



REPUBLIC of TURKEY
ESKİŞEHİR OSMANGAZİ ÜNİVERSİTESY
INSTITUTE of HEALTH SCIENCE
DEPARTMENT OF MEDICAL PHARMACOLOGY

AN INVESTIGATION of the PROTECTIVE EFFECT of
BERBERINE on VANCOMYCIN-INDUCED
NEPHROTOXICITY in RATS

TAREK AL HOMSI

MASTER'S THESIS

ADVISOR

Prof. Dr. ENGİN YILDIRIM

Eskişehir, 2023



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ABSTRACT

Title: An Investigation of The Protective Effect of Berberine on Vancomycin-Induced Nephrotoxicity in Rats

Introduction: One limitation of vancomycin use is its nephrotoxicity due to the high doses of vancomycin used to treat resistant infections. Berberine is a natural product with a broad pharmacological activity including anti-inflammatory and antioxidant potential effects. The reno-protective effect of BBR has been investigated against nephrotoxicity induced by VCM.

Objective: biomarkers including Cr, BUN, KIM-1, SOD, MDA, IL-6, TNF- α , Bcl-2 and CAS-9 have been evaluated. Additionally, histological methods have been used. In this investigation, we have aimed to study the potential reno-protective effect of BBR against nephrotoxicity induced by VCM in wistar albino rats.

Methods: In this study, 35 adult male Wistar Albino rats were randomly assigned into five groups, each containing seven rats. The control group received oral normal saline SF for eight days and SF intraperitoneally for seven days. The other groups were administered VCM dissolved in SF for seven days intraperitoneally. BBR was administered via oral gavage over a period of eight consecutive days, commencing one day prior to the administration of VCM. Three distinct dosage levels of BBR, namely 50mg/kg, 100mg/kg, and 200mg/kg were used for each group of BBR respectively.

Result: The findings of the current investigation indicate that the administration of VCM has led to nephrotoxicity, as evidenced by elevated levels of inflammation and oxidative stress biomarkers, as well as significant histological damage. In addition, a minor increase of apoptosis biomarkers has been noted. However, VCM has showed insignificant apoptotic related kidney damage. The administration of BBR has shown the potential improvement of the oxidative stress and inflammation significantly and the reduction of apoptosis has not been significant.

Conclusion: Our results have showed that BBR might has the potential to reduce the nephrotoxicity induced by the administration of VCM at the dose of 400mg/kg for seven days intraperitoneally in male rats.

Keywords: Vancomycin (VCM), Berberine (BBR), Nephrotoxicity, Rat

ABSTRACT (TURKISH)

Başlık: Sıçanlarda Vankomisin Uygulamasına Bağlı Nefrotoksisitede Berberinin Koruyucu Etkilerinin Araştırılması.

Giriş: Dirençli enfeksiyonların tedavisinde kullanılan yüksek doz Vankomisin'e bağlı gelişen nefrotoksisite, Vankomisin kullanımını kısıtlayan önemli bir durumdur. Berberin, geniş bir farmakolojik aktiviteye sahip, anti-inflamatuvar ve antioksidan etkileri olan doğal bir üründür. BBR'nin VCM tarafından oluşturulan nefrotoksisiteye karşı koruyucu etkisi araştırılmıştır.

Amaç: Bu araştırmada, erkek wistar albino sıçanlarda VCM'nin uygulamasına bağlı gelişen nefrotoksisiteye karşı BBR'nin farklı dozlarda potansiyel koruyucu etkisinin araştırılması amaçlanmıştır. Böbrek fonksiyonları ve böbrek hasarı Cr, BUN, KIM-1, SOD, MDA, IL-6, TNF- α , Bcl-2 ve CAS-9 gibi biyobelirteçler ile ve ek olarak apoptoz, histolojik yöntemler ile değerlendirilmiştir.

Yöntem: 35 erkek wistar sıçanından oluşan bir grup oluşturuldu ve bu daha sonra her biri yedi sıçan içeren beş gruba ayrıldı. Kontrol grubuna sekiz gün oral normal salin (SF) ve yedi gün SF (i.p.) uygulandı. Diğer tüm gruplar için VCM, SF içinde çözülerek yedi gün (i.p) uygulandı. BBR (50mg/kg, 100mg/kg ve 200mg/kg) dozlarında, VCM uygulamasının başlamasından bir gün önce SF içinde çözülerek sekiz gün boyunca oral sonda ile uygulandı.

Bulgular: VCM uygulaması sonucu gelişen nefrotoksisite sonucunda, inflamasyon ve oksidatif stres biyobelirteçlerinde artış ve böbrek histolojinde önemli bozulma gözlenmiştir. Ek olarak, apoptoz biyobelirteçlerinde artış ile birlikte, apoptoza bağlı kısmi böbrek hasarı gözlenmiştir. BBR uygulanması, oksidatif stres ve enflamasyonun düzelmesinde potansiyel katkı sağlamış ancak apoptozun önlenmesinde yeterli katkı sağlamamıştır.

Sonuç: BBR'nin, sıçanlarda VCM uygulamasına bağlı gelişen nefrotoksisiteyi önleme potansiyeline sahip olabileceğini gözlenmiştir.

Anahtar Kelimeler: Vankomisin (VCM), Berberin (BBR), Nefrotoksisite, Sıçan

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INDEX of SYMBOLS and ABBREVIATIONS

AIN	: Acute Interstitial Nephritis.
AKI	: Acute kidney injury.
AMPK	: AMP-activated protein kinase.
ATN	: Acute Tubular Necrosis.
AUC	: Area under the curve.
BBB	: Blood-Brain Barrier.
BBR	: Berberine.
BUN	: Blood urea nitrogen.
CAS	: Caspase.
CAT	: Catalyzes enzyme.
Const.	: Concentration.
COX2	: Cyclooxygenase-2.
CSF	: Cerebral spinal fluid.
CYPs	: Cytochrome P450 isoforms.
DISC	: Death-inducing signaling complex.
DNA	: Deoxyribonucleic acid.
GSH	: Glutathione.
GSK-3	: Glycogen synthase kinase-3.
i.p.	: Intraperitoneal administration.
I.V.	: Intravenous administration.
ICAM-1	: Intercellular Adhesion Molecule 1.
IKK-β	: I κ B kinase- β .
IL	: Interleukin.
IM	: Intramuscular administration.
iNOS	: Inducible nitric oxide synthase.

INDEX of SYMBOLS and ABBREVIATIONS (continue)

IκB-α	: Inhibitory κ B- α .
KIM-1	: Kidney Injury Molecule -1.
LD50	: The dose causing lethal effect in 50% of the population.
LPS	: Lipopolysaccharide.
MAPK	: Mitogen-activated protein kinase.
MDA	: Malondialdehyde.
MRSA	: Methicillin-resistant Staphylococcus aureus.
NADPH	: Nicotinamide adenine dinucleotide phosphate.
NF-κB	: Nuclear factor- κ B.
NO	: Nitric oxide.
Nrf2	: Nuclear factor erythroid-2-related factor-2.
PARP-1	: Poly adenosine diphosphate ribose polymerase -1.
PI3K	: Phosphatidylinositol 3-kinase.
RMS	: Red man syndrome.
RNA	: Ribonucleic acid.
ROS	: Reactive oxygen species.
Scr	: Serum creatin.
SOD	: Superoxide dismutase enzyme.
T_{1/2}	: Plasma elimination half-life.
TGF	: Transforming growth factor.
TNF-α	: Tumor Necrosis Factor Alpha.
UGTs	: Glucuronosyltransferase enzymes.
VCM	: Vancomycin.
Vd	: Volume of distribution.
VIKI	: VCM induced kidney injury.

1. INTRODUCTION and PURPOSE

Vancomycin (VCM), a glycopeptide antibiotic, is extensively employed as the foremost treatment modality for the effective control and treatment of severe and time-critical infections instigated by methicillin-resistant *Staphylococcus aureus* (MRSA), a distinct variety of Gram-positive bacterial species (Álvarez et al., 2016). However, the administration of VCM has been associated with nephrotoxicity, which is a common concern (Filippone et al., 2017). Recent guidelines recommend higher VCM trough levels to effectively treat complicated MRSA infections (Álvarez et al., 2016). However, this therapeutic approach may augment the likelihood of nephrotoxicity, particularly in individuals with a heightened susceptibility to acute kidney injury. (Álvarez et al., 2016; Elyasi et al., 2012; Wong-Beringer et al., 2011).

Berberine (BBR) is an isoquinoline alkaloid naturally occurring in a range of plant species, including *Berberis vulgaris*, *Berberis thunbergii*, *Berberis aquifolium*, and *Coptis chinensis* (Imanshahidi & Hosseinzadeh, 2008). The pharmacological properties of BBR encompass a broad spectrum, encompassing antioxidant, anti-inflammatory, and anti-apoptotic effects, as evidenced by the recent conducted study (Adil et al., 2016). The restorative potential of BBR for glutathione, glutathione peroxidase, and superoxide dismutase levels has been observed in animal studies (Lao-Ong et al., 2012). Additionally, the inhibitory effects of BBR on superoxide anion production in macrophages have been demonstrated in a separate study (Sarna et al., 2010).

Numerous studies have indicated the potential of BBR to alleviate inflammatory responses in both laboratory and animal models (Y. H. Wu et al., 2012). BBR has also been found to suppress proinflammatory mediators and mitigate acute liver injury in mice (Domitrović et al., 2011). In addition, BBR has shown antiapoptotic properties by alleviating mitochondrial stress in renal proximal tubule cells (W. Yu et al., 2013), and reducing mitochondrial dysfunction in doxorubicin-induced cardiomyocytes (X. Lv et al., 2012). Animal studies have further suggested a protective effect of BBR against nephrotoxicity (Adil et al., 2016; S. Ahmad et al., 2019; Allameh et al., 2020; Ashrafizadeh et al., 2020; Hasanein & Riahi, 2018; Hassanein et al., 2019; Ibrahim Fouad & Ahmed, 2021; Qi et al., 2020).

Despite the growing evidence suggesting the potential of BBR's on the reduction of oxidative stress, inflammation, and apoptosis, its potential nephroprotective effects against VCM-induced kidney injury have not been investigated in live subjects. Therefore, this investigation was aimed to explore the potential of BBR in mitigating oxidative stress, inflammatory response, and apoptosis as underlying molecular mechanisms for its renal protective effects against acute VCM-induced kidney injury in Wistar albino rats.



2. GENERAL INFORMATION

2.1. Vancomycin

Vancomycin (VCM), classified as a glycopeptide antibiotic, has been utilized in medical practice since its discovery in 1952 and subsequent therapeutic use in 1958. It is primarily employed for the treatment of bacterial infections caused by Gram-positive microorganisms, with a particular emphasis on patients who have allergies to penicillin-based antibiotics.(C. Liu et al., 2011). However, it is often considered as a "drug of last resort," meaning that it is used only after other antibiotics have failed. VCM is widely recognized as the preeminent antibiotic for the successful treatment of severe infections, particularly those caused by Gram-positive microorganisms exhibiting high levels of resistance, notably *Methicillin-Resistant Staphylococcus aureus* (MRSA) and *Enterococcus faecium* (Ingram et al., 2008).

However, VCM is associated with some side effects. One of the most serious side effects of the administration of VCM is that it may increase the risk of developing nephrotoxicity, which have been reported in patients on a VCM-containing therapy plan. However, most of the reported toxicities associated with VCM were formerly linked to the presence of impurities, hence VCM was known as "Mississippi mud" (Bailie & Neal, 1988; Hodoshima et al., 2007). With better fermentation techniques and processing, the final purity of the drug has grown from 70% to approximately be approximately 95%, significantly lowering its reported toxicity severities. This is particularly important as the nephrotoxicity has also been reduced (Bailie & Neal, 1988; Elting et al., 1998; Lodise et al., 2008, 2009). VCM reported to be among the regularly used antibiotics in clinical practice and has been linked to the occurrence of AKI, with an incidence of up to 43% (Jeffres et al., 2007; S. M. Kim et al., 2021).

VCM is mostly used at dosing regimen of (1 g every 12 hours). However, this dose has been frequently reported to result in plasma concentration values below the target of 10 to 20 mg/l, which is the level of the drug that is recommended by recent guidelines (Electronic Medicines Compendium, 2022). Therefore, to achieve the optimal target concentration, it has been recommended that the VCM dose should be individually adjusted according to weight, age, and the underlying type and severity of the infection (Electronic Medicines Compendium, 2022). However, high-dose therapy aimed at achieving a serum concentration of 15-20mg/L has been proposed in

various published guidelines to eliminate MRSA strains with lower susceptibility. On the other hand, increased risk of nephrotoxicity have been associated with such high dose (Society & America, 2005).

Nevertheless, VCM has numerous limits regarding the dose and length of administration (Naughton, 2008; Nishino et al., 2002), this is particularly important because of its association with AKI (Rhodes et al., 2016). More notably with the increased VCM levels especially when its level is close to $>20 \mu\text{g/ml}$ or at doses reaching more than 4 gram per day. Risk factors including the co-administration of VCM with other nephrotoxic agent, prolonged treatment plan continued for more than 7 days, and the prolonged hospital admission especially in the intensive care unit are reported to potentiate the nephrotoxicity following the administration of VCM (Elyasi et al., 2012). Aside from that, the longer the administration period of VCM is, the more likely it is to develop nephrotoxicity (Rostas et al., 2014). Furthermore, it has been reported that acute kidney damage is among the most important reason of increasing morbidity and mortality in hospitalized patients.

Although studies have suggested that VCM may lead to nephrotoxicity through free radical generation and consequent oxidative stress and mitochondrial damage, the precise mechanism remains yet unknown (Elyasi et al., 2012; King & Smith, 2004). Over the past decade, evidences have indicated that formation of reactive oxygen metabolites are greatly involved in the pathogenesis of a variety of renal diseases (Baliga et al., 1999). It has been documented that VCM is eliminated almost exclusively renally, where its elimination has been noted to be via glomerular filtration for the majority of the dose given, in addition to a smaller percent eliminated via active tubular secretion (Nakamura et al., 1996b). The filtration processes and the energy-dependent transport mechanisms which are found in the proximal tubule epithelium are rendering the kidney to be highly susceptible to toxicant-induced renal injury (Inoue et al., 1999).

Certainly, various studies have presented compelling evidence suggesting that oxidative stress, in combination with an exacerbated inflammatory response, holds a pivotal role in the advancement of kidney damage. Recent investigations conducted both in vivo and in vitro have additionally demonstrated that the administration of high doses of VCM can result in heightened levels of oxidative stress, inflammation, and apoptotic cell death (Filippone et al., 2017; Mehanna et al., 2022; Sakamoto et al.,

2017). Severe VCM renal toxicity may present histologically as tubulointerstitial nephritis, sometimes with granulomas (Uckun et al., 2020). Moreover, changes of function of have been reported in VCM-induced nephrotoxicity (H. Li et al., 2021). Furthermore, a recent study have showed that the reported anti oxidative stress, apoptosis and inflammation potentials of vitamin C have reduces VCM-related nephrotoxicity in mice (J. He et al., 2021). Moreover, the increased generation of ROS, together with the increased level of inflammatory cytokines and chemokines in addition to the increased in membrane lipid peroxidation and the reduction of defensive antioxidants have been associated with the administration of VCM (Fouad et al., 2014).

2.1.1. The structure of VCM and its mechanism of action

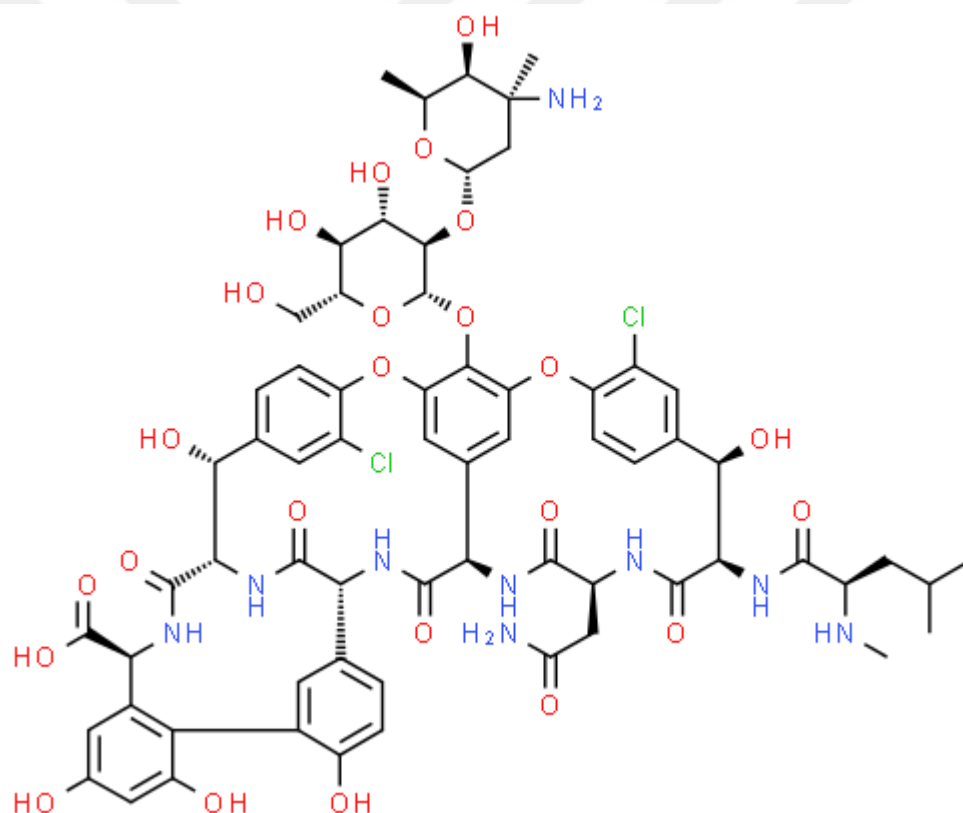


Figure 2.1. The structure of VCM (Chemspider, 2022b).

It is a large molecule (1448 dalton), composed of a disaccharide polymer, containing glucose and vancosamine with a seven-subunit peptide chain consisting of two beta-hydroxychlorothiazines, three phenylglycines, one N-methicillin, and one aspartic acid amide. VCM cannot reach the target molecules at the gram-negative bacteria by penetrating through the cell wall's outer membrane due to their molecular

size. As a result, gram-negative bacteria are intrinsically resistant to VCM. However, VCM is a highly stable compound and water soluble (Bryskier & Veyssier, 2005).

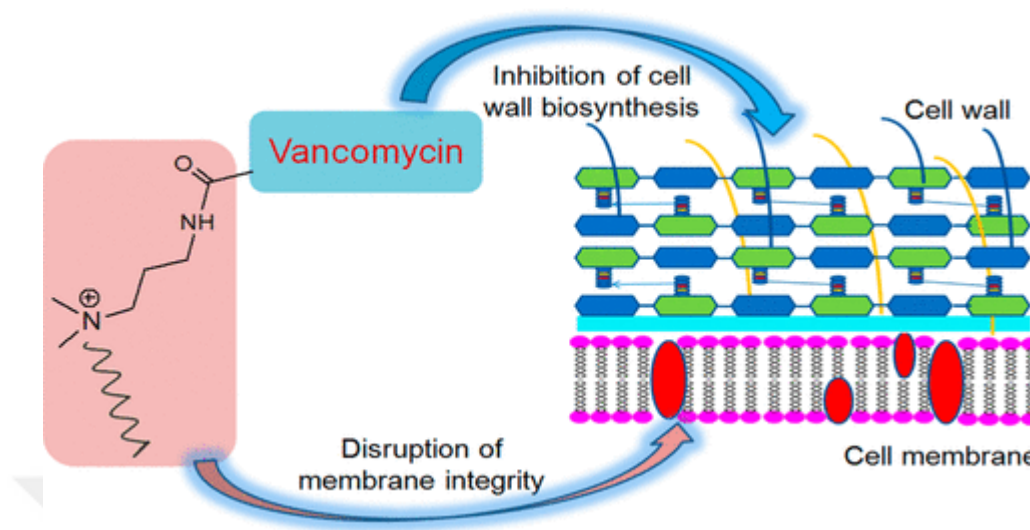


Figure 2.2. The mechanism of action of VCM on gram-positive bacterial cell wall (Yarlagadda et al., 2014).

