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Evaluation the effectiveness of *Andrographis paniculata* aqueous extract on hepatotoxicity and CD44 gene expression in male rabbits treated with butylated hydroxytoluene (BHT)

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***Abstract*---**This study is aimed at knowing the protective role of the aqueous extract of *Andrographis paniculata* on toxic effects that are induced by butylated hydroxytoluene (BHT) in domestic male rabbits. The study was conducted in College of Education for Pure Sciences / Univ. of Karbala from the beginning of November 2021 until February 2022. Forty adult male rabbits have been used, which were divided randomly into four equal groups (ten animals per group), the first group(G1) was administered 1ml/kg of corn oil and considered as the control group, the 2nd group (G2) administrated 1mg/kg of BHT, the third group (G3) administrated 100 mg/kg of *A. paniculata*, while the animals of the fourth group (G4) administrated 1mg/kg of BHT and 100mg/kg of *A. paniculata* extract orally for a month daily. Fasting Blood samples were collected after the end of experimentation in order to study the concentration of the following parameters: alanine transaminase (ALT), aspartate transaminase (AST), Tumor necrosis factor alpha (TNF-α), alkaline phosphatase (ALP), Cytochrome P450, and Interleukin 6 (IL6) in addition to measurement of the gene expression of CD44. The results showed that oral administration of BHT caused significant increase (P<0.01) in concentrations of AST,

ALP, ALT, TNF-α and IL-6, while there has been significant decrease (P<0.01) in concentration of Cytochrome P450 in comparison to the controls. The group that was treated with *A. paniculata* extract showed a significant increase (P<0.01) in the concentration of Cytochrome P450, With no significant difference (P<0.01) in concentrations of AST, ALP, ALT, TNF-α and IL-6. The experiment also showed that oral administration of BHT with oral administration of *A. paniculata* extract caused no significant difference (P<0.01) in concentration of ALP, AST, ALT, TNF-α, IL-6 and Cytochrome P450.The results of the Molecular gene expression of CD44 gene showed significant increase in gene expression in the group treated with BHT when compared to the control group, while there was no significant increase in gene expression in the group treated with *A. paniculata* and BHT compared to the control group. It has been concluded from this study that BHT causes clear pathological effects on the liver, and confirms that treatment with 100 mg/kg of aqueous *A. paniculata* extract has a protective role against damage caused by BHT in male rabbits.

***Keywords*---**Aqueous extract, BHT, Male rabbits.

Introduction

Butylated hydroxytoluene (BHT) is a white crystalline substance (EFSA, 2012) with the chemical formula C15H24O, molecular weight 220.36g/mol, melting point of 70 degrees Celsius, trade symbol E 321, and it is a lipophilic compound (Husøy et al., 2019), BHT is one of the most common antioxidants used in many applications to this day (Higgins et al, 2019), small amounts of preservatives are added for the purpose of improving the quality of taste and prolonging the shelf life of food during its production and to protect food from spoilage or rancidity (Pandey & Kumar, 2021; Zamzam et al., 2019). Butylhydroxytoluene (BHT) is an important compound and one of the food additives that is commonly utilized in food industry. It is a phenolic derivative with antioxidant properties that prevents the self-oxidation of non-organic compounds. (Sharma &Singh, 2019), oxidation is a big and dangerous problem facing the food manufacturing process because it has a clear impact on food quality and negatively affects the flavor, color, taste and smell of food as well as reduces the nutritional value In manufactured foods (Alebic & Richter, 2021), BHT is a very common type of the food additives that are used to improve the taste of food, and it is a substance that was approved by US Food and Drug Administration (FDA) and has been recognized as being classified by the European Union as safe when utilized in small amounts, and based on the recommendations of The Food and Agriculture Organization of US and the World Health Organization (WHO) the daily intake of the BHT in the foods should limit to 0-0.125mg/kg, whereas acceptable range for BHT by the European Economic Community was 0-0.05mg/kg (Ghosh *et al.*, 2020), the incorrect and excessive use of the synthetic phenolic anti-oxidants is one of the causes of cytotoxicity, carcinogenicity, oxidative stress induction and endocrine disrupting effects (Xu et al., 2021).humans are exposed to these chemicals in food, and with the increase in their use for longer periods, many health problems that threaten the life of the individual appear (Pandey & Kumar, 2021), as it has been confirmed that BHT

accumulates in fatty tissues and causes a disturbance in metabolism. Diet (Mean et al, 2018) has also been found it causing many pulmonary disorders and acute poisoning (Liu *et al*, 2016). Some doses of BHT can also cause adenomas, in addition to increasing the weight of glands such as the thyroid gland, BHT can also interfere with the activity of vitamin K-dependent blood clotting factors in mice (Derks *et al.*, 2021). also affects the rate of hormonal regulation (Pop *et al.*,2018)

