THE EFFECT OF \( \gamma \)-CARYOPHYLLENE ON ISOPROTERENOL-INDUCED MYOCARDIAL INFARCTION

Farah M. Jamal Laham

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United Arab Emirates University

College of Medicine and Health Sciences

Department of Pharmacology

THE EFFECT OF β-CARYOPHYLLENE ON ISOPROTERENOL-INDUCED MYOCARDIAL INFARCTION

Farah M. Jamal Laham

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Pharmacology and Toxicology)

Under the Supervision of Dr. Shreesh Kumar Ojha

May 2020
Declaration of Original Work

I, Farah M. Jamal Laham, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “The Effect of β-Caryophyllene on Isoproterenol-Induced Myocardial Infarction”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Shreesh Kumar Ojha, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Cannabinoid type 2 receptors (CB2), a key member of the endocannabinoid system has recently emerged as a crucial therapeutic target for cardiovascular diseases (CVDs). Downregulation of CB2 receptors has been witnessed in various cancers, neurological and cardiovascular disorders. Thus, the activation of CB2 receptors may protect against ISO induced myocardial infarction (MI) in rats. The present study investigates the cardioprotective effect of a selective cannabinoid type 2 receptor agonist β-caryophyllene (BCP), a dietary phytocannabinoid and a natural bicyclic sesquiterpene against isoproterenol (ISO)-induced MI in rats. Male albino Wistar rats were pre- and co-treated with β-caryophyllene (50 mg/kg, orally) twice daily for 10 days along with the subcutaneous injection of ISO (85 mg/kg) at an interval of 24 h for two days (9th and 10th day). AM630 (1 mg/kg), a CB2 receptor antagonist was injected intraperitoneally as a pharmacological challenge prior to BCP treatment to demonstrate CB2 receptor-mediated cardioprotective mechanisms of BCP. ISO induced MI showed a significant decline in cardiac function, elevated levels of serum cardiac marker enzymes and enhanced oxidative stress markers with increased lipid peroxidation. Isoproterenol also induced pro-inflammatory cytokines release following activation of the nuclear factor kappa-B. Furthermore, a significant rise was also observed in the levels of inflammatory mediators, namely cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in ISO-challenged rats. Additionally, Isoproterenol also increased expression of proapoptotic (Bax, and active caspase-3) proteins along with the decreased expression of anti-apoptotic protein, Bcl2 and Bcl-xL in the myocardium. β-caryophyllene treatment resulted in significant protective effects on all biochemical and molecular parameters analyzed.
Histopathological and ultrastructural evidence was found in line with these findings. Treatment with AM630, a potent CB2 receptor antagonist abrogates protective effects of BCP on all the biochemical and molecular parameters analyzed in ISO induced MI in rats. Thus, this study revealed that BCP protects the myocardium against ISO induced MI by attenuating oxidative stress, apoptosis and inflammation and the underlying mechanism of this protection is the activation of CB2 receptors. CB2 receptor selective compounds may provide a new potential class of cardioprotective drugs. The pharmacophore of these compounds could be used for synthesizing leads in drug discovery and development.

**Keywords**: Myocardial infarction, Phytochemical, Natural product, Cannabinoids, β-Caryophyllene, Cannabinoid receptor type 2, Isoproterenol, Catecholamine.
Title and Abstract (in Arabic)

تأثير بيتا-كاريوفايلين (β-Caryophyllene) على إحشاء عضلة القلب الناتج عن الأيزوبرينالن (Isoproterenol)

المتخصص

تعتبر مستقبلات الكانابينويد من النوع الثاني عضو أساسي في نظام الإندوكنابينويد. وقد ظهر مؤخراً كهدف علاجي حاسم للأمراض القلبية الوعائية. وقد شهد خفض تنظيم مستقبلات CB2 (Isoproterenol) في الجرذان. وتحقق هذه الدراسة في تأثير البيتا-كاريوفايلين (β-Caryophyllene) في إحشاء عضلة القلب الناتج عن الأيزوبرينالن (Isoproterenol) في الجرذان. وقد عولجت جرذان ويستار الذكور بجرعة (50 ملغ/كج) (β-Caryophyllene) (Isoproterenol) مرتين في اليوم لمدة عشرة أيام، بالإضافة إلى الحقن تحت الجلد بالأيزوبرينالن بجرعة (85 ملغ/كج) وقد أعطي جرعتين في فترات زمنية فاصلة تبلغ 24 ساعة لمدة يومين (اليوم التاسع والعشرين). وأيام (AM630) الذي يعتبر ضاد للمستقبل المذكور، وقد حققت الجرذان بجرعة (1 ملغ/كج) داخل الصفع لتأكيد أليات الوقاية للقلب عبر نهض مستقبلات الكانابينويد من النوع الثاني. سبب الأيزوبرينالن (Isoproterenol) من ارتفاع مستويات الأنزيمات القلبية وارتفاع مؤشرات الإجهاد التأكسدي وبيروكسيدات الدهون. وقد سبب أيضاً بحث إطلاق السيتوكينات البادئة للالتهاب بعد تفعيل العامل النووي كابا-B (kappa-B) وحمض النتريك سينثاز المحرر (iNOS). بالإضافة إلى ذلك، سبب الأيزوبرينالن (Isoproterenol) من ارتفاع مستوى بروتينات الموتية للإسثماط (proapoptotic proteins) (Bax) وكاسباز 3 النشط (active caspase-3) (Bcl-xL). انخفاض مقدار البروتين المضاد للإسثماط بي سي ال 2 (Bcl2) وبي سي ال-أكس ال 2 (Bcl-xL) (β-Caryophyllene) في عضلة القلب. وقد أدى العلاج بالبيتا-كاريوفايلين (β-Caryophyllene) إلى وفاة كبرى على جميع المعايير الكيميائية الحيوية والجزيئية. وقد تمكنت النتائج التي توصلنا لها مع الأدلة والصور المأخوذة تحت المجهر. وعلاوة على ذلك، قد سبب حقن الجرذان ب أيام AM630.
هو يعتبر ضاب لمستقبل الكانابينويد من النوع الثاني، بإلغاء التأثيرات الوقانية على جميع المعاملات الكيميائية الحيوية والجزئية المدرسة في الجرذان المحونة بـ BCP للأيزوبرينالن (Isoproterenol). وهكذا كشفت دراستنا أن BCP يحمي عضلة القلب من الإحتشاء (oxidative stress) والالتهاب (inflammation) والالتهاب (apoptosis) الإسعافية لهذه الحماية هي، تنشيط مستقبلات CB2 وقد توفر المركبات المحفزة لمستقبلات CB2 فئة جديدة من الأدوية القلبية الوقائية ويمكن استخدام حامل الخاصة الدوائية لهذه المركبات في اكتشاف وتطوير العقاقير.

مفهوم البحث الرئيسي: احتشاء عضلة القلب، كيميائيات نباتية، المنتجات الطبيعية، كانابينويدات، بيتا-كاروفيلين، مستقبل كنابينويد من النوع الثاني، أيزوبرينالن، كاتيكولامينات.
Acknowledgements

In the name of Allah, the Most Gracious and the Most Merciful Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. First off all, I would like to acknowledge everyone who played a role in my academic accomplishments, my parents, my husband and my friends, who supported me with love and understanding. Without you, I could never have reached this current level of success.

Secondly, my supervisor, Dr. Shreesh Ojha for his continuous support, motivation and untiring guidance have made this dream come true. His vast knowledge, calm nature and positive criticism motivated me to starve for nice results. It is my great opportunity to thank him for his kind co-operation, generous help and constant encouragement for the successful completion of my thesis. I also would like to thank the Chair of the Department Prof. Salim Bastaki and my thesis committee members, each of whom has provided patient advice and guidance throughout the research process. Thank you all for your unwavering support.

I am grateful to Dr. M.F. Nagoor Meeran. And I would like to thank him for encouraging my research and for helping and mentoring me in the lab work and for always being there when I required help.

Also, I would like to extend my thanks to Dr. Sheikh Azimuth, for his constant advice and guidance in many issues that I faced during my research.

Special thanks also go to Dr. Rami Beiram, Assistant Dean for Scientific Research and Graduate Studies, and all the other faculty members and non-teaching staff members of the Department of Pharmacology and Therapeutics, UAEU for their support and co-operation in all possible ways.
Dedication

I dedicate this thesis to my beloved parents and family
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List of Abbreviations

2-AG  2-Arachidonoylglycerol
ACEI  Angiotensin-Converting Enzyme Inhibitors
ADP   Adenosine Diphosphate
AEA    Anandamide
AMP   Adenosine Monophosphate
ATP   Adenosine Triphosphate
BAX   BCL2-Associated X Protein
BP    Blood Pressure
BCP   Beta Caryophyllene
Bcl-2  B-Cell Lymphoma 2
Bcl-xl B-Cell Lymphoma-Extra Large
cAMP  Cyclic Adenosine Monophosphate
CAT   Catalase
CB1, CB2 Cannabinoid Receptor 1, Cannabinoid Receptor 2
CBD   Cannabidiol
CBR   Cannabinoid Receptors
CCB   Calcium Channel Blockers
CK    Creatine Kinase
CNS   Central Nervous System
COX-2 Cyclooxygenase-2
CVD   Cardiovascular Disease
DP    Diastolic Pressure
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty Acid Amide Hydrolase</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSTs</td>
<td>Glutathione S-Transferases</td>
</tr>
<tr>
<td>HMG-COA</td>
<td>β-Hydroxy β-Methylglutaryl-CoA</td>
</tr>
<tr>
<td>HR</td>
<td>Heart Rate</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 Beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ISO</td>
<td>Isoproterenol</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LOOH</td>
<td>Lipid Hydroperoxide</td>
</tr>
<tr>
<td>MAGL</td>
<td>Monoacylglycerol Lipase</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean Arterial Pressure</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated Fatty Acids</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>SP</td>
<td>Systolic Pressure</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric Acid Reactive Substances</td>
</tr>
<tr>
<td>Acronym</td>
<td>Name</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-Like Receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TRBV1</td>
<td>Transient Receptor Potential Cation Channel Subfamily V Member 1</td>
</tr>
<tr>
<td>VR1</td>
<td>Vanilloid Receptor 1</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cells</td>
</tr>
<tr>
<td>Δ-9-THC</td>
<td>Delta-9-Tetrahydrocannabinol</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Cardiovascular diseases and myocardial infarction (MI)

Cardiovascular diseases (CVDs) are the leading causes of morbidity and mortality worldwide and it accounts for 17.3 million deaths per year with expected growth of more than 23.6 million by 2030 (Laslett et al., 2012). CVDs, which are disorders of the heart and blood vessels, include:

- Coronary artery disease also known as coronary heart disease, or ischemic heart disease, is the most common type of heart disease. The disease is caused by the building up of plaques in the inner walls of the arteries of the heart, which blocks blood flow and heightens the risk for heart attack and stroke.

- Peripheral vascular disease, also known as peripheral artery disease, refers to the blockage of large arteries not within the coronary, aortic arch vasculature, or brain. It can result from atherosclerosis, inflammatory processes leading to the formation of thrombus. It causes either acute or chronic ischemia (lack of blood supply).

- Rheumatic heart disease describes a group of short-term (acute) and long-term (chronic) heart disorders that can occur because of rheumatic fever. One common result of rheumatic fever is heart valve damage. This damage to the heart valves may lead to valve disorders.

- Cerebrovascular disease refers to a group of conditions that affect the circulation of blood to the brain, causing limited or no blood flow to the affected areas of the brain.
• Congenital heart disease is a defect in the structure of the heart and vessels which is present at birth (Wallace, 2011).

The deposition of fatty substances in the inner walls of the blood vessels causes blockage and insufficient blood flow to the heart or brain results in heart attack and stroke (Rauch et al., 2001). The coronary arteries supply nutrients and oxygen rich blood to the heart muscle for the proper functioning of heart. A dearth of blood supply to the heart due to the accumulation of fatty deposits such as cholesterol and fats called plaques in the inner walls of the arteries resulting in pain or angina (Marcus et al., 2007).

Myocardial infarction (MI) occurs when coronary blood flow is inadequate, hence the oxygen/nutrients supply is insufficient for the demand of the myocardium due to the formation of blood clot by the rupturing of lipid-laden plaques, resulting in irreversible damage to the heart which in turn results in death of that portion of the heart muscle (Rauch et al., 2001). The heart is a muscular organ about the size of a closed fist that functions as the body’s circulatory pump which has four chambers that pumps blood to all parts of the body (Iaizzo, 2009).

1.1.1 Myocardial infarction

MI is one of the major causes of mortality and morbidity in the world. It commonly known as heart attack occurs when the blood supply to a part of the heart is interrupted causing some heart cells to die. This is commonly due to the blockage of a coronary artery following the rupture of a vulnerable atherosclerotic plaque, which defined as an unstable collection of lipids (cholesterol) and WBC (especially macrophage) in the wall of an artery. The resulting ischemia and oxygen shortage, if left untreated for a sufficient period of time, can cause damage and/or death
(infarction) of the myocardium (Upaganlawar, Gandhi & Balaraman, 2009). The structure of the infarcted heart is shown in Figure 1.

1.1.1.1 Classification of myocardial infarction

There are two basic types of MI:

- **Transmural**: A transmural MI is characterized by ischemic necrosis of the full thickness of the affected muscle segment(s), extending from the endocardium through the myocardium to the epicardium. Transmural infarcts extend through the whole thickness of the heart muscle and are usually a result of complete occlusion of the area's blood supply (Fabricius et al., 1979).

- **Subendocardial**: A subendocardial MI involves a small area in the subendocardial wall of the left ventricle, ventricular septum, or papillary muscles. The subendocardial area is particularly susceptible to ischemia supply (Fabricius et al., 1979).

![Figure 1: Structure of infarcted heart](image-url)
1.1.1.2 Signs and symptoms of myocardial infarction

Chest pain is the common symptom of MI and is often described as a sensation of tightness, pressure, or squeezing. The pain may radiate to the left arm, neck, shoulders, jaw and epigastrium where it may mimic heartburn (Arora & Bittner, 2015). Other symptoms include diaphoresis (excessive sweating), weakness, light headedness, nausea, vomiting and palpitations. Immediate administration of thrombolytics (clot-dissolving drugs) after an acute MI reduces the risk of death. One-fourth of all MI are silent which can be identified by electrocardiogram (ECG), echocardiogram and enzyme tests (Valensi et al., 2011).

1.1.1.3 Risk factors of myocardial infarction

Diabetes (with or without insulin resistance)  
Tobacco smoking  
Hypercholesterolemia (more accurately Hyperlipoproteinemia, especially high (LDL-C), (VLDL-C) and low (HDL-C))  
Age: Men acquire an independent risk factor at age 45; Women acquire an independent risk factor at age 55  
Stress (occupations with high stress index are known to have susceptibility for MI)  
Hyperhomocysteinemia  
Hypertension  
Obesity  
Exposure to air pollution  
Males are more at risk than females

Figure 2: Risk factors of myocardial infarction (Iaizzo, 2009)
Many of these risk factors that shown in Figure 2 are modifiable and so many heart attacks can be prevented by maintaining a healthier lifestyle. Physical activity is associated with a lower risk profile (Buttar et al., 2005).

1.1.1.4 Myocardial metabolism

Normal and Ischemic myocardium:

Carbohydrates and lipids are the major substrates of normal heart metabolism. Normally, the increased levels of free fatty acids (FFAs) in the blood lead to the inhibition of glucose oxidation by the heart. Fatty acids then become the major source of energy and any glucose taken up is increasingly converted to glycogen by the glucose-sparing effect of fatty acid oxidation. About 60-80% of the heart’s energy supply is derived from fatty acid metabolism under normoxic conditions. About 20-40% of the adenosine triphosphate (ATP) which is necessary for contractile function is provided by glucose metabolism (Taegtmeyer, 1994).

The collapse of most of the cyclic processes is due to the sudden interruption of oxygen. This collapse is apparent in the rapid cessation of the rhythmic contractions of heart muscle in the area supplied by the affected coronary vessel. The collapse of cycles which leads to the accumulation of the metabolic end-products is a salient feature of MI. These include lactate, protons, succinate, alanine and fatty acid metabolites fatty acyl carnitine, fatty acyl-CoA and 2-hydroxy fatty acids (Garfein, 1990).