VCM is effective against all Gram (+) aerobic and anaerobic bacteria. Figure 2.2 depicts the mechanism of action of VCM against Gram (+) bacteria (Yarlagadda et al., 2014). The large hydrophilic molecule N-acetyl glucosamine, which forms the basis of peptidoglycan synthesis in these bacteria, demonstrates its effectiveness when it forms multiple hydrogen bond interactions, where it plays a crucial role in the binding of VCM to the D-alanyl-D-alanine terminal dipeptide located at the end of the pentapeptide chain attached to the muramic acid in the N-acetyl muramic acid disaccharide (Nagarajan, 1991). With this binding, VCM inhibits the transglycosylation and transpeptidation processes, which are critical for peptidoglycan synthesis. Therefore, VCM prevents peptidoglycan elongation and cross-linking. As a result, the peptidoglycan structure deteriorates, and cell wall formation is hindered. Hence, the drug's mode of action is the suppression of bacterial cell wall production. VCM has bactericidal properties against developing and growing bacteria (AJ, 2012).

2.1.2. Pharmacokinetics

2.1.2.1. Pharmacokinetic properties

Since its absorption by the gastrointestinal tract is very low (Spitzer & Eliopoulos, 1984) and it causes considerable local pain as a result of IM administration, so its recommended for parenteral use by IV route (Griffith, 1981). Although oral VCM can be used as an alternative to metronidazole in the treatment of colitis due to *Clostridium difficile* infection (McDonald et al., 2018), as for now there is no oral form in Turkey.

Following IV administration, VCM is broadly disseminated throughout body tissues and diffused, it could be traced in body several fluids including the pericardial, pleural, ascitic, and synovial fluid. Smaller amounts could be traced within bile. Unless the serum concentration gets quite high and in the absence of inflammation, traces of VCM have not been reported to readily diffuse into CSF (Beach et al., 2017). However low levels could be detected in CSF if the meninges are undergoes inflammation, although most patients with uninflamed meninges have negligible amounts measured in CSF. The association between CSF concentrations and VCM's therapeutic efficacy in the treatment of meningitis is not fully clear (Beach et al., 2017).

2.1.2.2. Absorption

Oral VCM can be used as an alternative to metronidazole in colitis treatment, which is caused by *Clostridium difficile* infection and the oral absorption might express a notable increase during the inflammation time (McDonald et al., 2018). However, oral VCM is not as effective as IV administration of VCM for systemic infections. Hence, there is no need for dose adjustment in renal impairment patients taking oral VCM due to its limited systemic absorption (Gerding et al., 2018).

2.1.2.3. Distribution

The Protein binding of VCM is reported to be approximately 55% when VCM serum concentrations range from 10 to 100mcg/L, where 0.4-1.0L/kg is its volume of distribution (M. Rybak et al., 2009). It has a limited ability to permeate the meninges, resulting in restricted penetration into the CSF, therefore higher dose could be used to sustain a clinically effective concentration of this antibiotic in the blood through IV administration. However, therapeutic VCM serum concentrations could be reached in patients on oral VCM for active pseudomembranous colitis (Powder et al., 2019).

2.1.2.4. Metabolism

Following 24 hours of IV administration of VCM, almost 80% of VCM is excreted unchanged in the urine has been reported. Therefore, seemingly there is no important metabolism of VCM to be noted (Keller, 2014). Additionally, levels of VCM at or below the detection limits in the liver and within the bile has been noted following the administration by 24 hours (Matzke et al., 1986).

2.1.2.5. Elimination

Within 24 hours of the IV administration, glomerular filtration excretes 70-90% of the medication unchanged. The liver may possibly play a role in the disposition of VCM; however, only 5% of the drug appears in bile following intravenous delivery (Wilhelm & Estes, 1999). In people with normal kidney functions, indeed, the elimination half-life of VCM from the plasma is ranging from 4 up to 6 hours. Approximately 80% of VCM is reported to be eliminated in the urine by glomerular filtration within 24 hours following its administration (Powder et al., 2019). The mean plasma clearance has been reported to be nearly 0.06L/kg/hour, while its renal clearance could reach 0.05L/kg/hour (Powder et al., 2019). However, VCM excretion is slowed in renal impairment. The average elimination half-life in anephric patients is 7.5 days (Powder et al., 2019).

2.1.3. Pharmacodynamic

Extensive investigations conducted both in vitro and in clinical studies consistently demonstrate the effectiveness of VCM against a wide range of microorganisms. These encompass *Listeria monocytogenes*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* (including penicillin-resistant strains), *Streptococcus agalactiae*, *Actinomyces* species, and *Lactobacillus* species (Keller, 2014). Additionally, previous studies have indicated the synergistic activity of VCM when it is co-administered with an aminoglycoside against various strains of *Staphylococcus aureus*, *Streptococcus bovis*, and *enterococci* (Keller, 2014).

2.1.4. Therapeutic indications of VCM

VCM is primarily recommended for the management of infections that pose a potential threat to life and cannot be effectively treated with alternative antimicrobial agents such as penicillins and cephalosporins (Powder et al., 2019), which are also less toxic. It is frequently included in the treatment regimen for severe infections caused

by methicillin-resistant staphylococci. This is particularly crucial in patients who are unable to receive or have not responded to penicillins and cephalosporins, or in cases where the infecting staphylococci exhibit resistance to other antibiotics (Powder et al., 2019). However, bacterial susceptibility tests should be taken to specifically determine the pathogen causing the infection, in addition to the susceptibility to VCM test (Powder et al., 2019). The most common clinical use of VCM is in: Endocarditis (Keller, 2014), Meningitis (Ahmed, 1997), Respiratory tract infections, Septicemia and Skin infections. In addition to Clostridium difficile-associated diarrhea and colitis (Powder et al., 2019).

2.1.5. Side effects and toxicity

2.1.5.1 Toxicity on animal

Experiments assessing the toxicity of Vancomycin (VCM) have been performed using a wide range of animal models, encompassing mice, rats, dogs, cats, and rhesus monkeys (Wold & Turnipseed, 1981). Acute toxicity experiments have revealed that rats perished in a clonic convulsion, shortly after the IV administration of VCM resulting in LD50 up to 319 mg/kg because of central nervous system poisoning. However, this could be explained as at that time, the drug had not yet been refined, since contaminants might be related to this adverse outcome (Bailie & Neal, 1988). Intraperitoneal VCM injection have been reported to be better tolerated, and large dosages were required to elicit deadly effects at LD50 of 2218 mg/kg (Anderson et al., 1956). Nevertheless, it should be noted that escalating the VCM dosage to 450mg/kg through intraperitoneal (i.p.) administration and prolonging the treatment period to 28 days is associated with more pronounced histopathological damage and elevated levels of acute kidney injury (AKI) renal biomarkers (Fuchs et al., 2012; Vaidya et al., 2010). Furthermore, the proximal tubule was noted to be the most damaged part of the nephron, which is corroborated by renal biomarkers such as KIM-1 (Pais, Avedissian, Nicholas O'Donnell, et al., 2019). In VIKI animal models, biomarkers such as KIM-1 was documented to be the significant predictors of the histopathologic damage that had been noted 24 hours following the administration of VCM (Pais, Avedissian, Nicholas O'Donnell, et al., 2019).

2.1.5.2. Intravenous VCM injection

Nausea, stomach discomfort, and hypokalemia are the most prevalent side effects of IV administration of VCM (Keller, 2014). Indeed, individuals who are 65 years old and above are more susceptible to experiencing hypokalemia, urinary tract infections, peripheral edema, insomnia, constipation, anemia, depression, vomiting, and hypotension compared to those who are younger than 65 years old.(Keller, 2014). Furthermore, nephrotoxicity including reports of renal failure, renal impairment, high blood creatinine, and other symptoms have been reported with IV administration of VCM in several investigations, where it has been recorded to occur during or after the course of medication. However, the risk of such nephrotoxicity increases in persons above the age of 65 (Keller, 2014).

Patients who have undergone VCM treatment have reported occurrences of ototoxicity, characterized by damage to the auditory branch of the eighth cranial nerve and the manifestation of symptoms such as vertigo, dizziness, and tinnitus (Bailie & Neal, 1988; Bergman et al., 1988) and it can be temporary or chronic. This effect has mostly been recorded in patients who were received high intravenous dosages, have renal disease, have an underlying hearing loss, or are under concomitant therapy with another ototoxic agent such as an aminoglycoside (Keller, 2014). Neutropenia has also been documented in several dozen individuals, generally starting one week or more after the IV VCM therapy, or after a cumulative dosage of greater than 25mg. However, as soon as the administration of VCM stopped, the neutropenia appears to be quickly reversible. Moreover, thrombocytopenia has also been reported (Keller, 2014).

Moreover, hypersensitivity causes a condition known as 'Red Man Syndrome' in some people. If the medicine is required in RMS, which is frequently caused by fast VCM infusion, an antihistamine containing diphenhydramine should be administered, and the infusion rate should be lowered. Otherwise, switching to alternate medication therapy is advised (Sivagnanam & Deleu, 2003). However, taking into consideration the changes in renal function, volume of distribution and accumulation related to aging, elderly patients are considered to be more vulnerable to VCM toxicity with IV administration. These patients must be closely monitored and must be given a more restrictive dosing regimen (Keller, 2014).

2.1.5.3. Oral VCM

The oral administration of Vancomycin (VCM) has been documented to result in gastrointestinal adverse effects, including abdominal discomfort and nausea, as reported in scientific literature. Furthermore, dysgeusia, or a confused perception of taste, has also been noted to be a common side effect of oral VCM. Moreover, emergent medical assistance should be considered when these side effects get severe and troublesome in patients (Patel et al., 2022). However, it should be noted that many of these side effects are quite transient, peripheral edema, diarrhea, flatulence, urinary tract infection, and fever are some of the less common side effects of oral VCM (Patel et al., 2022). Moreover, numerous cases have been reported in the literature regarding uncommon occurrences of elevated serum creatinine, red man syndrome, interstitial nephritis, nephrotoxicity, ototoxicity, thrombocytopenia, and vasculitis associated with the administration of VCM (Patel et al., 2022).

2.1.5.4. Considerations for pregnancy

For use during pregnancy, oral VCM is classified as a category B medication, intravenous VCM injection, on the other hand, is classified as category C (Powder et al., 2019). It is advised that VCM should be avoided during pregnancy unless the benefits outweigh the dangers. If the administration of Vancomycin (VCM) is deemed necessary during pregnancy, it is recommended to implement diligent monitoring of maternal blood to mitigate the potential risks of fetal ototoxicity and nephrotoxicity, (Powder et al., 2019). Furthermore, it has been documented that VCM can cross the placenta, resulting in its presence in fetal serum, amniotic fluid, and cord blood (Powder et al., 2019). Moreover, it should be noted that pregnant women may require higher doses of VCM to attain therapeutic concentrations, owing to pharmacokinetic alterations such as increased volume of distribution (Vd) and total plasma clearance, which may further heighten the risk of fetal harm (Powder et al., 2019).

2.1.5.5. Considerations for breastfeeding

Following IV administration, VCM could be traced in breast milk. Oral VCM, on the other hand, is reported to have a poor systemic absorption thus limited excretion through breast milk (Patel et al., 2022). Breastfeeding mothers who are undergoing a therapeutic plan commencing IV administration of VCM should check with their practitioner before continuing the medication plan, that's because it could resemble a

potential harm for their infant (Patel et al., 2022). However, VCM could be prescribed for the treatment of *Clostridium difficile* infections in nursing mothers (Patel et al., 2022).

2.1.5.6. Drug interactions

When VCM is administered concurrently with certain medications, there is a heightened risk of experiencing adverse effects and toxicity. Consequently, it is of utmost importance to thoroughly assess and adjust dosages, incorporate additional monitoring protocols, and carefully deliberate alternative treatment approaches when utilizing VCM alongside other drugs (Patel et al., 2022). This precautionary measure becomes particularly critical when combining VCM with other nephrotoxic agents such as aminoglycosides, amphotericin derivatives, and intravenous contrast agents (Patel et al., 2022).

2.1.5.7. VCM and nephrotoxicity

Nephrotoxicity is considered as a major complaint following the administration of VCM. This is particularly important when patients under high doses of VCM are considered, or VCM is co-administered with other antibiotics such as aminoglycoside (M. Rybak et al., 2009). Recently published guideline are recommending a therapeutic level of VCM up of 15 to 20mg/L in order to eradicate infections be MRSA strains (M. J. Rybak et al., 2020). However, such high concentration of VCM could also increase of the risk of inducing nephrotoxicity as a side effect (Wong-Beringer et al., 2011). The incidence of recorded VCM-associated nephrotoxicity have been reported to be as high as 33% and 20% in those with trough levels of VCM of >20µg/ml and 15–20µg/ml respectively (Lodise et al., 2009). Adverse effects on the kidneys, known as nephrotoxicity, have been reported in association with the use of Vancomycin (VCM). These clinical presentations encompass increased concentrations of blood urea nitrogen (BUN) or serum creatinine (Cr), the presence of hyaline and granular casts in urine, albuminuria, severe uremia leading to fatality, and the occurrence of acute interstitial nephritis. Previous studies have linked these kidney-related complications to the administration of VCM (Bailie & Neal, 1988; Bergman et al., 1988; Hong et al., 2007). Renal toxicity is predominantly observed in patients with pre-existing renal dysfunction, individuals receiving high doses or prolonged intravenous administration of VCM, or those concurrently receiving other nephrotoxic

medications, specifically aminoglycoside-derived drugs. (Keller, 2014).

Recent data on animals studies have demonstrated that VCM could significantly increase the oxidative stress in the proximal renal tubule, therefore suggesting the usage of antioxidants could provide a protection against VCM-induced kidney injury (Uckun et al., 2020). The increased oxidative stress and potentiated immune inflammatory response have been reported to be associated with the progression of kidney damage (J. He et al., 2021). Increasing the generation of ROS resembled in the increased of lipid peroxidation, the potentiated inflammatory response including higher levels of cytokines and chemokines, and the increased consumption of antioxidant defense have been reported to be significantly important in the development and the progression of VCM-induced nephrotoxicity (Fouad et al., 2014). Hence, studies have shown that the urinary excretion of renal MDA has increased following VCM administration in rats. In contrast the activity of SOD as antioxidant enzyme have decreased in those animals (J. He et al., 2021).

VCM could facilitate the increased production of superoxide radical (Humanes et al., 2015). As a result, augmenting the depolarization of the mitochondrial membrane potential leads to the liberation of cytochrome C, thereby triggering the activation of caspases 3 and 9. These cascades of events are critical in initiating apoptotic cell death (Humanes et al., 2015). Furthermore, the increased oxidative stress resulting in nitrosative stress and the activation of inflammatory cascades in addition to the up regulation of caspase family of proteases, therefore resulting in apoptotic cell death eventually (Fouad et al., 2014). Moreover, VCM has also been reported to alter the energy-dependent renal reabsorption function of the proximal tubule cells, in addition to the alteration of mitochondrial function (King & Smith, 2004). Mitochondrial function alteration has been noted to be a significantly important in the of kidney diseases progression (Che et al., 2014), where in VCM-induced nephrotoxicity, excess production and formation of ROS derived from respiratory chain has been documented, therefore resulting in structural and functional alteration of the mitochondria (Filippone et al., 2017).

In this investigation, the mRNA expression of Kim-1 have been measured as it is known to be counted as one of the new markers of kidney injury (Vaidya et al., 2010). Additionally, we have measured the creatinine and urea nitrogen levels within serum samples collected from the animals involved in this investigation.

2.1.5.8. The mechanism(s) of nephrotoxicity related to VCM

VCM is eliminated by glomerular filtration (Golper et al., 1988; H. E. Nielsen et al., 1975) as well as tubular secretion. The organic cation transporter system actively transports VCM through the basolateral membrane into proximal tubule cells (Nakamura et al., 1996a; Sokol, 1991). The presence of substantial levels of VCM at the brush boundary suggests that the medication is being secreted or reabsorbed at the apical side -from the urine- (Beauchamp et al., 1992; Fujiwara et al., 2012). The VIKI pathology could take place through different routes. Figure 2.3 demonstrates the nephrotoxicity routes by the administration of VCM (Uckun et al., 2020).

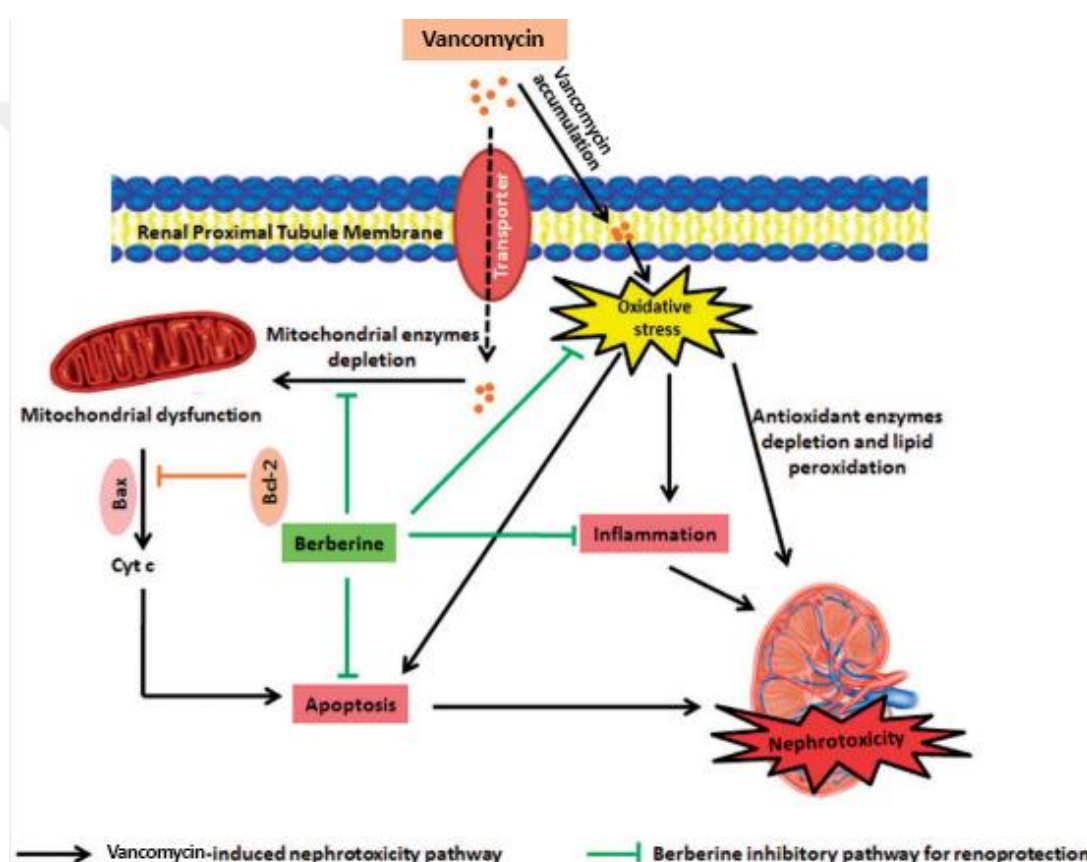


Figure 2.3. The nephrotoxicity routs of VCM (Adil et al., 2016).