Andrographis paniculata is an annual herbaceous plant which grows in India and Sri Lanka that is widely cultivated in regions of Asia and utilized as a traditional herbal remedy in Hong Kong, the Philippines, Malaysia and Indonesia (Firdous *et al.*, 2020). It was also used in ancient traditional medicine around the world (Sharma & Sharma, 2018), *A. paniculata* contains a high percentage of saponins, alkaloids, terpenoids, phenols, flavonoids and tannins (Nagajothi *et al.*, 2018), and about 135 compounds have been isolated from this plant, including 40 flavonoids, 82 terpenes, as well as 3 steroids, in addition to 10 other compounds in the plant (Aminah *et al.*, 2021). Andrographolide contains many phytochemicals including Andrographolide, It is a dicyclic diterpene lactone and is considered the biologically active substance in the plant (Ketterman *et al.*, 2020). The activity of this main compound is due to the fact that it contains aliphatic hydrogen groups on C-11 carbon atoms that can play the role of the hydrogen donor and thus displace free radicals (ANDRIANI et al., 2018), and contains several other major active compounds in the leaves such as neoandrographolide and 14-deoxy-12-hydroxyandrographolide as well as 14-deoxyandrographolide (Lim et al., 2021). The plant also contains the compounds Andrographosterin and stigmasterol (Rasool *et al.*, 2018), Andrographidine, apigenin and luteolin (Rafi *et al.*, 2020).Therefore, in the present study, the first focus has been to investigate effects of the BHT on kidney tissue and function and study the effects of *Andrographis paniculata* against kidney failure that induced by BHT.

Materials and Methods
Experience design

A total of40 local male rabbits have been used, divided to 4 groups (10 animals / group): the first group was orally dosed with 1 ml of corn oil and a control group was made, the 2nd group has been orally dosed with a dose of 1mg/kg of BHT, the 3rd group was orally dosed with 100mg/ kg of andrographis aqueous extract, group IV dosed orally with 1mg/kg of BHT + 100mg/kg of andrographis aqueous extract. Blood samples have been drawn after 30 days, the blood was placed in special tubes and the serum has been separated by a centrifuge at 3000rpm for 15 min. to measure the following parameters :(AST) Aspartate transaminase, ALT ،ALP, TNF-α, Interleukin 6 (IL-6) and Cytochrome P450, in addition to the measurement of the gene expression of CD44. Plasma concentration of AST, ALT, ALP, TNF-α, IL-6 and Cytochrome P450were measured by ELISA using commercially available kits. CD44 gene expression was also measured using Quantitative Reverse Transcription Real-Time PCR (qRT-PCR).

Preparation of the aqueous extract

Twenty grams of dry powder has been utilized, mixed with 400ml of the distilled water, an electric grinder was used and left at room temperature for 24 hours. Several layers of medical gauze were used for the purpose of filtering the mixture, then centrifuging at a speed of 3000 rpm. For 10 min., the extract was filtered with filter paper to obtain a clear solution. An electric oven was used to dry the extract at a temperature of 40 °C and kept in refrigerator until it is used (Hernandezi *et al* .,1994).

The above method was adopted for the preparation of *A. paniculata* extract, with some modifications, as follows: The dry leaves were crushed well using an electric grinder and soaked in distilled water for 24 hours with constant stirring and filtered using layers of medical gauze and pieces of soft and clean cloth, then poured into Clean and sterilized glassware for drying it on fan air only, then keeping the extract in the refrigerator until it is used.

