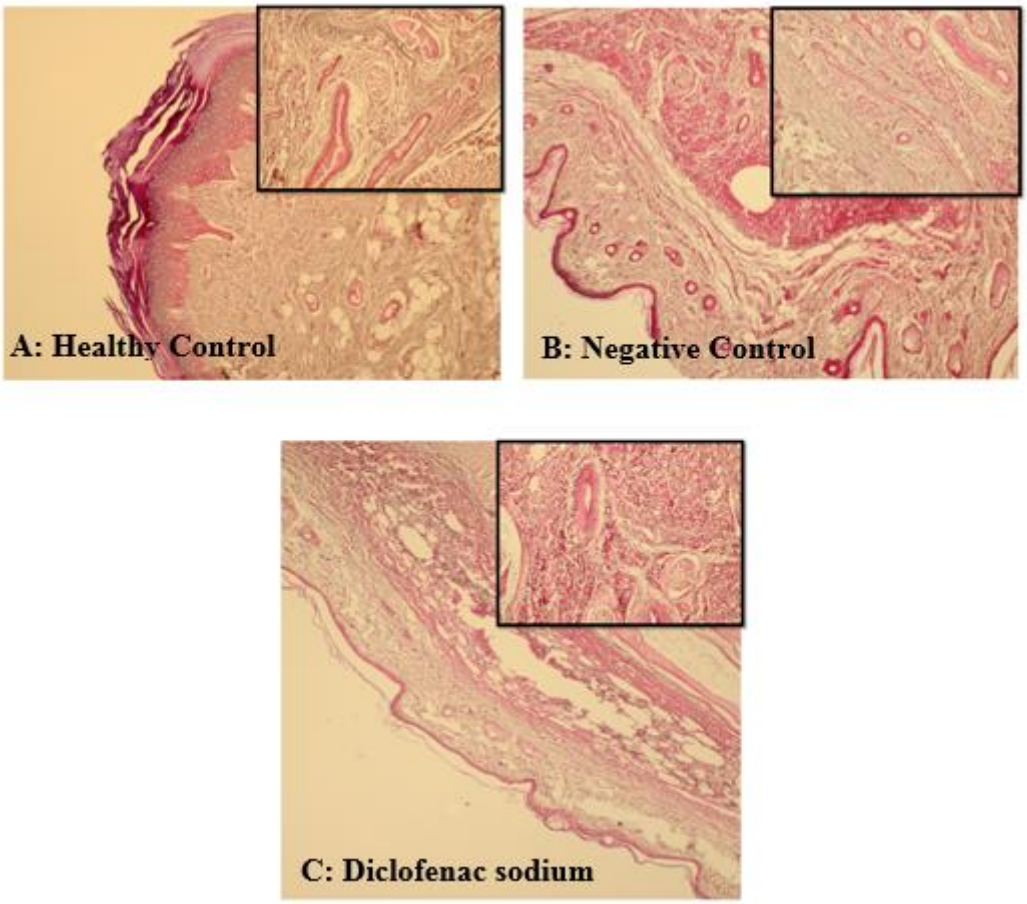


Figure 3.9 Micro sections of histopathological examinations of ankle joints from the experimental groups stained with hematoxylin & eosin. A: Healthy control group B: Negative control group (CFA induced) C: Diclofenac sodium treated group.

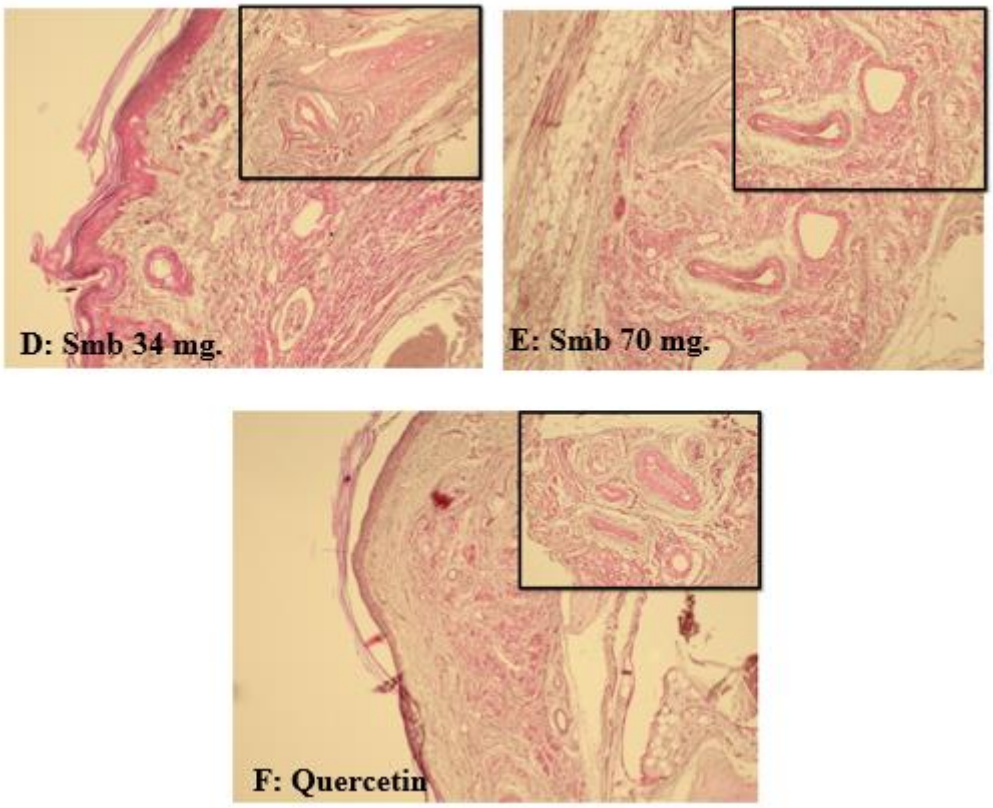


Figure 3.10. Micro sections of histopathological examinations of ankle joints from the experimental groups stained with hematoxylin & eosin D: 34 mg *Smb* 34 mg treated group, E: 70 mg. *Smb* treated group, E: Quercetin treated group.

3.6 Immunohistochemical Examinations of Joint Tissues

To examine inflammatory cell influx (TNF-alpha and IL-12) into the ankle joints and synovial cells, immunohistochemical examinations were done in joint tissues. The immunoperoxidase staining method was used for the examinations—this method was based on the analysis of the H&E stained area of sectioned slides. H&E stained areas refer to as the "immunoreactivity degree of TNF-alpha and/or IL-12" inflammatory cells.

Possible suppressing effects of *Smb*, quercetin, and diclofenac sodium, on chronic inflammation were characterized by examining the immunoreactivity degree of TNF-alpha and IL-12 inflammatory cells in joint tissues.

The infiltrated and stained TNF-alpha and IL-12 cells were quantified as cell numbers per unit area by counting on four high-power random fields (HPF, magnification*400). All image analysis, cell counts, and fluorescence measurements were performed "off-line" position on captured images and taken from the stained sections. The total numbers of positively identified immunoreactive TNF-alpha and IL-12 cells were counted manually in individual joint tissue sections from each experimental group. For immunohistochemical analysis, all obtained data were evaluated with % immunopositive stained areas and interpreted by Leica QWin image analysis software. Captured images taken from the stained sectioned or immunopositive stained areas of microsections for TNF-alpha and IL-12 were shown in Figure 3.11 and Figure 3.12, respectively.

According to the obtained data, in the healthy control group, very low levels of TNF-α and IL-12 expressions were observed among all experimental groups. The CFA-induced negative control group has the highest TNF-α and IL-12 expression levels ($p < 0.005$) compared to other experimental groups. 34mg/kg *Smb* treated group has a reducing effect on both TNF-α and IL-12 levels ($p < 0.005$). The same result was also observed ($p < 0.005$) for the diclofenac sodium-treated group. 70mg/kg *Smb* treated group has lower TNF-α and IL-12 expression level ($p < 0.05$) compared to 34mg/kg *Smb* treated and diclofenac sodium group. Quercetin treated group has the lowest TNF-α and IL-12 levels ($p < 0.005$) among all experimental groups. Graphical representation of the " % area values of TNF-α and IL-12 immunohistochemistry analysis of experimental groups" with their statistical comparison was given in Figure 3.13 and Figure 3.14, respectively.

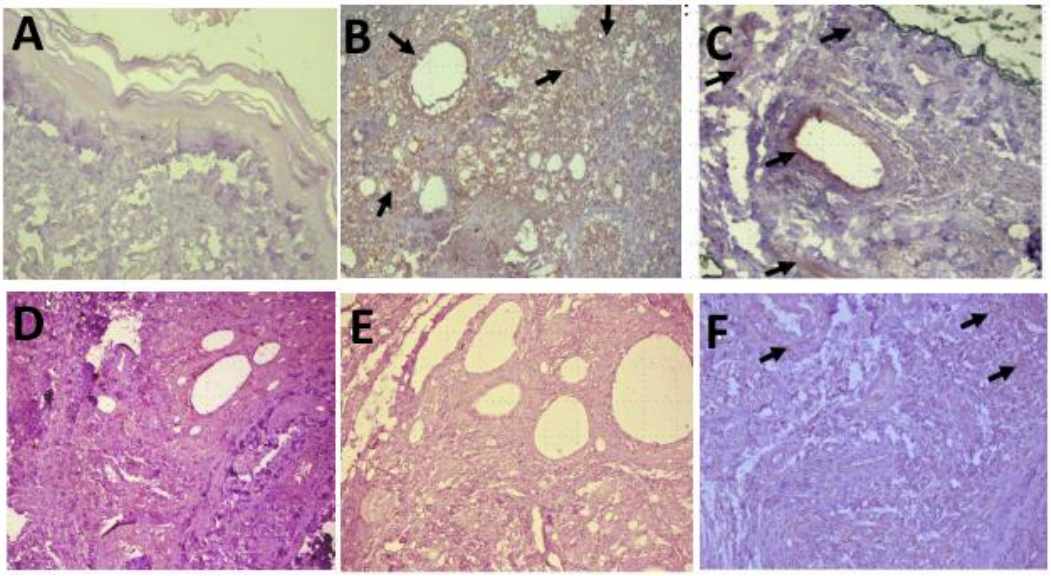


Figure 3.11. Immunopositive stained areas of microsections for TNF-α A: healthy control, B: 34 mg *Smb*, C: 70 mg *Smb*, D: CFA induced negative control, E: diclofenac sodium, F: quercetin.