2.1.5.8.1. Acute interstitial nephritis

VCM has been associated with AIN (Blumenthal et al., 2012; Htike et al., 2012). It has been imposed that VCM-induced AIN is mainly due to the cellular hypersensitivity (Azar et al., 1996). Furthermore, in a renal biopsy, interstitial edema has been documented in addition to an infiltration of inflammatory cells including eosinophils, lymphocytes, and macrophages (Htike et al., 2012). However, the exact

mechanism underlying VCM-induced AIN has not been comprehensively elucidated thus far (Blumenthal et al., 2012). In addition, it has been proposed that VIKI is resulted from the inflammatory response (Uckun et al., 2020). Hence many study have demonstrated that the kidneys of rats who have been receiving high-dose of VCM have showed signs of VIKI resembled in the elevation of inflammation biomarkers (J. He et al., 2021; Uckun et al., 2020).

2.1.5.8.2. Acute tubular necrosis

VCM have been documented to induce dose-dependent ATN (Sawada et al., 2018). The main contributors to this phenomenon are primarily attributed to oxidative stress in proximal tubule cells, autophagy, and the formation of obstructive casts. (Luque et al., 2017).

2.1.5.8.2.1. Oxidative stress

Oxidative stress refers to a state of imbalance between the generation of reactive oxygen species (ROS) or free radicals and the body's capacity to counteract them using antioxidants. This imbalance can result in mitochondrial dysfunction and cellular apoptosis, contributing to various pathological processes. In cultured renal cells, VCM has been reported to promote the oxygen consumption and to induce a dose-dependent rise in ATP concentrations (King & Smith, 2004), indicating that VCM could accelerate the mitochondrial oxidative phosphorylation (Nishino et al., 2003a). Moreover, the increase in oxygen consumption increases the generation of free radicals, therefore could have an important role in the pathophysiology of VIKI by decreasing the anti-oxidant enzymes activity such as SOD (Nishino et al., 2003a). It has been reported that 2,3-dihydroxybenzoic acid which is an iron chelator, has shown a protection effect against VIKI, thus providing evidence that VIKI could be ameliorated by oxidative stress (Naghibi et al., 2007). The formation of an iron complex has been observed to catalyze the generation of ROS, particularly the hydroxyl radical. Iron plays a crucial role in the formation of the hydroxyl radical, which contributes to oxidative stress. (Naghibi et al., 2007).

Free radicals also facilitate lipid peroxidation, which is a chain process that damages unsaturated fatty acids in the cell membrane and produces malondialdehyde (F. Nielsen et al., 1997). Elevated levels of MDA which is used as an indication of lipid peroxidation, have been documented in the kidney tissue of rats following VCM

administration, indicating oxidative stress (J. He et al., 2021). Additionally, a reduction in the activity of antioxidative enzymes, such as glutathione peroxidase and superoxide dismutase, has been observed, further supporting the presence of oxidative stress in the kidneys (J. He et al., 2021). Antioxidants, including a superoxide dismutase, and Vit C have reversed or prevented these effects and have mitigated the histopathologic damage (J. He et al., 2021; Nishino et al., 2003a). Moreover, the generation of superoxides by VCM has been found to result in the progression of the mitochondrial membrane depolarization and the subsequent release of cytochrome C. This process activates downstream caspases, which ultimately triggers cellular apoptosis (Arimura et al., 2012; Humanes et al., 2015).

Furthermore, endogenous inducers such as superoxide anions are involved in the initiation of DNA single strand breaks, which are necessary for the activation of the enzyme PARP-1 (Heller et al., 1995). This enzyme is normally involved in the DNA repair process. During this process, the enzyme PARP-1 requires NAD⁺ as a substrate, therefore cells use its ATP stock to rebuild NAD⁺ storage (Dalaklioglu et al., 2010). In the presence of extreme DNA damage, PARP-1 overactivation results in NAD⁺/ATP depletion and eventually necrosis. Therefore, the overactivation of PARP-1 in rats given VCM and the reduction of kidney damage in rats given 1,5-isoquinolinediol, as an PARP-1 inhibitor, provides an additional evidence for the oxidative stress pathway in VCM related kidney injury (Dalaklioglu et al., 2010).

2.1.5.8.2.2. Autophagy

Autophagy is a cellular mechanism in which cellular components are degraded through lysosomal-mediated processes, and it is widely acknowledged as an adaptive and defensive mechanism promoting cell survival (C. He & Klionsky, 2009). Autophagy within the proximal tubules has been documented to play a protective role against AKI (M. Jiang et al., 2012). However, Massive and dysregulated autophagy on the other hand, could result in cell death (Maiuri et al., 2007).

The sequestration of VCM within endosomes is initiated through the process of receptor-mediated endocytosis, which occurs when VCM is taken up from the urine. Additionally, the transporters facilitate the secretion of VCM from the bloodstream into the tubular epithelial cells. Endosomes merge with lysosomes, leading to VCM accumulation in lysosomes (Fujiwara et al., 2012). VCM accumulation in proximal

tubule cell lysosomes was initially seen using immunoelectron microscopy (Beauchamp et al., 1992), and was later validated by another group (Fujiwara et al., 2012). VCM build up within the lysosomes results in a deleterious autophagy process (Boya & Kroemer, 2008). In addition, different stresses, and the formation of ROS leads lysosomes that are near the mitochondria to permeabilize (Boya & Kroemer, 2008). The rupture of lysosomal membranes leads to the liberation of proteases, specifically cathepsins, into the cytosol. These proteases are involved in triggering the activation of apoptotic effectors, including mitochondria and caspases (Boya & Kroemer, 2008). Moreover, the complete lysosome disruption can cause cytosolic acidity, which can lead to uncontrolled cell death via necrosis (Boya & Kroemer, 2008).

2.1.5.8.2.3. Cast formation

Uromodulin is the most abundant renal protein, can modulate salt transportation, help in the protection against urinary tract infection and kidney stones in addition to some immunomodulatory activities (Devuyst et al., 2017). Uromodulin produces filaments in the tubular lumen that form the matrix of hyaline casts (Devuyst et al., 2017). Uromodulin has been documented to interact with VCM aggregates, therefore resulting in the production of intratubular casts, inflammation, and ATN (Luque et al., 2017). VCM has been found in uromodulin casts from nine ATN patients (Stokes, 2017). The presence of VCM supersaturation and subsequent precipitation within the tubular lumen can be attributed to various factors, such as increased concentrations of VCM and acidic pH levels in the urine (Stokes, 2017). Furthermore, VCM buildup near the brush border has been reported, VCM has been observed to localize within the tubular lumen, microvilli, and cytoplasmic compartments. (Fujiwara et al., 2012).

2.2. Berberine

Berberine (BBR) is an alkaloid that has been used for more than 3000 years by the traditional medical culture of Ayurvedic India and China (Neag et al., 2018). It could be isolated from a variety of commonly used plants, including *Coptis chinensis* Franch, *Berberis vulgaris*, and *Berberis aristate* (K. Wang, Feng, et al., 2017). BBR was originally used in the treatment of various infections of the ears, eyes, and mouth. In addition to its usage in mending wounds, curing hemorrhoids, indigestion and

dysentery (Dev, 2006). BBR has also been used in the management of diarrhea and intestinal parasites in traditional Chinese medicine (C. Chen et al., 2014; I. P. Singh & Mahajan, 2013). Several studies in recent years have documented the potentials of BBR as a hypoglycemic, hepatoprotective, and anti-inflammatory agent as well as its hypolipidemic and anti-obesity reported properties (Tillhon et al., 2012; K. Wang, Feng, et al., 2017).



Figure 2.4. *Berberis vulgaris* (Plantus.com, 2022).

BBR is a yellow crystalline powder with a strong bitter flavor (Xiaojun Feng et al., 2019). Free BBR slowly dissolves in water, quickly in ethanol with heat, and in low-polar organic solvents such as benzene and chloroform is insoluble (Xiaojun Feng et al., 2019). The hydrochloride salt form of BBR is characterized of being less soluble in water but in boiling water it is more easily dissolved, whereas the phosphate salt forms are somewhat water soluble in addition to the sulfate salt form of BBR (Xiaojun Feng et al., 2019).



Figure 2.5. *Coptis chinensis* (Made-in-china.com, 2022).

Although there is no drug containing BBR as the main active ingredient approved by the drug authorities just yet (Vuddanda et al., 2010), it is used as a food supplement in the USA in doses ranging from 400 mg to 1200 mg (Tew et al., 2020). When it comes to worries about the possible riskiness in using BBR, it is documented to be characterized by high degree of safety in both humans and animals (Imenshahidi & Hosseinzadeh, 2016).

In recent studies, BBR has been extensively characterized for its diverse pharmacological properties, which encompass anti-inflammatory, anti-oxidative, and anti-apoptotic effects (Ayati et al., 2017; Moghaddam et al., 2014), which is particularly important to notice, since it is reflected in the BBR neuroprotective, hepatoprotective, and cardioprotective potentials (Eissa et al., 2018; Fang et al., 2020; X. Liu et al., 2016), as well as its potential effects reno-protective in addition to some anti-hyperglycemic properties (Javad-Mousavi et al., 2016; J. Tang et al., 2009). One of its most Important potential is its protective effect against nephrotoxicity that have been documented in several animal studies (Adil et al., 2016; S. Ahmad et al., 2019; Allameh et al., 2020; Ashrafizadeh et al., 2020; Hasanein & Riahi, 2018; Hassanein et al., 2019; Ibrahim Fouad & Ahmed, 2021; Qi et al., 2020). Furthermore, multiple investigations have shown that BBR could have some anti-tumor properties to consider, which is particularly important to notice since its capability of inducing apoptosis and inhibiting cancer cell proliferation, migration, and angiogenesis without causing cytotoxicity in non-cancer cells have been reported in recent investigations (D. Liu et al., 2019; Ren et al., 2021; Zhao et al., 2017). In addition, BBR has been

found in a clinical investigation of 90 individuals with renal atherosclerosis to enhance their kidney functions, since using a dose of 500mg t.i.d. for 4 weeks has been reported to significantly lowered BUN and serum creatinine levels as well as improving the renal function in these patients (Xiaopeng et al., 2017).

Moreover, BBR has the ability to inhibit the generation of reactive oxygen species (ROS) and concurrently enhance the activity of antioxidant enzymes. Furthermore, BBR has been shown to improve mitochondrial function by decreasing oxygen consumption rate and improving mitochondrial membrane potential against oxidative damage (Imenshahidi & Hosseinzadeh, 2019). In addition to its reported effect of inhibiting and a significant reduction of the generation of superoxide anion in macrophages (Sarna et al., 2010). it has recovered some of the anti-oxidant enzyme including glutathione and SOD levels in a study conducted on mice (Lao-Ong et al., 2012).

2.2.1. Structure

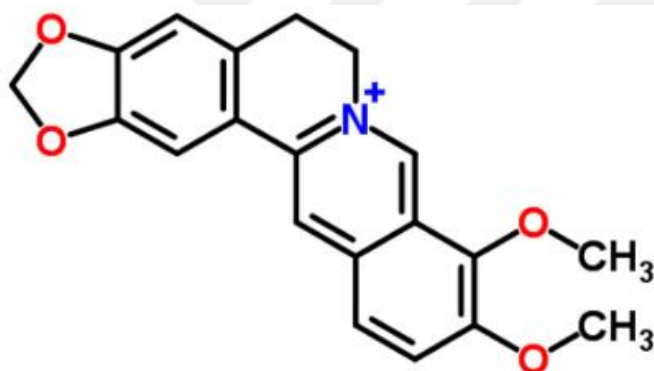


Figure 2.6. BBR structure (Chemspider, 2022a).

BBR is a nonbasic quaternary benzyloquinoline alkaloid, which chemically known as *2,3-methylenedioxy-9,10-dimethoxy-protoberberine* (Neag et al., 2018).

2.2.2. Pharmacokinetics

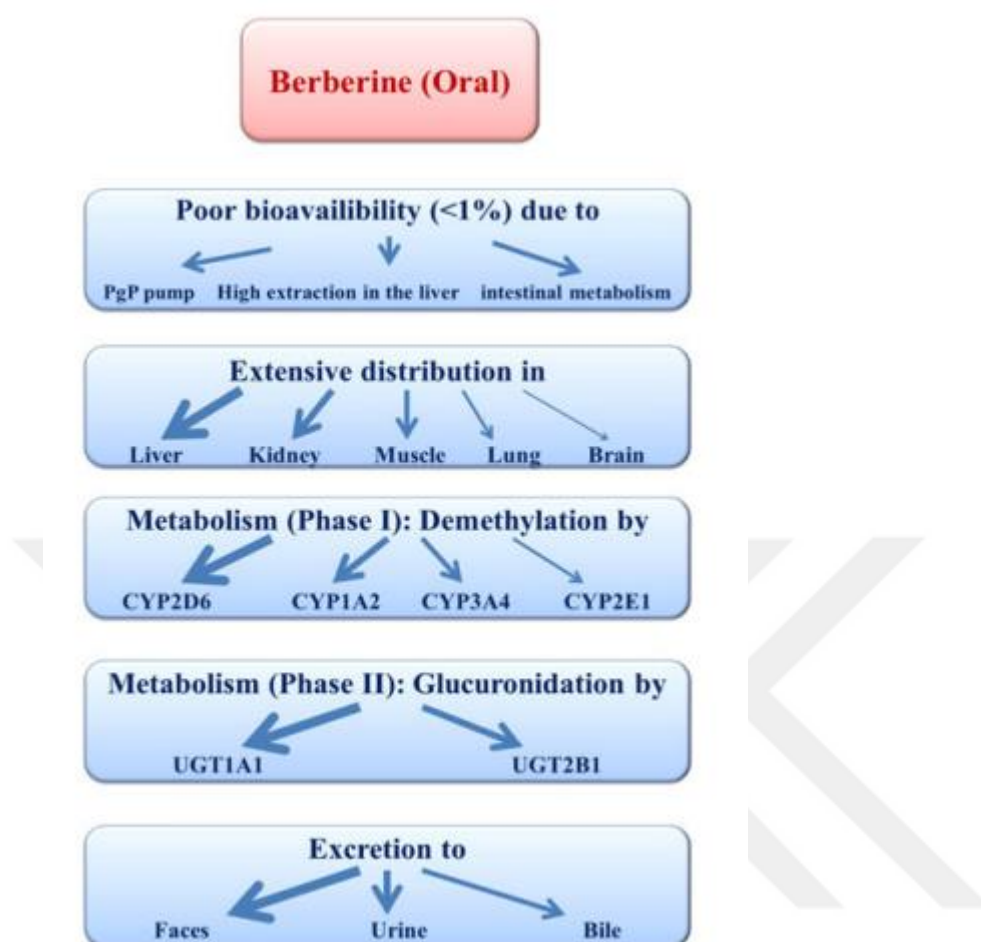


Figure 2.7. Pharmacokinetics of BBR (Imenshahidi & Hosseinzadeh, 2016).

2.2.2.1. Absorption

BBR is documented to have a noticeably poor oral bioavailability, mostly due to its poor solubility and dissolution in aqueous solutions (Y. Zhang et al., 2013). It has been demonstrated to be quite low ranging less than 5% in recent reports (Neag et al., 2018). Studies using 1,000-1,500mg of BBR have reported pharmacologically intended benefits following oral BBR administration (R. Feng et al., 2015). However, enhancing the absorption using different pharmaceutical techniques has been reported to reduce the dose of BBR required to reach its wanted effects (R. Feng et al., 2015). Additionally, microbiota within the gut facilitated by nitroreductases could chemically convert BBR into its more readily absorbable form of dihydroberberine, which could enhance its absorption by a huge rate reaching of almost five-folds in rats. This conversion. Nonetheless, research findings have demonstrated that upon permeating the tissues of the intestinal wall, dihydroberberine undergoes prompt oxidation,

resulting in its immediate conversion back into BBR. (R. Feng et al., 2015).

Moreover, BBR has been noted to be affected by P-Glycoprotein mediated efflux from the intestines (Chae et al., 2008) and liver (Tsai & Tsai, 2002). Studies have indicated that nearly 90% of the reduced transportation of BBR in the intestines is related to P-Glycoprotein efflux (Chae et al., 2008), since BBR has also been reported to induce the activity of P-Glycoprotein transporters in the intestine (X. Zhang et al., 2011). Therefore resulting in the reduction of absorption for other compounds subject to P-Glycoprotein, such as Ciprofloxacin (Hwang et al., 2012). However for reaching a higher absorption of BBR, an analogue that is not subject of efflux by P-Glycoprotein could be used (Shan et al., 2013). In addition, inhibition of P-Glycoprotein by combining BBR with an agent like Milk Thistle (di Pierro et al., 2012) or Ketoconazole (Zhou et al., 2012) could therefore enhance the biological activity of BBR. Moreover, BBR absorption has also been enhanced by Sodium Caprate, which is an agent that increases the size of tight junctions between intestinal cells thus an improvement in the AUC of BBR by almost 28% have been reported (X. Y. Lv et al., 2010). This can prove evidence that enhancing the absorption of BBR precedes greater desired therapeutic post-absorptive effects (M. Zhang et al., 2012).

2.2.2.2. Distribution

BBR have been be to have s high tissue distribution, despite its low oral bioavailability (Kumar et al., 2015). Moreover, it has been reported that BBR can cross the blood-brain barrier (BBB). Therefore, a rapid accumulation in the brain, particularly in the hippocampus (X. Wang et al., 2005). However, this accumulation has been reported to be followed by slow elimination in rats (X. Wang et al., 2005). The distribution of BBR in organs has been noted to be fast and higher than its concentration in the blood after oral administration (Tan et al., 2013). Its accumulation has been documented mostly in the liver, kidneys, muscle, lungs, brain, heart and the pancreas while it was lease distributed in fat (Tan et al., 2013).

2.2.2.3. Metabolism

BBR is metabolized in the liver by cytochrome P450 isoforms extensively. This has been noted as it goes through phase I oxidative demethylation followed by phase II glucuronidation where BBR would be conjugated with glucuronic acid or sulfuric acid (C. S. Liu et al., 2016). Moreover, the formation of 97 metabolites of

BBR have been documented in rats (K. Wang, Chai, et al., 2017). However, the existing literature on this topic is limited and primarily concentrates on the unaltered form of BBR and its phase I metabolites, including berberrubine, demethyleneberberine, jatrorrhizine, thalifendine, and columbamine (Xinchi Feng et al., 2021). Dihydroberberine has been shown as the much more absorbable form of BBR and will be converted by the gut microbiota. However, shortly after that, it will be reverted back to BBR through oxidation (R. Feng et al., 2015).