The balance between ATP production and consumption is disturbed as in myocardial ischemia when oxygen delivery is interrupted, leads to the ATP concentration decline and a cascade phenomenon results (Zarco & Zarco, 1996).
- Adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine and P, levels increase.
- Shift from aerobic respiration to anaerobic glycolysis
- Acidosis
- Osmolar load with increased levels of sodium (Na+) and calcium (Ca^{2+})
- Production of lactate

**Intracellular acidosis:**

A crucial role has been played by intracellular acidosis in contractile failure and in glycolysis inhibition that leads to an irreversible ischemic damage. Cellular acidosis reduces cardiac pressure and arouses cardiac arrhythmias. A major cause for force reduction is an acidosis-induced decrease in the response of contractile proteins to Ca^{2+}. Protons compete with Ca^{2+} at Ca^{2+} binding sites resulting in decreased binding of Ca^{2+} to troponin-C. Acidosis brings down the Ca^{2+} current which triggers the release of Ca^{2+} from sarcoplasmic reticulum (Murphy et al., 1991).

### 1.1.1.5 Diagnosis of myocardial infarction

**Diagnostic criteria:**

The World Health Organization criteria have classically been used to diagnose MI, a patient is diagnosed with MI, if two (probable) or three (definite) of the following criteria are satisfied (Thygesen et al., 2012):

1. Clinical history of ischemic type chest pain lasting for more than 20 minutes.
2. Changes in serial ECG tracings.
3. Rise and fall of serum cardiac biomarkers such as creatine kinase-MB (CK-MB) and troponins.
Physical examination:

The general appearance of patients may vary according to the experienced symptoms; the patient may be uncomfortable, or restless and in severe distress with an increased respiratory rate. A cool and pale skin is common and points to vasoconstriction. Blood pressure may be elevated or decreased, and the pulse rate may be irregular (Kasper, 2005).

Electrocardiography:

Electrocardiogram is a central tool used to establish the diagnosis of myocardial ischemia or infarction. A simple, painless test records the heart's electrical activity (Schweitzer, 1990). The increase in ST-segments could be due to ischaemic or neurogenically mediated myocardial injury. Elevations of the ST-segment and changes in the T wave are indication of MI. Q waves can be seen on the ECG signifying damaged heart tissue after MI (Schweitzer, 1990). Electrocardiogram waves are shown in Figure 3.

Figure 3: Electrocardiogram
Echocardiography:

Echocardiogram, often referred to as a cardiac echo is a sonogram of the heart. Echocardiography uses standard two-dimensional, three-dimensional and doppler ultrasound to create images of the heart. It has become routinely used in the diagnosis, management and follow-up of patients with any suspected or known heart diseases. Severe ischemia produces regional wall motion abnormalities that can be visualized echocardiographically within seconds of coronary artery occlusion (Hauser et al., 1985; Wohlgelernter et al., 1986). Immediately after coronary occlusion, myocardium ceases to function in the area served by the involved vessel. The affected left ventricular wall fails to thicken during systole and in fact may thin, producing systolic bulging of the ischemic segment can be seen by using echocardiography. Such abnormality of a regional wall motion can be detected in acute MI with approximately 90% sensitivity by echocardiography (Kloner & Parisi, 1987). So, it is one of the most widely used diagnostic tests in cardiology.

Markers of myocardial infarction:

Myocardial infarction is accompanied by the release of cellular enzymes in the bloodstream. Thus, evaluation of these markers provides an excellent way to diagnose MI.

- Total creatine kinase:

Creatine kinase (CK) is an intracellular enzyme present in large amounts in skeletal muscle, brain and myocardium. Disruption of cell membranes due to hypoxia or other injury releases CK from the cellular cytosol into the systemic circulation. On this basis, elevated serum levels of CK have been used as a sensitive but nonspecific test for MI. The poor specificity reflects the ubiquity of CK in many tissues other than
the myocardium (Cabaniss, 1990). A scientific study reports that the activity of total CK is found to be increased in the serum of isoproterenol (ISO) induced myocardial infarcted rats (Kannan & Quine, 2011a).

- **Creatine kinase - MB:**

  Creatine kinase is subdivided into three isoenzymes: MM, MB, and BB. The MM fraction is present in both cardiac and skeletal muscle, but the MB fraction is much more specific for cardiac muscle. About 15 to 40% of CK in cardiac muscle is MB, while less than 2% in skeletal muscle is MB. CK-MB is a very good marker for acute myocardial injury, because of its excellent specificity, and it rises in serum within 2 to 8 h of onset of acute MI. The CK-MB is also useful for diagnosis of reinfarction or extensive of an MI, because it begins to fall after a day, dissipating in 1 to 3 days, so subsequent elevations are indicative of another event (Laboratory Diagnosis of Myocardial Infarction, 1999). Serum CK-MB activity is found to be raised in the ISO induced myocardial infarcted rats (Upaganlawar et al., 2009).

- **Cardiac troponins:**

  Troponins-T and I are structural components of the muscle of the heart. They are released into the blood stream in MI. They are specific for MI. Troponin levels will begin to increase following MI within three to twelve h and remain elevated up to five to nine days for troponin-I and up to two weeks for troponin-T. This makes troponins a superior marker for diagnosing MI (Skeik & Patel, 2007). The levels of serum troponins (I and T) are found to be elevated in ISO induced MI (Kumaran & Prince, 2010).
• **Lactate dehydrogenase:**

  The enzyme lactate dehydrogenase (LDH) begins to rise in 12 to 24 h following MI, and peaks in two to three days, gradually dissipating in 5 to 14 days. Measurement of LDH is necessary for greater specificity for cardiac injury (Jaffe et al., 1996). The LDH activity are found to be elevated in ISO induced myocardial infarcted rats (Ouyang et al., 2019).

1.1.1.6 Prevention of MI

• Stop smoking.
• Regular exercise within tolerance level.
• Keep the body weight within recommended level.
• Sodium chloride intake should be less than 6 g per day.
• Control of diabetes mellitus.
• Intake of low levels of fat and cholesterol in diet (Doyle et al., 2012).

1.1.1.7 Treatment

**Non-pharmacological therapy:**

Dietary intake of fruits, vegetables (Law & Morris, 1998), fiber (Pietinen et al., 1996) and fish (Albert et al., 1998) may able to protect the heart, while a high intake of saturated fat (found in meat and dairy fat) and trans fatty acids (found in margarine and processed foods containing hydrogenated vegetable oils) and its dietary intake may contribute to heart disease (Hu et al., 1999).

**Cardiovascular drugs:**

The development of MI is a dynamic process, which reflects abnormal hormonal, metabolic and hemodynamic effects, as well as increasing demand of
myocardial oxygen and decreasing coronary blood flow. Recent approaches include the use of pharmacological agents to dissolve occluding thrombi and mechanical procedures to recanalize occluded coronary arteries.

Most of the currently available cardiovascular drugs used in clinical practice are divided into groups depending upon their action or what they treat, and it include statins, diuretics, anticoagulants, beta blockers (BB), digitalis drugs, vasodilators, calcium channel blockers (CCB) and angiotensin converting enzyme inhibitors (ACEI). Examples of cardiovascular drugs and their pharmacological action are shown in Table 1.

Table 1: Cardiovascular drugs

<table>
<thead>
<tr>
<th>Category</th>
<th>Drug Action</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statins</td>
<td>Cholesterol lowering drugs, used to control cholesterol levels. Act by inhibiting HMG-CoA reductase enzyme.</td>
<td>Atorvastatin</td>
</tr>
<tr>
<td>Diuretics</td>
<td>Help to reduce fluid retention. These may also reduce pressure. When the body is retaining fluid, though, can often make the heart harder, using diuretics will reduce heart workload.</td>
<td>Furosemide</td>
</tr>
<tr>
<td>Anticoagulants</td>
<td>Lengthen the time it takes for blood to clot, which can prevent the formation of blood clots that might cause a stroke. People who have artificial valves, who have had a stroke or the ones at risk for MI, may need an anticoagulant to minimize further risk.</td>
<td>Warfarin</td>
</tr>
<tr>
<td>Anti-platelets</td>
<td>Reduce the tendency of platelets in the blood to clump and cause clotting.</td>
<td>Aspirin</td>
</tr>
</tbody>
</table>
Table 1: Cardiovascular drugs (continued)

<table>
<thead>
<tr>
<th>Category</th>
<th>Drug Action</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta blockers</td>
<td>Control pressure and reduce chest pain associated with angina. BB slow</td>
<td>Metoprolol</td>
</tr>
<tr>
<td></td>
<td>heartbeat that may control numerous heart disease symptoms which may</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reduce future risk of MI.</td>
<td></td>
</tr>
<tr>
<td>Cardiac Glycosides</td>
<td>Stimulates the heart to beat more forcefully. Used for arrhythmia and in</td>
<td>Digitalis</td>
</tr>
<tr>
<td></td>
<td>congestive heart failure.</td>
<td></td>
</tr>
<tr>
<td>Vasodilators</td>
<td>Are like beta-blockers may reduce the work of heart and they are often</td>
<td>Nitroprusside,</td>
</tr>
<tr>
<td></td>
<td>prescribed to treat chest pain resulting from angina.</td>
<td>nitroglycerin,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and hydralazine.</td>
</tr>
<tr>
<td>Calcium channel</td>
<td>Used in the treatment of some forms of angina and to treat certain</td>
<td>Nifedipine</td>
</tr>
<tr>
<td>blockers</td>
<td>arrhythmias or hypertension.</td>
<td></td>
</tr>
<tr>
<td>ACEI</td>
<td>Decrease some blood supply to the heart, which reduces its work. Cardiovascular drugs that fall into this category might lower blood pressure and increase heart function.</td>
<td>Captopril</td>
</tr>
</tbody>
</table>

1.1.2 Free radicals

Free radicals are defined as a group of atoms with an odd (unpaired) number of electrons and it formed when oxygen interacts with certain molecules. Once formed they will act as a highly reactive radicals and will start a chain or reactions. Their main danger comes from the damage resulted from the reactions with important cellular components such as DNA or cell membrane. Selected radical and non-radical oxidants in vivo are presented in Table 2. The major contributors for oxidative stress in the human body (Huang et al., 2005) are marked with an ash (#).
Table 2: Selected radical and non-radical oxidants with significance in vivo

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide radical</td>
<td>O$_2^-$</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>OH$^-$</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>ONOO$^-$</td>
</tr>
<tr>
<td>Nitric oxide radical</td>
<td>NO$^-$</td>
</tr>
<tr>
<td>Nitrogen dioxide radical</td>
<td>NO$_2^-$</td>
</tr>
<tr>
<td>Alkoxyl radical</td>
<td>RO$^-$</td>
</tr>
<tr>
<td>Peroxyl radical</td>
<td>ROO$^-$</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H$_2$O$_2$</td>
</tr>
<tr>
<td>Hypochlorous acid</td>
<td>HOCl</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>$^{1}$O$_2$</td>
</tr>
</tbody>
</table>

1.1.2.1 Lipid peroxidation

Lipid peroxidation in vivo is a fundamentally deteriorative reaction occurs in polyunsaturated fatty acids (PUFAs) and involves the direct reaction of oxygen and lipids to form free radical intermediates and semi stable peroxides. The overall process of lipid peroxidation consists of three stages: initiation, propagation and termination. Which presented in Figure 4.

Initiation:

Lipid peroxidation is initiated by the attack on a fatty acid or fatty acyl side chain of any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain. PUFAs are particularly susceptible to peroxidation because of greater number of double bonds in a fatty acid side chain and
their hydrogen atoms are easily removable. The resulting carbon-centered lipid radical can have several fates. But in aerobic cells, it undergoes molecular rearrangement and form conjugated dienes, which are able to combine with O$_2$ and produce peroxyl (ROO•) radical mutagens (Halliwell & Chirico, 1993).

**Propagation:**

Peroxyl radicals can combine with each other or they can attack the membrane proteins. They are also capable of abstracting hydrogen from another lipid molecule (adjacent fatty acid), especially in the presence of metals such as copper or iron, thus causing an autocatalytic chain reaction and so propagating the chain reaction of lipid peroxidation. The ROO• combines with free hydrogen molecule to give a lipid hydroperoxide (LOOH). Probable alternative fates of ROO• are to be transformed into cyclic peroxides or even cyclic endoperoxides (from PUFAs such as arachidonic or eicosapentanoic acid). The length of the propagation chain depends on many factors including the lipid-protein ratio in a membrane (the chance of a radical reacting with a membrane protein will increase as the protein content of the membrane rises), the fatty acid composition, oxygen concentration and the presence of chain breaking antioxidant mutagens (Halliwell & Chirico, 1993).

**Termination:**

Termination (formation of a hydroperoxide) is most often achieved by the reaction of a ROO• with α-tocopherol, which is the main lipophilic "chain-breaking molecule" in the cell membranes. Furthermore, any kind of alkyl radical’s lipid free radical (L•) can react with lipid peroxide radical (LOO•) to give non-initiating and non-propagating species such as the relatively stable dimers or two peroxide molecules combining to form hydroxylated derivatives. The resulting LOOH can easily
decompose into several reactive species including lipid alkoxyl radical (LO•), aldehydes (e.g., malondialdehyde, HOC–CH2–CHO), alkanes, lipid epoxides and alcohols in which most of them are toxic and active mutagens (Halliwell & Chirico, 1993).

1.1.3 Antioxidants

Compounds disposing the reactive oxygen species (ROS), scavenging them, suppressing their formation or opposing their actions are antioxidants. There are two types of antioxidants namely

(i) Enzymatic antioxidants, and

(ii) Non-enzymatic antioxidants

Figure 4: The chemistry of oxygen radical induced membrane lipid peroxidation (Halliwell & Gutteridge, 1990)
1.1.3.1 Enzymatic antioxidants

Superoxide dismutase:

Superoxide dismutase is present in all aerobic organisms and in most subcellular compartments that generates activated oxygen. It has a central role in the defense against oxidative stress (Scandalios, 1993). It catalyzes the dismutation of superoxide anion ($O_2^{•−}$) to hydrogen peroxide which will be subsequently removed by the reaction of catalase or glutathione peroxidase (GPx).

\[
2 \text{O}_2^{•−} + 2\text{H} \xrightarrow{\text{Superoxide dismutase}} \text{H}_2\text{O}_2 + \text{O}_2
\]

The activity of superoxide dismutase is found to be decreased in ISO treated MI (Saravanan et al., 2013).

Catalase:

Catalase is a tetrameric hemeprotein of molecular weight 240 kDa, consisting of four identical subunits of 60 kDa. It is present in all mammalian cells and is abundantly present in subcellular organelles such as the peroxisomes of the liver and kidney. It catalyzes the reduction of hydrogen peroxide and thereby protects the cellular constituents from oxidative damage by highly reactive hydroxyl radicals ($\text{OH}^{•}$) (Lalitha et al., 2013).

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2
\]

The activity of catalase is found to be decreased in ISO induced MI (Saravanan et al., 2013)
Glutathione peroxidase:

Glutathione peroxidase, an important antioxidant enzyme whose foremost biological function is to protect the organism from oxidative damage. It catalyses the decomposition of both hydrogen peroxide and organic peroxides at the expense of reduced glutathione (GSH) with the formation of glutathione disulfide, water and organic alcohol (Vural et al., 2004).

\[
\text{ROOH} + 2\text{GSH} \xrightarrow{\text{GPx}} \text{GSSG} + \text{ROH} + \text{H}_2\text{O}
\]

A decrease in the heart GPx activity is reported in the ISO induced myocardial infarcted rats (Saravanan et al., 2013).

Glutathione S-transferase:

The Glutathione S-transferases (GSTs) form a group of multi-gene isoenzymes involved in the cellular detoxification of both xenobiotic and endobiotic compounds. It catalyses the conjugation of reduced glutathione GSH via the sulfhydryl group to electrophilic centers on a wide variety of substrates. This activity is involved in the detoxification of endogenous compounds such as peroxidised lipids (Leaver & George, 1998) as well as the metabolism of xenobiotics. The activity of GST is found to be decreased in ISO treated MI (Saravanan et al., 2013).