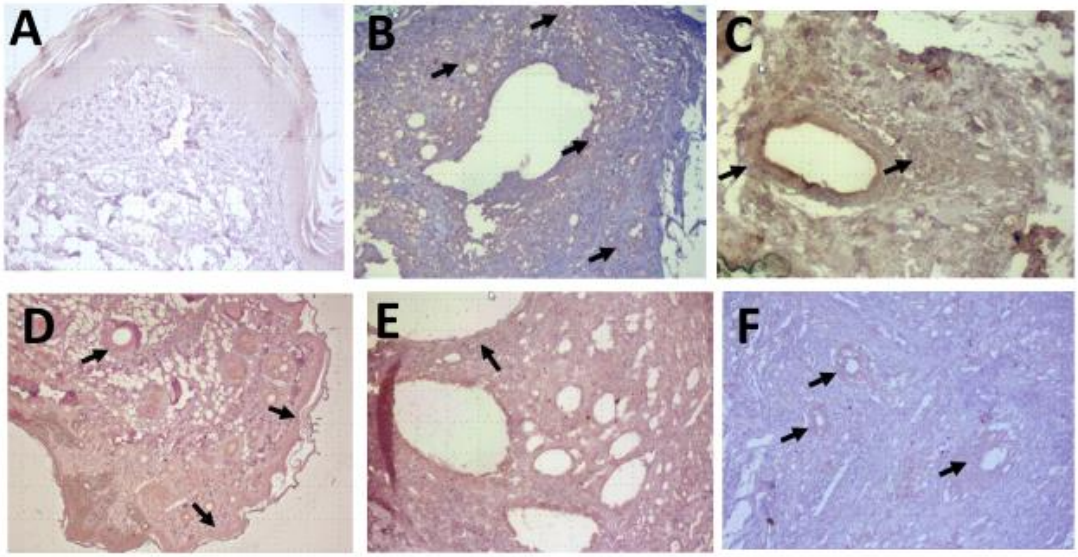


Figure 3.12. Immunopositive stained areas of microsections for IL-12 A: healthy control, B: 34 mg *Smb*, C: 70 mg *Smb*, D: CFA induced negative control, E: diclofenac sodium, F: quercetin.

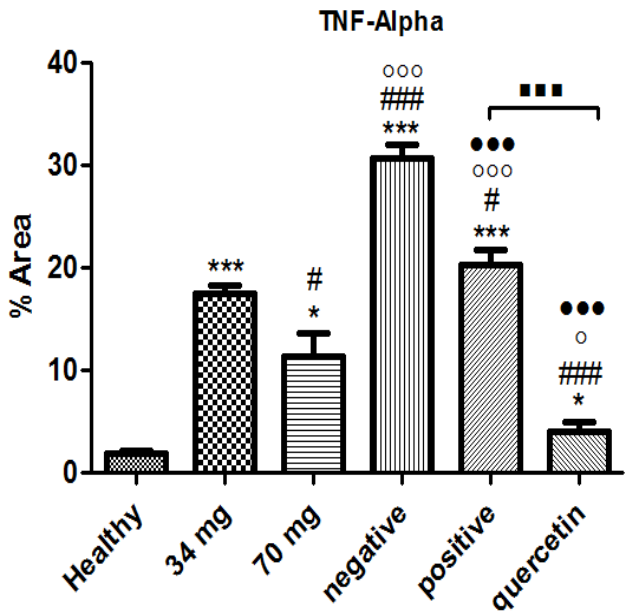


Figure 3.13. % Area values of TNF-α from the immunohistochemistry analysis of experimental groups. Values are expressed as mean ±S.D. analyzed by one-way ANOVA followed by Tukey comparison test. * p< 0.05, *** p< 0.005 compared with healthy control group. # p< 0.05, ### p< 0.005 compared with 34 mg. *Smb*. treated group. ooo p < 0.005, o p< 0.05 compared with 70 mg. *Smb*. treated group. ●●● p< 0.005 compared with negative control group. ■■■ p < 0.005 compared with positive control and quercetin treated group

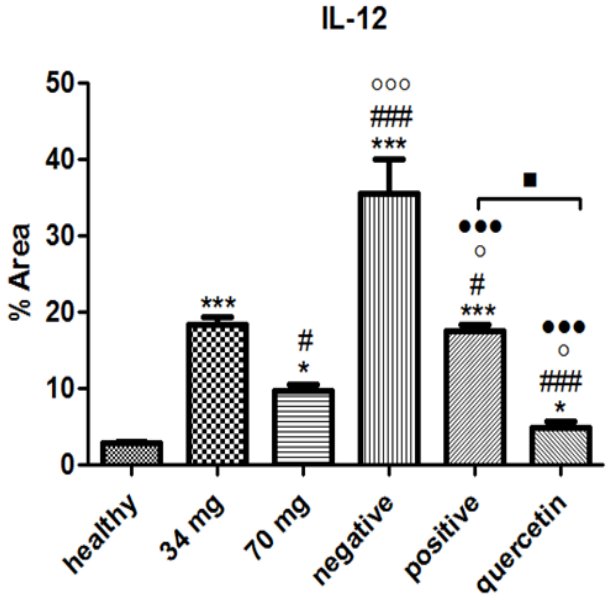


Figure 3.14. % Area values of IL-12 from the immunohistochemistry analysis of experimental groups. Values are expressed as mean ±S.D. analyzed by one-way ANOVA followed by Tukey comparison test. * p< 0.05, *** p< 0.005 compared with healthy control group. # p< 0.05, ### p< 0.005 compared with 34 mg. *Smb.* treated group. ooo p < 0.005, o p< 0.05 compared with 70 mg. *Smb.* treated group. ●●● p< 0.005 compared with negative control group. ■ p < 0.05 compared with positive control and quercetin treated group.

3.7 Protein Content and CYP450s Isoform Expression by Immunoblotting of Rat Liver Microsomal Fractions

Protein levels of the inflammation-induced rat liver microsomal fractions were measured by the "Western blotting" method. After the blotting step, protein band images were detected by chemiluminescence as described in the materials and methods section. Photographed immunoreactive protein band images were quantified by the densitometric scanning method using the Image J program. Before this step, band images were cropped and corrected for brightness and contrast. Beta-actin (43 kDa) house-keeping protein was

used as a protein loading control (internal standard). The ratio of each targeted band/β-actin was calculated and stated as the expression level of the target protein.

Investigation of the inflammation-related CYPs protein expressions of liver microsomal fractions after following treatments (*Sanguisorba minor subsp. balearica,* quercetin and Diclofenac sodium) were done with these selected CYP proteins; CYP1A2 (58 kDa), CYP3A4 (57 kDa), CYP2C9 (55 kDa) and CYP2E1 (57 kDa). Based on the broad literature survey, it is indicated that each of these selected CYP proteins has important roles during inflammatory and/or xenobiotic metabolism processes (Hammer et al., 2021; Mcgraw, 2014; Redlich et al., 2008; Shahabi et al., 2014; Walsky & Obach, 2004; Williams et al., 2000; Zanger & Schwab, 2013).

Changes in the CYP1A2, CYP3A4, CYP2C9, and CYP2E1 protein expression levels of inflammation-induced rat liver microsomal fractions after the following treatment were analyzed by the ratio of each targeted CYP protein band/β-actin. After subsequent analysis, this calculated ratio is stated as the expression level of the target protein. Beta-actin (43 kDa) house-keeping protein was used as a protein loading control (internal standard) for the analysis.

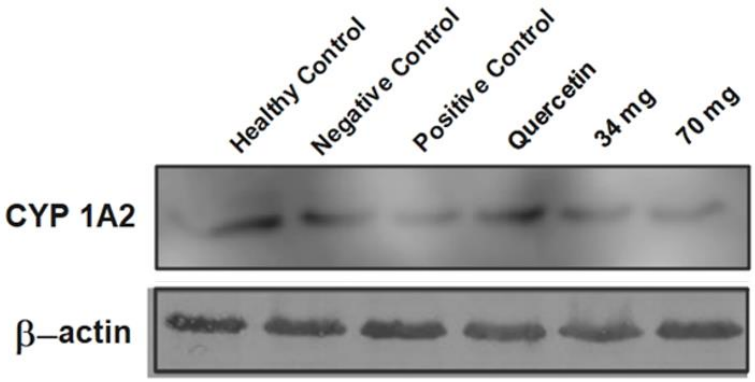
All obtained results were expressed as means with their "Standard Error of Means (SEM)." The correlation between treated and control animals was tested, and a possibility of 0.05 and 0.005 was stated depending on the statistical significance level.

3.7.1 Effects of *Sanguisorba minor subsp. balearica,* Quercetin and Diclofenac sodium on Rat Liver Microsomal CYP1A2 Protein Expressions

The effects of *Sanguisorba minor subsp. balearica,* quercetin, and Diclofenac sodium on inflammation-induced rat liver microsomal CYP1A2 protein expressions were analyzed by the western-blot technique. CYP1A2 protein levels for each treatment group were compared relative to β-actin protein expression. In addition, all treatment groups were compared with each other.

The intensity of each band was analyzed as an arbitrary unit, relative peak area (RPA), by using the Image J program. Relative expression levels were obtained from the densitometric values of the proteins of control and treated rats (Figure 3.15 (A)). The expression levels of CYP1A2 protein in all treated groups were lower than the healthy control group, and there is a statistically significant difference in terms of the CYP1A2 protein expressions between the control and all treated groups. The negative control group has a 0.23 fold decrease in CYP1A2 protein expressions compared to the healthy control group, the positive control group has a 0.55 fold decrease, and 34 and 70 mg/kg *Smb* treated groups have 0.15 and 0.22 fold decrease in CYP1A2 protein expressions, respectively. Quercetin group has 0.11fold decrease in CYP1A2 expression (Figure 3.15 (B)).

(A)



(B)

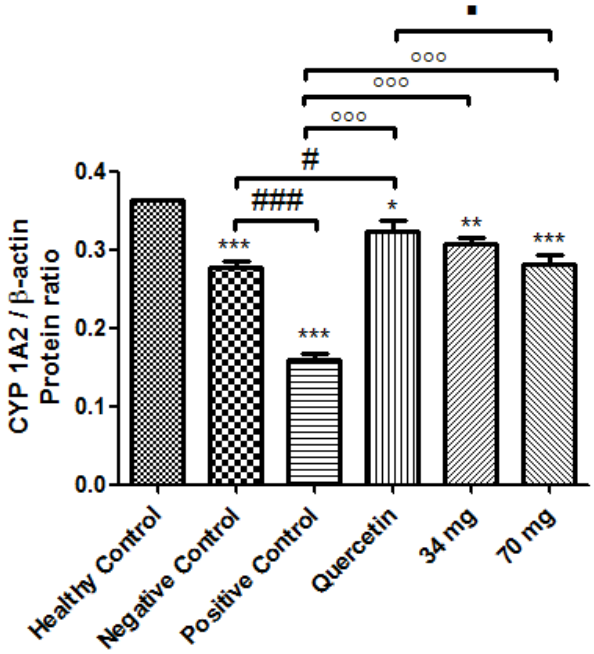


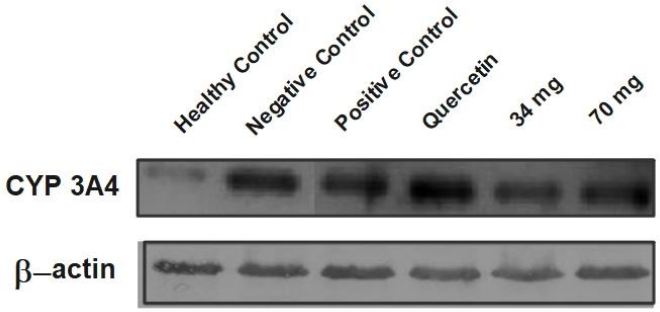
Figure 3.15. **(A)** Representative immunoblot protein bands of rat liver microsomal CYP1A2 (58 kDa) and β-actin (43 kDa) protein in all experimental groups. Healthy control, negative control (CFA treated), positive control (CFA+5mg/kg diclofenac sodium treated), quercetin (CFA+25mg/kg quercetin treated), 4mg (CFA+34mg/kg *Smb* treated) and 70mg (CFA+70mg/kg *Smb* treated) respectively (line 1→6). **(B)** Comparison of CYP1A2 protein expressions in all experimental groups (each group n=6). For each experimental group, band quantification was expressed as the mean ±SEM analyzed by one-way ANOVA followed by Tukey comparison test. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.005$ healthy control group compared with all treated groups. # $p< 0.05$, ### $p< 0.005$ compared with negative control, positive control and quercetin groups. ooo $p < 0.005$ compared with positive control, quercetin treated, 34 mg. *Smb.* and 70 mg. *Smb.* treated groups. ■ $p < 0.05$ compared with quercetin and 70 mg *Smb.* treated groups. Band quantification was expressed as the mean ±SEM of the relative intensity with respect to that of the internal control β-actin (43 kDa) protein.