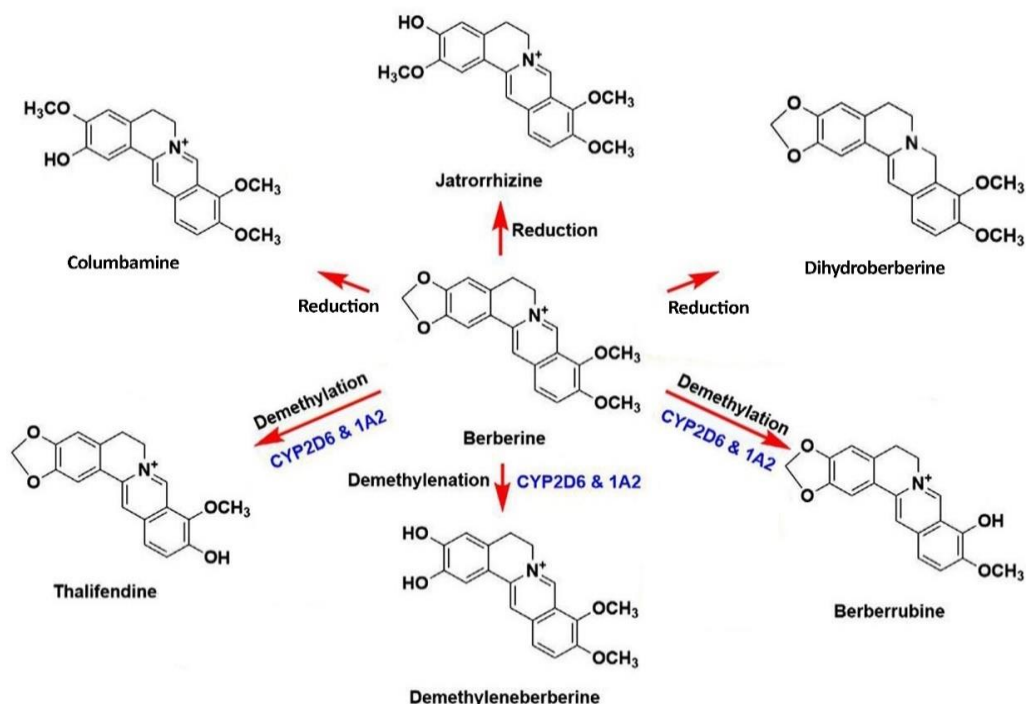


Figure 2.8. BBR and its main metabolites (Xinchi Feng et al., 2021).

Studies have reported that the main enzymes noted to be considered significant regarding the metabolism of BBR were CYP2D6 and CYP1A2, while UGT2B1 and UGT1A1 were mainly involved during the glucuronidation processes (Xinchi Feng et al., 2021). As a result, the inhibition of BBR metabolism can be significantly observed with the use of quinidine, a prominent specific inhibitor of CYP2D6, and furafylline, a leading specific inhibitor of CYP1A2 (CHEN, 2013).

2.2.2.4. Elimination

In rats, following the metabolization of BBR is mainly excreted by the hepatobiliary system in addition to the role of the kidneys (Tan et al., 2013). Eleven different metabolite of BBR have been seen in mouse urine and feces, and most of these metabolites were demethylated products (Y. Li et al., 2011). However, only

0.036% of the total oral dose of BBR have been traced as the unchanged form of BBR in rats (A. M. Ahmad, 2007). Moreover, in a study conducted on BBR and its most relevant metabolites, it was noted that BBR was mainly excreted in feces and mostly in its ionized form. Most likely the unabsorbed form of BBR after its oral administration, since only a small amount of the ionized form could be absorbed by the intestine (Xinchi Feng et al., 2021). Furthermore, 18.6% of BBR has been reported to be traced in feces as berberrubine. While the accumulative biliary excretions of berberrubine and its glucuronate metabolites were approximately 2.976% (Xinchi Feng et al., 2021).

2.2.3. Pharmacodynamic

BBR has been characterized by exhibiting anti-inflammatory, anti-oxidative, and anti-apoptotic potential activities, which was reflected in its broad pharmacological effects (Ayati et al., 2017; Moghaddam et al., 2014). Furthermore, BBR can reduce the generation of ROS (Imenshahidi & Hosseinzadeh, 2019). As its effect of improving the mitochondrial function have been documented to be through the reduction of oxygen consumption rate (Imenshahidi & Hosseinzadeh, 2019). Additionally, BBR has also been reported to recover the glutathione and superoxide dismutase levels in mice (Lao-Ong et al., 2012), as well as inhibiting the superoxide anion production (Sarna et al., 2010).

The anti-inflammatory effect of BBR have been documented in vivo in addition to in vitro studies (Y. H. Wu et al., 2012). Moreover the direct and indirect antioxidant actions, scavenging free radicals have also been noted (X. Chen et al., 2016; Tillhon et al., 2012). Furthermore, the reduction of oxidative stress markers such as MDA and NO levels have also reported (Y. Feng et al., 2010; Othman et al., 2014). BBR's nephroprotective properties in the proximal renal tubular injury via the attenuation of oxidative stress has been significant (Visnagri et al., 2015a).

2.2.3.1. Anti-inflammatory effects of BBR

Inflammation is an immune response of tissues exposed to various simulations, which could include pathogens invasion, damaged cells or a xenobiotic molecule (Ferrero-Miliani et al., 2007). Inflammation is a protective response regulated by the immune system, it could involve immune cells, blood vessels and molecular mediators with the primary objective to eliminate the noxious stimuli and to promote healing

(Ferrero-Miliani et al., 2007). However, uncontrolled and progressive inflammation results in a variety of disorders, including the development and the facilitation of kidney injuries (L. Chen et al., 2018). The activation of inflammatory involves the regulation of inflammatory cytokines and chemokines (Fouad et al., 2014). It has been shown that BBR could significantly reduce the expressions of TNF- α and IL-6 in acute kidney failure animal models, as well as attenuating the inflammatory mediators like IL-1 β and IL-8 (Lou et al., 2011; S. Zhang et al., 2011; Z. Zhang et al., 2016).

Table 2.1. the effect of BBR on inflammatory cytokines (Chueh & Lin, 2012; W. C. Lin & Lin, 2011; Pang et al., 2015; Shang et al., 2010; Yi et al., 2008).

The used cell type or animal model	The dose of BBR and time treatment interval	The examined samples	The BBR Effect
3T3-L1 adipocytes	10 μ M (18 hours)	3T3-L1 adipocytes	↓: TNF- α , IL-6
HepG2 cells,	0.1–10 μ M (24 hours)	Cultured media	↓: TNF- α , IL-6
Mouse	0.8–3.3 μ M for (48 hours)	Cultured media	↓: TNF- α , IL-6
Mouse	50 and 150mg/kg/d, orally (2 weeks)	Serum	↓: TNF- α , IL-6
Mouse	50, 150 and 500mg/kg/d, orally for (14 weeks)	Kidney and liver	↑: IL-10/IL-6 and IL-10/TNF- α ratios of mRNA levels
Rats	200 mg/kg/d, orally for (12 weeks)	Kidney	↓: ICAM-1, TGF- β 1 protein expression

2.2.3.1.1. The Pathways of BBR as an anti-inflammatory

The cellular mechanisms underlying the anti-inflammatory effects of BBR exhibit some similarities with its antioxidant pathways. The AMPK pathway has been identified as a significant factor in reducing inflammation in addition to its important role in BBR's antioxidant activity. Scientists have demonstrated that the suppressive impact of BBR on the synthesis of proinflammatory cytokines in macrophages can be annulled by inhibiting AMP-activated protein kinase (AMPK) (Jeong et al., 2009; Mo et al., 2014).

The effect of BBR as an anti-inflammatory has also been associated with its ability to inhibit the effect of MAPK signaling pathways, which normally is activated following the inflammatory stimulations (Jeong et al., 2009; Q. Wang et al., 2012; Y.

Wang, 2014). Reports have documented that BBR suppressed inflammation through inhibiting MAPKs (Jeong et al., 2009; Q. Wang et al., 2012; Y. Wang, 2014). In addition to its involvement in the activation of P38 (Bae et al., 2013; D. Lee et al., 2013). Since it has been demonstrated that in unstimulated cells that BBR had the ability to increase the P38 phosphorylation (D. Lee et al., 2013), as well as decreasing the MAPK phosphorylation in cells that have been subjected to an inflammatory stimuli activating MAPKs, this could include agents like lipopolysaccharide (LPS) as well as TNF- α (Jeong et al., 2009; Q. Wang et al., 2012; Y. Wang, 2014).

The impact of BBR on the transcription factor Nrf2 has been recognized as a significant pathway contributing to the anti-inflammatory activity of BBR. In macrophages, it has been observed that blocking Nrf2 diminishes the inhibitory effect of BBR on the production of proinflammatory cytokines. BBR treatment has the potential to activate AMPK and P38, leading to the nuclear translocation of Nrf2, ultimately resulting in the suppression of proinflammatory cytokine production (D. Lee et al., 2013; Mo et al., 2014).

The transcription factor NF- κ B is recognized as a pivotal mediator in facilitating the transcriptional activation of a wide range of proinflammatory cytokines, including TNF- α and IL-6, thereby exerting significant influence on the regulation of inflammatory processes (Goldin et al., 2006; Gratas-Delamarche et al., 2014). Consequently, it represents a key target for the anti-inflammatory activity of BBR. In the NF- κ B signaling pathway, an inflammatory stimulus like TNF- α can activate I κ B kinase- β (IKK- β) (Goldin et al., 2006; Gratas-Delamarche et al., 2014). However, the administration of BBR has been observed to significantly reduce the phosphorylation and activation of IKK- β (Pandey et al., 2008). This inhibition of IKK- β by BBR leads to the stabilization of inhibitory κ B- α (I κ B- α) (Delhase et al., 1999; Mercurio et al., 1997), preventing its phosphorylation by IKK- β and subsequent degradation (Delhase et al., 1999; Q. Jiang et al., 2011). Consequently, the blockage of the nuclear translocation of NF- κ B in a variety tissues including pancreatic β -cells (Y. Wang, 2014), lung cells (C.-H. Lee et al., 2007), and rats kidney (Q. Jiang et al., 2011; Wan et al., 2013). Hence, the suppressive impact of BBR on the synthesis of proinflammatory cytokines can be ascribed to its capacity to exert negative regulation on the NF- κ B signaling pathway.

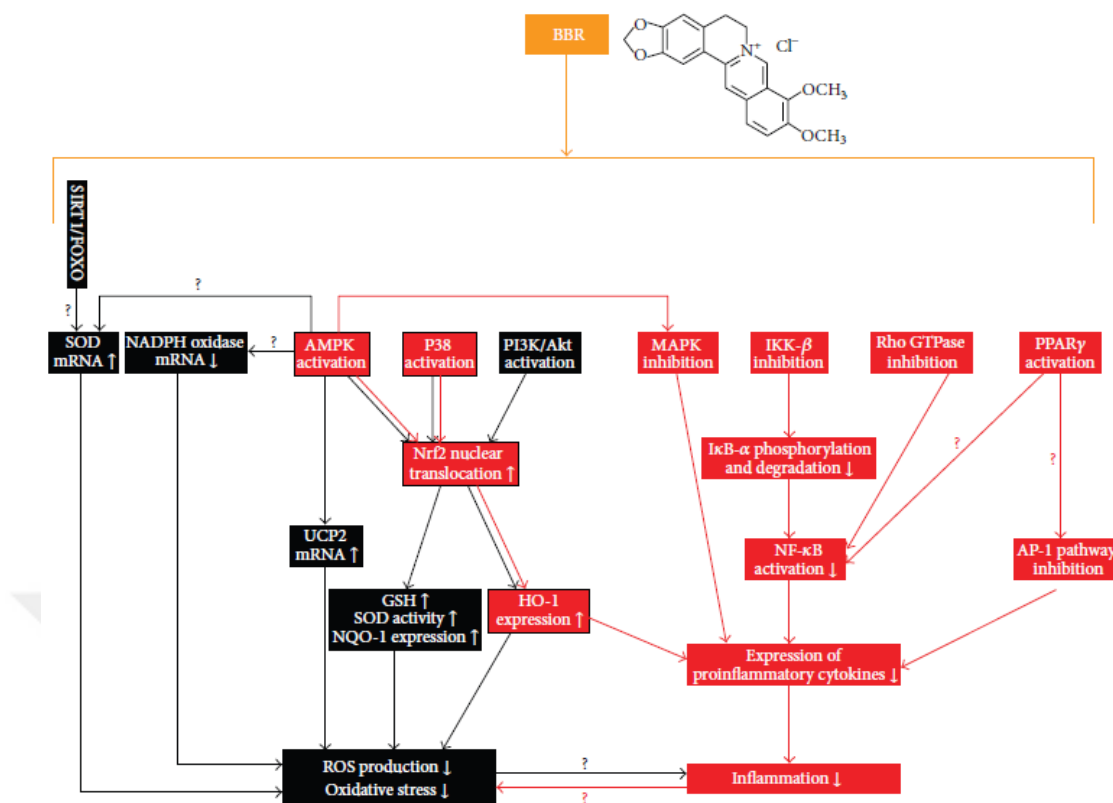


Figure 2.9. The anti-inflammatory mechanism of action of BBR (Z. Li et al., 2014).

2.2.3.2. Oxidative stress

Reactive oxygen species (ROS), which encompass hydrogen peroxide (H₂O₂), superoxide anion radical (O₂⁻), and hydroxyl radical (OH⁻), represent free radicals that can be encountered through diverse origins, including infections, environmental pollution, dietary intake, or generated intrinsically within mitochondria during aerobic metabolism (Bui & Nguyen, 2017; Pongkittiphan et al., 2015). While a mild level of ROS plays a role in cellular functions like cell proliferation, signal transduction, and programmed cell death, an excessive level of ROS poses a challenge to the cellular antioxidant defense system and can cause damage to vital biomolecules such as DNA, RNA, proteins, and lipids, leading to cellular deterioration (Pongkittiphan et al., 2015). The imbalance between ROS production and the antioxidative defense system is known as oxidative stress (Bui & Nguyen, 2017). Normally, cells maintain an optimal level of ROS through intrinsic enzymatic and nonenzymatic systems (Pongkittiphan et al., 2015). Key intrinsic enzymatic antioxidants involved in this process include catalase, superoxide dismutase, and glutathione reductase (Pongkittiphan et al., 2015).

The excessive production of ROS has been linked to the development of various kidney diseases, including drug-induced acute kidney injury (Kwiatkowska et al., 2021). The augmentation of antioxidant enzymes, including catalase and superoxide dismutase, has been documented as a viable approach for safeguarding renal tissues against oxidative stress inducers (Visnagri et al., 2015a).

2.2.3.2.1. Antioxidant activity of BBR

Many natural compounds have been documented to have the potential to protect cells from oxidative stress, and prevent or ameliorate various diseases caused by oxidative stress (Jia et al., 2018). The reported broad range of BBR's biological activities could be explained in an important part, by its remarkably diverse antioxidant activities. The antioxidative properties of BBR have been extensively investigated in various studies, demonstrating its ability to modulate markers of oxidative stress and antioxidant enzymes. These markers include MDA, a byproduct of lipid peroxidation that typically rises under conditions of oxidative stress, as well as GSH and SOD, which tend to decrease during oxidative stress (Hassanein et al., 2019).

In addition to the reduction of ROS generating activity by BBR, BBR's ability to compensate the intrinsic antioxidant enzymes has also been demonstrated (Siow et al., 2011). In addition, BBR has exhibited the capacity to maintain non-enzymatic antioxidant levels. When administered in conjunction with the carcinogenic agent azoxymethane, BBR has been reported to preserve normal levels of antioxidants, including glutathione, vitamin C, and vitamin E, therefore resulting in the reduction of the lipid peroxide generation, and the reduction of cellular apoptosis in rats (Thirupurasundari et al., 2009). Furthermore, BBR has the ability to modulate the activity of numerous endogenous antioxidant enzymes. Studies have demonstrated that pretreatment with BBR significantly enhances the activity of superoxide dismutase (SOD) in macrophages stimulated with lipopolysaccharide (LPS), leading to a decrease in intracellular levels of reactive oxygen species (ROS) such as O₂ (Sarna et al., 2010).

Table 2.2. The effects of BBR on oxidative stress parameters. ↑: increased, ↓: decreased (Chatuphonprasert et al., 2014; W. Liu et al., 2008; Moghaddam et al., 2014; L.-Q. Tang et al., 2006; D. Wu et al., 2012).

The used dose of BBR	Tissues examined	Oxidative stress marker (MDA)	Antioxidant enzyme (SOD)
200mg/kg per day, orally. (12 weeks)	Serum	↓	↑
100mg/kg per day, orally for (2 weeks)	Liver and kidney	↓	↑
100 and 200mg/kg per day, orally (8 weeks)	Kidney	↓	↑
100 and 200mg/kg per day, orally (21 days)	Heart	↓	↑
50 and 100mg/kg per day, orally (8 weeks)	Hippocampus	↓	↑

However, BBR's antioxidant properties, are more than just a direct chemical scavenging of ROS (Siow et al., 2011). PI3K/Akt/Bcl-2 and Nrf2/HO-1 pathways have been involved in reducing the oxidative stress by BBR (Hsu et al., 2012).

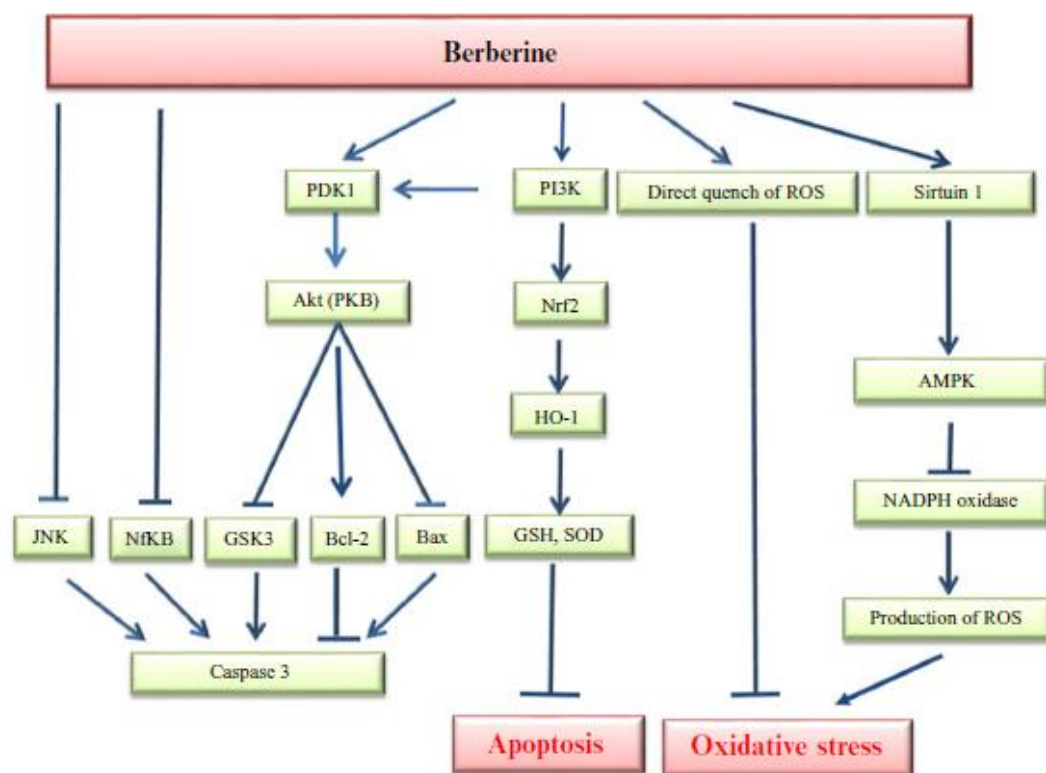


Figure 2.10. Signaling pathways of antiapoptotic and antioxidant effects of BBR (Imenshahidi & Hosseinzadeh, 2020).