1.1.3.2 Non-enzymatic antioxidants

Reduced Glutathione:

Glutathione is a tripeptide (Glu-Cys-Gly), whose antioxidant function is facilitated by the sulphhydryl groups of cysteine (Rennenberg, 1980). It can react chemically with singlet oxygen, \(\text{O}_2^{\bullet-}\), and \(\text{OH}^{\bullet}\) and therefore function directly as a free radical scavenger. It may stabilize membrane structure by removing acyl peroxides
formed by lipid peroxidation reactions. Decreased levels concentrations of GSH were observed in ISO-induced myocardial infarcted rats (Saravanan et al., 2013).

Isoproterenol induces oxidative stress and results in alterations of cardiac function and ultra-structure in experimental rats (Kumaran & Prince, 2010a). The positive inotropic and positive chronotropic response of ISO augments myocardial oxygen consumption. The increase in energy demand (Prabhu et al., 2000) and the decrease in blood flow induce an energy imbalance by the overload of Ca$^{2+}$ (Chagoya de Sanchez et al., 1997). This is accompanied by the disruption of mitochondria (Xia et al., 2002), with inactivation of tricarboxylic acid cycle enzymes and altered mitochondrial respiration (Prabhu et al., 2006). In acute myocardial injury, ROS toxicity shuts down mitochondrial oxidative phosphorylation and the tricarboxylic acid cycle, blocking mitochondrial ATP production and cardiac contraction.

1.1.4 Pro-inflammatory cytokines and myocardial infarction

The release of proinflammatory cytokines such as TNF-$\alpha$, interleukin-6 (IL-6) and interleukin-1$\beta$ (IL-1$\beta$) have been observed consistently in the experimental models of MI. The functional pleiotropy and redundancy is a characteristic feature of cytokines which indicates its wide range of biological effects on various cell types (Kishimoto et al., 1995). The multifunctional, overlapping and often contradictory effects of cytokines have been impeded because of its role in cardiac injury and repair.

**Tumor necrosis factor-$\alpha$:**

Tumor necrosis factor-$\alpha$ is a potent proinflammatory cytokine has a crucial role in cardiomyocytes injury and repair that acts on vascular endothelial cells to induce expression of leukocyte adhesion molecules. Moreover, it decreases cardiac
contractility (Grandel et al., 2000) and enhances apoptosis in the cardiomyocytes (Song et al., 2000). In cardiac fibroblasts, TNF-α regulates the extracellular matrix metabolism by increasing the activity of matrix metalloproteinases (MMPs) and by decreasing the synthesis of collagen (Siwik et al., 2000). Previous reports have shown the effects of TNF-α signaling in cardiac dysfunction (Sugano et al., 2004).

**Interleukin-1β family:**

Interleukin 1β family is a group of cytokines, which plays a central role in the regulation of immune and inflammatory responses to infections or sterile insults. It consists of interleukin-1α, IL-1β (agonists) and interleukin-1 receptor antagonist (specific receptor antagonist) (Dinarello, 1996). The levels of IL-1β are increased in the plasma of acute myocardial infarcted patients (Guillen et al., 1995). IL-1 shows an injurious role in the ischaemic myocardium. This signaling activates inflammatory and fibrogenic pathways in the healing infarct heart and may play a vital role in the pathogenesis of postinfarction remodeling (Bujak et al., 2008).

**Interleukin-6:**

Interleukin-6 is a member of structurally related cytokines acts as both pro-inflammatory and anti-inflammatory cytokine with overlapping physiological and biological effects. It is secreted by T cells and macrophage to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation. Under hypoxic conditions, cardiomyocytes produce IL-6 which could contribute to the myocardial dysfunction observed in ischemia reperfusion injury (Sharma & Das, 1997).
1.1.5 Apoptosis

Apoptosis, the programmed cell death is a process by which cells undergo inducible non-necrotic cellular suicide that may occur in multicellular organisms (Song et al., 2000). Biochemical events lead to characteristic cell changes (morphology) and death, which shown in Figure 5. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation.

![Apoptosis diagram](Image)

Figure 5: Apoptosis (Abou-Ghali & Stiban, 2015)

1.1.5.1 Apoptosis in myocardial infarction

Apoptosis, one of the major forms of cell death, has been implicated in different CVDs. Several studies have reported cardiomyocyte apoptosis during human MI (Baldi et al., 2002; Saraste et al., 1997). The discovery of apoptosis sheds a new light on the role of cell death in MI and other CVDs. There is mounting evidence that
apoptosis plays an important role at multiple points in the evolution of MI, and comprises not only cardiomyocytes but also inflammatory cells, as well as cells of granulation tissue and fibrous tissue (Zidar et al., 2007). The two pathways that lead to apoptosis are an extrinsic Pathway and an intrinsic Pathway, shown in Figure 1.6. The extrinsic pathway begins outside the cell through activation of pro-apoptotic receptors on the cell surface whereas the intrinsic or mitochondrial pathway is initiated within the cell.

Involvement of reactive oxygen species in the pathogenesis of myocardial apoptosis:

Reactive oxygen species may play an important pathophysiological role in cardiac diseases characterized by apoptotic cell death and suggest that different ROS-induced activations of the apoptotic cell death program in cardiomyocytes involve distinct signaling pathways (von Harsdorf et al., 1999). A scientific study has observed significant linear relationship between apoptotic myocytes number, macrophages and transmigrated neutrophils during early and prolonged reperfusion (Zhao et al., 2001). Also, ischemia followed by reperfusion induces myocardial injury through apoptotic pathway.
Figure 6: Intrinsic and extrinsic pathway of apoptosis (Kumar, 2015)
1.1.6 Experimental myocardial infarction in animal models

For the experimental MI studies, doxorubicin, cyclophosphamide and ISO are used, but in this research, I will focus mainly on isoproterenol.

Isoproterenol:

Isoproterenol (1-[3, 4 dihydroxyphenyl]-2-isopropyl amino ethanol hydrochloride) is a synthetic catecholamine and β-adrenergic agonist, which has been found to cause severe stress in the myocardium resulting in infarct like necrosis of heart muscles (Wexler & Greenberg, 1978). ISO-induced MI serves as a well-standardized and common model to find out the beneficial effects of many drugs (Ithayarasi & Devi, 1997). The supramaximal doses of catecholamines result in necrotic lesions development in the myocardium of experimental animals (Knufman et al., 1987). The pathophysiological changes that take place in rat's heart following MI induced by ISO are comparable to those changes taking place in MI in humans (Geng et al., 2004). Chemical structure of ISO is shown in Figure 7.

![Structure of isoproterenol](image)

Figure 7: Structure of isoproterenol

The hazardous effect of catecholamine is due to the oxidation of hydroxyl groups leading to their conversion into quinones and the subsequent formation of adrenochromes, as shown in Figure 8. ISO induced MI results in increased oxidative stress, hypoxia, mitochondrial dysfunction, hyperlipidemia, intracellular depletion of
ATP increased Ca\textsuperscript{2+} influx, and cyclic adenosine monophosphate levels. The free radicals initiate the peroxidation of membrane bound PUFAs resulting in functional and structural myocardial injury (Thompson & Hess, 1986). During ISO induced MI, enhanced free radical formation and lipid peroxide accumulation have been proposed as one of the possible biochemical mechanisms for myocardial damage (Sushamakumari et al., 1989).

The enzymes, catechol-o-methyl transferase and monoamine oxidase are involved in the degradation of catecholamines by methylation of the 3-hydroxyl group of the catechol ring and removal of the amino group under physiological conditions results in the formation of non-toxic metabolites such as metanephrine, dihydroxy mandelic and vanillyl mandelic acids (Yates, Beamish & Dhalla, 1981). However, high concentration of catecholamines saturates these enzyme systems. Free radicals such as O\textsuperscript{2-} and hydrogen peroxide were formed by the metal catalyzed Fenton reaction of OH\textsuperscript{-} from the oxidation of catecholamines to quinones. Moreover, adrenochrome, a toxic metabolite is formed by the intra cyclization of quinone (Yates et al., 1981).
1.2 Cannabinoids as drug target for MI

Cannabinoids are mainly defined as the active compounds in marijuana, which extracted from different part of the hemp plant Cannabis sativa, including flowers, leaves and resin. It has been used in China for medical purposes throughout the history from around five-thousand years ago (Mendizabal & Adler-Graschinsky, 2007). The medical marijuana has been documented to be used as analgesic, anti-inflammation, muscle relaxant, anticonvulsant, antiemetic, appetite stimulants and as recreational use due to its euphoric effect, which has limited its medical application (Iversen, 2001; Mechoulam, 1986).

Cannabinoids could also be defined as a class of diverse chemical compounds that act on cannabinoid receptors (endocannabinoid system), which alter the release of

Figure 8: Proposed mechanism of auto-oxidation of isoproterenol (Singal et al., 1983)
neurotransmitters in the brain. The ligands for these receptors are often grouped into three types.

1.2.1 Endocannabinoids

Endocannabinoids, are the endogenous lipids that bind cannabinoid receptors and lead to affecting the behavior in a way partially mimicked by the effects produced by the psychoactive components of cannabis, mainly Delta 9-THC ((-)-trans-delta-9-tetrahydrocannabinol) (Lu & Mackie, 2016). The first discovered endocannabinoid is anandamide (N-arachidonoylethanolamine; AEA), it has been discovered by (Devane et al., 1992). According to this report, anandamide is an arachidonic acid derivative that function as a natural ligand for the cannabinoid receptor. Three years later another endocannabinoid was discovered by (Mechoulam et al., 1995), 2-arachidonoylglycerol (2-AG). Since then, a number of other endocannabinoid-like-activity lipids was discovered and reported in Table 3, but the most well studied and best characterized are AEA and 2-AG.
Table 3: The pharmacological activity of endogenous cannabinoids

<table>
<thead>
<tr>
<th>Type</th>
<th>Activity</th>
<th>References</th>
</tr>
</thead>
</table>
| Anandamide (AEA)                  | CB1>>CB2 agonist, TRBV1 agonist | (Mechoulam et al., 1995)  
|                                   |                           | (Khanolkar et al., 1996) |
| 2-Arachidonoylglycerol (2-AG)     | CB1≈CB2 agonist           | (Mechoulam et al., 1995) |
| 2-Arachidonylglycerol ether       | CB1>>CB2 agonist, TRPV1 partial agonist | (Hanus et al., 2001) 
|                                   |                           | (Duncan et al., 2004) |
| O-Arachidonoyl ethanolamine       | CB1 antagonist, CB2 agonist | (Porter et al., 2002) |
| (Virodhamine)                     |                           |                                 |
| N-Arachidonoyl dopamine           | CB1>>CB2 agonist, TRBV1 agonist | (Bisogno et al., 2000) 
|                                   |                           | (Huang et al., 2002) |

Anandamide is an N- (polyunsaturated fatty acyl) ethanolamine that formed from the formal condensation of carboxy group of arachidonic acid with amino group of ethanolamine. It play a role as a neurotransmitter, vasodilator and as a blood serum metabolite (PubChem, 2019b).

Anandamide is a highly potent endogenous agonist of both cannabinoid receptors (CB1 and CB2 receptors) (PubChem, 2019b). Previous studies show that AEA might inhibit tumor cell proliferation or induce apoptosis independently of CB1 and CB2 receptors, this action mediated by interaction with the type 1 vanilloid receptor (VR1). VR1 is an ion channel expressed almost exclusively by sensory neurons, it’s activated by pH, noxious heat (>48 degree °C) and plant toxins and it
plays an important role in nociception. Cervical cancer cells are sensitive to AEA-induced apoptosis via VR1 that is aberrantly expressed in vitro and in vivo while CB1 and CB2 receptors shows to play a protective role (Contassot et al., 2004).

2-Arachidonoylglycerol (2-AG), is an endocannabinoid and an endogenous agonist of both cb1 and cb2 cannabinoid receptors. It is isolated in 1995 from rat brain and canine gut as an endogenous ligand for the cannabinoid receptors. 2-AG is an ester formed from omega-6-arachidonic acid and glycerol. It play a role as a human metabolite and it’s derived from arachidonic acid-containing phospholipids. (PubChem, 2019a).

A vital feature of these endocannabinoids is that their precursors are present in lipid membranes. Upon demand (typically by activation of certain G protein-coupled receptors or by depolarization), endocannabinoids are synthesized in one or two rapid enzymatic steps and then released into the extracellular space. Unlike the classical neurotransmitters that are synthesized ahead of time and stored in vesicles. Intrinsic efficacy of the endogenous cannabinoids varies, THE 2-AG show a high efficacy agonist for both CB1 and CB2 receptors, while AEA show a low efficacy agonist at CB1 receptors and a very low efficacy at CB2 receptors (Gonsiorek et al., 2000). The endocannabinoids structures are represented in Figure 9.

The activity of both AEA and 2-AG at their receptors is limited by cellular uptake. This action mediated by anandamide membrane transporter (AMT), followed by intracellular degradation. Fatty acid amide hydrolase (FAAH) is the main enzyme that degrade AEA, whereas a monoacylglycerol lipase (MAGL) is critical in degrading 2-AG (Sugiura et al., 2006). The Endocannabinoids synthesis and degradation are represented in Figure 10.
1.2.2 Synthetic cannabinoid

Synthetic CBR agonists are a large group of legal and illegal compounds that bind to one or both known CBR. Many of these substances were initially researched in the 1950s and 1960s for their potential therapeutic action in a variety of diseases. Adverse effects, such as depression and increased potential of suicide stopped these substances from reaching therapeutic use (Tai & Fantegrossi, 2017). Examples of synthetic cannabinoids are shown in Table 4.
Synthetic cannabinoids have been developed which can act as selective agonists or antagonists to CB receptors. Synthetic analogues are produced to either stimulate or block the CB receptors specifically. Specific CB2 agonists are useful in avoiding the undesirable effects caused by CB1 stimulation (Xavier, 2013).

The endocannabinoid levels can be increased by:

- Inhibiting the degrading enzymes (FAAH inhibitors).
- Inhibiting the reuptake of endocannabinoids (Reuptake inhibitors- Inhibiting reuptake transporters).
- Specific agonists and antagonists are useful in the research in finding out the receptors involved in specific function.

Table 4: Examples of synthetic cannabinoids (Xavier, 2013)

<table>
<thead>
<tr>
<th>Group</th>
<th>Synthetic analogues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cannabinoid receptor agonists</td>
<td>Classical cannabinoids: Delta-8-THC, HU-210, AM411</td>
</tr>
<tr>
<td></td>
<td>Non-classical cannabinoids: CP-55940</td>
</tr>
<tr>
<td></td>
<td>Specific CB2 agonists: AM-1241, HU-308</td>
</tr>
<tr>
<td>Cannabinoid receptor antagonists</td>
<td>SR141716A (Rimonabant): CB1 antagonist.</td>
</tr>
<tr>
<td></td>
<td>AM251: CB1 antagonist.</td>
</tr>
<tr>
<td></td>
<td>SR147778: CB1 antagonist.</td>
</tr>
<tr>
<td>Uptake blockers</td>
<td>AM404, UCM707, AM1172, VDM 11</td>
</tr>
<tr>
<td>Inhibitors of fatty acid amide hydrolase (FAAH)</td>
<td>OL-135, URB 597</td>
</tr>
</tbody>
</table>
1.2.3 Phytocannabinoids

More than 60 organic compounds are derived from the cannabis plant and called cannabinoids. Among the plant cannabinoids extracted from cannabis, THC is the most active ingredient and considered the main psychotropic constituent of cannabis. Δ-THC and cannabidiol (CBD) are the mostly studied. Other cannabinoids are cannabinol, Δ9-tetrahydrocannabivarin, cannabigerol. Amount of the cannabinoids present in each plant strain are different. For example, THC levels of the plant strains bred for recreational purpose is much more than that of the plants bred for the hemp. The affinity of the cannabinoids to their receptors is different since their effect (Xavier, 2013). Examples of plant derived cannabinoids are summarized in Table 5.

- THC act as CB1 and CB2 partial agonist and show equal affinity to both CB1 and CB2 receptors.
- CDB act as CB2 receptor antagonist (inverse agonist).