3.7.2 Effects of *Sanguisorba minor subsp. balearica,* Quercetin and Diclofenac sodium on Rat Liver Microsomal CYP3A4 Protein Expressions

The effects of *Sanguisorba minor subsp. balearica,* quercetin, and diclofenac sodium on inflammation-induced rat liver microsomal CYP3A4 protein expression were analyzed by the western-blot technique. CYP3A4 protein levels for each treatment group were compared relative to β-actin protein expression. In addition, all treatment groups were compared with each other.

The intensity of each band was analyzed as an arbitrary unit, relative peak area (RPA), by using the Image J program. Relative expression levels were obtained from the densitometric values of the proteins of control and treated rats (Figure 3.16 (A)). The expression levels of CYP3A4 protein in all treated groups were higher than the healthy control group, and there is a statistically significant difference in terms of the CYP3A4 protein expressions between control and treated groups. The negative control group has a 6.4 fold increase in CYP3A4 protein expressions compared to the healthy control group ($p < 0.005$), positive control group has a 4.9 fold increase in CYP3A4 protein expressions ($p < 0.005$). 34 and 70 mg/kg *Smb* treated groups have 3.08 and 3.43 fold increase in CYP3A4 protein expressions, respectively ($p < 0.005$). Quercetin group has a 7.09 fold increase (Figure 3.16 (B)).

(A)



(B)

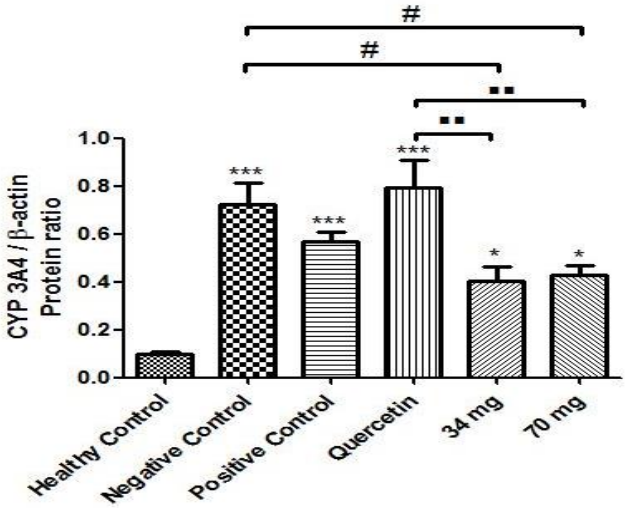


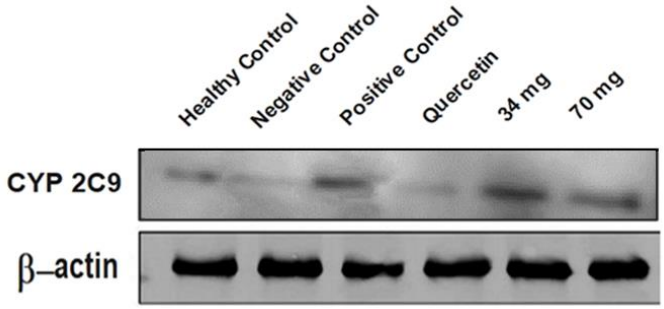
Figure 3.16. **(A)** Representative immunoblot protein bands of rat liver microsomal CYP3A4 (58 kDa) and β-actin (43 kDa) protein in all experimental groups. Healthy control, negative control (CFA treated), positive control (CFA+5mg/kg diclofenac sodium treated),quercetin (CFA+25mg/kg quercetin treated), 34mg (CFA+34mg/kg *Smb* treated) and 70mg (CFA+70mg/kg *Smb* treated) respectively (line 1→6). **(B)** Comparison of CYP3A4 protein expressions in all experimental groups (each group n=6). For each experimental group, band quantification was expressed as the mean ±SEM analyzed by one-way ANOVA followed by Tukey comparison test. Healthy control group compared with all treated groups. *** $p< 0.005$ compared with the positive control, negative control, and quercetin treated groups. * $p< 0.05$ compared with 34mg and 70mg *Smb* treated groups. # $p< 0.05$ compared with negative control, 34 mg and 70 mg *Smb* treated groups. ooo $p < 0.005$ compared with positive control, quercetin treated, 34 mg. *Smb.* and 70 mg. *Smb.* treated groups. ■■ $p < 0.01$ compared with quercetin, 34 mg. *Smb*. and 70 mg. *Smb*. treated groups. Band quantification was expressed as the mean ±SEM of the relative intensity with respect to that of the internal control β-actin (43 kDa) protein.

3.7.3 Effects of *Sanguisorba minor subsp. balearica,* Quercetin and Diclofenac sodium on Rat Liver Microsomal CYP2C9 Protein Expressions

The effects of *Sanguisorba minor subsp. balearica,* quercetin, and diclofenac sodium on inflammation-induced rat liver microsomal CYP2C9 protein expression were analyzed by the western-blot technique. CYP2C9 protein levels for each treatment group were compared relative to β-actin protein expression. In addition, all treatment groups were compared with each other.

The intensity of each band was analyzed as an arbitrary unit, relative peak area (RPA), by using the Image J program. Relative expression levels were obtained from the densitometric values of the proteins of control and treated rats (Figure 3.17(A)). The expression levels of CYP2C9 protein in positive control and 34mg and 70 mg/kg *Smb* treated groups were higher than healthy control rats, and there is a statistically significant difference in terms of the CYP2C9 protein expressions. Both negative control and quercetin-treated groups have a 0.64 fold decrease in CYP2C9 protein expressions compared to the healthy control group. On the other hand, the positive control group has a 0.3 fold increase in CYP2C9 protein expressions. 34 and 70 mg/kg *Smb* treated groups have 0.4 and 0.6 fold increase in CYP2C9 protein expressions, respectively ($p < 0.05$) (Figure 3.17 (B)).

(A)



(B)

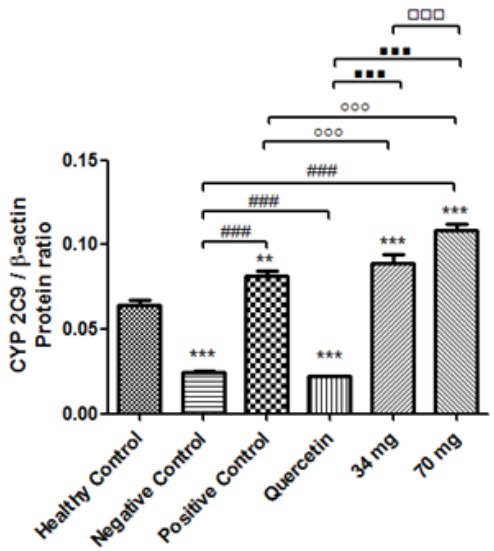


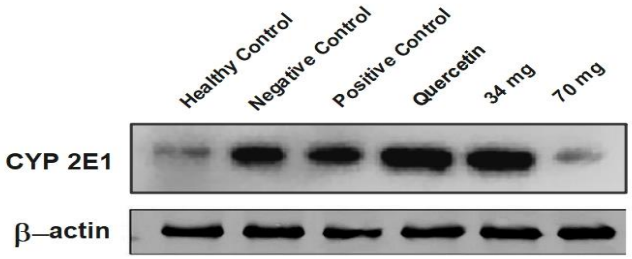
Figure 3.17. **(A)** Representative immunoblot protein bands of rat liver microsomal CYP2C9 (58 kDa) and β-actin (43 kDa) protein in all experimental groups. Healthy control, negative control (CFA treated), positive control (CFA+5mg/kg diclofenac sodium treated), quercetin (CFA+25mg/kg quercetin treated), 34mg (CFA+34mg/kg *Smb* treated) and 70mg (CFA+70mg/kg *Smb* treated) respectively (line 1→6). **(B)** Comparison of CYP2C9 protein expressions in all experimental groups (each group n=6). For each experimental group, band quantification was expressed as the mean ±SEM analyzed by one-way ANOVA followed by Tukey comparison test. Healthy control group compared with all treated groups ** $p < 0.01$, *** $p < 0.005$. ### $p < 0.005$ negative control compared with positive control quercetin and 70mg *Smb* treated groups. ooo $p < 0.005$ positive control group compared with 34mg and 70mg *Smb* treated groups. ■■■ $p < 0.005$ quercetin treated group compared with 34mg and 70mg *Smb* treated groups. □□□ $p < 0.005$ 34 mg *Smb* treated group compared with 70 mg *Smb* treated group. Band quantification was expressed as the mean ±SEM of the relative intensity with respect to that of the internal control β-actin (43 kDa) protein.

3.7.4 Effects of *Sanguisorba minor subsp. balearica,* Quercetin and Diclofenac sodium on Rat Liver Microsomal CYP2E1 Protein Expressions

The effects of *Sanguisorba minor subsp. balearica,* quercetin, and Diclofenac sodium on inflammation-induced rat liver microsomal CYP2E1 protein expressions were analyzed by the western-blot technique. CYP2E1 protein levels for each treatment group were compared relative to β-actin protein expression. In addition, all treatment groups were compared with each other.

The intensity of each band was analyzed as an arbitrary unit, relative peak area (RPA), by using the Image J program. Relative expression levels were obtained from the densitometric values of the proteins of control and treated rats (Figure 3.18 (A)). The expression levels of CYP2E1 protein in all treated animals were higher than in healthy control rats, and there is a statistically significant difference in terms of the CYP2E1 protein expressions between control and negative control, positive control, and quercetin treated groups. Negative control and positive control groups have 1.6 fold and 1.7 fold increase in CYP2E1 protein expressions, respectively, compared to the healthy control group ($p < 0.05$). On the other hand, the quercetin group has a 2.6 fold increase in CYP2E1 protein expressions ($p < 0.005$). 34 and 70 mg/kg *Smb* treated groups have 2.5 and 0.2 fold increase in CYP2E1 protein expressions, respectively (Figure 3.18 (B)).