The activation of Akt, also known as protein kinase B, by PI3K stimulates the PI3K/AKT signaling pathway, which in turn increases the activity of the Nrf2/HO-1 signaling pathway (X. Lin & Zhang, 2018). Nrf2, a transcription factor, is essential in coordinating cellular responses to oxidative stress (X. Lin & Zhang, 2018). When translocated to the nucleus, Nrf2 activates the antioxidant response by increasing transcription of Nrf2-regulated genes such as HO-1, an antioxidant enzyme that protects cells from oxidative stress-induced cytotoxicity and apoptosis (J.-H. Chen et al., 2012). BBR has been shown to activate Nrf2 via the PI3K/AKT pathway and enhance HO-1 mRNA expression in astrocytes (J.-H. Chen et al., 2012). Studies have demonstrated that BBR can provide neuroprotection by upregulating the PI3K/AKT/Bcl-2 and Nrf2/HO-1 antioxidative pathways in PC12 cells and zebrafish, where BBR have improved dopaminergic neuronal disorders by inducing HO-1 expression via the PI3K/Akt/Nrf2 pathways (C. Zhang et al., 2017).

The SIRT1/AMPK pathway is another mechanism involved in BBR's antioxidant effect (X. Lin & Zhang, 2018). BBR has been shown to reduce ROS levels by activating the SIRT1/AMPK pathway. where AMPK activation has been

documented to negatively regulate NADPH oxidase (Fusi et al., 2018), an enzyme responsible for the production of ROS by transferring electrons from NADPH to oxygen atoms (Kumar et al., 2015).

However, while BBR has shown substantial antioxidant properties in numerous models and approaches, there are also indications that BBR promotes oxidative stress in some scenarios. BBR's potential anticancer effects are mostly related to its ability to trigger oxidative stress in cancer cells which has not been noted in normal cells (Siow et al., 2011).

2.2.3.3. BBR and apoptosis.

2.2.3.3.1. Apoptosis

Apoptosis is an innate process of programmed cell death found in multicellular organisms. It is activated by a variety of biochemical events that induce specific changes within the cell, ultimately resulting in its demise (Jabbarzadeh Kaboli et al., 2014). The initiation of apoptosis is tightly controlled by precise mechanisms. Once initiated, the process progresses irreversibly, leading to the ultimate death of the cell (Böhm & Schild, 2003). Apoptosis, or programmed cell death, can be initiated through two distinct pathways. The intrinsic pathway is activated by intracellular signals that arise from cellular stress, resulting in the release of proteins from the intermembrane space of mitochondria. Conversely, the extrinsic pathway is triggered by the binding of extracellular ligands to death receptors located on the cell surface, leading to the formation of the death-inducing signaling complex (DISC) (Böhm & Schild, 2003, p. 1023,1024,1032). The TNF- α signaling is an essential example of the extrinsic pathway (G. Chen & Goeddel, 2002). Both intrinsic and extrinsic pathways of apoptosis activate initiator caspases, which in turn activate executioner caspases to degrade proteins and ultimately cause cell death (Jabbarzadeh Kaboli et al., 2014). Apoptosis is a critical biological process, and defects in apoptotic mechanisms have been associated with various diseases (Waller et al., 2007).

Excessive apoptosis can lead to tissue atrophy, whereas insufficient apoptosis can result in uncontrolled cell proliferation, as observed in cancer (Waller et al., 2007). Caspases serve as promoters of apoptosis, while specific members of the Bcl-2 protein family act as inhibitors (Waller et al., 2007). The regulation of the mitochondrial-mediated apoptotic pathway is a complex process involving various members of the

Bcl-2 protein family. Within this family, Bcl-2, an anti-apoptotic protein, and Bax, a pro-apoptotic protein, are of particular importance in governing the release of cytochrome C and the subsequent activation of downstream caspases. Disturbances in this pathway can lead to an imbalance, resulting in excessive apoptosis. This imbalance is commonly characterized by increased Bax expression, decreased Bcl-2 expression, and enhanced caspase-9 activity (Waller et al., 2007).

2.2.3.3.2. *Anti-antiapoptotic effect of BBR*

BBR has been reported to have antiapoptotic potential, especially in neurodegenerative diseases. Where it has been documented to prevent the hippocampal apoptosis in addition to lowering the dopaminergic neuron loss, which have been associated with memory impairment and motor dysfunction in Parkinson's disease (M. I. A. Kim et al., 2014). It has also been found that BBR has antiapoptotic activity against cerebral ischemia-perfusion injury through Akt activation (Hu et al., 2012). Therefore, the PI3K/Akt pathway could provide evidence for the antiapoptotic mechanisms of BBR.

2.2.3.3.2.1. *PI3K/AKT/Bcl-2 pathway*

Akt/PKB is involved in a range of cellular processes by phosphorylating various cellular substrates, ultimately leading to the inhibition of apoptosis (Sen et al., 2003). Especially, the upregulation of Bcl-2 expression as it is an important anti-apoptotic pathway (Pugazhenthir et al., 2000). The regulation of mitochondrial outer membrane permeability by Bcl-2 family proteins ultimately leads to the activation of the caspase cascade (Zheng et al., 2016). The antiapoptotic proteins such as Bcl-2, proapoptotic proteins such as Bax, and a class of cysteine proteases known as caspases are all part of the Bcl-2 family (Shi et al., 2018). However, The increase in the levels of Bcl-2 is often used to evaluate the induction of apoptosis, where the phosphorylation of Bcl-2 inhibits the anti-cell death function (Adil et al., 2016; Shi et al., 2018). A suppression in the phosphorylation of Bcl-2 has been documented in hippocampal slice cultures of mice receiving BBR (Cui et al., 2009). In addition, an increased Bcl-2 and a decreased apoptotic proteins such as cytochrome C, Bax and caspases have also been reported (Hsu et al., 2012). Also seen in a reported improvement in short term memory impairments (M. I. A. Kim et al., 2014). As well as noted benefits in Alzheimer's disease related (Liang et al., 2017). In short, the upregulation of antiapoptotic proteins

such as Bcl-2 in addition to the downregulation of proapoptotic factors such as caspase and cytochrome C by BBR gives evidence of its potential anti-apoptotic activity.

2.2.3.3.2.2. *PI3K/AKT/GSK3 β* signaling pathway

The Glycogen synthase kinase -3 (GSK-3) plays an important role in apoptosis, cell survival, cell growth, stress response and proliferation, since Bcl-2 and the NF- κ B signaling are among its the main targets, while its activity is inhibited by PI3K/Akt signaling (Maurer et al., 2014). The reversed activation of GSK-3 β have been reported to be effective in improving Alzheimer's disease, which was noted following BBR administration (G. Yu et al., 2011). Additionally, following the inhibition of the phosphorylation of the amyloid precursor protein via the PI3K/AKT/GSK3 β signaling pathway.

2.2.3.3.2.3. *AMPK* pathway

The AMP- activated protein kinase known as AMPK functions as a sensor of cellular energy and has been reported to be excessively active in various neurodegenerative diseases (Krishan et al., 2015). Moreover, the inhibition of AMPK has been associated with neuroprotective effect in stroke (J. Li et al., 2007; McCullough et al., 2005). BBR has been documented to be able to regulate AMPK by inhibiting its activity depending on the cellular energy status where both the activation on AMPK has been noted to reduce inflammation as well as inhibiting it to prevent apoptosis (W. Chen et al., 2016; Lu et al., 2015).

2.2.4. Toxicology

BBR is considers as a low toxicity agent with minimal side effects (Pang et al., 2015). The route of administration can significantly affect its acute toxicity, where in mice IV and i.p. injection of BBR have LD50 values of 9.0 and 57.6 g/kg respectively, while no notable LD50 was observed with intragastric BBR (Kheir et al., 2010). Clinical studies evaluating the safety of BBR in humans have reported only mild gastrointestinal reactions such as diarrhea and constipation (Pang et al., 2015). However, transient elevation in serum bilirubin level has also been noted (Linn et al., 2012). Nevertheless, additional investigations have yielded diverse observations concerning the effects of BBR. These include its impact on the distribution of sex hormone synthesis (Thinnakorn Lao-Ong, 2013). Furthermore, studies have reported the suppressive influence of BBR on both cellular and humoral immune functions

(Mahmoudi et al., 2016). Additionally, the prothrombotic effects associated with BBR has been documented (Holy et al., 2009). Additionally, animal studies have reported the following effects, depending on the type of animal, the doze given, and for how long BBR given: a reduction in white blood cells, liver and kidney enlargement, gastric ulcers and bleeding, diarrhea ,vomiting, nausea, mild muscle tremor (Kamrani Rad et al., 2017; N. Singh & Sharma, 2018).

2.3. Nephrotoxicity Models Created with VCM.

VCM has been associated with the increased generation of ROS and lipid peroxidation of the membrane and resulting in the loss of the membrane's structural and functional integrity. (Ahmida, 2012). In addition to its effect on increasing the oxidative stress in tissues (Habib & Ali, 2011). Biomarkers related to the lipid peroxidation of the membrane such as MDA have been documented to be increased resembling nephrotoxicity models induced by VCM administered at 200-400 mg/kg/day (i.p). for 7 days, as well as decreased levels of SOD as one of the main intrinsic anti-oxidant enzyme within kidney tissues (Basarslan et al., 2012; Cetin et al., 2007; Nishino et al., 2003b; Ocak et al., 2007; Öktem et al., 2005). Furthermore, recent studies have used VCM to create a model in order to induce nephrotoxicity on rats, where its results have showed significant histological changes on the groups that have been administrated VCM compared to other groups, in addition apoptosis, inflammation and oxidation biomarkers have been tested to assess the toxicity of VCM at 400 mg/kg for 7 days (J. He et al., 2021; Uckun et al., 2020). In addition, a study that has been done in vitro investigating the potential effect of BBR to reduce VCM-induced nephrotoxicity has revealed that BBR has reduced the mRNA expression of antioxidant and proliferative markers such as CAS-9 and Cytochrome-c (Malkani et al., 2022). While also BBR as an antioxidant and an anti-inflammatory agent has also been investigated against nephrotoxicity induced by cyclophosphamide in mice (Mombeini et al., 2022).

Moreover, biomarkers such as BUN and Cr have been reported to be significantly increased following the administration of VCM in some studies (J. He et al., 2021; Uckun et al., 2020). While the administration of VCM have showed no significant difference of BUN and Cr compared to the control group in another study (Öktem et al., 2005) hence the usage of a newer biomarkers have emerged where biomarkers such as KIM-1 have been used in recent studies to assess the kidneys tissue

damage and function precisely, therefore compare it to other groups, KIM-1 have detected the kidney injury more accurately and showed higher sensitivity toward the detection of kidney injury (Mehanna et al., 2022; Mombeini et al., 2022; Uckun et al., 2020).



3. MATERIALS and METHODS

3.1. Materials

3.1.1. Animals

The experiments presented in this study were conducted in accordance with the guidelines and regulations set by the Animal Experimentation Ethics Committee of Eskisehir Osmangazi University. Approval was obtained under File Registration No. 2021/859 before the commencement of any experimental procedures. Male adult Wistar Albino rats, weighing within the range of 180-220 g, were selected as the subjects for the experiments. To ensure proper adaptation to the experimental conditions, the rats were housed in a well-ventilated room maintained at a temperature of $22\pm1^{\circ}\text{C}$, with a light-dark cycle of 12 hours, for a period of one week. Throughout both the adaptation period and the entire duration of the experiment, the animals were provided with standard animal food and access to tap water.

3.1.2. Devices used

Automatic pipette	Eppendorf, Germany.
Centrifuge	Neofuge 13R-Refrigerated Centrifuge-Heal Force Bio.
Elisa reader	BiotekPowerWave XS, USA.
Homogenizer	Heidolp, Germany.
Incubator	Lab. Companion S1-600, USA.
Magnetic Stirrer	DAIHAN, South Korea
Nanodrop	Thermo Scientific™, NanoDrop™ 2000/2000c, Spectrophotometers.
Pipette set	Brand, Germany.
Precision Scales	Ohaus, USA.
Real Time PCR Master Mix	Thermo Scientific™ Maxima SYBR Green/ROX qPCR Master Mix (2X).
Real Time PCR	CFX96 TOUCH BIORAD Real-Time PCR System.

Refrigerated centrifuge	Eppendorf (5810R), Germany.
Vortex Heidolp	REAX, Germany.
3.1.3. Chemicals and kits	
Berberine	Fluoro Chem, England.
cDNA Kit	High-Capacity cDNA Reverse Transcription Kit.
Ketamine (Ketalar®)	Pfizer, Turkey.
Nuclease Free, Ultra Distilled Water	Biochrom AG Ultra Pure Water.
PBS	Gibco™ PBS, pH 7.2.
Primer	50 nmol OPC Grade.
Rat MDA ELISA Kit	Bioassay Technology LAB, China.
Rat SOD ELISA Kit	Bioassay Technology LAB, China.
Vancomycin	VCM Lyomark, Germany.
Water	Thermo Scientific™ Water, Nuclease-free, Molecular Biology Grade, Ultrapure.
Xylazine (Rompun®)	Bayer, Turkey.

3.2. Method

3.2.1. Nephrotoxicity model

VCM has been injected intraperitoneally (ip) into animals at the dose of 400 mg/kg for 7 consecutive days, which is known to cause nephrotoxicity in rats (J. He et al., 2021; Konishi et al., 2013; Uckun et al., 2020).

3.2.2. Test protocol

The planned dose of BBR is 50mg/kg has been chosen as it has been used to investigate the potential protective effect of BBR against gentamicin-induced renal injury in male wistar rats (Allameh et al., 2020). The dose of 100mg/kg has been chosen as it has been used to investigate the potential protective effect of BBR against cisplatin-induced renal injury in male wistar rats (Adil et al., 2016). The dose of

200mg/kg has been chosen to overcome the reported low bioavailability of BBR. All the doses have been dissolved in normal saline (SF) and have been prepared to be given by oral gavage (p.o.). However, the elimination half-life of BBR is 4.8 ± 1.4 h according to (Gupta et al., 2010). Therefore, BBR has been planned to be administered one day before the administration of VCM.

In this investigation, 35 adults male wistar albino rats were used and divided into five groups with seven rats in each group. The control group received oral SF for eight days, which is the solvent of BBR, and SF was given intraperitoneally (i.p.) for seven days, which is the solvent of VCM. The remaining groups received VCM dissolved in SF intraperitoneally for seven days. BBR was dissolved in SF and administered orally by gavage (p.o.) for eight days, starting one day before the VCM administration. The allocation of rats to each group was randomized.

Group 1; Rats have been administered oral SF for eight days. In addition, SF has been administered intraperitoneally starting one day after the first oral SF administration and continued for seven days (n: 7) labeled as CONTROL.

Group 2; Rats have been administered oral SF for eight days, 400 mg/kg VCM dissolved in saline have been administered intraperitoneally starting one day after the first administration of SF and continued for seven days (n: 7) labeled as VCM.

Group 3; Rats have been administered 50 mg/kg BBR dissolved in SF for eight days, 400 mg/kg VCM dissolved in saline have been administered intraperitoneally starting one day after the first administration of SF and continued for seven days (n: 7) labeled as BBR 50.

Group 4; Rats have been administered 100 mg/kg oral BBR, dissolved in SF for eight days, 400 mg/kg VCM dissolved in saline has been administered intraperitoneally starting one day after the first SF administration and continued for seven days (n: 7) labeled as BBR100.

Group 5; Rats have been administered 200 mg/kg oral BBR dissolved in SF for eight days, 400 mg/kg VCM dissolved in saline have been administered intraperitoneally starting one day after the first SF administration and continued for seven days (n: 7) labeled as BBR200.

Table 3.1. The groups of animals used and experimental protocol.

Days	Group				
	Control	VCM	BBR50	BBR100	BBR200
1	SF (p.o.)	SF (p.o.)	50 mg/kg BBR (p.o.)	100 mg/kg BBR (p.o.)	200 mg/kg BBR (p.o.)
2-8	SF (p.o.) + SF (i.p.)	SF (p.o.) + VCM (i.p.)	50 mg/kg BBR (p.o.) + VCM (i.p.)	100 mg/kg BBR (p.o.) + VCM (i.p.)	200 mg/kg BBR (p.o.) + VCM (i.p.)

Intracardiac blood samples (2 cc) have been taken 24 hours after the last administered dose and both kidneys have been removed under Ketamine/Xylazine anesthesia in animals. Post-euthanasia, the right kidney has been used for histological examination under the light microscope in addition to the evaluation of apoptosis, while the left kidney has been used to prepare the homogenate for biochemical tests.

Renal function biomarkers including BUN and Cr in the serum have been evaluated. In addition, in the kidney homogenates the renal function biomarker KIM-1, oxidative stress markers such as MDA and SOD, as well as cytokines levels IL-6 and TNF- α , apoptosis marker levels Cas-9, Bcl-2 have been evaluated. Additionally, tissue apoptosis has been assessed using histological examination.

3.2.3. Sample collection

The protocols for sample collection 24 hours after the last drug dose after 8 days of administration to the groups are as follows:

1. At the end of the 8 days experiment period, rats belonging to the experimental groups were euthanized by cervical dislocation after anesthetization with 60 mg/kg-ketamine and 6 mg/kg-xylazine intraperitoneally.

2. Blood samples (2 cc) have been taken directly as intracardiac blood, following the samples collection, they have been centrifuged at 3000 rpm for 20 minutes to obtain the serum and they have been stored at -20 °C to be used for biochemical analysis.

3. Immediately afterwards, both kidneys were surgically isolated, weighed, decapsulated and the left kidney was divided equally into two longitudinal segments.

4. The right kidney has been placed in 10% formalin solution for histopathological examination.

5. The left kidney has been washed with saline and stored at -80 °C until analysis, where it has been homogenized in cold phosphate buffer (0.05 M, pH 7.4).

6. Kidney homogenates have been centrifuged at 5000 rpm for 5 minutes. The resulting supernatant has been used for biochemical analysis of the selected biochemical parameters.

3.2.4. Biochemical analysis

3.2.4.1. Serum markers of kidney function:

Serum creatinine level (Cr) and blood urea nitrogen (BUN) were measured using diagnostic kits. Cr and BUN levels were evaluated using an automated biochemical analyzer with specific test kits (bioassay technology laboratory, China) according to the manufacturer's instructions.

3.2.4.2. Real time PCR

The mRNA levels were assessed using a reverse transcription-polymerase chain reaction (RT-PCR) method, following the previously described protocol (Kandhare et al., 2014). To generate single-stranded cDNA, the reverse transcriptase High-Capacity cDNA Reverse Transcription Kit was utilized in accordance with the provided instructions. β -actin amplification was utilized as a control to ensure proper sample loading and integrity. The primer sequences for KIM-1, Caspase-9, IL-6, TNF- α , Bcl-2, and β -actin were selected based on a previously reported method (Visnagri et al., 2015b). The RT-PCR was conducted using (CFX96 TOUCH BIORAD Real-Time PCR System). For each PCR, 2 μ L of cDNA, 10 μ L of universal PCR master mix, 2 μ L of primers, and 6 μ L of DNA-free water were mixed, for a final volume of 20 μ L. The PCR amplification consisted of 40 cycles where temperature of 60°C for 30 seconds, 72°C for 30 seconds, 95°C for 15 seconds cycles were utilized. The relative RNA copy numbers were determined using standard curves generated by serial dilution of quantified template cDNA. The relative gene expressions were calculated using the $2^{-\Delta\Delta C_t}$ method, as explained previously (Pfaffl, 2007). The fold change was calculated as $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ represents (Ct Sample - Ct β -actin) - (Ct Control - Ct β -actin). The expression of each target gene was normalized with respect to the expression of the housekeeping gene β -actin. The primers used are shown in Table 3.2.