Results and Discussion

Effect of BHT on ALP, ALT and AST

Results in table (1) indicates that oral administration of 1mg/kg BHT caused significant increase ($P<0.01$) in concentration of ALP, ALT and AST in the serum of male rabbits compared with the control group (G1). The significant elevation in liver enzymes, which the current study referred to, may be due to the effects of BHT, which may exert an oxidative effect, causing hepatotoxicity as a result of oxidative stress. Oxidative stress is produced when ROS and RNS are generated in large quantities, which include superoxide anion, hydrogen peroxide (H2O2), hydroxyl radicals (OH), superoxide (O_2-.), nitric oxide (NO) and peroxynitrite (ONOO-) due to the enzymatic effect of xanthine oxidase, an enzyme Monoamine oxidase, NADPH oxidase, nitric oxide synthase, electron transport chain In addition to spontaneous oxidation or chemical reactions that may produce other unstable species (Stoia et al., 2022), these free radicals contain unpaired electrons. These (single) electrons are energetic and highly dangerously reactive with other species, causing destructive changes within the mitochondrion. and other places in the cell, the superoxide anion is known as a species of free radical and it is produced from O2 by hydroperoxyl radicals, which interferes with new molecules forming secondary ROS through other enzymatic pathways. It also participates in other functions, such as transmitting signals that bring cytokines and nuclear factor (NF-KB), and its accumulation is harmful and dangerous, as it attacks DNA and destroys nitrogenous bases and causes carcinogenesis, neurological damage, aging, immune and cardiovascular diseases (Neha *et al.*, 2019). ROS can also break down peptide chains, oxidation of a few amino acids and lipid peroxidation by cell membrane disruption. Unsaturated aldehydes, isoprostanes and thiobarbituric acid reactive substances are capable of inducing oxidative stress and disrupting proteins and cellular molecules(Firuzi *et al.*,2011). The free radicals and oxidative stress generated by BHT caused clear liver damage, which causes impaired liver cell functions and plasma membrane rupture, and thus this may be one of the reasons for the increase in the concentration of liver enzymes as well as the enzymatic leakage that occurs

across the plasma membrane (Mean *et al.*, 2018), It was found that BHT causes necrosis in hepatocytes, which in turn leads to a defect in the amount of enzymes, and this enzymatic defect may be due to the dangerous metabolites of BHT, which can attack hepatocytes, causing clear damage to the liver tissue, which leads to The release of liver enzymes into the blood serum in large quantities (Abou-Hadeed *et el.*, 2021; Altuntaş & Değer., 2017).

Table (1) Effects of butyl hydroxytoluene and aqueous extract of *A. paniculata* on liver enzymes in male rabbits

	ALP(U/L)	AST(U/L)	ALT(U/L)
G1((	244.30±5.61	137.90±1.78	56.20±1.57
Control	A	A	A
)(G2	476.40±9.85	289.40±7.64	107.70±2.52
1 mg/kg BHT	B	B	B
(G3)	234.10±3.61	136.00±2.15	56.60±1.25
100 mg/kg Andrographis	A	A	A
G4)(	249.30±5.18	138.80±2.90	57.10±1.47
1 mg/kg BHT+10 mg/kg Andrographis	A	A	A
LSD	18.626	12.402	5.1552

mean ± standard error, n = 10/ group. The different letters are an indication of the presence of significant differences vertically under the probability level P<0.01

Effects of *A. paniculata* on ALP, ALT and AST

Table (1) indicates that oral administration with a 100mg / kg dose of *A. paniculata* extract led to no significant differences in the rate of liver enzymes (ALP, ALT and AST), and this is agreement with (Owoade et al., 2022; Salunkhe & Patil (2018; Shi *etal.*, 2020; Dey *etal.*, 2020; Nasir *etal.*, 2013)

The absence of a significant difference in liver enzymes in the current paper may be due to the role of the plant used as an effective drug in treating many different diseases and disorders to the organs, such as the liver (Sundhani *et al.*, 2022), During the study period, BHT caused the harmful effect on the liver through the production of free radicals and stimulation of oxidative stress. It has been proven that the aqueous extract of *A. paniculata* played an effective role in scavenging free radicals, as this plant contains many active active compounds such as andrographiold, Andrographolide, Andographoside and Newandrographolide in addition to many other important active compounds that scavenge free radicals and protect the liver from oxidative stress caused by harmful substance and thus regulate the work of liver enzymes (Saunkhe & Patil, 2018 (Benny *et al.*, 2021;). It can be seen the role of the andrographiold compound, which is the main compound that has a distinctive molecular formula. It has aliphatic hydrogen atoms on its carbon number 11 (C-11) atoms, which works to give hydrogen to unstable free radicals and thus reduce these radicals through a series of Reactions and lead to the breakdown of the lipid peroxide chain, which shows its