(A)



(B)

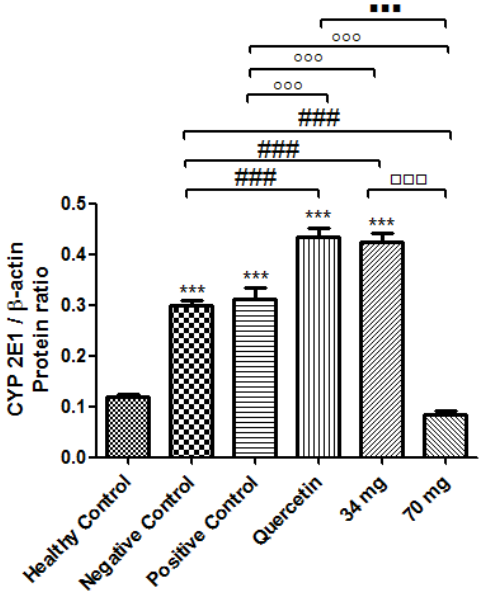


Figure 3.18. **(A)** Representative immunoblot protein bands of rat liver microsomal CYP2E1 (58 kDa) and β-actin (43 kDa) protein in all experimental groups. Healthy control, negative control (CFA treated), positive control (CFA+5mg/kg Diclofenac sodium treated), quercetin (CFA+25mg/kg quercetin treated), 34mg (CFA+34mg/kg *Smb* treated) and 70mg (CFA+70mg/kg *Smb* treated) respectively (line 1→6) **(B)** Comparison of CYP2E1 protein expressions in all experimental groups (each group n=6). For each experimental group, band quantification was expressed as the mean ±SEM analyzed by one-way ANOVA followed by Tukey comparison test. *** $p < 0.005$ healthy control group compared with all groups except 70 mg *Smb* treated group. ### $p < 0.005$ negative control compared with quercetin, 34mg and 70 mg *Smb* treated groups. ooo $p < 0.005$ positive control compared with quercetin, 34mg and 70 mg *Smb* treated groups. ■■■ $p < 0.005$ quercetin treated group compared with 70mg *Smb* treated group. □□□ $p < 0.005$ 34mg *Smb* treated group compared with 70mg *Smb* treated group. Band quantification was expressed as the mean ±SEM of the relative intensity with respect to that of the internal control β-actin (43 kDa) protein.

CHAPTER 4

DISCUSSION

Studies investigating the new biologically active compounds for the discovery of new therapeutic agents have been carried out around the world since the emergence of diseases. Drug development research has been going on for centuries, so the treatment of many diseases is possible today. Even some diseases have disappeared completely.

Since the human being, raw therapeutical materials originating from nature have been used for the treatment of the different types of diseases. In the last decade, these nature-derived products are named "Medicinal Plants" and have admitted great attention in the literature. Considering the side effects of conventional drugs, medicinal plants can be addressed as safe therapeutical agents due to their naturally derived compounds, such as phytochemicals (Abad et al., 2000;(Zbikowska et al., 2016). Phytochemical compounds are responsible for the therapeutic properties of medicinal plants. Phytochemicals or secondary metabolites produced by plants are classified into different groups. Some of these are polyphenols, flavonoids, alkaloids, and terpenoids (Ferreira et al., 2006; Gatto et al., 2013).

In the literature, different types of phytochemicals and herbal products have been investigated, and their therapeutical effects are proven both in the experimental animal model and/or cell line culture studies up to now. Number of mechanisms have been investigated to understand both their therapeutical properties and also inhibitory responses to the pathogenesis. (Ferreira et al., 2006; Guarrera & Savo, 2013), (Goun et al., 2002), (Cuccioloni et al., 2012), (Karkanis *et al.*, 2015). Mechanisms that attract the attention and are intensively studied in the literature are anti-proliferative mechanisms, mechanism of apoptosis, protection mechanisms

from oxidation, induction or inhibition of detoxification enzymes, regulatory mechanisms of the immune system, mechanisms in cellular signaling pathways, and targeting mechanisms of specific enzymes (Ranfa *et al.*, 2014).

Sanguisorba minor, in the family of Rosaceae, belongs to the *Sanguisorba* genus and consists of approximately 27 species. *Sanguisorba officinalis* L. (great burnet) and *Sansguisorba minor* Scop. (small burnet) are the most widespread and most well-known *Sanguisorba* species in the literature. Medicinal properties of Sanguisorba species have been known since the 16th century, and they have been used in folk medicine since then (Karkanis et al., 2015). *Sanguisorba minor*, known as a medicinal plant, is traditionally used in the treatment of diseases such as diarrhea, hemorrhoids, goiter in Anatolia. It has been determined that these therapeutic properties of *Sanguisorba minor* plants are originated from their high phytochemical content. (Bedoya et al., 2001; Ferreira et al., 2006; Gürbüz et al., 2005).

Sanguisorba minor plant is mentioned as a "Promising Medicinal Plant" due to its high amount of bioactive compounds (Guarrera & Savo, 2013). More than 120 phytochemical compounds belonging to *Sanguisorba* genus plants, especially *S. officinalis* and *S. minor*, have been identified, and major phytochemical compounds of plant extract have been described by different researchers (Ayoub, 2003), (Liu et al., 2005),(Cuccioloni et al., 2012),(Hu et al., 2012). These identified phytochemicals are grouped as phenolics, flavonoids, neolignans, and terpenoids. Ranfa et al., 2014, reported that *Sanguisorba minor* exhibited the highest total polyphenolic (258 mg/100g) content among the other *Sanguisorba* genus. Vanzani et al., 2011, also found that the number of flavonoids present in *Sanguisorba minor* was also particularly high (98.2 mmol total phenols/kg).

The first part of the present study includes qualitative and quantitative analysis of the phenolic content of the plant extract. Then extraction, lyophilization, and dissolution studies of *Smb* were carried out. The water-soluble plant extract was used in further experimental steps. Although phytochemical contents of the plant are

known, they may vary according to the regions where the plant is derived from. Therefore, quantitative determination of the active constituents of the plant needs to be performed.

HPLC analysis of *Smb* water extract showed that plant extract includes 4.1288 ppm ellagic acid and 2.5594 ppm gallic acid. There was also a low amount of quercetin hydrate and p-coumaric acid as 0.0657 and 0.0633 ppm, respectively. Catechin hydrate, coumarin, and kaempferol were other detected phenolic compounds, but their concentrations were less than ≤ 0.005ppm.

Extracts of the genus of *Sanguisorba minor* were compositionally characterized, and it was reported in a number of studies. Some of its therapeutical properties were also proved by these studies. Some of the described therapeutical properties are anti-viral, anti-ulcerogenic, anti-cancer, anti-acetylcholinesterase, radioprotective, antiallergic, antioxidant, immunomodulatory, and anti-inflammatory effects (Abad et al., 2000; Ferreira et al., 2006; Goun et al., 2002; Shin et al., 2002), (Cai et al., 2012; Ravipati et al., 2012; Yu et al., 2011; Zbikowska et al., 2016; L. Zhang et al., 2012). It is emphasized that the therapeutical effects of the *Sanguisorba* species, which have been studied extensively in the literature, were largely due to their polyphenolic and flavonoid content (Hachiya et al., 2001; Liu et al., 2005; Shin et al., 2002). In addition, it has also been shown that there is a relationship between the flavonoid content of the plant and its anti-inflammatory effects (Y. H. Kim et al., 2008; Ravipati et al., 2012; Yu et al., 2011).

Our HPLC results obtained from *Smb* water extract were in correlation with the results in the literature. HPLC data revealed that *Smb* extract is composed of a high amount of phenolic content (ellagic acid, gallic acid, and coumaric acid) as well as flavonoid (quercetin) content. Hence, in this study, we hypothesized that "Sanguisorba *minor* subsp. *balearica,* which has a high phenolic and flavonoid content, is more likely to have an anti-inflammatory effect on an inflammation-induced rat model," and we tested the hypothesize using different methods.

“Anti-inflammatory effect” of *Smb* was investigated in the present study. In the literature, a small number of studies were carried out on the anti-inflammatory effects of the *Sanguisorba* genus. In a case study, it was revealed that ethanolic extract of *Sanguisorba officinalis* exerts inhibitory effects on PGE production and suggests a potent anti-inflammatory activity mediated by NF-κB and AP-1 inhibitory properties (Yu T. et al., 2011). Anti-inflammatory effect of ethanolic extract of *Sanguisorba officinalis L.* on skin disorders was tested in human keratinocyte HaCaT cells, and the obtained data revealed that ethanolic extract of *Sanguisorba officinalis L.* exerts anti-inflammatory effects by suppressing the expression of TNF-α/IFN-γ stimulated chemokines and pro-inflammatory molecules in human keratinocyte HaCaT cell lines (Yang et al., 2015). In another study, anti-inflammatory effects of the aqueous extract of *Sanguisorba minor* subsp *balearica* were examined on an inflammation-induced rat model, and anti-inflammatory effects of *Smb* in a dose-dependent manner were observed on model animals (Arıhan, and Özkan, 2015).

Inflammation and inflammatory processes are normal physiological phenomena of the biological systems. In response to tissue injury, infection or tissue stress, or disfunction, biological systems alert a network of chemical signals. These chemical signal cascades then trigger two different physiological responses. One of them stimulates host defense against the infection or tissue repair response, and it is called “acute inflammatory response.” The acute inflammatory response is a temporary, reversible process. On the other hand, when metabolism adapts to this harmful condition, and it takes a long time, then it is called “chronic inflammation.” At this level, the normal inflammation process turns into a detrimental stage, and it has deleterious effects on tissue, organ, and systemic level (Wellen & Hotamisligil, 2005; Y. Zhang et al., 2016). It is a known fact today that chronic inflammation plays an important role in the pathogenesis of many diseases. The most important of them are; cancer, diabetes, obesity, and cardiovascular diseases (Chiurchiù & MacCarrone, 2011; Frey et al., 2015).

The inflammatory response is a properly working network controlled by the body’s extracellular molecules and regulators. These extracellular events work in a

correlation with the complex intracellular signaling pathways. In case of tissue injury or infection, epithelial and endothelial cells around these target areas release factors that induce the inflammatory cascade and immune response immediately. The first immune cell arrived at the target injured site are; neutrophils, followed by monocytes, lymphocytes (Natural Killer cells [NK-cells]), T-cells, B-cells and mast cells. Inflammation-mediated immune cells can differentiate into a specific type of inflammatory cells (Zhang J.M. & An J., 2007,(Libby, 2007; Slaviero, Clarke, & Rivory, 2003).

Cytokines are key inflammatory signaling molecules and have an important role during the inflammatory response. Two main types of cytokines have an important role in the regulation of inflammatory response; these are called "Pro-inflammatory cytokines" and "Anti-inflammatory cytokines." Pro-inflammatory cytokines are produced predominantly by activated macrophages and involved upregulation of the inflammation. The most abundantly contributory pro-inflammatory cytokines are; interleukin-1 (IL-1), interleukin-6 (IL-6), and Tumour Necrosis Factor (TNF-α). On the other hand, anti-inflammatory cytokines are a group of cytokines that control the proinflammatory cytokine response and help the resolution of inflammation. Major anti-inflammatory cytokines include interleukin-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13.