Table 5: Plant derived cannabinoids (Xavier, 2013)

<table>
<thead>
<tr>
<th>Phytocannabinoids</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ 9-tetrahydrocannabinol type</td>
<td>Cannabicyclol type</td>
</tr>
<tr>
<td>Δ 8- tetrahydrocannabinol type</td>
<td>Cannabielsoin type</td>
</tr>
<tr>
<td>Cannabidiol type</td>
<td>Cannabitriol type</td>
</tr>
<tr>
<td>Cannabigerol type</td>
<td>Miscellaneous type</td>
</tr>
<tr>
<td>Cannabichromene type</td>
<td>Cannabinol and cannabinodiol type</td>
</tr>
</tbody>
</table>
Phytocannabinoids found in plants other than cannabis:

In the last few years, several non-cannabinoid plant products have been discovered and reported to act as cannabinoid receptor ligands, this allow us to define the “phytocannabinoids” as any plant constituents not specifically from cannabis sativa capable to interact directly with CB receptors or share chemical similarity of cannabinoids or both (Gertsch, Pertwee & Marzo, 2010).

Figure 11: Phytocannabinoids possess at least some of these properties (Gertsch et al., 2010).

In Table 6, some examples of natural products are summarized that have been reported to interact with the ECS:
Table 6: Natural products that have been reported to interact with the ECS

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Description and Function</th>
</tr>
</thead>
</table>
| β-caryophyllene                      | ![Structure](image) | - Selectively target the CB2 receptor at nM concentrations (Ki = 155 nM) and to act as a full agonist (Gertsch, 2008a).  
  - Orally administration (<5 mg·kg⁻¹), strong anti-inflammatory and analgesic effects in wildtype mice but not in CB2 receptor knockout mice (Gertsch et al., 2010). |
| Pristimerin                          | ![Structure](image) | - Naturally occurring quinonoid triterpenoids  
  - Potent reversible monoacylglycerol lipase inhibitor (IC₅₀ < 100 nM) (King et al., 2009)                                                                                                        |
| Rutamarin                            | ![Structure](image) | - Coumarin derivative from the medicinal plant Ruta graveolens L (Rollinger et al., 2009).  
  - Selective CB2 affinity (Ki value <10 mM) in human (Rollinger et al., 2009).                                                                                                           |
| DIM                                  | ![Structure](image) | - Anti-carcinogenic metabolite generated by ingestion of indole-3-carbinol (Yin et al., 2009).  
  - It shown to act as a weak CB2 receptor partial agonist (Yin et al., 2009).                                                                                                          |
1.3 Cannabinoid receptors

Initially it was thought that the psychotropic effects mediated by cannabinoids are due to their high lipophilicity. At the middle of 1980’s, they found that the cannabinoids activity is highly stereospecific and this led them to discover the both types of CB receptors, cannabinoid type 1 (CB1) and type 2 (CB2) receptors (Pertwee, 2006). Among many new therapeutic targets, the endogenous cannabinoid system represents one of the newest and most promising drug targets (Dias Dde et al., 2012). Both receptors are members of the GPCR family, and its activation leads to adenylate cyclase inhibition (Gi/o) or MAPK activation, as shown in Figure 12. CB1 receptors are mainly distributed in the brain and CB2 in the periphery, as shown in Table 7. (Pertwee, 2006).

Figure 12: Cannabinoid pathway (Xavier, 2013)
CB2 receptors are encoded by the gene CNR2, which consists of 360 amino acid in humans. At the protein level, it shares 44% sequence homology with CB1 receptors. The CB2 receptors show a greater species differences among humans and rodents in comparison to CB1 receptors, as the amino acid sequence homology is slightly above 80% between humans and rodents (Liu et al., 2009; Zhang et al., 2015).

Structurally both CB1 and CB2 receptors composed of seven transmembrane protein domains (Shao et al., 2016), shown in Figure 13, which predominantly couple to the inhibitory G proteins Gi and Go (Mackie, 2008). Activation of G protein causes intracellular consequences include the inhibition of adenylate cyclase and certain voltage gated calcium channels, the activation of mitogen activated protein kinase (MAP kinase) and of inwardly rectifying potassium channels (Howlett et al., 2002). In the central and peripheral nervous system, the net effect of these processes is to lessen neuronal excitability and to negatively modulate neurotransmission. In immune cells, activation of CB2 receptor results in a suppressive effects which including impaired

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Distribution</th>
</tr>
</thead>
</table>
| Cannabinoid 1 | In CNS: Hippocampus, basal ganglia, cerebellum, cerebral cortex, periaqueductal gray, certain parts of spinal cord.  
In periphery: Heart, GIT, urinary tract, leukocytes, spleen, endocrine glands, salivary glands, reproductive tract. |
| Cannabinoid 2 | Immune cells (leukocytes), spleen, tonsils, thymus, and bone.                |
antigen presentation, reduced cytokine release and disruption of immunocyte migration (Eisenstein & Meissler, 2015).

1.4 CB2 receptors and heart diseases

Cardioprotective effects of endocannabinoid-mediated CB2 receptor activation were first reported in LPS-induced preconditioning (Sultana & Saify, 2013). Thereafter, several recent reports using synthetic CB2 receptor agonists in vitro, ex vivo or in vivo showed protective effects on remote preconditioning, infarct size, arrhythmias while counterbalancing chronic heart failure-induced structural changes, inhibiting atherogenesis and preventing myocyte enlargement (Liu et al., 2013; Oda et al., 2011; Wang et al., 2010). The anti-inflammatory effects of CB2 receptor activation in the endothelium and its inhibitory effect on monocytes/macrophages and/or
leukocyte migration were reversed by pharmacological antagonism of CB2 receptors with AM630 (Liu et al., 2015). Pretreatment with the CB2 receptor antagonist AM630 or SR144528 abolished the cardioprotective effects and reversed cardioprotection. CB2 receptors have also been implicated in the modulation of endoplasmic reticulum stress and immune cell migration involving the MAPK, PI3K/Akt and ERK½ pathways (Liu et al., 2015; Liu et al., 2013; Oda et al., 2011; Wang et al., 2010). Many studies with synthetic cannabinoid ligands of CB2 receptors paved the way for CB2 receptor activation as a strategy in cardioprotection.

The adverse effects, abuse liability and addictive properties of synthetic CB2 receptor ligands in humans generated interest in targeting CB2 receptors by phytocannabinoids, which are ligands of natural source. To date, several phytocannabinoids have been pharmaceutically developed and this is demonstrated by the availability of Sativex, nabilone and dronabinol. Phytocannabinoids have been widely studied as potential protective agents in chronic diseases because of their multiple targets, time tested efficacy and low cytotoxicity (Bento et al., 2011). However, in general, the adverse psychoactive effects of phytocannabinoids derived from cannabis also limit their therapeutic use and bring legal concerns. Recently, several of the phytocannabinoids derived from plants other than cannabis have received interest for their favorable physicochemical, pharmacokinetic and pharmacological properties and their anti-inflammatory and antioxidant effects which make them promising for pharmaceutical development (Wu et al., 2014).
1.5 Beta-Caryophyllene

Beta-caryophyllene (BCP) is a volatile bicyclic sesquiterpene which found abundantly in the essential oils of many edible plants and spices such as cloves (*Syzygium aromaticum*), cinnamon (*Cinnamomum spp.*), oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), hemp (*Cannabis sativa*), germander (*Teucrium spp.*), Copaiba oil (*Copaiba spp.*), hops (*Humulus lupulus*) and black pepper (*Piper nigrum*) (Sharma et al., 2016). Sources of BCP are shown in Figure 14.

![Some natural sources of BCP](image)

Figure 14: Some natural sources of BCP

BCP has been approved by USFDA and European Food Safety Authority (EFSA) for use as food additive and flavoring agent (Sharma et al., 2016), and this attracted the scientific community to explore it for its additional possible therapeutic benefits (Gertsch, 2008a; Gertsch et al., 2008b). It exhibited significant therapeutic potential in numerous diseases due to multiple pharmacological properties to name a few like antioxidant, anti-inflammatory (Gertsch et al., 2008b), antimicrobial (Donati et al., 2015), anticancer and chemopreventive properties (Sain et al., 2014).
BCP is mainly found in the essential oils obtained from different parts or whole plant mainly aromatic plants. It has been reported to exist in more than one thousand plants which provide an extensive natural availability and accessibility. It is also one of the significant constituents (about 35%) in the essential oil of *Cannabis sativa L*, which considered a major source of plant derived cannabinoids or phytocannabinoids (Calleja et al., 2013; Hendriks et al., 1975). Table 8 shows the medicinal plants that are recognized to contain the amount of BCP equal to or more than 30% of total phytoconstituents.

Table 8: Plants that contain BCP (presented as % of BCP amongst constituent phytochemicals) (Sharma et al., 2016)

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Parts</th>
<th>% BCP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acanthospermum hispidum</strong></td>
<td>Oil</td>
<td>35.2</td>
</tr>
<tr>
<td><strong>Callicarpa integerrima</strong></td>
<td>Leaves</td>
<td>33.7</td>
</tr>
<tr>
<td><strong>Canarium parvum Leen</strong></td>
<td>Stem</td>
<td>30.4</td>
</tr>
<tr>
<td><strong>Copaifera multijuga</strong></td>
<td>Trunks</td>
<td>36.0</td>
</tr>
<tr>
<td><strong>Copaifera multijuga Hayne</strong></td>
<td>Trunks</td>
<td>57.5</td>
</tr>
<tr>
<td><strong>Copaifera reticulata Ducke</strong></td>
<td>Trunks</td>
<td>40.9</td>
</tr>
<tr>
<td><strong>Croton heliotropifolius</strong></td>
<td>Oil</td>
<td>35.8</td>
</tr>
<tr>
<td><strong>Euphorbia cotinifolia</strong></td>
<td>Leaves</td>
<td>39.3</td>
</tr>
<tr>
<td><strong>Glechon marifolia</strong></td>
<td>Oil</td>
<td>32.2</td>
</tr>
<tr>
<td><strong>Guarea humatensis</strong></td>
<td>Leaves</td>
<td>33.3</td>
</tr>
<tr>
<td><strong>Hyptis pectinata</strong></td>
<td>Oil</td>
<td>54.1</td>
</tr>
<tr>
<td><strong>Lantana montevidensis</strong></td>
<td>Leaves</td>
<td>31.5</td>
</tr>
<tr>
<td><strong>Leucas indica</strong></td>
<td>Aerial parts</td>
<td>51.1</td>
</tr>
<tr>
<td><strong>Leucas aspera</strong></td>
<td>Aerial parts</td>
<td>34.2</td>
</tr>
</tbody>
</table>
Table 8: Plants that contain BCP (presented as % of BCP amongst constituent phytochemicals) (Sharma et al., 2016) (continued)

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Parts</th>
<th>% BCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melampodium divaricatum</td>
<td>Aerial parts</td>
<td>56.0</td>
</tr>
<tr>
<td>Mydocarpus fraxinifolius</td>
<td>Leaves</td>
<td>63.0</td>
</tr>
<tr>
<td>Murraya paniculata</td>
<td>Leaves</td>
<td>30.0</td>
</tr>
<tr>
<td>Myrica rubra</td>
<td>Leaves</td>
<td>89.9</td>
</tr>
<tr>
<td>Newbouldia laevis</td>
<td>Leaves</td>
<td>36.0</td>
</tr>
<tr>
<td>Peristrophe bicalyculata</td>
<td>Oil</td>
<td>33.9</td>
</tr>
<tr>
<td>Phoenix dactylifera</td>
<td>Buds</td>
<td>44.2</td>
</tr>
<tr>
<td>Pinus halepensis</td>
<td>Oils</td>
<td>30.0</td>
</tr>
<tr>
<td>Piper guineense, white</td>
<td>Fruits</td>
<td>51.7</td>
</tr>
<tr>
<td>Piper guineense, black</td>
<td>Fruits</td>
<td>57.6</td>
</tr>
<tr>
<td>Piper duckei</td>
<td>Leaves</td>
<td>30.0</td>
</tr>
<tr>
<td>Plectranthus rugosus</td>
<td>Aerial parts</td>
<td>36.0</td>
</tr>
<tr>
<td>Plectranthus neochilus</td>
<td>Leaves</td>
<td>30.0</td>
</tr>
<tr>
<td>Stachys cretica</td>
<td>Aerial parts</td>
<td>51.0</td>
</tr>
<tr>
<td>Tabernaemontana catharinensis</td>
<td>Leaves</td>
<td>56.9</td>
</tr>
<tr>
<td>Teucrium chamaedrys</td>
<td>Aerial parts</td>
<td>47.6</td>
</tr>
<tr>
<td>Teucrium polium</td>
<td>Aerial parts</td>
<td>52.0</td>
</tr>
<tr>
<td>Teucrium flavum</td>
<td>Aerial parts</td>
<td>32.5</td>
</tr>
<tr>
<td>Teucrium divaricatum</td>
<td>Aerial parts</td>
<td>30.1</td>
</tr>
<tr>
<td>Valeriana alliiarifolia</td>
<td>Aerial parts</td>
<td>38.9</td>
</tr>
</tbody>
</table>
BCP is usually found to be concentrated mostly in the aerial parts, leaves, flowers and florescence and in traces in roots, rhizomes, stems and barks of different plants (Dias Dde et al., 2012; Lucca et al., 2015).

Chemically, BCP is a bicyclic sesquiterpene which is named (trans-(1R,9S)-8-Methylene-4,11,11-trimethylbicyclo(7.2.0) undecene), it is also recognized by several other synonyms such as caryophyllene, trans caryophyllene, (-)-trans-caryophyllene, β-(E)-caryophyllene and L-caryophyllene (Budavari et al., 1996). BCP present in plants as a mixture of two pharmacologically active isomers E-BCP and Z-BCP, together with substantially inactive sesquiterpenes such as alpha-humulene and derivatives such as BCP oxide. Natural sources include a greater proportion of E-BCP than Z-BCP (Gertsch et al., 2008c). Optically, β-Caryophyllene and iso-caryophyllene are trans- and cis- double isomers respectively, while α-humulene is a ring-opened isomer. In plants, the BCP is generally found in their essential oils as a mixture with iso-caryophyllene or α-humulene (Budavari et al., 1996). Structure of β-Caryophyllene is shown in Figure 15.

BCP is being used since 1930 in different forms in foods and cosmetics, however the first total synthesis was in 1964 and to date several approaches including semi-synthetic and microbial biotransformation have been adopted in order to meet the industrious demand for the production and supply of BCP (Oda et al., 2011; Sultana & Saify, 2013).

![Figure 15: Structure of β-Caryophyllene (BCP) (Javed et al., 2016)](image-url)
BCP, which is a sesquiterpene, is effective on the endocannabinoid system in terms of binding to the CB2 receptor. Due to its lipophilicity, BCP can cross through the plasma membranes (Gertsch et al., 2008b; Sharma et al., 2016). CB2 receptor agonists have been reported to repress inflammation (Gertsch et al., 2008b). BCP and β-Caryophyllene oxide (BCPO), which is an oxide of BCP, show both anticancer and analgesic features (Fidyt et al., 2016). Moreover, BCP has anti-inflammatory (Medeiros et al., 2007; Sain et al., 2014), antimicrobial (Sabulal et al., 2006), antioxidative (Kubo et al., 1996; Singh et al., 2006), antispasmodic (Leonhardt et al., 2010), and antiviral (Astani et al., 2011) effects. A research which was carried out in 2012 revealed that BCP was effective in alleviating cisplatin-induced nephrotoxicity (Horvath et al., 2012b). The researchers found CB2 receptors in the rat heart using the Western Blot method (Bouchard, Lépicier & Lamontagne, 2003). The research also revealed that endocannabinoids (endogenous cannabinoids) exhibited a strong cardioprotective effect mainly by the activation of a CB2 receptor in a rat model of cardiac ischemia-reperfusion (I/R) injury (Bouchard et al., 2003). Besides this, CB2 expression was upregulated in cardiomyocytes of the ischemic mouse hearts (Duerr et al., 2014). Due to all these promising features of BCP, it has been chosen as a perfect candidate to be used in this study.

BCP is not only a volatile substance but also its solubility in water is quite low. It is also quite sensitive to ambient factors such as temperature, humidity, light, oxygen, and moisture (Sköld et al., 2006; Wang et al., 2010). The animal studies show that BCP, which is orally bioavailable, reaches its peak concentration in serum/plasma within an hour after administration (Liu et al., 2013). BCP is not metabolized instantly like the popularly used polyphenolic compounds which have been recently researched. However, its volatility and poor solubility in the water cause a decrease in the
bioavailability of BCP (Liu et al., 2015). The pharmacokinetic characteristics of BCP, including its bioavailability, have been enhanced by delivering BCP in a β-cyclodextrin inclusion complex. Thus it renders a hydrophilic surface to BCP improving its dissolution and solubility in the water (Liu et al., 2015). The Physico-chemical properties and pharmacokinetics of BCP are summarized in Table 9.