action as an antioxidant (Andriani et al., 2018), while the flavonoid compound contains in its molecular structure a phenol hydroxyl group (OH), which also participates in the synthesis of glutathione (GSH) and is thus It activates endogenous antioxidants (Youssef *etal.*, 2010), The protective role of *A. paniculata* extract in many researches have confirmed its role in regulating levels of liver enzymes ALT, AST, and ALP by working to raise the antioxidant enzymes in the body in animals treated with many oxidizing and harmful substances and its role in reducing lipid peroxidation and controlling stress. (Venmathi Maran *et al.*, 2022).

Effect of BHT on IL-6, TNF-α and cytochrome P450 (CYP)

The results showed that oral administration of BHT led to a significant ($P<0.01$) increase in concentration of IL-6, TNF-α, and a significant decrease ($P<0.010$) in concentration of Cytochrome P450.

The significant increase in concentration of IL-6, which was indicated by the current study, could be due to the negative effects produced by BHT, which had caused an oxidative effect, causing hepatotoxicity as a result of the oxidative stress that occurred. As the treatment of the body with foreign chemicals or toxic and harmful substances can stimulate the generation of a large number of free radicals and the occurrence of oxidative stress, resulting in the occurrence of lipid peroxidation, inflammation and necrosis in the liver, and as a result of this, hepatocytes release inflammatory cytokines like the IL-6, and this is evident Through elevated and abnormal concentrations of IL-6 (Permata et al., 2020), liver injury occurs through several inducers, pathways and factors, for example nuclear factor NF-κB, a multifunctional transcription regulator that plays a significant and vital role in pro-inflammatory pathways. And hepatic injury, and this result is likely due to oxidative stress due to ROS that has been shown to activate NF-κB, and liver damage leads to the process of activation of the liver Kupffer cell and an increase in the proportion of phagocytic macrophage cells that lead to increased oxidative stress and an increase in pro-inflammatory factors such as IL-6 and activation of other inflammatory cells (infiltrating the macrophage and neutrophils) (Sun *et al.*,2022),

TNF-alpha is a pro-inflammatory cytokine which activates a broad range of cellular signalling path-ways such as mitotic responses or cell death such as apoptosis and may be involved in being a cytotoxin especially at high concentrations of it as it can be detrimental and promote inflammation and multi-organ injury (Steeland. et al., 2018). BHT used in the current study may be able to disrupt, toxic and generate reactive oxygen species (ROS) whose effects are clear in many organs (Kagan *et al.*, 1990). Specifically, it is highly sensitive when exposed to various toxic substances, as the occurrence of hepatic damage is related to the release of pro-inflammatory cytokines such as TNF through multiple mechanisms (Wree *et al.*, 2018). Interactions in the immune system may occur as a result of oxidative stress and the inflammatory response of the system Immunological (activation and increase of macrophage, neutrophil, epithelial cells, TNF, and other pro-inflammatory cytokines) due to ingestion of foreign substances and their entry into the palpation This leads to immune reactions and weakness which in turn works to secrete pro-inflammatory cytokines such as

TNF. The development of tissue damage, and the high level of this cytokine is evidence that the toxic substances stimulated the immune system through the infiltration of inflammatory cells to the site of injury and in this case, the immune cells become responsible for the development of cellular injury through release of pro-inflammatory cytokines like the TNF (Alayunt *et al.*, 2019).