The second part of our study includes "Animal experiments." In this part of the study, we analyzed both the local and systemic effects of the treatments on model animals. We first created an environment of inflammation on model animals which is called "Complete Freund's Adjuvant (CFA). This model has been the most commonly used immunization stimulant method in the literature. It is used in many experimental studies to mimic chronic inflammatory diseases (Escobedo-Martínez et al., 2017; Ruckmani et al., 2018; Shi et al., 2015). Before further biochemical and immunohistochemical analysis, we evaluated the animal model whether it properly works or not. To be sure of similar inflammation degree after CFA injection on animals hind paw; paw thickness was measured by plethysmometer during acute and

subacute phase (on days 0 and 7) in all experimental groups. An increase in paw thickness was observed in all experimental groups except the healthy control group. On the 7th day after CFA injection, severe inflammation was observed on local injection sites of all animals. From this point, the "chronic phase of inflammation" has begun. At this stage, treatments were started orally (p.o.) for 21 consecutive days for all experimental groups. The decrease of chronic inflammation was ascertained by measuring the paw thickness periodically after the treatments on the 10th, 18th, 24th, and 28th days. Swelling of the CFA-injected hind paw was determined to follow the course of the disease.

After treatments, a decrease in paw thickness was observed both in the negative control (CFA) and treated groups. A decrease in paw thickness in all experimental groups on day 28 was found to be significant when compared to CFA control. *Smb* 70mg/kg and quercetin groups have similar and the highest decrease in paw thickness compared to diclofenac and *Smb* 34 mg/kg groups.

Based on this data, percentage inhibition of the paw thickness (paw edema) was calculated to determine which treatment group has the highest efficacy. Percentage inhibition of edema was calculated using the formula given below:

% decrease in foot thickness = $(1-T0/Tt)\times100$, where [T0 is the mean of foot thickness at day 0; Tt is the mean of foot thickness at a particular time].

Inhibitory effects of *Smb* extract at the doses of 34 and 70 mg/kg and diclofenac sodium (5 mg/kg) and quercetin (25 mg/kg) on paw edema were examined against the CFA-control group and found to be significant at $p<0.001$ and $p<0.05$ respectively. The highest percent of inhibition was expressed by quercetin and *Smb* 70 mg/kg group, especially at chronic phase at (70.08% and 61.23%, respectively) and even higher than diclofenac sodium (49%). *Smb* 34 mg/kg also shows more percent inhibition degree (56%) than diclofenac sodium group and followed *Smb* quercetin and 70 mg/kg group respectively. At this step of our study, we tested the local anti-inflammatory effects of treated reagents. Based on the obtained data, both of the plant extract doses (*Smb* 34 mg/kg, *Smb* 70 mg/kg),

antioxidant agent (quercetin 25 mg/kg), and non-steroidal anti-inflammatory drug (diclofenac sodium 5 mg/kg) caused a decrease in varying degrees on local injection area of inflammation.

From a similar study, conducted with *Sanguisorba minor Scop.* subsp.*muricata* (Spach) *Brig.* plant and examined its local anti-inflammatory effect on carrageenan-induced inflammation model rats. In this study, it was defined that, *Smb* plant extract at a dose of 25, 50, and 100mg/kg have an anti-inflammatory activity with 41.9, 76.4, and 83.4% degrees, respectively, and they concluded that, *Sanguisorba minor Scop.* subsp.*muricata* (Spach) *Brig*. has an anti-inflammatory effect on model animals in a dose-dependent manner (Arihan et al., 2015).

After examining the local effects of all the treatments on experimental groups, we started to examine the possible systemic effects of the treatments on all experimental groups. For this purpose, ELISA analysis, immunohistopathological and immunohistochemical analysis, and finally, western blot analysis was performed.

The degree of recovery of chronic inflammation can be detected from the concentration of specific cytokine markers in the serum samples. TNF-alpha and IL-1 beta were targeted inflammatory markers in this study. After the sacrification step, isolated blood serum samples were analyzed in all experimental groups on day 28. Enzyme-Linked Immunosorbent Assay (ELISA) method was used for the determination of TNF-alpha and IL-1 beta levels from isolated serum samples.

According to obtained results, all CFA-induced animals show a dramatic increase in both TNF-α and IL-1beta levels compared to the healthy control group ($p < 0.005$). Furthermore, all *Smb*, quercetin, and diclofenac sodium-treated groups show a low level of TNF-alpha and IL-1 beta concentrations ($p < 0.005$, $p < 0.05$) compared to CFA induced negative control group. Very low levels of TNF-alpha and IL-1beta concentrations were observed in the healthy control group as expected. The CFA-induced negative control group has the highest TNF-alpha and IL-1 beta levels ($p < 0.005$) compared to other experimental groups. *Smb* treatment induced a

significant and dose-dependent decrease in both TNF-alpha and IL-1 beta levels as compared to the negative control group ($p < 0.05$). Quercetin treated group also showed a significant decrease in TNF-alpha as compared to *Smb* treated group ($p < 0.05$); on the other hand, *Smb* treated group shows a significant decrease in IL-1 beta as compared to quercetin treated group.

In one of the similar studies in the literature (Ruckmani et al., 2017), the anti-inflammatory activity of *Sesamum indicum* seed extract on CFA-induced rats was examined. IL-6 and TNF-alpha were chosen as the target proinflammatory cytokines. It was observed that during chronic inflammation state (on day 28), both IL-6 and TNF-alpha levels were significantly increased compared to healthy control rats. In the CFA group, the levels of these two cytokines were 68.04± 0.84pg/ml and 66.5± 0.04 pg/ml, respectively. 800 mg/kg *SI* seed extract-treated groups showed a decrease in both IL-6 and TNF-alpha levels, 39.87± 0.29 pg/ml and 40.46± 0.19 pg/ml, respectively, and this decrease is found to be greater than diclofenac sodium treated group and equal to antioxidant treated group.

In another study, a medicinal plant extract ingredient "gastrodin" derived from the *Gastrodia elata Blume,* where the protein expression levels of TNF-alpha and IL-6 of CFA induced mice were examined. Fourteen days after the CFA injections, western blot analyses were performed on paw tissue fractions. Obtained data revealed that both TNF-alpha and IL-6 protein expression levels are significantly induced (2.04± 0.25 and 3.19± 0.31 fold respectively) compared to the control group. Furthermore, gastrodin treatment during the course of 14 days inhibited the protein levels of TNF-alpha and IL-6 (1.49 ± 0.29 and 0.92± 0.16 fold of the control) and IL-6 (2.02± 0.24 and 1.35± 0.16 fold of the control) compared to the control group (Sun et al., 2016).

In another case study, the effect of vitamin B analog thiamine and riboflavin on CFA induced rat model was examined for proinflammatory cytokine production and/or inhibition degree. According to obtained data from the referred study, TNF-alpha and IL-6 concentration were increased in paw tissue fraction on day 15 after

the treatments with riboflavin and thiamine, in addition, concentration levels of these two cytokines were reduced to a similar extent (Menezes et al., 2017).

Based on also similar studies on different plant species in the literature, it can be concluded that the *Smb* extract at doses of 34 and 70 mg/kg inhibited the pro-inflammatory cytokines, TNF-alpha, and IL-1 beta levels resulting in their anti-inflammatory activities. These obtained results are also comparable to both the diclofenac sodium and quercetin treatments.

Immunohistopathological and immunohistochemical examinations are the second part of our study by which effects of the treatments at the tissue level were analyzed. Histopathological changes are reliable evidence of tissue injury during pathological conditions. In our study, histopathological changes of joint tissue microsections were evaluated microscopically and were defined as the cellular changes in the synovial membrane and in the ankle joints of rat's joint tissues. The histological damage is basically defined as cartilage and bone destruction, mononuclear cell infiltration, and vascularity in synovial tissues.

The histopathological examination of the ankle joints stained with H&E revealed that the healthy control group showed normal histological appearance with structural integrity without any inflammatory sign. However, the negative control group (CFA) exhibited thickening of the cartilages plate and synovial membrane with severe necrotic changes and inflammation. Treatment with diclofenac sodium (5 mg/kg) has a mild recovery effect on arthritic signs; multifocal bordered inflammation was observed around the cartilage and synovial tissues. Treatment with *Smb* 34 and *Smb* 70 mg/kg ameliorated the severity of arthritis (joint destruction) associated with CFA injection. Histopathological recovery was defined as the varying degree of reduction in inflammation and also regeneration and repaired of the area between joint spaces. Treatment with quercetin lessened the severity of arthritis with a significant reduction of "mean histopathological scores" accompanying necrosis and inflammatory infiltration in comparison with the negative control (CFA) and other treated groups.

Histopathologically examined, and stained tissues were evaluated by a visual scoring system on a scale of 0-5 in terms of the severity of pathological damage of tissues (Bais et al., 2017; Impellizzeri et al., 2016). Scoring was done according to four parameters which are; edema, inflammation, bleeding, and necrosis. Comparison of the histopathological score on the stained tissue area in all experimental groups was given in Table 4.1.

Table 4.1 Histopathological Score of the Stained Tissue Area of Experimental Groups

Group Name	Edema	Inflammation	Bleeding	Necrosis	Comment
Healthy Control	0	0	0	0	Any arthritic sign
Negative Control (CFA)	3.5	4	2.5	4.75	Extensive necrotic changes in muscular tissue.Severe necrosis and inflammation in the vessel wall. Inflammation covering all interstitial tissue

Table 4.1 (continued) Histopathological Score of the Stained Tissue Area of Experimental Groups

Positive Control 5 mg/kg diclofenac sodium	1.8	3	1.5	1	Multifocal bordered inflammation. Decreased level of blood vessel necrosis. Inflammatory changes only injection area
Quercetin 25 mg/kg	1.8	1.5	0	0	There isn't any pathologic pattern except a minimal level of edema. Low level of inflammation btw interstitial space
Smb 34 mg/kg	2.5	2.5	2	2	Localized inflammation. Intraplantar side. Capsule formation bordered the side: regeneration and repair.
Smb 70 mg/kg	2.2	2.2	1	0.75	High level of repair. New fibroblast formation. Minimal and bordered level of inflammation

To examine inflammatory cell influx (TNF- α and IL-12) into the ankle joints and synovial cells, immunohistochemical examinations were performed. Possible suppressing effects of *Smb*, quercetin, and diclofenac sodium, on chronic inflammation were characterized by examining the immunoreactivity degree of TNF-α and IL-12 inflammatory cells. H&E stained areas refer to as the "immunoreactivity degree of TNF-α and IL-12" inflammatory cells. The infiltrated and stained TNF-α and IL-12 cells were quantified as cell number per unit area, and all obtained data were evaluated with % immunopositive stained areas.

According to obtained data, in the healthy control group, very low levels of TNF-α and IL-12 expressions were observed among all experimental groups. The CFA-induced negative control group has the highest TNF-α and IL-12 expression levels ($p< 0.005$) compared to other experimental groups. 34mg/kg *Smb* treated group has a reducing effect on both TNF-α and IL-12 levels ($p< 0.005$). The same result was also observed ($p< 0.005$) for the diclofenac sodium-treated group. 70mg/kg *Smb* treated group has lower TNF-α and IL-12 expression level ($p< 0.05$) compared to 34mg/kg *Smb* treated and diclofenac sodium group. Quercetin treated group has the lowest TNF-α and IL-12 levels ($p< 0.005$) among all experimental groups.

In a recent study carried out with the same animal model, the anti-inflammatory effect of Melittin on chronic prostatitis induced by CFA was examined. It was found that intraprostatic injection of CFA resulted in the accumulation of inflammatory cells in the prostate tissue. CFA rats were shown to exhibit an extensive influx of inflammatory cells, of which the quantified level was significantly higher than that of the healthy control group ($p< 0.005$). In this study, melittin, which is a nature-derived substance, demonstrated a profound anti-inflammatory effect that resulted in less severe infiltration of inflammatory cells (Lin et al., 2017).