Table 9: Physico-chemical properties and pharmacokinetics of BCP (Sharma et al., 2016)

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>β-Caryophyllene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synonym:</td>
<td>Caryophyllene</td>
</tr>
<tr>
<td>Physical state:</td>
<td>Colorless to yellow oily liquid</td>
</tr>
<tr>
<td>Solubility:</td>
<td>Insoluble in water</td>
</tr>
<tr>
<td>Odor:</td>
<td>Woody/terpene and cloves odor</td>
</tr>
<tr>
<td>Density:</td>
<td>0.90 g/mL at 20°C</td>
</tr>
<tr>
<td>Boiling point:</td>
<td>256-259°C at 760 mmHg</td>
</tr>
<tr>
<td>Specific gravity:</td>
<td>0.899 to 0.908 at 25°C</td>
</tr>
<tr>
<td>Molecular weight:</td>
<td>204.35 g/mol</td>
</tr>
<tr>
<td>Molecular formula:</td>
<td>C15H25</td>
</tr>
<tr>
<td>CAS number:</td>
<td>87-44-5</td>
</tr>
<tr>
<td>XLogP3-AA:</td>
<td>4.4</td>
</tr>
</tbody>
</table>

1.5.1 Pharmacological properties of BCP

BCP is considered as a multifunctional and poly pharmacological agent for therapeutic development in complex diseases (Chicca et al., 2014). The pharmacological targets of BCP are exemplified in Figure 16.
It is seen that BCP regulate the expression and release of a variety of pro-inflammatory cytokines, chemokine’s, growth factors, transcription factors, genes, enzymes, adhesion molecules, receptors and heat shock proteins and apoptosis and also proteins associated with the cell cycle (Bento et al., 2011; Gertsch et al., 2008c; Horvath, et al., 2012b; Jung et al., 2015; Sain et al., 2014). It has been stated that BCP regulated multiple molecular targets in various diseases and disorders and this is performed by modifying gene expression, molecular and cellular signaling pathways or by direct interaction with the targets to thwart the progression and development of various disease processes (Sharma et al., 2016).

Figure 16: The multiple receptor targets of β-caryophyllene
It has been manifested that BCP has various pharmacological targets. BCP functions by adjusting cellular and molecular signaling tracts, modifying gene expression and also interacting with biochemical and/or molecular targets (Sharma et al., 2016). Table 10, display some of the molecular mechanisms, which BCP is involved in.

Table 10: Molecular mechanisms that BCP has an effect on (Sharma et al., 2016)

<table>
<thead>
<tr>
<th>Molecular mechanisms that BCP has an effect on (Sharma et al., 2016)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of intracellular Ca²⁺</td>
</tr>
<tr>
<td>Voltage-dependent Ca²⁺ channel blockade</td>
</tr>
<tr>
<td>Inhibition of proinflammatory cytokines</td>
</tr>
<tr>
<td>Inhibition of activation of the toll-like receptor complex CD14/TLR4/MD2</td>
</tr>
<tr>
<td>Activates the mitogen-activated kinases Erk1/2 and p38</td>
</tr>
<tr>
<td>Blocker of STAT3 signaling cascade</td>
</tr>
<tr>
<td>Enhanced phosphorylation of AMP-activated protein kinase (AMPK)</td>
</tr>
<tr>
<td>Enhanced phosphorylation of cAMP responsive element-binding protein (CREB)</td>
</tr>
<tr>
<td>Stimulates SIRT1/PGC-1α-dependent mechanism</td>
</tr>
<tr>
<td>Down-regulation of anti-apoptotic genes (bcl-2, mdm2, cox2 and cmyb)</td>
</tr>
<tr>
<td>Up-regulation of pro-apoptotic genes (bax, bak1, caspase-8, caspase-9 and ATM)</td>
</tr>
</tbody>
</table>

In several experimental studies (in vitro, in vivo and in silico), it has been manifested that BCP interacts with and binds to CB2 receptors and serves as a full selective functional agonist (Gertsch, 2008a). It was revealed that BCP specifically binds with Δ9-tetrahydrocannabinol binding site in the CB2 receptor. It is also exceptional in terms of having an atypical cyclobutane ring, a unique scaffold that enables an interacting geometry for its binding to CB2 receptors (Gertsch et al.,...
The CB2 receptors are G-protein coupled receptors that generally bind with G-proteins in the Gi/o family. Triggering of the CB2 receptors leads to multiple intracellular effects that may be of cell type and ligand specific and that involve the suppression of diverse voltage gated Ca\(^{2+}\) channels and adenylate cyclase activity and the triggering of K\(^{+}\) channels, leading to lower levels of cAMP along with the triggering of MAPK pathways (Felder et al., 1995). The CB2 receptor combined with Gi intercedes their cellular effects through the suppression of adenylyl cyclase and regulation of transcription factors (Felder et al., 1995). Activation of CB2 receptors symbolize an important therapeutic target in numerous diseases (Calleja et al., 2013; Gertsch et al., 2008c; Horvath et al., 2012b). The cyclobutane pharmacophore of BCP was further utilized as a template for the discovery of new drugs and exposed to structural modifications. The modifications brought forth a series of new monocyclic amides which preserved CB2 receptor agonism property in addition to its quality in order to suppress fatty acid amide hydrolase (FAAH), an endocannabinoid degrading enzyme of the endocannabinoid system that improves the tone of endocannabinoid signaling. The generated molecules also evoke the suppressive properties on endocannabinoid substrate-specific metabolizing enzyme, cyclooxygenase-2 (COX-2), which is an important interceder of inflammatory pathways in the metabolism of arachidonic acid for the generation of prostanglandins (Chicca et al., 2014). This shows that the pharmacophore of BCP is disposed to preserve the polypharmacological nature.

It has also been determined that BCP regulate the nuclear receptors and transcription factor, peroxisome proliferator-activated receptors (PPARs) subtype, PPAR-\(\gamma\) and PPAR-\(\alpha\). The triggering of PPARs by cannabinoid affiliated molecules evokes multiple beneficial physiological effects and therapeutic advantages (Bento et
The PPARs are among the significant members of nuclear receptor superfamily, which function as ligand triggered transcription factors and play a vital role in the differentiation and proliferation of cells, organogenesis as well as inflammation. They also found to regulate the expression of hepatic enzymes and participate in glucose homeostasis, insulin sensitivity as well as lipid metabolism (Kota et al., 2005). There are three separate isoforms of PPARs namely PPAR-α, PPAR-γ and PPAR-δ which show different ligand selectivity and specific distribution in the tissues. Among these different isoforms, PPAR-α is primarily expressed in heart, liver, intestine and macrophages and is triggered by polyunsaturated fatty acids and leukotrienes. While PPAR-γ is essentially expressed in adipocytes, but its transcript has also been specified in various other tissues, though in low abundance. PPAR-γ plays a critical role in adipocyte differentiation and lipid accumulation as it is triggered by polyunsaturated fatty acids and 15d-prostaglandin J2 (Inagaki et al., 2007). The PPAR isoforms are like those of the steroid receptors and are associated with several functions started by nutrients, nutraceuticals and phytochemicals.

The ligand-binding to cannabinoid receptors activate mitogen-activated protein kinase (MAPK), which adjusts the activation of PPAR even more through direct phosphorylation. Although the accurate mechanisms about cannabinoid and PPARs interactions are not known, the PPAR stimulation by cannabinoid ligands materialize as partners that act together to obtain the therapeutic benefits (O'Sullivan & Kendall, 2010). This presents a basis through which cannabinoids can regulate gene transcription triggered by the dietary interferences. Since BCP is highly lipophilic, it can go across the membranes and access the nuclear receptors; that is, it has the capacity to regulate both the surface and nuclear receptors.
BCP was also observed to suppress the pathways activated by the initiation of the toll like receptor complex (CD14/TLR4/MD2) and curtailed the immune-inflammatory processes in multiple autoimmune diseases (Gertsch, 2008a). The extra μ-opioid receptor activity (Katsuyama et al., 2013; Paula-Freire et al., 2014) and effective antagonist activity on homomeric nicotinic acetylcholine receptors (α7-nAChRs) strengthen its potent anti-inflammatory activity. Multiple studies have shown the in vitro (Donati et al., 2015; Lee et al., 2013; Vinholes et al., 2014) and in vivo antioxidant mediated protection of different organs (Calleja et al., 2013; Guo et al., 2014; Horvath et al., 2012b; Molina-Jasso et al., 2009). BCP displayed an effective chain breaking antioxidant and free radical scavenger activity against the highly reactive free radicals such as hydroxyl and superoxide anions (Molina-Jasso et al., 2009; Vinholes et al., 2014). BCP has also been observed to regenerate the glutathione redox cycle and efficiently impede lipid peroxidation, a significant pathogenic event and a critical culprit in most of the chronic degenerative and acute organ injuries. BCP has also been observed to be distinguished compared to various standard antioxidants such vitamin C and E, available for clinical use (Vinholes et al., 2014). BCP (32.5%) extracted from essential oil of Teucrium flavum L. subsp. flavum displayed free radical scavenging activity and antioxidant potential in DPPH assay (Hammami et al., 2015). It has also been observed to impede 5-lipoxygenase, an important enzyme of arachidonic acid pathways that is efficiently involved in the production of inflammatory mediators and institution of inflammation in multiple inflammatory diseases (Sain et al., 2014). The GST inhibitory and lipoxygenase inhibitory activity of BCP was further supported by in silico data (Babu et al., 2012). The antioxidant activities interceded by the CB2 receptors were also displayed and they implied the
additional antioxidant properties of BCP in therapeutic benefits other than anti-inflammatory activity (Assis et al., 2014; Vinholes et al., 2014).

Regarding BCP, it has been stated that it exercises strong anti-inflammatory effects in various in vitro and in vivo studies (Bento et al., 2011; Gertsch et al., 2008c; Ma et al., 2015; Paula-Freire et al., 2014; Rufino et al., 2015). Moreover, these effects positively alter many of its biological and therapeutic activities in diseases where low grade and long term inflammation play a significant role in etiopathogenesis (Fine & Rosenfeld, 2013; Guo et al., 2014). The mostly attested favorable effects of BCP are its capacity to regulate pro-inflammatory cytokines and chemokines and avoid the occurrence and progression of immune-inflammatory disorders interceded by CB2 receptors (Gertsch et al., 2008a).

Several of the studies demonstrate that the anti-inflammatory effects of BCP are mediated by the activation of CB2 receptors. BCP upon binding to CB2 receptors inhibit the enzyme, adenylate cyclase which leads to intracellular Ca^{2+} transients and further activates the signaling pathways mediated by Erk1/2 and p38 (Gertsch, 2008a). As is the case with various known CB2 receptor specific ligands, BCP also impedes the pathways triggered by the activation of the toll-like receptor (TLR) complex; CD14/TLR4/MD2, which results in the expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) and incites TH1 interceded immune response interceded by CB2 receptor mechanism (Gertsch, 2008b; Gertsch et al., 2008c). Moreover, BCP exhibited significant anti-inflammatory effects in multiple in vivo models such as xylene-induced mice ear edema (Okoye et al., 2011), lipopolysaccharides (LPS)-induced inflammation (Medeiros et al., 2007) and carrageenan-induced rat paw inflammation (Gertsch et al., 2008c). The anti-inflammatory activity of various plants including Copaifera multijuga has been associated with the presence of a high amount
of BCP (Dias Dde et al., 2012). The essential oil of Hyptis pectinate, which involves 54.07% of BCP was developed in an inclusion complex with β-cyclodextrin and exhibited intensified analgesic and anti-inflammatory pharmacological effects in formalin-incited pain protocol in mice (Menezes Pdos et al., 2015). BCP is observed to be an important natural agent that treats diseases where immune-inflammatory alterations are the common accompaniment of the pathogenesis of diseases because of its multimodal anti-inflammatory mechanisms (Gertsch et al., 2008c). Besides, in addition to the antioxidant and anti-inflammatory activities, BCP exhibited effective immunomodulatory potential through the inhibition of T-cell immune responses in mouse primary splenocytes (Ku & Lin, 2013). The repressive effects of BCP on both, TH1/TH2 cytokines point to the potential possible benefits of BCP in various autoimmune diseases interceded by CB2 receptors activation and subsequent inhibition of toll like receptors (Ku & Lin, 2013). The CB2 receptors are mainly found on the cells of immune origin involving spleen and thymus and also circulating inflammatory cells including T and B-lymphocytes, natural killer cells, monocytes and neutrophils, which indicates a significant possible role of BCP in multiple diseases associated with the modulation of the immune system (Galiègue et al., 1995). As a result, the specific activation of CB2 receptors by BCP could offer encouraging therapeutic applications in multiple autoimmune and immune-inflammatory diseases.
1.5.2 Chemical properties and SAR of BCP

(E)-BCP is generally found together with small amounts of its isomers (Z)-β caryophyllene ((Z)-BCP) and α-humulene or in a mixture with its oxidation product, BCP oxide (Gertsch et al., 2008c). Chemical structures of the bicyclic sesquiterpenes is manifested in Figure 17.

Quantitative radioligand binding experiments performed by Gertsch et al. (2008c) revealed that (E)-BCP and its isomer, (Z)-BCP, dose-dependently displaced [3H]CP55,940 from hCB2 receptors expressed in HEK293 cells with apparent Ki values in the nM range. (E)-BCP exhibited a slightly higher CB2 receptor binding affinity (Ki=155 ± 4 nM) than (Z)-BCP (Ki= 485 ± 36 nM). Nevertheless, BCP oxide and the ring-opened isomer α-humulene did not remove [3H]CP55,940 from the hCB2 receptor (Ki > 20 µM). In line with the data obtained with Cannabis essential oils, BCP isomers did not exhibit significant binding affinity to the hCB1 receptor.

In silico docking Analysis of the (E)-BCP CB2, Receptor Binding Interaction was performed by Gertsch et al. (2008c). It indicates that (E)-BCP binds into the hydrophobic region of the water-accessible cavity. The apparent binding site of CB2
receptor ligands is situated next to helices III, V, VI, and VII at the near extracellular site of the 7TM bundles, as shown in Figure 18. In this model, the lipophilic (E)-BCP docks into the hydrophobic cavity of the amphipathic binding pocket and the binding mode of (E)-BCP seems to be alleviated by π–π stacking interactions with residues F117 and W258. Moreover, (E)-BCP closely interacts with the hydrophobic residues I198, V113, and M265, as shown in Figure 18.

Figure 18: In Silico Docking Analysis of the (E)-BCP CB2 Receptor Binding Interaction (Gertsch et al., 2008c)

1.5.3 Safety and toxicity of BCP

The FDA has classified BCP as a flavoring substances and it is designated “generally recognized as safe” by the USFDA and several other regulatory agencies worldwide for human consumption (Gertsch et al., 2008c). Recently, BCP has been approved by European Union (EU) for food preservation and adopted in EU for food additive legislation. The safety profile of BCP has been shown by several in vitro and in vivo studies revealing low toxicity for humans (Di Sotto et al., 2008; Di Sotto et al.,
2013; Di Sotto et al., 2010; LaVoie et al., 1986; Molina-Jasso et al., 2009; Seifried et al., 2006). It is reported as a safe functional non-psychoactive and a macrocyclic anti-inflammatory CB2 receptor ligand in foodstuffs (Gertsch, 2008a).

The LD50 of acute oral doses of BCP in rats and the LD50 of acute dermal doses in rabbits found more than 5000 mg/kg (Opdyke, 1973). BCP in intratracheal doses (12-48 mg/kg) was found non-toxic to the respiratory system including lungs in rats. Also, BCP in ointments upon dermal exposure did not found to cause skin irritation or sensitization in humans at concentrations up to 4% (LaVoie et al., 1986). Most of findings in literature have reported absence of genotoxic or mutagenic effects with BCP in preclinical studies (Di Sotto et al., 2008; Di Sotto et al., 2010; Molina-Jasso et al., 2009).