Table 3.2. The primers used in the RT-PCR test.

PRIMARY NAME	PRIMARY SERIES	BASE PAIR (Bp)
B AKTIN FORWARD PRIMER	AGCAAGAGAGGCATCCTCACC	21
B ACTIN REVERSE PRIMER	ACAGGGATAGCACAGCCTGGA	21
BCL2 FORWARD PRIMER	GGTGAAGTGGGGGAGGATTG	20
BCL2 REVERSE PRIMER	AGAGCGATGTTGTCCACCAG	20
CAS9 FORWARD PRIMER	AATTCTGAGCCCGGAGTTGG	20
CAS9 REVERSE PRIMER	CTGCACTTTGGGAGGCTAAG	20
IL6 FORWARD PRIMER	TCTGGTCTTCTGGAGTTCCGT	21
IL6 REVERSE PRIMER	GGAAGTTGGGGTAGGAAGGAC	21
TNF ALFA FORWARD PRIMER	CATCCGTTCTCTACCCAGCC	20
TNF ALFA REVERSE PRIMER	AATTCTGAGCCCGGAGTTGG	20
KIM-1 FORWARD PRIMER	CTGCCAGATGTGCCAAAAACA	21
KIM-1 REVERSE PRIMER	TCTCTTAGTGCCAAAGCGTCT	21

3.2.4.3. The estimation of the antioxidant effect of BBR

The assessment of the anti-oxidation effect in kidney tissue homogenates was conducted using commercially available ELISA kits for superoxide dismutase (SOD) and malondialdehyde (MDA) as a marker of lipid peroxidation. Specifically, the Rat SOD ELISA Kit and Rat MDA ELISA Kit from Bioassay Technology LAB in China were employed. The evaluation was carried out following the test procedure provided by the manufacturer of the kits.

3.2.4.4. Histological evaluation

The right kidneys of animals taken from rats under anesthesia were fixed in 10% formaldehyde solution for 24 hours. After that, it was turned into paraffin blocks by going through routine tissue follow-up processes (dehydration, transparency, paraffin impregnation and paraffin embedding). 5µm thick sections were taken from paraffin blocks and routine hematoxylin-eosin (HE) staining (deparaffinization, hydration, HE dyes steps and dehydration) was performed and the slides were closed with entellam. The kidney tissues, which were turned into histological preparations,

were examined under a microscope with a digital camera, and representative pictures of the groups were taken. Histopathological grading was conducted by our histopathologist using a semiquantitative scale. The assessment scale comprised four distinct categories to evaluate the extent of histopathological damage. The first category, represented by (-), indicated the absence of significant damage. The second category, denoted as (+), indicated a mild degree of damage. The third category, marked as (++), indicated a moderate degree of damage. Lastly, the fourth category, represented by (+++), indicated a severe degree of damage. This scale allowed for the classification and grading of histopathological damage based on the observed severity. The grading process involved assessing the extent of inflammation, tubular dilation, tubular necrosis, and tubular glomerular damage/decreased Bowman's space.

3.2.4.5. Statistical analysis

The statistical analysis was performed using the SPSS 25.0 software package. For variables that did not exhibit a normal distribution, the Kruskal-Wallis one-way analysis of variance on rank test was employed. The significance level was set at $P < 0.05$ to determine statistical significance. This approach allowed for the evaluation of data and the identification of any significant differences among the variables being analyzed.

4. RESULTS

4.1. Biochemical Findings

Table 4.1. Biochemical Results, Kruskal-Wallis One-way Analysis has been applied.

+: (p<0.05) compared to the VCM group, *: (p<0.05) compared to the control group.

		N	Mean±SD	Median (%25-%75)	P value
BUN	Control	7	21.57 ± 3.32	18 (17-21)	Control- BBR200 P = 0.0025 VCM-BBR200 P = 0.0420
	VCM	7	25 ± 1.41	24 (23.5-27.5)	
	BBR50	7	36.14 ± 4.2	33 (31.5-42.0)	
	BBR100	5	42 ± 11.3	32 (30-34)	
	BBR200	7	49.86 ± 6.03	57 (37-59) ^{+, *}	
Cr	Control	7	0.32 ± 0.04	0.29 (0.26-0.30)	P = 0,164 NS (P>0.05).
	VCM	7	0.35 ± 0.03	0.35 (0.29-0.41)	
	BBR50	7	0.39 ± 0.05	0.38 (0.30-0.42)	
	BBR100	5	0.63 ± 0.33	0.31 (0.30-0.32)	
	BBR200	7	0.67 ± 0.15	0.62 (0.37-0.90)	
KIM-1	Control	3	1.12± 0.64	0.95 (0.76-1.39)	Control-VCM P = 0.041
	VCM	5	7.85 ± 2.35	7.15 (6.69-8.03) [*]	
	BBR50	3	3.36 ± 2.37	2.36 (2.01-4.22)	
	BBR100	2	1.36 ± 0.57	1.36 (0.95-1.76)	
	BBR200	3	2.58 ± 1.66	3.12 (1.92-3.51)	
SOD	Control	6	2.41±0.15	2.38 (2.27-2.49)	VCM-BBR100 P = 0.029
	VCM	6	2.05 ± 0.11	2.05 (1.94-2.13)	
	BBR50	6	2.23 ± 0.43	2.31 (1.88-2.52)	
	BBR100	5	2.54 ± 0.24	2.57 (2.44-2.66) ⁺	
	BBR200	6	2.32 ± 0.24	2.28 (2.14-2.50)	
MDA	Control	5	3.28 ± 0.28	3.31 (3.05-3.35)	VCM-BBR100 P = 0.048
	VCM	5	3.73 ± 0.30	3.71 (3.59-3.84)	
	BBR50	4	3.26 ± 0.40	3.28 (2.98-3.53)	
	BBR100	2	2.51 ± 0.58	2.51 (2.10-2.93) ⁺	
	BBR200	2	3.08 ± 0.17	3.08 (2.96-3.20)	
IL-6	Control	7	1.33 ± 0.92	1.36 (0.69-1.80)	P = 0.435 NS (P>0.05).
	VCM	5	5.16 ± 5.23	4.35 (1.18-5.39)	
	BBR50	7	2.93 ± 3.89	1.23 (0.98-2.92)	
	BBR100	5	1.28 ± 0.73	1.08 (0.98-1.84)	
	BBR200	7	3.03 ± 2.48	3.52 (0.61-5.02)	

Table 4.1. (continuation) Biochemical Results. Kruskal-Wallis One-way Analysis has been applied. +: (p<0.05) compared to the VCM group, *: (p<0.05) compared to the control group.

		N	Mean $\pm$ SD	Median (%25-%75)	P value
TNF-α	Control	7	1.05 $\pm$ 0.29	1.17 (0.91-1.23)	VCM-BBR50 P = 0.002
	VCM	7	2.44 $\pm$ 1.47	2.21 (1.31-3.38)	
	BBR50	7	0.58 $\pm$ 0.27	0.59 (0.51-0.69) ⁺	
	BBR100	5	1.19 $\pm$ 0.38	1.17 (0.91-1.53)	
	BBR200	7	0.86 $\pm$ 0.23	0.88 (0.81-1.01)	
CAS-9	Control	6	0.89 $\pm$ 0.29	0.90 (0.66-1.15)	P = 0,173 NS (P>0.05).
	VCM	7	1.70 $\pm$ 0.94	1.58 (1.10-2.25)	
	BBR50	6	0.71 $\pm$ 0.34	0.70 (0.40-0.97)	
	BBR100	5	0.85 $\pm$ 0.58	0.62 (0.50-0.90)	
	BBR200	7	0.88 $\pm$ 0.76	0.51 (0.42-1.28)	
BCL-2	Control	5	1.79 $\pm$ 1.39	1.40 (0.90-2.05)	P = 0,697 NS (P>0.05).
	VCM	7	0.86 $\pm$ 0.48	0.75 (0.61-1.05)	
	BBR50	7	1.34 $\pm$ 1.10	1.17 (0.49-1.95)	
	BBR100	5	1.69 $\pm$ 1.76	1.08 (0.54-2-28)	
	BBR200	7	2.13 $\pm$ 1.66	3.19 (0.46-3.30)	

4.1.1. Renal function biomarkers

4.1.1.1. Serum creatin

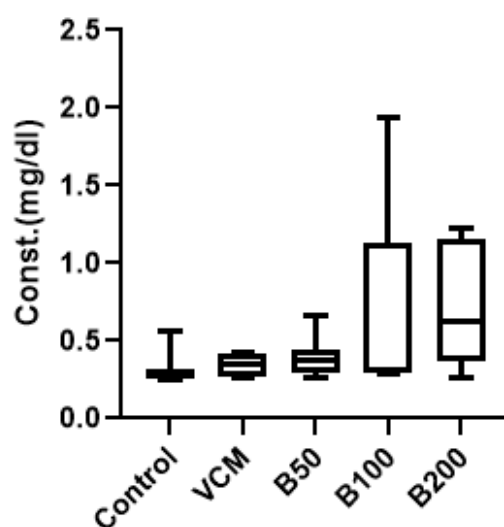


Figure 4.1. Serum Creatin.

The analysis of serum creatinine concentration revealed a marginal increase in the animals belonging to the B200 group compared to the other animal groups. Nevertheless, no statistically significant differences were observed between the groups ($p > 0.05$).

4.1.1.2. Serum BUN

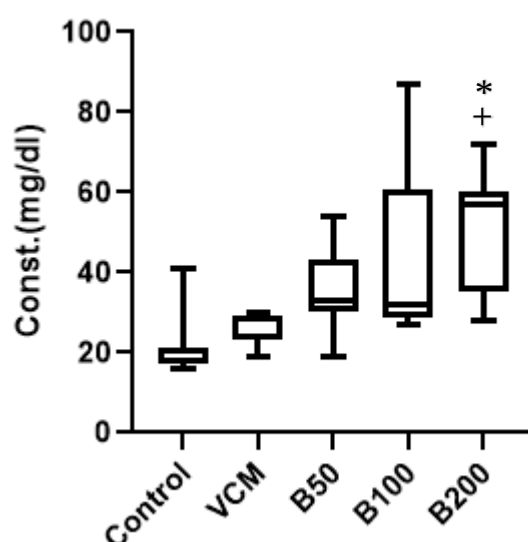


Figure 4.2. Serum BUN. *: ($p < 0.05$) compared to the control group, +: ($p < 0.05$) compared to the VCM group.

The assessment of blood urea nitrogen (BUN) levels revealed a significant increase in the animals belonging to the BBR200 group when compared to both the VCM group (+, $p<0.05$) and the control group (*, $P<0.05$). In contrast, the other experimental groups did not exhibit statistically significant differences in comparison to the respective reference groups.

4.1.1.3. KIM-1

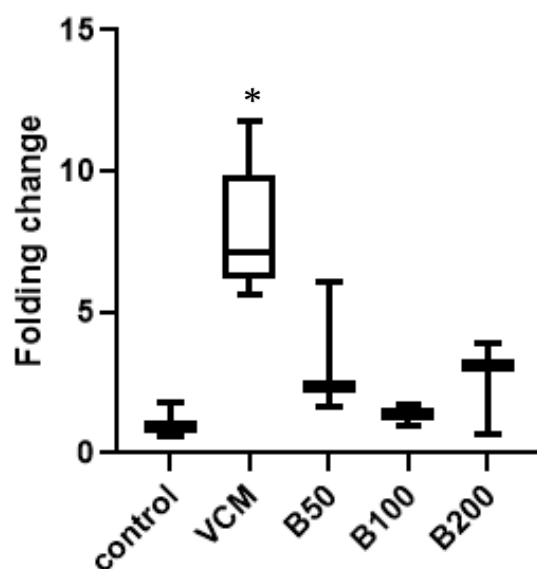


Figure 4.3. The folding change of KIM-1. *: ($p<0.05$) compared to the control.

The evaluation of folding change in KIM-1 levels revealed a significant increase in the animals belonging to the VCM group when compared to the control group (*, $P<0.05$). Notably, the BBR groups exhibited a decreasing trend in the folding change levels of KIM-1, approaching levels similar to the control group. However, these changes did not reach statistical significance in comparison to the VCM group.

4.1.2. Oxidative stress markers

4.1.2.1. MDA

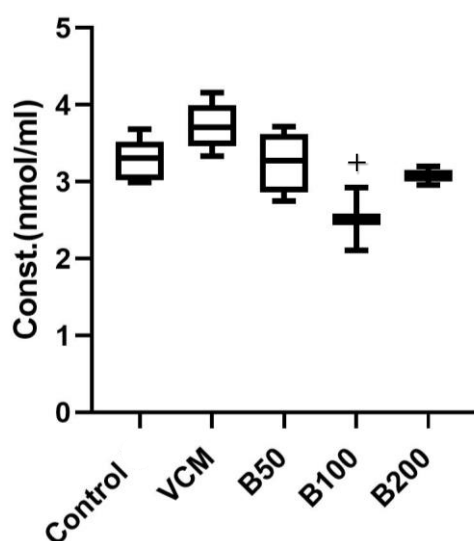


Figure 4.4. MDA concentration in the Tissue. +: ($p < 0.05$) compared to the VCM.

The quantification of MDA levels in kidney homogenates was performed using the ELISA method. Interestingly, the animals in the VCM group displayed a slight elevation in MDA levels when compared to the control group, although this difference did not reach statistical significance ($p > 0.05$). However, the B100 group demonstrated a significant reduction in MDA levels ($p < 0.05$) in comparison to the VCM group.

4.1.2.2. SOD

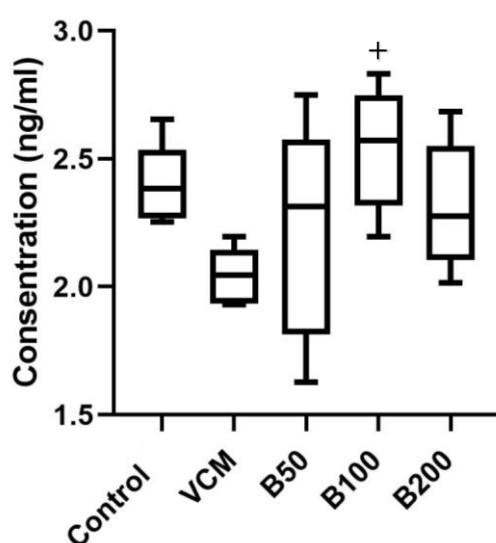


Figure 4.5. SOD concentration in the Tissue. +: ($p < 0.05$) compared to the VCM.

The assessment of SOD levels in kidney tissues was conducted using the ELISA method on kidney homogenates. Notably, the animals in the VCM group exhibited a noticeable decrease in SOD levels when compared to the control group, although no statistically significant difference was observed ($p>0.05$). However, the BBR100 group displayed a significant alteration in SOD levels ($p<0.05$) in comparison to the VCM group. In contrast, no significant differences were observed in SOD levels between the other experimental groups and the VCM group.

4.1.3. Inflammation markers

4.1.3.1. IL-6

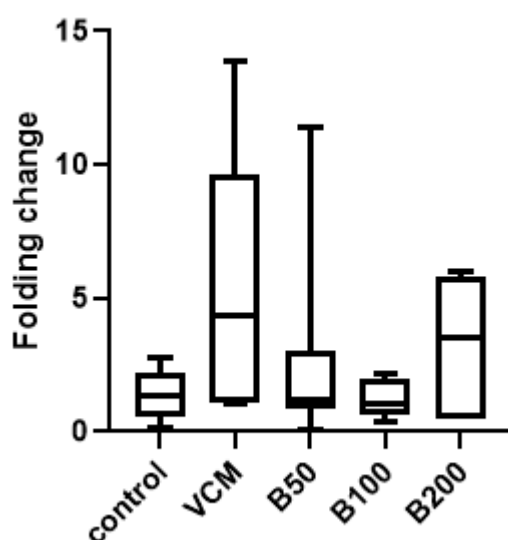


Figure 4.6. The folding change of IL-6.

The IL-6 folding change exhibited a marginal increase in the VCM group when compared to the control group, while the BBR100 group displayed a slight decrease in IL-6 folding change relative to the VCM group. Animals in the BBR200 group demonstrated a minor elevation in the folding change of IL-6. However, no statistically significant differences were observed in the IL-6 folding change levels among any of the groups ($p>0.05$).

4.1.3.2. *TNF- α*

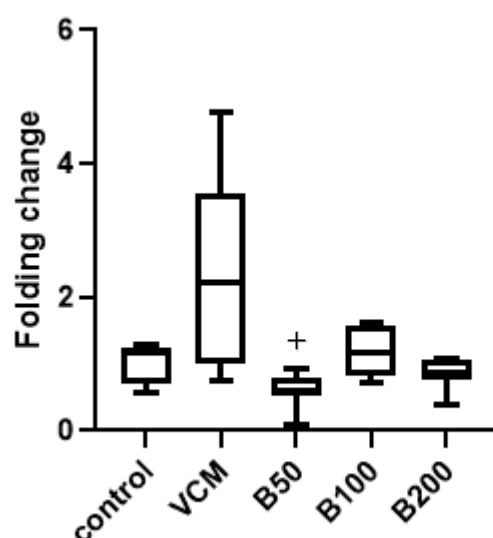


Figure 4.7. The folding change of *TNF- α* . +: ($p < 0.05$) compared to the VCM group.

The analysis of *TNF- α* folding change in kidney homogenates revealed a modest elevation in the VCM group compared to the control group. Notably, the BBR50 group exhibited a significant reduction in *TNF- α* folding change ($p < 0.05$) when compared to the VCM group.

4.1.4. Apoptosis markers

4.1.4.1. *Caspase-9*

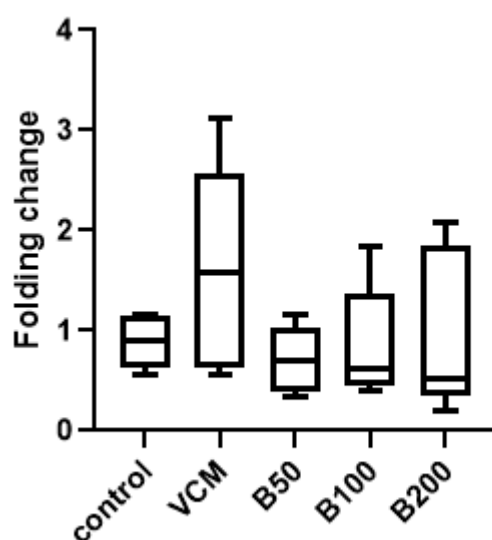


Figure 4.8. The folding change of *Caspase-9*.

While observing a marginal increase in the CAS-9 folding change level in the VCM group compared to the control group, and a slight decrease in the BBR-administered groups compared to the VCM group, no statistically significant difference in CAS-9 folding change was detected among any of the experimental groups ($p>0.05$). Moreover, the results indicated minimal variation between the different doses of BBR utilized in the study.