According to histopathological and immunohistochemical examinations, it can be concluded that *Smb* extract with both doses has reducing effects on the

severity of histopathological scores as compared with the CFA control group. This effect is also comparable to diclofenac sodium and quercetin treatment (quercetin has the best-reducing effect among all treatment groups). Basically, immunohistochemical examinations reveal the underlying mechanism of the recovery of histopathological scores. Immunohistochemical examinations indicated that *Smb* extract with both the doses alleviated infiltration of inflammatory cells, TNF-alpha and IL-12, as well as ameliorated the joint destruction. These immunohistochemical results were also comparable to both diclofenac sodium and quercetin-treated groups.

In addition to their role in xenobiotic metabolism, CYP450 enzymes metabolize numerous endogenous substrates, including steroid hormones, vitamin D, cholesterol, and fatty acids, to biologically active mediators. Most of the CYP enzymes belonging to CYP1, 2, 3, and 4 families are responsible for the metabolism of endogenous substrates and the majority of xenobiotics. Basically, some CYP450 enzymes are "constitutively" expressed forms, and some are "inducible" expressed. Constitutively expressed CYP enzymes catalyze the reaction involving "endogenous substrates." On the other hand, inducible expressed CYP enzymes often have "various substrate specificities" and have a role in the biotransformation of 75% of xenobiotics and pharmaceuticals in human metabolism (Tesfaigzi et al., 2001; Zanger & Schwab, 2013).

It is well-established that acute inflammatory stimuli alter hepatic CYP expression. Cytokines suppress hepatic CYP expression via pre-translational mechanisms. It was found in a study that administration of IL-6, IL-1 beta, and TNF-alpha in human hepatocyte culture significantly decreased the expression levels of CYP1A2, CYP2C, CYP2E1, and CYP3A (Morgan, 2001). In the early stages of the inflammatory response, cytokines, mainly IL-1 beta, TNF-alpha, and IL-6, infiltrated into the systemic circulation. Hepatocytes are influenced by these inflammatory cytokines and alter the synthesis of plasma proteins. Pieces of evidence from the number of in vitro studies revealed that cytokines, especially proinflammatory cytokines, are responsible for the down-regulation of the hepatic CYP genes.

Obtained data from the comparable studies revealed that CYP3A4 expression was downregulated by IL-6 family, IL-1, and IL-6. Besides, CYP2C9 was down-regulated by IL-6 and TGF. It was also found that CYP2B6 level is sensitive to the presence of IL-6 and IFN cytokines (Aitken et al., 2006; Chun et al., 2001; Jenny & Caro, 2011; Jover, Bort, Gómez-Lechón, & Castell, 2002; Kalsotra et al., 2003; Kawase, Wada, & Iwaki, 2013).

Cytochrome P450 (CYP450) monooxygenase-dependent metabolism of Arachidonic acid (AA) produces biologically active eicosanoids enzymatically or non-enzymatically. During arachidonic acid metabolism, activation and inactivation of inflammatory response is conducted by CYP epoxygenase and ω-hydroxylase pathways epoxygenases of AA is carried out by the CYP2C and CYP2J isoforms and generates EETs. Conversely, ω-hydroxylases of AA are carried out by the CYP4A and CYP4F subfamilies and produce HETEs. Both EETs and HETEs regulate numerous physiological and pathophysiological processes in biological systems. In fact, they have antagonist effects on different physiological processes. Regulation of "inflammatory response" by CYP450 monooxygenases is an interesting topic in this study. Accumulating evidence has demonstrated that CYP-derived metabolites of the arachidonic acid pathway by epoxyeicosatrienoic acids (EETs) have potent anti-inflammatory properties by reducing the stimulation of leukocyte adhesion by cytokines via inhibition of nuclear factor-kB (NF-kB). Conversely, 20-HETEs have pro-inflammatory properties which activate NF-kB signaling and induces expression of cellular adhesion molecules and cytokines, thereby promoting the inflammation (Fan & Roman, 2017; Fava & Bonafini, 2018; Node et al., 1999; Panigrahy et al., 2010; Quispe-Tintaya, 2017; Shahabi et al., 2014; Thomson et al., 2012; Xu et al., 2011).

In this study, effects of *Sanguisorba minor* subsp. *balearica,* quercetin, and diclofenac sodium on inflammation-induced rat liver microsomal CYP1A2, CYP3A4, CYP2C9, and CYP2E1 protein expressions were analyzed by western blot technique. These targeted CYP protein levels for each treatment group were

quantified by densitometry, normalized to β-actin, and expressed relative to the healthy control group. In addition, all treatment groups were compared with each other.

Expression levels of CYP1A2 protein in treated animals were lower than in healthy control, and there is a statistically significant difference in terms of the CYP1A2 protein expressions between control and all treated groups. The positive control group (5mg/kg diclofenac sodium treated) have 0.55 fold decrease in CYP1A2 protein expressions compared to the healthy control group, negative control group have 0.23 fold decrease, on the other hand, quercetin (25mg/kg quercetin treated) group have 0.11 fold decrease and 34 and 70 mg/kg *Smb* treated groups have 0.15 and 0.22 fold decrease in CYP1A2 protein expressions respectively.

CYP1A2 is a constitutively expressed CYP isoform in the liver. The number of non-genetic factors has an acting effect on the CYP1A2 expression level. Due to its relatively high expression in the liver, CYP1A2 plays a significant role in the metabolism of several drugs. Endogenous substrates of CYP1A2 include; arachidonic acid, prostaglandin, estrogens, melatonin, and retinoic acid. Treatment of the CYP1A2 substrates with specific drugs leads to sensitivity for drug interactions, and this caused an inhibitory effect on the CYP1A2 expression (Kawase et al., 2013; Klein, Winter, Turpeinen, Schwab, & Zanger, 2010; Nebert & Karp, 2009).

In our study, all the treated groups have a decrease in CYP1A2 protein expression. Observed results revealed that, in a chronic inflammatory state, treatment of two different doses of *Smb*, diclofenac sodium and quercetin, have an inhibitory effect on CYP1A2 expression. The underlying mechanism of this result could be explained by its role in arachidonic acid metabolism as well as its sensitivity to the drug treatment on this pathway. So based on this assumption, we can say *Smb* extract with both doses might show their anti-inflammatory effects via arachidonic acid metabolism by the epoxygenase pathway. In other aspect diclofenac sodium also

show their anti-inflammatory effect via arachidonic acid metabolism, except, it uses cyclooxygenase (COX) pathway. All these mentioned mechanisms under the inflammatory state may have a suppressor effect on the hepatic CYP1A2 expression. It could also be interpreted that quercetin might also show its anti-inflammatory effects via arachidonic acid metabolism.

The effects of *Sanguisorba minor* subsp. *balearica,* quercetin, and diclofenac sodium on inflammation-induced rat liver microsomal CYP3A4 protein expressions were analyzed by the western-blot technique. CYP3A4 protein levels for each treatment group were quantified by densitometry, normalized to β-actin, and expressed relative to the healthy control group. In addition, all treatment groups were compared with each other. The expression levels of CYP3A4 protein in treated animals were higher than in healthy control rats, and there is a statistically significant difference in terms of the CYP3A4 protein expressions between control and all treated groups. The positive control group (5mg/kg diclofenac sodium treated) have 4.96 fold increase in CYP3A4 protein expressions compared to the healthy control group ($p < 0.005$), on the other hand, negative control and quercetin (25mg/kg quercetin treated) groups have 6.4 and 7.09 fold increase in CYP3A4 protein expressions respectively ($p < 0.005$). 34 and 70 mg/kg *Smb* treated groups have 3.08 and 3.43 fold increase in CYP3A4 protein expressions, respectively ($p < 0.005$).

CYP3A4 is a major drug-metabolizing CYP450 isozyme. In the majority of individuals, it is abundantly expressed in the liver. CYP3A4 has an efficient role in the catabolism of several endogenous steroid hormones and bile acids also. CYP3A4 has an important function during inflammation. In the course of the inflammatory response, its downregulation is mediated by specific cytokines via JAK/STAT pathway (Bai et al., 2020; Chun, Park, & Yang, 2003; Jover et al., 2002; Sevrioukova & Poulos, 2017; Walsky & Obach, 2004; Zanger & Schwab, 2013).

In this study, all the treated groups have a significant increase in CYP3A4 protein expression. Observed results revealed that in a chronic inflammatory state, two different doses of *Smb,* diclofenac sodium, and quercetin treatment stimulate

CYP3A4 expression. The underlying mechanism of this result could be explained, its function during the inflammatory response. Normally, under inflammatory conditions, expression levels of CYP3A4 are decreased. Based on the results obtained, it could be interpreted that; in this current study on day 28, after the treatments, there wasn't a sufficient amount of specific cytokines in the circulatory system, which have a suppressing effect on the CYP3A4 expression. If we evaluate these results through its major drug-metabolizing CYP characteristics, it could be interpreted that, under experimental conditions, CYP3A4 isozymes perceive these applied substances as xenobiotics and/or drugs metabolites, and it leads to an increase in its expression level.

The effects of *Sanguisorba minor* subsp. *balearica,* quercetin, and diclofenac sodium on inflammation-induced rat liver microsomal CYP2C9 protein expressions were analyzed by the western-blot technique. CYP2C9 protein levels for each treatment group were quantified by densitometry, normalized to β-actin, and expressed relative to the healthy control group. In addition, all treatment groups were compared with each other. The expression levels of CYP2C9 protein in positive control and 34 and 70mg/kg *Smb* treated groups were higher than healthy control rats, and there is a statistically significant difference in terms of the CYP2C9 protein expressions. Both negative control and quercetin-treated groups have a 0.64 fold decrease in CYP2C9 protein expressions compared to the healthy control group. On the other hand, the positive control group has a 0.3 fold increase in CYP2C9 protein expressions. 34 and 70 mg/kg Smb treated groups have 0.4 and 0.6 fold increase in CYP2C9 protein expressions, respectively ($p < 0.05$).

Substrates of CYP2C9 are weakly acidic substances, such as non-steroidal anti-inflammatory drugs. CYP2C9 metabolizes endogenous substances including, steroid hormones as well as arachidonic acid. CYP2C9 catalyzes the arachidonic acid epoxygenation; it belongs to the "CYP epoxygenases" and has a critical role in arachidonic acid metabolism during the inflammatory state. It favors inactivation of the inflammatory response by the production of epoxyeicosatrienoic acids (EETs)

mediators (Coller et al., 2002; Fava & Bonafini, 2018; Redlich et al., 2008; Theken et al., 2011; Xu et al., 2011; H. Zhou, Josephy, Kim, & Guengerich, 2004; S. Zhou et al., 2003).

In our study, the expression level of CYP2C9 under experimental conditions increased after both *Smb* treatments as well as diclofenac sodium treatment. Increase expression in the diclofenac sodium group could be explained by its metabolic pathway via arachidonic acid by cyclooxygenases. On the other hand increase in the expression level of CYP2C9 after *Smb* treatment could be explained by its strong relationship with the epoxygenase pathway during arachidonic acid metabolism under inflammatory conditions. A high expression level of CYP2C9 inactivates inflammatory response via ETTs production. Based on this obtained interpretation, we can say *Smb* extract with both doses could have anti-inflammatory effects via the stimulation of the CYP epoxygenase pathway. Diclofenac sodium, which is a member of NSAID chosen in our study, shows this anti-inflammatory effect via the COX pathway.