1.5.4 AM630

AM630 (iodoprvadoline) is an aminoalkyindole which act as a CB2 receptor antagonist. It is commonly used in research to demonstrate the effect, or lack thereof, of CB2 receptor inhibition (Javed et al., 2016). In this study AM630 was chosen as a CB2 receptor antagonist to demonstrate the protective effects of BCP mediated by CB2 receptors. Structure of AM630 is shown in Figure 19.

![Figure 19: Structure of AM630 (Javed et al., 2016)](image-url)
1.6 Aims and objectives of the study

A search for novel pharmacotherapy for MI is in progress for last decade. This is reflected by a large number of studies on natural as well as synthetic compounds to find out a suitable substitute for those drugs that have been used nowadays to treat myocardial infarction.

In recent years, the endocannabinoid system consisting of cannabinoid type 1 and 2 receptors (CB1 & CB2) has emerged as an important therapeutic target for cardioprotection (Pacher et al., 2006). Among the cannabinoid receptors, the CB2 receptor subtype has gained enormous attention as an important therapeutic target due to its multipharmacological properties and lack of psychoactivity, a common feature which manifest with cannabinoids (Mukhopadhyay et al., 2010). Among the ligands which target CB2 receptors, β caryophyllene (BCP), a dietary sesquiterpene garnered attention being widely found in various essential oils including cinnamon, oregano, black pepper, basil and cloves (Gertsch et al., 2008c) and pharmacologically showing a fully selective CB2 receptor agonist activity with a Ki value of 155nmol/L for human CB2 receptors, with no affinity for CB1 receptors, which causes activation of Gi/Go subtype of G-proteins (Gertsch et al., 2008c). The wide availability, dietary accessibility and favourable physicochemical and potent pharmacological properties makes it an outstanding candidate for therapeutic interventions. The therapeutic benefits of BCP through the activation of CB2 receptors have been revealed in various diseases affecting numerous organs such as liver (Calleja et al., 2013), kidney (Horvath et al., 2012b; Javed et al., 2016) and brain (Javed et al., 2016).
**Aim 1:** To determine the optimal dose of BCP from different doses (25, 50 and 100 mg/kg) in ISO-induced MI model in rats.

- To study the effect of BCP at different doses on cardiomyocyte injury marker (CK) in the serum, and to determine the optimum effective dose for further studies.

**Aim 2:** To investigate the therapeutic potential of natural CB2 receptor agonist, BCP in ISO induced MI in rats.

- To study the effect of BCP on cardiomyocyte injury markers in serum.
- To study the effect of BCP on hemodynamic measurements.
- To study the effect of BCP on oxidative stress and lipid peroxidation in the heart.
- To study the effect of BCP on myocardial pro-inflammatory cytokines and inflammatory mediators.
- To study the effect of BCP on apoptosis in the heart.
- To study the effect of BCP on NF-κB expressions in the myocardium.
- To study the effect of BCP on CB2/PPARγ expressions.
- To study the effect of BCP on structural changes in heart tissue.
- To elucidate the CB2 receptor mediated mechanism of BCP.
- Pretreatment with CB2 receptor antagonist AM630 should abolish/reverse the cardioprotective effects of BCP.
Chapter 2: Materials and Methods

2.1 Experimental animals

Male albino Wistar rats weighing (200-250 g) acquired from the experimental animal research facility in the College of Medicine and Health Sciences (CMHS), United Arab Emirates University. Maximum of four rats were housed per cage in polypropylene cages (47 × 34 × 20 cm³) lined with husk (renewed every 24 h) in a 12-hour light/dark cycle at pathogen free environment and controlled temperature around 22°C. Rats were fed a standard rodent chow diet (National Feed and Flour Production and Marketing Company LLC., Abu Dhabi, UAE) and water ad libitum. The experimental protocol and procedures for animal experimentation was approved by the Animal Ethics Committee of United Arab Emirates University.

2.2 Induction of experimental myocardial infarction

Isoproterenol (85 mg/kg body weight) dissolved in saline and injected subcutaneously to rats at an interval of 24 h for two days (Malik et al., 2011; Ojha et al., 2011; Ojha et al., 2012; Ojha et al., 2013). ISO-induced MI was confirmed by increased activities of serum creatine kinase (CK) and lactate dehydrogenase (LDH) in rats.

2.3 Dose dependent study

A total of 60 rats divided into 10 groups of 6 rats in each group were used in the dose dependent study. Blood was collected after cervical decapitation; serum was separated and used for the assay CK to reveal the dose dependent effect of BCP.
Group I: Rats were orally given light olive oil in a similar quantity to BCP treatment and used as a normal control.

Groups II: Rats were orally treated with BCP (100 mg/kg body weight,) dissolved in light olive oil daily for a period of 10 days.

Group III: Rats were subcutaneously injected with ISO alone (85 mg/kg body weight) at an interval of 24 h for 2 days (on 9th and 10th day).

Group IV: Rats were orally pre and co-treated with BCP (25 mg/kg body weight) dissolved in light olive oil daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (on 9th and 10th day).

Group V: Rats were orally pre and co-treated with BCP (50 mg/kg body weight) dissolved in light olive oil daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (on 9th and 10th day).

Group VI: Rats were orally pre and co-treated with BCP (100 mg/kg body weight) dissolved in light olive oil daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (on 9th and 10th day).

Group VI: Rats were orally pre and co-treated with BCP (100 mg/kg body weight) dissolved in light olive oil daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (on 9th and 10th day).

Group VII: Rats were orally pre and co-treated with BCP (25 mg/kg body weight) dissolved in light olive oil daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (on
9th and 10th day). Rats were treated with AM630 (1 mg/kg), a selective CB2 receptor antagonist prior to the administration of BCP to reveal the functional CB2 receptor dependent mechanisms of BCP.

Group VIII: Rats were orally pre and co-treated with BCP (50 mg/kg body weight) dissolved in light olive oil daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (on 9th and 10th day). Rats were treated with AM630 (1 mg/kg), a selective CB2 receptor antagonist prior to the administration of BCP to reveal the functional CB2 receptor dependent mechanisms of BCP.

Group IX: Rats were orally pre and co-treated with BCP (100 mg/kg body weight) dissolved in light olive oil daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (on 9th and 10th day). Rats were treated with AM630 (1 mg/kg), a selective CB2 receptor antagonist prior to the administration of BCP to reveal the functional CB2 receptor dependent mechanisms of BCP.

Group X: Rats were intraperitoneally injected with AM630 (1 mg/kg body weight) for a period of 10 days.

**Assay of creatine kinase:**

Serum CK activity was assayed by using VetTest® 8008 Chemistry Analyzer (IDEXX Laboratories, Hoofddrop, Netherland).

**Statistical analysis:**

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 12.0. Results were expressed as mean
± standard deviation (S.D) for 6 rats in each group. P values <0.05 were considered significant.

2.4 Experimental design

The animals were randomly divided into six experimental groups each containing 15 rats. BCP (Sigma-Aldrich, St. LOUIS, MO, USA) was diluted in scientific grade light olive oil which has been used as a vehicle, based upon previous research studies. AM630 (Sigma-Aldrich, St. LOUIS, MO, USA) a selective CB₂ receptor antagonist, was prepared by using Dimethyl sulfoxide (DMSO) as solvent and saline as vehicle. ISO (Sigma-Aldrich, St. LOUIS, MO, USA) was used to induce myocardial infraction. BCP, AM630 and ISO were administered orally, intraperitoneally and subcutaneously, respectively. All treatments were prepared fresh just before dosing.

2.5 Experimental groups

The dose of BCP was given as (50 mg/kg body weight) and the dose of ISO was (85 mg/kg body weight). The dose selection in this experimental protocol was based in dose dependent study.

Group I: Rats were orally given light olive oil in a similar quantity to BCP treatment and used as a normal control.

Groups II: Rats were orally treated with BCP (50 mg/kg body weight) dissolved in light olive oil daily for a period of 10 days.

Group III: Rats were subcutaneously injected with ISO alone (85 mg/kg body weight) at an interval of 24 h for 2 days (on 9th and 10th day).
Group IV: Rats were orally pre and co-treated with BCP (50 mg/kg body weight, BID) dissolved in light olive oil daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (on 9th and 10th day).

Group V: Rats were orally pre and co-treated with BCP (50 mg/kg body weight, BID) dissolved in light olive oil daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (on 9th and 10th day). Rats were treated with AM630 (1 mg/kg), a selective CB2 receptor antagonist prior to the administration of BCP to reveal the functional CB2 receptor dependent mechanisms of BCP.

Group VI: Rats were intraperitoneally injected with AM630 (1 mg/kg body weight, OD) for a period of 10 days.

12 hours after the second dose of ISO injection (i.e. on 11th day), the rats were anesthetized by pentobarbital sodium (60 mg/kg body weight) and then sacrificed by cervical decapitation. For serum, blood was collected in dry tubes without anticoagulant and serum was separated by centrifugation at room temperature for 15-20 minutes at 3000 rpm. The heart was excised immediately, rinsed in ice chilled saline then snap frozen in liquid nitrogen and stored at -80°C, for biochemical analysis. Some hearts were collected and fixed in both 10% neutral buffered formalin and karnovsky fixative and stored at 4°C for histological studies such as light microscopy and transition electron microscopy respectively.

2.6 Hemodynamic measurements

The measurement of systolic blood pressure (SP), diastolic blood pressure (DP) and mean arterial pressure (MAP) were measured by tail cuff method using CODA
Non-invasive Blood Pressure System (Kent Scientific Corporation., U.S.A). Heart rate (bpm) was also measured using the CODATM monitor equipped with a volume pressure recording sensor. The conscious rats were well placed in a restrainer that was positioned on a heating pad to maintain the body temperature and establish ample blood flow to the tails. Each rat was allowed to adjust to the restrainer for 5-7 minutes and thereafter blood pressure was measured. The volume-pressure recording determines the tail blood volume using a volume pressure recording sensor and an occlusion tail-cuff. Twenty continuous cycles were performed and the average values were used for data analysis as mentioned in the Statistical Analysis section. 15 seconds between each cycle was programmed. The CODATM monitor system includes a controller, laptop computer, software, cuffs, animal holders, infrared warming pads and an infrared thermometer.

2.7 Biochemical estimations

2.7.1 Serological evaluation

Serum creatine kinase (CK) and lactate dehydrogenase (LDH) activity was assayed by using VetTest® 8008 Chemistry Analyzer (IDEXX Laboratories, Hoofddrop, Netherland).

2.7.2 Assessment of oxidative stress and lipid peroxidation

The concentrations of heart thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) were estimated.
Estimation of heart thiobarbituric acid reactive substances:

The concentration of TBARS in the heart was estimated by the method of (Fraga et al., 1988).

Reagents:

1. Thiobarbituric acid - 0.38% in hot distilled water.
2. Trichloro acetic acid - 15%.
3. Hydrochloric acid - 0.25 N.
4. Thiobarbituric acid-trichloro acetic acid-hydrochloric acid reagent: Solutions (1), (2) and (3) were mixed freshly in the ratio of 1:1:1.
5. Stock standard: 4.8 mM: 0.079 ml of 1, 1", 3, 3' tetra methoxy propane was diluted to 100 ml.
6. Working standard: Stock solution was diluted to get a concentration of 4.8 nM/ml.

Procedure:

The heart tissue homogenate was prepared in Tris-HCL buffer (pH 7.5). 100 µl of the tissue homogenate was treated with 200 µl of thiobarbituric acid-trichloro acetic acid-hydrochloric acid reagent and mixed thoroughly. The mixture was kept in a boiling water bath for 15 minutes. After cooling, the tubes were centrifuged for 10 minutes and the supernatant was taken for measurement. The absorbance was read at 535 nm against the reagent blank. The TBARS values were expressed as mmoles/100g wet tissue.
Estimation of lipid hydroperoxides:

The levels of LOOH in the heart was estimated by the method of (Jiang et al., 1992).

Reagents:

1. Fox reagent: 88 mg of butylated hydroxy toluene, 7.6 mg of xylenol orange and 9.8 mg of ammonium iron (II) sulphate were added to 90 ml of methanol and 10 ml of concentrated sulphuric acid.

2. Standard - 0.2 M, hydrogen peroxide.

Procedure:

180 \( \mu l \) of the Fox reagent was mixed with 20 \( \mu l \) of the heart homogenate/plasma and incubated for 30 minutes at room temperature. The colour developed was read at 560 nm.

The LOOH values were expressed as mmoles/100 g wet tissue for heart.

Assay of superoxide dismutase:

The activity of superoxide dismutase in the heart was assayed according to the procedure of (Kakkar et al., 1984).

Reagents:

1. Sodium pyrophosphate buffer - 0.025 M, pH 8.3

2. Phenazine methosulphate - 186 \( \mu M \)

3. Nitroblue tetrazolium - 300 \( \mu M \)

4. Nicotinamide adenine dinucleotide (reduced) - 780 \( \mu M \)

5. Glacial acetic acid

6. n-Butanol
7. Chloroform
8. Ethanol

Procedure:

20 µl of heart tissue homogenate was diluted to 100 µl with distilled water. Then, 250 µl of ethanol and 150 µl of chloroform (all reagents chilled) were added. This mixture was shaken for one minute at 4°C and then centrifuged. The enzyme activity in the supernatant was assayed. The assay mixture contained 120 µl of sodium pyrophosphate buffer (0.025 M, pH 8.3), 10 µl of 186 µM phenazine methosulphate, 30 µl of 30 µM nitroblue tetrazolium, 20 µl of 780 µM nicotinamide adenine dinucleotide (reduced), appropriately diluted enzyme preparation and distilled water in a total volume of 300 µl. The reaction was started by the addition of nicotinamide adenine dinucleotide (reduced). After incubation at 30°C for 90 seconds, the reaction was arrested by the addition of 100 µl of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 400 µl of n-butanol. The intensity of the chromogen in the butanol layer was measured at 560 nm against a blank containing n-butanol. The activity of superoxide dismutase was expressed as U/mg protein. One unit of the enzyme activity is defined as the enzyme concentration required inhibiting the optical density at 560 nm of chromogen production by 50% in one minute.

Assay of catalase:

The activity of catalase in the heart was assayed by the method of (Sinha, 1972).

Reagents:

1. Phosphate buffer - 0.01 M, pH 7.0.
2. Hydrogen peroxide - 0.2 M.
3. Potassium dichromate - 5%.

4. Dichromate-acetic acid reagent: Potassium dichromate and glacial acetic acid were mixed in the ratio of 1:3. From this, 1.0 ml was diluted again with 4.0 ml of glacial acetic acid.

5. Standard hydrogen peroxide: 0.1 ml of 0.2 M hydrogen peroxide was diluted to 100 ml with distilled water.

Procedure:

To 50 μl of phosphate buffer, 50 μl of heart tissue homogenate and 50 μl of hydrogen peroxide were added. After 60 seconds, 200 μl of dichromate-acetic acid mixture was added. The tubes were kept in a boiling water bath for 10 minutes and the color developed was read at 620 nm. The activity of catalase was expressed as μmoles of hydrogen peroxide consumed/minute/mg protein.

Estimation of reduced glutathione:

The levels of GSH in the heart was estimated by the method of (Ellman, 1959).

Reagents:

1. Phosphate buffer - 0.2 M, pH 8.0.
2. Trichloro acetic acid - 5%.
3. Ellman's reagent: 19.8 mg of dithio nitro bisbenzoic acid in 100 ml of 1% sodium citrate solution.
4. Disodium hydrogen phosphate - 0.3 M.
5. Standard glutathione solution: 10 mg of reduced glutathione was dissolved in 100 ml of distilled water.
Procedure:

A known weight of heart tissue was homogenized in phosphate buffer. From this, 50 µl of tissue homogenate was pipetted out and precipitated with 200 µl of 5% trichloro acetic acid. After centrifugation, 100 µl of the supernatant was taken. To this, 50 µl of Ellman’s reagent and 300 µl of phosphate buffer were added. The yellow color developed was read at 412 nm. The concentrations of GSH was expressed as mmoles/g wet tissue for heart.