4.1.4.2. BCL-2

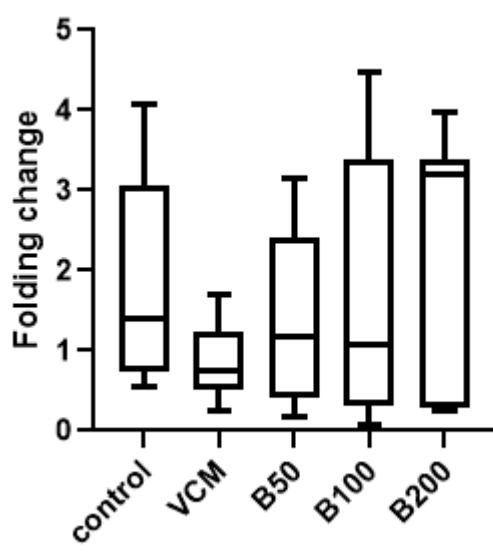


Figure 4.9. The folding change of BCL-2.

Despite observing a modest reduction in the level of BCL-2 folding change in the VCM group compared to the control group, and a slightly higher level in the BBR200 group relative to the VCM group, no statistically significant difference in BCL-2 folding change was detected among any of the experimental groups ($p>0.05$).

4.2. Histological Results

Histological results as Light microscopic images of rat's kidneys in all experimental groups at different magnifications (HE, scale bar: 500 μ m X4, 200 μ m X10, 100 μ m X20, 50.0 μ m X40).

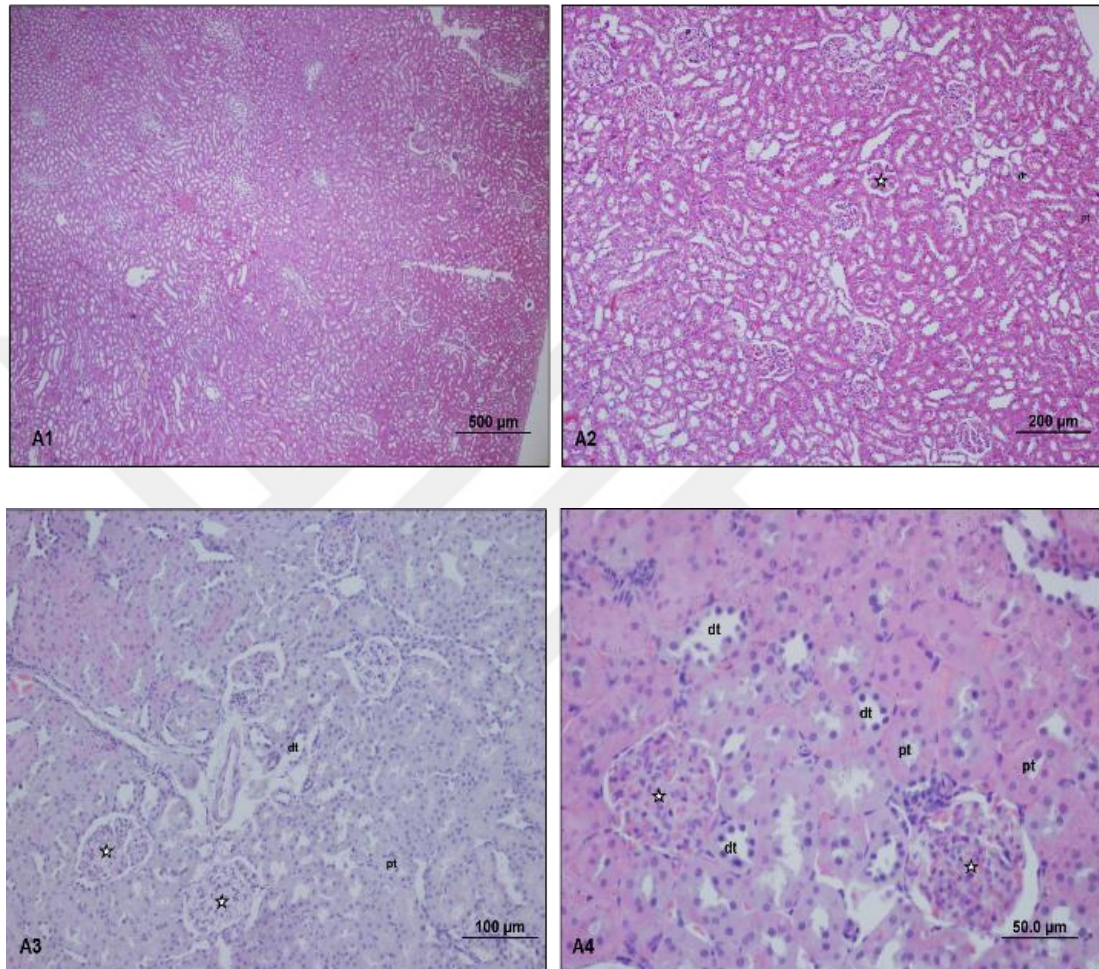


Figure 4.10. Histological results of the control group. (HE, scale bar: 500 μ m X4, 200 μ m X10, 100 μ m X20, 50.0 μ m X40).

A1-A4: Control Group, the kidneys of the control group are observed in normal histological structure with Malpighi bodies (☆), distal tubule (dt) and proximal tubule (pt) structures.

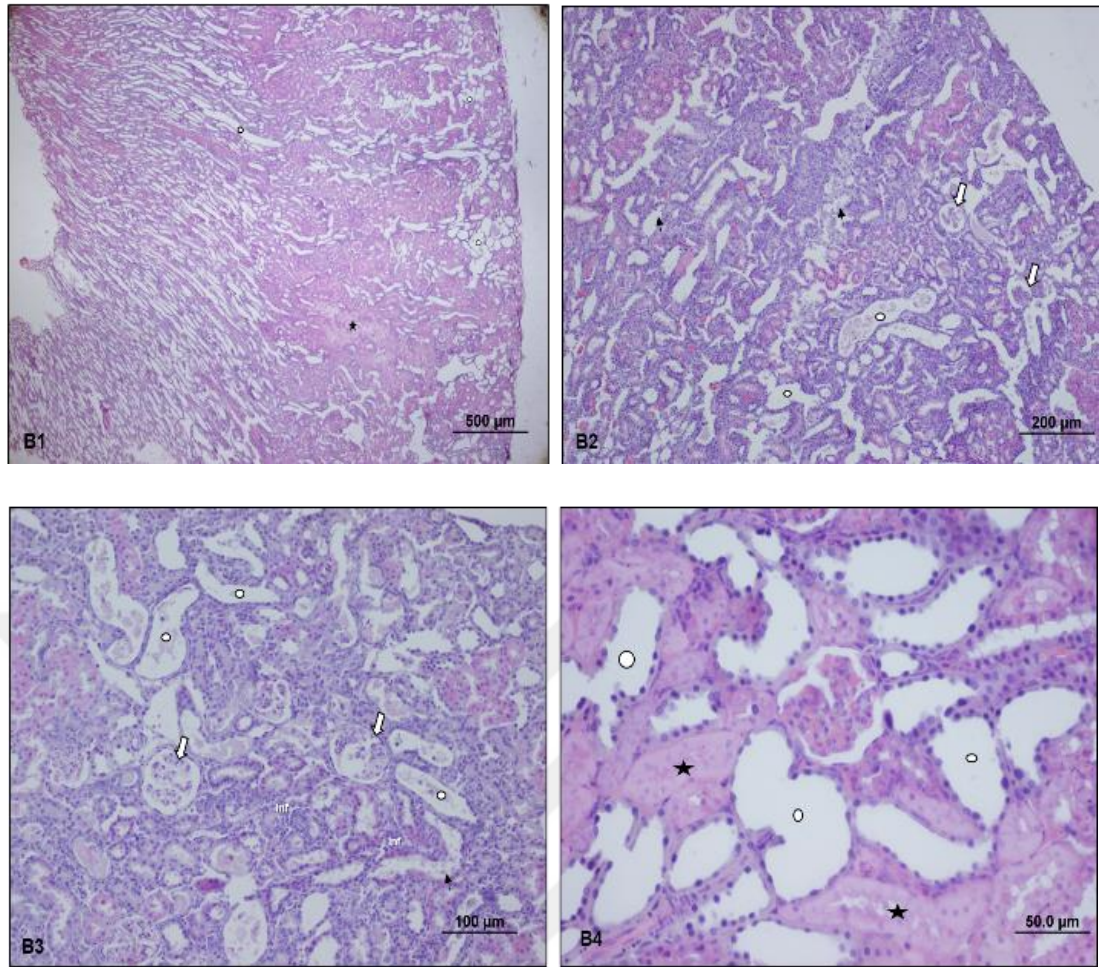


Figure 4.11. Histological results of VCM group. (HE, scale bar: 500µm X4, 200µm X10, 100µm X20, 50.0µm X40).

B1-B4: VCM Group, tubular necrosis areas in the renal cortex of rats belonging to the VCM group (★), dilatation in the distal tubules (o), tubular epithelial spills (→), glomerular damage (⇒), and cellular infiltration (inf) in the interstitial areas being seen.

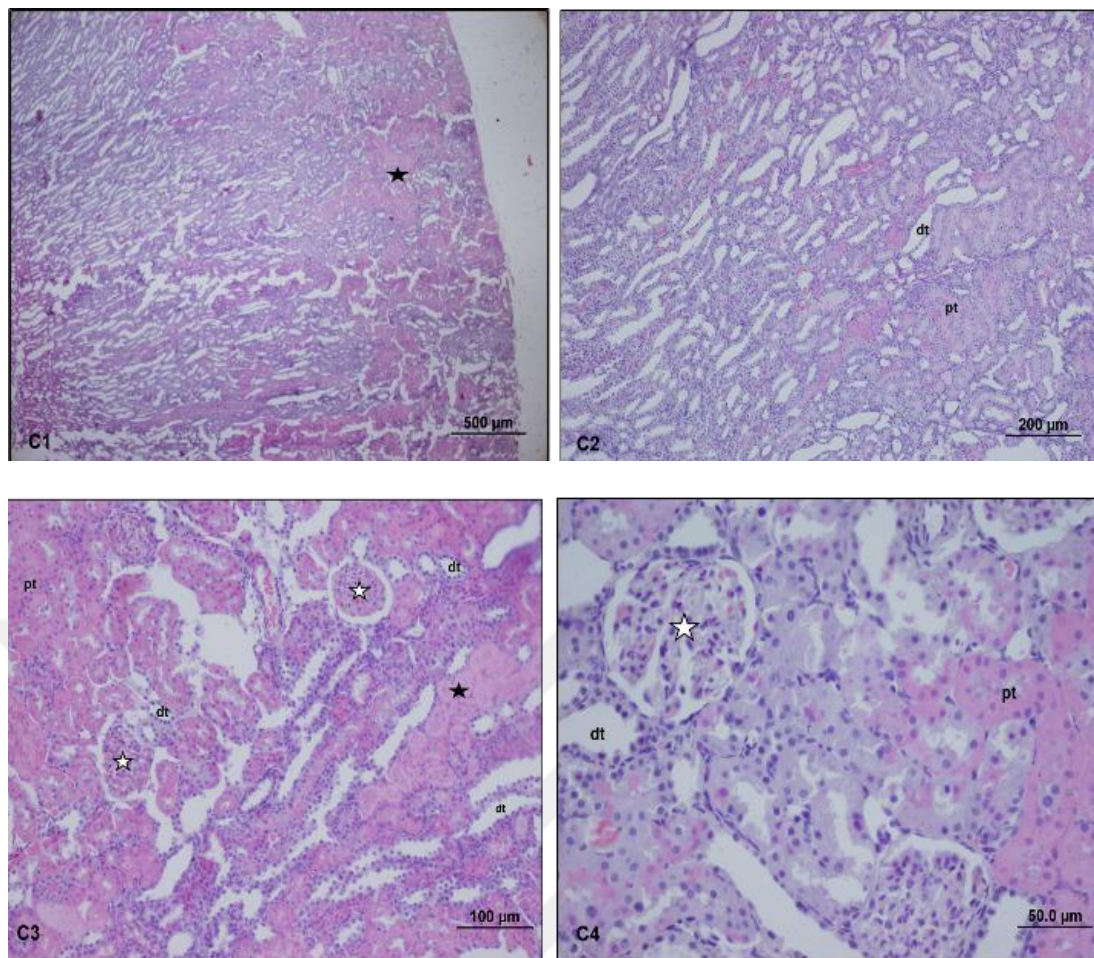


Figure 4.12. Histological results of BRB 50 group. (HE, scale bar: 500μm X4, 200μm X10, 100μm X20, 50.0μm X40).

C1-C4: BBR-50mg/kg group, although partial tubular necrosis areas (★) are observed in the renal cortex of BBR-50mg/kg group rats, near-normal distal tubule (dt) and proximal tubule (pt) structures and near-normal Malpighi bodies (☆) compared to the VCM group, notable reduced damage draws attention.

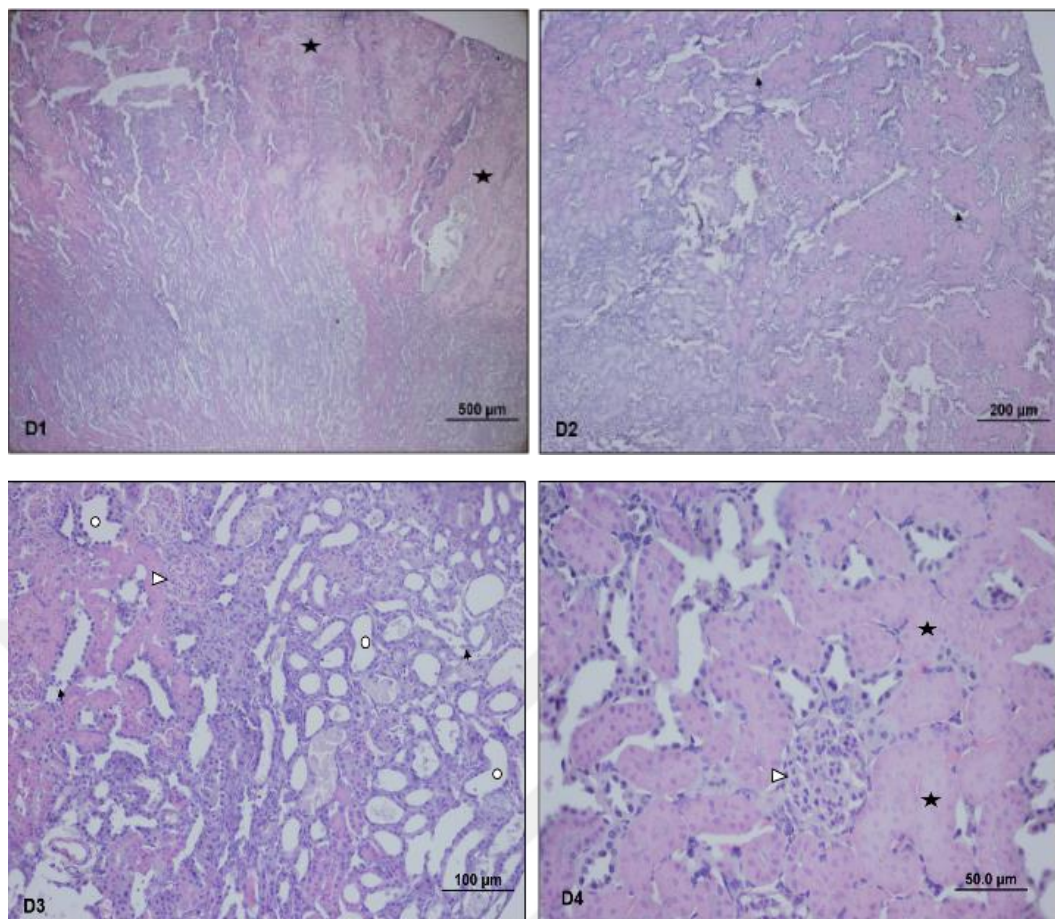


Figure 4.13. Histological results of BBR 100 group. (HE, scale bar: 500μm X4, 200μm X10, 100μm X20, 50.0μm X40).

D1-D4: BBR-100 mg/kg group, BBR-100mg/kg group rats have necrosis in the renal cortex, especially in the proximal tubules (★), dilatation in the distal tubules (o), tubular epithelial shedding (→), glomerular damage (⇒), and decrease in Bowman's space (▷) and similar damages are seen compared to the VCM group.

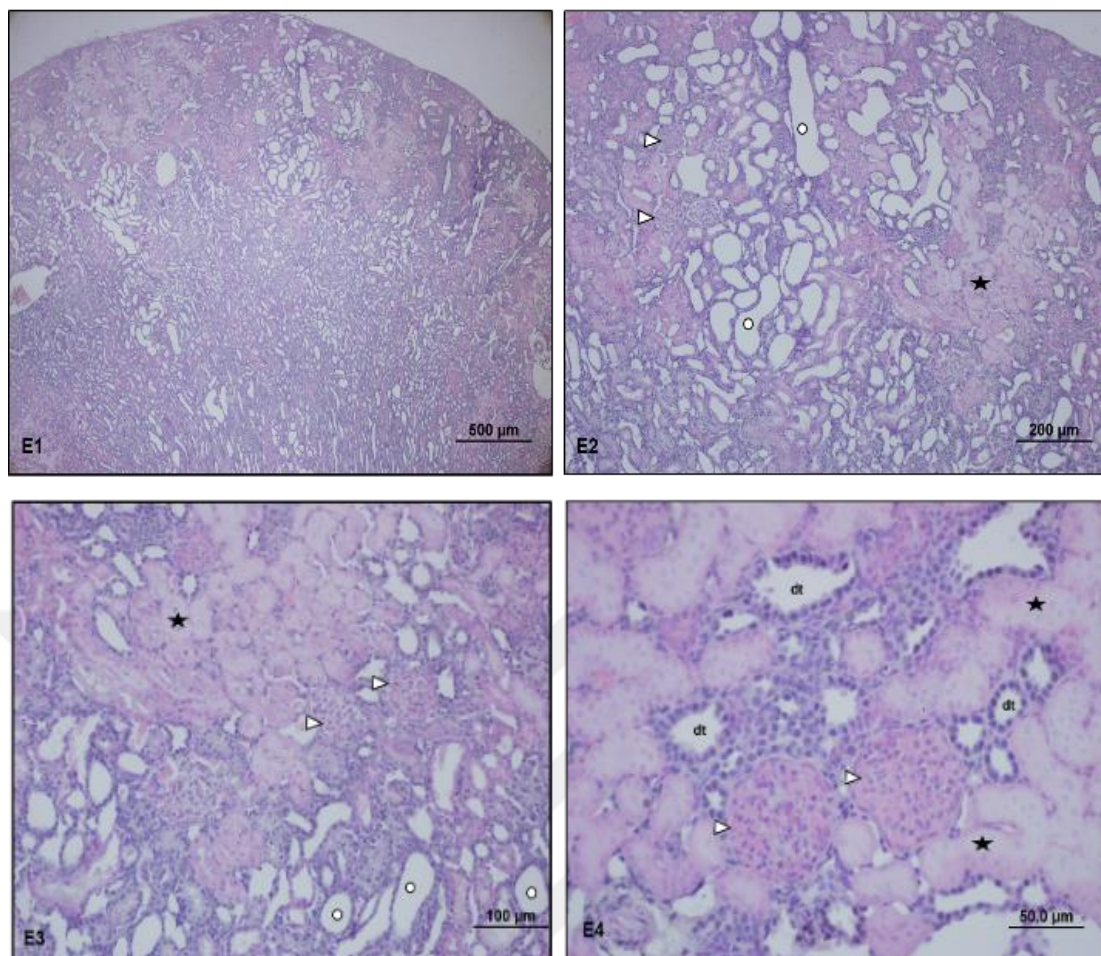


Figure 4.14. Histological results of BBR 200 group. (HE, scale bar: 500μm X4, 200μm X10, 100μm X20, 50.0μm X40).