The effects of *Sanguisorba minor* subsp. *balearica,* quercetin, and diclofenac sodium on inflammation-induced rat liver microsomal CYP2E1 protein expressions were analyzed by the western-blot technique. CYP2E1 protein levels for each treatment group were quantified by densitometry, normalized to β-actin, and expressed relative to the healthy control group. In addition, all treatment groups were compared with each other. The expression levels of CYP2E1 protein in all treated animals were higher than in healthy control rats, and there is a statistically significant difference in terms of the CYP2E1 protein expressions between control and negative control, positive control, and quercetin treated groups. Negative control and positive control groups have 1.6 fold and 1.7 fold increase in CYP2E1 protein expressions, respectively, compared to the healthy control group ($p < 0.05$). On the other hand, the quercetin group has a 2.6 fold increase in CYP2E1 protein expressions ($p < 0.005$). 34 and 70 mg/kg Smb treated groups have 2.5 and 0.2 fold increase in CYP2E1 protein expressions, respectively.

CYP2E1 displays a substrate specificity for low molecular weight molecules. Industrial chemicals, environmental toxicants, and procarcinogens are the main substrates of CYP2E1. Endogenous substrates of CYP2E1 are; fatty acid oxidation products (lauric acid, acetone). CYP2E1 also has an important role in ethanol oxidation. During ethanol oxidation by CYP2E1, reactive oxygen species (ROS) generation occurs, which contributes to the damage of liver cells. Because of its substrate specificity, it is admitted that CYP2E1 has a strong connection with toxicological processes. Due to its role in the metabolic activation of procarcinogens and chemical toxicants, it is associated with cancer and intensively investigated in the literature around this topic (Bolt, Roos, & Thier, 2003; Caro & Cederbaum, 2004; Kobayashi et al., 2002; Lu & Cederbaum, 2007; Neafsey et al., 2009; Trafalis, Panteli, Grivas, Tsigris, & Karamanakos, 2010).

In our study, all the treated groups have an increase in CYP2E1 protein expression level. Observed results revealed that in a chronic inflammatory state, two different doses of *Smb,* diclofenac sodium, and quercetin treatment stimulate CYP2E1 expression. The underlying mechanism of this result could be explained by its substrate specificity. Actually, under inflammatory conditions, an increase in its expression level is an expected result. CYP2E1 also has a strong connection with toxicological processes. Based on these results, it could be interpreted that; under experimental conditions, CYP2E1 isozymes converted these administered bioactive compounds and drugs into other metabolites in the liver. It is possible that these metabolites triggered the CYP2E1 activity in a correlation. It also leads to an increase in the CYP2E1 expression level. We do not have enough data on the anti-inflammatory effect of *Smb* extract to be interpreted through CYP2E1.

In a case study, the effects of the medicinal plant *Schisandra chinensis (SC)* on the activity and protein expression of CYP1A2, CYP3A4, and CYP2E1 were examined in rats. It was found that treatment with single or multiple doses of *SC* extract induced CYP3A4 enzyme activity and inhibited CYP1A2 enzyme activity. On the other hand, CYP2E1 enzyme activity was induced after treatment with a

single dose. These results obtained from this case study are very similar to those in our study (Su et al., 2013).

CHAPTER 5

CONCLUSION

The present study was aimed to investigate the possible therapeutic effects of *Sanguisorba minor* subsp *balearica* extracts against inflammatory conditions in rats**.** *Sanguisorba minor* subsp*. balearica (Smb)* belongs to the *Sanguisorba* genus in the family Rosaceae, known as a medicinal plant in Anatolia and has been used traditionally. Several scientific investigations of the *Sanguisorba* genus have been carried out, and these studies demonstrated their high quantity of phytochemical content and also their therapeutic potential.

Inflammation and inflammation-related disorders are areas of interest and are studied extensively in the literature. In that, chronic inflammation provides a unifying pathophysiological mechanism underlying many chronic diseases. Non-steroidal anti-inflammatory drugs are the main therapeutical agents for the current treatment approach to chronic inflammation. Since these drugs have some adverse effects on the gastrointestinal and cardiovascular system, there is a need to discover new therapeutical agents which have a high safety profile and low adverse effects for the treatment of chronic inflammation.

Cytochrome P450 enzymes (CYP450) are intracellular hemeproteins that activate molecular oxygen for the oxidative metabolism of lipophilic organic chemicals. CYP450s play a crucial role in the metabolism of thousands of endogenous and exogenous chemical compounds in biological systems. It was found that downregulation of some of the CYP450 isoforms is a pathophysiological consequence of the inflammatory process and inflammatory stimuli cause important changes in the activity and expression level of various forms of CYP450s.

There is no data available in the literature to clarify the possible effects of *Smb* on CYP450s expression and their relation with the anti-inflammatory response. Therefore, this current study aims to clarify *in vivo* possible anti-inflammatory effects of medicinal plant *Smb,* which modulate the specific proinflammatory cytokines activity and protein expression level on targeted CYP450 on an inflammation-induced rat model.

In this study, firstly, the polyphenolic content of *Smb* was analyzed by the HPLC method, then the inflammation-induced rat model was created and tested. After these initial stages, animal studies were conducted with the treatments of six different groups (healthy control, CFA control, CFA+5mg/kg Diclofenac sodium treated, CFA+25mg/kg quercetin treated, CFA+34mg/kg *Smb* treated, and CFA+70mg/kg *Smb* treated). The last stage of the experimental part included the biochemical, immunohistopathological and immunohistochemical, and also protein immunoblotting analysis on selected liver microsomal CYP450 (CYP1A2, CYP3A4, CYP2C9, CYP2E1) and pro-inflammatory cytokine molecules (TNF-alpha, IL-1 beta, and IL-12).

According to the HPLC analysis, *Smb* water extract includes high amounts of ellagic and gallic acid, whose concentrations are 4.1288 ppm. and 2.5594 ppm. respectively. Low amounts of quercetin hydrate and p-coumaric acid were also detected. The second part of the study includes "animal experiments." In this part of the study, we first created an environment of inflammation on model animals. To be sure of similar inflammation degree after CFA injection, paw thickness was measured by plethysmometer in all experimental groups. An increase in paw thickness was observed in all experimental groups except the healthy control group. At this stage, treatments were started orally (p.o.) for consecutive 21 days for all experimental groups. After the treatment period, a decrease in paw thickness was observed in both the negative control (CFA) and treated groups. *Smb* 70mg/kg and quercetin groups have similar and the highest decrease in paw thickness compared to diclofenac and *Smb* 34 mg/kg groups.

The degree of recovery of chronic inflammation can be detected by measuring the concentration of specific cytokine markers in the serum samples. ELISA method was used for the determination of TNF-alpha and IL-1 beta levels in the serum samples. According to ELISA results, all CFA-induced animals showed a dramatic increase in both TNF-α and IL-1beta levels compared to the healthy control group ($p < 0.005$ and $p < 0.005$, respectively). Furthermore, *Smb*, quercetin, and diclofenac sodium-treated groups showed a low level of TNF-alpha and IL-1 beta ($p < 0.005$) compared to the inflammation (CFA) induced group. Very low levels of TNF-alpha and IL-1beta concentrations were observed in the healthy control group as expected.

Immunohistopathological and immunohistochemical examinations were performed to understand the effects of treatments on tissue level. In our study, histopathological examination of the ankle joints revealed that; healthy control group showed normal histological appearance with structural integrity without any inflammatory sign. However, the negative control group (CFA) exhibited thickening of the cartilages plate and synovial membrane with severe necrotic changes and inflammation. Treatment with Diclofenac sodium has a mild recovery effect on arthritic signs. Treatment with *Smb* 34 mg/kg and *Smb* 70 mg/kg ameliorated the severity of arthritis associated with CFA injection. Treatment with quercetin ameliorated the severity of arthritis with a significant reduction of "mean histopathological scores" in comparison with the negative control and other treated groups. To examine inflammatory cell influx (TNF-alpha and IL-12) into the ankle joints and synovial cells, immunohistochemical examinations were done. According to obtained data, in the healthy control group, very low levels of TNF-α and IL-12 expressions were observed among all experimental groups. The negative control group has the highest TNF-α and IL-12 expression levels compared to other experimental groups. 34mg/kg *Smb* treated group has a reducing effect on both TNF-α and IL-12 levels. The same result was also observed for the diclofenac sodium-treated group. 70mg/kg *Smb* treated group has lower TNF-α and IL-12 expression

level compared to 34mg/kg *Smb* treated and Diclofenac sodium group. Quercetin treated group has the lowest TNF-α and IL-12 levels among all experimental groups.

Protein expression levels were analyzed by the western-blot technique. Expression levels of CYP1A2 protein in treated animals were lower than in healthy control rats. The negative control group has a 0.23 fold decrease in CYP1A2 protein expressions compared to the healthy control group, the positive control group has a 0.55 fold decrease, and 34 and 70 mg/kg Smb treated groups have 0.15 and 0.22 fold decrease in CYP1A2 protein expressions, respectively. The expression levels of CYP3A4 protein in treated animals were higher than in healthy control rats. The positive control group has a 4.4 fold increase in CYP3A4 protein expressions compared to the healthy control group; on the other hand, negative control and quercetin groups both have 6.6 fold increase in CYP3A4 protein expressions. 34 and 70 mg/kg *Smb* treated groups have 2.8 and 3.6 fold increase in CYP3A4 protein expressions, respectively. The expression levels of CYP2C9 protein in positive control and 34 and 70mg *Smb* treated groups were higher than in healthy control rats. Both negative control and quercetin-treated groups have a 0.64 fold decrease in CYP2C9 protein expressions compared to the healthy control group. On the other hand, the positive control group has a 0.3 fold increase in CYP2C9 protein expressions. 34 and 70 mg/kg Smb treated groups have 0.4 and 0.6 fold increase in CYP2C9 protein expressions, respectively ($p< 0.05$). Expression levels of CYP2E1 protein in all treated animals were higher than in healthy control rats. Negative control and positive control groups have 1.6 fold and 1.7 fold increase in CYP2E1 protein expressions, respectively, compared to the healthy control group ($p< 0.05$). On the other hand, the quercetin group has a 2.6 fold increase in CYP2E1 protein expressions ($p< 0.005$). 34 and 70 mg/kg Smb treated groups have 2.5 and 0.2 fold increase in CYP2E1 protein expressions, respectively.

All the presented pieces of evidence in this study point out that; *Smb* with doses 34 and 70 mg/kg has an anti-inflammatory effect comparable to diclofenac sodium and quercetin. This action could be explained by its inhibitory effect of the release of proinflammatory cytokines TNF-alpha, IL-1 beta, and IL-12. The

underlying mechanism of this inhibitory effect could be explained by the changes in CYP-mediated eicosanoid metabolism under inflammatory conditions after *Smb* treatment. Which also leads to a shift in specific CYP450 protein expression. Observations from this study suggested that modulating CYP-mediated eicosanoid metabolism may be a novel anti-inflammatory therapeutical strategy for a given therapeutical agent. At this point, *Smb* could show their anti-inflammatory effect by this metabolic pathway.