Table (2) Effects of the butyl hydroxytoluene and aqueous extract of *A. paniculata* on IL-6, TNFα and CYT P450 in male rabbits

	IL-6	TNFα	CYT P450
G1	35.57±0.52	2.81±0.15	1.12±0.08
Control	A	A	A
G2	39.49±0.48	4.68±0.39	0.65±0.09
1 mg/kg BHT	B	B	B
G3	34.11±0.44	2.67±0.12	1.27±0.08
100 mg/kg Andrographis	A	A	C
G4	35.57±0.44	2.87±0.14	1.04±0.07
1 mg/kg BHT+10 mg/kg Andrographis	A	A	A
LSD	1.3672	0.6728	0.2477

mean ± standard error, n = 10/ group. The different letters are an indication of the presence of significant differences vertically under the probability level P<0.01

The results also showed that oral administration of BHT led to a considerable decrease (P<0.01) in the mean concentration of Cytochrome P450 . CY P450 system is one of the most important toxic metabolic enzyme systems that are mainly found in the liver and intestinal tract. It works to stimulate the metabolism of various nutrients, including toxic substances, CY P450 can change the composition of the iron ion present in heme and oxidize foreign matter, and It enhances the solubility of toxins in water and thus makes them easier to expel from the body. (Wang *et al.*, 2021), and since most of the foreign compounds entering the body depend on CY P450 metabolism, these toxins often cause lipid peroxidation and the generation of a large number of free radicals, which leads to decreased activity of cytochrome P450. At the same time, these harmful and toxic substances directly inhibit cytochrome P450 CY synthesis, and decreased cytochrome P-450 CY activity directly or indirectly results in the decrease in the liver's ability to detoxify (Pan et al. 2018). The decrease in enzymatic activity of Cytochrome P-450 in detoxified animals increases oxidative stress, which eventually leads to increased toxicity (El Rabey et al., 2021). The cytochrome P450 family can be involved in metabolic activation of BHT with the help of Certain triggers result in the production of reactive oxidants that can cause cell death, especially in the absence of appropriate levels of glutathione (Powell & Connolly, 1991).

Effect of *A. paniculata* aqueous extract on the concentration of Interleukin 6 (IL-6), TNFα, and Cytochrome P450 (CYP)

The results showed that oral administration of *A. paniculata* aqueous extract resulted in no significant difference (P<0.01) in the concentration of IL-6 and TNF-

α, which is in agreement with (Sheeja & Kuttan, 2010; Sani *etal.*, 2019; Zhu *etal.*., 2018; Mussard *et al.*, 2020)

Andrographis plant contains many active compounds with various anti-inflammatory effects through regulation and control of pro-inflammatory cytokines, which include andrographolide, neoandrographolide, and 14-deoxy-11,12 didehydroandrographolide, in addition to the presence of flavonoids, uinic acids and xanthones.

A. paniculata extract and its active compounds proved anti-inflammatory properties, which made the plant extract able to reduce various inflammations and cellular injuries by inhibiting the expressions of pro-inflammatory cytokines like the tumor necrosis factor-α and IL. 6- Along with the modification and regulation of these cytokines and their receptors (Rahmi *et al.*, 2022), one of the most important causes of damage and inflammation that occurs in cells and tissues is due to the generation of free radicals and reactive oxygen species ROS, esulting in the oxidative stress and lipid peroxidation and resulted in clear damage in many parts of the body. The active compounds in *A. paniculata* play a vital role in activating antioxidant enzymes that reduce the effect of oxidative stress, and this contributes to the regulation of inflammation-stimulating cytokines and reduced injury (Chauhan *et al.*, 2019). It became clear that hepatocytes were affected by ROS and exogenous toxins that stimulated them to release inflammatory cytokines. The *A. paniculata* aqueous extract reduced the phagocytic activity by oxidative stress and free radicals and thus reduced the secretion of these cells of pro-inflammatory cytokines like the TNF-α And IL-6 (Weng *et al.*, 2018).

Effects of butyl hydroxytoluene on gene expression of CD44 gene

The results of the molecular examination of the CD44 gene had shown a considerable increase in gene expression in group that is treated with BHT compared with control group.