E1-E4: BBR-200mg/kg group, necrosis in the renal cortex of rats belonging to the BBR-200mg/kg group, especially in the proximal tubules (★), although decreased compared to the VCM group, distal tubules (dt) as well as distal tubule dilatation (o), tubular epithelial shedding (→), glomerular damage (⇔), and a decrease in Bowman's space (▷), similar damage is observed compared to the VCM group.

4.2.1. Histological assessment

Table 4.2. Histological assessment, -: none, +: mild, ++: moderate, +++: sever.

Group	Control	VCM	BBR50	BBR100	BBR200
Inflammation	-	+++	-	-	-
Tubular necrosis (proximal tubule was predominantly observed)	-	+++	+	++	++
Tubular dilatation (distal tubule was predominantly observed)	-	+++	+	++	+
Glomerular damage/decreased Bowman's space	-	+++	-	+++	+++

Table 4.2. shows a comparison between each of the groups in terms of inflammation, tubular necrosis, tubular dilatation, and glomerular damage/decreased Bowman's space. Where the animals belonging to the VCM group have shown incremental signs of kidney damage compared to the control group and animals belonging to BBR50 have shown the least sign of kidney damage out of the 3 different doses of BBR and have shown similar results to the control group. However, the BBR was able to eliminate the inflammation induced by VCM with all of its 3 different doses.

5. DISCUSSION

The current investigation aims to investigate the potential advantageous impact of a supplementary administration of BBR in mitigating the development of VCM-induced nephrotoxicity in male rats. where the evaluation of the oxidative stress, inflammation, histological changes, and apoptosis have been conducted in this investigation.

VCM is used in serious infections and against resistant strains of bacteria. However, studies have associated the administration of VCM with renal toxicity, this has been reported to particularly important with prolonged exposure to the drug and with higher doses aiming to reach its clinically effecting concentration in the blood (Elyasi et al., 2012; Rostas et al., 2014). This could be even followed by non-renal complications especially cardiovascular impairments (Chandrajay, 2010). The upregulation of the apoptotic and inflammatory markers by VCM has been reported to induce cell toxicity (Im et al., 2017). Moreover, the process of removing VCM from the body through the kidneys remains largely unaltered, but there is a propensity for VCM to accumulate in the kidney tissues, which can result in nephrotoxic effects (Im et al., 2017). Furthermore, the augmentation of ROS generation has been linked to renal cell damage caused by VCM (Filippone et al., 2017).

Several studies have demonstrated that the IV administration of VCM has resulted in a marked histopathological injury, including tubular epithelial alteration such as desquamation, dilatation and necrosis, in addition to vacuolization, casts, atrophy and interstitial edema in the tissues of rats' kidneys. The histopathological results of this study are compatible with the previously mentioned studies, which have unveiled that in rat kidney tissues, observable alterations were observed in the tubular epithelial structure along with the presence of interstitial inflammation. that have been administrated VCM. This could be linked with our results that have indicated the increase of the inflammation markers such as TNF- α and IL-6 within the VCM group, where tubular necrosis areas in the renal cortex of rats from the VCM group have been observed, as well as dilatation in the distal tubules, tubular epithelial leaks, glomerular damage, and cellular infiltration in the interstitial areas. In the out investigation the administration 50mg/kg of BBR has showed a noticeable reduction of the damage that has been induced following the administration of VCM compared to the group that have been administered VCM alone. A near-normal distal tubule along with proximal

tubule structures and near-normal Malpighi bodies have been noticed, which goes along with the results provided in this study where the BBR50 group have showed a reduction of oxidative stress markers in addition to the inflammation markers. This might suggest that the administration of BBR at the dose of 50 mg/kg could have the potential to protect against the kidney damage induced by the administration of VCM. Furthermore, the administration of BBR at dose of 100mg/kg and 200mg/kg have showed similar damage in comparison to the VCM group with no noticeable improvement. However, the inflammation has been eradicated from all the groups that have received BBR at all of the three different doses used in this study.

Serum biomarkers such as the levels of BUN and Cr are generally used to evaluate the acute kidney damage, since they can predict the filtration capacity of the glomerulus and could be generally utilized for the detection of renal injury (Salazar, 2014). However, these are reported to be affected and influenced by nonrenal factors unrelated to kidney damage or dysfunction (Sakamoto et al., 2017). Where biomarkers such as KIM-1, have been documented as more selective and sensitive since it produced primarily by the damaged kidney (Sakamoto et al., 2017).

Even though glomerular function markers such as serum Cr was normal in this study within all of the groups, results of this study have showed similarity to a result shown in another study, which have been made investigating VCM induced nephrotoxicity where both Cr and BUN were normal after the administration of VCM (Öktem et al., 2005). However, in this investigation, BUN levels in the animals that have been taken BBR at dose of 200mg/kg were found to be significantly higher in comparison to the group of animals that have been administrated VCM only ($p < 0.05$). Additionally, comparing BBR200 to the control group have also showed a significant difference ($p < 0.05$), but there was no significant difference between the other groups in comparison. This result might suggest that BBR could increase the level of BUN depending on the given dose. However, that could be explained as the exponential increase of BUN level was in parallel to the dose of BBR, where it continued to increase until reaching its highest level at the dose of 200 mg/kg of BBR. We suggest that this incremental increase of BUN level was due to gastric bleeding that have been noticed within the animals intestine, not from the degradation of the kidney functions, this side effect of BBR has been reported in some animal studies (Kamrani Rad et al., 2017). Furthermore gastric bleeding has been reported as one of the main reason to

increase BUN levels (Zia Ziabari et al., 2019), which could explain the increased level of BUN in all of the animal groups that have been administrated BBR, and the increased level BUN in parallel with the increased dose of BBR.

In this study, the increased folding change of KIM-1 has been seen in the VCM-group, indicating that VCM may have an influence on the renal proximal tubule. The increasement of MDA levels and the reduction of SOD could also be an indication of proximal tubule damage. Similarly, this data supports the notion that tubular cell damage associated with VCM administration is resulted by oxidative stress in kidneys. Furthermore, BBR administration has been reported to reduce the MDA levels and to increase the SOD levels in rat's kidneys in this investigation. This data implies that BBR might have the potential to protect the tubulointerstitial tissue in VIKI.

In our investigation there was no meaningful change in the level of serum Cr between the groups of the experiment. Even when comparing the VCM group to the other groups there was no significant elevation in the level of serum Cr or BUN. Throughout this investigation, we employed KIM-1 and our histological results as the primary indicators for measuring the kidney function and to assess the kidney damage. Furthermore, urinary KIM-1 is the first injury biomarker of kidney toxicity qualified to be utilized by the FDA. additionally it has been significantly outperforming other biomarkers such as serum Cr and BUN in multiple models of kidney injury, as the diagnostic performance of renal KIM-1 was compared to other traditional biomarkers as predictors of kidney tubular histopathologic changes (Vaidya et al., 2010).

Under normal circumstances, the expression of KIM-1 in kidney tissue is infrequent. However, its notable upregulation has been documented in cases of acute kidney injury resulting from ischemia, hypoxia, or chemically induced renal toxicity. Moreover, the levels of KIM-1 in the kidneys exhibit a positive correlation with the extent of renal damage (Song et al., 2019). Therefore, it has been reported in many studies that VCM administration has increased the KIM-1 levels as an indication of the nephrotoxicity side effect of VCM (Mehanna et al., 2022; Pais, Avedissian, O'Donnell, et al., 2019; Westhoff et al., 2017). Our results in this study have showed that the folding change of KIM-1 is higher in the animal's group that have been administered VCM alone, compared to the control group. The animals that have been administered BBR have showed a somewhat noticeable reduction in the folding change of KIM-1 especially at the dose of 100 mg/kg where it was close to the KIM-

1 folding change level of the control group. However, the folding change of KIM-1 has been found to be significantly higher in the animals belonged to the VCM group compared to the control animals ($P < 0.05$). Thus, suggesting an improvement in the kidney function following the administration of BBR in rats.

Given that reactive ROS are widely recognized to exert a significant role in the nephrotoxicity induced by VCM (Im et al., 2017), it has been demonstrated that VCM possesses the ability to augment cellular ATP levels and stimulate oxygen consumption. This phenomenon suggests that VCM acts as a stimulant for oxidative phosphorylation, leading to an increased generation of free radicals. Consequently, the cell membrane undergoes lipid peroxidation, resulting in the disruption of its structural and functional integrity (Ahmida, 2012). Moreover, the breakdown of peroxidized lipids gives rise to various end products, including MDA (Vardi et al., 2005). In the present investigation, the detection of lipid peroxidation using the biomarker MDA serves as an indicator, with elevated MDA concentrations indicating damage caused by free radicals to cellular membrane components. Remarkably, there was a significant rise in MDA concentration within the renal tissues of the group of animals administered VCM alone, compared to the control group. This finding suggests the involvement of oxidative stress-induced lipid peroxidation in the observed renal tissue damage.

The intracellular ROS concentration depends mainly on the production and/or removal by the antioxidant system. Naturally, normal healthy cells are able to maintain a balanced storage of enzymatic antioxidants, that is in order to prevent or repair the damage caused by ROS on the cells, one of the most important antioxidant enzymes that are produced within the cells is superoxide dismutase (SOD). The SODs convert superoxide radicals to hydrogen peroxide and oxygen (O_2). It has been noted that BBR has showed an important anti-oxidant activity by reducing the level of MDA as well as increasing the level of SOD in many studies that have been conducted on heart, kidney, liver and hippocampus (Chatuphonprasert et al., 2014; W. Liu et al., 2008; Moghaddam et al., 2014; L.-Q. Tang et al., 2006; D. Wu et al., 2012). The antioxidant effect of BBR has been noticed in our investigation as well, where the level of MDA measured from the kidney homogenate has been found to be the highest in the animals belonged to the VCM group, whereas BBR100 group has showed a significant decrease of the MDA level in comparison to the level of MDA within the VCM group

($p < 0.05$). This is probably due to the potential capacity of BBR to reduce free oxygen radicals. The suggested protection effect of renal toxicity by BBR might be also related to the reduction of lipid peroxidation within the renal tubular cells of the rat's kidney at the dose of 100 mg/kg. Moreover, the SOD level within the kidney homogenate has been found to be slightly lower in the animals belonging to the VCM group compared to the control animals. Therefore, suggesting that the increase in ROS production in relation with the administration of VCM has consumed the natural reserve of the body's SOD to counter act the effect of the increased production of ROS on the kidney tissues. Furthermore, BBR100 group has showed a significant increase of SOD level ($p < 0.05$) in comparison to the VCM group, which could suggest that BBR at a dose of 100mg/kg has been able to maintain the level of SOD in the kidney tissues despite the effect of VCM. However, the levels of SOD within the BBR groups have shown some similarity with that of the control group. Moreover, a very slight elevation in the level of SOD compared to the control group has been noticed, which might suggest the increased body's reserve of SOD.

Cytokines are extracellular proteins or glycoproteins that are produced by the immune system during its defense of the body at various occasions. However, they have been reported to play an important pathophysiological role in several diseases. It has been reported that BBR could function as an anti-inflammatory by a process involved the NF- κ B pathway. The NF- κ B pathway has been reported to play an important role in controlling inflammation (Gratas-Delamarche et al., 2014). In NF- κ B signaling pathway, I κ B kinase- β (IKK- β) could be activated by an inflammatory stimuli like TNF- α (Goldin et al., 2006; Gratas-Delamarche et al., 2014). Whereas it has been reported that the administration of BBR could greatly reduce the phosphorylation and the activation of IKK- β (Pandey et al., 2008). However, the inhibitory κ B- α (I κ B- α) has been reported to be phosphorylated by IKK- β and then degraded (Delhase et al., 1999; Mercurio et al., 1997). Hence, the inhibition of IKK- β by BBR has been reported to maintain the stabilization of I κ B- α (Delhase et al., 1999; Q. Jiang et al., 2011), which as a result blocks the nuclear translocation of NF- κ B (Y. Wang, 2014; Yi et al., 2008). In its role as a transcription factor, NF- κ B facilitates the transcriptional activation of numerous proinflammatory cytokines, including TNF- α and IL-6 (Goldin et al., 2006; Gratas-Delamarche et al., 2014).

The anti-inflammatory properties of BBR have been extensively reported in both in vitro and in vivo (W. C. Lin & Lin, 2011; Lou et al., 2011). Notably, BBR has exhibited the ability to mitigate the levels of proinflammatory cytokines and acute phase proteins, indicating its effectiveness in suppressing inflammatory responses (Lou et al., 2011). The anti-inflammatory effects of BBR have been observed across diverse tissues, such as serum, liver, adipose tissue, and kidney (Chueh & Lin, 2012). The levels of many cytokines such as TNF- α and IL-6 have been found to be reduced after the administration of BBR as a sign of its anti-inflammatory activity (Chueh & Lin, 2012). In this investigation it has been noted that the administration of BBR at the dose of 50 mg/kg has been able to significantly reduce the folding change of TNF- α compared to the group of animals that have been only administered VCM ($p < 0.05$), which could suggest that BBR has showed an anti-inflammatory effect within the tissues taken from the animals of that group. In addition, the folding change of IL-6 has been found to be slightly reduced in animals that have taken BBR at a dose of 100 mg/kg, which goes along with other studies suggesting that the anti-inflammatory effect of BBR has involved the (NF- κ B) pathway.

Apoptosis has been associated with several inflammatory renal illnesses, particularly drugs induced nephrotoxicity including VCM (AlBasher et al., 2020). The VCM-induced Acute Interstitial Nephritis has been explained by the immune response to VCM, this was observed in interstitial infiltrate of eosinophils, mast cells, plasma cells, lymphocytes, and macrophages (Azar et al., 1996). Furthermore VCM elevated intracellular reactive oxygen species generation, resulting in mitochondrial membrane depolarization and caspase-9 and 3/7 activation (Arimura et al., 2012), as well as downregulating Bcl-2 (Mehanna et al., 2022). In addition, VCM induces an imbalance between free radicals and antioxidants, where it has stimulated the oxygen consumption and resulted in a dose-dependent increase of ATP concentrations within the cells, ultimately leading to mitochondrial dysfunction and cellular apoptosis in cultured renal cells (King & Smith, 2004). Moreover, VCM has been also associated with Acute Tubular Necrosis (ATN) (Sawada et al., 2018). This could be explained as elevated levels of mitochondrial ROS results in DNA damage, on the other hand it is required for the activation of PARP-1 which is an enzyme utilized to repair the DNA, however the overactivation of PARP-1 activity results in NAD⁺/ATP depletion and therefore necrosis (Dalaklioglu et al., 2010).

BBR has been reported to have an anti-apoptotic effect, where it increased the levels of the anti-apoptotic BCL-2 in gentamycin induced nephrotoxicity (Adil et al., 2016). Furthermore BBR has attenuated the apoptosis through mitochondrial-dependent pathway in a study made on human renal proximal tubular cells undergoing a hypoxia/reoxygenation injury (W. Yu et al., 2013). Moreover, excessive lipid accumulation promoted hepatocyte apoptosis, which has been reported to be inhibited following the administration of BBR via reducing the expression of caspase-3 and caspase-9 in a study conducted on induced liver injury (Yang et al., 2019). It has also been proven in PC12 cells and zebrafish that even the modest doses of BBR can exert neuroprotective efficacy against induced neurotoxicity models via the upregulating of P13K/AKT/Bcl-2 and Nrf2/HO-1 antioxidative pathways (C. Zhang et al., 2017). A similar study has found that BBR could improve dopaminergic neuronal disorders by inducing HO-1 expression via the PI3K/Akt/ Nrf2 pathways (J.-H. Chen et al., 2012).

In an in vitro study, the expression of cytochrome c has been shown to be increased in VCM-treated cells and a reduction has been noted following the administration of BBR. Where VCM administration has promoted caspase-9 overexpression, therefore resulting in cellular cytotoxicity. However, BBR has reduced the expression of this marker, protecting the cells from the antibiotic's nephrotoxic effects (Malkani et al., 2022). In addition, BBR, has been found to drastically lower the expression of apoptotic and inflammatory markers in cisplatin-induced nephrotoxic mice model (Domitrović et al., 2013). Furthermore, our results in this investigation have showed some similar results with the previously mentioned studies, where the CAS-9 folding change was slightly higher in the VCM group compared to the control group. Since the administration of VCM alone has shown the tendency to increase the caspase-9 folding change slightly. Additionally, it was slightly lower in the groups administered BBR compared to the VCM group. However, there was no significant change in the CAS-9 folding change between any of the groups ($p>0.05$), and there was no important disparity in the results between the different doses of BBR. Moreover, the administration of VCM alone has showed a small tendency to reduce the folding change of BCL-2 compared to the folding change of the control group, whereas the administration of BBR has showed only a slight elevation of the BCL-2 folding change in the group that have been administered BBR at the dose of 200 mg/kg in comparison with the VCM group. However, there was no

significant difference in the BCL-2 folding change between any of the groups ($p>0.05$).

Our findings have concluded that BBR administration has caused some important changes in oxidative stress and cytokine levels, implying a potential protective effect of the kidney tissues against the nephrotoxicity induced by the intraperitoneal dose of 400mg/kg of VCM for 7 days. Furthermore, BBR has been shown to slightly reduce the folding change of apoptotic biomarkers. However, these findings of our study have revealed no significant change on BCL-2 nor Caspase-9 folding change.



6. CONCLUSIONS and RECOMMENDATIONS

The results of the present study suggest that VCM has resulted in nephrotoxicity damage resembled in the increase of the inflammation and the oxidative stress biomarkers. Additionally, the administration of VCM has resulted in a histological deterioration of the tissues in addition to a minor increase of the apoptotic biomarkers within the kidneys of the rats. The kidney injury induced by VCM has been evaluated in terms of oxidative stress, inflammation, and apoptosis. These evaluations have demonstrated a significant inflammatory and oxidative stress, and an insignificant apoptotic effect on the kidney tissues.

The administration of BBR has shown the potential toward the improvement of the oxidative stress and inflammation significantly and has showed a small reduction in apoptosis but not significantly, however with the increase of the BBR dose, the lower the protection effect has been noticed. With the expanding literatures on the potential beneficial effect of BBR as a reno-protective agent, which has showed its effect against many nephrotoxicity-induced by various agents, our results are suggesting that BBR might have the potential to protect against the nephrotoxicity induced by VCM in male rats receiving 400 mg/kg intraperitoneal injection of VCM for 7 days.

We would like to recommend more investigation regarding the effect of BBR as a reno-protective agent. If we get chance, we would like to test the potential of BBR as a reno-protective agent against multi different nephrotoxicity agents and compare the results to some other reno-protective agents, to get some deeper details about the potentials of BBR as a reno-protective agent. We recommend extending the findings obtained from animal studies to human subjects through prospective controlled clinical investigations involving patients undergoing VCM treatment. Such studies aim to address the issue of nephrotoxicity associated with the administration of VCM at high dosages.

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