From the past decades, many different therapeutic approaches such as prostaglandin, glucocorticoids, and COX-2 inhibitors have been used to ameliorates the deleterious effects of inflammation. Beyond having good therapeutical effects of the conventional anti-inflammatory drugs, these agents also have reported numerous side effects (Tabas et al., 2013), (Blumenthal et al., 2017). Hence, *Smb* extract can be further evaluated for its use as an effective future alternative to conventional anti-inflammatory drugs. Observations from this study also suggested that modulating CYP-mediated eicosanoid metabolism may be a novel anti-inflammatory therapeutical strategy for a given therapeutical agent, but the detail of this pathway in vivo has not been investigated. Hence, *Smb* extract should be further evaluated at this aspect for its use as an effective alternative to anti-inflammatory drugs.

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APPENDICES

A. Appendix A

T.C.
SAĞLIK BİLİMLERİ ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURUL-GÜLHANE KARARI

ETİK ONAYI GEÇERLİLİK SÜRESİ: 36 Ay	KARAR TARİHİ	: 28 Mart 2017
	KARAR SAYISI	: 17/13

Ortadoğu Teknik Üniversitesi Biyolojik Bilimler Bölüm Başkanlığından **Prof.Dr.Orhan ADALI'nın 28.03.2017 gün ve ETİK-2017/12 no'lu "'Tıbbi Bitki Sanguisorba Minör Subsp. Balearica'nın Anti-Enflamatuvar Etkinliğinin Enflamasyon İndüklenmiş Rat Modelinde CYP450 ve Antioksidan Enzimler Düzeyinde Araştırılması"** isimli başvurusu değerlendirilmiştir.

Araştırmadaki Deney Hayvanlarının Tür, Sayı ve kullanım amaçları projenin deney hayvanlarına ilişkin yönleri SBÜ-HADYEK Gülhane Yönergesinin 10 uncu maddesinde belirtilen "Hayvan Deneyleri ile İlgili Etik İlkeler" dikkate alınarak hazırlandığı saptanmıştır.

Yönergede belirtilen "Başvuruda İstenilen Bilgilere" uygun olarak hazırlanan Proje, Etik Kurul'un **28 Mart 2017 gün ve 3 sayılı** toplantısında değerlendirilmiş ve çalışmanın 36. **adet Rat** ile yapılmasına oy birliği / ~~oy çokluğu~~ ile uygun / ~~uygun değildir~~ / şartlı olarak uygun kararı verilerek **kabul edilmiştir.** / **kabul edilmemiştir.**

Başkan Ömer AZALI Prof.Dr.	Üye Cüneyt GÖKSOY (İZİNLİ) Prof.Dr.
Üye Metin ŞENÇİMEN Prof.Dr.	Üye İlker TAŞÇI Prof.Dr
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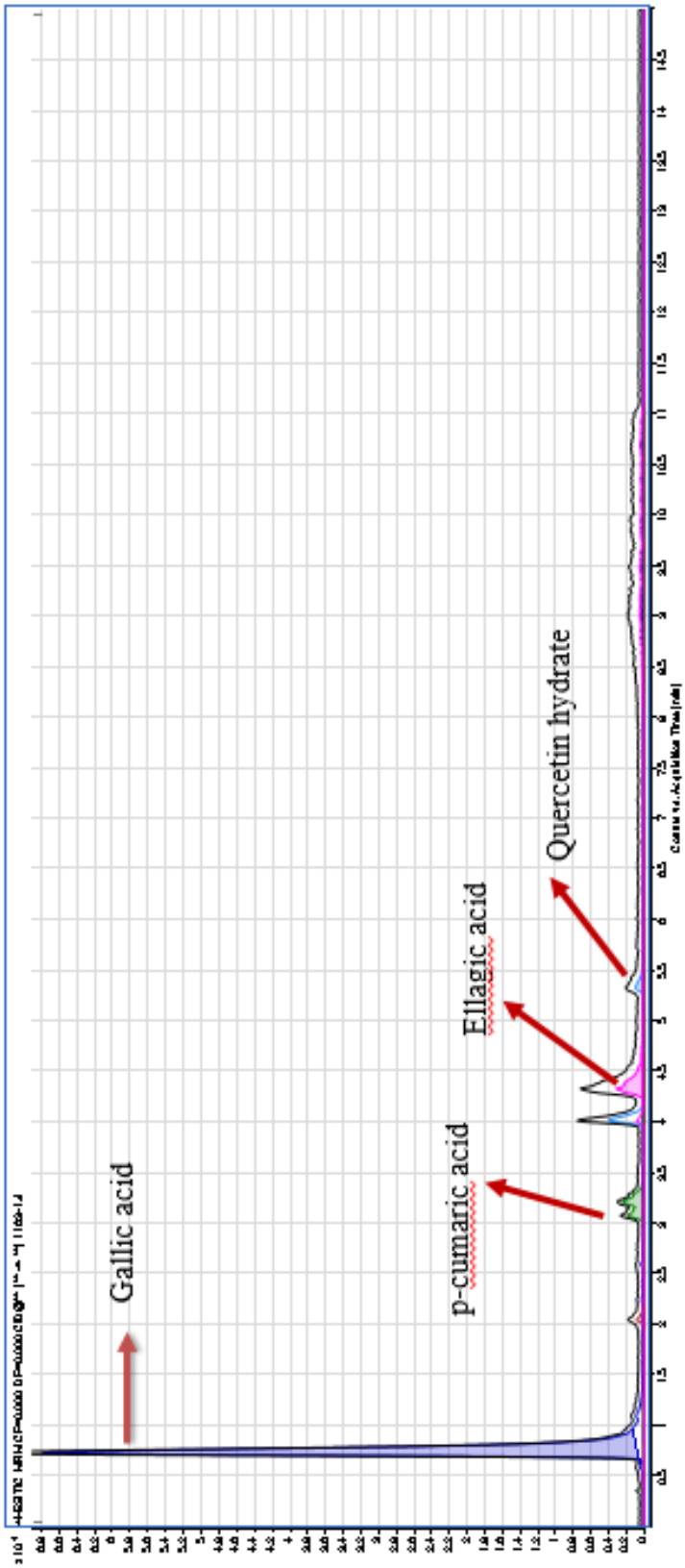
*SBÜ HADYEK Gülhane Yönergesinin 7.Maddesinin 7. Fıkrasına göre "Şartlı Olarak Uygun" kararı verilen projeler, SBÜ-HADYEK-G'nin belirlediği ve kararında belirttiği bir süre boyunca "Hayvan Refahı Birimi" tarafından izlenir. "Hayvan Refahı Birimi" istenen şartların sağlanıp sağlanmadığına ilişkin SBÜ-HADYEK-G'ye rapor verir. SBÜ-HADYEK-G, bu raporu değerlendirdikten sonra başvuruyu nihai karara bağlar.

B. Appendix B



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WORK EXPERINCE

Year	Place	Enrollment
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2009-2018	METU	Research Assistant
2004-2008	Novartis Pharmaceuticals	Clinical Research Associate

FOREIGN LANGUAGES

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PROJECTS

Molecular characterization of the effects of COX-2 inhibitor group drugs on colon cancer cells by FTIR Spectroscopy	ÖYP-DPT Project	2011 - 2014
Medicinal plant Sanguisorba minor subsp. Investigation of the anti-inflammatory activity of balearica at the level of CYP450 and pro-inflammatory cytokines in an inflammation-induced rat model.	ÖYP-DPT Project	2015 - 2019

PUBLICATIONS

1- INAN GENÇ A., GENÇLER ÖZKAN A.M., ADALI O., KUL O., "*Sanguisorba minor* subsp. *Balearica* inhibit production of cytokines in a chronic model of inflammation induced by complete Freund's adjuvant", Toxicology Letters, 314 (S1) S1–S339 (2019). (International)

2- INAN GENÇ A., GOK S., BANERJEE S., SEVERCAN F.; "Valdecoxib recovers the lipid composition, order and dynamics in colon cancer cell lines independent of COX-2 expression", Toxicology Letters, 280S, S224 (2017). (International)

3- INAN GENÇ A., GOK S., BANERJEE S., SEVERCAN F.; "Valdecoxib Recovers the Lipid Composition, Order and Dynamics in Colon Cancer Cell Lines: An ATR-FTIR Spectroscopy Study", Applied Spectroscopy, Vol. 71(1), 105–117, (2016). (International)

POSTER PRESENTATIONS IN NATIONAL AND INTERNATIONAL CONGRESSES

1-INAN GENÇ A., GENÇLER ÖZKAN A.M., ADALI O., KUL O., *Sanguisorba minor subsp. Balearica* inhibit production of cytokines in a chronic model of inflammation induced by complete Freund's adjuvant; September 2019. EUROTOX 2019 (55rd Congress of European Societies of Toxicology) **(International)**

2- INAN GENÇ A., GENÇLER ÖZKAN A.M., ADALI O., KUL O. Proinflammatory Cytokine Mediated Anti-inflammatory Effect of Sanguisorba minor subsp. Balearica on Inflammation Induced Rat Model; May 2019. International Molecular Medicine Symposium by the Bosphorus. **(International)**

3- INAN GENÇ A., GOK S., BANERJEE S., SEVERCAN F.; Selektif COX-2 Inhibitörü Valdekoksib'in Kolon Kanserinde Protein Parametreleri Üzerine Etkilerinin ATR-FTIR Spektroskopisi ile Yorumlanması; Kasım 2017. 2. Ulusal Proteomik Kongresi – TuPA. **(Ulusal)**

4- INAN GENÇ A., GOK S., BANERJEE S., SEVERCAN F.; Valdecoxib recovers the lipid composition, order and dynamics in colon cancer cell lines independent of cox-2 expression; September 2017. EUROTOX 2017. (53rd Congress of European Societies of Toxicology) **(International)**

5- INAN GENÇ A., GOK S., BANERJEE S., SEVERCAN F.; Molecular Characterization of the Effects of Valdecoxib on Colon Cancer Cell Lines (HT29 & SW620) Using ATR-FTIR Spectroscopy; July 2014. Conference: Frontiers in Medical Sciences: Diabetes, Cancer and Their Connection. **(International)**

AREAS OF INTEREST

- Toxicology
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- Drug Metabolism
- Molecular Pharmacology

CERTIFICATES

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DNA Damage, Repair and Tandem Mass Spectrometry Training	Dokuz Eylul Univ. Faculty of Medicine, Dept. of Medical Biochemistry and Molecular Medicine.	5 -8 June 2018
Embryonic Stem Cell Culture Training	TÜBİTAK Marmara Research Center Gen Eng. and Biotechnology Institute	4 – 8 June 2012
Experimental Animal Use in Scientific Research Course	Gulhane Military Medical Academy Animal Experiments Ethics Committee	21 November – 2 December 2011

TEZ İZİN FORMU / THESIS PERMISSION FORM

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TEZİN TÜRÜ / DEGREE: **Yüksek Lisans** / Master [ ] **Doktora** / PhD [X]

1. **Tezin tamamı dünya çapında erişime açılacaktır.** / Release the entire work immediately for access worldwide. [X]
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A copy of the Decision of the Institute Administrative Committee will be delivered to the library together with the printed thesis.

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