2.7.3 Assessment of inflammatory markers in cardiac tissue

Heart tissue was homogenized in lyss buffer using Tris–HCl buffer (0.1 M; pH 7.4) and 1X HaltTM phosphatase inhibitor cocktail (Thermo Fisher Scientific). A handheld homogenizer (TH tissue homogenizer, OMNI, USA) was used and the homogenate was centrifuged at 4°C for 30 minutes at 12,000 rpm. Supernatant was collected and used to quantify inflammatory markers via ELISA assay kits for TNF-α, IL-6 and IL1β (R&D Systems, Minneapolis, MN, USA). The assays were carried out as instructed by the manuals provided in the respective kits.

2.7.4 Western Blot analysis

Protein extractions have been obtained by homogenizing heart samples in an ice-cold RIPA buffer (Sigma Aldrich, St. Louis, MO, USA). The homogenate was centrifuged at 4°C for 30 minutes at 12,000 rpm. The clear supernatant was mixed with laemmlili sample buffer (Bio-Rad, USA) along with 2-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA). The protein contents in the sample were estimated using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Samples containing equal amount of proteins has been loaded and separated by
gel electrophoresis using SDS polyacrylamide (12%). Then it transferred onto PVDF membranes (Amersham Hybond P 0.45, PVDF, GE Healthcare Life Sciences, Germany) and incubated overnight at 4°C with primary antibodies against COX-2 (1:500), iNOS (1:2000), (Sigma Aldrich, St. Louis, MO, USA), B-cell lymphoma-2 associated-x (Bax) (1:1000), anti-apoptotic B-cell lymphoma-2 (Bcl-2) (1:1000), B-cell lymphoma-extra-large (bcl-xL) (1:1000), active caspase-3 (1:500), (Abcam, Cambridge, MA, USA), CB2 receptor ((1:500) dilution; Abcam, Cambridge, UK) PPARγ (1:500) (Cell signaling technology, USA), NF-κB-p65 (1:1000), p-NF-κB-p65 (1:1000), cTnI (1:1000) and β-actin ((1:2000) dilution; MERCK Millipore, USA) was used as a loading control. The samples were then incubated with their corresponding secondary antibodies (anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase) for one hour at room temperature and the proteins were visualized by using an enhanced chemiluminescence pico kit (Thermo Fisher Scientific, Rockford, IL, USA). The intensity of bands was quantified using Image J software public domain Java image processing software.

2.8 Histopathological evaluation

After fixation of heart tissue in 10% neutral buffered formalin for a week, the tissue was gradually dehydrated in increasing concentrations of ethanol, cleared in xylene and finally embedded in paraffin wax. Sections of 10 µm thickness were cut using a microtome (RM2125 RTS, Leica Biosystems, Nussloch, Germany) and stained with hematoxylin and eosin (H&E). The H&E sections were mounted on glass slides and evaluated under a light microscope (BX41, Olympus).
2.9 Transmission electron microscopy (TEM)

Tissue samples from the apex of the heart were fixed in Karnovsky’s fixative for TEM imaging. After fixation and subsequent washing in buffer solution, samples were dehydrated in increasing concentration of ethanol. This was followed by treatment with propylene oxide and staining with osmium tetroxide. Samples were treated with propylene oxide and resin mixtures before finally being embedded in resin for ultrathin slicing and examination.

2.10 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was done using one-way analysis of variance (ANOVA), followed by the Duncan’s Multiple Range Test (DMRT) using IBM SPSS statistics v24.0. A value of \( P < 0.05 \) was considered statistically significant.
Chapter 3: Results

3.1 Effect of BCP on serum CK (Dose dependent study)

A pilot study was conducted with three different doses of BCP (25, 50, and 100 mg/kg body weight) to determine its dose dependent effect in ISO induced myocardial infarcted rats, as shown in Figure 20. ISO injections showed a significant rise in the serum levels of CK. However, treatment with BCP at all the doses prevented the leakage of CK into the serum as evidenced by the reduced serum CK levels in the treatment groups as compared to the ISO group. BCP (50 mg/kg body weight) treatment showed more promising effect compared to the other doses of BCP on the activity of serum CK in ISO induced myocardial infarcted rats compared to ISO alone induced myocardial infarcted rats. BCP (100 mg/kg body weight) alone treated rats showed no significant effects compared to normal control rats. Since, 50 mg/kg body weight of BCP showed the highest effect, this dose has been chosen for the further studies.
3.2 Effect of BCP on cardiomyocyte injury marker enzymes in serum

The effects of BCP on the activities of CK and LDH in the serum of normal and ISO induced rats is presented in Figure 21. ISO injections increased the serum levels of CK and LDH as compared to the normal control rats. However, treatment with BCP prevented the injury induced release of CK, and LDH into the serum as evidenced by the significant (P<0.05) decrease in the levels of these cardiac marker enzymes in the serum compared to ISO control rats. Interestingly, this protective effect of BCP was significantly (P<0.05) reversed by prior administration of CB2 receptor antagonist AM630 to the rats treated with ISO and BCP. Rats treated with AM630 alone did not show any alterations in the levels of CK and LDH as compared to normal control rats.

Figure 20: Activity of serum CK (dose dependent study). Each column is mean ± SEM for six rats in each group; *P<0.05 compared to normal control, $P<0.05 compared to ISO control, #P<0.05 compared to BCP+ISO (DMRT).
3.3 Effect of BCP on hemodynamic measurements

The effect of BCP on hemodynamic parameters was presented in Figure 22. ISO injections to the rats caused a significant (P<0.05) decrease in the heart rate, systolic, diastolic, and mean arterial pressure compared to the normal control rats. BCP treatment significantly (P<0.05) restored the heart rate, systolic, diastolic, and mean arterial pressure compared to ISO control rats. However, treatment with AM630 prior to the administration of BCP considerably (P<0.05) alters the hemodynamics of rats administered ISO when compared to ISO control rats. Rats treated with AM630 alone did not show significant alterations in the hemodynamics of rats.
3.4 Effect of BCP on lipid peroxidation

The levels of lipid peroxidation in the heart of normal and experimental rats is presented in Figure 23. Rats induced with ISO showed significant (P<0.05) increase in the levels of lipid peroxidation products in the heart compared to normal control rats. Treatment with BCP to ISO induced rats near normalized the levels of heart lipid peroxidation products such as TBARS and LOOH compared to ISO alone induced rats. The administration of AM630 before BCP treatment significantly abrogated the effect of BCP on heart TBARS and LOOH. Furthermore, rats treated with AM630 alone did not showed any alterations in the lipid peroxidation parameters compared to normal control rats.
The activities of the antioxidant enzymes (catalase and SOD) and non-enzymatic antioxidant (GSH) are presented in Figure 24. Rats challenged with ISO showed a significant (P<0.05) decrease in the activities/concentrations of SOD, catalase and GSH compared to normal control rats. However, rats treated with BCP showed significant (P<0.05) increase in the activities/concentration of SOD, catalase and GSH compared to ISO control rats. Administration of AM630 before BCP treatment significantly reduced the impact of BCP in ISO challenged rats. Furthermore, rats treated with AM630 alone did not showed any alterations in the above-mentioned parameters compared to normal control rats.

3.5 Effect of BCP on antioxidants

The activities of the antioxidant enzymes (catalase and SOD) and non-enzymatic antioxidant (GSH) are presented in Figure 24. Rats challenged with ISO showed a significant (P<0.05) decrease in the activities/concentrations of SOD, catalase and GSH compared to normal control rats. However, rats treated with BCP showed significant (P<0.05) increase in the activities/concentration of SOD, catalase and GSH compared to ISO control rats. Administration of AM630 before BCP treatment significantly reduced the impact of BCP in ISO challenged rats. Furthermore, rats treated with AM630 alone did not showed any alterations in the above-mentioned parameters compared to normal control rats.
The effect of BCP on pro-inflammatory cytokine levels is presented in Figure 25. The levels of TNF-α, IL-6, IL-1β were significantly (P<0.05) increased in ISO induced myocardial infarcted rats compared to normal control rats. BCP treatment showed significant (P<0.05) decrease in the levels of TNF-α, IL-1β, IL-6 in ISO induced myocardial infarcted rats compared to ISO alone induced rats. Administration of AM630 before BCP treatment significantly (P<0.05) decreased the effect of BCP in ISO-induced MI in rats. Rats treated with AM630 alone did not show any alterations in the levels/expressions of cytokines and inflammatory mediators compared to normal control rats.

3.6 Effect of BCP on pro-inflammatory cytokines

The effect of BCP on pro-inflammatory cytokine levels is presented in Figure 25. The levels of TNF-α, IL-6, IL-1β were significantly (P<0.05) increased in ISO induced myocardial infarcted rats compared to normal control rats. BCP treatment showed significant (P<0.05) decrease in the levels of TNF-α, IL-1β, IL-6 in ISO induced myocardial infarcted rats compared to ISO alone induced rats. Administration of AM630 before BCP treatment significantly (P<0.05) decreased the effect of BCP in ISO-induced MI in rats. Rats treated with AM630 alone did not show any alterations in the levels/expressions of cytokines and inflammatory mediators compared to normal control rats.
3.7 Effect of BCP on inflammatory mediators and NF-κB expressions

The expression of iNOS and COX-2 is presented in Figure 26. The expression of COX-2 and iNOS increased in ISO-challenged rats as compared to control rats. However, BCP treatment significantly decreased the elevated expression of COX-2 and iNOS as compared to ISO-injected animals. AM630 pretreatment abrogated the protective effect of BCP with respect to COX-2 expression. The expression of NF-κB is presented in Figure 26, (A, D). Administration of ISO caused a remarkable increase in the expression of NF-κB while treatment with BCP significantly reduced NF-κB expression.
The effect of BCP on apoptosis is presented in Figure 27. ISO-induced MI in rats showed a significant (P<0.05) increase in the myocardial protein expressions of Bax and active caspase-3 along with significant (P<0.05) decrease in the expressions of Bcl-2 and bcl-xL compared to normal control rats. Treatment with BCP to ISO-induced MI in rats showed significant (P<0.05) decrease in the expressions of Bax and active caspase-3 with significant (P<0.05) increase in the expressions of Bcl-2 and bcl-
xL which in turn was significantly (P<0.05) abrogated by the CB2 receptor antagonist, AM630. AM630 alone treated rats did not show any alterations in the apoptotic markers compared to normal control rats.

Figure 27: Effect of BCP on apoptosis. (A), represents the images of Western immunoblot analysis for Bax, Bcl2, Bcl-xL and active caspase-3 in the myocardium. (B-E), represents the densitometric analysis of myocardial protein expressions of (B) Bax, (C) Bcl2, (D) Bcl-xL and (E) active caspase-3 assessed by Western blot analysis. *P<0.05 compared to normal control, $P<0.05$ compared to ISO control, #P<0.05 compared to BCP+ISO (DMRT).
3.9 Effect of BCP on CB2/PPARγ expressions and troponin-I

The effect of BCP on CB2/PPARγ expressions and troponin-I is presented in Figure 28, (A-D). ISO-induced MI in rats exhibited a significant (P<0.05) decrease in the myocardial expression of CB2 receptors, PPARγ and troponin-I compared to normal control rats, whereas BCP treatment showed significant (P<0.05) increase in the expression of CB2 receptors, PPARγ and troponin-I compared to ISO control rats. However, treatment with the selective CB2 receptor antagonist, AM630, prior to the administration of BCP caused a considerable decline in the expression of CB2 receptors, PPARγ and troponin-I in ISO-induced MI in rats. Additionally, rats treated with AM630 alone showed a subtle insignificant decrease in the expression of CB2 and PPARγ in the myocardium.
3.10 Effect of BCP on Histopathology of myocardium

Histopathological changes in the heart tissues of all groups were shown in Figure 29. The heart of normal control rats (i) showed a normal intact muscle with no edema, and inflammation. BCP alone treated rat's heart (ii) also showing normal intact muscle fibers without degradation and inflammatory cells. However, rats treated with ISO (iii) showed extensive muscle fiber degradation with inflammatory cells. Treatment with BCP to ISO-induced MI in rats (iv) protects the myocardial
architecture as evidenced by less muscle fiber degradation without inflammatory cells. Furthermore, treatment with CB2 receptor antagonist AM630 (v) abrogated the protective effects of BCP against ISO-induced pathological changes in the myocardium. Treatment with AM630 (vi) did not show any pathological changes compared to normal control rats.

Figure 29: Effect of BCP on myocardial structure. (i) NOR, (ii) BCP, (iii) ISO, (iv) ISO+BCP, (v) ISO+BCP+AM630 and (vi) AM630. (X10 magnification)
3.11 Effect of BCP on cardiomyocyte ultrastructure (TEM)

The ultrastructure of heart tissue examined by electron microscopy is shown in Figure 30. The heart of normal and BCP alone treated rats, (i) and (ii), showed intact myofibrils without mitochondrial damage. However, rats treated with ISO (iii) showed extensive myofibrillar loss and mitochondrial degeneration. ISO-induced MI in rats treated with BCP (iv) showed near normal mitochondrial architecture without any degeneration. Treatment with CB2 receptor antagonist AM630 (v) abrogated the protective effects of BCP against ISO induced mitochondrial damage as evidenced by mitochondrial degradation with myofibrillar loss in the myocardium clearly revealed the functional CB2 receptor dependent mechanism behind the protective effects of BCP on heart mitochondria. Treatment with CB2 receptor agonist AM630 alone (vi) did not show any changes in the mitochondrial architecture compared to normal control rat's heart mitochondria.
Figure 30: TEM study on heart mitochondria. (i) NOR, (ii) BCP, (iii) ISO, (iv) ISO+BCP, (v) ISO+BCP+AM630 and (vi) AM630. (X16,500 Magnification), (↑ Myofibril, ↑ Mitochondria)
Chapter 4: Discussion

4.1 Importance and relevance of BCP cardioprotective effect findings

Myocardial infarction is the irreversible necrosis of heart muscle occurs as a result of imbalance between coronary blood supply and myocardial demand. Production of toxic ROS such as $\text{O}_2^{-*}$ and $\text{OH}^*$ causes myocardial cell damage (Vaage & Valen, 1993). Catecholamines undergo auto-oxidation which results in the generation of highly cytotoxic free radicals (Cohen & Heikkila, 1974), which could initiate peroxidation of membrane bound PUFAs, leading to both functional and structural myocardial injury (Thompson & Hess, 1986). In this study, ISO was used to induce MI in male Wistar rats. The effects of ISO on heart are mediated through B1 and B2 adrenoceptors and they mediate the positive inotropic and chronotropic effects of b-adrenoceptor agonists (Brodde, 1991). Thus, ISO produces relative ischemia or hypoxia due to coronary hypotension, myocardial hyperactivity and induce myocardial ischemia due to overload of cytosolic $\text{Ca}^{2+}$ (Senthil et al., 2007). The toxic dosage of ISO causes characteristic myocardial damage that subsequently leads to heart failure (Grimm et al., 1998).

Among the many new therapeutic targets, the endogenous cannabinoid system, which comprises cannabinoid ligands and cannabinoid type 1 (CB1) and type 2 (CB2) receptors represents one of the newest and most promising drug targets. Among cannabinoid receptors, CB2 receptors are G-protein coupled receptors that, upon activation, induce members of the MAPK family, which trigger expression of genes including those involved in stress response, inflammation, cell survival or proliferation (Howlett, 2005). CB2 receptor activation is also associated with nuclear translocation of the transcription factor NF-κB (Derocq et al., 2000). In contrast to CB1 receptors,
CB2 receptor activation does not modulate ion channel function (Bosier et al., 2010). As a result, CB2 receptor mediated Ca\(^{2+}\) responses are less pronounced (Schuehly et al., 2011) than the potent CB1 receptor-mediated effects on Ca\(^{2+}\) fluxes (Bosier et al., 2010). Cardioprotective effects of endocannabinoid-mediated CB2 receptor activation were first reported in LPS-induced preconditioning (Lagneux & Lamontagne, 2001). Thereafter, several recent reports using synthetic CB2 receptor agonists \textit{in vitro, ex vivo or in vivo} showed protective effects on remote preconditioning, infarct size, arrhythmias and counterbalanced chronic heart failure-induced structural changes, inhibited atherogenesis and prevented myocyte enlargement (Horvath et al., 2012b; Montecucco et al., 2009; Ramirez et al., 2012; Steffens et al., 2005; Weis et al., 2010; Zarruk et al., 2012). The anti-inflammatory effects of CB2 receptor activation in the endothelium, and its inhibitory effect on monocytes/macrophages and/or leukocyte migration were diminished by pharmacological antagonism of CB2 receptors with AM630 (Zhao et al., 2010). Pretreatment with the CB2 receptor antagonist AM630 or SR144528 abolished the cardioprotective effects and reversed cardioprotection. CB2 receptors have also been implicated in the modulation of endoplasmic reticulum stress and immune cell migration (Miller & Stella, 2008) involving the PI3K/Akt and ERK1/2 pathways. Many studies with synthetic cannabinoid ligands of CB2 receptor paved the way for CB2 receptor activation as a strategy in cardioprotection.