BHT is a synthetic antioxidant added to various foods and materials to prevent oxidation. Because it is ubiquitous, humans and animals are highly exposed to this substance through the environment, eating food containing the substance, or when using personal care products. The substance is an antioxidant, but increased exposure to it makes it a toxic and oxidizing substance, which may cause diseases and necrosis or tumors in some organs like the liver and kidney (Liu and Maburi., 2020). A study by (Vikis *et al.*, 2012) agreed with the results of our study and indicated that using BHT as an antioxidant in animals leads to an increase in the level of T cells. It was found that after measuring CD44 gene expression, the activation of CD44 cells increased with the increase of this expression in response to exposure to toxic substances, as T cells accumulate twice in the Treated animals compared to non-BHT treated animals, and that CD44 gene expression is normal in normal liver, while its expression increases significantly in the liver. This is observed mainly in (unfiltered) lymphocytes or Kupffer hepatocytes (Satoh *et al.*, 2000) and hepatocellular carcinoma (Fang *etal.*, 2020). Uno et al., 2021 indicated in their study. That some diets may cause harmful fatty changes in liver and cirrhosis with oxidative stress and a group of

changes, which stimulates the response of the CD44 gene due to the occurrence of hepatitis as it activates T cells, and expression of this gene increases in the advanced stages of infection up to stage of cirrhosis of the liver. Hepatitis is one of the reasons for the increase in the expression of CD44. It continues to rise throughout the development of inflammation (Egan *et al.*, 2013), where CD44 is expressed mainly on leukocytes and other cells during inflammation because it is one of the adhesion molecules involved in migration of cells like the neutrophils to the inflammation site and thus increases it in the site of damaged tissues of the liver. When using an antibody to CD44 leads to a decrease in the migration and infiltration of neutrophils in the damaged tissues, reducing infection and inflammation of the liver and regulating its function. Exposure to toxins can lead to the expression and increased activation of other types of CD44 that have a role In the immune response, its evolution and regulation (Kim *et al.*, 2011).

Effect of the aqueous extract of *A. paniculata* on CD44. gene expression

The molecular assay results for the CD44 gene showed no significant differences in gene expression in the group treated with A. paniculata aqueous extract and BHT in comparison with control group.

The use of *A. paniculata* aqueous extract reduces damage when exposed to BHT, as this plant is a powerful immune stimulator, enhances immune responses, and is an antioxidant that helps treat many diseases. It also affects many genes and has a high ability to reduce efficacy toxicity (Wang *etal.*, 2021; Niranjan *et al.*, 2010), Chan et al., 2020) indicated in their study that using plant extracts from herbs when exposed to toxic chemicals has a role in treating organs affected by this exposure. Reducing their damage, decreasing apoptosis, and improving cell renewal of damaged organs, as the CD44 gene helps this regeneration process through its contribution to cellular pathways that work on the rapid renewal of cells damaged by chemicals, as indicated (Olayeriju *et al.*,2021) in his study that The use of plant extracts to treat damage in some treated organs when exposed to toxic chemicals, prevents CD44 gene expression from rising above the normal limit or may reduce its height due to Toxic pain substance, that is, it regulates the gene expression and may maintain its normal existence, this is an agreement with the results of our study.

The results of the study (Fan *et al.*, 2020) showed that using medicinal plants and their extracts has a high ability to treat cancerous tumors, prevent their formation and migration, and reduce gene expression for genes associated with tumors, such as the CD44 gene, which is associated with stem cells responsible for cell proliferation, the use of these active substances reduces injuries. It stimulates apoptosis by targeting CD44, where the extracts maintain the normal expression of this gene or reduce it. Leukocytes play a significant impact on pathogenesis of a variety inflammatory diseases. Lymphocytes' adhesion to vascular endothelium and consequent migration activate lymphocytes at inflammatory sites. Secretion of the CD44 protein involved in immune cell adhesion and migration if these extracts inhibit adhesion of activated T lymphocytes. The plant compounds also contribute to reducing the production of tumor necrosis factor-alpha, which is involved in adhesion and inflammatory processes and partially in the activation of CD44 protein (Yi *et al.*, 2008).

Table (3) Effect of butylhydroxytoluene and aqueous extract of *A. paniculata* on levels of gene expression of CD44 gene in local male rabbits

	G1 Control	G2 1 mg/kg BHT	G3 100 mg/kg Andrographis	G4 1 mg/kg BHT+10 mg/kg Andrographis	P value
	20±0.949	11.5±1.581	_	_	<0.001*
CD44	20±0.949	-	_	19±0.667	0.093
	_	11.5±1.581	_	19±0.667	<0.001*
	_	_	20.3±0.949	19±0.667	0.343
	_	11.5±1.581	20.3±0.949		<0.001
	20±0.949	_	20.3±0.949		0.0181

Mean ± standard error, n = 10/group, * sign indicates significant differences.

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