In the present study, the cardioprotective effect of BCP has been studied, a dietary phytocannabinoid that has attracted attention after its approval by European regulatory agencies and the USFDA for use as food additive and flavoring agent. Chemically, BCP is a bicyclic sesquiterpene and pharmacologically, it is a selective CB2 receptor agonist. It is found abundantly in various flowering plants and spices which makes it one of the more widely available and accessible agents devoid of
psychoactive properties (Gertsch et al., 2008c). It has been recognized as a key ingredient in several traditional Chinese, European, Eastern and Indian medicines and is reported to possess a wide range of potent biological activities, including potent anti-inflammatory, antioxidant and analgesic effects. BCP’s structure contains a cyclobutane ring which provides a potent and rigid geometry to this molecule for binding to cannabinoid receptors and largely contributes to BCP’s anti-inflammatory properties. The therapeutic benefits of BCP in inflammation has been shown to be mediated by potent CB2 receptor activation (Gertsch, 2008a). Additionally, the neuroprotective effects of BCP in Parkinson’s disease, cerebral ischemia, epilepsy, Alzheimer’s disease, depression, anxiety and addiction have been reported recently (Donati et al., 2015; Sain et al., 2014).

To the best of my knowledge, this is the first study to report the cardioprotective effects of BCP against ISO-induced MI via activation of CB2 receptors in rats. It has been found that ISO administration downregulates the activation of myocardial CB2 receptors which proves that CB2 receptors activation is an important strategy to safeguard the myocardium against MI. BCP, natural CB2 receptor agonist upregulates the CB2 receptors activation and protects the myocardium against ISO-induced MI in rats. The crosstalk between CB2 and PPARγ pathways has been demonstrated recently in which the binding of natural and synthetic cannabinoids to PPARγ has been demonstrated (Burstein, 2005; Meeran et al., 2019). This study has revealed that ISO-challenge downregulates the expression of PPARγ whereas BCP treatment unregulates its expression in ISO-induced MI in rats. Upregulation of PPARγ along with the expression of CB2 receptors clearly revealed the CB2/PPARγ receptor mediated cardioprotection offered by BCP against ISO-induced MI in rats.
The myocardium contains plentiful concentrations of diagnostic markers of MI and once metabolically damaged, it releases its contents into the extra cellular fluid (Upaganlawar et al., 2009). Serum CK-MB, a golden standard diagnostic marker of MI along with CK, LDH and troponins are the other diagnostic markers of myocyte injury or death are found to be increased in ISO induced rats. Due to poor oxygen or glucose supply the myocardial cells are damaged making the cardiac membrane permeable resulting in the leakage of these enzymes. These enzymes enter into the blood stream thus increasing their concentration in the serum (Mathew, Menon & Kurup, 1985). The activities of CK, LDH and troponin-I are declined in the heart tissue of ISO induced rats. This might be due to the damage caused to the sarcolemma by the β-agonist, which has rendered it leaky (Mathew et al., 1985). BCP defends the myocardium by inhibiting the leakage of cardiac diagnostic marker enzymes into the circulation in ISO induced myocardial infarcted rats by virtue of its potent membrane stabilizing property. The protective effect of BCP was abrogated by the administration of CB2 antagonist AM630 has clearly revealed the CB2 receptor dependent mechanism behind the protective effects of BCP against MI in rats.

Administration of ISO induces impaired hemodynamics (decreased SP, DP, MAP and heart rate) followed by the left ventricular dysfunction (Ojha et al., 2008). Alterations in hemodynamics after ISO injections reflects systolic and diastolic dysfunction in rats (Malik et al., 2011). BCP treatment reinstates normal heart rate and hemodynamics in ISO-induced MI in rats whereas the protective effects of BCP were attenuated by the prior administration of CB2 antagonist AM630. Thus, BCP protects the myocardium in a CB2 dependent mechanism.

Lipid peroxidation is an important pathogenic event that has been linked to altered membrane structure and enzyme inactivation in MI. Lipid peroxidation of
membranes is regulated by the availability of inducers such as free radicals, and the increased levels of lipid peroxides in ISO induced damage might be due to free radical-mediated chain reactions that could damage the myocardium. It is an indication of the severity of ISO induced necrotic damage of the heart (Albayrak et al., 2009). The degree of lipid peroxidation was reflected by increased levels of TBARS, LOOH and conjugated dienes in the plasma and heart of ISO induced myocardial infarcted rats. An increase in the lipid peroxidation products level injures blood vessels, thereby increasing adherence and platelets collection in the injured sites. Pre and co-treatment with BCP near normalized excess lipid peroxidation products level in ISO induced rats. Hence, BCP scavenges the excess lipid peroxidation products generated by ISO and safeguards the heart by its anti-lipid peroxidation effect. The protective effect of BCP was abrogated by the administration of CB2 antagonist AM630 which has clearly revealed the CB2 receptor dependent mechanism behind the protective effects of BCP against MI in rats.

Pharmacological augmentation of endogenous myocardial antioxidants has been identified as a promising therapeutic approach in diseases associated with increased oxidative stress. Endogenous antioxidant enzymatic defense is a very important source to neutralize the oxygen free radical-mediated tissue injury (Saravanan et al., 2013). Superoxide dismutase and catalase, the primary free radical scavenging enzymes, are the first line of cellular defense against oxidative injury, decomposing oxygen and hydrogen peroxide before their interaction to form the more reactive OH•. In this study, significantly lowered activities of superoxide dismutase and catalase were observed in heart of ISO induced rats. The observed decrease in the activities of these enzymes might be due to their increased utilization for scavenging ROS and their inactivation by excessive ISO oxidants. Treatment with BCP improved
the activities of superoxide dismutase and catalase by scavenging O$_2^-$ and OH$^-$ produced by ISO. The protective effect of BCP was abrogated by the administration of CB2 antagonist AM630 which has clearly revealed the CB2 receptor dependent mechanism behind the protective effects of BCP against MI in rats.

GSH level is commonly used marker for oxidative stress. The decreased GSH levels during ISO induction inactivate GSH related enzymes (Sathish et al., 2002) which are responsible for degrading hydrogen peroxide and O$_2^-$ during ischemia or injury and significantly reduce the activities of GPx and GST (Kumaran & Prince, 2010b). GSH is important in protecting the myocardium against oxygen free radical injury and thus a reduction in cellular glutathione content could impair recovery after short period of ischemia (Saravanan & Prakash, 2004). The observed decrease in GSH levels might be due to increased utilization in protecting thiol ROS oxygen species. Treatment with BCP near normalized the activities/concentrations of GSH in ISO induced rats. This shows the antioxidant property of BCP. The protective effect of BCP was abrogated by the administration of CB2 antagonist AM630 has clearly revealed the CB2 receptor dependent mechanism behind the protective effects of BCP against MI in rats.

Mediators of myocardial inflammation predominantly cytokines in ISO induced MI were evaluated, because cytokines have been implicated in the healing process after infarction. Pro-inflammatory cytokines, namely TNF-α, IL-6 and IL-1β are elevated in acute myocardial injury and infarction. (Cusack et al., 2002) reported that the cytokines have been implicated in the initiation and maintenance of vascular and systemic inflammation associated with the coronary artery disease. Furthermore, pro-inflammatory cytokines alter proliferation of cells, collagen extra-cellular matrix production, and generation of mediator substances by the cardiac fibroblast (Koudssi
et al., 1998). Pro-inflammatory cytokines have been implicated as pathological cardiac remodeling mediators with evidence linking these molecules to heart failure progression in chronic heart failure (Ono et al., 1998). In coronary heart disease, TNF-α is a relevant cytokine in the course of the inflammation process and affects a number of mediators of the pathological process involved in MI. Recently, cardiomyocytes have been shown to be a main source of TNF-α production in conditions such as MI and heart failure (Kannan & Quine, 2011a).

TNF-α inhibits contractility of myocardium, induces cardiomyocyte apoptosis and acts directly on cardiomyocytes as well as the vascular endothelium to increase the adhesion of leukocytes during inflammation (Sharma & Das, 1997). Moreover, it is thought to contribute to the progression of the atheroma by augmenting the local inflammatory response (Olivieri et al., 2006).

IL-6 is another proinflammatory cytokine involved in cardiodepression. There is evidence that in patients suffering from acute MI, IL-6 may affect the progression and the healing process of this CVDs, because serum IL-6 levels to be elevated in these myocardial infarcted patients (Ikeda et al., 1992). IL-6 is predominantly produced by myocardial fibroblasts, although it has also been reported to be produced by cardiomyocytes as a result of myocardial ischemia or cardiotoxicity by beta-adrenergic agonists. Also, IL-6 up-regulation is an early and important phenomenon in ISO-induced MI (Mikaelian et al., 2008).

Interleukin-1 is a multifunctional cytokine, primarily involved in the regulation of inflammatory process. Two genes are expressed for IL-1: IL-1α and IL-1β. IL-1β gene is one of the key pro-inflammatory cytokines because its elevated levels are correlated with disease severity. An enhanced level of IL-1β mRNAs in ischemic
myocardium indicates the role of cytokine in myocardial inflammation (Sharma & Zimmerman, 1993).

Higher expressions of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) has been observed in the myocardium of ISO-induced myocardial infarcted rats. The observed increased expression of these proinflammatory cytokines mediates inflammation in the myocardium thereby reducing cardiac muscle function and extensive damage to the myocardium (Kannan & Quine, 2011b). The present study confirmed that treatment with BCP downregulated the expression of pro-inflammatory cytokines in the myocardium of ISO-induced myocardial infarcted rats, thereby inhibiting inflammation by its anti-inflammatory effect. The protective effect of BCP was abrogated by the administration of CB2 antagonist AM630 has clearly revealed the CB2 receptor dependent mechanism behind the protective effects of BCP against MI in rats.

Although the inflammatory response considered as a defense mechanism against infection or injury, sustained inflammation is a pathological condition. Over expression of several pro-inflammatory enzymes and reactive species like superoxide (O$_2^-$) and nitric oxide (NO) radicals, are produced during inflammation (Okoli & Akah, 2004). Nitric oxide is formed from an oxygen and L-arginine by inducible nitric oxide synthase (iNOS), this enzyme become up-regulated during the inflammatory process (Tayeh & Marletta, 1989).

Prostaglandins (PGs) is a group of physiologically active mediators which also over-expressed during inflammation. Prostaglandins are bioactive signaling molecules derived from cyclooxygenase (COX) and subsequent PG synthase activity on arachidonic acid. Like iNOS, COX-2 is highly up-regulated in response to infection, atherosclerosis and a number of cancers (Simmons et al., 2004). It plays a critical role
in the mediation of inflammation, and catalyzes the rate limiting step in prostaglandin biosynthesis. PG synthases will form important signaling molecules, including PGI₂, thromboxane A₂, PGE₂, PGD₂ and PGF₂α (Buczynski, Dumlaо & Dennis, 2009). The up-regulation of iNOS and COX-2 during inflammation is controlled by NF-κB which is a pro-inflammatory transcription factor (Kim et al., 2008; Raso et al., 2001).

BCP treatment reduced COX-2, iNOS and proinflammatory cytokine levels. This reduction in proinflammatory cytokines seems to be in line with the results of other studies conducted with BCP (Cho et al., 2015; Horvath et al., 2012a). The protective effect of BCP was abrogated by the administration of CB2 antagonist AM630 which has clearly revealed the CB2 receptor dependent mechanism behind the protective effects of BCP against MI in rats. CB₂ receptors are expressed in immune cells and many studies have shown that the CB₂ receptor plays a role in immunomodulation (via both endocannabinoids and cannabinoids) (Turcotte et al., 2016). However the exact mechanisms and pathways are still not fully understood or known (Pacher & Mechoulam, 2011).

Cardiomyocyte apoptosis produces hypoxia and ischemia after increased myocardial contractions during β1-adrenergic receptor activation (Nagoor Meeran et al., 2019). Bax activation due to the overproduction of ROS plays a major role in promoting apoptosis by creating pore in the mitochondria which leads to the release of mitochondrial cytochrome-C into the cytosol (Sahu et al., 2014). CHOP is known to down regulate the expression of Bcl2 and triggers apoptosis through cytochrome-C release by inhibiting mitochondrial Bcl2 family proteins and by creating mitochondrial permeability transition pore (MPTP) opening (Roy et al., 2009). Mst1 triggers apoptosis by inhibiting YAP by promoting the phosphorylation of LATS1/2. During ischemic conditions under severe oxidative stress, activation of MST1 activates
intrinsic pathway of apoptosis by dissociating Bcl-xL from Bax through Bcl-xL phosphorylation (Nakamura et al., 2016). Released cytochrome-C activates executioner caspases by the formation of apoptosomes (Radhiga et al., 2012). Isoproterenol activates intrinsic pathway of apoptosis by releasing cytochrome-C followed by the activation of caspases whereas BCP treatment protects the myocardium against ISO induced apoptotic cell death of the myocardium. The antagonistic effects of AM630 against the anti-apoptotic effects of BCP clearly revealed the functional CB2 receptor dependent mechanisms behind the anti-apoptotic properties of BCP against ISO induced myocardial apoptosis in rats.

ISO causes myocardial injury which is characterized by various morphological features which include necrosis, myocardial hypertrophy, blood capillary distortion, and interstitial edema (Al-Rasheed et al., 2015). The histopathological findings show extensive muscle degradation in ISO induced myocardial infarcted heart, which shows necrosis, edema, and inflammatory cells. The histopathological findings of BCP treated ISO induced myocardial infarcted heart displayed a near normal morphology of cardiac muscle without necrosis, edema and inflammatory cells as compared to ISO induced myocardial infarcted heart. The histopathology of BCP treated myocardium established the protective nature of BCP in ISO induced rats.

BCP could be used for nutritional supplementation over other phytocannabinoids due to its unique properties as being natural, dietary, readily bioavailable, non-psychoactive and safe with a wide presence in numerous plants. Translating the outcomes in humans would be promising as these agents have not only shown the ability to activate CB2 receptors only, but they also activate PPAR-γ, which are the target of thiazolidinedione class of drugs that used clinically to treat diabetes. BCP is of more therapeutic value than the other phytocannabinoids due to it being
found in plants other than cannabis which may help in the legal logistics of its use. CB2 receptor selective compounds may provide a new potential class of cardioprotective drugs. The pharmacophore of these compounds could be used for synthesizing leads in drug discovery and development.
Chapter 5: Conclusion

The present study revealed that BCP, a plant-derived sesquiterpene have pharmacotherapeutic significance due to its multi pharmacological properties. Activation of CB2 receptors is the major underlying mechanism behind the cardioprotective property of BCP which has shown to attenuate oxidative stress, inflammation and apoptosis in ISO induced MI.

This study is the first study so far that report the cardioprotective effects of BCP against ISO-induced MI via activation of CB2 receptors in rats. I observed that ISO administration down-regulates the activation of myocardial CB2 receptors which proves that CB2 receptors activation is an important strategy to safeguard the myocardium against MI. BCP also demonstrated a positive effect by reducing oxidative stress, inflammation and inflammatory mediators, apoptosis along with reducing the serum levels of the cardiomyocyte injury marker enzymes CK and LDH. The histological and ultra-structural evidences are found in line with the biochemical and molecular findings. Treatment with AM630, a potent CB2 receptor antagonist abrogates protective effects of BCP on all the biochemical and molecular parameters analyzed in ISO-induced MI in rats. The present study findings clearly demonstrate that BCP may be an attractive natural compound which can bestow protective effects to the myocardium via CB2 receptor-dependent manner against ISO induced MI. Furthermore, BCP could be the first plant-derived CB2 receptor agonist in the quest for development of novel class of drugs that might improve conventional therapies as well as provide novel disease modifying agents.
References


List of Publications
